

EVALUATION OF THE INFLUENCE OF THE INNATE AND ADAPTIVE IMMUNE
RESPONSE IN THE PATHOGENESIS OF POST-TRAUMATIC OSTEOARTHRITIS
BASED ON AN IN-DEPTH ANALYSIS OF THE CELLULAR INFLAMMATION
PATTERN AND ITS MOLECULAR DRIVING FACTORS.

A thesis
submitted in fulfillment of the requirements for
the degree of
Doctor of Philosophy

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Statement of originality and authorship attribution

This is to certify that to the best of my knowledge; the content of this thesis is my own work. This thesis has not been submitted for any degree or other purposes. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

Chapter 1 of this thesis includes a published literature review as:

Haubruck P, Pinto MM, Moradi B, Little CB, Gentek R. Monocytes, Macrophages, and Their Potential Niches in Synovial Joints - Therapeutic Targets in Post-Traumatic Osteoarthritis? *Front Immunol.* 2021 Nov 4;12:763702. doi: 10.3389/fimmu.2021.763702. eCollection 2021. I performed the literature search, created the figures, drafted the manuscript, and approved the final version of the manuscript.

Chapter 2 of this thesis includes two published manuscripts:

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Haubruck P, Heller R, Blaker CL, Clarke EC, Smith SM, Burkhardt D, Liu Y, Stoner S, Zaki S, Shu CC, Little CB. Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models. *Osteoarthritis Cartilage.* 2023 Dec;31(12):1602-1611. doi: 10.1016/j.joca.2023.07.013.

I designed the study with guidance from CB Little. I performed the animal experiments, harvested all tissues, and scored all histological slides. Histological sample preparation as well as staining was performed by Sue Smith, Daniel Burkhardt, Yolanda Liu, Shihani Stoner. Statistical analysis was performed by Raban Heller and me. I created the figures, drafted the manuscript, and approved the final version of the manuscript.

Chapter 3 of this thesis includes a drafted manuscript as:

Haubruck P, Colbath AC, Blaker CL, Clarke EC, Moradi B, Shu C, Little CB. Innate and adaptive immune response to joint injury and the onset and progression of post-traumatic osteoarthritis: a comparison of common murine models.

I performed the animal surgeries with assistance of AC Colbath, I conducted the flow cytometry experiments with assistance of AC Colbath. I performed all data acquisition and conducted the statistical analysis. S Smith and D Burkhardt performed histological processing, sectioning, and staining. I created the figures, drafted the manuscript, and approved the final version of the manuscript.

Chapter 4 of this thesis includes a drafted manuscript as:

Haubruck P, Blaker CL, Hill C, Smith S, Burkhardt D, Liu Y, Clarke EC, Shu C, Little CB. Characterising the role of the IL12/23 pathway during onset and progression of post-traumatic osteoarthritis.

I performed the animal surgeries; I conducted the flow cytometry and in vitro experiments. PCR analysis was performed by Y Liu, S Smith and D Burkhardt performed histological processing, sectioning, and staining. I performed all data acquisition and conducted the statistical analysis. I created the figures, drafted the manuscript and approved the final version of the manuscript.

Chapter 5 of this thesis includes a drafted manuscript as:

Haubruck P, Li L, Xia T, Blaker CL, McCracken S, Smith S, Burkhardt D, Liu Y, Clarke EC, Shu C, Little CB. Targeting different myeloid/innate-immune cell subsets has differential effects on joint inflammation and structural damage associated with post-traumatic osteoarthritis.

I performed all animal experiments and surgeries; I conducted the flow cytometry experiments and harvested the samples for RNAseq analysis. RNAseq samples were analyzed by L Li and T Xia under my guidance. S Smith and D Burkhardt performed histological processing, sectioning, and staining. Otherwise, I performed all data acquisition and conducted the statistical analysis. I created the figures, drafted the manuscript, and approved the final version of the manuscript.

Chapter 6 of this thesis includes a drafted manuscript as:

Haubruck P, Blaker CL, McCracken S, Smith S, Burkhardt D, Liu Y, Clarke EC, Shu C, Little CB. In-vivo evaluation of CD40 ligand as an early potential target in the treatment of post-traumatic osteoarthritis.

I performed the animal surgeries; I conducted the flow cytometry and in vitro experiments. PCR analysis was performed by Y Liu; S Smith and D Burkhardt performed histological processing, sectioning, and staining. I performed all data acquisition and conducted the statistical analysis. I created the figures, drafted the manuscript and approved the final version of the manuscript.

In addition to the statements above, in cases where I am not the corresponding author of a published item, permission to include the published material has been granted by the corresponding author.

Patrick Haubruck, 27th September 2023

As supervisors for the candidature upon which this thesis is based, I can confirm that the authorship attribution statements above are correct.

Christopher Little, 27th September 2023

Elizabeth Clarke, 27th September 2023

Abstract

Post-traumatic osteoarthritis (ptOA) remains a common and debilitating musculoskeletal disease affecting a large population of patients. While initially it was believed that ptOA was purely caused by a biomechanical mismatch resulting in an accelerated wear and tear of the cartilage and surrounding tissue, in recent years studies derived from rheumatoid arthritis and subsequently osteoarthritis (OA) as a whole, without further distinction regarding the underlying cause (primary vs secondary), highlighted the role of an aberrant immune response as a contributing factor and furthermore implicated pro-inflammatory immune cells subsets of the innate and adaptive immune system as key players in the aetiology of OA. At the same time, studies revealed that although joint injuries increase the lifetime risk of ptOA not all injuries with similar biomechanical instabilities will go on to develop ptOA. In addition, restoration of biomechanics alone has been shown to be insufficient to prevent ptOA development. Thus, the concept of a critical role of the immune system has gained importance in the field ptOA research, however studies providing robust evidence supporting this concept have been missing. Therefore, the central scope of this thesis is to investigate the role of the immune system during onset and development of ptOA and thereafter to identify cellular key players that drive the immune response, and finally test if immunomodulation could be used to prevent ptOA from developing after joint injury.

Therefore, this body of works starts with an exhaustive literature review regarding the role of monocytes and macrophages and how the “macrophage niche” theory provides explanations for the intricate crosstalk between macrophages and non-hematopoietic stroma cells and their perturbations following joint injury. Following the review of the literature, questions and aims were defined and a set of consecutive studies were designed and tailored to address those questions. New laboratory techniques were developed specifically for these studies and validated prior to their implementation. In an initial study, a variety of murine ptOA models injuring differing joint tissues both surgically and non-surgically were utilized and the innate and adaptive immune response characterized immediately after joint injury, during progression of ptOA and finally at the stage of established ptOA. In this study we were able to identify cellular key players of the innate and adaptive immune systems that were associated with the inflammatory immune reaction. In order to further decipher the role of the individual cellular players and their molecular pathways of activation, a study was conducted that modulated the IL12/23 pathway, which has immediate impact on pro-inflammatory T-helper cell subsets (Th1, Th17), prior to joint injury and development of ptOA. In addition, a second study was

conducted that used antibody mediated depletion of pro-inflammatory subsets of monocytes (Ly6C^{high} monocytes) as well as drug mediated depletion of synovial tissue resident macrophages (STRM) to evaluate the individual role of these key components of the innate immune response in context with joint injury. While these studies were tailored to specifically address either the innate or adaptive immune response, we observed an immunomodulation going beyond the individual and targeted arm of the immune system in each study, which highlighted the possibility that an early immunomodulation that targets factors orchestrating the interplay between cellular key players of the immune system rather than the individual cell subsets themselves, may provide a new therapeutic approach. Therefore, we used an antibody mediated inhibition of CD40L as an early treatment to modulate macrophage activation and decrease promotion of the Th1 response.

The key findings of this thesis are: What you injure (ACL vs meniscus) rather than how you injure it (surgery vs non-surgical) determines the temporal pattern and systemic involvement of both the innate and adaptive immune response and the structural ptOA progression and severity. The IL12/23 pathway plays an important role in orchestrating the immune response following joint injury and during development of ptOA while promoting a production of IL17 producing Th17 cells and thereby perpetuating the inflammatory response initiated by joint disease. Inhibition of IL12/23 *in-vivo* leads to a persistently decreased inflammatory response on a cellular level and the mitigation of histopathological changes associated with wild-type ptOA development. Depletion of Ly6C^{high} monocytes leads to a persistent anti-inflammatory response both in blood originated macrophages as well as STRM. Importantly, depletion of STRM had the opposite effect on the immune response thereby supporting the theory of a joint protective function of STRMs. Finally, anti-CD40L treatment results in an anti-inflammatory polarization of not only migrating macrophages but also STRM while histologically a reduction of joint pathology was achieved.

The role of the immune response in context with ptOA is highly complex and a plethora of intricate pathways are involved. Only by improving the understanding of the immune response and what perpetuates and drives the inflammatory reaction, can novel targets be identified that might serve as future disease-modifying osteoarthritis drugs (DMOAD). The results of this thesis provide a first step along the way in deciphering the role of innate and adaptive cellular key players, the underlying molecular pathways and a how to use this knowledge in identifying potential targets for treatment.

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Chapter 1

1 Introduction and literature review

1.1 Structure of this thesis

This thesis is presented in a “hybrid” format, where most chapters are consisting of either published papers or manuscripts that have been submitted or are in the final stage of being submitted. Some sections contain additional information and explanatory text to provide context and to highlight important aspects of each individual chapter with regarding to the entire thesis framework. The introduction section will provide a brief overview of the relevant literature stating the current knowledge regarding the relevance and pathogenesis of post-traumatic osteoarthritis (ptOA), followed by an introduction of the role of the adaptive immune response in context with ptOA and finally a published literature review regarding the role of relevant myeloid cell subsets and their potential as therapeutic targets in treating ptOA. Thereafter, each chapter is a self-contained manuscript including a review of the respective relevant literature and methods used. Lastly, an overarching discussion was included to discuss the findings of each chapter in context with the overarching aim of this thesis and to summarize the combined findings of the program of research.

1.2 Background

1.2.1 Osteoarthritis

Osteoarthritis (OA) is a common degenerative musculoskeletal (MSK) disease affecting synovial joints in a large population of patients (Chen, D. et al. 2017). Initially OA was believed to be purely caused by “wear and tear” of the cartilage and surrounding tissue, and only during the last decades has a paradigm shift occurred introducing a multifactorial aetiology including traumatic, hereditary and patient related factors (e.g. age and obesity) (Loeser, R. F. et al. 2012). Despite considerable research advances, to date effective early treatment modalities are missing due at least in part, to an incomplete understanding of the cellular and molecular mechanisms involved in the onset and development of OA. This chapter will provide a brief overview of the prevalence, aetiology, and pathology of OA and thereafter will introduce current concepts with regard to the role of the adaptive and innate immune system.

1.2.1.1 Prevalence of Osteoarthritis

The majority of studies investigating the prevalence of OA only investigate clinical cases of established OA, and thus information regarding underlying etiology or disease-phenotype (e.g. genetic, metabolic, post-traumatic) is missing. In addition, most studies report only cases where OA was diagnosed based on radiographic imaging alone and studies investigating the prevalence of OA defined through joint pain are scarce (Pereira, D. et al. 2011). Taken together this highlights the difficulty in assessing the true prevalence of OA due to heterogeneity in reporting and disease definition. Therefore, while the focus of this thesis is post-traumatic (pt)OA, the general term OA is used when no distinction was made between phenotypes in the cited studies. OA is one of the most common MSK diseases (Loeser, R. F. et al. 2012; Swain, S. et al. 2020) affecting more than 25% of the population of over 18 years-old (Chen, D. et al. 2017) and is only surpassed by the prevalence of lower back pain which is estimated to be 9.4% in the global population (March, Lyn et al. 2014). Interestingly, while the age-standardized incidence rate of lower back pain decreased between 1990 and 2017 it showed a steady increase for OA during the same time, thereby highlighting the future need for affective treatment modalities (Ziyi, Jin et al. 2020). While OA can affect all synovial joints, 80% of its disease burden derives from disease of the knee (KOA) (Cui, Aiyong et al. 2020; Wallace, Ian J. et al. 2017). Although age remains a common risk factor for the development of OA (Chen, D. et al. 2017) it has been shown that the prevalence for KOA peaks around the age of 50 years (Ackerman, Ilana N. et al. 2017) indicating the clinical importance of this disease in a younger population of patients. Epidemiological studies have provided evidence regarding a rising

prevalence of OA in younger patients and it is expected that the number of patients with OA younger than 55 years will increase by 20% in Australia alone over the next 15 years (Ackerman, Ilana N et al. 2016). OA affects patients on a personal level and symptoms vary from moderate pain to severe disability; in particular 80% of patients with OA are limited to a certain extent and 25% of patients are unable to carry out activities of daily life (Blaker, C. L. et al. 2016). On a socioeconomic level, OA was shown to cost the Australian health system an estimated \$3.5 billion annually, accounting for 28% of the disease expenditure in MSK diseases and 3% of total disease expenditure (AIHW 2020). Further adding to the enormous socioeconomic costs of OA are work-related costs which are expected to increase to \$9.4 billion dollar annually in Australia by the year 2030 (Ackerman, Ilana N. et al. 2017). Taken together this evidence highlights the impact of OA on an individual and socioeconomic level, which will be worsened by the increase in prevalence in younger patient populations that is predicted to occur in the coming decades.

1.2.1.2 Aetiology of Osteoarthritis

“Primary” OA is defined as cartilage and joint degeneration without any known cause, while the term “secondary” OA is used when disease occurs due to a recognized or identifiable trigger (Hsu, H. et al. 2022). The most important risk factors include age, sex, obesity, misalignment of the joint surfaces, altered biomechanics, metabolic diseases and joint trauma/ sports injuries (Mobasheri, Ali et al. 2016). In particular post-traumatic (pt)OA occurs after a joint injury and accounts for at least 12 % of all OA and up to 25% of OA in susceptible joints such as the knee (Little, C. B. et al. 2013; Riordan, E. A. et al. 2014). It is known that joint injury increases the lifetime risk of ptOA by more than five times (Muthuri, S. G. et al. 2011). Furthermore, 10-20 years after injuries to the anterior cruciate ligament (ACL) or menisci around 50% of affected patients develop ptOA which has been termed “the young patient with an old knee” by Lohmander et al in 2007 (Lohmander, L. Stefan et al. 2007). In recent years, research centering around mitigating the effect of joint injury on the development of ptOA has been conducted and patients at risk were identified. In addition, person-centered interventions to promote self-management and exercise to reduce known risk factors have been promoted to mitigate this lifetime risk (Jackie, L. Whittaker et al. 2022). Although fundamental differences in the aetiology of OA (primary vs secondary) have been well defined, it is also known that, for example, not all injuries with apparently similar instability will go on to develop ptOA.

Therefore, a new paradigm was born that conceptualizes OA as phenotypic subsets related to a primary abnormality (Sun, Bob H. et al. 2007). In ptOA, injuries to the joint are varied in severity and tissues affected, and can include fractures, tearing of the supporting soft tissues (ligaments, menisci) causing significant joint instability, and bruising and inflammation of the synovium and capsule (Blaker, C. L. et al. 2016), and defining what factors drive the long-term ptOA-risk in individuals remains challenging.

1.2.1.3 Phenotypes of Osteoarthritis and their Pathology

Progressive cartilage loss, subchondral bone thickening, osteophyte formation and joint space narrowing characterize all forms of OA. However, the new concept regarding individual phenotypes of OA opened up the opportunity for a more targeted investigation into disease pathophysiology and the potential treatments (Sun, Bob H. et al. 2007).

A recent study furthered the understanding of OA phenotypes by defining clinical phenotypes of OA, such as ‘Chronic Pain Phenotype’, ‘Inflammatory Phenotype’. ‘Mechanical Overload Phenotype’, and ‘Minimal Joint Disease Phenotype’ (Dell’Isola, A. et al. 2016; Mobasheri, A. et al. 2019). Subsequently the concept of “OA molecular endotypes” was developed: endotype means a specific molecular pathway that explains the observable properties of a phenotype, such as elevated pro-inflammatory cytokines, chemokines, MMPs, and cathepsins that are contributing towards the development of the clinical phenotype (Mobasheri, A. et al. 2019). Combining this knowledge, the authors proposed diverse pathophysiological OA phenotypes and their subsets: 1) Age-related and systemic phenotypes 2) Extra-articular phenotypes 3) Intra-articular phenotypes 4) Secondary phenotypes (Mobasheri, A. et al. 2019). While based on a review of the literature and thus missing much mechanistic and causative evidence, this proposal introduced an intriguing concept highlighting the complexity of OA and emphasizing the importance of molecular pathways and their contribution to each phenotype. To further complicate this new paradigm subsequent studies found different structural phenotypes restricted to KOA (Roemer, Frank W. et al. 2023), different pain phenotypes in KOA (Kittelson, Andrew J. et al. 2021) and finally genetic phenotypes of OA (Aubourg, G. et al. 2022).

To date, ptOA has been grouped under the secondary OA phenotypes, and whether ptOA itself is a completely independent phenotype is still under debate (Watt, Fiona E. 2021). Some authors argue that there are potentially unique features in ptOA, depending of the specific

injury, including mechanical instability, acute hemorrhage into the joint and/or joint tissues, tissue injury and wound-healing responses (inflammation, fibrosis), and acute structural changes in the cartilage, soft tissue and subchondral bone (Robinson, W. H. et al. 2016). On the other hand, the clinical endpoint of primary OA and ptOA looks similar, the structural appearances look the same and the molecular changes within the joint are similar during the developing disease (Watt, Fiona E. 2021). Therefore, the possibility remains that a person with ptOA has simply brought forward the OA that they would have developed eventually (Watt, Fiona E. 2021).

Be that as it may, the clinically significant joint injury initiating ptOA development, provides the opportunity for prevention and outcome modification, and thus a better understanding of the pathomechanisms following joint injury is needed. In particular, as differing clinically relevant joint injuries lead to differences in ptOA development and not all injuries with apparently similar instability will go on to develop ptOA, it is likely that phenotypes of ptOA itself exist driven by different joint injuries. Growing evidence implicates a significant role of inflammation in the pathogenesis of ptOA regardless of injury (Mathiessen, A. et al. 2017). In particular synovitis and the overproduction of cytokines and growth factors can influence the production of degradative enzymes that lead to structural damage and the clinical syndrome of ptOA (Scanzello, C. R. 2017). However, if other phenotype specific differences within ptOA on a cellular or endotype level exist is unknown and the subject of this thesis.

1.2.1.4 Available Treatment Modalities for Osteoarthritis

Current treatments of ptOA and OA can be stratified into non-pharmacological management, pharmacologic management and surgical modalities (Nelson, Amanda E. et al. 2014). Non-pharmacological management includes education and self-management, as well as exercise and weight loss. Weight loss, in particular, has been shown to be effective in relieving ptOA/OA symptoms as well as improving the quality of life (Bliddal, H. et al. 2014). Ultimately, patients may choose to treat advanced stages of OA via surgical joint replacement or fusion of the effected joints. Despite the overall success of this surgical procedure an inherent risk associated with anaesthesia and the surgical procedure itself remains. Furthermore 10-34% of patients after total knee replacement (Ditton, Elizabeth et al. 2020) and 7-23% of patients after total hip replacement (Judge, Andy et al. 2010) show limited clinical improvement.

Clinical data from Chua et al. have highlighted the ongoing and unaltered need for effective,

preventative and disease modifying drugs in the treatment of OA (DMOAD), by showing a similar initial disease burden in patients suffering from rheumatoid arthritis (RA) or OA, whereas after 6 months of treatment the disease burden in OA patients was significantly higher (Chua, J. R. et al. 2019) due to a lack of effective treatment options. Established treatment modalities such as non-steroidal-anti-inflammatory-drugs (NSAIDs), corticosteroid injections and opioids, alleviate pain and are standard in many guidelines (Kloppenburg, M. et al. 2020). However, due to the chronic nature of OA long-term use of these agents is necessary, increasing the risk of side effects and co-morbidities, thus limiting their utility over time.

Novel treatment modalities include biologicals, such as monoclonal antibodies blocking specific pathways and factors. To date, no DMOAD has been approved by any of the regulatory bodies as the approval guide requires a potential DMOAD to demonstrate a slowed disease progression and improved clinical symptoms (Oo, W. M. et al. 2021). Nevertheless, a variety of phase 2/3 clinical trials are ongoing as part of active drug development pipelines. Potential novel DMOADs that are targeting synovial inflammation currently in phase 2/3 include inhibitors of IL-1, IL-1 β , and TNF- α (Oo, W. M. et al. 2021). Diacerein (targeting IL-1 β) showed low efficacy similar to an NSAID, while trials using inhibitors of IL-1 and TNF- α failed as they did not possess symptomatic benefits irrespective of the joint or the inflammatory OA phenotype (Oo, W. M. et al. 2021). The failure of these biologicals was attributed to a more complicated interaction amongst various cytokines in the pathogenesis of OA (Oo, W. M. et al. 2021). Further mixed results were reported in a recent systemic review and the authors concluded that studies investigating treatment strategies specifically addressing a more nuanced concept of inflammation with specific anti-inflammatory treatments are warranted (Kloppenburg, M. et al. 2020).

In addition, despite the advances in understanding the influence of the role of inflammation in the pathogenesis of OA, evidence regarding specific pathophysiological pathways in ptOA and if these differ from non-OA-inducing joint injury is scarce. Furthermore, it is paramount to analyze which pathological changes are associated with the injury itself and which pathological changes result in ptOA development. Taken together this evidence indicates that a more nuanced anti-inflammatory approach is most likely needed that accounts for the complex interplay between cytokines during ptOA development.

1.3 Current Concepts Regarding the Role of the Immune Response during Development of Osteoarthritis

1.3.1 Phases of Joint Injury and the Transition into Posttraumatic Osteoarthritis

Joint injury leads to a distinct mechanobiological response that is characterized by three temporally separated phases. The immediate phase is characterized by the mechanical effects on a molecular, microscopic and macroscopic level deriving from the underlying injury (Anderson, D. D. et al. 2011; Punzi, L. et al. 2016). Here, within seconds, collagen disruption, cartilage swelling, and hemarthrosis is happening which initializes the second phase: the acute inflammatory phase (Lieberthal, J. et al. 2015). This phase is characterized by acute cell death and a distinct inflammatory response involving both cellular and molecular reactions from the innate and adaptive immune system (Higuchi, H et al. 2006; Irie, Kazunori et al. 2003; Kandahari, A. M. et al. 2015; Struglics, André et al. 2015) and lasts between hours and days. The final phase in physiological cases is the spontaneous resolution and return to normal joint biology. These phases have been well established both in animal models as well as in clinical cases. In contrast, the underlying reasons why joint injury leads to ptOA is still under investigation.

Recent studies (Kandahari, A. M. et al. 2015; Lieberthal, J. et al. 2015; Punzi, L. et al. 2016) utilizing pre-clinical data postulated that the acute inflammatory response acts as a “fork in the road” with respect to the development of ptOA. Although it has been well established the inflammatory response is a crucial part of the wound healing cascade (Maruyama, Masahiro et al. 2020) a timely resolution of inflammation might prevent the onset of ptOA while a perpetuation of inflammation may contribute towards the risk of disease development (Lieberthal, J. et al. 2015; Lotz, M. K. et al. 2010). Possible reasons for an ongoing inflammatory reaction include a continuously disrupted equilibrium of pro- and anti-inflammatory factors (Anderson, D. D. et al. 2011) or inadequate post-injury inflammation control (Lieberthal, J. et al. 2015). In addition, biomechanical factors, such as ongoing instability and recurring joint injury (Lotz, M. K. et al. 2010), resulting in a continuous activation of the post-injury mechanobiological response may ultimately contribute towards an inflammatory vicious circle. The chronic and final phase of the ptOA response is defined by a

persistent low-level synovitis (Jackson, M. T. et al. 2014) and continuous remodeling processes that ultimately lead to progressive destruction of the joint surface (Chen, L. et al. 2020). Studies (Lotz, M. K. et al. 2010) identified the acute inflammatory response as a potential target for DMOADs, therefore it is important to understand what initiates the inflammatory reaction and which cellular and molecular key factors are involved.

1.3.2 The Immune System and its Key Players for Inflammation and its Resolution

The immune system is a highly complex and specialised defence mechanism that protects the body against a variety of threats, such as microbes, injuries or malignancies (Woodell-May, Jennifer E. et al. 2020) and it consists of two major factions, the innate and adaptive immunity. Immediately after injury macrophages and neutrophils from the innate immune system (non-antigen-specific responses) are activated via signals such as damage-associated molecular patterns (DAMPs), inflammatory cytokines or complement factors (Woodell-May, Jennifer E. et al. 2020). Besides complementing the phagocyte compartment, macrophages also serve inflammatory functions when activated. Activation and polarization of macrophages was initially thought to be dichotomous with a classically activated and pro-inflammatory state (M1) and an alternatively activated anti-inflammatory state (M2) (Gordon, S. et al. 2010; Sica, A. et al. 2012). Since then polarization of macrophages has been intensively studied and emerging evidence suggested further intricacies in this oversimplified classification system (Martinez, F. O. et al. 2014) by introducing several intermediated stages of polarization suggesting that there is a complex spectrum of activation and the M1 and M2 stage represent the extreme poles in such a spectrum (Haubruck, Patrick et al. 2021; Hume, D. A. 2015; Murray, P. J. 2017). The adaptive immune system consists of T lymphocytes (T cells), B lymphocytes (B cells), and antibodies (antigen-specific or acquired response). Depending on their designated function T-cells can be further divided into T-helper (Th) cells (CD4+) and cytotoxic T-cells (CD8+): Th-cells contribute towards guidance and orchestration of the immune response while cytotoxic T-cells are responsible for eliminating the targeted cells (Woodell-May, Jennifer E. et al. 2020). Subsets of the Th-cell system can be categorized as pro-inflammatory (Th1, Th17) and anti-inflammatory (Th2, Treg) (Li, Y. S. et al. 2017). Postulated roles for the pro- and anti-inflammatory immune cell subsets during the onset and development of pTOA are further discussed in **Chapter 1.3.3** and **Chapter 1.3.5**.

1.3.3 The Role of the Adaptive Immune Response

T-cells are the main cell type of the adaptive immune system and their migration to peripheral tissues is tightly regulated via a widely accepted homing mechanism that is orchestrated by a variety of chemokines and chemoattractants (Buckley, C. D. 2003; Burman, A. et al. 2005). To date, the majority of data that has analyzed the T-helper cell profile and polarization derives from late-stage OA samples (Dolganiuc, A. et al. 1999; Haynes, M. K. et al. 2002), thus little is known about the mechanisms that contribute towards polarization of CD4⁺ T-helper cells acutely after their migration into the joint in OA. In rheumatoid arthritis (RA) a pro-inflammatory polarization is attributed to an interaction between T-cells and synovial dendritic cells via specific chemokines (CXCL12) (Tran, C. N. et al. 2005) and secretion of soluble cytokines (Hussein, M. R. et al. 2008) (IL-12, IL-23, IL-27, IL-1, IL-6 and TNF- α) (Tran, C. N. et al. 2005). Furthermore, direct stimulation of T-cells by monocytes using a functional autoantigen-bound MHC II complex was postulated for RA (Tran, C. N. et al. 2005; Tsark, E. C. et al. 2002; Wagner, U. et al. 2004). Whether the same pathomechanisms result in activation and polarization of T-cells in OA remains unknown. Compelling evidence exists regarding the presence of relevant T-helper cell subsets in OA joints and thus their potential involvement in disease pathogenesis. Overall a predominantly pro-inflammatory T-helper cell polarization was found in the synovial compartment, with increased numbers of pro-inflammatory Th1 (Ishii, H. et al. 2002; Li, Y. S. et al. 2017; Yamada, H. et al. 2011), Th17 (Faust, Heather J. et al. 2020; Li, Y. S. et al. 2017; Zhang, L. et al. 2012) and Th9 (Li, Y. S. et al. 2017; Qi, C. et al. 2016), and decreased numbers of anti-inflammatory Th2 (Haynes, M. K. et al. 2002; Li, Y. S. et al. 2017) and Tregs (Guo, S. Y. et al. 2015; Li, Y. S. et al. 2017) T-cell subsets. However, as most studies were of a descriptive nature in established OA, the individual role and/or contribution of each cell type to ptOA pathophysiology remains poorly understood.

1.3.4 Literature Review

A comprehensive literature review was carried out regarding the role of monocytes, macrophages, and their subsets in the development of ptOA. In addition, this review explored a potential therapeutic application for modulating individual subsets and pathways and pointed out existing gaps in knowledge and resulting research questions that need to be addressed.

1.3.5 Published Literature Review on the Role of Monocyte and Macrophages in Post-Traumatic Osteoarthritis



Monocytes, Macrophages, and Their Potential Niches in Synovial Joints – Therapeutic Targets in Post-Traumatic Osteoarthritis?

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Synovial joints are complex structures that enable normal locomotion. Following injury, they undergo a series of changes, including a prevalent inflammatory response. This increases the risk for development of osteoarthritis (OA), the most common joint disorder. In healthy joints, macrophages are the predominant immune cells. They regulate bone turnover, constantly scavenge debris from the joint cavity and, together with synovial fibroblasts, form a protective barrier. Macrophages thus work in concert with the non-hematopoietic stroma. In turn, the stroma provides a scaffold as well as molecular signals for macrophage survival and functional imprinting: “a macrophage niche”. These intricate cellular interactions are susceptible to perturbations like those induced by joint injury. With this review, we explore how the concepts of local tissue niches apply to synovial joints. We introduce the joint micro-anatomy and cellular players, and discuss their potential interactions in healthy joints, with an emphasis on molecular cues underlying their crosstalk and relevance to joint functionality. We then consider how these interactions are perturbed by joint injury and how they may contribute to OA pathogenesis. We conclude by discussing how understanding these changes might help identify novel therapeutic avenues with the potential of restoring joint function and reducing post-traumatic OA risk.

Keywords: osteoarthritis, monocyte - macrophage, inflammation, niche, native immune functions, synovitis, immunomodulation

INTRODUCTION

Osteoarthritis (OA) is the most common joint disorder (1). Its prevalence is expected to increase further (2) due to rising societal levels of ageing, obesity and injury, key risk factors for OA. While the disease commonly affects knees, hips, hands and feet, OA of the knee accounts for more than 80% of the disease burden (1, 3). The knee is particularly susceptible to injury, with approximately

40% of patients that suffer a traumatic knee injury developing so-called “post-traumatic” (pt)OA, and surgical reconstruction and restoration of joint biomechanics insufficient to prevent its development (4). Treatment options for OA are very limited, and there is a particular need for effective preventive and disease modifying drugs (DMD). This is highlighted by clinical data showing comparable disease burden at diagnosis but significantly higher burden 6 months later in OA compared to rheumatoid arthritis (RA) patients (5). Owing to this paucity of treatment options and the high and rising prevalence, OA contributes substantially to the global burden of disease. In a 2015 survey, OA was identified as the second most prevalent cause for years lived with disability (2), highlighting the impact OA has on both individuals and society (2).

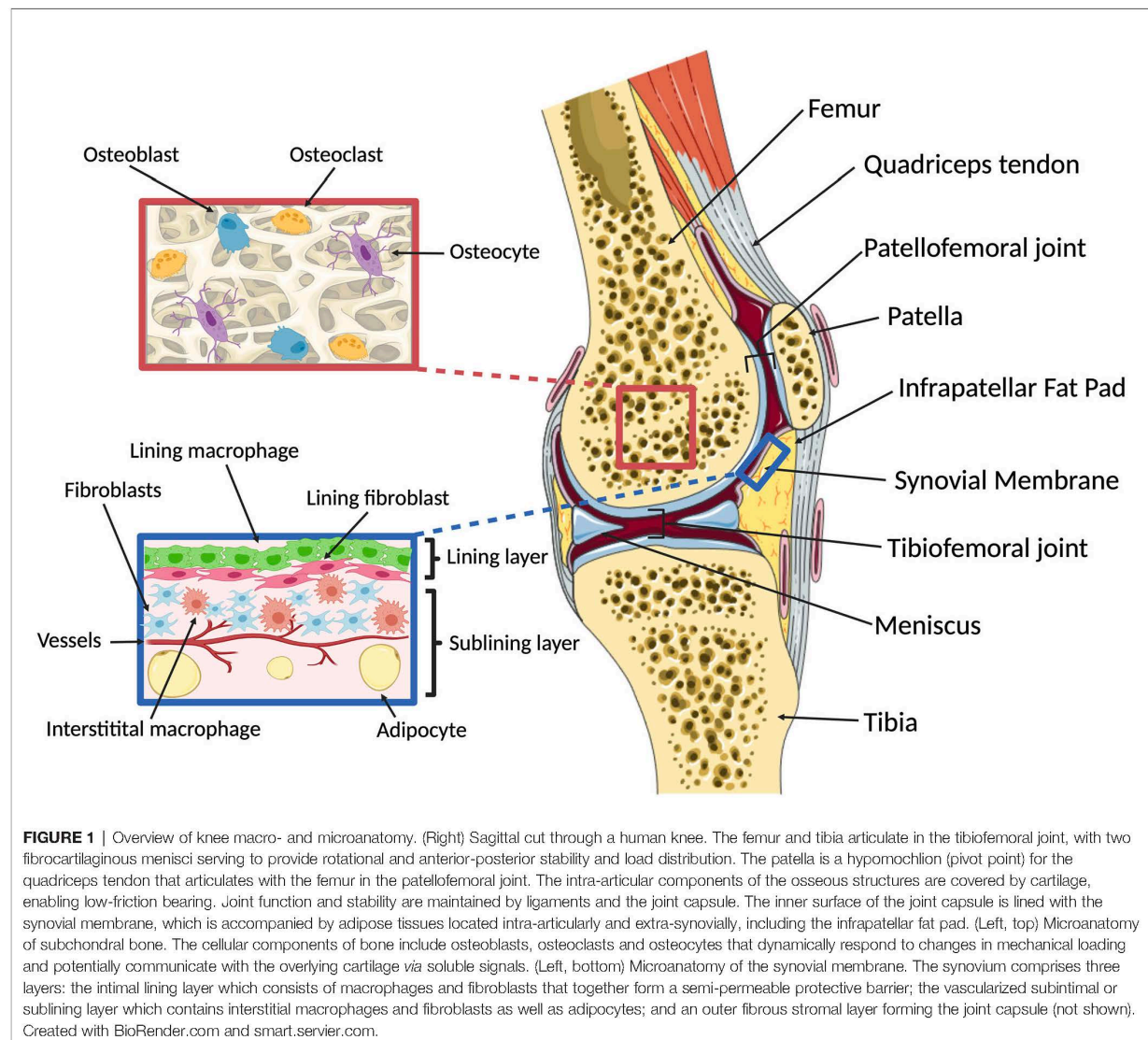
Although the name osteoarthritis implies an inherent inflammatory process (6), it was historically believed that OA had purely biomechanical causes (7). Indeed, OA was regarded a disease of the elderly, inevitably caused by years of “wear and tear”. Breaking with this previously held view, we now know that OA development involves a complex active biological response with local interaction between joint tissues and their resident cells, and these with systemic mediators. This includes an inflammatory response (8) that is accompanied by complex metabolic changes, which contribute to cartilage degradation and activation of bone remodeling (9). Although innate immune cells, and monocytes and macrophages in particular have been implicated (7, 10, 11), the exact nature of the inflammatory response in OA, its underlying mechanisms and its relative contribution to onset or progression of structural pathology and symptoms remain incompletely resolved (12).

Much like OA etiology, our understanding of the complex development and functional heterogeneity of macrophages and monocytes as well as their interactions within local tissue “niches” has dramatically changed in recent years. It is now firmly established that the long-held paradigm of discrete, polarized monocyte and macrophage activation states is an oversimplification of what in reality is a spectrum of cell states. Likewise, it is now recognized that macrophages established from fetal progenitors can persist in adult tissues, and that many macrophages self-maintain independently of monocytes (13–16). Lastly, we are beginning to appreciate that macrophages engage in bidirectional crosstalk with other cell types within their local niches, interactions that are of mutual benefit and implicate macrophages as gatekeepers of tissue function (17, 18). While much remains to be learned and confirmed, these concepts developed in other organs and tissues appear to also apply to macrophages in joints (19, 20). Indeed, macrophages found in the healthy synovium are predominantly monocyte-independent, and they protect and contribute to joint homeostasis in several ways, including barrier formation, clearance of debris and even lubrication (20). Under inflammatory conditions, such as may occur with joint injury however, monocytes are recruited to the affected joint and can differentiate into macrophages, which retain a more inflammatory phenotype (21). These joint macrophage populations thus not only differ in their origins, but also exert distinct functions (22, 23).

Modifying the developmental, functional and *in situ* dynamics of joint macrophages and monocytes might therefore represent an attractive avenue for novel therapeutic approaches in OA. This may be particularly relevant in ptOA, where causal initiation and subsequent temporal changes in monocytes, macrophages and their activation with disease onset and progression may be targeted. This review aims to explore this notion, with a focus on the synovial rather than osseous joint tissue niche. We will summarize experimental and clinical studies on macrophages and monocytes in healthy and diseased joints and interpret these in the context of current paradigms of myeloid biology. Our emphasis in this review is on joint injury and ptOA, as this represents the major OA phenotype studied in pre-clinical research, and as noted above, it has the most well demarcated disease stages and thus potentially the broadest therapeutic opportunity. In doing so, we hope to bridge persisting gaps between bench and bedside and highlight research questions with the potential to pave the way towards better treatment options for ptOA, but also other OA phenotypes more broadly.

MACRO- AND MICROANATOMY OF THE KNEE JOINT

Synovial joints provide critical motion segments that allow low friction movement between rigid (osseous) skeletal components. They enable diverse and essential bio-mechanical functions ranging from fine movements of arms, hands and fingers through to walking, running and jumping. The knee represents an anatomically complex example of a joint (**Figure 1**) that enables locomotion in a variety of terrains, while minimizing muscular energy requirements and absorbing and redistributing forces that originate from the contact between the walking surface and the foot (24). Its main osseous components are the femur, tibia and patella, that articulate in two locations: the tibiofemoral and patellofemoral joints. The menisci, two C-shaped fibro-cartilaginous structures, absorb and distribute load between the femoral and tibial surfaces. Together with a multitude of extra- and intra-articular ligaments and the fibrous joint capsule, the menisci also provide stability in flexion/extension and rotation, enabling the unique biomechanical function of the knee (25). As in all joints, the osseous surfaces in the knee are covered by hyaline cartilage, a sparsely cellular, deformable connective tissue matrix with key components of collagen type II and highly hydrated proteoglycans. Cartilage is heterogenous and can be broadly divided into three zones based on depth from the surface. These have distinct composition, biomechanical properties and functions (26). Chondrocytes make up about 2% of the articular cartilage volume (27) and are responsible for the maintenance and repair of the cartilage extracellular matrix. They are highly specialized cells derived from mesenchymal stem cells that have limited potential for replication *in situ*, but can react to a plethora of mechanical and molecular stimuli (26). The knee also harbors several adipose tissues. These are located intra-articularly and extra-synovially,



and include the infrapatellar fat pad, which can be considered a highly specialized compartment in the sublining interstitial tissue [in humans known as Hoffa's fat pad (28)]. Beyond filling the space in the joint cavity and absorbing shock, adipose tissues also secrete cytokines and adipokines (29, 30) and are therefore potent immune-modulators. It is also believed that the infrapatellar fat pad engages in intimate crosstalk with the synovial membrane, a specialized connective tissue that lines the inner surface of the joint capsule (31). The synovial membrane consists of three layers: The intimal lining layer is found closest to the joint cavity and consists mainly of macrophages ("type A cells") and fibroblasts ("type B cells") that show low degrees of cell division (31). Beneath this is the vascularized subintimal layer, also referred to as sublining interstitial tissue, and finally a fibrous stromal layer forming

the joint capsule. The synovial membrane maintains joint homeostasis by providing lubrication and nutrition to the cartilage. It also forms a semi-permeable protective barrier that controls the molecular traffic in and out of the joint (32) and renders the synovial cavity relatively immune-privileged (20). Because of its critical role in joint homeostasis, this review will largely focus on monocyte and macrophage biology of the synovial membrane, including its sublining interstitial layer.

A REVISED VIEW OF MONOCYTE AND MACROPHAGE BIOLOGY

Many historically held views of monocyte and macrophage biology have been overhauled in recent times, including their phenotypic

and functional heterogeneity, developmental dynamics as well as their crosstalk and functional interdependence with other cell types in the same tissue microenvironment.

Monocyte and Macrophage Development

It was previously believed that the key (if not sole) function of monocytes was to produce macrophages, and that in turn, all macrophages found in peripheral tissues originate exclusively from monocytes (33). Elegant studies exploiting genetic fate mapping have since shown that most tissue-resident macrophages are in fact of fetal origin and self-maintain in adult tissues independently of bone marrow (BM)-derived monocytes (33). Indeed, macrophages colonize tissues concomitantly with their development in what appears to be a demand-driven way. They are generated from successive, but overlapping waves of hematopoietic progenitors produced at distinct anatomical sites (34). The majority of fetal macrophages originate from erythro-myeloid progenitors (EMP) produced in the extra-embryonic yolk sac (15, 16). EMP are fetal-restricted progenitors that differentiate into macrophages either directly or *via* fetal liver intermediates, but as an uncommitted entity do not persist into adulthood.

This new paradigm of predominantly fetal origins of tissue macrophages notwithstanding, monocytes can still complement tissue phagocyte compartments on demand (33). While this applies to some tissues at homeostasis (e.g. skin and gastro-intestinal tract), it is particularly true and important in inflammatory conditions. Importantly, in both scenarios, monocytes themselves have a number of key effector functions (35).

Monocytes differentiate from BM hematopoietic stem cells (HSC) in a strictly hierarchical, tree-like maturation process (35). They share a common progenitor with dendritic cells (DCs) known as “monocyte-macrophage DC progenitor” (MDP) (36, 37), which gives rise to a monocyte-committed intermediate, designated the “common monocyte progenitor” (cMoP) (38). The downstream “transitional pre-monocytes” (TpMos) (38, 39) are believed to be the final intermediate stage in monocyte differentiation (39). They are capable of rapid proliferation and express high levels of C-X-C motif chemokine Receptors (CXCR) 4, which anchors them to the BM. Based on differential expression of Lymphocyte antigen 6C (Ly6C), CX3CR1 and C-Chemokine Receptor type 2 (CCR2) in mice (40) or Cluster of Differentiation (CD)14 and CD16 in humans (37), mature monocytes can be broadly classified into classical (mice: Ly6C^{high} CX3CR1^{low} CCR2^{high}; humans: CD14^{high} CD16⁻) and non-classical monocytes (mice: Ly6C^{low} CX3CR1^{high} CCR2^{low}; humans: CD14^{low} CD16⁺) (35, 41–44). This binary classification is now widely established (45) and has more recently been backed up by extensive high-dimensional studies, the latter also revealing a previously underappreciated heterogeneity (46). A third monocyte population with an intermediate phenotype is exclusive to humans (CD14⁺ CD16⁺) (35). Classical and non-classical monocytes differ in a number of features, including their relative abundance and the regulatory mechanisms governing their retention in and egress from the BM (47). The mature monocyte

compartment in the BM is vastly predominated by Ly6C^{high} monocytes, which downregulate CXCR4 (48, 49) and highly express CCR2 (50, 51), collectively enabling their egress from the BM. Ly6C^{low} monocytes, on the other hand, only express very low levels of CCR2, and while still under investigation, Sphingosine-1-Phosphate Receptor 5 (S1PR5) signaling has been implicated in orchestrating their BM egress (52).

Once released into the blood stream, classical Ly6C^{high} monocytes have a relatively short half-life lasting for a mere 20–24 hours in mice (53–55), whereas their non-classical counterparts are slightly longer-lived with a half-life of around 2 days in mice and 7 days in humans (53). The two populations are also developmentally connected: lineage tracing indicating that Ly6C^{low} monocytes originate from aging Ly6C^{high} monocytes (41, 53), a gradual conversion that is dependent on Nuclear Receptor subfamily 4 group A member 1 (NR4A1) signaling (56) and involves direct cellular contact with endothelial cells and Notch signaling (57, 58). Similar mechanisms appear to be at play in human monocytes (59, 60). At homeostasis, Ly6C^{low} monocytes do not normally extravasate but instead patrol the luminal side of the endothelium (61). They roll along the vascular endothelium, independent of the direction of the blood flow, *via* CX3CR1, β 2 integrin (58, 62) and interactions between Lymphocyte Function-associated Antigen-1 (LFA-1) and IntraCellular Adhesion Molecule 1 (ICAM1) and ICAM2 (58, 62). They have thus been considered the “tissue-resident” macrophages of blood vessels. In non-homeostatic conditions, Ly6C^{low} monocytes are thought to promote resolution of inflammation, however, they can also contribute to autoimmunity and chronic inflammatory diseases (58), as we will discuss further below. Intriguingly, experiments using bleomycin-induced lung fibrosis in mice identified an alternative pathway to Ly6C^{low} monocytes, consisting of a separate progenitor referred to as a “Segregated-nucleus-containing atypical Monocyte (SatM)”, whose production depends on the transcription factor C/EBP β (63). Whether this pathway is relevant to other pathologies remains to be determined.

Unlike their non-classical counterparts, Ly6C^{high} monocytes do traffic into peripheral tissues even at steady state (64). In tissues that (partially) rely on homeostatic renewal from the BM, such as the skin and gastro-intestinal tract (65, 66), the majority of recruited Ly6C^{high} monocytes gradually differentiate into macrophages, a process phenotypically characterized as a “monocyte waterfall” (67). These macrophages are functionally imprinted in response to local cues that superpose tissue-specific identity onto a transcriptional core lineage program (68, 69). Provided monocytes encounter a homeostatic environment and are allowed sufficient time in the tissue, monocyte-derived macrophages are phenotypically, transcriptomically and epigenetically indistinguishable from pre-existing tissue-resident macrophages (70, 71). However, this is not the case following inflammation or other insults resulting in perturbed homeostasis, which might have important functional implications. Indeed, different and sometimes even opposing roles have been reported for developmentally distinct macrophages in conditions like cancer (72–74) and stroke (75), and this might also be the case in joint pathology, as we will discuss below.

Monocyte Effector Functions

In addition to representing an “on-demand” source for macrophages, monocytes also have important effector functions in their own right. Indeed, a fraction of classical monocytes recruited at steady state maintains their monocytic phenotype with minimal transcriptional changes (76). In the parenchyma of non-lymphoid organs like the skin, lung, and heart (43, 66), they contribute to immune surveillance. During sterile inflammatory responses, as would occur following closed traumatic knee injury, Ly6C^{high} and Ly6C^{low} monocytes are recruited in a highly orchestrated manner facilitated by differential chemokine release. Under such conditions, Ly6C^{low} monocytes have primarily been attributed beneficial, anti-inflammatory roles. In the ischemic heart and kidney for example, deficiency in Ly6C^{low} monocytes results in higher inflammatory levels and impaired restoration of organ function (77–79). In line with this, Ly6C^{low} monocytes predominantly produce anti-inflammatory mediators like Interleukin (IL)-10 (80, 81) as well as Vascular Endothelial Growth Factor (VEGF) and other pro-angiogenic factors (82), as observed during spinal cord injury and myocardial infarction (82), respectively.

Somewhat contradictory evidence exists regarding the role of Ly6C^{high} monocytes. Historically, these classical monocytes have been recognized as potent pro-inflammatory effector cells. Indeed, CCR2 knockout mice, which are largely deficient in classical monocytes in the periphery, show decreased levels of IL-1 β and Tumor Necrosis Factor (TNF)- α and an increase in the anti-inflammatory cytokines IL-4, IL-5 and IL-13 at the site of inflammation during cerebral ischemia (83). Ly6C^{high} monocytes also show high levels of reactive oxygen species, TNF- α and IL-6 (84) in the context of liver ischemia reperfusion injury (84). In line with this pro-inflammatory phenotype, Ly6C^{high} monocytes mediate tissue damage in the ischemic liver as well as the heart following myocardial infarction, and contribute to progression of atherosclerosis (84–87). At the same time however, Ly6C^{high} monocytes have also been implicated in regression of atherosclerosis (88), although this may be attributable to anti-inflammatory effects of monocyte-derived macrophages, rather than a true monocyte effector function. Nonetheless, these findings collectively suggest that instead of being globally pro- and anti-inflammatory, classical and non-classical monocytes differentially shape the local inflammatory response *via* the tailored production of cytokines and other cellular mediators. Although similarities exist between tissues and insults, their exact trafficking patterns and effector functions appear to be context-dependent, and therefore need to be delineated specifically in the homeostatic, injured and OA joint.

Tissue Adaptation and Activation of Monocytes and Macrophages

Monocytes and macrophages dynamically respond to a variety of cues in their microenvironment, which shape their local tissue adaptation and activation state. Consequently, although they share a lineage-defining core transcriptomic signature, macrophages in different tissues are transcriptionally, phenotypically and functionally very diverse (70, 71, 89). The core macrophage

program is initiated in committed fetal progenitors or BM-derived monocytes and driven by lineage-determining transcription factors (68–71).

Acquisition of tissue-specific identity and function is subsequently orchestrated by additional transcription factors in response to signals present in the local microenvironment (69). In the spleen, for example, heme from senescent red blood cells induces expression of the transcription factor SPI-C, which in turn activates a transcriptional program inducing differentiation of red pulp macrophages (90). Experimental data from adoptive transfer experiments demonstrate that exposure to different environments partially, though not fully, rewires the tissue-specific identity of macrophages, indicating a limited degree of plasticity even under such non-physiological conditions (70, 91).

At the same time, the activation state of terminally differentiated macrophages can vary as a function of microenvironmental signals, in particular cytokines. Historically, it has been thought that macrophages polarize into either classically activated, pro-inflammatory (“M1”) or alternatively activated, anti-inflammatory (“M2”) (92, 93) subtypes in response to cytokines associated respectively with type I or type II immunity (94, 95). However, it is now abundantly clear that this strict dichotomy is a drastic oversimplification of real-life *in vivo* physiology. Rather, these opposing polarization states represent extremes (94, 95) of a much wider and more fluid spectrum of activation states (96, 97). Understanding the different activation states of macrophages and monocytes in OA and the signals that drive them will be paramount in delineating their respective contribution to disease pathogenesis. Since circulating monocytes represent a modifiable source, they – and their relationship with macrophages found in the joints – are of particular translational relevance.

Macrophage Niches

The intricate developmental dynamics between monocytes and macrophages and their adaptation to tissue-derived signals illustrate that these cell types actively engage with each other and their immediate environment or “niche”. Research into such niches represents a current focus in the field of myeloid cell biology. The niche concept postulates that macrophages are not only functionally imprinted by tissue-specific cues, but that their niches also provide them with a physical scaffold for anchoring and survival factors (17, 18). In turn, macrophages support appropriate functioning of their cellular partners. They thus form mutually beneficial cellular circuits (18) with their niches. In line with this, organ function is heavily impaired in mice lacking numerous tissue-resident macrophages owing to genetic deficiency in Colony Stimulating Factor (CSF)1, a key macrophage survival signal, or its receptor (98–100). Niches consist of macrophages and other, often non-hematopoietic stromal cell types, as well as the extracellular matrix surrounding them, and they can also “call” monocytes for replenishment. In the liver, for example, hepatocytes, endothelial and stellate cells together provide numerous signals to resident Kupffer cells and incoming monocytes, including CSF1, IL-34, CCL2 and Notch ligands (101), whereas in the red pulp of the spleen, macrophages depend on CSF1 produced by fibroblasts (102). In return, macrophages help facilitate tissue-specific functions and homeostasis. Beyond their

role in immune surveillance and protective immunity, macrophages have been implicated in diverse physiological processes ranging from haemoglobin recycling, intestinal motility, surfactant degradation in the lung, to cardiac conduction (64, 102–109). The circuits underlying some of these less-traditional macrophage effects are starting to be deciphered. For example, macrophages located in the interstitial space of the testis produce cholesterol, which stimulates steroidogenesis in Leydig cells (110–112).

Whilst their cellular partners, signaling circuitry and functions remain incompletely understood, it is highly conceivable that distinct macrophage niches also exist in the joint. In the following, we will thus discuss how the current concepts of monocyte and macrophage biology in other tissues and organs reviewed above, apply to synovial joints, with particular emphasis on molecular and cellular mechanisms bearing potential for translational exploitation in OA. By interpreting the dynamics between these pleiotropic cell types and their functions within their potential joint-associated niches, we aim to provide an integrative view of their contribution to joint health and disease.

MONOCYTES AND MACROPHAGES IN JOINT HOMEOSTASIS

Bone and Adipose Tissue

The bone-resident macrophages are known as osteoclasts, peculiar large and multinucleated cells whose primary function is bone resorption. They are essential for skeleton remodeling and maintenance of the hematopoietic environment in the BM. Consequently, defects in osteoclasts cause osteopetrosis and hematopoietic failure, while their overactivation leads to osteoporosis. Osteoclasts allow for homeostatic bone turnover in joint-associated subchondral bone in response to loading. Osteoclasts first colonize the ossification centers of developing bones in the fetus from EMP, where they form long-lived syncytia that are maintained throughout life by low-grade fusion with incoming monocytes (113, 114). Adding to this complexity, elegant recent intra-vital imaging has shown that osteoclasts do not necessarily undergo apoptosis following activation and bone resorption, but instead, can fission into daughter cells termed “osteomorphs” (115). These can be recycled by fusion with osteoclasts but remain transcriptionally distinct from both osteoclasts and other macrophages.

As in many other tissues, adipose tissue-resident macrophages are developmentally and functionally heterogeneous. In the healthy adipose tissue of lean mice and likely humans (116, 117), monocyte-derived macrophages co-exist with long lived, fetal yolk sac EMP-derived macrophages and regulate appropriate development of adipose tissues and lipid storage during homeostasis (116, 118). Of note, it is currently unclear whether the macrophage compartments within joint-specific adipose tissues, such as the infra-patellar fat pad, are developmentally and functionally equivalent to those in more commonly studied adipose depots, such as the subcutaneous or inguinal fat.

The infrapatellar fat is highly vascularized and innervated, and thus more reminiscent of visceral than subcutaneous fat (Reviewed in Urban and Little 2018) (30). It is also interesting to note that although generally considered a type of white adipose tissue, the infrapatellar fat may not always behave like other adipose tissues, for example in conditions of obesity. Although the infrapatellar fat pad increases in volume, vascularization and adipocyte size in response to obesity like other adipose tissues, it may be more protected from obesity-induced inflammation (119–121). This suggests that infrapatellar fat may show features of both white and brown adipose tissue in response to obesity, and distinct responses to other white adipose deposits have also been observed in OA (122). In end-stage knee OA patients the infrapatellar fat pad had significantly less macrophages, toll-like receptor 4 expression and fibrosis compared with other peri-synovial adipose tissue. In these same patients both adipose tissues had increases in adipocyte size and haematopoietic and M2 macrophage cell infiltration correlated with body mass index. This complex interplay between systemic and local joint factors related to post-traumatic OA, and how these affect and are affected by infrapatellar fat pad macrophage polarization, has been demonstrated in mouse models (123). The infrapatellar fat has been implicated as a major player in sustaining and perpetuating inflammation in OA (29). While macrophage deregulation has been associated with pathological changes in other adipose depots, those in the infrapatellar fat can contribute directly or indirectly to OA pathogenesis and future research is needed to better characterize which macrophage features it shares with other adipose tissues and which are unique.

Synovial Membrane and Interstitial Connective Tissue

At homeostasis, macrophages are virtually the only immune cells in the synovial membrane (124, 125), and whilst the underlying interstitial connective tissue does harbor other lineages like mast cells and lymphocytes, macrophages predominate by far (126). Importantly, both the steady state synovial membrane and interstitium are largely devoid of monocytes. Healthy synovial tissue contains three populations of macrophages that are dynamically interconnected: lining macrophages gradually turn over from proliferating MHCII⁺ macrophages found in the sub-lining connective tissue, which also generate a second population of interstitial macrophages characterized by expression of Hypoxia-Induced Mitogenic Factor (Resistin-like alpha; RELMa) (22). The exact sources from which synovial macrophages are originally established during development remain to be determined with appropriate additional fate mapping systems. However, chimeras and parabiosis have now firmly established that all three populations receive minimal if any monocyte input in the adult steady state (22).

Despite their developmental interdependence, the distinct populations of synovial macrophages are phenotypically and functionally highly specialized. In addition to being a source of other synovial macrophages, MHCII⁺ sub-lining macrophages are particularly well-equipped for antigen presentation, while the RELMa⁺ population shows a regulatory phenotype and

abundantly expresses scavenger receptors like CD206 and CD163 (22). Lining macrophages protect joint functionality and the immune privilege of the joint space through a multitude of mechanisms: they act as sentinels for molecular and cellular changes in the joint cavity (124) and facilitate clearance of cartilage and bone debris, highly immunogenic and hence dangerous signals that are constantly shed into the synovial fluid due to mechanical shear stress. In both mice and humans, lining macrophages express high levels of scavenger receptors, in particular Triggering Receptor Expressed on Myeloid Cells 2 (TREM2) and CD163 and are highly phagocytic and anti-inflammatory (22, 124, 127–129). Lining macrophages also actively participate in production of extracellular matrix (ECM) components and synovial fluid (22). Finally, sophisticated genetic and imaging approaches recently revealed that reminiscent of epithelial cells, lining macrophages form tight junctions with one another and thereby constitute a structural and immunological barrier (22). This barrier limits immune cell trafficking across the synovial membrane and thereby protects the avascular cavity from systemic threats. Conversely, it shelters the synovial connective tissue from immunogenic stimuli present in the joint space. Collectively, these features make synovial macrophages key regulators of joint homeostasis.

Potential Macrophage Niches and Signals in Healthy Joints

The exact cellular interactions and molecular signals comprising macrophage niches in healthy joint tissues remain to be deciphered with state-of-the-art approaches, however, fibroblasts are likely key players. This is the case for the spleen and peritoneal cavity (102, 130) and may also be particularly true for the synovial lining, where in the absence of a basement membrane they are in intimate contact with lining macrophages. Synovial fibroblasts and macrophages have been characterized individually in great detail over the last several years (22, 131, 132), and their potential interplay has been discussed in excellent recent reviews (133–135). Fibroblasts are ideally suited to provide anchorage to macrophages, and they are also a recognized source of key macrophage survival factors, such as CSF1 (Figure 2). Synovial lining macrophages are lacking in CSF1-deficient osteopetrotic (“op/op”) mice (99) demonstrating their CSF1-dependence, at least during development. Intriguingly, systemic administration of CSF1 does not restore synovial macrophages, whereas transgenic overexpression of the full-length transmembrane protein does, suggesting they depend on the membrane-bound isoform of the growth factor and thus, local sources (99, 136). Whilst synovial lining macrophages express the receptor for CSF1 receptor (CSF1R) at steady state (22), it is currently unclear whether they also rely on CSF1 for their homeostatic maintenance and turnover.

Fibroblasts could also act to bring macrophages in proximity of tissue-specific cues that imprint their functional identity, although this process may be orchestrated by additional stromal cell types in the joint, such as adipocytes, endothelial cells and chondrocytes. Such complexity is seen in the liver, where incoming monocytes

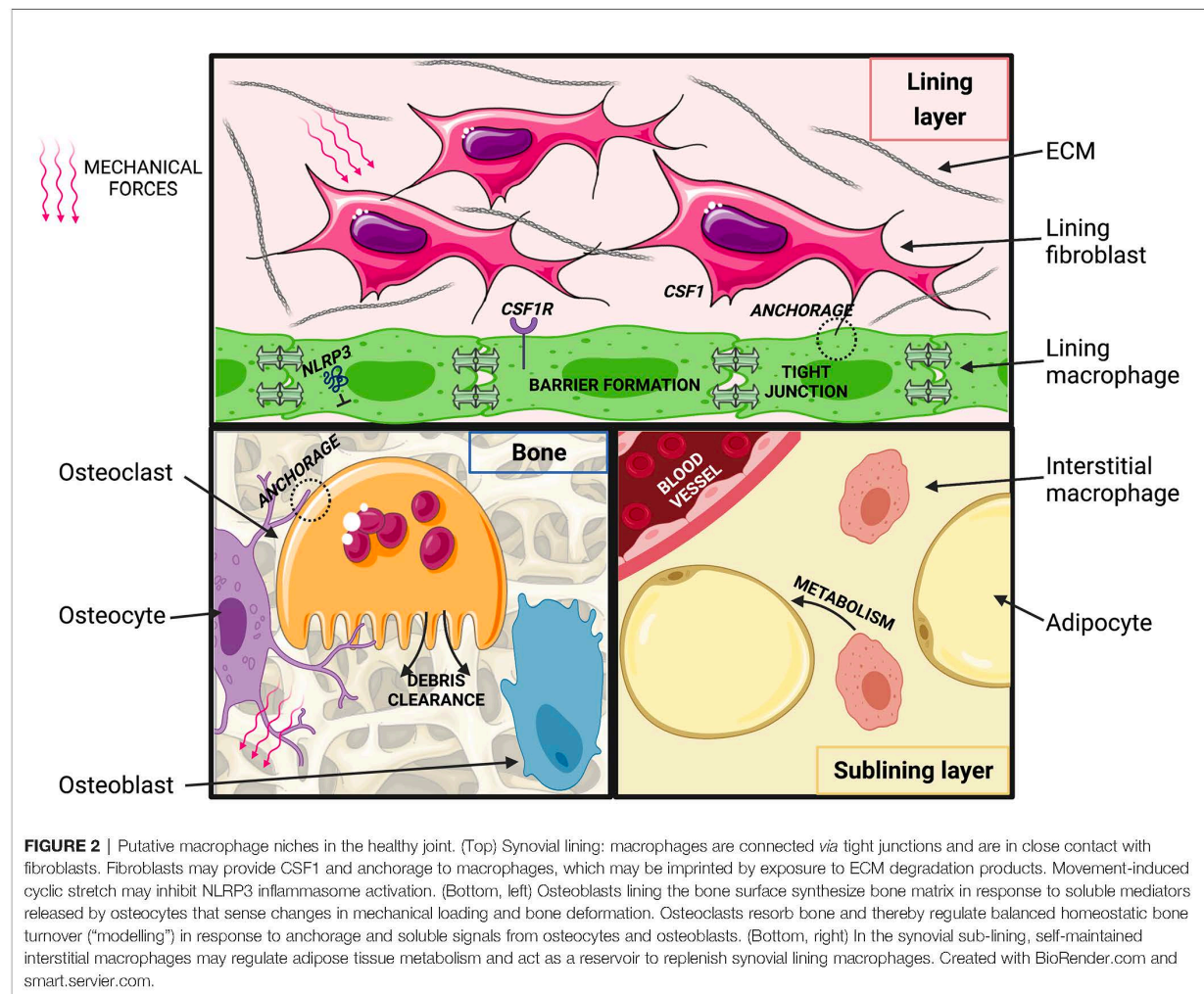
are functionally imprinted by a triad of hepatocytes, endothelial cells and stellate cells (101). Joint tissues are constantly exposed to shear stress and tensile forces that dynamically change with variations in joint loading. Mechanical stimuli are amongst the candidate cues that could play a particularly important role in instructing the specific identity of macrophages in joint tissues. Macrophages are in principle capable of sensing mechanical forces. Human BM-derived macrophages for example respond to substrates with different stiffness with changes in their shape, migration and proliferation (137). Mechanotransduction can also directly modulate their inflammatory cytokine production (138, 139). This latter effect is dependent on the NLRP3 (NOD-, LRR- and pyrin domain containing 3) inflammasome and can also involve signaling through TRPV1 and 4 (Transient receptor potential vanilloid-type 1 and 4) cation channels, which have been implicated in ptOA pathophysiology in mice (140–142). Transduction of mechanical signals through TRPV4 has also been implicated in the formation of multinucleated giant cells, inflammatory and destructive multinucleated macrophages (143). In addition to mechanical stress, normal ECM turnover products represent candidate signals that could imprint joint macrophage identities. Indeed, synovial lining macrophages appear to be highly phagocytic and constantly scavenge cartilage debris from the joint cavity (144–146). Joint biomechanics are altered during OA pathogenesis, and joint-tissue ECM degradation products more prevalent than at homeostasis, thus these pathways likely also impact macrophage identities and functions in arthritic joints.

MONOCYTE AND MACROPHAGE FUNCTIONS IN THE INJURED AND OSTEO-ARTHRITIC JOINT

Macrophage Functions in ptOA Pathogenesis

In addition to self-maintained lining and interstitial sub-lining macrophages already present at steady state, the arthritic synovium contains inflammatory monocyte-derived macrophages (147, 148). Similar changes also occur in other tissues within the joint (e.g. subchondral bone), regional (e.g. lymph node) and in remote tissues (e.g. spleen, peripheral blood). The necessity to delineate the specific roles of these distinct macrophage populations is highlighted by discrepant findings on the consequences of macrophage depletion depending on the experimental approach. Although the precise contribution of the various populations remains to be shown, systemic depletion of macrophages in mice in which apoptosis is induced in cells expressing CSF1R exacerbates experimental ptOA, whereas local clodronate liposome-mediate depletion within the joint is beneficial (149).

In RA, the respective functions of the distinct macrophage subsets have now been well explored, and macrophages originating from recruited monocytes appear to have overall disease-promoting functions (150, 151). Similarly, the majority of studies on OA-affected joints have identified inflammatory, monocyte-derived macrophages as the main culprit in



promoting and sustaining inflammation (124, 152). These cells produce pro-inflammatory cytokines and release additional signaling molecules associated with tissue-injury, which can attract lymphocytes that further propagate inflammation. However, exploiting these findings therapeutically is currently hindered by a lack of detailed understanding of the exact interplay between monocyte-derived macrophages and different types of lymphocytes, and how these change in the distinct stages of ptOA pathogenesis. Monocyte-derived macrophages also participate in cartilage destruction via production of IL-1 β and TNF- α , which suppress synthesis of the ECM components aggrecan and collagen by chondrocytes and upregulate expression of catabolic enzymes like ADAMTS-4 and MMP-13 (153–155). Soluble matrix degradation products in turn can activate resident synovial macrophages via Toll-Like Receptors (TLRs) and other pattern-recognition receptors (156). As this example illustrates, different macrophage populations in the joint can be functionally interlinked.

Another effector by which macrophages might contribute to ptOA pathogenesis is B cell Activating Factor (BAFF), a member of the TNF superfamily. BAFF is a crucial B cell survival factor, but also exerts co-stimulatory effects on T cell activation via upregulation of B-cell lymphoma 2 (BCL-2) (157). Furthermore, BAFF promotes T-helper-cell (Th)1 and suppresses Th2 responses (158), and drives Th17 differentiation via IL-6 signaling (87, 158–161). BAFF levels are elevated in serum and synovial fluid from RA patients (162), and BAFF appears to have a pathogenic role in RA (163, 164). During established RA, BAFF promotes pro-inflammatory polarization of CD4⁺ T cells, DC maturation as well as proliferation of inflammatory fibroblasts (163). In the inflamed joint, macrophages (165) are the main source of BAFF, although it is unclear if these are monocyte-derived or resident macrophages, or both. This compelling evidence led to the development of BAFF antagonists as DMDs for RA, which are currently being tested in early phase clinical trials (163). Whether BAFF production is also a

mechanism by which macrophages contribute to pathogenesis of OA has not been determined but elevated BAFF levels have been detected in OA synovial fluid (166).

Unlike their monocyte-derived counterparts, and some controversy notwithstanding, self-maintained resident synovial macrophages have largely been attributed protective roles in arthritis. The barrier generated by synovial lining macrophages is disrupted in both RA patients and experimental RA (22). In mice, this occurs rapidly upon induction of serum transfer-mediated arthritis, and thus constitutes an early event in disease development. In this model, barrier breakdown occurs following phagocytosis of immune complexes containing auto-antibodies, which activate lining macrophages and induce structural joint pathologic changes. Consequently, depletion of lining macrophages or specific disruption of their tight junctions worsens experimental RA. In turn, drug-mediated stabilization of tight junctions protects mice from RA (22), a finding that is translationally promising. Whilst the role of synovial lining macrophages has not yet been addressed specifically in OA pathogenesis, it is worthwhile noting that targeting lining macrophages or tight junctions not only exacerbates RA but may also result in spontaneous inflammation in the joint cavity in otherwise healthy animals (20, 22). With respect to ptOA, one could thus envision a scenario in which following injury, mechanical disruption of the synovial lining macrophage barrier enables rapid influx of inflammatory cells and hence, transition to the inflammatory phase of OA pathogenesis. Unlike in RA however, this barrier breach might be transient in nature, since the barrier appears more intact in patients with established OA compared to RA (22). This might be due to differences between immune complex-mediated and mechanical barrier-breakdown and could contribute to the, often considerable, lag phase between joint injury and ptOA onset.

Monocyte Functions in Joint Pathogenesis

Monocytes are critical players in OA pathogenesis, both as effector cells and a source of additional macrophages. As described earlier, it is widely accepted that $\text{Ly6C}^{\text{high}}$ and Ly6C^{low} monocytes can differentiate into macrophages with distinct polarization profiles in response to the cytokine milieu encountered in the tissue microenvironment. Reflecting this complexity, the overall impact of classical and non-classical monocytes on joint disease pathogenesis remains unclear. On the one hand, adoptive transfer of Ly6C^{low} monocytes following pan monocyte depletion increases the development of serum-transfer induced arthritis (58, 167, 168). In this model, Ly6C^{low} monocytes are actively recruited to the joint, where they are critical for the initiation of sterile joint inflammation and differentiate into inflammatory macrophages (169). On the other hand, Ly6C^{low} monocytes were also found to limit excessive inflammation in arthritic mice *via* enhanced recruitment of regulatory T-cells (Tregs) (58, 170). This seemingly contradictory evidence regarding the role of Ly6C^{low} monocytes in RA underscores the need for further studies that improve the understanding of the complex role of monocytes in inflammatory arthritis, and similar considerations apply to OA. The diverse roles of monocytes and macrophages in ptOA pathogenesis will be

discussed in more detail in the following section, focusing on molecular and cellular factors shaping their respective functions.

SIGNALS AND CELLULAR INTERACTIONS SHAPING MONOCYTES AND MACROPHAGES IN PTOA

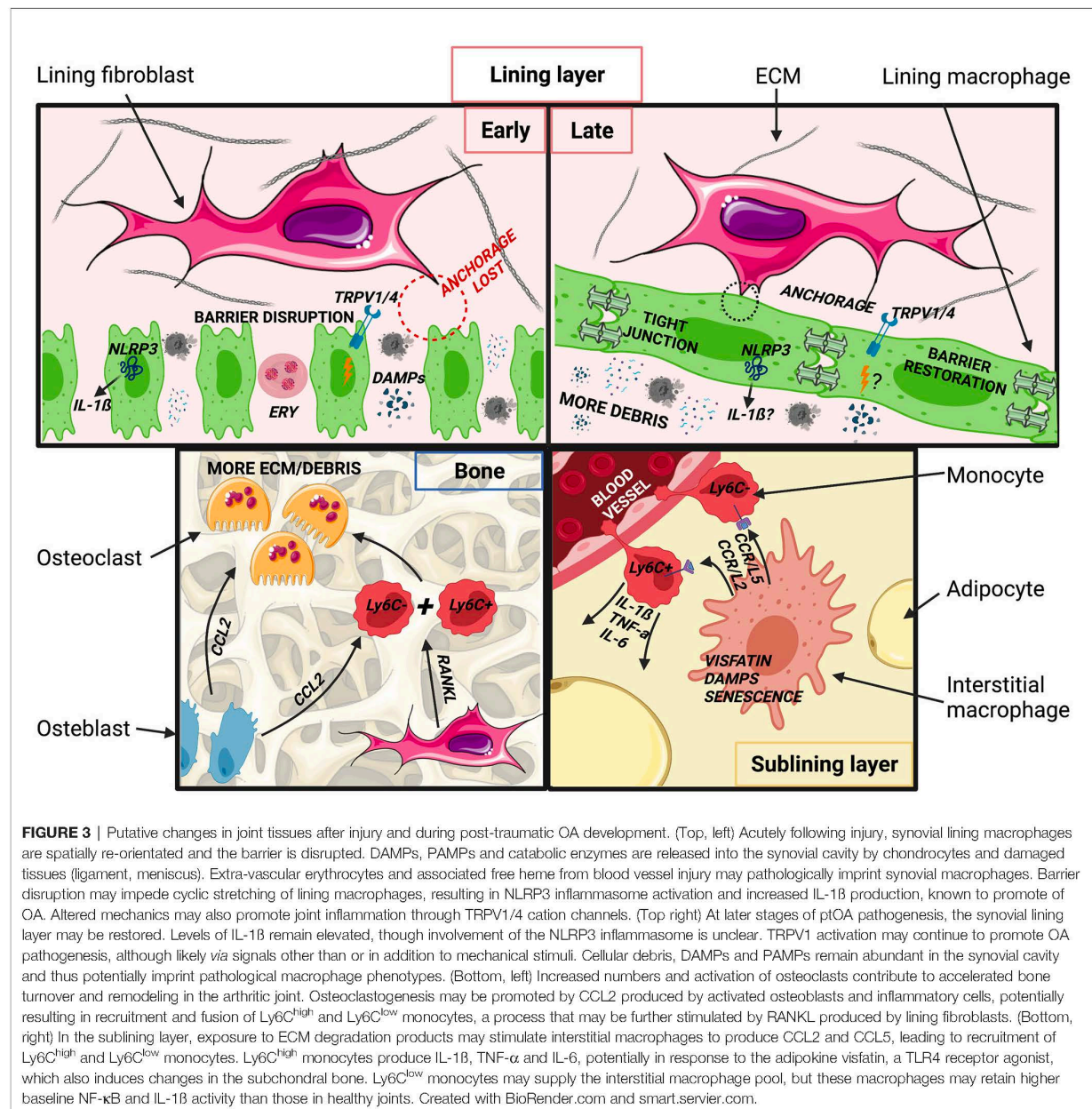
Depending on severity, joint injury can induce marked mechanical, anatomical and immunological changes, initially resulting in recruitment of monocytes and other inflammatory cells. Pathological changes persist throughout ptOA development and in established disease, and impact both incoming monocytes and previously resident macrophages. This section discusses how the perturbed joint tissue environment might affect monocytes and macrophages (Figure 3). An overview of murine and human monocytes and macrophages found in the synovial tissue during homeostasis, rheumatoid arthritis and in as far as known osteoarthritis, can be found in Table 1.

Perturbations Following Joint Injury

Joint injury triggers a series of complex mechano-biological and immunological changes, which can be broadly separated into three successive phases. Immediately after injury, mechanical perturbation effects predominate. These are direct results of the injury (9, 171) and may include tissue disruption (e.g., subchondral bone (micro)fractures, ligament tearing), collagenous matrix disruption and cartilage swelling, blood-vessel injury and hemarthrosis i.e. the presence of blood in the synovial cavity. This immediate joint-tissue injury is followed by an acute inflammatory phase (172), which is characterized by abundant cell death and pro-inflammatory signaling involving both innate and adaptive lineages (173). The nature and duration of this inflammatory response have been identified as major determinants for the risk of developing ptOA post injury (9, 172, 173). While appropriate control and resolution of inflammation is essential for normal wound-healing and might prevent ptOA development, perpetuated inflammation leads to the chronic and final phase of OA pathogenesis, which is defined by fluctuating low-level synovitis (174) and continuous tissue remodeling processes that ultimately lead to destruction of the cartilage and joint failure (175). Delayed or failed resolution of inflammation can be due to a permanently disrupted equilibrium of pro- and anti-inflammatory factors (171) or inadequate post-injury inflammation control (172). In addition, biomechanical factors such as instability or recurring joint injuries (176) can result in continuous re-triggering of acute mechano-biological responses, which initiate an inflammatory vicious circle. These considerations identify the acute inflammatory phase as a potential target for ptOA DMDs and highlight the need for a better understanding of its cellular and molecular regulators.

Mechanisms Underlying Monocyte Recruitment During OA Pathogenesis

The exact nature of the inflammatory response subsequent to joint injury is still under investigation, and differences might



exist depending on the type of injury and/or tissues injured, as we will discuss below. Overall, however, a growing body of evidence implicates chemokines and their receptors in monocyte recruitment during OA pathogenesis. As reported for other pathologies (177), classical and non-classical monocytes differentially depend on CCL2/CCR2 or CCL5/CCR5 and CX3CL1/CX3CR1. CCL2 (also known as MCP-1) is a key regulator of Ly6C^{high} monocyte egress from the BM and their recruitment to peripheral tissues (178). Following joint injury, release of ECM degradation products and complement factors

temporally induces CCL2 production by chondrocytes, resident synovial macrophages (179) and endothelial cells (180). This occurs *via* a positive feedback loop, stimulated by increased IL-1 β and TNF- α expression in synovial macrophages (181) and fibroblasts (182). In keeping with this, clinical studies have reported elevated levels of CCL2 in synovial fluid immediately after traumatic joint injury (183) and subsequent to meniscal tears (184), and concentrations correlate with severity of OA (185, 186). Its expression is also significantly elevated in the serum of OA patients (187), and CCL2 might also affect other

TABLE 1 | Markers, origin and putative function of monocytes and synovial macrophage subsets in mouse and human.

Population, location	Phenotype	Origin, maintenance	Functions at homeostasis	Functions in osteoarthritis
Macrophages, synovial lining	Mouse: TREM2 ⁺ CX3CR1 ^{high} MHCII ⁺	Long-lived, BM-independent, not proliferating ^{2,3} , replenished from sublining interstitial	Protective barrier ²	Protective barrier ² , limiting disease development (RA), immune regulation ³
	"mTOR activated M1 macrophages" iNOS ⁺	Unknown	Unknown (low number)	Chondrocyte differentiation ¹
	Human: TREM2 ⁺ CD68 ⁺ MerTK ⁺ LYVE1 ⁺ FOLR2 ⁺ ^{4,2}	Proliferation ^{4,5}	Protective barrier ² Control local immune responses ⁴	Protective barrier ² Resolution of inflammation, induce reparative fibroblast
Macrophages, synovial sublining/ interstitial	CD14 ⁺ HLADR ⁺ FOLR2 ⁺ CD86 ⁺ ⁵ K167 ^{high} ULK1 ⁺	Proliferation ⁵	Unknown	Cartilage remodelling ⁵
	CD14 ⁺ HLADR ⁺ FOLR2 ⁺ CD86 ⁺ K167 ^{low} HTRA1 ⁺ (location uncertain)	Uncertain	Unknown	
	Mouse: RELMa ⁺ CD206 ⁺ CD163 ⁺ CX3CR1 ⁺ MHCII ⁺ CSF1R ⁺	BM-independent ² Proliferation, BM-independent (RA) ² / BM (arthritis) ³	Joint homeostasis Generate lining and sublining macrophages ²	Generate lining and sublining macrophages ² , Inflammation Fibroblast inflammatory response ⁴
Macrophages, synovial tissue of the "bare area"	Human TREM2 ⁺ MerTK ⁺ CD206 ⁺ CLEC10a ⁺ ^{4,2}	Unknown	Unknown	
	Mouse: CX3CR1 ⁺ MHCII ⁺ Ly6C ^{int} F4/80 ⁺	BM ⁶	Absent	Osteoclastogenesis ⁶
	Human: CX3CR1 ⁺ HLA-DR ^{high} CD11c ⁺ CD80 ⁺ CD86 ⁺	Unknown	Unknown	Osteoclastogenesis ⁶
Monocytes, synovial tissue	Mouse: Ly6C ⁺ CD64 ^{int} Ly6C ⁺	BM ³ BM ³	Tissue patrolling Absent	Generate Ly6C ⁺ monocytes Initiation of sterile joint inflammation ³ Generate MHCII ⁺ macrophages ³
	Human: CD14 ⁺ CD11c ⁺ CD38 ⁺ IL1B ⁺ IFN-activated SPP1 ⁺ ⁷		Unknown	Pro-inflammatory ⁷
	CD14 ⁺ CD11c ⁺ NUPR1 ⁺ ⁷		Joint homeostasis ⁷	Inversely correlated with tissue inflammation, bone remodelling ⁷

TREM2, triggering receptor expressed on myeloid cells 2; CXCR1, C-X3-C Motif Chemokine Receptor 1; MHCII, major histocompatibility complex class II; mTOR, mammalian target of rapamycin; iNOS, inducible nitric oxide synthase; BM, bone marrow; RA, rheumatoid arthritis; MerTK, MER Proto-Oncogene, Tyrosine Kinase; LYVE1, Lymphatic Vessel Endothelial Hyaluronan Receptor 1; FOLR2, Folate Receptor Beta; CD, cluster of differentiation; HLADR, Human Leukocyte Antigen – DR isotype; ULK1, Unc-51 Like Autophagy Activating Kinase 1; HTRA1, HtrA Serine Peptidase 1; RELMa, Resistin-like molecule a; CSF1R, colony-stimulating factor 1 receptor; CLEC10a, C-type lectin domain family 10; Ly6, lymphocyte antigen 6; IL, interleukin; IFN, interferon; SPP1, Secreted phosphoprotein 1; NUPR1, Nuclear Protein 1.

¹Zhang, H. et al. Synovial macrophage M1 polarisation exacerbates experimental osteoarthritis partially through R-spondin-2. *Ann. Rheum. Dis.* 77, 1524–1534 (2018).

²Culemann, S. et al. Locally renewing resident synovial macrophages provide a protective barrier for the joint. *Nature* 572, 670–675 (2019).

³Misharin, A. V. et al. Nonclassical Ly6C⁺ monocytes drive the development of inflammatory arthritis in mice. *Cell Rep.* 9, 591–604 (2014).

⁴Alivernini, S. et al. Distinct synovial tissue macrophage subsets regulate inflammation and remission in rheumatoid arthritis. *Nat. Med.* 26, 1295–1306 (2020).

⁵Wood, M. J. et al. Macrophage proliferation distinguishes 2 subgroups of knee osteoarthritis patients. *JCI Insight* 4, (2019).

⁶Hasegawa, T. et al. Identification of a novel arthritis-associated osteoclast precursor macrophage regulated by FoxM1. *Nat. Immunol.* 20, 1631–1643 (2019).

⁷Zhang, F. et al. Defining inflammatory cell states in rheumatoid arthritis joint synovial tissues by integrating single-cell transcriptomics and mass cytometry. *Nat. Immunol.* 20, 928–942 (2019).

cells relevant to OA pathogenesis. In chondrocytes, for example, CCL2 increases expression of the catabolic enzymes MMP3 and MMP13 (179) and inhibits proliferation and enhances apoptosis. CCL2 might thus promote OA pathogenesis *via* attracting monocytes to the joint, but also by directly promoting cartilage destruction (179).

Unlike CCL2, which is found mainly in the intimal lining of the synovium, CCL5 [also known as RANTES (Regulated on Activation, Normal T Cell Expressed and Secreted)] is distributed more diffusely throughout the synovial tissue (188). Conflicting evidence exists regarding the role of CCL5 and its receptor CCR5, which possess strong chemo-attractive properties for Ly6C^{low} monocytes. In line with a disease-promoting role of Ly6C^{low} monocytes, CCR5^{-/-} mice were initially reported to exhibit reduced cartilage destruction (189), however, a recent study by Raghu and colleagues found that neither deficiency in CCL5, nor

CCR5 protects mice from ptOA (21). This was further corroborated by a clinical study of synovial biopsy specimens, which found significantly higher levels of CCL5 in RA compared to OA patients, whilst expression of all other chemokines and receptors is comparable (188). This may suggest that the inflammatory responses underlying these different arthritides have some unique molecular signatures or phenotypes. Considering its role in attracting Ly6C^{low} monocytes, which are known to promote pathogenesis of inflammatory arthritis, it seems surprising that depletion of CCR5 has no protective function in ptOA (21). However, CX3CL1/CX3CR1 signaling also participates in recruitment of non-classical monocytes, and elevated levels of CX3CL1 have been found in peripheral blood (190) and synovial fluid (186) of OA patients. Functional experiments revealed that in addition to its chemo-attractive properties, CX3CL1 also stimulates inflammation specifically at

the early stages of OA (190). Finally, soluble CX3CL1 induces production of the pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α in recently recruited monocytes (191). Taken together these data indicate that the CX3CL1/CX3CR1 axis predominates in recruitment and pro-inflammatory activation of Ly6C^{low} monocytes in the context of OA initiation. Ly6C^{high} monocytes recruited and activated *via* CCL2/CCR2, on the other hand, might help sustain inflammation at later stages (192). Temporal changes in chemokine expression and associated monocyte sub-population recruitment and accumulation, may in part explain the recently described loss with time post-injury, of an initially protective/anabolic effect of injured synovium on chondrocytes (193).

Osteoclasts have been implicated in progressive joint destruction. Osteoclastogenesis is controlled by RANK (Receptor Activator of Nuclear factor Kappa B (NF- κ B) (194). Its ligand (RANKL) is expressed by fibroblast-like synoviocytes and Th17 cells, and expression is regulated by pro-inflammatory cytokines secreted by monocytes and macrophages (IL-1 β , IL-6, IL-17 and TNF- α) (195). A particularly interesting mechanism by which monocytes could contribute to increased osteoclastogenesis in the context of arthritis was proposed by Hirose *et al.* They postulated that CCL2 secreted by osteoblasts leads to fusion of Ly6C^{high} with Ly6C^{low} monocytes stimulated by RANKL, resulting in mature, multinucleated osteoclasts (195). In addition to osteoblasts, activated inflammatory cells produce large amounts of CCL2 and this might explain the accelerated osteoclastogenesis and joint destruction in an inflammatory setting (195).

Signals Governing Monocyte Differentiation, Activation, and Functions in OA

Following their recruitment to the tissue, monocyte functions can be shaped by a variety of signals present in the non-homeostatic joint. Calcium binding proteins can act as damage- or pathogen-associated molecular patterns (DAMPs and PAMPs, respectively) and have multi-faceted effects on OA pathogenesis. Damage to the cartilage leads to increased levels of S100A8 and its binding partner S100A9 specifically in synovial pro-inflammatory macrophages, but not fibroblasts (196). Secretion of these factors induces the production of pro-inflammatory cytokines in these macrophages in an autocrine manner (196). In addition, in a murine collagenase-induced OA model, which has a more inflammatory phenotype than surgically-induced disease, release of S100A8/9 elicits influx of Ly6C^{high} monocytes *via* upregulation of CCL2 (197) and increased egress of Ly6C^{high} monocytes from the BM (197). In turn, activated monocytes are a major source for S100A8/9, which might thus constitute a positive feedback loop. Finally, S100A8/9 might also be derived from chondrocytes and directly contribute to cartilage disruption in OA by inducing production of ADAMTS-4 and -5, MMP-1, -3, -9 and -13 and the pro-inflammatory cytokines IL-6, IL-8 and CCL2 in chondrocytes in a TLR4-dependent manner (198, 199). Interestingly, chondrocyte-derived S100A8/9 may play a predominant role in the acute post-injury phase, as expression and protein levels in cartilage decrease with post-traumatic OA disease progression while levels are maintained in immune-mediate inflammatory arthropathy (199). S100A8/9 thus fuel the

initial pro-inflammatory microenvironment in the joint, provide chemotactic cues for Ly6C^{high} monocytes and exert direct catabolic effects within the cartilage, processes which are further amplified by several feedback loops. Another class of OA-associated DAMPs are basic calcium phosphate (BCP) crystals. Whilst *in vivo* data on their relevance to OA are currently lacking, *in vitro* exposure of monocyte-derived macrophages to BCP crystals leads to a classically activated, pro-inflammatory phenotype, a bioenergetic switch towards glycolysis and increased expression of S100A8 (200). Promisingly, both BCP-induced phenotypic polarization and S100A8 expression are inhibited by a glycolytic inhibitor (2DG) indicating that metabolic reprogramming might be underlying these effects (200).

The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT), Mitogen-Activated Protein Kinase (MAPK) and NF- κ B pathways are involved in differentiation of monocytes into macrophages and their functional polarization. The latter depends on interferon regulatory factors (IRFs) (201). IRF5 is a also downstream target of Granulocyte-Macrophage Colony-Stimulating Factor Receptor (GM-CSFR), and plays a critical role in pro-inflammatory macrophage polarization (202). A recent clinical study investigated the role of IRF5 in OA and found it to be overexpressed in synovial macrophages, but not circulating monocytes (203). However, exposure to synovial fluid from OA patients induced expression of IRF5 and IL-12 (via the individual subunits IL-12p35 and IL-12p40) in monocytes, in turn making them potent inducers of a Th1 response characterized by expression of IFN- γ and Tbx21 in co-cultured naïve CD4⁺ T-cells (203). This suggests that patient synovial fluid contains soluble factors capable of inducing IRF5 in monocytes, thus contributing towards a Th1 inflammatory response.

TLR 4 is a receptor for PAMPs and DAMPs expressed on monocytes that plays an important role in the activation of innate immunity (204), and has been implicated in the inflammatory reaction associated with OA (205). The adipokine visfatin was recently identified as a TLR4 receptor agonist capable of evoking inflammatory responses (206). In addition, visfatin stimulates production of IL-1 β , TNF- α and IL-6 by monocytes (207), and the resulting inflammatory environment displays higher levels of circulating visfatin, thus constituting a positive feedback loop (208, 209). Visfatin is also involved in inter-tissue joint communication underlying changes in the subchondral bone (210). These have long been described in OA, but the exact mechanisms of this remodeling and pathways of its activation has remained elusive. Emerging evidence now points towards direct communication between the subchondral bone and cartilage *via* diffusion (211). Liguillon *et al.* found that visfatin is produced in cartilage, synovium and subchondral bone and exerts an enzymatic effector function selectively inducing a pro-inflammatory phenotype in chondrocytes, osteoblasts and synoviocytes, characterized by increased secretion of IL-6 and CCL2 (210, 212–214). Because of its contribution to the inflammatory response and tissue remodeling, inhibition of visfatin might be a promising DMD approach.

The gene expression profiles of monocytes and monocyte-derived macrophages, and hence, their functional polarization,

might also be shaped by microRNAs expressed in response to environmental stimuli. A recent study identified miR-155 as a potential genomic switch in monocyte-derived macrophages generated *in vitro*, which regulates their inflammatory profile (215). Intriguingly, this phenomenon is partially reversed by treatment with monoclonal anti-TNF antibodies, but not a soluble TNF receptor (Etanercept) (215). MiRNAs expressed by monocytes and their macrophage progeny, such as miR-155, might therefore represent promising candidate DMD targets.

While we have focused primarily on joint injury and ptOA, monocytes and macrophages in the arthritic joint might also be affected by age and cellular senescence, as has been demonstrated for RA. In this context, an elegant mouse study from Misharin *et al.* is of note, where the role of different monocyte subsets in RA pathogenesis using serum-transfer induced arthritis was investigated. Ly6C^{low} monocytes are recruited to the joint and initially develop into classically activated macrophages, but the macrophage compartment gradually undergoes a switch towards a more alternatively activated phenotype (169). The initial highly pro-inflammatory nature of the Ly6C^{low} monocytes could be caused by a senescence-associated secretory phenotype, which is associated with high baseline NF- κ B and IL-1 α activity (216). In addition, accumulation of Ly6C^{low} monocytes is found in the elderly. These findings suggest that senescence might correlate with increased numbers and pro-inflammatory skewing of Ly6C^{low} monocytes, which might further exaggerate the inflammatory response unfolding during RA progression. Whether similar mechanisms might be at play in ptOA requires evaluation, but it is interesting to note the accelerated disease progression, increased expression of inflammatory genes and inhibitory effects of MIF ablation following medial meniscal destabilization in older versus younger mice (217, 218).

Immunogenic and Imprinting Signals in the Injured Joint

As introduced, the synovial membrane plays a key role in maintaining joint homeostasis as it guarantees the relative immune privilege of the synovial cavity. However, while the joint space itself is not vascularized, the synovial membrane also features a vascular net located just below the intima. This comprises capillaries, venules, arterioles and lymphatics (219) through which systemic and local inflammatory stimuli can be sensed (125, 220). Hemarthrosis and cartilage damage are direct consequences of joint trauma, which affect the joint not only macroscopically, but also on a cellular and molecular level (176). Of note, the presence of blood in synovial fluid is an independent predictor or poorer 2-year outcome following joint injury (221). It is tempting to speculate that heme could be a cellular cue shaping macrophages in the early stages of ptOA pathogenesis, but unlike in the homeostatic spleen, it may instruct more inflammatory cell states (222). Moreover, hemarthrosis directly activates the complement system, leading to production of complement anaphylatoxins (C3a and C5a) and formation of the membrane attack complex (223). Intriguingly, genetic deficiency for individual components of the complement system in mice leads to either attenuated [C5 and C6 (224)] or

aggravated [CD59, also known as protectin (224)] ptOA joint damage following injury.

As discussed above, mechanical forces may directly shape macrophage functions in the homeostatic joint. It is therefore plausible that the mechanical changes following joint injury, impact on resident and recruited macrophages. This appears to be the case at least in experimental RA, where the extent of mechanical loading determines the local distribution of inflammation and degree of damage (225). Mechanical damage to the cartilage also leads to substantial ECM degradation. Collagen fibers fail to contract (226, 227) and ECM degradation is further enhanced by the lack of maintenance and repair (228) following chondrocyte death. ECM-derived tissue fragments are widely recognized as pro-inflammatory and immunogenic (229, 230). These fragments and the complement anaphylatoxin C5a can act as effective chemo-attractants for innate and adaptive immune cells (231, 232) and directly activate macrophages *via* NF- κ B signaling (233). Released cartilage destruction products such as matrilin-3 (229, 234), tenascin-C (235), fragmented biglycan (236) and fibronectin (237) can also potentially activate resident synovial macrophages. Finally, cartilage and other joint tissue degradation can induce release of additional DAMPs (238) capable of activating innate immune cells *via* TLR2 and 4 (239) and NF- κ B signaling. The mechano-biological damage induced by injury thus generates an inflammatory microenvironment in the joint space, which is characterized by an increase in soluble inflammatory mediators and chemo-attractants that might induce transition to the acute inflammatory phase, and importantly shape subsequent responses of both recruited monocytes and resident macrophages.

T Cell-Mediated Monocyte and Macrophage Activation in OA?

Other immune cells may provide signals amplifying the effects of monocytes and macrophages. In the context of joint injury, recruitment and activation of lymphocytes have traditionally been thought of as secondary events that follow monocyte influx and changes in macrophages (11). However, T cells are found in synovium at higher levels in early versus late OA (240) and might actively contribute to monocyte and macrophage activation *via* co-stimulatory pathways. One such pathway relies on interactions between CD40, a member of the TNF receptor family found primarily on antigen-presenting cells and monocytes, and its ligand CD40L (CD154), which is almost exclusively expressed by activated CD4⁺ T cells (241, 242). CD40/CD40L interactions elicit a broad pro-inflammatory response (243) that involves B cell differentiation (244) and macrophage activation. In turn, activated macrophages and other antigen-presenting cells enhance immunoglobulin antigen affinity (241), activate cytotoxic T cells and promote a Th1 immune response (245). This co-stimulatory pathway therefore has the potential to initiate a powerful amplification loop that propagates joint inflammation. In keeping with this notion, exaggerated CD40/CD40L signaling contributes to autoimmunity (246), including RA. Multiple studies have

shown overexpression of both CD40L (247–249) and CD40 (246) in RA, and levels of CD40L are associated with disease activity (248) and perpetuation (247). Based on these findings, biological treatments targeting this axis in RA have been developed, which are currently undergoing early phase clinical trials (250). Despite differences in the pathogenesis and mechanisms involved in the development of RA and OA, shared elements in the underlying inflammatory response seem plausible (251). The effects of targeting the CD40/CD40L axis in OA remain to be determined, however we have found that CD40L mRNA levels are elevated exclusively in the synovium immediately after ACL rupture and during early onset of OA development (252). These preliminary findings support the notion that CD40/CD40L may be an early driver of T cell-mediated synovial macrophage activation and warrant future research into the CD40/CD40L axis specifically in ptOA.

Additional Candidate Pathways and Mechanisms Leading to Macrophage Dysregulation During OA Pathogenesis

In addition to the factors discussed above, obesity is a well-established risk factor contributing to OA development (253), through increased mechanical loading but also *via* dysregulated secretion of adipokines and other metabolic factors (254). In mice, high-fat diet (HFD) results in elevated leptin-induced levels of lysophosphatidylcholine (lysoPC), which in turn increases MMP13 production by chondrocytes. As a consequence, obese mice show an earlier onset and progressive course of spontaneous OA (254). Direct links between obesity and OA have also been shown in mouse models of ptOA. HFD was associated with inflammation in the infrapatellar fat pad, characterized by macrophage crown-like structures, which may have a priming effect on the fat pad leading to a metabolic state of progressive OA following injury (123). HFD was also shown to aggravate inflammation of the synovial membrane post-injury, which was marked by increased macrophage infiltration (255). These findings are in line with the notion that obesity contributes to aberrant macrophage activation in OA pathogenesis. Intriguingly, these detrimental effects of HFD on OA persisted even after a normal diet was resumed (254), indicating long-lasting effects and potential windows or particular susceptibility. Some of these effects may even be programmed in early life and transmitted across generations. Indeed, increased higher susceptibility to experimental ptOA has been reported in the first and second generation offspring of mice fed a HFD during breeding (256). Immune cells have been implicated as mediators of such programming and transgenerational effects of obesity in offspring (256), and epigenetic dysregulation has been postulated as a central mechanism.

While some epigenetic modifications are stable and passed on across generations, others are more dynamic and responsive to environmental stimuli (257). These are believed to play a significant role in OA development. Of the studies that have investigated epigenetic changes in OA development, most have focused on epigenetic mechanisms modulating chondrocyte

biology and inflammatory mediators (258). Evidence for epigenetic modifications of macrophage remains scarce in the context of OA. In principle, epigenetic processes govern various aspects of macrophage biology, including their development, differentiation, and activation, as well as the specification of their tissue identity (259–261). For example, active DNA demethylation occurs during monocyte to macrophage differentiation *in vitro* (262) and the identity of tissue-resident macrophages is shaped by unique enhancer landscapes in response to microenvironmental cues (70, 71). They also activate genes governing embryonic stem cell-like self-renewal through macrophage-specific enhancers (263). Fully differentiated macrophages are maintained in a “balanced” state through a combination of activating (such as PU.1, H3K4me1 and open chromatin) (264) and repressive (such as H3K9me3, H3K27me3 and H4K20me3) (265) epigenetic marks and regulators (262). These repressive marks are removed upon stimulation of macrophages through TLR, and specifically TLR4, ultimately resulting in the production of inflammatory cytokines such as IL-1 β , CXCL10, IL-6 and TNF (262). TLR4 signaling has also been implicated in low-grade inflammation mediated by plasma proteins present in the synovial fluid of OA patients (266). Whether epigenetic changes in macrophages contribute to this remains to be formally shown, however.

Activation *via* TLR4 also initiates metabolic reprogramming of macrophages, and distinct metabolic states have been linked to functional differences in macrophage subsets. For example, metabolic reprogramming towards increased glycolysis promotes pro-inflammatory polarization (267). In OA, increased glucose uptake correlates with disease progression, and the hypoxic environment in the OA synovium may enhance osteoclastogenesis (267, 268). Osteoclastogenesis appears to also be promoted by metabolic syndrome through NF- κ B activation and advanced glycation end products (269). A bioenergetic switch towards glycolysis is also induced in macrophages by basic calcium phosphate crystals, which are specifically found in OA (220), further supporting the notion that macrophages may undergo metabolic reprogramming during OA pathogenesis.

Finally, epigenetic and immunometabolic changes are also hallmarks of “trained immunity”. This recently coined concept (270) acknowledges that innate immune cells, including macrophages, show increased responsiveness to secondary stimuli following “training” by primary exposures. Whilst trained immunity has not specifically been studied in the context of OA, many of the cellular signals that impact on macrophages during OA pathogenesis – or even preceding disease onset – could mediate long-term effects through inducing this type of innate memory in macrophages. Thus, obesity/HFD may be primary exposures that train heightened or specific OA inducing immune responses to a secondary stimulus such as injury (123, 256). It is interesting to speculate that this may also be relevant in the context of prior even minor joint injuries increasing the risk and/or severity of ptOA following a critical/destabilizing injury such as anterior cruciate ligament (ACL) rupture (see Blaker et al., 2021 and references therein) (271).

HARNESSING MONOCYTE AND MACROPHAGE BIOLOGY FOR OA RISK STRATIFICATION, DIAGNOSIS, AND THERAPY?

It is now well recognized that ptOA development features an early inflammatory response. This involves systemic processes resulting in monocyte recruitment, as well as a local disbalance within the immune “niches” of the affected joint, whose immune privilege is therefore compromised. This recognition has several implications that in the future could be exploited for prognostic, diagnostic and therapeutic benefit, examples of which we discuss below.

Monocytes and Macrophages as Biomarkers for OA

To this day, OA diagnosis largely depends on clinical presentation/symptoms and conventional imaging methods like x-ray, computerized tomography (CT) scans or magnetic resonance imaging (MRI) (272). Diagnostic biomarkers are currently missing, as are reliable predictive markers. Access to synovial fluid and hence, the search for useful biomarkers, is limited by the invasive nature of the acquisition procedure. Nonetheless, advances have been made recently in the search for cellular and molecular biomarkers with diagnostic and/or predictive potential (221, 273), using synovial fluid where available, or peripheral blood, which can be more readily obtained.

On the cellular level, a growing body of data implicates monocytes and monocyte-derived macrophages in OA pathogenesis, as discussed in this review. Several recent clinical studies therefore investigated the prognostic value of peripheral immune cell ratios. While the exact ratios differ between studies, monocytes represent a common denominator. In particular, the neutrophil to monocyte ratio is independently and inversely associated with OA severity as classified using the Kellgren-Lawrence scale (274). Similarly, the monocyte to lymphocyte ratio reliably predicts OA progression (275). MicroRNA analysis of peripheral blood mononuclear cells (PBMCs) from OA patients showed elevated expression of miRNA-146a and miRNA-155 (276), which influence inflammatory cell signaling via the NF- κ B pathway (277, 278). Moreover, transcriptomic analysis of PBMCs from OA patients identified more than 1000 differently expressed genes, pathway analysis of which implicated inhibition of chondrocyte differentiation, increased osteoclastogenesis and MAPK activation (279). These data collectively indicate that peripheral blood monocytes of OA patients differ from healthy controls both quantitatively and qualitatively. It is tempting to speculate that specific OA-primed inflammatory monocytes exist in the peripheral blood during disease progression, and even potentially prior to onset. This notion is corroborated by data from Loukov and colleagues, who demonstrated that following *in vitro* exposure to DAMPs, peripheral blood monocytes from women with knee OA produced higher levels of the pro-inflammatory cytokines IL-1 β and TNF- α than monocytes from healthy controls (280). The same group also demonstrated significantly higher levels of

CD14 expression on monocytes of OA patients, further implicating non-classical activated monocytes.

Within the synovial fluid of knee OA patients, monocytes and macrophages constitute the second most abundant cell population after T cells, and a large proportion of these are CD16⁺, thus further implicating non-classical monocytes (281). Liu et al. investigated the relative abundance of phenotypically distinct macrophages in synovial fluid of knee OA patients and found an increased ratio of “classically” compared to “alternatively” activated macrophages (282). This ratio further correlated with disease severity, suggesting that despite the limitations of this simplistic dichotomy, such analyses can yield clinically relevant data.

A wealth of experimental and clinical studies has analysed inflammatory parameters and markers in synovial fluid for their potential to serve as biomarkers. These studies have shown that levels of CCL2, IL-6 and IL-8 accurately distinguish OA from normal joints (283–285) and inflammatory markers can even predict the outcome of ACL reconstruction. Similarly, the presence and severity of synovitis following meniscal injury are associated with the risk of progressive cartilage damage, even if inflammation subsequently resolves (286). Elevated levels of several additional synovial fluid biomarkers associate not only with radiographic OA severity (sVCAM-1, sICAM-1, TIMP-1 and VEGF) and OA symptoms (VEGF, MMP-3, TIMP-1, sVCAM-1, sICAM-1 and MCP-1) but are also highly correlated with levels of neutrophil elastase (287). This highlights a potential role for neutrophil activation in the onset of OA. These initial findings were further corroborated by a recent study indicating that expression levels of TGF- β 1 and elastase were associated with radiographic severity scores and predictive of knee OA progression (288).

Based on such findings, Jayadev *et al.* used a novel machine learning approach to develop a “cytokine fingerprint” for end-stage OA. Using a panel of eight biomarkers (PIIINP, TIMP-1, ADAMTS-4, CCL2, IP-10 and TGF- β 3), this model distinguishes between OA, knee injury and inflammatory knee arthritis (i.e. RA or psoriatic arthritis) with almost 100% efficacy (289). Interestingly, knee/hip arthroplasty further increases the levels of angiogenic and pro-inflammatory cytokines, but leaves anti-inflammatory cytokines unaffected, suggesting underlying changes specifically in pro-inflammatory pathways, which might be further exacerbated with surgical treatment (290).

In summary, biomarker research has both leveraged and fueled the notion that monocytes contribute to OA pathogenesis, and that OA-primed non-classical monocytes might exist. Although this progress is encouraging, the majority of recently identified biomarkers are associated with disease progression, rather than onset. There remains thus a pressing, unmet clinical need for biomarkers instrumental in diagnosis and stratification of individuals at risk of developing ptOA following knee injury.

Disease Modifying Drugs for OA: Where Are We on the Clinical and Pre-Clinical Level?

To target the early inflammatory phase that follows joint injury and initiates OA pathogenesis, adjuvant DMDs are urgently

needed. A variety of agents with the potential to serve as DMDs are currently being tested, but unfortunately, with limited success (291). Some studies on local inhibition of IL-1 in animal models showed promising results. In particular after closed articular fracture, intra-articular injection of an antagonist for IL-1 receptor (IL-1Ra) reduces post-traumatic OA, cartilage degeneration and synovitis (292–294). However, the clinical efficacy of this approach remains controversial. While an early clinical study found that intra-articular administration of IL-1Ra performed within 1 month of severe knee injury led to reduced knee pain and improved function over a 2-week follow-up period (295), other clinical studies using either IL-1Ra (Anakira) or a dual variable domain immunoglobulin that simultaneously inhibits IL-1 α and IL-1 β (Lutikizumab) have yielded no benefit in OA patients compared to placebo controls (291). Interestingly, retrospective secondary analysis of a large-scale clinical trial of a monoclonal antibody targeting IL-1 β for cardiovascular disease found reduced incidence of hip and knee replacement in patients with high C-reactive protein, suggesting a potential effect on OA progression in an inflammatory setting (296).

It is noteworthy, that the OA-specific studies of DMD candidates described above were tested mostly in patients with advanced OA with the intention of reducing established clinical and radiological disease progression. In line with the current difficulties in risk stratification and early diagnosis outlined above, to date, no drugs have been advanced to the clinical stage that target the inflammatory response at the onset or early stages of disease. Experimentally, however, attempts to advance in this direction have been made recently. One study explored the role of incretin hormone receptors *in vitro*. It found that an analogue for the human Glucagon-Like Peptide-1 (GLP-1), liraglutide, reduced production of reactive oxygen species, IL-6 and CCL2, reduced collagen and aggrecan degradation and inhibited inflammation *via* deactivation of NF- κ B signaling (297). Similarly, administration of dexamethasone, rapamycin or BMP-7 results in a more anti-inflammatory macrophage phenotype *in vitro*. Whilst these findings await *in vivo* confirmation, these drugs might hold potential of modulating synovial inflammation in patients (298).

One of the few *in vivo* studies investigating the impact of anti-inflammatory therapy immediately after joint injury utilized a porcine model. Here, the authors induced OA *via* transection of the anterior cruciate ligament (ACL) and immediately provided corticosteroids by intraarticular injection, which resulted in mitigated collagen degradation, reduced monocyte recruitment and a less inflammatory macrophage profile/phenotype (299). Our group has investigated the respective effects of intraarticular administration of hyaluronan or BM-derived mesenchymal stem cells in a murine OA model. We found that hyaluronan therapy increased anti-fibrotic macrophages and decreased pain sensitization, while treatment with MSCs did not impact pain, but led to long-term chondroprotection (300). Along similar lines, another recent study demonstrated that administration of Alpha defensin-1 renders macrophages less inflammatory and attenuates OA in a surgical model (301). Collectively, these data suggest that specific anti-inflammatory treatment immediately

after knee injury might represent a promising future therapeutic approach, and thus, justify additional experimental and ultimately clinical studies.

CONCLUDING REMARKS, OPEN QUESTIONS, AND FUTURE DIRECTIONS

Distinct Inflammatory Responses in Different Joint Injuries?

Post-traumatic OA accounts for nearly 12% of all cases of symptomatic OA (302) and a recent longitudinal cohort study showed that the risk of developing OA is almost sixfold increased by knee injury at a young age (303). Further stratification of these data revealed distinct risks for different injury types: ACL injury, meniscal tears and articular fractures of the tibia (risk difference (RD) of 19.5%, 10.5% and 6.6%, respectively) were associated with the highest risk (303). In trying to identify possible reasons for these differences, the simplest explanation of variable biomechanical aberration is insufficient, as an abundance of data has shown that restoration of biomechanics alone does not prevent ptOA development (4). An alternative hypothesis is that phenotypic differences exist in the pathogenesis of ptOA depending on the type of tissue affected by injury. Thus, metabolic and immunobiological differences may determine the individual risk of developing ptOA. Although comprehensive studies investigating the tissue- and phenotype-specific immune response after joint injury are missing, existing evidence supports an immunological role for the meniscus (304), which engages in a pro-inflammatory crosstalk with the synovium in OA (305). Furthermore, synovial fluid, cartilage tissue and isolated cartilage cells display distinct pro-inflammatory cytokine profiles depending on the type of pathology, further supporting the notion that a phenotype-specific cytokine topography exists in the joint (306). Future studies should therefore be designed to examine the inflammatory reaction associated with different types of joint injury.

Could Restoring Joint Immune Homeostasis Hold the Key to OA?

Owing to decades of research, we have come to understand that OA cannot simply be attributed to “wear and tear” resulting from biomechanical changes. Rather, OA results from a complex biological response of different cells in multiple joint tissues, with “inflammation” playing a crucial role in this process. Monocytes and macrophages are emerging as key players in the inflammatory process associated with OA. Circulating monocytes are particularly attractive as druggable targets, but selective targeting of tissue-resident macrophages might be equally feasible, for example using antibody-conjugated lipid nanoparticles (72). It is tempting to speculate that dysregulated dynamics between monocytes, macrophages and other cell types they cross-react with in the joint not only fuels pathology, but in fact represents an underlying cause of OA. To harness these intricate cellular interactions for diagnostic and therapeutic

purposes, we need to further improve our understanding of monocyte and macrophage biology in both healthy and arthritic joints. Deciphering their developmental and functional dynamics harbours the potential of one day being able to restore synovial immune homeostasis and thus, finally provide a causative treatment for this debilitating disease.

Can Findings From ptOA Be Translated to Other OA Phenotypes?

A wide range of small and large animal models for OA have been developed, and these have recently been reviewed (307). Far and away the most commonly used models are those induced by joint injury. They share a comparably rapid onset and highly standardized disease progression, and allow investigation of underlying molecular and cellular mechanisms, as well as evaluation of potential treatments at different stages of disease progression. However, the translation of findings made in these models to clinical settings has remained challenging (308). One explanation is the current discrepancy between preclinical research predominantly using ptOA models and clinical studies, the majority of which investigate late-stage “primary OA” which occurs in the absence of prior trauma or disease (309). There is emerging evidence that the pathophysiologic mechanisms of structural and symptomatic OA differs depending on the key initiating factors or disease phenotype (310, 311). How different the complex cellular inflammatory response is in different OA phenotypes remains to be resolved. In a first attempt to overcome this issue, findings from preclinical ptOA studies should be tested in preclinical models of primary OA, such as spontaneous age-associated and metabolic/obesity-induced disease. Whilst the associated immune response may be different in strength and spatio-temporal patterns, it is likely that at least some aspects of monocyte and macrophage biology relevant to ptOA apply to other OA phenotypes, such as their roles in ECM degradation and chondrocyte death, which are universal OA disease features. Likewise, some molecular signals identified in ptOA models as regulators of macrophage activation and polarization, such as specific cytokines and chemokines, and are also present in multiple OA phenotypes (312).

Open Questions and Future Directions for Research

- Does the initial inflammatory response following joint injury pathologically imprint monocytes, macrophages and their stromal niche in the joint?
- Do disease-specific, “imprinted” populations of monocytes and macrophages emerge prior to disease onset? Do they mediate (or: propagate) OA pathogenesis?

REFERENCES

1. Spitaels D, Mamouris P, Vaes B, Smeets M, Luyten F, Hermens R, et al. Epidemiology of Knee Osteoarthritis in General Practice: A Registry-Based Study. *BMJ Open* (2020) 10(1):e031734. doi: 10.1136/bmjopen-2019-031734

- Are monocyte and macrophage dynamics permanently altered following joint injury?
- Can these populations be targeted therapeutically?
- Is there an optimal ratio between pro- and anti-inflammatory monocyte/macrophage subsets that mitigates the risk of OA after joint injury?
- Can a threshold be determined that governs the future direction of either resolution of inflammation and restoration of joint function or ongoing inflammation that contributes towards development of OA?
- Do similar considerations also apply to non-traumatic forms of OA?

Addressing these questions will provide the critical scientific understanding necessary to improve diagnosis, risk prognosis, and underpin development of specific targeted therapies to prevent OA onset and/or slow its progression following joint injury. Recognizing that the targets may differ depending on injury and change with time and being able to identify these therapeutic stages/windows, will be key to providing effective individualized patient management.

AUTHOR CONTRIBUTIONS

Conception and design of study: PH, CL, BM, and RG. Acquisition of data: PH, RG, CL, and MP. Drafting of article: PH, RG, MP, CL, and BM. Revising it critically for important intellectual content: PH, RG, MP, CL, and BM. All authors have approved the final version of the manuscript.

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2. Kloppenburg M, Berenbaum F. Osteoarthritis Year in Review 2019: Epidemiology and Therapy. *Osteoarthritis Cartilage* (2020) 28(3):242–8. doi: 10.1016/j.joca.2020.01.002
3. Vos T, Flaxman AD, Naghavi M, Lozano R, Michaud C, Ezzati M, et al. Years Lived With Disability (YLDs) for 1160 Sequelae of 289 Diseases and Injuries 1990–2010: A Systematic Analysis for the Global Burden of Disease

- Study 2010. *Lancet* (2012) 380(9859):2163–96. doi: 10.1016/S0140-6736(12)61729-2
4. Barton KI, Shekarforoush M, Heard BJ, Sevik JL, Vakili P, Atarod M, et al. Use of Pre-Clinical Surgically Induced Models to Understand Biomechanical and Biological Consequences of PTOA Development. *J Orthop Res* (2017) 35(3):454–65. doi: 10.1002/jor.23322
 5. Chua JR, Jamal S, Riad M, Castrejon I, Malfait AM, Block JA, et al. Disease Burden in Osteoarthritis Is Similar to That of Rheumatoid Arthritis at Initial Rheumatology Visit and Significantly Greater Six Months Later. *Arthritis Rheumatol* (2019) 71(8):1276–84. doi: 10.1002/art.40869
 6. Dequeker J, Luyten FP. The History of Osteoarthritis-Osteoarthrosis. *Ann Rheum Dis* (2008) 67(1):5–10. doi: 10.1136/ard.2007.079764
 7. Orlowsky EW, Kraus VB. The Role of Innate Immunity in Osteoarthritis: When Our First Line of Defense Goes on the Offensive. *J Rheumatol* (2015) 42(3):363–71. doi: 10.3899/jrheum.140382
 8. Sokolove J, Lepus CM. Role of Inflammation in the Pathogenesis of Osteoarthritis: Latest Findings and Interpretations. *Ther Adv Musculoskelet Dis* (2013) 5(2):77–94. doi: 10.1177/1759720X12467868
 9. Punzi L, Galozzi P, Luisetto R, Favero M, Ramonda R, Oliviero F, et al. Post-Traumatic Arthritis: Overview on Pathogenic Mechanisms and Role of Inflammation. *RMD Open* (2016) 2(2):e000279. doi: 10.1136/rmdopen-2016-000279
 10. Berenbaum F. Osteoarthritis as an Inflammatory Disease (Osteoarthritis is Not Osteoarthrosis!). *Osteoarthritis Cartilage* (2013) 21(1):16–21. doi: 10.1016/j.joca.2012.11.012
 11. Woodell-May JE, Sommerfeld SD. Role of Inflammation and the Immune System in the Progression of Osteoarthritis. *J Orthop Res* (2020) 38(2):253–7. doi: 10.1002/jor.24457
 12. Mora JC, Przkora R, Cruz-Almeida Y. Knee Osteoarthritis: Pathophysiology and Current Treatment Modalities. *J Pain Res* (2018) 11:2189–96. doi: 10.2147/JPR.S154002
 13. Schulz C, Gomez Perdiguero E, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, et al. A Lineage of Myeloid Cells Independent of Myb and Hematopoietic Stem Cells. *Science* (2012) 336(6077):86–90. doi: 10.1126/science.1219179
 14. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-Resident Macrophages Self-Maintain Locally Throughout Adult Life With Minimal Contribution From Circulating Monocytes. *Immunity* (2013) 38(4):792–804. doi: 10.1016/j.immuni.2013.04.004
 15. Gomez Perdiguero E, Klapproth K, Schulz C, Busch K, Azzoni E, Crozet L, et al. Tissue-Resident Macrophages Originate From Yolk-Sac-Derived Erythro-Myeloid Progenitors. *Nature* (2015) 518(7540):547–51. doi: 10.1038/nature13989
 16. Hoeffel G, Chen J, Lavin Y, Low D, Almeida FF, See P, et al. C-Myb(+) Erythro-Myeloid Progenitor-Derived Fetal Monocytes Give Rise to Adult Tissue-Resident Macrophages. *Immunity* (2015) 42(4):665–78. doi: 10.1016/j.immuni.2015.03.011
 17. Williams M, Scott CL. Does Niche Competition Determine the Origin of Tissue-Resident Macrophages? *Nat Rev Immunol* (2017) 17(7):451–60. doi: 10.1038/nri.2017.42
 18. Williams M, Thierry GR, Bonnarde J, Bajenoff M. Establishment and Maintenance of the Macrophage Niche. *Immunity* (2020) 52(3):434–51. doi: 10.1016/j.immuni.2020.02.015
 19. Ginhoux F, Williams M. Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity* (2016) 44(3):439–49. doi: 10.1016/j.immuni.2016.02.024
 20. Culemann S, Gruneboom A, Kronke G. Origin and Function of Synovial Macrophage Subsets During Inflammatory Joint Disease. *Adv Immunol* (2019) 143:75–98. doi: 10.1016/bs.ai.2019.08.006
 21. Raghu H, Lepus CM, Wang Q, Wong HH, Lingampalli N, Oliviero F, et al. CCL2/CCR2, But Not CCL5/CCR5, Mediates Monocyte Recruitment, Inflammation and Cartilage Destruction in Osteoarthritis. *Ann Rheum Dis* (2017) 76(5):914–22. doi: 10.1136/annrheumdis-2016-210426
 22. Culemann S, Gruneboom A, Nicolas-Avila JA, Weidner D, Lammle KF, Rothe T, et al. Locally Renewing Resident Synovial Macrophages Provide a Protective Barrier for the Joint. *Nature* (2019) 572(7771):670–5. doi: 10.1038/s41586-019-1471-1
 23. Boersma GSA, Utomo L, Bayon Y, Kops N, van der Harst E, Lange JF, et al. Monocyte Subsets in Blood Correlate With Obesity Related Response of Macrophages to Biomaterials. *Vitro Biomater* (2016) 109:32–9. doi: 10.1016/j.biomaterials.2016.09.009
 24. Masouros SD, Bull AMJ, Amis AA.(i) Biomechanics of the Knee Joint. *Orthopaedics Trauma* (2010) 24(2):84–91. doi: 10.1016/j.mporth.2010.03.005
 25. Blackburn TA, Craig E. Knee Anatomy: A Brief Review. *Phys Ther* (1980) 60(12):1556–60. doi: 10.1093/ptj/60.12.1556
 26. Sophia Fox AJ, Bedi A, Rodeo SA. The Basic Science of Articular Cartilage: Structure, Composition, and Function. *Sports Health* (2009) 1(6):461–8. doi: 10.1177/1941738109350438
 27. Alford JW, Cole BJ. Cartilage Restoration, Part 1: Basic Science, Historical Perspective, Patient Evaluation, and Treatment Options. *Am J Sports Med* (2005) 33(2):295–306. doi: 10.1177/0363546504273510
 28. Hoffa A. The Influence Of The Adipose Tissue With Regard To The Pathology Of The Knee Joint. *J Am Med Assoc* (1904) XLIII(12):795–6. doi: 10.1001/jama.1904.92500120002h
 29. Jiang LF, Fang JH, Wu LD. Role of Infrapatellar Fat Pad in Pathological Process of Knee Osteoarthritis: Future Applications in Treatment. *World J Clin Cases* (2019) 7(16):2134–42. doi: 10.12998/wjcc.v7.i16.2134
 30. Urban H, Little CB. The Role of Fat and Inflammation in the Pathogenesis and Management of Osteoarthritis. *Rheumatol (Oxford)* (2018) 57(suppl_4):iv10–21. doi: 10.1093/rheumatology/kex399
 31. Kung MS, Markantonis J, Nelson SD, Campbell P. The Synovial Lining and Synovial Fluid Properties After Joint Arthroplasty. *Lubricants* (2015) 3(2):394–412. doi: 10.3390/lubricants3020394
 32. Scanzello CR, Goldring SR. The Role of Synovitis in Osteoarthritis Pathogenesis. *Bone* (2012) 51(2):249–57. doi: 10.1016/j.bone.2012.02.012
 33. Varol C, Mildner A, Jung S. Macrophages: Development and Tissue Specialization. *Annu Rev Immunol* (2015) 33:643–75. doi: 10.1146/annurev-immunol-032414-112220
 34. Mass E, Gentek R. Fetal-Derived Immune Cells at the Roots of Lifelong Pathophysiology. *Front Cell Dev Biol* (2021) 9:648313. doi: 10.3389/fcell.2021.648313
 35. Williams M, Mildner A, Yona S. Developmental and Functional Heterogeneity of Monocytes. *Immunity* (2018) 49(4):595–613. doi: 10.1016/j.immuni.2018.10.005
 36. Fogg DK, Sibon C, Miled C, Jung S, Aucouturier P, Littman DR, et al. A Clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic Cells. *Science* (2006) 311(5757):83–7. doi: 10.1126/science.1117729
 37. Auffray C, Fogg DK, Nami-Mancinelli E, Senechal B, Trouillet C, Saederup N, et al. CX3CR1+ CD115+ CD135+ Common Macrophage/DC Precursors and the Role of CX3CR1 in Their Response to Inflammation. *J Exp Med* (2009) 206(3):595–606. doi: 10.1084/jem.20081385
 38. Hettinger J, Richards DM, Hansson J, Barra MM, Joschko AC, Krijgsvelde J, et al. Origin of Monocytes and Macrophages in a Committed Progenitor. *Nat Immunol* (2013) 14(8):821–30. doi: 10.1038/ni.2638
 39. Chong SZ, Evrard M, Devi S, Chen J, Lim JY, See P, et al. CXCR4 Identifies Transitional Bone Marrow Premonocytes That Replenish the Mature Monocyte Pool for Peripheral Responses. *J Exp Med* (2016) 213(11):2293–314. doi: 10.1084/jem.20160800
 40. Geissmann F, Jung S, Littman DR. Blood Monocytes Consist of Two Principal Subsets With Distinct Migratory Properties. *Immunity* (2003) 19(1):71–82. doi: 10.1016/s1074-7613(03)00174-2
 41. Teh YC, Ding JL, Ng LG, Chong SZ. Capturing the Fantastic Voyage of Monocytes Through Time and Space. *Front Immunol* (2019) 10:834. doi: 10.3389/fimmu.2019.00834
 42. Ginhoux F, Jung S. Monocytes and Macrophages: Developmental Pathways and Tissue Homeostasis. *Nat Rev Immunol* (2014) 14(6):392–404. doi: 10.1038/nri3671
 43. Jakubczak C, Gautier EL, Gibbins SL, Sojka DK, Schlitzer A, Johnson TE, et al. Minimal Differentiation of Classical Monocytes as They Survey Steady-State Tissues and Transport Antigen to Lymph Nodes. *Immunity* (2013) 39(3):599–610. doi: 10.1016/j.immuni.2013.08.007
 44. Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, et al. Analysis of Fractalkine Receptor CX3CR1 Function by Targeted Deletion and Green Fluorescent Protein Reporter Gene Insertion. *Mol Cell Biol* (2000) 20(11):4106–14. doi: 10.1128/mcb.20.11.4106-4114.2000
 45. Kratochvil RM, Kubes P, Deniset JF. Monocyte Conversion During Inflammation and Injury. *Arterioscler Thromb Vasc Biol* (2017) 37(1):35–42. doi: 10.1161/ATVBAHA.116.308198

46. Zhao M, Tuo H, Wang S, Zhao L. The Roles of Monocyte and Monocyte-Derived Macrophages in Common Brain Disorders. *BioMed Res Int* (2020) 2020:9396021. doi: 10.1155/2020/9396021
47. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-Dependent Ly6C(low) Monocytes Monitor Endothelial Cells and Orchestrate Their Disposal. *Cell* (2013) 153(2):362–75. doi: 10.1016/j.cell.2013.03.010
48. Jung H, Mithal DS, Park JE, Miller RJ. Localized CCR2 Activation in the Bone Marrow Niche Mobilizes Monocytes by Desensitizing Cxcr4. *PLoS One* (2015) 10(6):e0128387. doi: 10.1371/journal.pone.0128387
49. Liu Q, Li Z, Gao JL, Wan W, Ganesan S, McDermott DH, et al. CXCR4 Antagonist AMD3100 Redistributes Leukocytes From Primary Immune Organs to Secondary Immune Organs, Lung, and Blood in Mice. *Eur J Immunol* (2015) 45(6):1855–67. doi: 10.1002/eji.201445245
50. Tsou CL, Peters W, Si Y, Slaymaker S, Aslanian AM, Weisberg SP, et al. Critical Roles for CCR2 and MCP-3 in Monocyte Mobilization From Bone Marrow and Recruitment to Inflammatory Sites. *J Clin Invest* (2007) 117(4):902–9. doi: 10.1172/JCI29919
51. Serbina NV, Pamer EG. Monocyte Emigration From Bone Marrow During Bacterial Infection Requires Signals Mediated by Chemokine Receptor CCR2. *Nat Immunol* (2006) 7(3):311–7. doi: 10.1038/nri1309
52. Debieu E, Mayol K, Biaisoux V, Dausy C, De Agüero MG, Taillardet M, et al. S1PR5 is Pivotal for the Homeostasis of Patrolling Monocytes. *Eur J Immunol* (2013) 43(6):1667–75. doi: 10.1002/eji.201343312
53. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate Mapping Reveals Origins and Dynamics of Monocytes and Tissue Macrophages Under Homeostasis. *Immunity* (2013) 38(1):79–91. doi: 10.1016/j.immuni.2012.12.001
54. Scott CL, Zheng F, De Baetselier P, Martens L, Saeys Y, De Prijck S, et al. Bone Marrow-Derived Monocytes Give Rise to Self-Renewing and Fully Differentiated Kupffer Cells. *Nat Commun* (2016) 7:10321. doi: 10.1038/ncomms10321
55. Varol C, Landsman L, Fogg DK, Greenshtein L, Gildor B, Margalit R, et al. Monocytes Give Rise to Mucosal, But Not Splenic, Conventional Dendritic Cells. *J Exp Med* (2007) 204(1):171–80. doi: 10.1084/jem.20061011
56. Hanna RN, Carlin LM, Hubbeling HG, Nackiewicz D, Green AM, Punt JA, et al. The Transcription Factor NR4A1 (Nur77) Controls Bone Marrow Differentiation and the Survival of Ly6C⁺ Monocytes. *Nat Immunol* (2011) 12(8):778–85. doi: 10.1038/ni.2063
57. Gamrekelashvili J, Giagnorio R, Jusoffie J, Soehnlein O, Duchene J, Brisenio CG, et al. Regulation of Monocyte Cell Fate by Blood Vessels Mediated by Notch Signaling. *Nat Commun* (2016) 7:12597. doi: 10.1038/ncomms12597
58. Narasimhan PB, Marcovecchio P, Hamers AAJ, Hedrick CC. Nonclassical Monocytes in Health and Disease. *Annu Rev Immunol* (2019) 37:439–56. doi: 10.1146/annurev-immunol-042617-053119
59. Schmidl C, Renner K, Peter K, Eder R, Lassmann T, Balwiercz PJ, et al. Transcription and Enhancer Profiling in Human Monocyte Subsets. *Blood* (2014) 123(17):e90–9. doi: 10.1182/blood-2013-02-484188
60. Buenrostro JD, Corces MR, Lareau CA, Wu B, Schep AN, Aryee MJ, et al. Integrated Single-Cell Analysis Maps the Continuous Regulatory Landscape of Human Hematopoietic Differentiation. *Cell* (2018) 173(6):1535–48 e16. doi: 10.1016/j.cell.2018.03.074
61. Quintar A, McArdle S, Wolf D, Marki A, Ehinger E, Vassallo M, et al. Endothelial Protective Monocyte Patrolling in Large Arteries Intensified by Western Diet and Atherosclerosis. *Circ Res* (2017) 120(11):1789–99. doi: 10.1161/CIRCRESAHA.117.310739
62. Thomas G, Tacke R, Hedrick CC, Hanna RN. Nonclassical Patrolling Monocyte Function in the Vasculature. *Arterioscler Thromb Vasc Biol* (2015) 35(6):1306–16. doi: 10.1161/ATVBAHA.114.304650
63. Satoh T, Nakagawa K, Sugihara F, Kuwahara R, Ashihara M, Yamane F, et al. Identification of an Atypical Monocyte and Committed Progenitor Involved in Fibrosis. *Nature* (2017) 541(7635):96–101. doi: 10.1038/nature20611
64. Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, et al. Embryonic and Adult-Derived Resident Cardiac Macrophages are Maintained Through Distinct Mechanisms at Steady State and During Inflammation. *Immunity* (2014) 40(1):91–104. doi: 10.1016/j.immuni.2013.11.019
65. Bain CC, Bravo-Bias A, Scott CL, Perdiguer EG, Geissmann F, Henri S, et al. Constant Replenishment From Circulating Monocytes Maintains the Macrophage Pool in the Intestine of Adult Mice. *Nat Immunol* (2014) 15(10):929–37. doi: 10.1038/ni.2967
66. Tamoutounour S, Williams M, Montanana Sanchis F, Liu H, Terhorst D, Malosse C, et al. Origins and Functional Specialization of Macrophages and of Conventional and Monocyte-Derived Dendritic Cells in Mouse Skin. *Immunity* (2013) 39(5):925–38. doi: 10.1016/j.immuni.2013.10.004
67. Tamoutounour S, Henri S, Lelouard H, de Bovis B, de Haar C, van der Woude CJ, et al. CD64 Distinguishes Macrophages From Dendritic Cells in the Gut and Reveals the Th1-Inducing Role of Mesenteric Lymph Node Macrophages During Colitis. *Eur J Immunol* (2012) 42(12):3150–66. doi: 10.1002/eji.201242847
68. Mass E, Ballesteros I, Farlik M, Halbritter F, Gunther P, Crozet L, et al. Specification of Tissue-Resident Macrophages During Organogenesis. *Science* (2016) 353(6304):353–85. doi: 10.1126/science.124238
69. T'Jonck W, Williams M, Bonnardel J. Niche Signals and Transcription Factors Involved in Tissue-Resident Macrophage Development. *Cell Immunol* (2018) 330:43–53. doi: 10.1016/j.cellimm.2018.02.005
70. Lavin Y, Winter D, Blecher-Gonen R, David E, Keren-Shaul H, Merad M, et al. Tissue-Resident Macrophage Enhancer Landscapes are Shaped by the Local Microenvironment. *Cell* (2014) 159(6):1312–26. doi: 10.1016/j.cell.2014.11.018
71. Gosselin D, Link VM, Romanoski CE, Fonseca GJ, Eichenfield DZ, Spann NJ, et al. Environment Drives Selection and Function of Enhancers Controlling Tissue-Specific Macrophage Identities. *Cell* (2014) 159(6):1327–40. doi: 10.1016/j.cell.2014.11.023
72. Etzerodt A, Tsalkitzi K, Maniecki M, Damsky W, Delfini M, Baudoin E, et al. Specific Targeting of CD163(+) TAMs Mobilizes Inflammatory Monocytes and Promotes T Cell-Mediated Tumor Regression. *J Exp Med* (2019) 216(10):2394–411. doi: 10.1084/jem.20182124
73. Zhu Y, Herndon JM, Sojka DK, Kim KW, Knolhoff BL, Zuo C, et al. Tissue-Resident Macrophages in Pancreatic Ductal Adenocarcinoma Originate From Embryonic Hematopoiesis and Promote Tumor Progression. *Immunity* (2017) 47(3):597. doi: 10.1016/j.immuni.2017.08.018
74. Loyer PL, Hamon P, Laviron M, Meghraoui-Kheddar A, Goncalves E, Deng Z, et al. Macrophages of Distinct Origins Contribute to Tumor Development in the Lung. *J Exp Med* (2018) 215(10):2536–53. doi: 10.1084/jem.20180534
75. Werner Y, Mass E, Ashok Kumar P, Ulas T, Handler K, Horne A, et al. Cxcr4 Distinguishes HSC-Derived Monocytes From Microglia and Reveals Monocyte Immune Responses to Experimental Stroke. *Nat Neurosci* (2020) 23(3):351–62. doi: 10.1038/s41593-020-0585-y
76. Swirski FK, Nahrendorf M, Etzerodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of Splenic Reservoir Monocytes and Their Deployment to Inflammatory Sites. *Science* (2009) 325(5940):612–6. doi: 10.1126/science.1175202
77. Li L, Huang L, Sung SS, Vergis AL, Rosin DL, Rose CE Jr., et al. The Chemokine Receptors CCR2 and CX3CR1 Mediate Monocyte/Macrophage Trafficking in Kidney Ischemia-Reperfusion Injury. *Kidney Int* (2008) 74(12):1526–37. doi: 10.1038/ki.2008.500
78. Huen SC, Cantley LG. Macrophages in Renal Injury and Repair. *Annu Rev Physiol* (2017) 79:449–69. doi: 10.1146/annurev-physiol-022516-034219
79. Furuichi K, Wada T, Iwata Y, Kitagawa K, Kobayashi K, Hashimoto H, et al. CCR2 Signaling Contributes to Ischemia-Reperfusion Injury in Kidney. *J Am Soc Nephrol* (2003) 14(10):2503–15. doi: 10.1097/01.asn.0000089563.63641.a8
80. Shechter R, Raposo C, London A, Sagi I, Schwartz M. The Glial Scar-Monocyte Interplay: A Pivotal Resolution Phase in Spinal Cord Repair. *PLoS One* (2011) 6(12):e27969. doi: 10.1371/journal.pone.0027969
81. Shechter R, London A, Varol C, Raposo C, Cusimano M, Yovel G, et al. Infiltrating Blood-Derived Macrophages are Vital Cells Playing an Anti-Inflammatory Role in Recovery From Spinal Cord Injury in Mice. *PLoS Med* (2009) 6(7):e1000113. doi: 10.1371/journal.pmed.1000113
82. Nahrendorf M, Swirski FK, Aikawa E, Stangenberg L, Wurdinger T, Figueiredo JL, et al. The Healing Myocardium Sequentially Mobilizes Two Monocyte Subsets With Divergent and Complementary Functions. *J Exp Med* (2007) 204(12):3037–47. doi: 10.1084/jem.20070885
83. Dimitrijevic OB, Stamatovic SM, Keep RF, Andjelkovic AV. Absence of the Chemokine Receptor CCR2 Protects Against Cerebral Ischemia/Reperfusion Injury in Mice. *Stroke* (2007) 38(4):1345–53. doi: 10.1161/01.STR.0000259709.16654.8f

84. Bamboat ZM, Ocun LM, Balachandran VP, Obaid H, Plitas G, DeMatteo RP. Conventional DCs Reduce Liver Ischemia/Reperfusion Injury in Mice. *Via IL-10 Secretion J Clin Invest* (2010) 120(2):559–69. doi: 10.1172/JCI40008
85. Spahn JH, Kreisel D. Monocytes in Sterile Inflammation: Recruitment and Functional Consequences. *Arch Immunol Ther Exp (Warsz)* (2014) 62(3):187–94. doi: 10.1007/s00005-013-0267-5
86. Robbins CS, Chudnovskiy A, Rauch PJ, Figueiredo JL, Iwamoto Y, Gorbakov R, et al. Extramedullary Hematopoiesis Generates Ly-6C(High) Monocytes That Infiltrate Atherosclerotic Lesions. *Circulation* (2012) 125(2):364–74. doi: 10.1161/CIRCULATIONAHA.111.061986
87. Zhou X, Liu XL, Ji WJ, Liu JX, Guo ZZ, Ren D, et al. The Kinetics of Circulating Monocyte Subsets and Monocyte-Platelet Aggregates in the Acute Phase of ST-Elevation Myocardial Infarction: Associations With 2-Year Cardiovascular Events. *Med (Baltimore)* (2016) 95(18):e3466. doi: 10.1097/MD.0000000000003466
88. Rahman K, Vengrenyuk Y, Ramsey SA, Vila NR, Girgis NM, Liu J, et al. Inflammatory Ly6Chi Monocytes and Their Conversion to M2 Macrophages Drive Atherosclerosis Regression. *J Clin Invest* (2017) 127(8):2904–15. doi: 10.1172/JCI75005
89. Gautier EL, Shay T, Miller J, Greter M, Jakubczak C, Ivanov S, et al. Gene-Expression Profiles and Transcriptional Regulatory Pathways That Underlie the Identity and Diversity of Mouse Tissue Macrophages. *Nat Immunol* (2012) 13(11):1118–28. doi: 10.1038/ni.2419
90. Haldar M, Kobayama M, So AY, Kc W, Wu X, Briseno CG, et al. Heme-Mediated SPI-C Induction Promotes Monocyte Differentiation Into Iron-Recycling Macrophages. *Cell* (2014) 156(6):1223–34. doi: 10.1016/j.cell.2014.01.069
91. van de Laar L, Saelens W, De Prijck S, Martens L, Scott CL, Van Isterdael G, et al. Yolk Sac Macrophages, Fetal Liver, and Adult Monocytes Can Colonize an Empty Niche and Develop Into Functional Tissue-Resident Macrophages. *Immunity* (2016) 44(4):755–68. doi: 10.1016/j.immuni.2016.02.017
92. Gordon S, Martinez FO. Alternative Activation of Macrophages: Mechanism and Functions. *Immunity* (2010) 32(5):593–604. doi: 10.1016/j.immuni.2010.05.007
93. Sica A, Mantovani A. Macrophage Plasticity and Polarization: *In Vivo* Veritas. *J Clin Invest* (2012) 122(3):787–95. doi: 10.1172/JCI59643
94. Hume DA. The Many Alternative Faces of Macrophage Activation. *Front Immunol* (2015) 6:370. doi: 10.3389/fimmu.2015.00370
95. Murray PJ. Macrophage Polarization. *Annu Rev Physiol* (2017) 79:541–66. doi: 10.1146/annurev-physiol-022516-034339
96. Martinez FO, Gordon S. The M1 and M2 Paradigm of Macrophage Activation: Time for Reassessment. *PLoS One* (2014) 9(12):e112703. doi: 10.1371/journal.pone.0112703
97. Xue J, Schmidt SV, Sander J, Draffehn A, Krebs W, Quester I, et al. Transcriptome-Based Network Analysis Reveals a Spectrum Model of Human Macrophage Activation. *Immunity* (2014) 40(2):274–88. doi: 10.1016/j.immuni.2014.01.006
98. Wiktor-Jedrzejczak W, Bartocci A, Ferrante AW Jr, Ahmed-Ansari A, Sell KW, Pollard JW, et al. Total Absence of Colony-Stimulating Factor 1 in the Macrophage-Deficient Osteopetrotic (Op/Op) Mouse. *Proc Natl Acad Sci U.S.A.* (1990) 87(12):4828–32. doi: 10.1073/pnas.87.12.4828
99. Cecchini MG, Dominguez MG, Mocci S, Wetterwald A, Felix R, Fleisch H, et al. Role of Colony Stimulating Factor-1 in the Establishment and Regulation of Tissue Macrophages During Postnatal Development of the Mouse. *Development* (1994) 120(6):1357–72. doi: 10.1242/dev.120.6.1357
100. Dai XM, Ryan GR, Hapel AJ, Dominguez MG, Russell RG, Kapp S, et al. Targeted Disruption of the Mouse Colony-Stimulating Factor 1 Receptor Gene Results in Osteopetrosis, Mononuclear Phagocyte Deficiency, Increased Primitive Progenitor Cell Frequencies, and Reproductive Defects. *Blood* (2002) 99(1):111–20. doi: 10.1182/blood.v99.1.111
101. Bonnardel J, T'Jonck W, Gaubomme D, Browaeys R, Scott CL, Martens L, et al. Stellate Cells, Hepatocytes, and Endothelial Cells Imprint the Kupfer Cell Identity on Monocytes Colonizing the Liver Macrophage Niche. *Immunity* (2019) 51(4):638–54 e9. doi: 10.1016/j.immuni.2019.08.017
102. Bellomo A, Mondor I, Spinelli L, Lagueyrie M, Stewart BJ, Brouilly N, et al. Reticular Fibroblasts Expressing the Transcription Factor WT1 Define a Stromal Niche That Maintains and Replenishes Splenic Red Pulp Macrophages. *Immunity* (2020) 53(1):127–42 e7. doi: 10.1016/j.immuni.2020.06.008
103. Mortha A, Chudnovskiy A, Hashimoto D, Bogunovic M, Spencer SP, Belkaid Y, et al. Microbiota-Dependent Crosstalk Between Macrophages and ILC3 Promotes Intestinal Homeostasis. *Science* (2014) 343(6178):1249288. doi: 10.1126/science.1249288
104. Kana V, Desland EA, Casanova-Acebes M, Ayata P, Badimon A, Nabel E, et al. CSF-1 Controls Cerebellar Microglia and is Required for Motor Function and Social Interaction. *J Exp Med* (2019) 216(10):2265–81. doi: 10.1084/jem.20182037
105. Muller PA, Kosco B, Rajani GM, Stevanovic K, Berres ML, Hashimoto D, et al. Crosstalk Between Muscularis Macrophages and Enteric Neurons Regulates Gastrointestinal Motility. *Cell* (2014) 158(2):300–13. doi: 10.1016/j.cell.2014.04.050
106. Yoshida M, Ikegami M, Reed JA, Chronos ZC, Whitsett JA. GM-CSF Regulates Protein and Lipid Catabolism by Alveolar Macrophages. *Am J Physiol Lung Cell Mol Physiol* (2001) 280(3):L379–86. doi: 10.1152/ajplung.2001.280.3.L379
107. Williams M, De Kleer I, Henri S, Post S, Vanhoutte L, De Prijck S, et al. Alveolar Macrophages Develop From Fetal Monocytes That Differentiate Into Long-Lived Cells in the First Week of Life via GM-CSF. *J Exp Med* (2013) 210(10):1977–92. doi: 10.1084/jem.20131199
108. De Schepper S, Verheijden S, Aguilera-Lizarraga J, Viola MF, Boesmans W, Stakenborg N, et al. Self-Maintaining Gut Macrophages Are Essential for Intestinal Homeostasis. *Cell* (2018) 175(2):400–15.e13. doi: 10.1016/j.cell.2018.07.048
109. Hulsman M, Clauss S, Xiao L, Aguirre AD, King KR, Hanley A, et al. Macrophages Facilitate Electrical Conduction in the Heart. *Cell* (2017) 169(3):510–22 e20. doi: 10.1016/j.cell.2017.03.050
110. Bergh A, Damber JE, van Rooijen N. Liposome-Mediated Macrophage Depletion: An Experimental Approach to Study the Role of Testicular Macrophages in the Rat. *J Endocrinol* (1993) 136(3):407–13. doi: 10.1677/joe.0.1360407
111. Hutson JC, Garner CW, Doris PA. Purification and Characterization of a Lipophilic Factor From Testicular Macrophages That Stimulates Testosterone Production by Leydig Cells. *J Androl* (1996) 17(5):502–8.
112. Lukyanenko YO, Chen JJ, Hutson JC. Production of 25-Hydroxycholesterol by Testicular Macrophages and its Effects on Leydig Cells. *Biol Reprod* (2001) 64(3):790–6. doi: 10.1095/biolreprod64.3.790
113. Yahara Y, Barrientos T, Tang Y, Puviandran V, Nadesan P, Zhang H, et al. Erythromyeloid Progenitors Give Rise to a Population of Osteoclasts That Contribute to Bone Homeostasis and Repair. *Nat Cell Biol* (2020) 22(1):49–59. doi: 10.1038/s41556-019-0437-8
114. Jacome-Galarza CE, Percin GI, Muller JT, Mass E, Lazarov T, Eitler J, et al. Developmental Origin, Functional Maintenance and Genetic Rescue of Osteoclasts. *Nature* (2019) 568(7753):541–5. doi: 10.1038/s41586-019-1105-7
115. McDonald MM, Khoo WH, Ng PY, Xiao Y, Zamerli J, Thatcher P, et al. Osteoclasts Recycle via Osteomorphs During RANKL-Stimulated Bone Resorption. *Cell* (2021) 184(5):1330–47 e13. doi: 10.1016/j.cell.2021.02.002
116. Cox N, Geissmann F. Macrophage Ontogeny in the Control of Adipose Tissue Biology. *Curr Opin Immunol* (2020) 62:1–8. doi: 10.1016/j.coi.2019.08.002
117. Zeyda M, Farmer D, Todoric J, Aszmann O, Speiser M, Gyori G, et al. Human Adipose Tissue Macrophages are of an Anti-Inflammatory Phenotype But Capable of Excessive Pro-Inflammatory Mediator Production. *Int J Obes (Lond)* (2007) 31(9):1420–8. doi: 10.1038/sj.ijo.0803632
118. Cox N, Crozet L, Holtman IR, Loyher PL, Lazarov T, White JB, et al. Diet-Regulated Production of PDGF α by Macrophages Controls Energy Storage. *bioRxiv* (2021) 373(6550):eabe9383. doi: 10.1126/science.abe9383
119. Barboza E, Hudson J, Chang WP, Kovats S, Townner RA, Silasi-Mansat R, et al. Proinflammatory Infrapatellar Fat Pad Remodeling Without M1 Macrophage Polarization Precedes Knee Osteoarthritis in Mice With Diet-Induced Obesity. *Arthritis Rheumatol* (2017) 69(6):1221–32. doi: 10.1002/art.40056
120. Fujisaka S, Usui I, Bukhari A, Ikutani M, Oya T, Kanatani Y, et al. Regulatory Mechanisms for Adipose Tissue M1 and M2 Macrophages in Diet-Induced Obese Mice. *Diabetes* (2009) 58(11):2574–82. doi: 10.2337/db08-1475
121. Iwata M, Ochi H, Hara Y, Tagawa M, Koga D, Okawa A, et al. Initial Responses of Articular Tissues in a Murine High-Fat Diet-Induced

- Osteoarthritis Model: Pivotal Role of the IPFP as a Cytokine Fountain. *PLoS One* (2013) 8(4):e60706. doi: 10.1371/journal.pone.0060706
122. Harasymowicz NS, Clement ND, Azfer A, Burnett R, Salter DM, Simpson A. Regional Differences Between Perisynovial and Infrapatellar Adipose Tissue Depots and Their Response to Class II and Class III Obesity in Patients With Osteoarthritis. *Arthritis Rheumatol* (2017) 69(7):1396–406. doi: 10.1002/art.40102
 123. Warmink K, Kozijn AE, Bobeldijk I, Stoop R, Weinans H, Korthagen NM. High-Fat Feeding Primes the Mouse Knee Joint to Develop Osteoarthritis and Pathologic Infrapatellar Fat Pad Changes After Surgically Induced Injury. *Osteoarthritis Cartilage* (2020) 28(5):593–602. doi: 10.1016/j.joca.2020.03.008
 124. Kurowska-Stolarska M, Alivernini S. Synovial Tissue Macrophages: Friend or Foe? *RMD Open* (2017) 3(2):e000527. doi: 10.1136/rmdopen-2017-000527
 125. de Lange-Brokaar BJE, Ioan-Facsinay A, van Osch GJVM, Zuurmond AM, Schoones J, Toes REM, et al. Synovial Inflammation, Immune Cells and Their Cytokines in Osteoarthritis: A Review. *Osteoarthritis Cartilage* (2012) 20(12):1484–99. doi: 10.1016/j.joca.2012.08.027
 126. Buckley CD, Ospelt C, Gay S, Midwood KS. Location, Location, Location: How the Tissue Microenvironment Affects Inflammation in RA. *Nat Rev Rheumatol* (2021) 17(4):195–212. doi: 10.1038/s41584-020-00570-2
 127. Ambarus CA, Noordenbos T, de Hair MJ, Tak PP, Baeten DL. Intimal Lining Layer Macrophages But Not Synovial Sublining Macrophages Display an IL-10 Polarized-Like Phenotype in Chronic Synovitis. *Arthritis Res Ther* (2012) 14(2):R74. doi: 10.1186/ar3796
 128. Fonseca JE, Edwards JC, Blades S, Goulding NJ. Macrophage Subpopulations in Rheumatoid Synovium: Reduced CD163 Expression in CD4+ T Lymphocyte-Rich Microenvironments. *Arthritis Rheum* (2002) 46(5):1210–6. doi: 10.1002/art.10207
 129. Smith MD, Barg E, Weedon H, Papangelis V, Smeets T, Tak PP, et al. Microarchitecture and Protective Mechanisms in Synovial Tissue From Clinically and Arthroscopically Normal Knee Joints. *Ann Rheum Dis* (2003) 62(4):303–7. doi: 10.1136/ard.62.4.303
 130. Buechler MB, Kim KW, Onufer EJ, Williams JW, Little CC, Dominguez CX, et al. A Stromal Niche Defined by Expression of the Transcription Factor WT1 Mediates Programming and Homeostasis of Cavity-Resident Macrophages. *Immunity* (2019) 51(1):119–30 e5. doi: 10.1016/j.immuni.2019.05.010
 131. Alivernini S, MacDonald L, Elmesmari A, Finlay S, Tolusso B, Gigante MR, et al. Distinct Synovial Tissue Macrophage Subsets Regulate Inflammation and Remission in Rheumatoid Arthritis. *Nat Med* (2020) 26(8):1295–306. doi: 10.1038/s41591-020-0939-8
 132. Croft AP, Campos J, Jansen K, Turner JD, Marshall J, Attar M, et al. Distinct Fibroblast Subsets Drive Inflammation and Damage in Arthritis. *Nature* (2019) 570(7760):246–51. doi: 10.1038/s41586-019-1263-7
 133. Schuster R, Rockel JS, Kapoor M, Hinz B. The Inflammatory Speech of Fibroblasts. *Immunol Rev* (2021) 302:126–46. doi: 10.1111/imr.12971
 134. Hannemann N, Appayilay F, Courties G. Synovial Macrophages: From Ordinary Eaters to Extraordinary Multitaskers. *Trends Immunol* (2021) 42(5):368–71. doi: 10.1016/j.it.2021.03.002
 135. Franklin RA. Fibroblasts and Macrophages: Collaborators in Tissue Homeostasis. *Immunol Rev* (2021) 302:86–103. doi: 10.1111/imr.12989
 136. Ryan GR, Dai X-M, Dominguez MG, Tong W, Chuan F, Chisholm O, et al. Rescue of the Colony-Stimulating Factor 1 (CSF-1)-Nullizygous Mouse (Csf1^{flp}/Csf1^{flp}) Phenotype With a CSF-1 Transgene and Identification of Sites of Local CSF-1 Synthesis. *Blood* (2001) 98(1):74–84. doi: 10.1182/blood.V98.1.74
 137. Adlerz KM, Aranda-Espinoza H, Hayenga HN. Substrate Elasticity Regulates the Behavior of Human Monocyte-Derived Macrophages. *Eur Biophys J* (2016) 45(4):301–9. doi: 10.1007/s00249-015-1096-8
 138. Escolano JC, Taubenberger AV, Abuhattum S, Schweitzer C, Farrukh A, Del Campo A, et al. Compliant Substrates Enhance Macrophage Cytokine Release and NLRP3 Inflammasome Formation During Their Pro-Inflammatory Response. *Front Cell Dev Biol* (2021) 9:639815. doi: 10.3389/fcell.2021.639815
 139. Maruyama K, Nemoto E, Yamada S. Mechanical Regulation of Macrophage Function - Cyclic Tensile Force Inhibits NLRP3 Inflammasome-Dependent IL-1 β Secretion in Murine Macrophages. *Inflamm Regen* (2019) 39:3. doi: 10.1186/s41232-019-0092-2
 140. O'Connor CJ, Ramalingam S, Zelenski NA, Benefield HC, Rigo I, Little D, et al. Cartilage-Specific Knockout of the Mechanosensory Ion Channel TRPV4 Decreases Age-Related Osteoarthritis. *Sci Rep* (2016) 6(1):29053. doi: 10.1038/srep29053
 141. Lv Z, Xu X, Sun Z, Yang YX, Guo H, Li J, et al. TRPV1 Alleviates Osteoarthritis by Inhibiting M1 Macrophage Polarization via Ca²⁺/CaMKII/Nrf2 Signaling Pathway. *Cell Death Dis* (2021) 12(6):504. doi: 10.1038/s41419-021-03792-8
 142. Michalick L, Kuebler WM. Trpv4—A Missing Link Between Mechanosensation and Immunity. *Front Immunol* (2020) 11:413(413). doi: 10.3389/fimmu.2020.00413
 143. Arya RK, Goswami R, Rahaman SO. Mechanotransduction via a TRPV4-Rac1 Signaling Axis Plays a Role in Multinucleated Giant Cell Formation. *J Biol Chem* (2020) 296:100–29. doi: 10.1074/jbc.RA120.014597
 144. Bhattaram P, Chandrasekharan U. The Joint Synovium: A Critical Determinant of Articular Cartilage Fate in Inflammatory Joint Diseases. *Semin Cell Dev Biol* (2017) 62:86–93. doi: 10.1016/j.semcdb.2016.05.009
 145. Kennedy A, Fearon U, Veale DJ, Godson C. Macrophages in Synovial Inflammation. *Front Immunol* (2011) 2:52. doi: 10.3389/fimmu.2011.00052
 146. van Lent PL, Licht R, Dijkman H, Holthuysen AE, Berden JH, van den Berg WB. Uptake of Apoptotic Leukocytes by Synovial Lining Macrophages Inhibits Immune Complex-Mediated Arthritis. *J Leukoc Biol* (2001) 70(5):708–14.
 147. Goldring MB, Otero M. Inflammation in Osteoarthritis. *Curr Opin Rheumatol* (2011) 23(5):471–8. doi: 10.1097/BOR.0b013e328349c2b1
 148. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM. M-1/M-2 Macrophages and the Th1/Th2 Paradigm. *J Immunol* (2000) 164(12):6166–73. doi: 10.4049/jimmunol.164.12.6166
 149. Wu CL, McNeill J, Goon K, Little D, Kimmerling K, Huebner J, et al. Conditional Macrophage Depletion Increases Inflammation and Does Not Inhibit the Development of Osteoarthritis in Obese Macrophage Fas-Induced Apoptosis-Transgenic Mice. *Arthritis Rheumatol* (2017) 69(9):1772–83. doi: 10.1002/art.40161
 150. Tu J, Wang X, Gong X, Hong W, Han D, Fang Y, et al. Synovial Macrophages in Rheumatoid Arthritis: The Past, Present, and Future. *Mediators Inflammation* (2020) 2020:1583647–. doi: 10.1155/2020/1583647
 151. Udalova IA, Mantovani A, Feldmann M. Macrophage Heterogeneity in the Context of Rheumatoid Arthritis. *Nat Rev Rheumatol* (2016) 12(8):472–85. doi: 10.1038/nrrheum.2016.91
 152. Shi C, Pamer EG. Monocyte Recruitment During Infection and Inflammation. *Nat Rev Immunol* (2011) 11(11):762–74. doi: 10.1038/nri3070
 153. Fernandes JC, Martel-Pelletier J, Pelletier JP. The Role of Cytokines in Osteoarthritis Pathophysiology. *Biorheology* (2002) 39(1–2):237–46.
 154. Schlaak JF, Pfers I, Meyer Zum Buschenfelde KH, Marker-Hermann E. Different Cytokine Profiles in the Synovial Fluid of Patients With Osteoarthritis, Rheumatoid Arthritis and Seronegative Spondylarthropathies. *Clin Exp Rheumatol* (1996) 14(2):155–62.
 155. Blom AB, van der Kraan PM, van den Berg WB. Cytokine Targeting in Osteoarthritis. *Curr Drug Targets* (2007) 8(2):283–92. doi: 10.2174/138945007779940179
 156. Cui J, Chen Y, Wang HY, Wang RF. Mechanisms and Pathways of Innate Immune Activation and Regulation in Health and Cancer. *Hum Vaccin Immunother* (2014) 10(11):3270–85. doi: 10.4161/21645515.2014.979640
 157. Huard B, Schneider P, Mauri D, Tschopp J, French LE. T Cell Costimulation by the TNF Ligand BAFF. *J Immunol* (2001) 167(11):6225–31. doi: 10.4049/jimmunol.167.11.6225
 158. Chen M, Lin X, Liu Y, Li Q, Deng Y, Liu Z, et al. The Function of BAFF on T Helper Cells in Autoimmunity. *Cytokine Growth Factor Rev* (2014) 25(3):301–5. doi: 10.1016/j.cytogfr.2013.12.011
 159. Lai Kwan Lam Q, King Hung Ko O, Zheng BJ, Lu L. Local BAFF Gene Silencing Suppresses Th17-Cell Generation and Ameliorates Autoimmune Arthritis. *Proc Natl Acad Sci U.S.A.* (2008) 105(39):14993–8. doi: 10.1073/pnas.0806044105
 160. Zheng SG, Wang J, Horwitz DA. Cutting Edge: Foxp3+CD4+CD25+ Regulatory T Cells Induced by IL-2 and TGF- β are Resistant to Th17

- Conversion by IL-6. *J Immunol* (2008) 180(11):7112–6. doi: 10.4049/jimmunol.180.11.7112
161. Serada S, Fujimoto M, Mihara M, Koike N, Ohsugi Y, Nomura S, et al. IL-6 Blockade Inhibits the Induction of Myelin Antigen-Specific Th17 Cells and Th1 Cells in Experimental Autoimmune Encephalomyelitis. *Proc Natl Acad Sci USA* (2008) 105(26):9041–6. doi: 10.1073/pnas.0802218105
 162. Bosello S, Youinou P, Daridon C, Tolusso B, Bendaoud B, Pietrapertosa D, et al. Concentrations of BAFF Correlate With Autoantibody Levels, Clinical Disease Activity, and Response to Treatment in Early Rheumatoid Arthritis. *J Rheumatol* (2008) 35(7):1256–64.
 163. Wei F, Chang Y, Wei W. The Role of BAFF in the Progression of Rheumatoid Arthritis. *Cytokine* (2015) 76(2):537–44. doi: 10.1016/j.cyto.2015.07.014
 164. Weyand CM. Immunopathologic Aspects of Rheumatoid Arthritis: Who is the Conductor and Who Plays the Immunologic Instrument? *J Rheumatol Suppl* (2007) 79:9–14.
 165. Seyler TM, Park YW, Takemura S, Bram RJ, Kurtin PJ, Goronzy JJ, et al. BLYS and APRIL in Rheumatoid Arthritis. *J Clin Invest* (2005) 115(11):3083–92. doi: 10.1172/JCI25265
 166. Yang L, Chen Z, Guo H, Wang Z, Sun K, Yang X, et al. Extensive Cytokine Analysis in Synovial Fluid of Osteoarthritis Patients. *Cytokine* (2021) 143:155546. doi: 10.1016/j.cyto.2021.155546
 167. Kouskoff V, Korganow AS, Duchatelle V, Degott C, Benoist C, Mathis D. Organ-Specific Disease Provoked by Systemic Autoimmunity. *Cell* (1996) 87(5):811–22. doi: 10.1016/s0092-8674(00)81989-3
 168. Korganow AS, Ji H, Mangialaio S, Duchatelle V, Pelanda R, Martin T, et al. From Systemic T Cell Self-Reactivity to Organ-Specific Autoimmune Disease via Immunoglobulins. *Immunity* (1999) 10(4):451–61. doi: 10.1016/s1074-7613(00)80045-x
 169. Misharin AV, Cuda CM, Saber R, Turner JD, Gierut AK, Haines GK3rd, et al. Nonclassical Ly6C(-) Monocytes Drive the Development of Inflammatory Arthritis in Mice. *Cell Rep* (2014) 9(2):591–604. doi: 10.1016/j.celrep.2014.09.032
 170. Brunet A, LeBel M, Egarnes B, Paquet-Bouchard C, Lessard AJ, Brown JP, et al. NR4A1-Dependent Ly6C(low) Monocytes Contribute to Reducing Joint Inflammation in Arthritic Mice Through Treg Cells. *Eur J Immunol* (2016) 46(12):2789–800. doi: 10.1002/eji.201646406
 171. Anderson DD, Chubinskaya S, Guilak F, Martin JA, Oegema TR, Olson SA, et al. Post-Traumatic Osteoarthritis: Improved Understanding and Opportunities for Early Intervention. *J Orthop Res* (2011) 29(6):802–9. doi: 10.1002/jor.21359
 172. Lieberthal J, Sambamurthy N, Scanzello CR. Inflammation in Joint Injury and Post-Traumatic Osteoarthritis. *Osteoarthritis Cartilage* (2015) 23(11):1825–34. doi: 10.1016/j.joca.2015.08.015
 173. Kandahari AM, Yang X, Dighe AS, Pan D, Cui Q. Recognition of Immune Response for the Early Diagnosis and Treatment of Osteoarthritis. *J Immunol Res* (2015) 2015:192415. doi: 10.1155/2015/192415
 174. Jackson MT, Moradi B, Zaki S, Smith MM, McCracken S, Smith SM, et al. Depletion of Protease-Activated Receptor 2 But Not Protease-Activated Receptor 1 may Confer Protection Against Osteoarthritis in Mice Through Extracellular Matrix Mechanisms. *Arthritis Rheumatol* (2014) 66(12):3337–48. doi: 10.1002/art.38876
 175. Chen L, Zheng JY, Li G, Yuan J, Ebert JR, Li H, et al. Pathogenesis and Clinical Management of Obesity-Related Knee Osteoarthritis: Impact of Mechanical Loading. *J Orthop Translat* (2020) 24:66–75. doi: 10.1016/j.jot.2020.05.001
 176. Lotz MK, Kraus VB. New Developments in Osteoarthritis. Posttraumatic Osteoarthritis: Pathogenesis and Pharmacological Treatment Options. *Arthritis Res Ther* (2010) 12(3):211. doi: 10.1186/ar3046
 177. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbroek R, Llodra J, et al. Monocyte Subsets Differentially Employ CCR2, CCR5, and CX3CR1 to Accumulate Within Atherosclerotic Plaques. *J Clin Invest* (2007) 117(1):185–94. doi: 10.1172/JCI28549
 178. Conti P, DiGioacchino M. MCP-1 and RANTES are Mediators of Acute and Chronic Inflammation. *Allergy Asthma Proc* (2001) 22(3):133–7. doi: 10.2500/108854101778148737
 179. Yuan GH, Masuko-Hongo K, Sakata M, Tsuruoka J, Onuma H, Nakamura H, et al. The Role of C-C Chemokines and Their Receptors in Osteoarthritis. *Arthritis Rheum* (2001) 44(5):1056–70. doi: 10.1002/1529-0131(200105)44:5<1056::AID-ANR186>3.0.CO;2-U
 180. Tong X, Zeng H, Gu P, Wang K, Zhang H, Lin X. Monocyte Chemoattractant Protein1 Promotes the Proliferation, Migration and Differentiation Potential of Fibroblastlike Synoviocytes via the PI3K/P38 Cellular Signaling Pathway. *Mol Med Rep* (2020) 21(3):1623–32. doi: 10.3892/mmr.2020.10969
 181. Tu J, Hong W, Guo Y, Zhang P, Fang Y, Wang X, et al. Ontogeny of Synovial Macrophages and the Roles of Synovial Macrophages From Different Origins in Arthritis. *Front Immunol* (2019) 10:1146. doi: 10.3389/fimmu.2019.01146
 182. Nadvig O, Beer Y, Goldberg M, Agar G, Loos M, Katz Y. Decreased Induction of IL-1beta in Fibroblast-Like Synoviocytes: A Possible Regulatory Mechanism Maintaining Joint Homeostasis. *Mol Immunol* (2007) 44(12):3147–54. doi: 10.1016/j.molimm.2007.02.001
 183. Watt FE, Paterson E, Freidin A, Kenny M, Judge A, Saklatvala J, et al. Acute Molecular Changes in Synovial Fluid Following Human Knee Injury: Association With Early Clinical Outcomes. *Arthritis Rheumatol* (2016) 68(9):2129–40. doi: 10.1002/art.39677
 184. Scanzello CR, McKeon B, Swaim BH, DiCarlo E, Asomugha EU, Kanda V, et al. Synovial Inflammation in Patients Undergoing Arthroscopic Meniscectomy: Molecular Characterization and Relationship to Symptoms. *Arthritis Rheum* (2011) 63(2):391–400. doi: 10.1002/art.30137
 185. Li L, Jiang BE. Serum and Synovial Fluid Chemokine Ligand 2/Monocyte Chemoattractant Protein 1 Concentrations Correlates With Symptomatic Severity in Patients With Knee Osteoarthritis. *Ann Clin Biochem* (2015) 52(Pt 2):276–82. doi: 10.1177/0004563214545117
 186. Endres M, Andreas K, Kalwitz G, Freymann U, Neumann K, Ringe J, et al. Chemokine Profile of Synovial Fluid From Normal, Osteoarthritis and Rheumatoid Arthritis Patients: CCL25, CXCL10 and XCL1 Recruit Human Subchondral Mesenchymal Progenitor Cells. *Osteoarthritis Cartilage* (2010) 18(11):1458–66. doi: 10.1016/j.joca.2010.08.003
 187. Ni F, Zhang Y, Peng X, Li J. Correlation Between Osteoarthritis and Monocyte Chemotactic Protein-1 Expression: A Meta-Analysis. *J Orthop Surg Res* (2020) 15(1):516. doi: 10.1186/s13018-020-02045-2
 188. Haringman JJ, Smeets TJ, Reinders-Blankert P, Tak PP. Chemokine and Chemokine Receptor Expression in Paired Peripheral Blood Mononuclear Cells and Synovial Tissue of Patients With Rheumatoid Arthritis, Osteoarthritis, and Reactive Arthritis. *Ann Rheum Dis* (2006) 65(3):294–300. doi: 10.1136/ard.2005.037176
 189. Takebe K, Rai MF, Schmidt EJ, Sandell LJ. The Chemokine Receptor CCR5 Plays a Role in Post-Traumatic Cartilage Loss in Mice, But Does Not Affect Synovium and Bone. *Osteoarthritis Cartilage* (2015) 23(3):454–61. doi: 10.1016/j.joca.2014.12.002
 190. Wojdasiewicz P, Poniatowski LA, Kotela A, Deszczynski J, Kotela I, Szukiewicz D. The Chemokine CX3CL1 (Fractalkine) and its Receptor CX3CR1: Occurrence and Potential Role in Osteoarthritis. *Arch Immunol Ther Exp (Warsz)* (2014) 62(5):395–403. doi: 10.1007/s00005-014-0275-0
 191. Yano R, Yamamura M, Sunahori K, Takasugi K, Yamana J, Kawashima M, et al. Recruitment of CD16+ Monocytes Into Synovial Tissues is Mediated by Fractalkine and CX3CR1 in Rheumatoid Arthritis Patients. *Acta Med Okayama* (2007) 61(2):89–98. doi: 10.18926/AMO/32882
 192. Yarmola EG, Shah YY, Lakes EH, Pacheco YC, Xie DF, Dobson J, et al. Use of Magnetic Capture to Identify Elevated Levels of CCL2 Following Intra-Articular Injection of Monoiodoacetate in Rats. *Connect Tissue Res* (2020) 61(5):485–97. doi: 10.1080/03008207.2019.1620223
 193. Lai-Zhao Y, Pitchers KK, Appleton CT. Transient Anabolic Effects of Synovium in Early Post-Traumatic Osteoarthritis: A Novel Ex Vivo Joint Tissue Co-Culture System for Investigating Synovium-Chondrocyte Interactions. *Osteoarthritis Cartilage* (2021) 29(7):1060–70. doi: 10.1016/j.joca.2021.03.010
 194. Takayanagi H. New Developments in Osteoimmunology. *Nat Rev Rheumatol* (2012) 8(11):684–9. doi: 10.1038/nrheum.2012.167
 195. Hirose S, Lin Q, Ohtsui M, Nishimura H, Verbeek JS. Monocyte Subsets Involved in the Development of Systemic Lupus Erythematosus and Rheumatoid Arthritis. *Int Immunol* (2019) 31(11):687–96. doi: 10.1093/intimm/dxz036
 196. van den Bosch MH, Blom AB, Schelbergen RF, Koenders MI, van de Loo PA, van den Berg WB, et al. Alarmin S100A9 Induces Proinflammatory and

- Catabolic Effects Predominantly in the M1 Macrophages of Human Osteoarthritic Synovium. *J Rheumatol* (2016) 43(10):1874–84. doi: 10.3899/jrheum.160270
197. Cremers NAJ, van den Bosch MHJ, van Dalen S, Di Ceglie I, Ascone G, van de Loo F, et al. S100A8/A9 Increases the Mobilization of Pro-Inflammatory Ly6C(high) Monocytes to the Synovium During Experimental Osteoarthritis. *Arthritis Res Ther* (2017) 19(1):217. doi: 10.1186/s13075-017-1426-6
 198. Schelbergen RF, Blom AB, van den Bosch MH, Sloetjes A, Abdollahi-Roodsaz S, Schreurs BW, et al. Alarmins S100A8 and S100A9 Elicit a Catabolic Effect in Human Osteoarthritic Chondrocytes That is Dependent on Toll-Like Receptor 4. *Arthritis Rheum* (2012) 64(5):1477–87. doi: 10.1002/art.33495
 199. Zreiqat H, Belluoccio D, Smith MM, Wilson R, Rowley LA, Jones K, et al. S100A8 and S100A9 in Experimental Osteoarthritis. *Arthritis Res Ther* (2010) 12(1):R16. doi: 10.1186/ar2917
 200. Mahon OR, Kelly DJ, McCarthy GM, Dunne A. Osteoarthritis-Associated Basic Calcium Phosphate Crystals Alter Immune Cell Metabolism and Promote M1 Macrophage Polarization. *Osteoarthritis Cartilage* (2020) 28(5):603–12. doi: 10.1016/j.joca.2019.10.010
 201. Schneider A, Weier M, Herderschee J, Perreau M, Calandra T, Roger T, et al. IRF5 Is a Key Regulator of Macrophage Response to Lipopolysaccharide in Newborns. *Front Immunol* (2018) 9:1597. doi: 10.3389/fimmu.2018.01597
 202. Krausgruber T, Blazek K, Smallie T, Alzabin S, Lockstone H, Sahgal N, et al. IRF5 Promotes Inflammatory Macrophage Polarization and TH1-TH17 Responses. *Nat Immunol* (2011) 12(3):231–8. doi: 10.1038/ni.1990
 203. Ni Z, Zhao X, Dai X, Zhao L, Xia J. The Role of Interferon Regulatory Factor 5 in Macrophage Inflammation During Osteoarthritis. *Inflammation* (2019) 42(5):1821–9. doi: 10.1007/s10753-019-01044-8
 204. Alonso-Perez A, Franco-Trepas E, Guillan-Fresco M, Jorge-Mora A, Lopez V, Pino J, et al. Role of Toll-Like Receptor 4 on Osteoblast Metabolism and Function. *Front Physiol* (2018) 9:504. doi: 10.3389/fphys.2018.00504
 205. Kalaitzoglou E, Lopes EBP, Fu Y, Herron JC, Flaming JM, Donovan EL, et al. TLR4 Promotes and DAP12 Limits Obesity-Induced Osteoarthritis in Aged Female Mice. *JBM R Plus* (2019) 3(4):e10079. doi: 10.1002/jbm.4.10079
 206. Franco-Trepas E, Guillan-Fresco M, Alonso-Perez A, Jorge-Mora A, Francisco V, Gualillo O, et al. Visfatin Connection: Present and Future in Osteoarthritis and Osteoporosis. *J Clin Med* (2019) 8(8). doi: 10.3390/jcm8081178
 207. Moschen AR, Kaser A, Enrich B, Mosheimer B, Theurl M, Niederegger H, et al. Visfatin, an Adipocytokine With Proinflammatory and Immunomodulating Properties. *J Immunol* (2007) 178(3):1748–58. doi: 10.4049/jimmunol.178.3.1748
 208. Dahl TB, Yndestad A, Skjelland M, Oie E, Dahl A, Michelsen A, et al. Increased Expression of Visfatin in Macrophages of Human Unstable Carotid and Coronary Atherosclerosis: Possible Role in Inflammation and Plaque Destabilization. *Circulation* (2007) 115(8):972–80. doi: 10.1161/CIRCULATIONAHA.106.665893
 209. Hong EH, Yun HS, Kim J, Um HD, Lee KH, Kang CM, et al. Nicotinamide Phosphoribosyltransferase Is Essential for Interleukin-1 β -Mediated Dedifferentiation of Articular Chondrocytes via SIRT1 and Extracellular Signal-Regulated Kinase (ERK) Complex Signaling. *J Biol Chem* (2011) 286(32):28619–31. doi: 10.1074/jbc.M111.219832
 210. Laiguelon MC, Houard X, Bougault C, Gosset M, Nourissat G, Sautet A, et al. Expression and Function of Visfatin (Nampt), an Adipokine-Enzyme Involved in Inflammatory Pathways of Osteoarthritis. *Arthritis Res Ther* (2014) 16(1):R38. doi: 10.1186/ar4467
 211. Mahjoub M, Berenbaum F, Houard X. Why Subchondral Bone in Osteoarthritis? The Importance of the Cartilage Bone Interface in Osteoarthritis. *Osteoporos Int* (2012) 23 Suppl 8:S841–6. doi: 10.1007/s00198-012-2161-0
 212. Brentano F, Schorr O, Ospelt C, Stanczyk J, Gay RE, Gay S, et al. Pre-B Cell Colony-Enhancing Factor/Visfatin, a New Marker of Inflammation in Rheumatoid Arthritis With Proinflammatory and Matrix-Degrading Activities. *Arthritis Rheum* (2007) 56(9):2829–39. doi: 10.1002/art.22833
 213. Meier FM, Frommer KW, Peters MA, Brentano F, Lefevre S, Schroder D, et al. Visfatin/pre-B-Cell Colony-Enhancing Factor (PBEF), a Proinflammatory and Cell Motility-Changing Factor in Rheumatoid Arthritis. *J Biol Chem* (2012) 287(34):28378–85. doi: 10.1074/jbc.M111.312884
 214. Evans L, Williams AS, Hayes AJ, Jones SA, Nowell M. Suppression of Leukocyte Infiltration and Cartilage Degradation by Selective Inhibition of Pre-B Cell Colony-Enhancing Factor/Visfatin/Nicotinamide Phosphoribosyltransferase: Apo866-Mediated Therapy in Human Fibroblasts and Murine Collagen-Induced Arthritis. *Arthritis Rheum* (2011) 63(7):1866–77. doi: 10.1002/art.30338
 215. Paoletti A, Rohmer J, Ly B, Pascaud J, Riviere E, Seror R, et al. Monocyte/Macrophage Abnormalities Specific to Rheumatoid Arthritis Are Linked to miR-155 and Are Differentially Modulated by Different TNF Inhibitors. *J Immunol* (2019) 203(7):1766–75. doi: 10.4049/jimmunol.1900386
 216. Ong SM, Hadadi E, Dang TM, Yeap WH, Tan CT, Ng TP, et al. The Pro-Inflammatory Phenotype of the Human non-Classical Monocyte Subset is Attributed to Senescence. *Cell Death Dis* (2018) 9(3):266. doi: 10.1038/s41419-018-0327-1
 217. Loeser RF, Olex AL, McNulty MA, Carlson CS, Callahan MP, Ferguson CM, et al. Microarray Analysis Reveals Age-Related Differences in Gene Expression During the Development of Osteoarthritis in Mice. *Arthritis Rheum* (2012) 64(3):705–17. doi: 10.1002/art.33388
 218. Rowe MA, Harper LR, McNulty MA, Lau AG, Carlson CS, Leng L, et al. Reduced Osteoarthritis Severity in Aged Mice With Deletion of Macrophage Migration Inhibitory Factor. *Arthritis Rheumatol* (2017) 69(2):352–61. doi: 10.1002/art.39844
 219. Smith MD. The Normal Synovium. *Open Rheumatol J* (2011) 5:100–6. doi: 10.2174/1874312901105010100
 220. Klein-Wieringa IR, de Lange-Brokaar BJ, Yusuf E, Andersen SN, Kwekkeboom JC, Kroon HM, et al. Inflammatory Cells in Patients With Endstage Knee Osteoarthritis: A Comparison Between the Synovium and the Infrapatellar Fat Pad. *J Rheumatol* (2016) 43(4):771–8. doi: 10.3899/jrheum.151068
 221. Garriga C, Goff M, Paterson E, Hrusicka R, Hamid B, Alderson J, et al. Clinical and Molecular Associations With Outcomes at 2 Years After Acute Knee Injury: A Longitudinal Study in the Knee Injury Cohort at the Kennedy (KICK). *Lancet Rheumatol* (2021) 3:648–58. doi: 10.1016/S2665-9913(21)00116-8
 222. Pradhan P, Vijayan V, Gueler F, Immenschuh S. Interplay of Heme With Macrophages in Homeostasis and Inflammation. *Int J Mol Sci* (2020) 21(3). doi: 10.3390/ijms21030740
 223. Struglics A, Okroj M, Sward P, Probell R, Saxne T, Lohmander LS, et al. The Complement System is Activated in Synovial Fluid From Subjects With Knee Injury and From Patients With Osteoarthritis. *Arthritis Res Ther* (2016) 18(1):223. doi: 10.1186/s13075-016-1123-x
 224. Wang Q, Rozelle AL, Lepus CM, Scanzello CR, Song JJ, Larsen DM, et al. Identification of a Central Role for Complement in Osteoarthritis. *Nat Med* (2011) 17(12):1674–9. doi: 10.1038/nm.2543
 225. Cambre I, Gaublumme D, Burssens A, Jacques P, Schryvers N, De Muynck A, et al. Mechanical Strain Determines the Site-Specific Localization of Inflammation and Tissue Damage in Arthritis. *Nat Commun* (2018) 9(1):4613. doi: 10.1038/s41467-018-06933-4
 226. Otsuki S, Brinson DC, Creighton L, Kinoshita M, Sah RL, D'Lima D, et al. The Effect of Glycosaminoglycan Loss on Chondrocyte Viability: A Study on Porcine Cartilage Explants. *Arthritis Rheum* (2008) 58(4):1076–85. doi: 10.1002/art.23381
 227. Quinn TM, Grodzinsky AJ, Hunziker EB, Sandy JD. Effects of Injurious Compression on Matrix Turnover Around Individual Cells in Calf Articular Cartilage Explants. *J Orthop Res* (1998) 16(4):490–9. doi: 10.1002/jor.1100160415
 228. D'Lima DD, Hashimoto S, Chen PC, Colwell CW Jr., Lotz MK. Impact of Mechanical Trauma on Matrix and Cells. *Clin Orthop Relat Res* (2001) 391 Suppl:S90–9. doi: 10.1097/00003086-200110001-00009
 229. Maldonado M, Nam J. The Role of Changes in Extracellular Matrix of Cartilage in the Presence of Inflammation on the Pathology of Osteoarthritis. *BioMed Res Int* (2013) 2013:284873. doi: 10.1155/2013/284873
 230. Adair-Kirk TL, Senior RM. Fragments of Extracellular Matrix as Mediators of Inflammation. *Int J Biochem Cell Biol* (2008) 40(6–7):1101–10. doi: 10.1016/j.biocel.2007.12.005
 231. Senior RM, Hinek A, Griffin GL, Pipoly DJ, Crouch EC, Mecham RP. Neutrophils Show Chemotaxis to Type IV Collagen and its 7S Domain and

- Contain a 67 kD Type IV Collagen Binding Protein With Lectin Properties. *Am J Respir Cell Mol Biol* (1989) 1(6):479–87. doi: 10.1165/ajrcmb.1.6.479
232. Duca L, Blanchevoye C, Cantarelli B, Ghoneim C, Dedieu S, Delacoux F, et al. The Elastin Receptor Complex Transduces Signals Through the Catalytic Activity of its Neu-1 Subunit. *J Biol Chem* (2007) 282(17):12484–91. doi: 10.1074/jbc.M609505200
 233. Noble PW, McKee CM, Cowman M, Shin HS. Hyaluronan Fragments Activate an NF-Kappa B/I-Kappa B Alpha Autoregulatory Loop in Murine Macrophages. *J Exp Med* (1996) 183(5):2373–8. doi: 10.1084/jem.183.5.2373
 234. Bonnans C, Chou J, Werb Z. Remodelling the Extracellular Matrix in Development and Disease. *Nat Rev Mol Cell Biol* (2014) 15(12):786–801. doi: 10.1038/nrm3904
 235. Patel L, Sun W, Glasson SS, Morris EA, Flannery CR, Chockalingam PS. Tenascin-C Induces Inflammatory Mediators and Matrix Degradation in Osteoarthritic Cartilage. *BMC Musculoskelet Disord* (2011) 12:164. doi: 10.1186/1471-2474-12-164
 236. Schaefer L, Babelova A, Kiss E, Hausser HJ, Babelova M, Krzyzankova M, et al. The Matrix Component Biglycan is Proinflammatory and Signals Through Toll-Like Receptors 4 and 2 in Macrophages. *J Clin Invest* (2005) 115(8):2223–33. doi: 10.1172/JCI23755
 237. Fan L, Wang Q, Liu R, Zong M, He D, Zhang H, et al. Citrullinated Fibronectin Inhibits Apoptosis and Promotes the Secretion of Pro-Inflammatory Cytokines in Fibroblast-Like Synoviocytes in Rheumatoid Arthritis. *Arthritis Res Ther* (2012) 14(6):R266. doi: 10.1186/ar4112
 238. Newton K, Dixit VM. Signaling in Innate Immunity and Inflammation. *Cold Spring Harb Perspect Biol* (2012) 4(3). doi: 10.1101/cshperspect.a006049
 239. Kim HA, Cho ML, Choi HY, Yoon CS, Jhun JY, Oh HJ, et al. The Catabolic Pathway Mediated by Toll-Like Receptors in Human Osteoarthritic Chondrocytes. *Arthritis Rheum* (2006) 54(7):2152–63. doi: 10.1002/art.21951
 240. Benito MJ, Veale DJ, FitzGerald O, van den Berg WB, Bresnihan B. Synovial Tissue Inflammation in Early and Late Osteoarthritis. *Ann Rheum Dis* (2005) 64(9):1263–7. doi: 10.1136/ard.2004.025270
 241. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular Mechanism and Function of CD40/CD40L Engagement in the Immune System. *Immunol Rev* (2009) 229(1):152–72. doi: 10.1111/j.1600-065X.2009.00782.x
 242. Graf D, Muller S, Korthauer U, van Kooten C, Weise C, Kroczeck RA. A Soluble Form of TRAP (CD40 Ligand) is Rapidly Released After T Cell Activation. *Eur J Immunol* (1995) 25(6):1749–54. doi: 10.1002/eji.1830250639
 243. Zirikli A, Maier C, Gerdes N, MacParlane L, Soosairajah J, Bavendiek U, et al. CD40 Ligand Mediates Inflammation Independently of CD40 by Interaction With Mac-1. *Circulation* (2007) 115(12):1571–80. doi: 10.1161/CIRCULATIONAHA.106.683201
 244. Perazzio SF, Soeiro-Pereira PV, Dos Santos VC, de Brito MV, Salu B, Oliva MLV, et al. Soluble CD40L is Associated With Increased Oxidative Burst and Neutrophil Extracellular Trap Release in Behcet's Disease. *Arthritis Res Ther* (2017) 19(1):235. doi: 10.1186/s13075-017-1443-5
 245. Soong RS, Song L, Trieu J, Lee SY, He L, Tsai YC, et al. Direct T Cell Activation via CD40 Ligand Generates High Avidity CD8+ T Cells Capable of Breaking Immunological Tolerance for the Control of Tumors. *PLoS One* (2014) 9(3):e93162. doi: 10.1371/journal.pone.0093162
 246. Guo Y, Walsh AM, Fearon U, Smith MD, Wechalekar MD, Yin X, et al. CD40L-Dependent Pathway Is Active at Various Stages of Rheumatoid Arthritis Disease Progression. *J Immunol* (2017) 198(11):4490–501. doi: 10.4049/jimmunol.1601988
 247. MacDonald KP, Nishioka Y, Lipsky PE, Thomas R. Functional CD40 Ligand is Expressed by T Cells in Rheumatoid Arthritis. *J Clin Invest* (1997) 100(9):2404–14. doi: 10.1172/JCI119781
 248. Roman-Fernandez IV, Garcia-Chagollan M, Cerpa-Cruz S, Jave-Suarez LF, Palafox-Sanchez CA, Garcia-Arellano S, et al. Assessment of CD40 and CD40L Expression in Rheumatoid Arthritis Patients, Association With Clinical Features and DAS28. *Clin Exp Med* (2019) 19(4):427–37. doi: 10.1007/s10238-019-00568-5
 249. Liu MF, Chao SC, Wang CR, Lei HY. Expression of CD40 and CD40 Ligand Among Cell Populations Within Rheumatoid Synovial Compartment. *Autoimmunity* (2001) 34(2):107–13. doi: 10.3109/08916930109001958
 250. Lai JH, Luo SF, Ho LJ. Targeting the CD40-CD154 Signaling Pathway for Treatment of Autoimmune Arthritis. *Cells* (2019) 8(8). doi: 10.3390/cells8080927
 251. Gierut A, Perlman H, Pope RM. Innate Immunity and Rheumatoid Arthritis. *Rheum Dis Clin North Am* (2010) 36(2):271–96. doi: 10.1016/j.rdc.2010.03.004
 252. Haubruck PSC, Liu Y, Moradi B, Blaker C, Clarke E, Little CB. Evaluating the Role and Origin of Pro-Inflammatory Macrophage Subsets as Part of the Cellular Immune Response During the Onset and Development of Posttraumatic Osteoarthritis in Mice. In: *ORS 2021 Annual Meeting* (2020). Virtual.
 253. Pottier P, Presle N, Terlain B, Netter P, Mainard D, Berenbaum F. Obesity and Osteoarthritis: More Complex Than Predicted! *Ann Rheum Dis* (2006) 65(11):1403–5. doi: 10.1136/ard.2006.061994
 254. Datta P, Zhang Y, Parousis A, Sharma A, Rossomacha E, Endisha H, et al. High-Fat Diet-Induced Acceleration of Osteoarthritis is Associated With a Distinct and Sustained Plasma Metabolite Signature. *Sci Rep* (2017) 7(1):8205. doi: 10.1038/s41598-017-07963-6
 255. Larrañaga-Vera A, Lamuedra A, Pérez-Baos S, Prieto-Potin I, Peña L, Herrero-Beaumont G, et al. Increased Synovial Lipodystrophy Induced by High Fat Diet Aggravates Synovitis in Experimental Osteoarthritis. *Arthritis Res Ther* (2017) 19(1):264. doi: 10.1186/s13075-017-1473-z
 256. Harasymowicz NS, Choi Y-R, Wu C-L, Iannucci L, Tang R, Guilak F. Intergenerational Transmission of Diet-Induced Obesity, Metabolic Imbalance, and Osteoarthritis in Mice. *Arthritis Rheumatol* (2020) 72(4):632–44. doi: 10.1002/art.41147
 257. Shen J, Abu-Amer Y, O'Keefe RJ, McAlinden A. Inflammation and Epigenetic Regulation in Osteoarthritis. *Connective Tissue Res* (2017) 58(1):49–63. doi: 10.1080/03008207.2016.1208655
 258. Raman S, FitzGerald U, Murphy JM. Interplay of Inflammatory Mediators With Epigenetics and Cartilage Modifications in Osteoarthritis. *Front Bioeng Biotechnol* (2018) 6:22. doi: 10.3389/fbioe.2018.00022
 259. Hoeksma MA, de Winther MP. Epigenetic Regulation of Monocyte and Macrophage Function. *Antioxid Redox Signal* (2016) 25(14):758–74. doi: 10.1089/ars.2016.6695
 260. Kapellos TS, Iqbal AJ. Epigenetic Control of Macrophage Polarisation and Soluble Mediator Gene Expression During Inflammation. *Mediators Inflammation* (2016) 2016:6591703. doi: 10.1155/2016/6591703
 261. Logie C, Stunnenberg HG. Epigenetic Memory: A Macrophage Perspective. *Semin Immunol* (2016) 28(4):359–67. doi: 10.1016/j.smim.2016.06.003
 262. Chen S, Yang J, Wei Y, Wei X. Epigenetic Regulation of Macrophages: From Homeostasis Maintenance to Host Defense. *Cell Mol Immunol* (2020) 17(1):36–49. doi: 10.1038/s41423-019-0315-0
 263. Soucie EL, Weng Z, Geirsdóttir L, Molawi K, Maurizio J, Fenouil R, et al. Lineage-Specific Enhancers Activate Self-Renewal Genes in Macrophages and Embryonic Stem Cells. *Science* (2016) 351(6274):aad5510. doi: 10.1126/science.aad5510
 264. Heinz S, Benner C, Spann N, Bertolino E, Lin YC, Laslo P, et al. Simple Combinations of Lineage-Determining Transcription Factors Prime Cis-Regulatory Elements Required for Macrophage and B Cell Identities. *Mol Cell* (2010) 38(4):576–89. doi: 10.1016/j.molcel.2010.05.004
 265. Kruidenier L, Chung CW, Cheng Z, Liddle J, Che K, Joberty G, et al. A Selective Jumonji H3K27 Demethylase Inhibitor Modulates the Proinflammatory Macrophage Response. *Nature* (2012) 488(7411):404–8. doi: 10.1038/nature11262
 266. Sohn DH, Sokolove J, Sharpe O, Erhart JC, Chandra PE, Lahey LJ, et al. Plasma Proteins Present in Osteoarthritic Synovial Fluid can Stimulate Cytokine Production via Toll-Like Receptor 4. *Arthritis Res Ther* (2012) 14(1):R7–R. doi: 10.1186/ar3555
 267. Rhoads JP, Major AS, Rathmell JC. Fine Tuning of Immunometabolism for the Treatment of Rheumatic Diseases. *Nat Rev Rheumatol* (2017) 13(5):313–20. doi: 10.1038/nrrheum.2017.54
 268. Hong YH, Kong EJ. (18)Fluoro-Deoxy-D-Glucose Uptake of Knee Joints in the Aspect of Age-Related Osteoarthritis: A Case-Control Study. *BMC Musculoskelet Disord* (2013) 14:141. doi: 10.1186/1471-2474-14-141
 269. Courties A, Gualillo O, Berenbaum F, Sellam J. Metabolic Stress-Induced Joint Inflammation and Osteoarthritis. *Osteoarthritis Cartilage* (2015) 23(11):1955–65. doi: 10.1016/j.joca.2015.05.016

270. Netea MG, Dominguez-Andrés J, Barreiro LB, Chavakis T, Divangahi M, Fuchs E, et al. Defining Trained Immunity and its Role in Health and Disease. *Nat Rev Immunol* (2020) 20(6):375–88. doi: 10.1038/s41577-020-0285-6
271. Blaker CL, Zaki S, Little CB, Clarke EC. Long-Term Effect of a Single Subcritical Knee Injury: Increasing the Risk of Anterior Cruciate Ligament Rupture and Osteoarthritis. *Am J Sports Med* (2021) 49(2):391–403. doi: 10.1177/0363546520977505
272. Wang X, Oo WM, Linklater JM. What is the Role of Imaging in the Clinical Diagnosis of Osteoarthritis and Disease Management? *Rheumatol (Oxford)* (2018) 57(suppl_4):iv51–60. doi: 10.1093/rheumatology/kex501
273. Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H, et al. Osteoarthritis. *Lancet* (2015) 386(9991):376–87. doi: 10.1016/S0140-6736(14)60802-3
274. Shi J, Zhao W, Ying H, Du J, Chen J, Chen S, et al. The Relationship of Platelet to Lymphocyte Ratio and Neutrophil to Monocyte Ratio to Radiographic Grades of Knee Osteoarthritis. *Z Rheumatol* (2018) 77(6):533–7. doi: 10.1007/s00393-017-0348-7
275. Gao K, Zhu W, Liu W, Ma D, Li H, Yu W, et al. Diagnostic Value of the Blood Monocyte-Lymphocyte Ratio in Knee Osteoarthritis. *J Int Med Res* (2019) 47(9):4413–21. doi: 10.1177/0300060519860686
276. Soyocak A, Kurt H, Ozgen M, Turgut Cosan D, Colak E, Gunes HV. miRNA-146a, miRNA-155 and JNK Expression Levels in Peripheral Blood Mononuclear Cells According to Grade of Knee Osteoarthritis. *Gene* (2017) 627:207–11. doi: 10.1016/j.gene.2017.06.027
277. Taganov KD, Boldin MP, Chang KJ, Baltimore D. NF- κ B-Dependent Induction of microRNA miR-146, an Inhibitor Targeted to Signaling Proteins of Innate Immune Responses. *Proc Natl Acad Sci U.S.A.* (2006) 103(33):12481–6. doi: 10.1073/pnas.0605298103
278. Ceppi M, Pereira PM, Dunand-Sauthier I, Barras E, Reith W, Santos MA, et al. MicroRNA-155 Modulates the Interleukin-1 Signaling Pathway in Activated Human Monocyte-Derived Dendritic Cells. *Proc Natl Acad Sci U.S.A.* (2009) 106(8):2735–40. doi: 10.1073/pnas.0811073106
279. Shi T, Shen X, Gao G. Gene Expression Profiles of Peripheral Blood Monocytes in Osteoarthritis and Analysis of Differentially Expressed Genes. *BioMed Res Int* (2019) 2019:4291689. doi: 10.1155/2019/4291689
280. Loukov D, Karampatos S, Maly MR, Bowdish DME. Monocyte Activation is Elevated in Women With Knee-Osteoarthritis and Associated With Inflammation, BMI and Pain. *Osteoarthritis Cartilage* (2018) 26(2):255–63. doi: 10.1016/j.joca.2017.10.018
281. Kriegova E, Manukyan G, Mikulkova Z, Gabcova G, Kudelka M, Gajdos P, et al. Gender-Related Differences Observed Among Immune Cells in Synovial Fluid in Knee Osteoarthritis. *Osteoarthritis Cartilage* (2018) 26(9):1247–56. doi: 10.1016/j.joca.2018.04.016
282. Liu B, Zhang M, Zhao J, Zheng M, Yang H. Imbalance of M1/M2 Macrophages is Linked to Severity Level of Knee Osteoarthritis. *Exp Ther Med* (2018) 16(6):5009–14. doi: 10.3892/etm.2018.6852
283. Allen PI, Conzemius MG, Evans RB, Kiefer K. Correlation Between Synovial Fluid Cytokine Concentrations and Limb Function in Normal Dogs and in Dogs With Lameness From Spontaneous Osteoarthritis. *Vet Surg* (2019) 48(5):770–9. doi: 10.1111/vsu.13212
284. Monibi F, Roller BL, Stoker A, Gerner B, Bal S, Cook JL. Identification of Synovial Fluid Biomarkers for Knee Osteoarthritis and Correlation With Radiographic Assessment. *J Knee Surg* (2016) 29(3):242–7. doi: 10.1055/s-0035-1549022
285. Cuellar VG, Cuellar JM, Kirsch T, Strauss EJ. Correlation of Synovial Fluid Biomarkers With Cartilage Pathology and Associated Outcomes in Knee Arthroscopy. *Arthroscopy* (2016) 32(3):475–85. doi: 10.1016/j.arthro.2015.08.033
286. MacFarlane LA, Yang H, Collins JE, Jarraya M, Guermazi A, Mandl LA, et al. Association of Changes in Effusion-Synovitis With Progression of Cartilage Damage Over Eighteen Months in Patients With Osteoarthritis and Meniscal Tear. *Arthritis Rheumatol* (2019) 71(1):73–81. doi: 10.1002/art.40660
287. Haraden CA, Huebner JL, Hsueh MF, Li YJ, Kraus VB. Synovial Fluid Biomarkers Associated With Osteoarthritis Severity Reflect Macrophage and Neutrophil Related Inflammation. *Arthritis Res Ther* (2019) 21(1):146. doi: 10.1186/s13075-019-1923-x
288. Hsueh MF, Zhang X, Wellman SS, Bolognesi MP, Kraus VB. Synergistic Roles of Macrophages and Neutrophils in Osteoarthritis Progression. *Arthritis Rheumatol* (2021) 73(1):89–99. doi: 10.1002/art.41486
289. Jayadev C, Hulley P, Swales C, Snelling S, Collins G, Taylor P, et al. Synovial Fluid Fingerprinting in End-Stage Knee Osteoarthritis: A Novel Biomarker Concept. *Bone Joint Res* (2020) 9(9):623–32. doi: 10.1302/2046-3758.99.BJR-2019-0192.R1
290. Shih L, Guler N, Syed D, Hopkinson W, McComas KN, Walborn A, et al. Postoperative Changes in the Systemic Inflammatory Milieu in Older Surgical Patients. *Clin Appl Thromb Hemost* (2018) 24(4):583–8. doi: 10.1177/1076029617747412
291. Ghouri A, Conaghan PG. Update on Novel Pharmacological Therapies for Osteoarthritis. *Ther Adv Musculoskelet Dis* (2019) 11:1759720X19864492. doi: 10.1177/1759720X19864492
292. Furman BD, Mangiapani DS, Zeitler E, Bailey KN, Horne PH, Huebner JL, et al. Targeting Pro-Inflammatory Cytokines Following Joint Injury: Acute Intra-Articular Inhibition of Interleukin-1 Following Knee Injury Prevents Post-Traumatic Arthritis. *Arthritis Res Ther* (2014) 16(3):R134. doi: 10.1186/ar4591
293. Kimmerling KA, Furman BD, Mangiapani DS, Moverman MA, Sinclair SM, Huebner JL, et al. Sustained Intra-Articular Delivery of IL-1RA From a Thermally-Responsive Elastin-Like Polypeptide as a Therapy for Post-Traumatic Arthritis. *Eur Cell Mater* (2015) 29:124–39; discussion 39–40. doi: 10.22203/ecm.v029a10
294. Olson SA, Furman BD, Kraus VB, Huebner JL, Guilak F. Therapeutic Opportunities to Prevent Post-Traumatic Arthritis: Lessons From the Natural History of Arthritis After Articular Fracture. *J Orthop Res* (2015) 33(9):1266–77. doi: 10.1002/jor.22940
295. Kraus VB, Birmingham J, Stabler TV, Feng S, Taylor DC, Moorman CT, et al. 3rd Effects of Intraarticular IL-1-Ra for Acute Anterior Cruciate Ligament Knee Injury: A Randomized Controlled Pilot Trial (NCT00332254). *Osteoarthritis Cartilage* (2012) 20(4):271–8. doi: 10.1016/j.joca.2011.12.009
296. Schieker M, Conaghan PG, Mindeholm L, Praetgaard J, Solomon DH, Scotti C, et al. Effects of Interleukin-1 β Inhibition on Incident Hip and Knee Replacement : Exploratory Analyses From a Randomized, Double-Blind, Placebo-Controlled Trial. *Ann Intern Med* (2020) 173(7):509–15. doi: 10.7326/m20-0527
297. Mei J, Sun J, Wu J, Zheng X. Liraglutide Suppresses TNF- α -Induced Degradation of Extracellular Matrix in Human Chondrocytes: A Therapeutic Implication in Osteoarthritis. *Am J Transl Res* (2019) 11(8):4800–8.
298. Utomo L, van Osch GJ, Bayon Y, Verhaar JA, Bastiaansen-Jenniskens YM. Guiding Synovial Inflammation by Macrophage Phenotype Modulation: An *In Vitro* Study Towards a Therapy for Osteoarthritis. *Osteoarthritis Cartilage* (2016) 24(9):1629–38. doi: 10.1016/j.joca.2016.04.013
299. Sieker JT, Ayturk UM, Proffen BL, Weissenberger MH, Kiapour AM, Murray MM. Immediate Administration of Intraarticular Triamcinolone Acetonide After Joint Injury Modulates Molecular Outcomes Associated With Early Synovitis. *Arthritis Rheumatol* (2016) 68(7):1637–47. doi: 10.1002/art.39631
300. Shu CC, Zaki S, Ravi V, Schiavinato A, Smith MM, Little CB. The Relationship Between Synovial Inflammation, Structural Pathology, and Pain in Post-Traumatic Osteoarthritis: Differential Effect of Stem Cell and Hyaluronan Treatment. *Arthritis Res Ther* (2020) 22(1):29. doi: 10.1186/s13075-020-2117-2
301. Xie JW, Wang Y, Xiao K, Xu H, Luo ZY, Li L, et al. Alpha Defensin-1 Attenuates Surgically Induced Osteoarthritis in Association With Promoting M1 to M2 Macrophage Polarization. *Osteoarthritis Cartilage* (2021) 4. doi: 10.1016/j.joca.2021.04.006
302. Thomas AC, Hubbard-Turner T, Wikstrom EA, Palmieri-Smith RM. Epidemiology of Posttraumatic Osteoarthritis. *J Athl Train* (2017) 52(6):491–6. doi: 10.4085/1062-6050-51.5.08
303. Snoeker B, Turkiewicz A, Magnusson K, Frobelt R, Yu D, Peat G, et al. Risk of Knee Osteoarthritis After Different Types of Knee Injuries in Young Adults: A Population-Based Cohort Study. *Br J Sports Med* (2020) 54(12):725–30. doi: 10.1136/bjsports-2019-100959
304. Cook AE, Cook JL, Stoker AM. Metabolic Responses of Meniscus to IL-1 β . *J Knee Surg* (2018) 31(9):834–40. doi: 10.1055/s-0037-1615821
305. Favero M, Belluzzi E, Trisolino G, Goldring MB, Goldring SR, Cigolotti A, et al. Inflammatory Molecules Produced by Meniscus and Synovium in Early and End-Stage Osteoarthritis: A Coculture Study. *J Cell Physiol* (2019) 234(7):11176–87. doi: 10.1002/jcp.27766

306. Tsuchida AI, Beekhuizen M, t Hart MC, Radstake TR, Dhert WJ, Saris DB, et al. Cytokine Profiles in the Joint Depend on Pathology, But are Different Between Synovial Fluid, Cartilage Tissue and Cultured Chondrocytes. *Arthritis Res Ther* (2014) 16(5):441. doi: 10.1186/s13075-014-0441-0
307. Zaki S, Blaker CL, Little CB. OA Foundations - Experimental Models of Osteoarthritis. *Osteoarthritis Cartilage* (2021) 3. doi: 10.1016/j.joca.2021.03.024
308. Cucchiaroni M, de Girolamo L, Filardo G, Oliveira JM, Orth P, Pape D, et al. Basic Science of Osteoarthritis. *J Exp Orthopaedics* (2016) 3(1):22. doi: 10.1186/s40634-016-0060-6
309. Little CB, Hunter DJ. Post-Traumatic Osteoarthritis: From Mouse Models to Clinical Trials. *Nat Rev Rheumatol* (2013) 9(8):485–97. doi: 10.1038/nrrheum.2013.72
310. Zaki S, Smith MM, Little CB. Pathology-Pain Relationships in Different Osteoarthritis Animal Model Phenotypes: It Matters What You Measure, When You Measure, and How You Got There. *Osteoarthritis Cartilage* (2021) 29(10):1448–61. doi: 10.1016/j.joca.2021.03.023
311. Zaki S, Smith MM, Smith SM, Little CB. Differential Patterns of Pathology in and Interaction Between Joint Tissues in Long-Term Osteoarthritis With Different Initiating Causes: Phenotype Matters. *Osteoarthritis Cartilage* (2020) 28(7):953–65. doi: 10.1016/j.joca.2020.04.009
312. Soul J, Barter MJ, Little CB, Young DA. OATargets: A Knowledge Base of Genes Associated With Osteoarthritis Joint Damage in Animals. *Ann Rheum Dis* (2020) 80(3):376–83. doi: 10.1136/annrheumdis-2020-218344

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1.4 Gaps in Knowledge

Despite considerable research efforts persisting gaps in knowledge exist regarding the contribution of the immune response towards pathogenesis of ptOA. Importantly, these gaps can be divided into cellular and molecular factors that: 1) drive inflammation post joint injury and during onset and development of ptOA; and 2) and of these, those might serve as promising targets for early immunomodulatory treatment options, capable of preventing or mitigating ptOA development. Important questions that have been identified and are not fully understood include:

- 1) Does the type of joint injury affect the resulting cellular inflammatory response or do phenotype specific differences in the inflammatory pattern exist?
- 2) Does in-vivo modulation of pro-inflammatory T-helper cell subsets in the peri-joint-injury period result in mitigated pathology or prevent ptOA from developing?
- 3) Does in-vivo modulation of pro-inflammatory myeloid cell subsets in the peri-joint-injury period result in mitigated pathology or prevent ptOA from developing?
- 4) Can molecular pathways/factors that impact crosstalk between the innate and adaptive immune response be used to reduce inflammation post joint injury and protect from developing ptOA?

This thesis uses a series of consecutive studies each including a variety of experiments designed to answer the above-mentioned research questions. Furthermore, studies were designed to not only provide answers on a cellular level but also provide explanations on a molecular level and to identify important pathways that might be implicated in immune activation and the pathogenesis of ptOA.

1.4.1 Thesis Aim

To address each research question, I have developed a set of specific aims and linked objectives:

1. **Aim:** To characterize the cellular inflammatory response in context with different phenotypes of ptOA using mouse models.

a. **Objectives:**

- i. Analyse the T-cell response during ptOA development in immunological regions of interest and identify relevant subsets (e.g. Th1, Th2, Th17, Treg).
- ii. Analyse the monocyte/macrophage response during ptOA development in immunological regions of interest and identify relevant subsets (e.g. M1, M2).
- iii. Compare the immune innate and adaptive immune response between different murine ptOA models to assess if phenotype-specific differences occur.

2. **Aim:** To investigate the individual impact of CD4⁺ T-helper cell subsets on the development of ptOA in one of the phenotypes from aim 1 and their mechanisms of activation.

a. **Objectives**

- i. Evaluate the impact of a targeted depletion/inhibition of various T-helper cell subsets on the progression of ptOA and its cellular inflammatory response.
- ii. Compare the influence of individual joint tissues from naïve and osteoarthritic animals on the polarization of T-helper cells with/without specific pathway blockade.

3. **Aim:** To investigate the individual impact of different monocyte/macrophage subsets on ptOA pathogenesis in one of the phenotypes from aim 1 and evaluate their mechanisms of activation

a. **Objectives**

- i. Evaluate the impact of depletion of various monocyte/macrophage subsets on the progression of ptOA and its cellular inflammatory response.
- ii. Characterise contributing factors on a genome wide expression level and define pathways of interest that influence the development of ptOA.

4. **Aim:** To investigate the therapeutic potential of an early inhibition of factors orchestrating the interplay between innate and adaptive inflammation post joint injury in one of the phenotypes from aim 1.
 - a. **Objective**
 - i. To test the influence on cellular, molecular, and histopathological outcome parameters of ptOA after inhibition of CD40L early post joint injury.

Chapter 2

2 Methodology

2.1 Development of a Consecutive Set of Studies Investigating Individual Aspects of the Role of the Immune Response in ptOA

In this thesis I developed a consecutive set of studies specifically designed to address each aim. To comprehensively study the cellular immune response associated with the onset and development of ptOA, flow cytometry was chosen as a powerful tool that allows simultaneous evaluation of a plethora of immune cell subsets. As no routinely used panel/protocol was available, I designed and established a designated multicolour antibody panel for use with ptOA mouse models and tissues. Furthermore, in order to facilitate scoring of histopathological changes associated with ptOA while maintaining reliable scores and comparability, an existing scoring technique was streamlined utilizing a statistics approach. Thereafter, the immune response was characterized on a cellular level implementing the developed techniques on a variety of murine ptOA models, both surgical and non-surgical, to compare immune response pattern between ptOA phenotypes. Cellular key players were identified and in subsequent studies inhibited/depleted to investigate the effect on the pathogenesis of ptOA. Finally, an orchestrating factor playing a crucial role interlinking the adaptive and innate immune response was identified and inhibited post injury to investigate the potential for an early preventative or mitigating treatment.

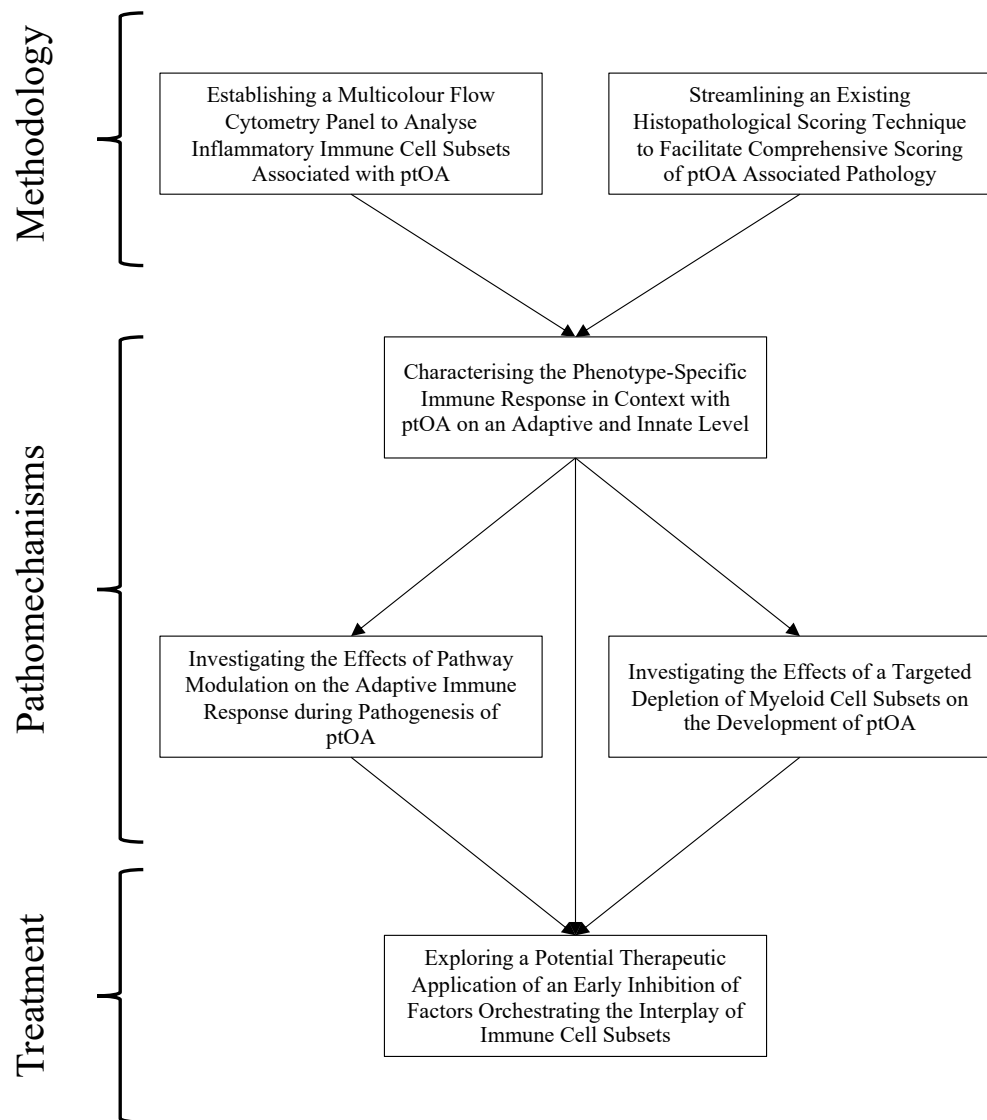


Figure 2.1: Flowchart of studies included in this thesis.

2.2 Utilization of Established and Novel Laboratory Techniques

2.2.1 Established Laboratory Techniques

A variety of established laboratory techniques have been included in this thesis. For each study the relevant techniques are described in the respective methods section and when needed a more detailed description was added as a supplementary method. In order to avoid duplication, established techniques are not described in this methodology section. All studies in this thesis investigated ptOA. This type of secondary OA was chosen as it provides the opportunity to study the development of OA from the underlying injury towards advanced stages of joint destruction in a comparably short time and offers a clear window for therapeutic intervention. All experiments conducted in this thesis were approved by the Northern Sydney Local Health District Animal Ethics Committee. For all experiments 10-12-week-old, male C57BL/6 mice were utilized, and mice were housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Health and Medical Research Council of Australia Revised 2010).

2.2.2 Animal Models used in this Thesis

In ptOA research animal models initiating ptOA development using a defined joint injury remain the gold standard as they allow the investigation of the cellular response together with the changed biomechanics that are characteristic for the disease (Blaker, C. L. et al. 2016). The most commonly used and established animal models are in the mouse, because this species provides a variety of advantages when investigating ptOA (Blaker, C. L. et al. 2017). In order to analyze if different injuries result in a differing inflammatory response and to evaluate its subsequent contribution to ptOA a reliable comparison of results from different ptOA models is crucial. In the mouse in particular, surgical and non-surgical OA models have been described (Blaker, C. L. et al. 2017): the most common remain the model utilizing a destabilization of the medial meniscus (DMM), transection of the anterior cruciate ligament model (ACLT), and non-surgical ACL rupture (ACLR) models, respectively (Lorenz, J. et al. 2014). These animal models are well suited for the investigation into the role of cellular inflammation during onset

and development of ptOA and therefore in this thesis all three animal models as well as their respective controls and naïve mice are used to allow for a comprehensive analysis of the inflammatory response.

DMM model of OA: One of the most established murine ptOA models uses surgical destabilization of the medial meniscus. The DMM model was established by Glasson et al (Glasson, S. S. et al. 2007) and showed reliable results regarding progressive cartilage erosion, osteophyte development and subchondral bone sclerosis not apparent in sham-operated animals. This model has been frequently used to demonstrate the role of specific proteins in ptOA pathogenesis. In particular, in a study by Jackson et al. (Jackson, M. T. et al. 2014) the DMM model was successfully used to show that deletion of protease-activated receptor 2 but not 1 resulted in a significant decrease in DMM induced cartilage damage in association with altered acute synovial inflammatory cell influx.

ACLT model of OA: The anterior cruciate ligament (ACL) is one of the most commonly injured structures in the knee, and more than 50% of patients sustaining an ACL rupture subsequently develop ptOA (Lohmander, L. S. et al. 2007). Multiple animal models inducing ptOA by surgical transection of the ACL (ACLT) have been introduced, with the murine model proven as a reliable and effective in inducing ptOA (Kamekura, S. et al. 2005; Little, C. B. et al. 2012). Kamekura et al. utilized this model to successfully study the cellular and molecular changes in the development of ptOA (Kamekura, S. et al. 2005).

Non-surgical anterior cruciate ligament (ACL) rupture model of OA: Besides surgical transection, the ACL can also be injured in mice non-surgically (Christiansen, B. A. et al. 2012) providing a reproducible ptOA model without the confounding issue of the initial operative trauma. A single compressive load is applied to the flexed knee resulting in posterior subluxation of the femur and ACL rupture without additional macroscopic damage to other structures within the joint (Blaker, C. L. et al. 2016). This model has the advantage of evaluating the immune response and inflammation in ptOA without the confounder of the initial incision and its associated inflammatory wound response (Blaker, C. L. et al. 2016).

In addition to these murine ptOA models appropriate controls are crucial when investigating the role of the immune system during ptOA development to account for potentially confounding factors. Therefore, surgical arthrotomy without any injury to the ACL or meniscus

(SHAM) was used to determine the effect of surgery alone and to control for DMM and ACLT mice. In addition, animals receiving mechanical ACL strain (ACLS) induced by a single compressive load to 75% of that required to rupture the ACL that does not induce progressive OA joint pathology (Blaker, C. L. et al. 2016), was used to control for effects of compressive joint loading in the ACLR group. Finally naïve non-operated control (NOC) mice were used to control for age related changes to the joint histology and the immune response and included when necessary.

2.2.3 Animal Ethics Compliance

NSLHD Animal Ethics Committee protocols that have been approved prior to the respective commencement of the study:

1. RESP/18/162 for **Chapter 2.2.2.1** and **Chapter 2.2.2.2** and **Chapter 3.2**.
2. RESP/20/083 for **Chapter 4.2**.
3. RESP/20/082 for **Chapter 5.2**.
4. RESP/19/067, RESP/20/083, RESP/20/082 and RESP/21/014 for **Chapter 6.2**.

2.2.4 Development of Novel Techniques

2.2.4.1 Published Manuscript Describing the Implementation and Testing a Novel Multicolor Flow Cytometry Analysis for Immune Cell Subsets in Context with ptOA.

Video Article

Flow Cytometry Analysis of Immune Cell Subsets within the Murine Spleen, Bone Marrow, Lymph Nodes and Synovial Tissue in an Osteoarthritis Model

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URL: <https://www.jove.com/video/61008>

DOI: [doi:10.3791/61008](https://doi.org/10.3791/61008)

Keywords: Medicine, Issue 158, Osteoarthritis, immunology, flow cytometry, lymphocyte subsets, T-cells, monocytes, macrophages, orthopedics, posttraumatic osteoarthritis

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Abstract

Osteoarthritis (OA) is one of the most prevalent musculoskeletal diseases, affecting patients suffering from pain and physical limitations. Recent evidence indicates a potential inflammatory component of the disease, with both T-cells and monocytes/macrophages potentially associated with the pathogenesis of OA. Further studies postulated an important role for subsets of both inflammatory cell lineages, such as Th1, Th2, Th17, and T-regulatory lymphocytes, and M1, M2, and synovium-tissue-resident macrophages. However, the interaction between the local synovial and systemic inflammatory cellular response and the structural changes in the joint is unknown. To fully understand how T-cells and monocytes/macrophages contribute towards OA, it is important to be able to quantitatively identify these cells and their subsets simultaneously in synovial tissue, secondary lymphatic organs and systemically (the spleen and bone marrow). Nowadays, the different inflammatory cell subsets can be identified by a combination of cell-surface markers making multi-color flow cytometry a powerful technique in investigating these cellular processes. In this protocol, we describe detailed steps regarding the harvest of synovial tissue and secondary lymphatic organs as well as generation of single cell suspensions. Furthermore, we present both an extracellular staining assay to identify monocytes/macrophages and their subsets as well as an extra- and intra-cellular staining assay to identify T-cells and their subsets within the murine spleen, bone marrow, lymph nodes and synovial tissue. Each step of this protocol was optimized and tested, resulting in a highly reproducible assay that can be utilized for other surgical and non-surgical OA mouse models.

Introduction

Osteoarthritis (OA) is a debilitating and painful disease involving various pathologies of all tissues associated with the joint¹. Affecting approximately 3.8% of the global population², OA is one of the most prevalent musculoskeletal diseases and it is to become the 4th leading cause of disability worldwide by 2020³. Post-traumatic OA occurs after a joint injury and accounts for at least 12% of all OA and up to 25% of OA in susceptible joints such as the knee^{4,5}. Furthermore, joint injury increases the lifetime risk of OA by more than five times⁶. Not all injuries with apparently similar instability will go on to develop OA, and therefore defining factors that drive the long-term OA-risk remains challenging. It is crucial in order to develop effective treatments to prevent and/or treat post-traumatic OA, to investigate and better define the injury-specific pathology, causes, and mechanisms that predispose to OA¹.

OA and its defining cartilage destruction was previously attributed entirely to mechanical stress and, thus, OA was considered a non-inflammatory disease². However, more recent studies have shown an inflammatory infiltration of synovial membranes and an increase of inflammatory cells in the synovial tissue in patients with OA compared to healthy controls², shedding light on an inflammatory component as a potential driving force in OA. Further studies indicated that abnormalities in both the CD4+ and CD8+ T-cell profile as well as monocytes/macrophages of the innate immune system may contribute to the pathogenesis of OA^{2,7}. Detailed investigations into these abnormalities revealed relevant roles for various T cell subsets², such as Th1⁸, Th2⁹, Th17⁸ and T regulatory (Treg) populations^{10,11}. Despite this compelling evidence, the causal relationship between the alteration of T-cell responses and the development and progression of OA is still unknown².

In addition to specific T-cells having a role in OA, recent studies suggest that differentially polarized/activated macrophages may be associated with pathogenesis of OA¹². In particular, macrophages originating from blood monocytes accumulate in the synovium and polarize into either classically activated macrophages (M1) or alternatively activated macrophages (M2) during OA development, implying a correlation between monocyte derived macrophages and OA¹³. In contrast, certain subsets of macrophages populate organs early during development and self-sustain their numbers in a monocyte independent manner¹⁴. Recently, a joint protective function mediated by a tight-junction barrier was shown for these synovial-tissue-resident macrophages (STRMs)¹⁴. These findings indicate that abnormalities in particular macrophage subsets may play a crucial role during development of OA. However, the interactions between this inflammatory cellular response and the structural changes in the joint subsequent to trauma is unknown.

Historically, analysis of immune cells in the synovial tissue was restricted to immunohistochemistry (IHC) or mRNA expression by reverse-transcription polymerase chain reaction (RT-PCR) approaches^{15,16}. However, both IHC and RT-PCR lack the ability to identify multiple different

cell types and their subsets simultaneously, thus, limiting the applicability of these methods. Furthermore, IHC is limited to analysis of small samples of tissue and may miss focal inflammatory cell accumulations. Over the last several years, a myriad of surface markers for various cell types have been developed, and subsets of immune cells can now be reliably identified by distinct combinations of these markers. Due to steady technical progress, flow cytometers are now capable of identifying a multitude of different fluorochromes simultaneously enabling analysis of large multicolor antibody panels.

Flow cytometry provides investigators with a powerful technique that allows simultaneous identification and quantification of a multitude of immune cells and their subsets at the single cell level. We have developed and optimized both an extracellular staining assay to identify monocytes/macrophages and their subsets as well as an extra/intracellular staining assay to identify T-cells and their subsets within murine spleen, bone marrow, lymph nodes and synovial tissue. Each step of this protocol was optimized and tested resulting in a highly reproducible assay that can be utilized for other surgical and non-surgical OA mouse models¹⁷.

Protocol

Northern Sydney Local Health District Animal Ethics Committee has approved all procedures mentioned in this protocol. Mice are housed and cared for in accordance with the Guide for the Care and Use of Laboratory Animals (National Health and Medical Research Council of Australia Revised 2010). For all experiments 10-12-week-old, male C57BL/6 mice were utilized.

NOTE: To induce post-traumatic OA, surgical destabilization of the medial meniscus (DMM) in the right stifle joint was performed. Detailed information regarding this animal model was published by Glasson et al.¹⁸. In short, general anesthesia is induced in an induction chamber using isoflurane and thereafter maintained using a nose cone. The surgical leg is shaved with a razor blade and the surgical site is washed and swabbed with ethanol to minimize contamination. The animal is then moved to the operating microscope and placed on a sterile towel and the leg draped with sterile paper drape to isolate the surgical site and minimize contamination. Using the microscope, a 0.5 cm medial para-patella arthrotomy is made, the patella luxated laterally, and the infra-patella fat pad elevated to expose the medial menisco-tibial ligament, which is transected with dissecting forceps. The joint is flushed with sterile saline to remove any blood and the wound is closed in three layers – joint capsule, subcutaneous tissue (using suture material) and skin (using surgical tissue glue). Methods described in this protocol, however, can be applied to other models and methods for inducing OA. OA can be induced in either side of the animal, and when harvesting tissues, it is important to harvest the ipsilateral (draining) lymph nodes.

1. Isolation of the spleen, contralateral bone marrow, ipsilateral lymph nodes draining the stifle and synovial tissue

1. Euthanize the mouse by cervical dislocation. Place the mouse in a supine position under a dissecting microscope and wipe the chest, abdomen and legs with 70% ethanol. Carefully open the skin on the midline for the length of the abdomen using straight scissors leaving the abdominal cavity intact.
 2. Gently pull the skin on the right side of the animal away from the underlying muscle leaving the subcutaneous adipose tissue attached to the skin. Normally, gentle traction alone will separate the skin and underlying adipose tissue from the muscle. Sporadic adhesions should be cut through with fine scissors in order to keep the tension necessary to separate tissues to a minimum and reduce the chance of harming the lymph nodes. Identify a crossing of three vessels by gently teasing out the adipose tissue located at the thigh using two curved fine forceps. The inguinal lymph node is located at the crossing and can be identified by its ovoid shape and slightly darker color.
 3. Remove the inguinal lymph node using fine dissecting forceps. Be careful not to rupture the capsule. Remove the remaining fat on the surface of the lymph node with the forceps.
 4. Open the abdominal cavity and identify the spleen. Cut out the spleen with fine scissors. Gently pull the intestines aside to expose the aorta and its bifurcation being careful not to harm them, in order to minimize risk of contamination. The iliac lymph node is located at the terminal segment of the abdominal aorta and the origin of the common iliac artery. Remove right iliac lymph node and proceed as described in 1.3.
 5. Gently remove the skin of both hind limbs. Dissect the left femur by cleaning it from muscle tissue using the blade and fine scissors. Carefully disconnect both the stifle and hip joint leaving the whole bone intact and remove the femur.
 6. Identify the patella tendon of the right stifle joint, then remove the adjoining muscle tissue proximal to this using fine scissors until approximately 5mm of quadriceps tendon proximal to the patella is exposed. Thereafter, cut through the quadriceps tendon approximately 3-4mm proximal to the patella to form a handle and using fine forceps gently pull it away from the joint. This will render the edges of the joint capsule attachment to femur visible, and using a scalpel blade carefully cut along the edges of the joint capsule on both sides, starting at the femur going towards the tibia, in order to maximize the amount of synovial membrane that is harvested. Whilst cutting it is important to maintain a gentle traction on the quadriceps tendon and pause when the synovial tissue block is only attached to the tibia. At this stage the intraarticular fat pad is now clearly visible distal to the patella and can be gently detached from the joint and anterior aspect of the menisci using the blade. Thereafter, cut along the remaining part of the joint capsule (tibial portion) to remove the synovial tissue block.
- NOTE:** This step has to be done very precisely to allow reliable results. After completing the dissection, the "synovial tissue block" should consist of the patella, patella tendon, infrapatellar fat pad, supra- and infrapatellar recesses synovial lining and associated joint capsule anterior to the collateral ligaments. Keep all tissues moist during dissection using 0.9% saline solution.
- NOTE:** Place each synovial tissue block sample in a separate well of a labelled 24-well plate containing 1.5 ml of RPMI 1640 medium. Combine both the iliac and the inguinal lymph node into one well and pool tissues from two mice.

2. Generation of single cell suspensions from each tissue

NOTE: In order to ensure sufficient cell numbers for flow analysis synovial tissues from two mice need to be pooled. In the current protocol, pool all tissues from the same two mice in order to maintain analogy. Furthermore, iliac and inguinal lymph nodes were combined for each animal resulting in a total of 4 lymph nodes for each sample. In general, cell numbers in spleen, bone marrow and lymph nodes from one animal are

sufficient to conduct flow analysis and the protocol can be applied. However, when using tissues from only one animal lysing times might need to be adjusted.

1. The spleen
 1. Place the two pooled spleens onto a 70 μ m cell strainer on top of a 15 mL tube. Gently macerate the spleens through the mesh filter using a sterile 3 mL syringe plunger. Flush the strainer frequently with a total of 6 mL of RPMI 1640 medium supplemented with 10% FBS.
 2. Spin the cells (500 x g, 5 min, RT) and resuspend the pellet in 5 mL of red blood cell (RBC) lysis buffer. Incubate for 5 min at RT and stop the reaction by diluting the lysis buffer with 10 mL of PBS. Spin the cells (500 x g, 5 min, RT) and repeat this step once or until no more RBC are in the pellet.
NOTE: Refilter the suspension using a 30 μ m cell strainer into a new 15 mL tube between the two rounds of lysing to remove coagulated cells.
 3. After lysing is complete, spin the cells (500 x g, 5 min, RT), discard supernatant and resuspend pellet in 1 mL of PBS. Count the number of live cells on a hemocytometer using Trypan blue exclusion.
2. The lymph nodes
 1. Place the four pooled lymph nodes onto a 70 μ m cell strainer on top of a 15 mL tube. Gently tease the lymph nodes apart into a single cell suspension by pressing with a sterile 3 mL syringe plunger. Flush the strainer frequently with a total of 6 mL of RPMI with 10% FBS.
 2. Spin the cells (500 x g, 5 min, RT), discard supernatant and resuspend the pellet in 500 μ L of PBS. Refilter the suspension using a 30 μ m cell strainer into a new 15 mL tube to remove coagulated cells. Count the number of live cells on a hemocytometer using Trypan blue exclusion.
3. The bone marrow
 1. Carefully grasp the intact femur using a tissue thumb forceps without fracturing it. Cut off the very end of the proximal femur with a sharp scissor in order to facilitate flushing of the bone. Turn the femur around and position a 23 G needle in the middle of the intercondylar notch of the femur. Whilst applying gentle pressure rotate the needle between thumb and index finger in order to drill a hole in the intercondylar notch to enter the bone cavity.
NOTE: Sometimes particles of the bone can obstruct the needle after drilling the hole, to avoid unnecessary high pressure during flushing a change of needle before flushing is recommended.
 2. Flush the bone with 6 mL of RPMI with 10% FBS (or until the flush turns white) using a 10 mL syringe with a 23 G needle onto a 70 μ m cell strainer that is placed on a 15 mL tube. Gently press the bone marrow through the cell strainer with a plunger of a 3 mL syringe and rinse the strainer with another 3 mL of RPMI.
NOTE: The bones should appear white once all the marrow has been flushed out completely.
 3. Spin the cells (500 x g, 5 min, RT) and resuspend the pellet in 5 mL of RBC lysis buffer. Incubate for 5 min at RT and stop the reaction by diluting the lysis buffer with 10 mL of PBS.
 4. Spin the cells (500 x g, 5 min, RT), discard supernatant and resuspend the pellet in 1 mL of PBS. Refilter the suspension using a 30 μ m cell strainer into a new 15 mL tube to remove coagulated cells. Count the number of live cells on a hemocytometer using Trypan blue exclusion.
4. The synovial tissue
 1. Dice the two synovial tissue blocks into tiny pieces with a fine surgical scissor. Transfer the samples with medium into a 15 mL tube. Rinse the old well with additional 0.5 mL of RPMI to get remaining cells and synovial tissues, transfer to falcon tube (final volume 2 mL).
TIP: Use a transfer pipette and cut off the tip where the diameter widens in this step.
 2. Reconstitute enzyme and aliquot according to manufacturer instructions (e.g., Liberase). Add sufficient enzyme to result in a final concentration of 1 Unit/mL (a total of 2 Units per sample). Digest at 37 °C for 2 h using a MACS rotator.
 3. Stop the digestion by adding 8 mL of RPMI with 10% FBS and filter cell suspension through a 70 μ m cell strainer into a new 15 mL tube. Rinse the old 15 mL tube with another 5 mL of RPMI with 10% FCS medium and filter cell suspension through same cell strainer into the new tube (15 mL final volume).
 4. Spin the cells (500 x g, 10 min, RT), discard supernatant and resuspend the pellet in 500 μ L of PBS. Count the number of live cells on a hemocytometer using Trypan blue exclusion.

3. Allocation of cells

1. Label two 96-well plates (U-bottom shape) with type of tissue, animal ID, and the designated antibody panel. A total of two antibody panels are used in this protocol: Monocyte subset panel (extracellular staining) and T-cell subset panel (extra- and intracellular staining).
2. Provide 5×10^5 cells per well using the respective single cell suspensions.
NOTE: When setting up the experiment, assess the absolute number of cells that is expected per group and tissue type (treated animals have a higher cell count in tissues than control animals). When resuspending the cell pellet during the last step of generating single cell suspensions, choose an appropriate amount of PBS in order to end up with a concentration of 5×10^5 per 200 μ L. The 96-well plate used here can hold a maximum of 300 μ L and typically, 200 μ L is ideal to minimize the risk of cross-contamination due to spillage.
3. For each panel and tissue type, distribute at least 5×10^5 cells as unstained controls in wells that have been clearly marked.

4. Monocyte Subset Panel

1. Perform viability staining: Spin cells (500 x g, 5 min, 4 °C) using a plate spinner and wash the cells once with 200 μ L of 1x PBS. Prepare a stock solution of cell-impermeant amine-reactive dye (viability stain) diluted 1:50 in 1x PBS. Thereafter, resuspend the cell pellets with 100 μ L of this stock solution resulting in an absolute volume of 2 μ L of viability stain per well. Incubate for 15 min at 4 °C protected from light.
NOTE: The optimal amount of viability stain needed should be determined by performing a dose titration curve. In addition, diluted viability stain stock solution should be used in a single day and not be stored. Refer to manufacturer's instructions for more information on how to reconstitute, dilute and store the viability stain.
2. During the incubation, prepare the cocktail of antibodies in an appropriate volume of FACS buffer (Ca²⁺ and Mg²⁺ free PBS containing 0.1% BSA and 0.02% sodium azide). Wash the cells twice with 200 μ L of FACS buffer, centrifuge (500 x g, 5 min, 4 °C) and resuspend each pellet with 100 μ L of the antibody mixture or appropriate control mixture. Incubate for 30 min at 4 °C protected from light.
NOTE: Please be aware that sodium azide is toxic to cells. In the current protocol the concentration of sodium azide in the flow buffer is very low (0.02%) and samples are run immediately after staining thus, not causing any issue. If downstream functional assays of sorted cells are planned, it might be beneficial to make up fresh FACS buffer each day of experiments and not use any sodium azide. When using a multitude of antibodies, it is advised to add an appropriate amount of "Brilliant Stain Buffer" to the cocktail of antibodies to enhance results.
3. Wash the cells twice with 200 μ L of FACS buffer and resuspend the cells in 250 μ L of FACS + EDTA buffer (FACS buffer containing 1 mM EDTA). Transfer samples into labeled FACS tubes. Keep samples at 4 °C and protected from light until acquisition.
NOTE: Immune cells have the tendency to be sticky. In order to minimize both the risk of blockage and number of doublets it is recommended to add 1 mM EDTA to the final flow buffer.

5. T Cell subset panel

1. Perform viability staining: Spin cells (500 x g, 5 min, 4 °C) using a plate spinner and wash the cells once with 200 μ L of 1x PBS. Prepare a stock solution of cell-impermeant amine-reactive dye (viability stain) diluted 1:50 in 1x PBS. Thereafter, resuspend the cell pellets with 100 μ L of this stock solution resulting in an absolute volume of 2 μ L of viability stain per well. Incubate for 15 min at 4 °C protected from light.
2. Whilst incubating the samples, prepare the extracellular staining antibody cocktail in an appropriate volume of 1x FACS buffer. Wash the cells twice with 200 μ L of 1x FACS buffer, spin them down (500 x g, 5 min, 4 °C) and resuspend each pellet with 100 μ L of the antibody mixture or appropriate control mixture. Incubate for 30 min at 4 °C protected from light.
3. Perform the intracellular staining with a fixation and permeabilization kit following the manufacturer's instructions. Wash the cells twice with 200 μ L of 1x FACS buffer and resuspend in 200 μ L of fixation buffer. Incubate for 40 min at 4 °C protected from light.
4. During the incubation, prepare the cocktail of antibodies (intracellular staining) in an appropriate volume of 1x permeabilization and wash buffer. Collect cells by spinning (750 x g, 5 min, 4 °C) and wash cells twice with 200 μ L of 1x perm/wash buffer.
NOTE: Fixation and permeabilization results in cells that tend to be a bit harder to properly pellet. In order to minimize cell loss during the subsequent washing steps, increase the centrifugal force to 750 x g. Alternatively a longer spinning cycle could also be applied. However, this would result in a considerably longer time that is needed to prepare the cells.
5. Spin cells (750 x g, 5 min, 4 °C) and resuspend each pellet with 100 μ L of the antibody mixture or appropriate control mixture. Incubate for 40 min at 4 °C protected from light.
6. Wash the cells twice with 200 μ L of 1x perm/wash buffer and resuspend the cells in 250 μ L of FACS + EDTA buffer. Transfer samples into labeled FACS tubes. Keep samples at 4 °C and protected from light until acquisition.
NOTE: For each antibody the optimal concentration needs to be determined by performing a dose titration curve. Concentration between antibodies can differ drastically: CD3 and CD80 was used with a dilution factor of 1:1, while CD11b and CD4 was used with a dilution factor of 1:6400. When titrating the antibody concentration use the same number of cells that will be used during the experiments.

6. Compensation, appropriate controls and gating

1. Setting up the experiment
 1. Once the optimal antibody concentration has been determined run unstained and single stained controls for compensation to adjust for spectral overlap.
NOTE: Run all compensation controls with both cells and compensation beads. Use whatever generates the brightest results (highest MFI of positive events) for compensation. MFI stands for mean fluorescence intensity and is often used to describe and define the mean intensity of the generated signal and thus, level of antibody expression.
 2. Run fluorescence minus one (FMO) controls and isotype controls when starting a new multicolor experiment. Further details regarding FMO have been previously published¹⁹.
 3. Determine the optimal Forward Scatter Area (FSC-A) voltage and Side Scatter Area (SSC-A) voltage in order to detect the leukocyte population in unstained controls of each tissue type.
NOTE: The fixation and permeabilization process alters the dimensions of the cell. Thus, the FSC-A and SSC-A voltages for the Monocyte Subset Panel and T Cell Subset Panel differ considerably. In order to find the optimal voltages for the T Cell Subset Panel, use cells that have been single stained with CD3 and back gate towards the leukocyte populations while adjusting the FSC-A and SSC-A values.
2. Gating strategy
 1. Once the optimal FSC-A and SSC-A voltage has been determined, set up a primary gate on the leukocyte population.
NOTE: Prior to each experiment calibrate the cytometer using calibration beads as per manufacturer's instructions and run unstained beads. Leukocyte populations of different time points should have comparable FSC and SSC properties (slight differences between tissue types are expected and normal). If FSC and SSC varies considerable trouble shoot the cytometer and sample generation.
 2. Exclude doublets: Plot FSC-A (y-axis) and FSC-H (x-axis). Singlets appear as a diagonal of this plot. Gate on singlets.
 3. Exclude dead cell: Plot FVS510 (viability stain) (x-axis) and FSC-A (y-axis). Dead cells will appear as positive events, thus gate on live cells.

NOTE: True negative cells will be visible in unstained controls. Thus, adjust this gate for each set of samples when running the unstained controls prior to stained samples. Further gating depends on the antibody panel and cell type that is investigated. Gating strategies for each panel used in this protocol can be found in **Figure 1** and **Figure 4**, respectively.

Representative Results

Representative results from both the monocyte subset panel and T-cell subset panel are described below.

Figure 1 illustrates the hierarchical gating strategy for the monocyte subset panel on immune cells gathered from bone marrow of DMM treated animals. The same strategy was used and verified in all other tissue types. When setting up the experiment, the Forward Scatter Area (FSC-A) and Side Scatter Area (SSC-A) voltage was determined for each tissue type to identify monocytes/macrophages and exclude T-cells and debris (G1). During each experiment, unstained controls of each tissue type were analyzed, and FSC-A and SSC-A voltage adjusted when necessary. Voltages are expected to stay similar over time, if parameters change drastically a blockage of the cytometer is likely. Furthermore, unstained controls were used to determine the true negatives for the dead/alive stain and gates were adjusted each time the experiment was conducted accordingly (**Figure 2A**). When designing the experiment, fluorochromes should be chosen carefully, and normally surface markers with low expression are paired with bright fluorochromes (e.g., here Alexa Fluor 647 was used for CD206). Various dead/alive stains exist that can be detected by different wavelengths; here, FVS510 was used.

Figure 3 illustrates sample data from immune cells isolated from synovial tissues and stained with extracellular surface markers 6 weeks after animals received either DMM or sham-control surgery. All subsets can easily be identified using the protocol both in study and control animals. In particular, differences between groups can be seen for macrophage subsets (higher percentage of Ly-6C+/MHC-II- macrophages (G7) in the DMM group) and the expression of M1 and M2 macrophages (higher percentage of M2 macrophages in the DMM group).

Figure 4 visualizes the hierarchical gating strategy for the extra- and intracellular T-cell panel on immune cells isolated from the spleen of DMM treated animals. Principles are identical to the ones used for the monocyte panel. However, the fixing and permeabilization process changes the size and density of cells. Thus, typical FSC and SSC parameters need to be determined using a back-gating process from CD3+ cells when first setting up the experiment for each cell type. Some fluorochromes tend to aggregate over time (e.g., PE that was used with FoxP3 here). Aggregates can potentially modify the results due to the high brightness that influences the spectral-overlap and compensation. Thus, all antibodies were vortexed and spun down each time prior to their use in order to decrease aggregates. Additionally, a gating strategy was used to further reduce the influence of aggregates (G2). While setting up the experiments fluorescence minus one controls (FMOs) were performed for each antibody. Sample data is shown in **Figure 2B,2C**.

Figure 5 and **Figure 6** show immune cells that were isolated from lymph nodes (**Figure 5**) and synovial tissue (**Figure 6**) and stained using the T-cell panel protocol 4 weeks after animals received either DMM or sham-control surgery. The data shows a higher percentage of Th1 cells in DMM animals (G9) in both tissues. Furthermore, intracellular staining for T-regulatory cells (G11) and Th17 cells (G12) is successful using the protocol and differences can be detected between groups.

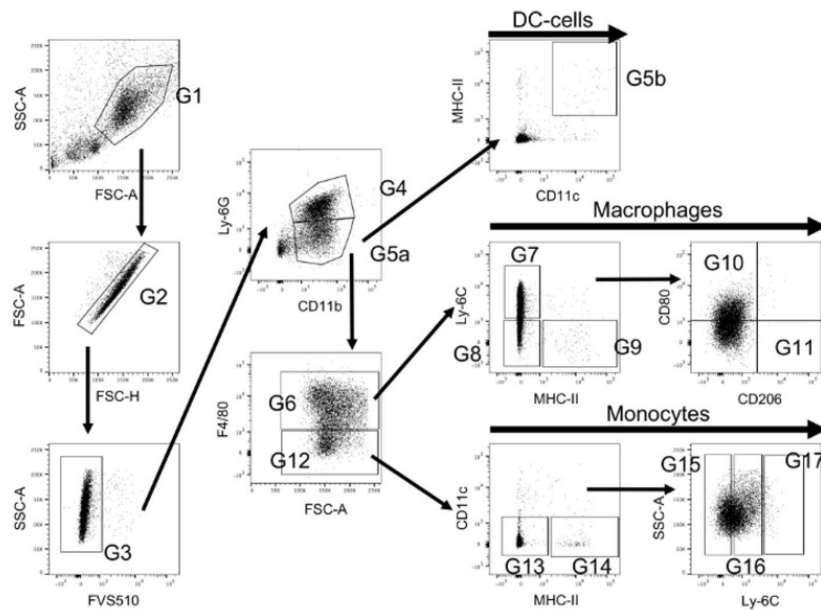


Figure 1: Flow cytometry hierarchical gating strategy using extracellular staining to identify monocytes/macrophages and their subsets. Myeloid cells are primarily identified using a forward/side scatter (FSC-A and SSC-A) dot plot (G1). Thereafter, singlets are detected using FSC-A and FSC-H (G2) and afterwards live cells are selected (G3). Cells from G3 are further classified using Ly-6G to identify neutrophils (G4) and CD11b for monocyte/macrophages (G5a). MHC-II is used to identify dendritic cells (G5b) amongst CD11b positive cells and F4/80 is used to select between macrophages (G6) and monocytes (G12). Macrophages are further classified into their subsets using Ly-6C and MHC-II (Ly-6C+/MHC-II- macrophages (G7); Ly-6C-/MHC-II- tissue resident macrophages (G8); Ly-6C+/MHC-II+ blood originated macrophages (G9)). More subsets can be selected from the entirety of macrophages and its respective subsets using CD206 and CD80 (M1: CD80+/CD206- (G10); M2: CD80-/CD206+ (G11)). Monocytes are further classified using MHC-II and CD11c (MHC-II-/CD11c- monocytes (G13); MHC-II+/CD11c- monocytes (G14)). The level of activation is then classified using the expression of Ly-6C and divided into low (G15), medium (G16) and high (G17). [Please click here to view a larger version of this figure.](#)

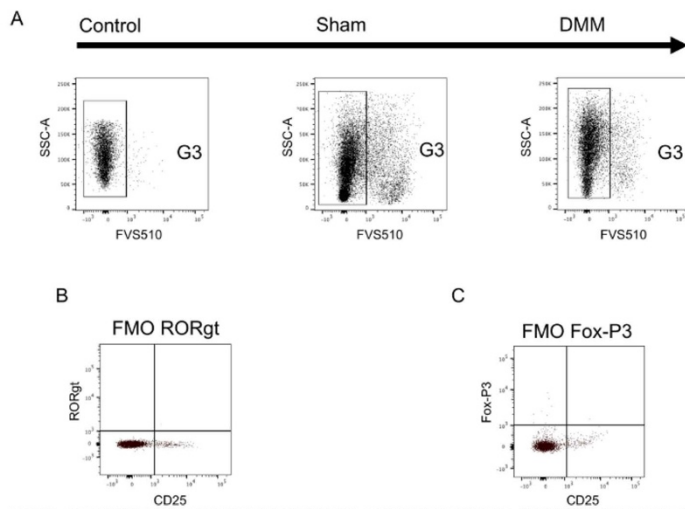


Figure 2: Sample data illustrating appropriate controls in both the monocyte and T-cell panel. (A) Synovial tissue was harvested 6 weeks after either DMM-surgery (DMM) or sham-control-surgery (sham) and a single cell suspension was stained using extracellular surface markers. During each experiment unstained cells were used to determine true negatives for the dead/alive stain and to set gates (Control). Setting of gates using unstained cells is shown in panel A. (B+C) Spleen cells were harvested from untreated control animals, a single cell suspension generated and stained using both extra- and intracellular markers. Fluorescents-minus-one (FMO) controls were generated by staining cells with the entire antibody panel missing only one antibody. Sample data is shown for both intracellular antibodies. (B) FMO -RORgt and (C) FMO -Fox-P3. FMOs were performed for both panels and used to set each gate. [Please click here to view a larger version of this figure.](#)

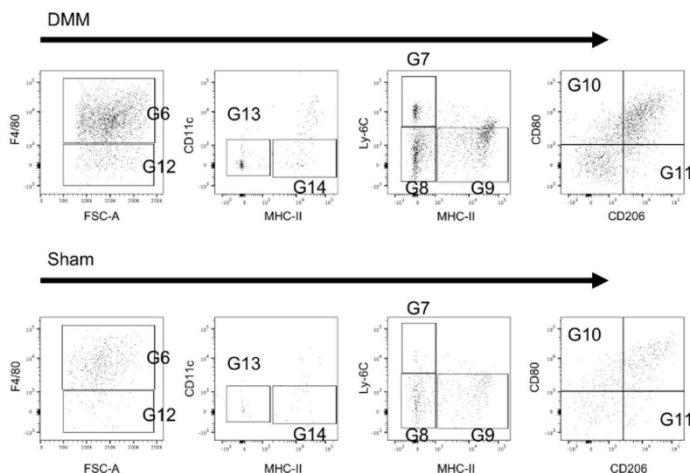


Figure 3: Extracellular staining of monocytes/macrophages isolated from the synovium of mice. Sample tissues were collected 6 weeks after mice received either DMM-surgery (DMM) or sham-control-surgery (sham). Further information regarding the utilized gates can be found in [Figure 1](#). Sample data shows that all subsets can be reliably identified and differences can be seen between groups. [Please click here to view a larger version of this figure.](#)

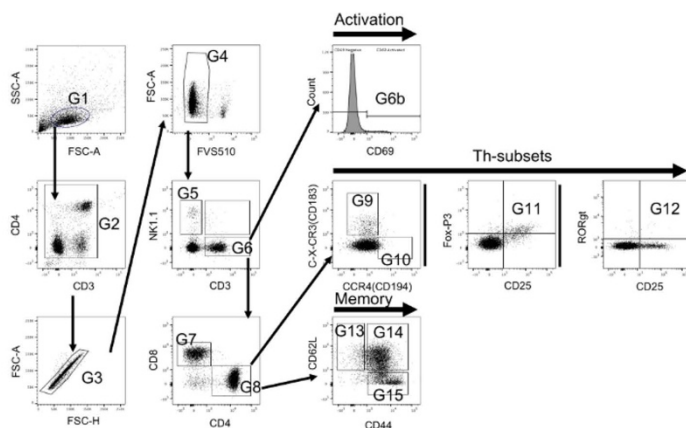
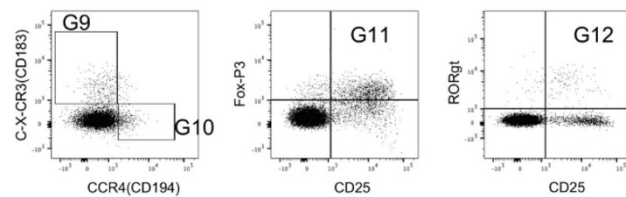


Figure 4: Flow cytometry hierarchical gating strategy using both extra- and intracellular staining to identify T-cells and their subsets. T-cells are primarily identified using a forward/side scatter (FSC-A and SSC-A) dot plot (G1). Due to the nature of the utilized antibodies aggregates should be excluded using CD3 and CD4 (G2). Thereafter, singlets are detected using FSC-A and FSC-H (G3) and afterwards live cells are selected (G4). Cells from G4 are further classified using NK1.1 to identify natural killer cells (G5) and CD3 to identify T-cells (G6). The level of activation is determined using CD69 (G6b). Thereafter, CD4 and CD8 are used to identify T-killer cells (CD4-/CD8+ (G7)) and T-helper cells (CD4+/CD8- (G8)). T-helper cells are classified into Th-1 (C-X-CR3+/CCR4- (G9)) and Th-2 cells (C-X-CR3-/CCR4+ (G10)) using C-X-CR3 (CD183) and CCR4 (CD194). In addition, Th-17 cells (CD25+/RORgt+ (G11)) and T-regulatory cells (CD25+/Fox-P3+ (G12)) are identified using intracellular markers. Furthermore, memory cell subsets are identified from T-helper cells using CD44 and CD62L (CD44-/CD62L+ naïve T-memory cells (G13); CD44+/CD62L+ central T-memory cells (G14); CD44+/CD62L- effector T-memory cells (G15)). [Please click here to view a larger version of this figure.](#)

DMM



Sham

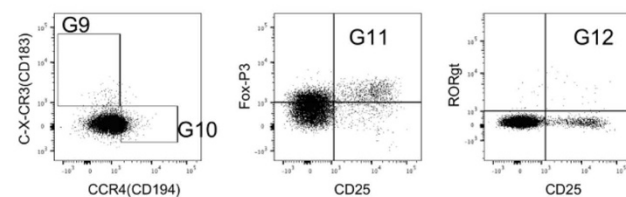


Figure 5: Extracellular and intracellular staining of T-cells isolated from draining lymph nodes of mice. Sample tissues were collected 4 weeks after mice received either DMM-surgery (DMM) or sham-control-surgery (sham). Further information regarding the utilized gates can be found in Figure 4. Sample data shows that all subsets can be reliably identified and differences can be seen between groups. [Please click here to view a larger version of this figure.](#)

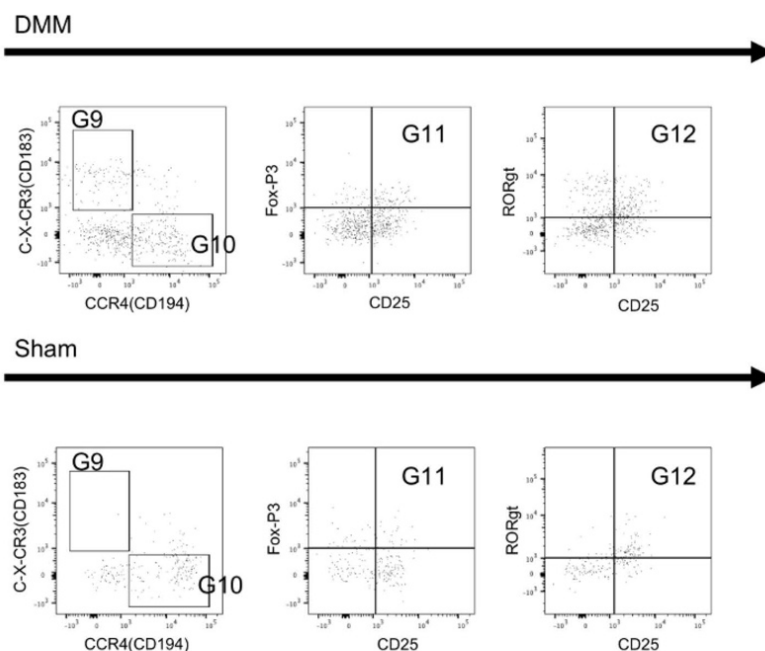


Figure 6: Extracellular and intracellular staining of T-cells isolated from synovial tissue of mice. Sample tissues were collected 4 weeks after mice received either DMM-surgery (DMM) or sham-control-surgery (sham). Further information regarding the utilized gates can be found in Figure 4. Sample data shows that all subsets can be reliably identified and differences can be seen between groups. [Please click here to view a larger version of this figure.](#)

Discussion

The methods described in this protocol have been designed and tested to reliably identify various subsets from both monocytes/macrophages and T-cells within the murine spleen, bone marrow, lymph nodes, and synovial tissue in a murine model of osteoarthritis (OA). The current protocol can easily be modified to investigate different tissue types, or other cell types by exchanging antibodies, and can be used for alternative murine models of OA. When testing other tissue types, it is critical to test the specificity of each antibody as expression of surface markers of immune cells vary in each tissue²⁰. In addition, when exchanging antibodies, it is necessary to perform a dose titration curve to establish the optimal concentration of antibody as well as repeating the compensation process to address changes in spectral overlap.

In the current protocol, OA was induced using the DMM mouse model¹⁸. The most commonly used and established animal models are in the mouse, because this species provides multifold advantages in investigating the pathophysiology of post-traumatic OA¹⁷. In the mouse in particular, surgical and non-surgical OA models have been described¹⁷, the most common being destabilization of the medial meniscus (DMM), surgical transection of the anterior cruciate ligament (ACLT) and non-surgical ACL rupture (ACLR), respectively²¹. All these animal models are well suited for the investigation into the role of cellular inflammation in the pathology of post-traumatic OA and the current protocol has been successfully tested for all previously mentioned animal models in the laboratory. Although all the above animal models are established and have been described in the literature, each has its own strengths and limitations that have been discussed in detail elsewhere¹⁷ and so are discussed only briefly below. All surgical models are subject to the surgical approach, wound healing process and its associated inflammatory response. When assessing the contribution of inflammatory cells to the development of post-traumatic OA, this inflammatory wound healing response might serve as a confounder, especially during the early time points after the intervention. In addition, DMM and ACLT procedures need to be conducted in a very standardized manner to minimize variability between animals. The ACLT surgery is much more difficult to learn than the DMM surgery and requires a greater surgical exposure than DMM to definitively identify and ensure injury only to the ACL and avoid iatrogenic damage to other joint tissues¹⁸. Non-surgical ACL rupture is a standardized and very efficient way of inducing post-traumatic OA. However, a specialized device that applies a controlled single compressive load to the tibia of the flexed knee is necessary. This device has to be calibrated and tested in order to get comparable and reliable results. Additionally, the ACLR model induces very severe and progressive joint damage in mice with marked erosion of the posterior medial tibial plateau²² that is not seen with ACL injury in other species including humans.

In order to comprehensively characterize the inflammatory process and its cellular component during development of OA, it is desirable to not only investigate the synovial tissue but also the local lymph nodes as well as secondary lymphoid organs, such as the spleen and bone marrow. Lymphatic drainage patterns of the knee joint have been characterized in mice and lymph fluid from the knee joint drains through both the iliac and inguinal lymph node at a varying dispersion^{23,24}. In order to facilitate comparability between animals, we decided in this protocol to pool the inguinal and iliac lymph node. In contrast, while in close proximity to the knee, the popliteal lymph node drains the hindfoot and does not play a role during inflammatory processes of the knee.

Mice have a small volume of intra-articular synovial tissues²⁵ and isolation of the immune cells herein remains challenging. In the current protocol, harvesting techniques for synovial tissues were adapted to allow isolation of the maximum number of immune cells. Therefore, the

harvesting technique includes the supra- and infrapatellar recesses as well as the infrapatellar fat pad, due to its high number of immune cells²⁶. The digestion process and choice of enzyme was optimized to fully digest the synovial membrane and fatty tissue, while leaving the tendon, and the patella and its cartilage unimpaired. Thus, the current protocol introduces a reproducible method of harvesting immune cells from synovial tissue.

Flow cytometry analysis has multiple advantages when investigating cellular immune processes during the development of OA; nonetheless, this technique has limitations. Due to the small number of immune cells in the synovial tissue, it is necessary to pool tissue samples from at least two animals to obtain one sample. Due to the large number of fluorochromes and colors used in this protocol, special attention has to be paid to a meticulous compensation of a possible spectral overlap for each tissue type and compensation needs to be re-evaluated consistently throughout the use of this technique. Due to the large number of available markers and considerable variation among studies to identify a certain population, another possible limitation is the choice of markers used to identify cells¹⁹. Flow cytometry allows quantification of "events", which does not necessarily coordinate to total cells. In order to obtain a truly quantitative analysis, one needs to either acquire counting beads concurrently when running flow analysis or count cells in single cell suspensions beforehand to obtain absolute numbers (as done here). In general, the principles of this protocol (e.g., how to design and set up a flow panel or techniques used to prepare single cell suspensions) could be potentially adapted for human samples. However, surface markers of human immune cells differ from murine cells and therefore, appropriate antibodies need to be selected and tested. In addition, duration of RBC lysis and appropriate volume of buffer needs to be determined as it is most likely different. Prior to adapting methods from this protocol to other species or tissues meticulous testing of each step is necessary to ensure that methods are working as intended.

Despite its limitations, flow cytometry analysis of immune cells remains a powerful technique that allows to identify both monocytic cell and T-cell subsets at the single cell level. In particular, the current protocol introduces a reliable and reproducible technique that can identify and quantify the cellular immune response during the development of OA in the synovial tissue and secondary lymphatic organs. In the future, this technique may help to characterize the immune response in various osteoarthritis inducing animal models and hereafter evaluate the efficacy of immune modulating drugs onto this debilitating disease.

In conclusion, this flow cytometry protocol describes a detailed and reproducible method to identify monocytes/macrophages and T cell subsets using both extra- and intracellular staining assays within murine spleen, bone marrow, lymph nodes and synovial tissue utilizing an established surgical osteoarthritis mouse model.

Disclosures

The authors have nothing to disclose.

Acknowledgments

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References

- Blaker, C. L., Clarke, E. C., Little, C. B. Using mouse models to investigate the pathophysiology, treatment, and prevention of post-traumatic osteoarthritis. *Journal of Orthopaedic Research*. **35** (3), 424-439 (2016).
- Li, Y. S., Luo, W., Zhu, S. A., Lei, G. H. T Cells in Osteoarthritis: Alterations and Beyond. *Frontiers in Immunology*. **8**, 356 (2017).
- Woolf, A. D., Pfleger, B. Burden of major musculoskeletal conditions. *Bull World Health Organ*. **81** (9), 646-656 (2003).
- Little, C. B., Hunter, D. J. Post-traumatic osteoarthritis: from mouse models to clinical trials. *Nature Reviews Rheumatology*. **9** (8), 485-497 (2013).
- Riordan, E. A., Little, C., Hunter, D. Pathogenesis of post-traumatic OA with a view to intervention. *Best Practice & Research: Clinical Rheumatology*. **28** (1), 17-30 (2014).
- Muthuri, S. G., McWilliams, D. F., Doherty, M., Zhang, W. History of knee injuries and knee osteoarthritis: a meta-analysis of observational studies. *Osteoarthritis and Cartilage*. **19** (11), 1286-1293 (2011).
- Leheita, O., Abed Elrazek, N. Y., Younes, S., Mahmoud, A. Z. Lymphocytes subsets in osteoarthritis versus rheumatoid arthritis. *Egyptian Journal of Immunology*. **12** (2), 113-124 (2005).
- Yamada, H. et al. Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints. *Journal of Rheumatology*. **38** (8), 1569-1575 (2011).
- Sakkas, L. I. et al. T cells and T-cell cytokine transcripts in the synovial membrane in patients with osteoarthritis. *Clinics and Diagnostics Laboratory Immunology*. **5** (4), 430-437 (1998).
- Guo, S. Y. et al. Correlation of CD4(+) CD2(5)(+) Foxp3(+) Treg with the recovery of joint function after total knee replacement in rats with osteoarthritis. *Genetics and Molecular Research*. **14** (3), 7290-7296 (2015).
- Moradi, B. et al. CD4(+)CD25(+)highCD127low(-) regulatory T cells are enriched in rheumatoid arthritis and osteoarthritis joints--analysis of frequency and phenotype in synovial membrane, synovial fluid and peripheral blood. *Arthritis Research & Therapy*. **16** (2), R97 (2014).
- Saito, I., Koshino, T., Nakashima, K., Uesugi, M., Saito, T. Increased cellular infiltrate in inflammatory synovia of osteoarthritic knees. *Osteoarthritis and Cartilage*. **10** (2), 156-162 (2002).
- Zhang, H. et al. Synovial macrophage M1 polarisation exacerbates experimental osteoarthritis partially through R-spondin-2. *Annals of the Rheumatic Diseases*. **77** (10), 1524-1534 (2018).
- Culemann, S. et al. Locally renewing resident synovial macrophages provide a protective barrier for the joint. *Nature*. **572**, 670-675 (2019).
- Mocellin, S. et al. Use of quantitative real-time PCR to determine immune cell density and cytokine gene profile in the tumor microenvironment. *Journal of Immunological Methods*. **280** (1-2), 1-11 (2003).

16. Ward, J. M. et al. Immunohistochemical markers for the rodent immune system. *Toxicologic Pathology*. **34** (5), 616-630 (2006).
17. Blaker, C. L., Clarke, E. C., Little, C. B. Using mouse models to investigate the pathophysiology, treatment, and prevention of post-traumatic osteoarthritis. *Journal of Orthopaedic Research*. **35** (3), 424-439 (2017).
18. Glasson, S. S., Blanchet, T. J., Morris, E. A. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis and Cartilage*. **15** (9), 1061-1069 (2007).
19. Laroumanie, F., Dale, B. L., Saleh, M. A., Madhur, M. S. Intracellular Staining and Flow Cytometry to Identify Lymphocyte Subsets within Murine Aorta, Kidney and Lymph Nodes in a Model of Hypertension. *Journal of Visualized Experiments*. 10.3791/55266 (119) (2017).
20. Yu, Y. R. et al. A Protocol for the Comprehensive Flow Cytometric Analysis of Immune Cells in Normal and Inflamed Murine Non-Lymphoid Tissues. *PLoS One*. **11** (3), e0150606 (2016).
21. Lorenz, J., Grassel, S. Experimental osteoarthritis models in mice. *Methods in Molecular Biology*. **1194**, 401-419 (2014).
22. Christiansen, B. A. et al. Non-invasive mouse models of post-traumatic osteoarthritis. *Osteoarthritis and Cartilage*. **23** (10), 1627-1638 (2015).
23. Shi, J. et al. Distribution and alteration of lymphatic vessels in knee joints of normal and osteoarthritic mice. *Arthritis & Rheumatology*. **66** (3), 657-666 (2014).
24. Harrell, M. I., Iritani, B. M., Ruddell, A. Lymph node mapping in the mouse. *Journal of Immunological Methods*. **332** (1-2), 170-174 (2008).
25. Zhao, J. et al. A protocol for the culture and isolation of murine synovial fibroblasts. *Biomedical Reports*. **5** (2), 171-175 (2016).
26. Belluzzi, E. et al. Contribution of Infrapatellar Fat Pad and Synovial Membrane to Knee Osteoarthritis Pain. *BioMed Research International*. **2019**, 18 (2019).

2.2.4.2 Published Manuscript Describing the Implementation and Testing of a Streamlined Histopathological Scoring Technique for Murine ptOA Models.

Osteoarthritis and Cartilage



Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models

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SUMMARY

Objectives: Histological scoring remains the gold-standard for quantifying post-traumatic osteoarthritis (ptOA) in animal models, allowing concurrent evaluation of numerous joint tissues. Available systems require scoring multiple sections/joint making analysis laborious and expensive. We investigated if a single section allowed equivalent quantitation of pathology in different joint tissues and disease stages, in three ptOA models.

Method: Male 10–12-week-old C57BL/6 mice underwent surgical medial-meniscal-destabilization, anterior-cruciate-ligament (ACL) transection, non-invasive-ACL-rupture, or served as sham-surgical, non-invasive-ACL-strain, or naïve/non-operated controls. Mice (n = 12/group) were harvested 1-, 4-, 8-, and 16-week post-intervention. Serial sagittal toluidine-blue/fast-green stained sections of the medial-femoro-tibial joint (n = 7/joint, 84 µm apart) underwent blinded scoring of 40 histology-outcomes. We evaluated agreement between single-slide versus entire slide-set maximum or median scores (weighted-kappa), and sensitivity/specificity of single-slide versus median/maximum to detect OA pathology.

Results: A single optimal mid-sagittal section showed excellent agreement with median (weighted-kappa 0.960) and maximum (weighted-kappa 0.926) scores. Agreement for individual histology-outcomes was high with only 19/240 median and 15/240 maximum scores having a weighted-kappa ≤0.4, the majority of these (16/19 and 11/15) in control groups. Statistically-significant histology-outcome differences between ptOA models and their controls detected with the entire slide-set were reliably reproduced using a single slide (sensitivity > 93.15%, specificity > 93.10%). The majority of false-negatives with single-slide scoring were meniscal and subchondral bone histology-outcomes (89%) and occurred in weeks 1–4 post-injury (84%).

Conclusion: A single mid-sagittal slide reduced the time needed to score diverse histopathological changes by 87% without compromising the sensitivity or specificity of the analysis, across a variety of ptOA models and time-points.

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Introduction

Osteoarthritis (OA) is the most common joint disease,¹ and the second highest cause for years lived with disability.² While various joints can be affected, knee OA is particularly disabling accounting for > 80% of total burden¹. Post-traumatic OA (ptOA) accounts for 12% of all symptomatic cases and more in susceptible joints such as the knee or ankle,³ with knee injury in adolescents increasing OA-risk up to sixfold.⁴ Given the scale of individual and socioeconomic cost and lack of effective treatments, there is a clear need for research to improve understanding of ptOA cellular and molecular pathophysiology to help define and develop novel and effective therapeutic approaches. Clinical studies of knee injury require a large initial sample size and decades of follow-up to account for patient heterogeneity, variable risk, and slow disease progression.⁵ In contrast, highly reproducible animal models of knee-ptOA allow longitudinal study of disease development from initiating injury to late/end-stage pathology, making them indispensable research tools.⁶

OA pathology has traditionally been characterised by marginal osteophyte development and progressive degradation of articular cartilage: in human patients typically through imaging (radiographs and/or magnetic resonance imaging),⁷ while in animal models histopathology remains the gold standard.^{6,8,9} In larger animal species, histopathologic analysis is limited to isolated chondral or osteochondral samples from selected joint locations often focusing on the most severe lesions.^{10–12} In smaller species evaluation of the entire joint in a single section is possible, and depending on the OA-model and sectioning plane, can simultaneously include affected and unaffected compartments.^{13–16} OA is now recognised as a joint-wide disease with variable pathological change in all tissues including: cartilage degradation, bone remodelling (sub-chondral sclerosis, marginal osteophytes/enthesophytes), synovial/joint-capsule inflammation and fibrosis, and meniscal and ligament tears and degradation. The ability to concurrently evaluate all these tissues in histology sections, along with development of numerous reproducible models and availability of genetically modified strains, has made the mouse knee one of the most widely used to evaluate factors influencing ptOA development and test therapeutic approaches.^{6,17–21}

A variety of scoring systems have been developed for histopathological evaluation of mouse knee OA.^{14,22–26} Despite considerable differences in how and what to evaluate, a common theme is the necessity/recommendation to score multiple sections to account for focal disease.²⁷ Translatability of pre-clinical research depends on meticulous study design respecting both accurate sample size calculations and appropriate control groups. This results in large mouse numbers and even larger numbers of serial histological sections that need to be scored by a qualified researcher, requiring considerable time and resources, and limiting research throughput.^{6,28} To address this, a recent study assessed applying two different scoring systems to multiple or single sections in mice 8-weeks following destabilization of the medial meniscus (DMM) or sham surgery.²⁷ They concluded that a single mid-coronal section can define OA cartilage pathology severity with equal sensitivity to summing pathology from multiple sections.

In line with recommendations to evaluate joint-wide pathology, we have developed a semi-quantitative mouse-OA histopathology scoring system that allows reproducible evaluation not only of cartilage but also osteophytes and enthesophytes, subchondral bone, meniscus, synovium, and cruciate ligaments.^{23,29–34} In the current study we used this grading scheme to score multiple sagittal sections in 3 different mouse models over 4 time-points spanning ptOA development and severity. Thereafter, we used statistical analyses to assess the agreement between scores derived from a single section

versus maximum and median scores from the entire slide-set, if this single-slide technique allows reproducible identification of ptOA histopathological changes in multiple joint tissues, and if this differed between models, joint tissues, or disease-stage.

Methods

Animals

The study was approved by the Northern Sydney Local Health District Animal Ethics Committee. For all experiments 10–12-week-old, male C57BL/6 mice were sourced housed, and cared for as previously detailed,^{29–33} in accordance with national guidelines.³⁵ A total of 288 mice underwent induction of ptOA in the right knee via DMM (n = 48), surgical anterior cruciate ligament transection (ACLT) (n = 48), or non-invasive/mechanically-induced ACL rupture (ACLR) (n = 48); or served as naïve/non-operated controls (NOC) (n = 48), SHAM surgery controls (n = 48), or non-OA inducing ACL-strain (ACLS) controls (n = 48). Mice (n = 12/group; enabling differences of 25–60% in the different joint pathology parameters to be detected using current serial-slide methodology using Kruskal-Wallis and/or Mann-Whitney-U with > 85% power with alpha 0.05^{23,29,32,33,36,37}), were harvested 1-, 4-, 8-, and 16-weeks post-intervention (equivalent ages for NOC). Animals were assigned to injury-group by random allocation of cages (n = 2–5 mice/cage; all mice in a cage were assigned the same injury-model/group and harvest time) but ensuring experimental numbers (as defined above) were met. Animals in all injury groups were simultaneously active throughout the study and were housed in the same room and cage-rack to minimise confounding.

Histology

Sample harvest, fixation, decalcification, paraffin-embedding, and staining were performed as described.^{23,29–33} A standardised sagittal-sectioning approach was based on a defined anatomical starting point: femoral articular cartilage surface first evident and anterior and posterior medial meniscal segments with a clear wedge shape but still connected by a thin band of tissue (Fig. 1). Thereafter slides were collected at 84 µm intervals, each with three 4 µm-thick sections (allowing for redundancy but only a single score/slide for any parameter was generated) and stained with Toluidine-blue/Fast-green. The anatomical location of slides 1–7 spanning the medial femoro-tibial joint width along with representative images for each model are shown in Fig. 1. Intervening sections were either discarded, or those from the central weight-bearing region in a randomly selected subset of animals in each group collected and stored for additional stains/immunohistochemistry (not presented in the current study).

Scoring

Histopathological changes predominantly occur in the medial femoro-tibial joint in the ptOA models evaluated in the current study, therefore only histopathology on slides spanning this compartment was scored. Slides were coded to blind the scorer (PH) to injury group and time-point (which were randomised), but all slides from a single mouse were scored together and section depth (i.e. slide #1–7) was not concealed. Clustering individual animal slides reduced potential bias from slides being scored at different times. This was critical in the present study where within-animal slide scoring was the primary comparison (between-model/time comparisons controlled by randomisation over the ~7 week scoring time-course). The scoring system evaluates five tissue/pathology domains (1-cartilage and subchondral bone; 2-osteophytes and

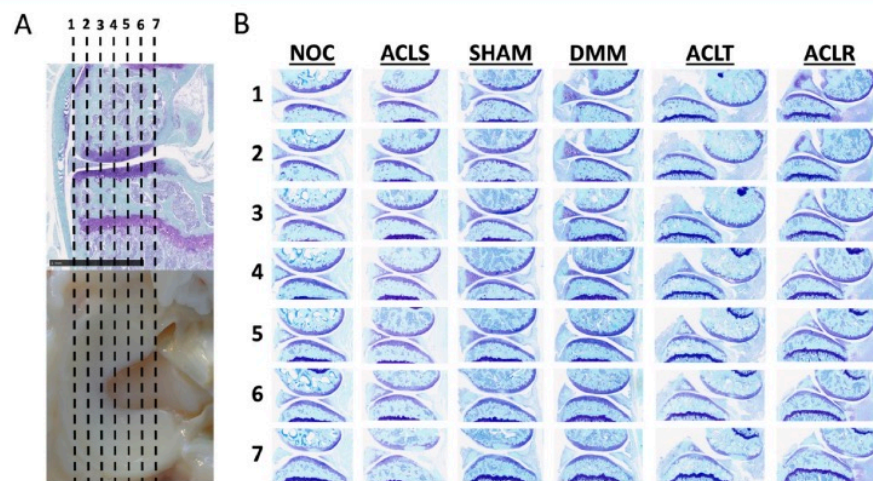


Figure 1

Osteoarthritis and Cartilage

A) The approximate position of the individual slides (1–7) is demonstrated on a mid-coronal histology section of the medial mouse femorotibial joint (top) and a macroscopic view of a sheep medial tibial plateau with meniscus (bottom). B) Representative histology images for each animal model and each slide (1–7) from mice harvested 1-week after ptOA induction and their respective controls. All images are oriented with left being anterior and the top being proximal.

enthesophytes; 3-meniscus; 4-synovium/joint-capsule; 5-cruciate ligaments) each with several tissue/cell-specific histological-parameters (Supplementary information).^{23,29–33} Domains 4 and 5 were not included in the current study; synovitis is already scored on a single section as changes are relatively uniform at all sectioning depths across the medial femoro-tibial joint^{29–32}; cruciate ligament changes are also scored on a single section and are not included in routine medial femorotibial ptOA evaluation as they require additional sections.³³ For the 3 other domains, maximum (most-severe/worst lesion/score in any of the 7 sections) and median scores from all 7 sections were generated for 40 histological-parameters (3 cartilage outcomes in femur and tibia; 4 subchondral bone outcomes in tibia; 3 meniscal outcomes in anterior and posterior segments; 3 osteophyte and 3 enthesophyte outcomes in anterior and posterior femur and tibia; see Supplementary information).

Statistics

Agreement between scores generated using a single slide versus the maximum or median score from multiple slides was performed for each histological-outcome separately within each ptOA model. As histological-parameters were categorical, weighted-kappa analysis was used to estimate agreement: > 0.8 considered “almost perfect”, 0.61–0.80 “substantial”, 0.41–0.60 “moderate”, and ≤ 0.4 as “parameters of concern” (POC).³⁸ Non-parametric analyses (Kruskal-Wallis and Mann-Whitney-U) were used to investigate differences between ptOA models and their controls (DMM vs Sham, ACLT vs Sham, ACLR vs ACLS) for all histological-outcomes at each time-point. Statistically significant differences ($p < 0.05$) detected using standard median or maximum scores from the entire slide-set were defined as the “ground truth”. These differences were compared with those detected using slide-5 to determine the sensitivity and specificity of single-slide scoring. The individual histology-outcomes within a joint were considered as independent variables, as prior analysis demonstrates they change differentially and with distinct temporal patterns in different OA models, and are independently

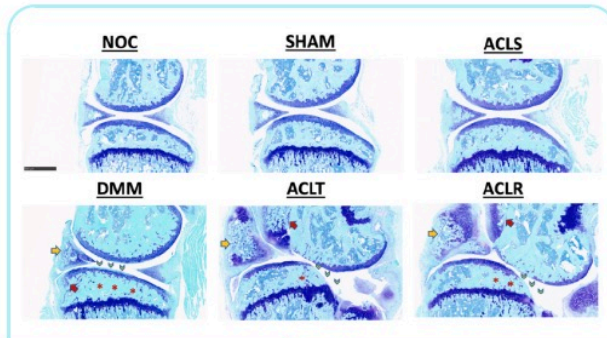
modified in response to treatment/genetic-modification.^{29–32,39} As an exploratory post-hoc analysis, all p-values in the current study were interpreted with no adjustment for multiple testing, with α set at 0.05. All statistical calculations were performed with R version 4.0.3⁴⁰ applying the *tidyr*,⁴¹ *dplyr*,⁴² and *PROC*⁴³ packages; *psych*⁴⁴ was utilised to estimate kappa including a penalty function of quadratic weights; Figures were created using *ggplot2*.⁴⁵

Results

All animals completed the study, with no adverse events, and no animals were excluded from the analysis. The three ptOA models (DMM, ACLT, ACLR) induced expected ptOA pathology including cartilage proteoglycan loss, cartilage structural damage/erosion, subchondral bone sclerosis, and marginal osteophytes/enthesophytes, with little or no structural OA pathology in controls (Fig. 2). The severity of pathology at a given time-point differed between models (DMM < ACLT/ACLR) but increased with time in all models (not shown).

Agreement between scores derived from a single slide versus the set of slides

The optimal single-slide for scoring was identified by calculating for each slide (1–7) the agreement (weighted-kappa) between the median or maximum scores from the entire slide-set with all time-points, groups and histological-outcomes pooled (Table 1), as well as each histological-outcome separately (to assess for bias associated with differing histopathology-outcome scoring scales (0–3 up to 0–12); Supplementary Table 1). All slides showed almost perfect agreement with pooled-sample weighted-kappas > 0.85 regardless of maximum or median comparator (Table 1). The lowest values were in sections towards the joint margin (slide 1 maximum weighted-kappa 0.856; median weighted-kappa 0.869) while the best agreement was in slides closer to the intercondylar notch (maximum: slide-7, weighted-kappa 0.928; median: slide-6, weighted-kappa 0.966).

**Figure 2****Osteoarthritis and Cartilage**

Representative images of slide-5 mid-sagittal sections from all groups/models demonstrating typical pathology at 4 weeks after destabilization of the medial meniscus (DMM), anterior cruciate ligament transection (ACLT) and mechanical ACL rupture (ACLR), compared with little or no structural abnormalities in non-operated controls (NOC), sham-operated controls (SHAM) or ACL strain (ACLS). Scale bar = 500 micro-metre. Slides stained with toluidine-blue and fast-green. Green arrow heads indicate areas of cartilage proteoglycan loss or structural damage (full-thickness osteo-chondral erosion in ACLT and ACLR); red arrow indicate marginal osteophyte or enthesophyte formation; red asterisks indicate areas of subchondral and epiphyseal bone sclerosis; orange arrows indicate regions of anterior meniscal marginal ossification.

Slide	Kappa-Value K [-95%,+95%]	Parameters > 0.80 agreement	Percentage of parameters scorable
Maximum			
Slide 1	0.856 [0.779,0.92]	27	68.0
Slide 2	0.857 [0.781,0.922]	27	76.0
Slide 3	0.913 [0.874,0.947]	33	83.4
Slide 4	0.921 [0.891,0.948]	34	88.6
Slide 5	0.926 [0.898,0.953]	34	87.3
Slide 6	0.922 [0.887,0.955]	34	56.9
Slide 7	0.928 [0.876,0.978]	34	23.5
Median			
Slide 1	0.869 [0.801,0.931]	28	67.9
Slide 2	0.886 [0.818,0.946]	29	76.0
Slide 3	0.953 [0.926,0.98]	36	83.4
Slide 4	0.955 [0.934,0.975]	37	88.5
Slide 5	0.96 [0.939,0.98]	39	87.2
Slide 6	0.966 [0.945,0.985]	38	56.9
Slide 7	0.954 [0.908,0.992]	39	23.5

Table I**Osteoarthritis and Cartilage**

Weighted kappa analysis of each slide in comparison to the

Slides with the most individual histological-outcome weighted-kappa's > 0.8 (Supplementary Table 1) were also toward the inter-condylar notch (maximum: 34/40 slides 4–7; median: 38–39/40 slides 5–7; Table I). However, there was a marked decrease in the number of histological-outcomes that could be scored in slides 6 and 7 (Table I), as topographically-distributed anatomic features were no longer present/scorable in all animals (notably anterior and posterior meniscus; Fig. 1). Slide-5 having the highest pooled- and individual-parameter agreement for both maximum and median after slide-6

and -7 while enabling scoring of all histological-outcomes in the majority of mice (Table I), was selected as optimum for further analysis.

Agreement of individual histological-outcomes stratified by experimental group

We compared scores derived from slide-5 with individual histological-outcome median and maximum scores from the entire set of slides (all time-points within each model). Weighted-kappa agreement was high regardless of the histological-outcome or comparator ($81\% > 0.8$, 8% 0.61 – 0.8). Only 19 of the 240 comparisons were identified as POCs (weighted-kappa ≤ 0.4) for slide-5 versus the median (Table II). POCs were observed in cartilage, subchondral bone, meniscus, and enthesophyte outcomes, with the majority (84.2% , $16/19$) in control groups (NOC 6, SHAM 5, ACLS 5). In ptOA mice only tibial subchondral bone sclerosis in ACLR, and anterior meniscal proteoglycan loss in DMM had poor agreement. Similarly, only 15 of the 240 comparisons between slide-5 and maximum scores were identified as POCs (Table II). Again most (73.3% , $11/15$) POCs were in control groups (NOC 4, SHAM 3, ACLS 4) and included cartilage, subchondral bone, and meniscal outcomes, while POCs in ptOA mice were restricted to anterior and posterior meniscal proteoglycan loss and damage in DMM and/or ACLR. Agreement of all available histological-outcomes in all animals at all time-points were visualised using scatter plots (Fig. 3). Agreement between slide-5 and both maximum and median scores were all excellent (weighted-kappa 0.81 – 0.99). Control groups displayed mild pathologies (low scores) in some animals: primarily superficial cartilage roughening and proteoglycan loss, mild subchondral bone sclerosis, meniscal bone formation, and superficial proteoglycan loss. Minor variations in the context of overall low scores in controls resulted in lower weighted-kappas compared with ptOA groups. Within ptOA models, histopathology-scores were lower in DMM compared with ACLT and ACLR, and again this was associated with reduced weighted-kappa particularly slide-5 versus maximum. The weighted-kappa agreements using slide-5 were equal-to or better than those for maximum versus median score from the full slide-set.

Inter-group detection of ptOA histopathology

We investigated if statistically significant differences between ptOA models and their controls (DMM vs Sham, ACLT vs Sham, ACLR vs ACLS) at different time-points were still detected using the single slide-5 approach (Table III). The majority of differences could be reliably reproduced, with sensitivity ranging from 93.15% (ACLT vs Sham maximum) to 98.19% (DMM vs Sham maximum). Specificity showed a similar range (93.10% in ACLT vs Sham maximum to 98.75% in ACLR vs ACLS median), indicating few “false-positives” using slide-5. Sensitivity and specificity using slide-5 were very similar (equal-to or better for 4/6 outcomes) to those using maximum score versus median score from the entire slide-set (Supplementary Table II).

The majority of statistically significant histology-outcome differences lost (“false-negatives”) using slide-5 involved the meniscus ($12/19$) or subchondral bone ($5/19$), and all false-positive differences were meniscal ($11/18$) or subchondral bone ($7/18$) histology-outcomes (Table IV). There was no clear difference between models in terms of the number of false-negatives or false-positives, or in the histology-outcomes affected. Failure to detect differences using slide-5 did not appear to be driven by effect-size (10 – 40% depending on the individual histology-outcome) which had a similar range to those detected as statistically significant using maximum or median from the entire slide set range, with $5/19$ incongruously larger with slide-5. Similarly, $13/18$ false-positive differences (12.5 – 50% effect

Median			Maximum		
POC	Animal Model	Kappa-Value K [–95%,+95%]	POC	Animal Model	Kappa-Value K [–95%,+95%]
Osteochondral Damage					
Proteoglycan Loss Tibia	SHAM	0.257 [–1, 1]	Proteoglycan Loss Tibia	SHAM	0.25 [–1, 1]
Cartilage Damage Femur	NOC	0.221 [–1, 1]	Subchondral Bone Vascular	NOC	0.208 [–1, 1]
	SHAM	0.371 [–1, 1]	Invasion Tibia	SHAM	0.362 [–1, 1]
	ACLS	0.179 [–1, 1]		ACLS	0.389 [–1, 1]
Cartilage Damage Tibia	NOC	0.107 [–1, 1]	Subchondral Bone Sclerosis Tibia	ACLS	0.333 [–1, 1]
	SHAM	0.189 [–1, 1]			
	ACLS	0.235 [–1, 1]			
Subchondral Bone Vascular Invasion Tibia	NOC	0 [–1, 1]			
	SHAM	0 [–1, 1]			
	ACLS	–0.036 [–1, 1]			
Subchondral Bone Sclerosis Tibia	ACLR	0.345 [–1, 1]			
Meniscal Pathology					
Anterior Damage	NOC	0 [–1, 1]	Anterior Damage	NOC	0 [–1, 1]
	DMM	0.008 [–1, 1]		DMM	0.011 [–1, 1]
Anterior Proteoglycan Loss	NOC	0.376 [–0.404, 0.853]	Anterior Proteoglycan Loss	NOC	0.245 [0.244, 0.244]
	DMM	0.224 [–1, 1]		DMM	0.253 [–0.209, 0.715]
	ACLS	0.213 [–1, 1]		ACLS	0.196 [–0.539, 0.752]
Posterior Damage	NOC	0 [–1, 1]	Bone Formation Anterior	NOC	0 [–1, 1]
	ACLS	0 [–1, 1]	Posterior Damage	DMM	0.25 [–1, 1]
				SHAM	0 [–1, 1]
			Posterior Proteoglycan Loss	ACLR	0.256 [–1, 1]
				ACLS	0.343 [–1, 1]
Osteophyte/Enthesophyte Pathology					
Posterior Tibial Enthesophyte Severity	SHAM	0.267 [–1, 1]			

Table II

Osteoarthritis and Cartilage

POC (Kappa ≤ 0.4) with single slide-scoring.

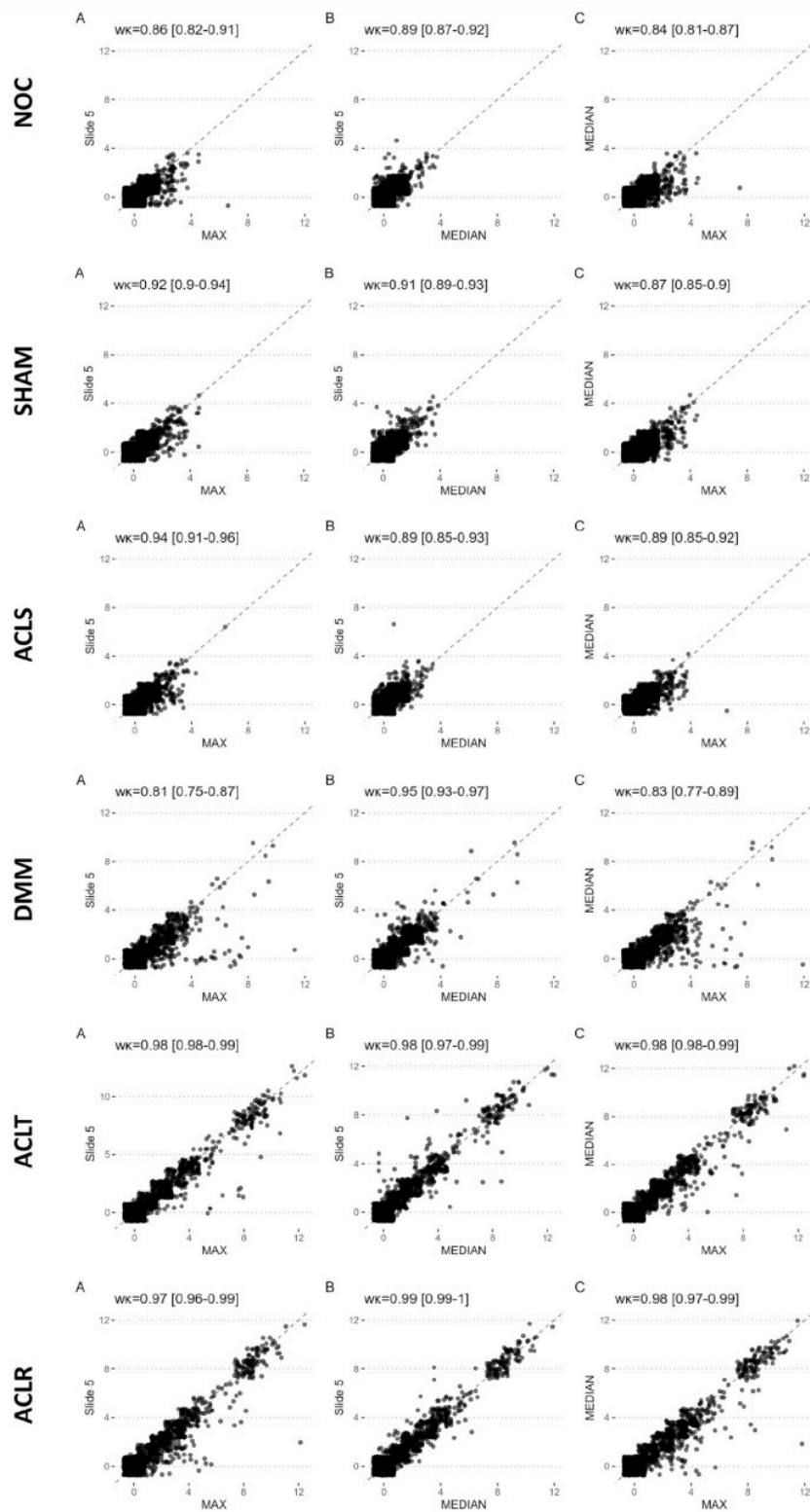
size) were smaller with slide-5 than with maximum/median. Most of the false-negative histology-outcomes using single slide-5 scoring were in early (16/19 wk-1 or -4) rather than late (wk-8 or -16) post-injury time-points, while false-positives were relatively evenly split between early and late times (7/18 and 11/18, respectively). The same patterns were seen with “false-negatives” and “false-positives” when using maximum versus median scores from the full slide set (Supplementary Table 3): all were meniscal or subchondral bone histology-outcomes, unrelated to effect size (14/19 larger/smaller with maximum), and 12/19 in early time-points.

Discussion

Animal models remain a cornerstone of discovery- and therapeutic-development-research across medical disciplines and diseases, with OA being no exception.^{6,20,28} In OA animal-model-based research, histopathology is the gold-standard for assessing structural disease. This is an exacting and time-consuming process, particularly with the current recommendations and widely accepted best-practice regarding evaluation of multiple serial sections in mouse OA models.^{14,22–26} Reducing the technical-, time- and personnel-burden of mouse OA-model histopathological evaluation without compromising the validity and reproducibility of findings would be of significant benefit to the field. In this regard, a recent study demonstrated that grading of a single mid-coronal section using either of two published histopathology schemes allowed equivalent definition of ptOA cartilage pathology severity to that determined by summing multiple sections in mice 8 weeks post-DMM.²⁷ However, within a “single disease phenotype” such as knee-ptOA there is considerable heterogeneity in injury type/mechanism, topographical distribution and severity of macroscopic/microscopic changes, and

disease progression. With no single “optimal” ptOA model, researchers need to select that/those most appropriate for their specific research question and target patient population.⁶ In the current study we have for the first time demonstrated that grading of a single rather than multiple serial sections can reliably detect ptOA-related changes in three of the most widely used ptOA mouse models: DMM, ACLT, and ACLR. Importantly, we show that this optimised histology evaluation was possible at multiple time-points representing acute-injury, early-, progressive- and late-stage disease in all models. Moreover, using a scoring system that evaluates joint-wide pathology,^{23,29–33} we showed that single-slide analysis was applicable not only for cartilage erosion (confirming previous findings²⁷) but also other aspects of cartilage pathology (proteoglycan loss, chondrocyte hypertrophy), subchondral bone remodelling, osteophyte/enthesophyte development, and meniscal pathology.

In the current study we used sagittal as opposed to the coronal sections evaluated by Armstrong et al.,²⁷ suggesting that single-slide ptOA histopathology optimisation is applicable to both sectioning approaches. In the Armstrong study a single section in the central load-bearing region of the joint was manually selected using histological landmarks, necessitating serial sectioning and staining to then enable identification of the optimal slide for scoring. We employed a consistent protocol for cutting and collecting sections based on a starting point defined by distinct anatomical landmarks visible in unstained sections, and standardised spacing between collected samples. This allowed us to determine whether mouse knee ptOA histopathology optimisation could potentially include not just scoring but also cutting and staining of a single section. Comparison of agreement derived from slides 1–7 to the median and maximum values from the entire serial slide set, identified slide-5 as the best-performing single slide. Slide-5 is mid-sagittal (Fig. 1), confirming



(caption on next page)

Figure 3

Osteoarthritis and Cartilage

Scatterplots showing the agreement of scores derived from slide-5 with maximum values (A) and median scores (B) from the entire set of slides per type of injury. Agreement of maximum and median scores both derived from the entire set of slides are shown in (C). The weighted kappa (w_k) and range of the 95% confidence interval (square brackets) is noted for each. Each dot represents the score for one of 40 histological-outcomes from an individual animal. All dots were plotted with a 0.5 jitter to allow visualisation of all data points. The large number of overlapping individual observations with complete score agreement will appear as boxes as data points are distributed across a range of ± 0.5 of the actual integer points (e.g. 0/0, 1/1 etc).

that the central load-bearing region of the medial-femoro-tibial joint is the optimal location for analysis in all three ptOA models evaluated in this study.

The uniform performance of the same single sagittal slide (#5) across ptOA models may not be true for coronal sections, which may require manual slide selection to capture the more anterior versus posterior lesion locations in DMM versus ACLT and ACLR, and potentially multiple slides to capture tibial and femoral pathology due to the marked posterior femoral subluxation in ACL models (Fig. 2).^{6,27,32,33,46} Nevertheless, disparity across the width of the medial-femoro-tibial joint in anatomical presence of and/or pathology localisation in specific tissues, likely explains variation in weighted-kappa and % of histological-outcomes that could be scored in sagittal-slides 1–7. The meniscal poles, the preferential bone formation location in mouse OA,⁴⁷ are visualised in slides closer to the intercondylar notch, while meniscal proteoglycan loss and structural damage are more prominent in the mid-body visualised in sagittal sections of the centre of the medial-femoro-tibial compartment

(Fig. 1). Given the topographically-distinct anatomy of the meniscus, researchers interested in histopathology of this tissue during onset and progression of ptOA should score multiple sagittal slides (e.g. 4, 5 and 6) across the joint width. Focal localisation of ptOA subchondral bone pathology towards the intercondylar region particularly in early ptOA, also accounted for the somewhat poorer performance of slide-5 in reproducing differences in this histology-outcome detected using the entire slide-set. Again, this issue could be overcome by scoring additional slides (e.g. 6 and 7). Despite different effects on joint mechanics and more rapid and severe disease in ACLT/ACLR than DMM,^{5,17,37,48} histology-outcome differences no longer detected using single-slide scoring (be that slide-5 or maximum score from the entire slide set) were predominantly seen early post-injury and were almost exclusively meniscal and subchondral bone in all models. This suggests a limited effect of ptOA model on the sagittal distribution of OA pathology, and that changes early in the disease course are more focal in all models. Sagittal pathological feature distribution and focal disease contribute to

		ACLR vs. ACLS Based on Median			
Based on Slide 5	Statistical Difference: Slide 5	Statistical Difference (80) 76	No Statistical Difference (80) 1	Sensitivity 95.00%	Specificity 98.75%
	No Statistical Difference: Slide 5	4	79		
Based on Slide 5	Statistical Difference: Slide 5	Based on Maximum Statistical Difference (78) 76	No Statistical Difference (82) 2	Sensitivity 97.44%	Specificity 97.56%
	No Statistical Difference: Slide 5	2	80		
Based on Slide 5	Statistical Difference: Slide 5	ACLT vs. SHAM Based on Median Statistical Difference (78) 74	No Statistical Difference (82) 2	Sensitivity 94.87%	Specificity 97.56%
	No Statistical Difference: Slide 5	4	80		
Based on Slide 5	Statistical Difference: Slide 5	Based on Maximum Statistical Difference (73) 68	No Statistical Difference (87) 6	Sensitivity 93.15%	Specificity 93.10%
	No Statistical Difference: Slide 5	5	81		
Based on Slide 5	Statistical Difference: Slide 5	DMM vs. SHAM Based on Median Statistical Difference (56) 53	No Statistical Difference (104) 4	Sensitivity 94.64%	Specificity 96.15%
	No Statistical Difference: Slide 5	3	100		
Based on Slide 5	Statistical Difference: Slide 5	Based on Maximum Statistical Difference (54) 53	No Statistical Difference (106) 3	Sensitivity 98.15%	Specificity 97.17%
	No Statistical Difference: Slide 5	1	103		

Table III

Osteoarthritis and Cartilage

Evaluation of test characteristics using a single slide compared to the median or maximum.

False Negative: Parameter	Time	False Positive: Parameter	Time
ACLR vs. ACLS			
Slide 5 vs. Median			
Subchondral Bone Sclerosis Tibia	wk 4	Subchondral Bone Sclerosis Tibia	wk 8
Subchondral Bone Vascular Invasion Tibia	wk 4		
Anterior Meniscal Proteoglycan Loss	wk 4		
Anterior Meniscal Proteoglycan Loss	wk 16		
Slide 5 vs. Maximum			
Posterior Meniscal Proteoglycan Loss	wk 1	Subchondral Bone Vascular Invasion Tibia	wk 1
Anterior Meniscal Proteoglycan Loss	wk 4	Subchondral Bone Sclerosis Tibia	wk 8
ACLT vs. SHAM			
Slide 5 vs. Median			
Proteoglycan Loss Femoral Cartilage	wk 4	Posterior Meniscal Damage	wk 1
Subchondral Bone Sclerosis Tibia	wk 4	Bone Formation Posterior Meniscal	wk 16
Bone Formation Posterior Meniscal	wk 4		
Posterior Meniscal Proteoglycan Loss	wk 8		
Slide 5 vs. Maximum			
Proteoglycan Loss Femoral Cartilage	wk 4	Subchondral Bone Sclerosis Tibia	wk 1
Subchondral Bone Sclerosis Tibia	wk 4	Anterior Meniscal Proteoglycan Loss	wk 4
Posterior Meniscal Proteoglycan Loss	wk 4	Subchondral Bone Vascular Invasion Tibia	wk 8
Bone Formation Posterior Meniscal	wk 4	Anterior Meniscal Proteoglycan Loss	wk 8
Posterior Meniscal Proteoglycan Loss	wk 8	Bone Formation Posterior Meniscal	wk 16
		Posterior Meniscal Damage	wk 16
DMM vs. SHAM			
Slide 5 vs. Median			
Subchondral Bone Vascular Invasion Tibia	wk 1	Subchondral Bone Sclerosis Tibia	wk 4
Bone Formation Posterior Meniscal	wk 1	Anterior Meniscal Damage	wk 4
Bone Formation Posterior Meniscal	wk 4	Anterior Meniscal Damage	wk 8
		Posterior Meniscal Proteoglycan Loss	wk 16
Slide 5 vs. Maximum			
Anterior Meniscal Proteoglycan Loss	wk 4	Subchondral Bone Sclerosis Tibia	wk 4
		Anterior Meniscal Proteoglycan Loss	wk 16
		Posterior Meniscal Proteoglycan Loss	wk 16

Table IV

Osteoarthritis and Cartilage

False discoveries using a single slide compared to the entire set of slides.

increased score variability when using a single slide, which appears to be more important than the effect-size in being able to detect statistically significant differences.

It is noteworthy that use of a single mid-sagittal slide performed better in ptOA than control joints (NOC/SHAM/ACLS). There was little difference between the three controls confirming lack of induced medial femoro-tibial OA-like pathology in SHAM and ACLS. While all three controls had low overall pathology, they had more POCs than the ptOA groups when comparing scores generated with slide-5 versus the complete set. The POC histology-outcomes in controls were superficial cartilage roughening and proteoglycan loss, mild subchondral bone sclerosis, and meniscal bone formation and superficial proteoglycan loss in some animals. The low-level histo-pathological features in these tissues in normal joints were more toward the intercondylar notch than the pathology induced by the joint injury models, which concentrate in the central load-bearing region. A single mid-coronal section may better enable detection of these normal histopathological features with dispersion across the medial compartment width, although visualisation of meniscal poles would still require multiple coronal sections. Simultaneous evaluation of pathology in medial and lateral femoro-tibial compartments is possible with a single mid-coronal slide, while requiring additional sagittal sectioning. This may be an important consideration in conditions where lateral femoro-tibial pathology is a feature such as spontaneous age-associated OA in male C57BL6 mice²⁷ and the ACLS model.³³

Optimising histopathology scoring of mouse OA models could have considerable benefit for the field. Experienced and trained researchers

(PH, CBL) require ~4 min on average to complete joint-wide scoring of a single slide. With a total of 288 animals or 2016 slides, the scoring time in the present study accumulates to > 134 h. Work health and safety recommendations in our facility restrict microscope use to no more than 4 h per day, thus ~34 workdays were needed to complete scoring. The single slide scoring system would reduce the time needed by 85.7% (~5 workdays). This is a dramatic improvement in research throughput and productivity, and with the average salary for an experienced post-doctoral researcher in Australia being \$93,422/annum (\$47.91/hour based on a 37.5-hour work-week), this would equate to a saving of ~\$5500 for the current study. Newer technologies enable scoring of digitised sections rather than slides. While this does not alter the time taken to score each section, it may allow for more sections to be scored in a single day. However, this is offset by the time and cost for slide scanning, data transfer, and storage: using our Nanozoomer-S210 (Hamamatsu Inc) ~6 h/200 slides. Our data demonstrate the potential for even further research productivity gain and workforce/financial savings through limiting sectioning and staining to a single mid-sagittal slide. This is only possible with highly reproducible embedding and sectioning methodology and establishing a consistent starting point based on easily identifiable anatomical landmarks. This could be affected by variable joint size and thus mouse age, strain, and sex, as well as injury-model or other factors that significantly alter anatomy/morphology (e.g. growth deformity in genetically modified mice). Researchers should optimise the sectioning technique relevant to their particular experimental mice/conditions to ensure reproducible slide-number and joint-location alignment and/or consider a modified approach where 3 sections

guaranteed to span the central weight-bearing region (slide 4, 5, and 6 in our method; Fig. 1) are cut and stained, before selecting and scoring a single mid-sagittal slide. This 3-slide approach would also allow capture of meniscal and subchondral bone histopathological features that have topographic and/or early disease-stage dispersity e.g. score meniscal pathology in all 3 slides and use maximum recorded value.

The current study strongly supports the utility of single sagittal slide histopathology scoring for mouse ptOA but is not without limitations. We used detection of statistically significant differences between ptOA models and their controls to evaluate the sensitivity/specificity of the single-slide approach. While researchers use scoring systems to quantify pathology and differences in these with e.g. time or treatment, the effects size and biological significance/importance of changes must be considered along with any statistical significance. Nevertheless, confidence that the optimised single-slide method will detect expected differences is important for its uptake and comparing future studies using a single-slide with previously published outcomes generated with current multi-slide maximum/median approaches. While shown to work in three of the most common ptOA models, the single-slide system may require modification in OA models that have distinct pathology distribution e.g. ACLS and ageing-OA as noted earlier. Researchers must be cognisant of topographic distribution of maximal joint pathology in the particular model they are using, which can vary with even subtle differences in induction/injury mechanism.^{6,17,49} Further research is necessary before establishing use of this system in females, and mice of different strains and ages, which could drive topographic variability in pathology localisation. Finally, particular pathological features may be less amenable to a single sagittal- or coronal-slide approach: our data highlights limitations when assessing OA meniscal changes, and researchers should validate the method for evaluating any pathologies not scored in our histopathology system.

Conclusion

While expansion in histopathology scoring to assess multiple joint tissues has advanced our understanding of joint-wide pathology and pathophysiology, the time needed to score many parameters on numerous serial sections (40 outcomes on each of 7 slides in our system) is a limiting factor in their widespread adoption. We show that using a single consistent mid-sagittal slide dramatically reduced the time needed to score diverse histopathological changes without compromising sensitivity or specificity, making it a fast, reliable, and detailed outcome assessment of joint-wide OA, across a variety of ptOA models and time-points within these.

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None of the funding agencies had any input into the conceptualisation or implementation of the study or analysis and interpretation of data.

Declaration of Competing Interest

The authors declare no competing interests for the work presented in this paper.

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Author contributions

Conception and design of study: PH, CBL. Acquisition of data: PH, CB, SZ, CS, EC, SS, DB, YL. Analysis and interpretation of data: PH, CBL, CB, RH. Drafting of article: PH, RH, CB. Revising it critically for important intellectual content: CBL, CS, SZ, EC. All authors have approved the final version of the manuscript. PH (patrick.haubruck@sydney.edu.au) and CBL (christopher.little@sydney.edu.au) take responsibility for the integrity of the work as a whole.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.joca.2023.07.013.

References

- Spitaels D, Mamouris P, Vaes B, Smeets M, Luyten F, Hermens R, et al. Epidemiology of knee osteoarthritis in general practice: a registry-based study. *BMJ Open* 2020;10, e031734.
- Kloppenburg M, Berenbaum F. Osteoarthritis year in review 2019: epidemiology and therapy. *Osteoarthr Cartil* 2020;28:242–8.
- Thomas AC, Hubbard-Turner T, Wikstrom EA, Palmieri-Smith RM. Epidemiology of posttraumatic osteoarthritis. *J Athl Train* 2017;52:491–6.
- Snoeker B, Turkiewicz A, Magnusson K, Frobell R, Yu D, Peat G, et al. Risk of knee osteoarthritis after different types of knee injuries in young adults: a population-based cohort study. *Br J Sports Med* 2020;54:725–30.
- Watt FE, Corp N, Kingsbury SR, Frobell R, Englund M, Felson DT, et al. Towards prevention of post-traumatic osteoarthritis: report from an international expert working group on considerations for the design and conduct of interventional studies following acute knee injury. *Osteoarthr Cartil* 2019;27:23–33.
- Zaki S, Blaker CL, Little CB. OA foundations - experimental models of osteoarthritis. *Osteoarthr Cartil* 2022;30:357–80.
- Newman S, Ahmed H, Rehmatullah N. Radiographic vs. MRI vs. arthroscopic assessment and grading of knee osteoarthritis - are we using appropriate imaging? *J Exp Orthop* 2022;9:2.
- Aigner T, Cook JL, Gerwin N, Glasson SS, Lavery S, Little CB, et al. Histopathology atlas of animal model systems - overview of guiding principles. *Osteoarthr Cartil* 2010;18(Suppl.3):S2–6.
- McCoy AM. Animal models of osteoarthritis: comparisons and key considerations. *Vet Pathol* 2015;52:803–18.
- Cook JL, Kuroki K, Visco D, Pelletier JP, Schulz L, Laferte FP. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the dog. *Osteoarthr Cartil* 2010;18(Suppl.3):S66–79.
- Little CB, Smith MM, Cake MA, Read RA, Murphy MJ, Barry FP. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in sheep and goats. *Osteoarthr Cartil* 2010;18(Suppl.3):S80–92.
- McIlwraith CW, Frisbie DD, Kawcak CE, Fuller CJ, Hurtig M, Cruz A. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the horse. *Osteoarthr Cartil* 2010;18(Suppl.3):S93–105.
- Gerwin N, Bendele AM, Glasson S, Carlson CS. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the rat. *Osteoarthr Cartil* 2010;18(Suppl.3):S24–34.

14. Glasson SS, Chambers MG, Van Den Berg WB, Little CB. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the mouse. *Osteoarthritis Cartil* 2010;18(Suppl.3):S17–23.
15. Kraus VB, Huebner JL, DeGroot J, Bende A. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the guinea pig. *Osteoarthritis Cartil* 2010;18(Suppl.3):S35–52.
16. Lavery S, Girard CA, Williams JM, Hunziker EB, Pritzker KP. The OARSI histopathology initiative - recommendations for histological assessments of osteoarthritis in the rabbit. *Osteoarthritis Cartil* 2010;18(Suppl.3):S53–65.
17. Blaker CL, Clarke EC, Little CB. Using mouse models to investigate the pathophysiology, treatment, and prevention of post-traumatic osteoarthritis. *J Orthop Res* 2017;35:424–39.
18. Little CB, Hunter DJ. Post-traumatic osteoarthritis: from mouse models to clinical trials. *Nat Rev Rheumatol* 2013;9:485–97.
19. Soul J, Barter MJ, Little CB, Young DA. OATargets: a knowledge base of genes associated with osteoarthritis joint damage in animals. *Ann Rheum Dis* 2020;80:376–83.
20. Malfait AM, Little CB. On the predictive utility of animal models of osteoarthritis. *Arthritis Res Ther* 2015;17, 225.
21. Moon PM, Beier F. Novel insights into osteoarthritis joint pathology from studies in mice. *Curr Rheumatol Rep* 2015;17, 50.
22. Chambers MG, Bayliss MT, Mason RM. Chondrocyte cytokine and growth factor expression in murine osteoarthritis. *Osteoarthritis Cartil* 1997;5:301–8.
23. Little CB, Barai A, Burkhardt D, Smith SM, Fosang AJ, Werb Z, et al. Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development. *Arthritis Rheum* 2009;60:3723–33.
24. McNulty MA, Loeser RF, Davey C, Callahan MF, Ferguson CM, Carlson CS. A comprehensive histological assessment of osteoarthritis lesions in mice. *Cartilage* 2011;2:354–63.
25. Sampson ER, Beck CA, Ketz J, Canary KL, Hilton MJ, Awad H, et al. Establishment of an index with increased sensitivity for assessing murine arthritis. *J Orthop Res* 2011;29:1145–51.
26. Pinamont WJ, Yoshioka NK, Young GM, Karuppagounder V, Carlson EL, Ahmad A, et al. Standardized histomorphometric evaluation of osteoarthritis in a surgical mouse model. *J Vis Exp* 2020;159:e60991.
27. Armstrong AR, Carlson CS, Rendahl AK, Loeser RF. Optimization of histologic grading schemes in spontaneous and surgically-induced murine models of osteoarthritis. *Osteoarthritis Cartil* 2021;29:536–46.
28. Smith MM, Clarke EC, Little CB. Considerations for the design and execution of protocols for animal research and treatment to improve reproducibility and standardization: "DEPART well-prepared and ARRIVE safely". *Osteoarthritis Cartil* 2017;25:354–63.
29. Jackson MT, Moradi B, Zaki S, Smith MM, McCracken S, Smith SM, et al. Depletion of protease-activated receptor 2 but not protease-activated receptor 1 may confer protection against osteoarthritis in mice through extracartilaginous mechanisms. *Arthritis Rheumatol* 2014;66:3337–48.
30. Shu CC, Jackson MT, Smith MM, Smith SM, Penm S, Lord MS, et al. Ablation of perlecan domain 1 heparan sulfate reduces progressive cartilage degradation, synovitis, and osteophyte size in a preclinical model of posttraumatic osteoarthritis. *Arthritis Rheumatol* 2016;68:868–79.
31. Shu CC, Zaki S, Ravi V, Schiavinato A, Smith MM, Little CB. The relationship between synovial inflammation, structural pathology, and pain in post-traumatic osteoarthritis: differential effect of stem cell and hyaluronan treatment. *Arthritis Res Ther* 2020;22, 29.
32. Zaki S, Smith MM, Smith SM, Little CB. Differential patterns of pathology in and interaction between joint tissues in long-term osteoarthritis with different initiating causes: phenotype matters. *Osteoarthritis Cartil* 2020;28:953–65.
33. Blaker CL, Zaki S, Little CB, Clarke EC. Long-term effect of a single subcritical knee injury: increasing the risk of anterior cruciate ligament rupture and osteoarthritis. *Am J Sports Med* 2021;49:391–403.
34. Little CB, Meeker CT, Golub SB, Lawlor KE, Farmer PJ, Smith SM, et al. Blocking aggrecanase cleavage in the aggrecan interglobular domain abrogates cartilage erosion and promotes cartilage repair. *J Clin Invest* 2007;117:1627–36.
35. National Health and Medical Research Council. Australian code for the care and use of animals for scientific purposes. 8th Edition Canberra: National Health and Medical Research Council; 2013.
36. Blaker CL, Ashton DM, Doran N, Little CB, Clarke EC. Sex- and injury-based differences in knee biomechanics in mouse models of post-traumatic osteoarthritis. *J Biomech* 2021;114, 110152.
37. Glasson SS, Blanchet TJ, Morris EA. The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse. *Osteoarthritis Cartil* 2007;15:1061–9.
38. McHugh ML. Interrater reliability: the kappa statistic. *Biochem Med* 2012;22:276–82.
39. Little CB, Hunter DJ. Post-traumatic osteoarthritis: from mouse models to clinical trials. *Nat Rev Rheumatol* 2013;9:485–97.
40. Team RC. R: a language and environment for statistical computing; 2013.
41. Wickham H, Wickham MH. Package 'tidyr'. Easily Tidy Data with 'spread' and 'gather' Functions; 2017.
42. Wickham H, Francois R, Henry L, Müller K. dplyr: a grammar of data manipulation. R Package Version; 2015.
43. Robin X, Turck N, Hainard A, Tiberti N, Lisacek F, Sanchez JC, et al. pROC: an open-source package for R and S+ to analyze and compare ROC curves. *BMC Bioinformatics* 2011;12, 77.
44. Revelle WR. Procedures for personality and psychological research; 2017.
45. Wickham H. ggplot2: elegant graphics for data analysis. Springer-Verlag New York; 2009.
46. Christiansen BA, Anderson MJ, Lee CA, Williams JC, Yik JH, Haudenschild DR. Musculoskeletal changes following non-invasive knee injury using a novel mouse model of post-traumatic osteoarthritis. *Osteoarthritis Cartil* 2012;20:773–82.
47. Ramos-Mucci L, Javaheri B, van 't Hof R, Bou-Gharios G, Pitsillides AA, Comerford E, et al. Meniscal and ligament modifications in spontaneous and post-traumatic mouse models of osteoarthritis. *Arthritis Res Ther* 2020;22, 171.
48. Christiansen BA, Guilak F, Lockwood KA, Olson SA, Pitsillides AA, Sandell LJ, et al. Non-invasive mouse models of post-traumatic osteoarthritis. *Osteoarthritis Cartil* 2015;23:1627–38.
49. Calkins MA, Read RA, Corfield G, Daniel A, Burkhardt D, Smith MM, et al. Comparison of gait and pathology outcomes of three meniscal procedures for induction of knee osteoarthritis in sheep. *Osteoarthritis Cartil* 2013;21:226–36.

Chapter 3

3 Characterization of the Innate and Adaptive Immune Response during Development of ptOA

3.1 Aim of the Study

In recent years specific immune cell changes were identified that are believed to drive the inflammatory response in context with ptOA. However, to date no study investigated if different joint pathologies result in a different acute inflammatory reaction and thus explain why some joint injuries cause rapid ptOA and others do not. Furthermore, the cellular inflammatory innate and adaptive immune response subsequent to joint injury and during development of ptOA is still not fully understood. The study presented in this chapter relates to **Aim 1** of this thesis.

In this study the loco-regional and systemic innate and adaptive inflammatory cell responses are compared in three of the most commonly used mouse models of ptOA: DMM, ACLT and ACLR. Using these models allows detailed temporal evaluation and direct comparison with non-OA-inducing injuries (SHAM, ACLS) not possible in human clinical studies, as well as the ability to explore the effect of injury to different joint-tissues (DMM *vs* ACLT) and surgery itself (ACLT *vs* ACLR) on the severity and phenotype of the inflammatory response.

3.2 Drafted Manuscript for Submission: Innate and adaptive immune response to joint injury and the onset and progression of post-traumatic osteoarthritis: a comparison of common murine models.

Innate and adaptive immune response to joint injury and the onset and progression of post-traumatic osteoarthritis: a comparison of common murine models.

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Introduction

Osteoarthritis (OA) is the most common joint disease [1] and the prevalence is expected to further increase as population-level risk-factors (e.g. age, obesity, joint injury) also increase [2]. OA contributes substantially to the global burden of disease being the second highest cause for years lived with disability in 2015 [2] and the leading condition contributing to healthcare expenditure in Australia [3]. OA has extraordinary individual and societal impact [2], with the knee accounting for >80% of the total burden [1]. While there are multiple underlying causes, post-traumatic-OA (ptOA) accounts for at least 12% of all cases of symptomatic OA [4] with knee injury at a young age increasing the risk of developing OA almost sixfold [5]. Restoration of biomechanics alone does not prevent ptOA development after injury [6, 7], suggesting other factors influence individual OA risk.

Studies investigating OA pathophysiology have increasingly provided evidence for a crucial immuno-inflammatory component involving both adaptive and innate immune systems [8-14]. A predominantly pro-inflammatory T-helper cell polarization was found in the synovial compartment in OA, with increased numbers of pro-inflammatory Th1 [8, 15, 16], Th17 [8, 17] and decreased numbers of anti-inflammatory Th2 [8, 18] and T-regulatory (Tregs) [8, 19] subsets. Studies investigating the innate immune response in OA have identified monocyte-derived (“blood-originated”) macrophages (BOM) as the main culprit in promoting and sustaining inflammation [20, 21]. In contrast, synovial tissue resident macrophages (STRM) maintain joint homeostasis [22] and act as sentinels for molecular and cellular changes in the joint cavity itself [20]. However, the majority of data in OA patients has analysed later-stage disease, thus relatively little is known about T-helper and monocyte/macrophage cell polarization subsequent to injury and during development and progression of ptOA, and if the inflammatory reaction initiates OA pathological changes or is a secondary response to it [23]. Pre-clinical animal models provide a key pathway to address these questions [24, 25].

While murine ptOA models allow the longitudinal study between disease onset and progression towards end-stage, results focusing on the inflammatory response need to be interpreted carefully as the majority of current widely used models use a surgical approach to induce joint injury which may influence the scale and type of immuno-inflammatory response [26]. Non-surgical mouse models, such as mechanical anterior cruciate ligament rupture (ACLR), have therefore been suggested as being superior in reflecting physiological joint injury mechanisms [26]. However, ACLR-induced OA in mice has a very rapid-onset and severe structural pathology that may not reflect slower onset human ptOA progression or degree of pathological change as well as the well-established surgical destabilisation of the medial meniscus (DMM) model [24, 25]. In order to account for these individual strengths and weaknesses, we evaluated surgical and non-surgical murine ptOA models and their respective sham-controls. Furthermore, we investigated the innate and adaptive immune response not only in the injured joint (synovial tissue) but also in primary (draining lymph node) and secondary (spleen and contra-lateral bone-marrow) lymphatic tissues providing an evaluation of loco-regional and systemic immuno-inflammatory response. The findings of the current study on the spatio-temporal immune reaction to joint injury and during onset and progression of ptOA, will help to better understand the role of the immune response in this complex disease and identify specific potential therapeutic targets.

Materials and Methods

Animals

All experiments were approved by the Northern Sydney Local Health District Animal Ethics Committee (protocol RESP/18/162), and the ARRIVE guidelines were followed [27]. For all experiments 10-12-week-old, male C57BL/6 mice were utilized, and mice were housed in individually-ventilated cages (up to 5/cage) with commercial bedding, environmental enrichment (perspex housing, wooden sticks, tissues), *ad-libitum* access to standard pelleted mouse feed and acidified water, in a dedicated SPF vivarium maintained at 20-22 °C with 12-hour light/dark cycles, and cared for in accordance with the Australian code [28].

Animals were assigned to one of 6 intervention groups by random allocation of cages (n=2-5 mice/cage; all mice in a cage assigned the same injury-model/group and harvest time) but ensuring experimental numbers (see below) were met. The 6 intervention groups were: (1) naïve mice that served as age-matched non-operated control (NOC) for all other groups; (2) destabilization of the medial meniscus (DMM) surgical model of slowly progressive ptOA [29]; (3) ACL transection (ACLT) [30] surgical model of rapidly progressive ptOA for comparison to group 2 and 6; (4) surgical arthrotomy without injury to the ACL or meniscus (SHAM) to determine the effect of surgery alone and control for groups 2 and 3; (5) mechanical ACL rupture (ACLR) induced by single compressive load applied to the flexed knee as a rapid-onset ptOA model [31], that by inducing ptOA without additional macroscopic damage to other structures within the joint [32] serves as a direct control for the confounding effects of surgery in group 3; and (6) mechanical ACL strain (ACLS) induced by a single compressive load to 75% of that required to rupture the ACL [33], that does not induce progressive OA joint pathology and serves as control for effects of compressive joint loading in group 5. Injury induction was staggered over 6-months but animals in all groups were simultaneously active throughout the study and housed in the same room and cage-rack to minimise confounding.

All interventions were done with animals under general anaesthesia (2% isoflurane in oxygen), and surgeries were conducted by two trained surgeons (PH and ACC) using standard sterile techniques (surgical site shaving and skin preparation; sterile drapes and surgical gloves; autoclaved instruments) and an operating microscope. Surgical incisions were closed in 3 layers using sterile absorbable suture (8/0 polyglactin 910; joint capsule and subcutaneous layer) and tissue glue (skin) to ensure anatomical reconstruction. To avoid confounding effects on the inflammatory response, animals received no other medication, rather they were monitored closely in the first 2 post-operative weeks for signs of lameness, wound dehiscence, or infection, which if detected required their euthanasia under the ethics protocol (0 mice had these issues).

Results from previous studies indicated that $n=12$ per group is necessary to detect statistical differences in histopathological scoring of murine ptOA cartilage pathology (the most variable study outcome measure) with $>80\%$ power [34-37]. A total of 8 time points were chosen at which mice from all intervention groups were sacrificed and tissues processed for histological analysis and flow cytometry. These correspond to the acute post-injury (day 1, 3 and 7), and early (2 weeks), progressive (4 and 6 weeks) and established/advanced (8 and 10 weeks) ptOA phases. Thus, the entire study cohort included a total of 576 mice (12 mice/group/time-point). Animals in groups 2, 3 and 5 that did not have complete ligament disruption or develop ptOA (based on gross evaluation at the time of joint harvest: $n = 8$), or any animals with other conditions requiring intervention or euthanasia ($n = 1$ for malocclusion), were excluded resulting in 567 mice being included in the final analysis.

Flow cytometry

Spleen, contralateral femoral bone-marrow, draining lymph-nodes (pooled inguinal and iliac) and synovial tissue were harvested from all animals as described [38] and see **Chapter 2.2.2.1**,

with tissues from two animals pooled to increase cell numbers resulting in n=6 samples/intervention/time. Compensation settings for spectral overlap on the multi-colour flow cytometer were set using the AbC Total Antibody Compensation Kit (Thermo Fischer), isotype control antibodies were used during optimization of the experiments to assess any non-specific binding, and “fluorescent minus one” (FMO) were used to set initial gates [39]. Respective mononuclear immune cell subsets were initially gated using a forward-/side-scatter (FSC-A and SSC-A) dot plot using FlowJo software (Tree Star), followed by a hierarchical antibody-based gating strategy. Two multi-color antibody panels were optimized, with an extra-cellular epitope/staining approach to identify monocytes/macrophages and their subsets, and extra- and intra-cellular epitope/staining to identify T-cells and their subsets (See Table S1 for Abs and Supplementary Figures 1 and 2). All gating and flow cytometry were done by a single researcher (PH), batched by timepoint but with randomization and concealment of intervention group in each batch.

Histology

After removal of the anterior synovium (for flow cytometry) the knee joints were fixed, decalcified, paraffin-embedded, and serial sagittal sections across the medial femoro-tibial joint cut and stained with toluidine blue-fast green as previously described [37, 40]. Utilizing a modified scoring system with a single mid-sagittal medial femoro-tibial compartment slide [41] and **Chapter 2.2.2.2**, summed femoral and tibial cartilage structural damage, tibial subchondral bone sclerosis, summed anterior and posterior femoral and tibial osteophyte and enthesophyte size and maturity, tibial osteochondral damage, and posterior femoral synovitis (individual parameters (sub-synovial inflammatory cell infiltration, synovial lining cell hyperplasia, panus outgrowth and bone erosion) and total score), were determined by a single

observer (PH) blinded to time and intervention-group. Median values for each histopathological feature for each group and time point were generated and compared.

Statistics

The individual histology-outcomes were considered as categorical and independent variables, as prior analysis demonstrates they change differentially and with distinct temporal patterns in different OA models, and are independently modified in response to treatment/genetic-modification [34, 36, 37, 40, 42]. Non-parametric analyses (Kruskal-Wallis and Mann-Whitney-U) were used to assess differences between groups for all histological-outcomes at each post-injury timepoint. Histological data is presented as median $\pm$ error. Flow cytometry data is presented as mean $\pm$ standard error of the mean (SEM). Two-way analysis of variance (ANOVA) with post-hoc Tukey's correction for multiple comparisons was used to compare all individual variables. All statistical tests used an α -level of 0.05. All statistical calculations were performed with GraphPad Prism (Version 9.0.0).

Results

Histological evaluation of ptOA osteochondral pathology onset and progression

Cartilage damage scores remained <2 in all control groups (NOC/ACLS/SHAM) for the duration of the study and significant differences were only observed at late-stage time points (ACLS $>$ NOC, $p=0.0003$) (Fig 1A). All ptOA models (DMM/ACLT/ACLR) had increased cartilage damage starting at day-3, with ACLT and DMM significantly greater than SHAM ($p<0.01$) and ACLR $>$ ACLS ($p=0.0233$), and thereafter all remaining greater than their respective controls (TABLE S2). Cartilage damage increased rapidly from day-1 in ACLT and ACLR peaking at day-28 and remaining stable thereafter with no difference between surgical and non-surgical models. Cartilage damage increased more slowly in DMM, with scores in ACLR and ACLT significantly higher than DMM scores at day-28 ($p<0.001$) and day-56 ($p<0.001$). Total osteochondral damage/erosion, calculated as cartilage damage and osteochondral damage, showed a similar pattern as cartilage damage alone and was predominantly observed in ACL-injury models (Fig. 1B). While osteo-/enthesophyte development was not observed in any of the control groups, ACLT and ACLR had significantly higher scores not only than their respective controls but also ACLT/ACLR $>$ DMM mice starting at day-7 and differences being significant at day-28 (Fig 1C; $p<0.0121$; Table S2). DMM had significantly higher scores than SHAM from day-42-70 (Fig. 1C). Subchondral bone sclerosis showed little change over time in controls nor difference between groups (Fig 1D, Table S2). In contrast to the model-specific pattern in cartilage damage, only DMM had significantly increased bone sclerosis compared with controls, and compared with ACLT (day-42: $p=0.0079$) (Fig. 1D). These data confirmed that ACLR and ACLT induce a similar rapid onset ptOA phenotype characterised by fast and severe tibial and femoral cartilage damage, low subchondral bone sclerosis, severe and progressive osteo-/enthesophyte development and osteochondral damage (Fig. 1). In contrast, DMM results in a less severe and slower

progression of cartilage damage and osteo-/ enthesophytes, accompanied by more subchondral bone sclerosis but no osteochondral erosion (Fig. 1B).

Histological evaluation of synovial inflammation.

The total synovitis score indicated that the surgical approach itself led to a significant inflammation that resolved by day-28 (scores in SHAM significantly higher than NOC, $p < 0.002$ day-1-14; Fig 2A, TABLE S3). Synovitis in the two surgical ptOA models (DMM/ACLT) were equivalent to SHAM acutely (day-1-7) but higher thereafter (ACLT from day-14 $p = 0.0003$; DMM from day-28 $p < 0.0001$). Interestingly despite no surgical intervention, synovitis in ACLR was equivalent to SHAM and DMM at day-1-14 and remained greater than SHAM and equivalent to DMM at day 28 ($p = 0.0167$) and day-56 ($p = 0.0161$). Synovitis in ACLT was greater early post-injury than both DMM (day-7 and -14; $p < 0.0136$) and ACLR (day-14 $p < 0.0332$) but then declined to be equivalent to other ptOA models from day-21. Thus, while surgery alone contributes acutely to synovial inflammation, it may be worsened by injury to specific joint tissues (ACLT vs DMM) and the maximum severity is no greater than non-surgical ptOA-inducing joint injury (ACLR).

The individual parameters generally showed similar temporal pattern to the total synovitis score but with some subtle model-specific differences. Surgery alone (SHAM) acutely increased synovial hyperplasia (Fig 2B) and sub-synovial cell infiltration (Fig 2C) with resolution by day-28. ACLR and ACLT induced comparable synovial hyperplasia which peaked at day-14 and then declined by day-28 remaining stably increased compared with controls thereafter. Synovial hyperplasia in DMM was less during early time points (DMM < ACLR/ACLT day-7 and -14; $p < 0.001$), similar during ptOA development (day-28 and -42), but then increased in later disease at a non-significant extent (Fig. 2B). Subsynovial immune cell infiltration was also similar in ACLR and ACLT during the entire course of the

study with an acute increase peaking at day-7 and then steadily declining but still significantly greater than controls (Fig. 2C). Subsynovial cell infiltration in DMM showed a similar temporal pattern but peaked earlier (day-3) and was significantly less than ACLR/ACLT between day-7-14 of ptOA development (Fig. 2C). Pannus formation was significantly more distinct in the surgical models with ACLT>DMM>ACLR from day-1-14 ($p<0.01$, Fig. 2D). These data again shows that the surgical approach itself leads to a significant histopathological synovial inflammation but that the specific tissues injured (ACL vs meniscus) can modify the temporal pattern and severity of these changes irrespective of the induction method (ACLR = ACLT). To better interrogate the inflammatory response in the different ptOA models, a hierarchical flow-cytometric gating strategy was used to determine loco-regional (synovial tissue and draining lymph-nodes) and systemic (spleen and contra-lateral bone-marrow) changes in adaptive- and innate-immune cell subsets.

Loco-regional inflammatory cell response to joint-injury and ptOA

There was little difference in synovial tissue CD4⁺ T-helper cells between the three control groups, with small transient increases in SHAM at day-3-7 ($p<0.01$) and ACLS at day-42 ($p<0.0001$; Fig 3A, Table S4). A significant increase in synovial tissue CD4⁺ T-helper cells was observed 3 days after ACLR and ACLT ($p<0.0001$ vs ACLS and SHAM), with levels peaking on day-7, declining by day-28 and remaining stable thereafter (ACLR>ACLS on days-3-28 $p<0.001$; ACLT>SHAM on days-3-70 $p<0.05$; Fig. 3A). In contrast, while synovial-tissue CD4⁺ T-helper cells were increased in DMM compared with SHAM on days-7-70 ($p<0.05$), the initial increase did not occur until day-7 and was less than ACLR/ACLT between day-3 and -14 ($p<0.0001$). The peak in synovial tissue CD4⁺ T-helper cells (comparable magnitude to ACLR/ACLT) did not occur until day-42 in DMM, with levels significantly higher than ACLR/ACLT day-28-56 days ($p<0.0001$). The pro- (Th17 and Th1) and anti-inflammatory

(Treg and Th2) subsets of CD4⁺ T-helper cells were evaluated and reported as relevant Th1/Th2 and Th17/Treg ratios (Fig 3B and C, respectively; values >1 indicating pro-inflammatory response). There was a short-lived pro-inflammatory effect of the surgical approach itself, with significantly higher Th1/Th2 and Th17/Treg ratios in SHAM compared NOC at day-1 (Th1/Th2: SHAM vs NOC $p<0.0001$; Th17/Treg: SHAM vs NOC $p=0.0002$). PtOA inducing injuries led to a much more marked pro-inflammatory T-helper cell polarization but with distinct model-specific temporal patterns. Equivalent peak increases in Th1/Th2 ratios in ACLR and ACLT were observed at day-7 before declining to control levels at day-42 day, followed by another small increase at day-56 then back to control levels by day-70. The Th1/Th2 ratio in ACLT was higher than ACLR at day-1 ($p=0.0008$) likely attributed to the surgical approach as levels were then not significantly different between the two ACL-injury models for the remaining study period. A day-1 increase in Th1/Th2 ratio due to the surgical approach was also observed in DMM, but thereafter ratios were similar to SHAM until day-28 when Th1/Th2 increased and peaked at day-42 and then progressively declined to baseline levels by day-70 (Fig. 3B). The Th17/Treg ratio had a similar initial temporal pattern to Th1/Th2, with the ACLR/ACLT increase peaking at day-7 (although ACLR was greater than ACLT; $p=0.0017$), whereas levels peaked at day-42 in DMM (Fig 3C). In contrast to Th1/Th2 ratios however, all three ptOA-inducing injuries led to a delayed pro-inflammatory Th17/Treg polarisation at day-56-70 (Fig. 3C).

Analysis of the innate immune response indicated an acute (day-1) influx of blood-derived (Ly6C^{high}) monocytes in all ACL injury groups ($p<0.01$ vs controls; Fig 3D) while in the meniscus injury group a significant influx was observed after day-7 ($p<0.0001$). Thereafter, levels of Ly6C^{high} monocytes declined in all three control groups reaching baseline levels at day-7 and remaining stable with no difference between groups for the study duration. In contrast, Ly6C^{high} monocytes in all three ptOA inducing injury models remained elevated

compared with controls to day-56 (with the exception of DMM at days-1-3). Ly6C^{high} monocytes in DMM were higher than ACLR/ACLT day-14-28 ($p<0.0001$), declined to be lower on day-42 ($p<0.0001$), and then as numbers progressively declined in both ACL injury models DMM was again higher on day-70 ($p<0.005$; Fig. 3D). In a consecutive analysis the origin of activated macrophages (CD11B+/F4/80+) in the synovium was designated using the intensity of expression F4/80 as cell surface marker (BOM=F4/80^{int}, STRM=F4/80^{high}), along with their M1/M2 polarization (CD80/CD206, respectively). This showed equivalent higher levels of BOM in all three ptOA-inducing models compared with controls, peaking at day-3 then gradually decreasing to baseline at day-42 (Fig 3E). During late time points (day-56-70) BOM showed a second increase exclusively in DMM ($p<0.0001$ vs SHAM; Fig. 3E). Surgery alone induced a transient pro-inflammatory polarization (increased M1/M2 ratio) of BOM and STRM (SHAM>NOC at day -1 (BOM $p<0.001$; STRM $p<0.05$) and day-3 (BOM $p<0.05$; STRM $p<0.05$); Fig. 3F and G). The BOM and STRM M1/M2 ratio remained elevated till day-14 in ACLR/ACLT and to day-42 in DMM. Importantly, no switch to an anti-inflammatory balance ($M1/M2 < 1$) was observed in BOM (Fig. 3F), while conversion to an anti-inflammatory polarization was observed in STRM at day-42 in ACLR and ACLT and day-70 in DMM ($p<0.0001$ vs controls; Fig. 3G). Taken together the data indicate that while surgery plays a significant role in inducing synovial inflammation, the tissue injured (ACL vs meniscus) has a major effect on both the magnitude and temporal pattern of the synovial tissue adaptive and innate immune response.

Analysis of the innate immune cell response in the draining lymph-nodes revealed no differences in Ly6C^{high} monocytes between the three control groups and no changes with time (Fig 4A, Table S5). In DMM there was a significant increase in lymph-node Ly6C^{high} monocytes day-1, and -7 ($p<0.05$ vs SHAM), a decrease at day-14 and then an increase and persistent elevation in DMM starting at day-28. Lymph-node Ly6C^{high} monocytes were also

increased acutely in ACLT (day-1, -3 and -14 vs SHAM; $p < 0.01$) and ACLR (day-1, -3 and -14; $p < 0.0001$ vs ACLS). However, unlike DMM, lymph-node Ly6C^{high} monocytes in both ACLT and ACLR returned to baseline by day-28 and remained unchanged thereafter. Lymph-node macrophages had higher pro-inflammatory M1/M2 ratios acutely with surgery alone (SHAM vs NOC day-1 and -3; $p < 0.05$). All three ptOA models also acutely increased M1/M2, DMM and ACLT greater than SHAM and ACLR > ACLS ($p < 0.01$). While M1/M2 ratios returned to baseline at day-28 in ACLR and ACLT, they steadily declined but stayed elevated compared with SHAM in DMM until day-56 ($p < 0.007$). Interestingly, an anti-inflammatory polarization switch was observed in ACLT and ACLR at day-70 but not DMM (Fig. 4B). Analysis of lymph-node CD4⁺ T-helper cell subsets revealed no change in Th1/Th2 ratios in any controls, consistently and persistently higher ratios in all ptOA inducing models, with DMM significantly higher than ACLT and ACLR at day-56 ($p < 0.0001$) and day-70 (DMM > ACLR $p = 0.0098$; DMM > ACLT $p = 0.0016$). In contrast to synovial tissue, no relevant differences were detected in the lymph-node Th17/Treg ratio over time, with all groups maintaining an anti-inflammatory balance (Fig. 4D). As with the synovial tissue response, the joint tissues injured rather than the method (ie. surgical vs non-surgical) had a more pronounced effect on the loco-regional lymph-node inflammatory response.

The systemic inflammatory response to joint-injury and ptOA

Surgery alone led to a very transient decrease in Ly6C^{high} monocytes in the contralateral bone-marrow on day-3 (SHAM vs NOC; $p < 0.0052$) before returning to baseline (Fig. 5A, Table S6). In contrast all three ptOA inducing injuries resulted in decreased Ly6C^{high} monocytes in bone-marrow day-1-14 ($p < 0.001$ vs controls) with the exception of DMM at day-3. While bone-marrow Ly6C^{high} monocytes in ACL injury models returned to baseline at day-28, they become elevated in DMM at day-28 (vs SHAM $p = 0.0011$) This same injury-specific pattern of change

in Ly6C^{high} monocytes was seen in the spleen, with surgery alone inducing a significant short-lived decrease in Ly6C^{high} monocytes (day-1-3 SHAM<NOC $p<0.0001$) whereas a more distinct and longer depletion of Ly6C^{high} monocytes was observed after ptOA inducing injury (Fig. 5B), and DMM significantly increasing Ly6C^{high} monocytes between day-14-42 ($p<0.0001$). Surgery alone also led to a transient (SHAM vs NOC day-1 and -3; $p<0.0001$) pro-inflammatory polarization of macrophages in both bone-marrow and spleen (Fig. 5C and D, respectively). Furthermore, ACLT and ACLR led to less marked and shorter duration (day-1-7 day) pro-inflammatory macrophage polarization compared to DMM mice in both bone-marrow and spleen. While M1/M2 ratios in DMM did ultimately return to baseline in the bone-marrow (day-42) they stayed significantly higher in the spleen for the duration of the study. In the bone-marrow an anti-inflammatory M1/M2 polarization switch occurred in ACLT and ACLR (day-56 and -70) but not DMM. In comparison to the innate response, the systemic adaptive immune response to joint injury was much more muted and transient and largely limited to surgical models (Fig 5 E-H). A pro-inflammatory response to all surgeries (SHAM/DMM/ACLT but not ACLR) in Th1/Th2 and Th17/Treg ratios was seen in spleen and bone-marrow at day-1. The only subsequent change was a higher Th1/Th2 ratio in DMM day-28 in bone-marrow ($p=0.0007$ vs SHAM; Fig. 5E) and spleen ($p=0.0108$ vs SHAM; Fig. 5F).

Summary of the systemic and loco-regional innate and adaptive immune response

In a final set of analyses, we calculated a fold difference between ptOA groups and their respective controls for all pro- and anti-inflammatory immune cell subsets and tissue to visualize the distinct spatio-temporal immune response (Fig. 6). This analysis and graphical representation highlights the limited systemic response except for the increase in M1 macrophages for 28 days in DMM, particularly in bone-marrow. In the draining lymph-nodes the temporal difference between the models in the M1 response can be seen, whereas a Th1-

driven pro-inflammatory response is observed in all models during the entire study period. The innate inflammatory response in the synovium is characterised by an early and comparatively short-lived M1 driven pro-inflammatory response (day-1-14 in ACLR and ACLT and day-1-28 in DMM), with a late increase in M2 predominantly in ACLR and ACLT. The temporal and model-specific difference in the synovial adaptive immune response is apparent, with an earlier increase and then decrease in Th1 cells in ACL injuries compared with DMM, and the initial increase in Th17 cells occurring earlier in ACL injuries than DMM but increasing again in all models at day 70.

Discussion

Current treatments for major/destabilizing joint injuries focus primarily on restoring biomechanics with surgery and/or muscle strengthening, and while improving clinical outcomes these approaches fail to prevent long-term ptOA [7]. The “non-mechanical mechanisms” underpinning ptOA risk remain to be defined but differences in severity and resolution of acute post-injury inflammation are strongly implicated [7, 43, 44]. Key to research efforts in this area are translationally relevant animal models [24, 25]. In this study we compared the loco-regional and systemic innate and adaptive inflammatory cell responses in three of the most commonly used mouse models of ptOA: DMM, ACLT and ACLR. Using these models allowed detailed temporal evaluation and direct comparison with non-OA-inducing injuries (SHAM, ACLS) not possible in human clinical studies, as well as the ability to explore the effect of injury to different joint-tissues (DMM *vs* ACLT) and surgery itself (ACLT *vs* ACLR) on the severity and phenotype of the inflammatory response. The key findings of these analyses were that: (1) non-critical/sprain loading injury (ACLS) does not incite a significant loco-regional or systemic immune/inflammatory activation; (2) while surgery alone induced acute synovial (but not lymph-node or systemic) inflammation this resolved rapidly (day-28 histology, day-7 innate and day-3 adaptive response), and there was minimal difference between surgical and non-surgical models (ACLT *vs* ACLR) in the magnitude or phenotype of the loco-regional and systemic innate and adaptive response; (3) however, there was a distinct temporal difference in the loco-regional and systemic innate and adaptive response depending of the joint-tissue injury (DMM *vs* ACLT). Model-specific differences in pathological, innate- and adaptive-immune response are discussed in further detail below.

Different ptOA pathology phenotypes.

Mouse models have been developed to recapitulate common injuries known to induce ptOA in the clinical setting [5]. Surgical ptOA models have the advantage of controlled injury to specific isolated joint-tissues under direct visualisation compared with joint loading models where damage to other tissues and/or knee compartments may occur [45, 46]. However, the surgical approach presents a limitation in potentially being a confounding “non-physiological” inflammatory stimulus [26]. Surgery alone did induce transient synovitis that resolved by 28-days consistent with previous findings [34] and with the higher synovitis scores for the first 21-days in ACLT vs ACLR in the current study. This increase in total synovitis score resulted from accumulation of smaller sometimes non-significant increases in each of the underlying individual parameters. Despite more synovitis there was little difference between ACLT and ACLR in the temporal pattern or severity of osteochondral pathology, suggesting histologically detected inflammation did not alter structural ptOA onset and progression. Previous studies in the rat also found no difference in cartilage histopathology in ACLR versus ACLT but subchondral bone remodeling and loss was greater in ACLT [47, 48]. This may indicate species-specific responses and/or differences in the surgical procedure and the degree of associated inflammation.

It is noteworthy that synovitis, particularly sub-synovial cellular infiltration and lining cell hyperplasia, was significantly greater in ACLT compared with DMM from very early post-injury (day-3) despite equivalent surgical intervention/induction. This suggests that the specific joint-tissue injured (ACL vs meniscus) may have a distinct impact on the inflammatory response prior to subsequent model-related differences in osteochondral damage that may induce secondary synovitis. The delayed synovial lining cell hyperplasia in DMM could be a secondary synovial response to the slower progression of cartilage pathology in this model [29]. However, the decrease in synovitis despite late osteochondral damage/erosion in ACLT/ACLR as a result of increased anterior/posterior laxity and posterior femoral

subluxation [49, 50], in concert with evidence of unique loco-regional and systemic innate and adaptive cell responses in DMM (see below), suggests an effect of the primary joint-tissue injury. This could be associated with increased synthesis of different inflammatory mediators from injured ACL and menisci [51, 52], and is consistent with cellular and molecular immune features of synovitis showing some differences in patients with ACL versus meniscal injury [53, 54]. The data from patients is confounded by the presence of concurrent/multiple joint-tissue injuries and marked variability and delay in time from injury to analysis, as well as age, sex and comorbidities. Animal model studies overcome these issues allowing a more detailed evaluation of the role of specific tissue-injuries on the local and systemic inflammatory response.

Characterisation of the adaptive immune response

An imbalance in the Th1/Th2 [55, 56] and Th17/Treg [57] ratios is encountered in OA patient joints. Whether and when these imbalances occur subsequent to joint injury, and if they are restricted to the synovium or can be found in loco-regional or systemic lymphoid tissues is unknown. We identified a very transient (1-3-day) Th1/Th2 and Th17/Treg imbalance in synovium and systemic but not draining lymph-tissues in SHAM, suggesting short-lived local activation as part of a normal wound-healing process [58]. The similar acute systemic Th1 response in SHAM, DMM and ACLT compared with a muted and delayed response in ACLS/ACLR could be a systemic response to general anaesthesia duration [59] but may also indicate specific effects of surgical tissue injury, which could also underpin the persistent/delayed rise in systemic Th1 in DMM compared with all other groups. That only DMM, ACLT and SHAM had a (1-day) systemic pro-inflammatory Th17/Treg response also suggests an acute transient effect of surgery on this pathway, although again the duration of anaesthesia cannot be excluded. While the precise mechanisms remain to be determined,

knowledge that these albeit transient systemic T-cell activation differences occur is important for researchers using surgical ptOA models to investigate the immune response or modulation thereof.

In the context of ptOA pathophysiology, the much more marked and prolonged pro-inflammatory Th1/Th2 imbalance in the synovium and draining lymph-node, and increased Th17/Treg ratio in the synovium were clear distinguishing features compared with non-ptOA-inducing injuries. The model-specific temporal differences in both Th1/Th2 and Th17/Treg indicated a major effect of the joint-tissue injured (ACL vs meniscus) rather than the mechanism (surgical vs non-surgical), with similar responses in ACLT and ACLR that differed significantly from DMM. The early acute pro-inflammatory synovial Th1/Th2 imbalance peak in ACLT/ACLR compared with slower onset delayed response in DMM, reflected the temporal differences in cartilage damage in these models. This may implicate an Th1-response to chondrocyte/cartilage-matrix derived molecules e.g. [60, 61], although as noted above different pro-inflammatory and immunomodulatory factors synthesised by injured ACL and menisci may also play a role [51, 52]. While a similar pattern of early and delayed peaks in synovial Th17/Treg ratios in ACLT/ACLR versus DMM were seen, rather than returning to baseline Th17 remained higher in all ptOA groups and increased in later (day-70) disease. The late-stage decrease in Th1 in our ptOA models contrasts with persistent elevation in end-stage patient OA knee joints [56]. The reason for this is unclear but could include differences in confounding factors such as age, sex, co-morbidities (e.g. obesity) in our mice versus patients. The prolonged synovial Th17 response is consistent with human clinical studies [12, 62] and ptOA in horses [63], and may suggest different and/or multiple cell/tissue-derived activating molecules/mechanisms that may be released over time/ptOA progression. For example, collagen-II-induced arthritis is predominantly Th17-driven while aggrecan-induced disease has a Th1 phenotype, but the latter can be modulated by different co-stimulatory agents, and

different epitopes with aggrecan can incite distinct T-cell activation signatures [60, 61, 64, 65]. Whether a difference in activating molecules/mechanisms and their distribution or presentation to T-cells also explains the draining lymph-node response only being Th1 and not Th17 is unclear. Nevertheless, our data indicates a distinct ptOA-inducing-model-specific spatiotemporal Th1 and Th 17 response that occurs predominantly on a locoregional level.

Characterisation of the innate immune response

All ptOA-inducing injuries led to a distinct pattern of loco-regional and systemic change in Ly6C^{high} monocytes compared with non-ptOA-inducing injuries. A short depletion of Ly6C^{high} monocytes in the bone-marrow and spleen with a concomitant influx in the synovial tissue in all ptOA models is consistent with targeted recruitment of systemic classically activated monocytes into the critically injured joint (but not SHAM or ACLS), as previously demonstrated in injured kidneys [66]. The restoration of systemic Ly6C^{high} monocytes after 2-4 weeks with persistent synovial elevation suggests a ptOA-induced increase in their generation in bone-marrow and spleen. Increased Ly6C^{high} monocyte generation in bone-marrow and spleen exceeding elevated efflux into synovium (and draining lymph-node) was evident from day-14-56 in DMM. The reason for this DMM-specific effect is not clear but could potentially be associated with prolonged cartilage degradation and/or sub-chondral bone remodeling/sclerosis compared with ACL-injury models. Along with Ly6C^{high} monocytes BOM were exclusively increased in synovium of ptOA-inducing joint injuries. This is consistent with evidence that blood/monocyte-derived macrophages are major contributors to promoting and sustaining inflammation during OA pathogenesis [20, 21]. The initial acute transient BOM influx (day-14-28) had the same magnitude and temporal pattern irrespective of induction method (surgical/non-surgical) or target joint-tissue (ACL/meniscus). While BOM influx was not temporally associated with any particular ptOA osteochondral

histopathology, the lack of their influx in SHAM implicates factors such chemokines (e.g. MCP-1/CCL2) synthesised and released from cells acutely injured in ACL, meniscus, or cartilage [67-70]. DMM was unique in having a second delayed increase in BOM, and as with the late increase in synovial Ly6C^{high} monocytes, this could be driven by prolonged cartilage degradation and/or sub-chondral bone remodeling/sclerosis in this model.

In vitro studies introduced the concept of a dichotomous macrophage polarization state being either classically activated and pro-inflammatory (M1) or alternatively activated and anti-inflammatory (M2) [71, 72]. This is now considered an oversimplified classification system with M1 and M2 representing the extreme poles of a complex spectrum of activation spectrum [11]. In the current study markers associated with a pro-inflammatory polarization (CD80) and anti-inflammatory polarization (CD206) were used to identify subsets of macrophages (F4/80+) subsequently labelled as M1 and M2 for simplicity. There was an effect of surgery alone inducing acute transient pro-inflammatory M1/M2 polarization loco-regionally and systemically. The large and more persistent increase in M1 in all tissues by ptOA-inducing injuries (albeit greater in surgical models) implicates a role for ptOA-associated tissue/cell generated molecules. That DMM induced a prolonged pro-inflammatory M1/M2 skew in all tissues may suggest a particular role for meniscal cells/proteins in macrophage activation, and/or be a reflection of the slower prolonged cartilage degradation and sclerotic subchondral bone remodeling.

There are numerous studies indicating that interventions targeting restoration of M1/M2 polarization balance are beneficial in mouse OA[73], and our data suggests that the therapeutic window may differ depending on the specific model used. Furthermore, the timing and efficacy in regulating both BOM and STRM M1/M1 polarization may be important considerations. STRM maintain joint homeostasis by protecting the joint space from detrimental stimuli via tight junctions in the synovial lining [22] and serve as sentinels for

molecular and cellular changes [20] acting as first responders to pathological changes happening in the joint. Activation of STRMs has been shown to occur via TLRs and a highly specialized pathogen recognition receptor (PRRs) induced pathway [74]. Our data indicates that despite their protective function STRM have a potential pathological role during the onset of joint-injury associated inflammation. Ultimately despite persistent and progressive ptOA pathology, synovial M1 macrophages decline with STRM displaying an anti-inflammatory M2 polarization. To the best of our knowledge the current study is the first study providing data regarding a natural temporal polarization switch of STRMs during the pathogenesis of ptOA, and may suggest targeting early M1/M2 polarization of STRMs in particular may be a good therapeutic target. Macrophage M1 or M2 polarization occurs in association with cytokines secreted from Th1 and Th2 T-cells [75, 76], respectively. In the current study the M1/M2 polarization switch in STRM was observed in concert with synovial Th1/Th2 ratio dropping below 2.7 in all three ptOA models. Targeting the Th1/Th2 balance may be beneficial for ptOA by directly reducing Th-1 catabolic factors and promoting improved M1/M2 STRM balance. In addition, the current study suggests that the M1/M2 polarization switch is not reserved to the joint and occurs in bone-marrow and regional lymph-nodes, and these may also be important targets in ptOA treatment.

Limitations and Conclusions

Flow cytometry has multiple advantages when investigating the inflammatory response during ptOA pathogenesis, however considerable antibody variation exists among studies limiting direct comparison of cell population, and quantitated flow cytometry “events” may not necessarily equate to total cells. In the current study established markers were utilized to maximise comparability and reduce variation, and the antibody panels were vigorously tested and optimized to ensure reproducibility. Recent studies have shown a marked sex-difference

in ptOA development after DMM surgery and the authors concluded that the male hormones likely exacerbate ptOA in male mice (Ma, H. L. et al. 2007). A recent study compared knee biomechanics between male and female mice (Blaker, Carina L. et al. 2021) and found that sex- and model-specific differences in ptOA progression are likely not influenced by differences in knee biomechanics (Blaker, Carina L. et al. 2021) thereby further highlighting a potential role of sex hormones. Our study only used young male mice, and future work should compare outcomes in females and older animals.

We were unable to include pain outcome evaluation and comparisons because of the number of animals used across time points and models, and the need to pool tissues from mice to ensure sufficient cells we could not do individual correlations between pathology outcomes and flow cytometry data. Finally, while our data allows assessment of association between innate and adaptive immune cell changes with injury and ptOA, determining the direction and causality of the interactions will require interventional studies. Despite these limitations, our detailed spatio-temporal analysis across multiple models has provided novel insights into innate and adaptive inflammation in response to joint injury and ptOA. Taken together this data indicates a predominant loco-regional and limited systemic response to joint injury, with the magnitude and duration of the initial transient increase in activated-monocyte and M1 macrophages and both acute and long-term Th1 and Th17 T-cell response differentiating pt-OA-inducing from non-ptO-inducing injury. Importantly, *what you injure* (ACL vs meniscus) rather than *how you injure it* (surgery vs non-surgical) determines the temporal pattern and systemic involvement of both the innate and adaptive immune response and the structural ptOA progression and severity.

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Conflict of interest

The authors declare no competing interests.

References

1. Spitaels D, Mamouris P, Vaes B, Smeets M, Luyten F, Hermens R, Vankrunkelsven P: **Epidemiology of knee osteoarthritis in general practice: a registry-based study.** *BMJ Open* 2020, **10**(1):e031734.
2. Kloppenburg M, Berenbaum F: **Osteoarthritis year in review 2019: epidemiology and therapy.** *Osteoarthritis Cartilage* 2020, **28**(3):242-248.
3. AIHW: **Australian Institute of Health and Welfare (2022) Disease expenditure in Australia 2019–20,**
<https://www.aihw.gov.au/reports/health-welfare-expenditure/disease-expenditure-in-australia-2019-20/contents/about>. In.; 2022.
4. Punzi L, Galozzi P, Luisetto R, Favero M, Ramonda R, Oliviero F, Scanu A: **Post-traumatic arthritis: overview on pathogenic mechanisms and role of inflammation.** *RMD Open* 2016, **2**(2):e000279-e000279.
5. Snoeker B, Turkiewicz A, Magnusson K, Frobell R, Yu D, Peat G, Englund M: **Risk of knee osteoarthritis after different types of knee injuries in young adults: a population-based cohort study.** *Br J Sports Med* 2020, **54**(12):725-730.
6. Barton KI, Shekarforoush M, Heard BJ, Sevvick JL, Vakil P, Atarod M, Martin R, Achari Y, Hart DA, Frank CB *et al*: **Use of pre-clinical surgically induced models to understand biomechanical and biological consequences of PTOA development.** *J Orthop Res* 2017, **35**(3):454-465.
7. Dilley JE, Bello MA, Roman N, McKinley T, Sankar U: **Post-traumatic osteoarthritis: A review of pathogenic mechanisms and novel targets for mitigation.** *Bone Rep* 2023, **18**:101658.
8. Li YS, Luo W, Zhu SA, Lei GH: **T Cells in Osteoarthritis: Alterations and Beyond.** *Front Immunol* 2017, **8**:356.
9. Nees TA, Rosshirt N, Zhang JA, Platzer H, Sorbi R, Tripel E, Reiner T, Walker T, Schiltenswolf M, Lorenz HM *et al*: **T Helper Cell Infiltration in Osteoarthritis-Related Knee Pain and Disability.** *J Clin Med* 2020, **9**(8).
10. Woodell-May JE, Sommerfeld SD: **Role of Inflammation and the Immune System in the Progression of Osteoarthritis.** *J Orthop Res* 2020, **38**(2):253-257.
11. Haubruck P, Pinto MM, Moradi B, Little CB, Gentek R: **Monocytes, Macrophages, and Their Potential Niches in Synovial Joints - Therapeutic Targets in Post-Traumatic Osteoarthritis?** *Frontiers in immunology* 2021, **12**:763702.
12. Rosshirt N, Trauth R, Platzer H, Tripel E, Nees TA, Lorenz HM, Tretter T, Moradi B: **Proinflammatory T cell polarization is already present in patients with early knee osteoarthritis.** *Arthritis Res Ther* 2021, **23**(1):37.
13. Trajerova M, Kriegova E, Mikulkova Z, Savara J, Kudelka M, Gallo J: **Knee osteoarthritis phenotypes based on synovial fluid immune cells correlate with clinical outcome trajectories.** *Osteoarthritis Cartilage* 2022, **30**(12):1583-1592.
14. Wang D, Chai XQ, Hu SS, Pan F: **Joint synovial macrophages as a potential target for intra-articular treatment of osteoarthritis-related pain.** *Osteoarthritis and Cartilage* 2022, **30**(3):406-415.
15. Yamada H, Nakashima Y, Okazaki K, Mawatari T, Fukushi J, Oyamada A, Fujimura K, Iwamoto Y, Yoshikai Y: **Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints.** *J Rheumatol* 2011, **38**(8):1569-1575.

16. Ishii H, Tanaka H, Katoh K, Nakamura H, Nagashima M, Yoshino S: **Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis.** *Osteoarthritis Cartilage* 2002, **10**(4):277-281.
17. Zhang L, Li YG, Li YH, Qi L, Liu XG, Yuan CZ, Hu NW, Ma DX, Li ZF, Yang Q *et al*: **Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis.** *PLoS One* 2012, **7**(4):e31000.
18. Haynes MK, Hume EL, Smith JB: **Phenotypic characterization of inflammatory cells from osteoarthritic synovium and synovial fluids.** *Clin Immunol* 2002, **105**(3):315-325.
19. Guo SY, Ding YJ, Li L, Zhang T, Zhang ZZ, Zhang ES: **Correlation of CD4(4)(+) CD2(2)(5)(+) Foxp3(3)(+) Treg with the recovery of joint function after total knee replacement in rats with osteoarthritis.** *Genet Mol Res* 2015, **14**(3):7290-7296.
20. Kurowska-Stolarska M, Alivernini S: **Synovial tissue macrophages: friend or foe?** *RMD Open* 2017, **3**(2):e000527.
21. Shi C, Pamer EG: **Monocyte recruitment during infection and inflammation.** *Nat Rev Immunol* 2011, **11**(11):762-774.
22. Culemann S, Gruneboom A, Nicolas-Avila JA, Weidner D, Lammle KF, Rothe T, Quintana JA, Kirchner P, Krljanac B, Eberhardt M *et al*: **Locally renewing resident synovial macrophages provide a protective barrier for the joint.** *Nature* 2019, **572**(7771):670-675.
23. Mora JC, Przkora R, Cruz-Almeida Y: **Knee osteoarthritis: pathophysiology and current treatment modalities.** *J Pain Res* 2018, **11**:2189-2196.
24. Blaker CL, Clarke EC, Little CB: **Using mouse models to investigate the pathophysiology, treatment, and prevention of post-traumatic osteoarthritis.** *J Orthop Res* 2017, **35**(3):424-439.
25. Zaki S, Blaker CL, Little CB: **OA foundations - experimental models of osteoarthritis.** *Osteoarthritis Cartilage* 2022, **30**(3):357-380.
26. Christiansen BA, Guilak F, Lockwood KA, Olson SA, Pitsillides AA, Sandell LJ, Silva MJ, van der Meulen MCH, Haudenschild DR: **Non-invasive mouse models of post-traumatic osteoarthritis.** *Osteoarthritis and cartilage* 2015, **23**(10):1627-1638.
27. Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M *et al*: **Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0.** *PLoS Biol* 2020, **18**(7):e3000411.
28. NHMRC: **Australian code for the care and use of animals for scientific purposes**, 8th edition edn: National Health and Medical Research Council; Canberra, Australia; 2013.
29. Glasson SS, Blanchet TJ, Morris EA: **The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse.** *Osteoarthritis Cartilage* 2007, **15**(9):1061-1069.
30. Kamekura S, Hoshi K, Shimoaka T, Chung U, Chikuda H, Yamada T, Uchida M, Ogata N, Seichi A, Nakamura K *et al*: **Osteoarthritis development in novel experimental mouse models induced by knee joint instability.** *Osteoarthritis Cartilage* 2005, **13**(7):632-641.
31. Christiansen BA, Anderson MJ, Lee CA, Williams JC, Yik JH, Haudenschild DR: **Musculoskeletal changes following non-invasive knee injury using a novel mouse model of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2012, **20**(7):773-782.

32. Blaker CL, Little CB, Clarke EC: **Joint loads resulting in ACL rupture: Effects of age, sex, and body mass on injury load and mode of failure in a mouse model.** *J Orthop Res* 2016.
33. Blaker CL, Little CB, Clarke EC: **Joint loads resulting in ACL rupture: Effects of age, sex, and body mass on injury load and mode of failure in a mouse model.** *Journal of Orthopaedic Research* 2017, **35**(8):1754-1763.
34. Jackson MT, Moradi B, Zaki S, Smith MM, McCracken S, Smith SM, Jackson CJ, Little CB: **Depletion of protease-activated receptor 2 but not protease-activated receptor 1 may confer protection against osteoarthritis in mice through extracartilaginous mechanisms.** *Arthritis Rheumatol* 2014, **66**(12):3337-3348.
35. Little CB, Barai A, Burkhardt D, Smith SM, Fosang AJ, Werb Z, Shah M, Thompson EW: **Matrix metalloproteinase 13-deficient mice are resistant to osteoarthritic cartilage erosion but not chondrocyte hypertrophy or osteophyte development.** *Arthritis Rheum* 2009, **60**(12):3723-3733.
36. Shu CC, Jackson MT, Smith MM, Smith SM, Penm S, Lord MS, Whitelock JM, Little CB, Melrose J: **Ablation of Perlecan Domain 1 Heparan Sulfate Reduces Progressive Cartilage Degradation, Synovitis, and Osteophyte Size in a Preclinical Model of Posttraumatic Osteoarthritis.** *Arthritis Rheumatol* 2016, **68**(4):868-879.
37. Zaki S, Smith MM, Smith SM, Little CB: **Differential patterns of pathology in and interaction between joint tissues in long-term osteoarthritis with different initiating causes: phenotype matters.** *Osteoarthritis Cartilage* 2020, **28**(7):953-965.
38. Haubruck P, Colbath AC, Liu Y, Stoner S, Shu C, Little CB: **Flow Cytometry Analysis of Immune Cell Subsets within the Murine Spleen, Bone Marrow, Lymph Nodes and Synovial Tissue in an Osteoarthritis Model.** *J Vis Exp* 2020(158).
39. Roederer M: **Compensation in Flow Cytometry.** *Current Protocols in Cytometry* 2002, **22**(1):1.14.11-11.14.20.
40. Shu CC, Zaki S, Ravi V, Schiavinato A, Smith MM, Little CB: **The relationship between synovial inflammation, structural pathology, and pain in post-traumatic osteoarthritis: differential effect of stem cell and hyaluronan treatment.** *Arthritis Res Ther* 2020, **22**(1):29.
41. Haubruck P, Heller R, Blaker CL, Clarke EC, Smith SM, Burkhardt D, Liu Y, Stoner S, Zaki S, Shu CC *et al*: **Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models.** *Osteoarthritis and Cartilage* 2023, **Accepted for publication**.
42. Little CB, Hunter DJ: **Post-traumatic osteoarthritis: from mouse models to clinical trials.** *Nat Rev Rheumatol* 2013, **9**(8):485-497.
43. Nieboer MF, Reijman M, Wesdorp MA, Bastiaansen-Jenniskens YM, Meuffels DE: **Improved Understanding of the Inflammatory Response in Synovial Fluid and Serum after Traumatic Knee Injury, Excluding Fractures of the Knee: A Systematic Review.** *Cartilage* 2023, **14**(2):198-209.
44. Khella CM, Asgarian R, Horvath JM, Rolauffs B, Hart ML: **An Evidence-Based Systematic Review of Human Knee Post-Traumatic Osteoarthritis (PTOA): Timeline of Clinical Presentation and Disease Markers, Comparison of Knee Joint PTOA Models and Early Disease Implications.** *Int J Mol Sci* 2021, **22**(4).
45. Poulet B, de Souza R, Kent AV, Saxon L, Barker O, Wilson A, Chang YM, Cake M, Pitsillides AA: **Intermittent applied mechanical loading induces subchondral bone**

- thickening that may be intensified locally by contiguous articular cartilage lesions. *Osteoarthritis Cartilage* 2015, **23**(6):940-948.
46. Christiansen BA, Guilak F, Lockwood KA, Olson SA, Pitsillides AA, Sandell LJ, Silva MJ, van der Meulen MC, Haudenschild DR: **Non-invasive mouse models of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2015, **23**(10):1627-1638.
 47. Maerz T, Kurdziel M, Newton MD, Altman P, Anderson K, Matthew HW, Baker KC: **Subchondral and epiphyseal bone remodeling following surgical transection and noninvasive rupture of the anterior cruciate ligament as models of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2016, **24**(4):698-708.
 48. Maerz T, Newton MD, Kurdziel MD, Altman P, Anderson K, Matthew HW, Baker KC: **Articular cartilage degeneration following anterior cruciate ligament injury: a comparison of surgical transection and noninvasive rupture as preclinical models of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2016, **24**(11):1918-1927.
 49. Adebayo OO, Ko FC, Goldring SR, Goldring MB, Wright TM, van der Meulen MCH: **Kinematics of meniscal- and ACL-transected mouse knees during controlled tibial compressive loading captured using roentgen stereophotogrammetry.** *Journal of orthopaedic research : official publication of the Orthopaedic Research Society* 2017, **35**(2):353-360.
 50. Blaker CL, Ashton DM, Doran N, Little CB, Clarke EC: **Sex- and injury-based differences in knee biomechanics in mouse models of post-traumatic osteoarthritis.** *J Biomech* 2021, **114**:110152.
 51. Brophy RH, Rai MF, Zhang Z, Torgomyan A, Sandell LJ: **Molecular analysis of age and sex-related gene expression in meniscal tears with and without a concomitant anterior cruciate ligament tear.** *J Bone Joint Surg Am* 2012, **94**(5):385-393.
 52. Brophy RH, Rothermich MA, Tycksen ED, Cai L, Rai MF: **Presence of meniscus tear alters gene expression profile of anterior cruciate ligament tears.** *J Orthop Res* 2018, **36**(10):2612-2621.
 53. Holers VM, Frank RM, Clauw A, Seifert J, Zuscik M, Asokan S, Striebich C, Clay MR, Moreland LW, Banda NK: **Potential causal role of synovial complement system activation in the development of post-traumatic osteoarthritis after anterior cruciate ligament injury or meniscus tear.** *Frontiers in immunology* 2023, **14**:1146563.
 54. Kim-Wang SY, Holt AG, McGowan AM, Danyluk ST, Goode AP, Lau BC, Toth AP, Wittstein JR, DeFrate LE, Yi JS *et al*: **Immune cell profiles in synovial fluid after anterior cruciate ligament and meniscus injuries.** *Arthritis Res Ther* 2021, **23**(1):280.
 55. Ishii H, Tanaka H, Katoh K, Nakamura H, Nagashima M, Yoshino S: **Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis.** *Osteoarthritis and Cartilage* 2002, **10**(4):277-281.
 56. Rosshirt N, Hagmann S, Tripel E, Gotterbarm T, Kirsch J, Zeifang F, Lorenz HM, Tretter T, Moradi B: **A predominant Th1 polarization is present in synovial fluid of end-stage osteoarthritic knee joints: analysis of peripheral blood, synovial fluid and synovial membrane.** *Clinical & Experimental Immunology* 2019, **195**(3):395-406.
 57. Ye X, Lu Q, Yang A, Rao J, Xie W, He C, Wang W, Li H, Zhang Z: **MiR-206 regulates the Th17/Treg ratio during osteoarthritis.** *Molecular Medicine* 2021, **27**(1):64.
 58. Raziyeve K, Kim Y, Zharkinbekov Z, Kassymbek K, Jimi S, Saparov A: **Immunology of Acute and Chronic Wound Healing.** *Biomolecules* 2021, **11**(5).
 59. Koksoy S, Sahin Z, Karsli B: **Comparison of the effects of desflurane and bupivacaine on Th1 and Th2 responses.** *Clin Lab* 2013, **59**(11-12):1215-1220.

60. Brand DD, Kang AH, Rosloniec EF: **Immunopathogenesis of collagen arthritis.** *Springer Semin Immunopathol* 2003, **25**(1):3-18.
61. Buzás EI, Végvári A, Murad YM, Finnegan A, Mikecz K, Glant TT: **T-cell recognition of differentially tolerated epitopes of cartilage proteoglycan aggrecan in arthritis.** *Cell Immunol* 2005, **235**(2):98-108.
62. Nees TA, Zhang JA, Platzer H, Walker T, Reiner T, Tripel E, Moradi B, Rosshirt N: **Infiltration Profile of Regulatory T Cells in Osteoarthritis-Related Pain and Disability.** *Biomedicines* 2022, **10**(9).
63. Keller LE, Tait Wojno ED, Begum L, Fortier LA: **T Helper 17-Like Regulatory T Cells in Equine Synovial Fluid Are Associated With Disease Severity of Naturally Occurring Posttraumatic Osteoarthritis.** *Am J Sports Med* 2023, **51**(4):1047-1058.
64. Boldizsar F, Tarjanyi O, Nemeth P, Mikecz K, Glant TT: **Th1/Th17 polarization and acquisition of an arthritogenic phenotype in arthritis-susceptible BALB/c, but not in MHC-matched, arthritis-resistant DBA/2 mice.** *Int Immunol* 2009, **21**(5):511-522.
65. Stoop JN, Tibbitt CA, van Eden W, Robinson JH, Hilkens CM: **The choice of adjuvant determines the cytokine profile of T cells in proteoglycan-induced arthritis but does not influence disease severity.** *Immunology* 2013, **138**(1):68-75.
66. Lin SL, Castaño AP, Nowlin BT, Lupher ML, Duffield JS: **Bone Marrow Ly6C^{high} Monocytes Are Selectively Recruited to Injured Kidney and Differentiate into Functionally Distinct Populations.** *The Journal of Immunology* 2009, **183**(10):6733.
67. Conti P, DiGioacchino M: **MCP-1 and RANTES are mediators of acute and chronic inflammation.** *Allergy Asthma Proc* 2001, **22**(3):133-137.
68. Watt FE, Paterson E, Freidin A, Kenny M, Judge A, Saklatvala J, Williams A, Vincent TL: **Acute Molecular Changes in Synovial Fluid Following Human Knee Injury: Association With Early Clinical Outcomes.** *Arthritis Rheumatol* 2016, **68**(9):2129-2140.
69. Scanzello CR, McKeon B, Swaim BH, DiCarlo E, Asomugha EU, Kanda V, Nair A, Lee DM, Richmond JC, Katz JN *et al*: **Synovial inflammation in patients undergoing arthroscopic meniscectomy: molecular characterization and relationship to symptoms.** *Arthritis Rheum* 2011, **63**(2):391-400.
70. Yuan GH, Masuko-Hongo K, Sakata M, Tsuruha J, Onuma H, Nakamura H, Aoki H, Kato T, Nishioka K: **The role of C-C chemokines and their receptors in osteoarthritis.** *Arthritis Rheum* 2001, **44**(5):1056-1070.
71. Gordon S, Martinez FO: **Alternative activation of macrophages: mechanism and functions.** *Immunity* 2010, **32**(5):593-604.
72. Sica A, Mantovani A: **Macrophage plasticity and polarization: in vivo veritas.** *J Clin Invest* 2012, **122**(3):787-795.
73. Zhang H, Cai D, Bai X: **Macrophages regulate the progression of osteoarthritis.** *Osteoarthritis Cartilage* 2020, **28**(5):555-561.
74. Cui J, Chen Y, Wang HY, Wang RF: **Mechanisms and pathways of innate immune activation and regulation in health and cancer.** *Hum Vaccin Immunother* 2014, **10**(11):3270-3285.
75. Hume DA: **The Many Alternative Faces of Macrophage Activation.** *Front Immunol* 2015, **6**:370.
76. Murray PJ: **Macrophage Polarization.** *Annu Rev Physiol* 2017, **79**:541-566.

Figure 1: Results of histopathological scoring of medial tibial and femoral cartilage and subchondral bone. Panel A shows combined tibiofemoral cartilage damage over time. Panel B visualizes the combined osseous and cartilage damage over time. Panel C shows the bony remodeling with a combined osteophyte and enthesophyte score. Panel D shows the subchondral bone sclerosis. E shows sample histological images of progression of OA. The scale bar shows a width of 2.5mm. Data is presented as median $\pm$ 95% CI and n = 12 animals/group/time point.

Figure 2: Results of the histopathological scoring of synovial inflammation over time. A shows the combined total synovitis, while B focuses on the synovial hyperplasia itself and C visualizes the extent of sub-synovial immune cell infiltration, whereas D depicts the bone erosion with pannus. E shows sample histological images of synovial inflammation in osteoarthritic animals. The scale bar shows a width of 500 μ m. Data is presented as median $\pm$ 95% CI and n = 12 animals/group/time point.

Figure 3: Results of the flow cytometry experiments of the synovial tissue block. Panel A-C visualize the adaptive immune response after joint injury and for respective controls. A shows the percentage of CD4⁺ T-helper cells of all T-cells in the synovial tissue block over time. Furthermore, the Th1/Th2 ratio is shown in panel B and the Th17/Treg ratio is shown in C. Panel D-G focus on the innate immune response during onset and development of OA as well as in the respective control groups. Panel D focuses on the Ly6C^{high} classically activated monocytes, while panel E shows the influx of macrophages that originated in the blood from monocytes. Panel F shows the polarization of macrophages that originated from blood monocytes. Polarization is shown as a ratio between either M1:M2 macrophages. Panel G

shows this polarization in synovial tissue resident macrophages (STRM). Data is presented as mean $\pm$ SEM and n = 6 biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 4: Results of the flow cytometry experiments of the draining lymph nodes. Panel A and B on the left-hand side show the innate immune response and Panel C and D show the adaptive immune response. Panel A shows the influx of Ly6C^{high} monocytes into the lymph node while panel B shows the M1/M2 polarization of macrophages over time. Regarding the adaptive immune response panel C shows the Th1/Th2 ratio of T-cells in the lymph node while panel D shows the Th17/Treg ratio. Data is presented as mean $\pm$ SEM and n = 6 biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 5: Results of the flow cytometry experiments of the systemic secondary immune tissues contralateral bone marrow (BM) and spleen (Sp). Panel A-D show the innate immune response. The expression pattern of Ly6Chigh monocytes is shown in bone marrow (A) and spleen (B) while the M1/M2 ratio is shown for bone marrow (C) and the spleen (D). Panel E-H show the adaptive immune response after joint injury. The Th1/Th2 ratio in both bone marrow (E) and spleen (F) are visualized as well as the Th17/Treg ratio in the same tissues (bone marrow panel G and spleen panel H). Data is presented as mean $\pm$ SEM and n = 6 biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 6: Fold differences were calculated between OA mice and their respective controls for both pro- and anti-inflammatory immune cells and results given for all three OA models as well as for all tissues over time. N = 6 biological replicates (pool of tissues from 2 mice)/group/time point.

Supplementary Figure 1: The hierarchical gating strategy used for analysing single cell suspensions via flow cytometry including the utilized antibodies is shown for the intra- and extracellular T-cell panel. ACLR mice derived spleen cells 1 day after injury were used as an example.

Supplementary Figure 2: The hierarchical gating strategy used for analysing single cell suspensions via flow cytometry including the utilized antibodies is shown for the extracellular myeloid panel. ACLR mice derived spleen cells 1 day after injury were used as an example.

Figure 1:

○ NOC □ SHAM ● DMM ■ ACLT △ ACLS ▲ ACLR

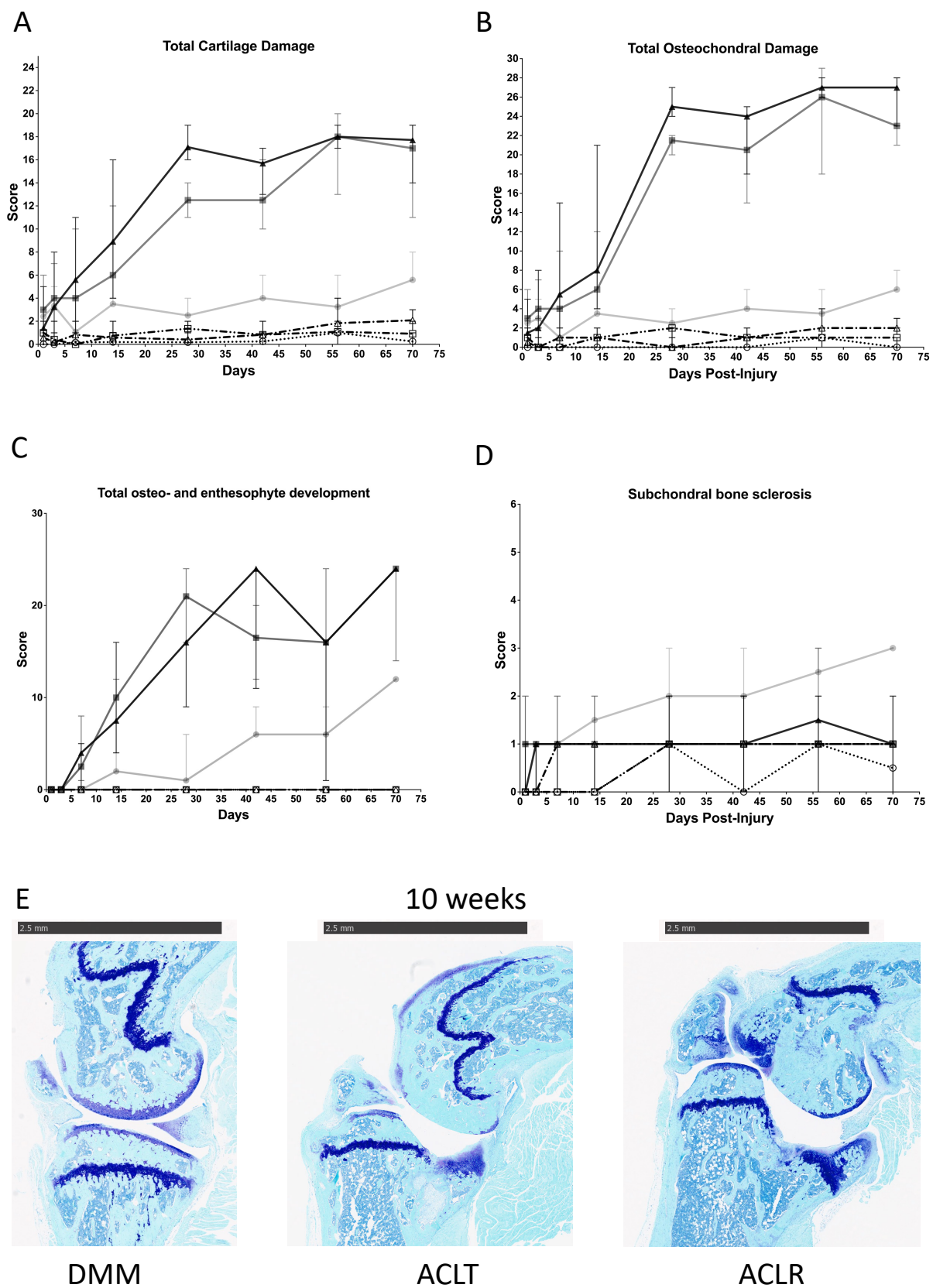


Figure 2:

○ NOC □ SHAM ● DMM ■ ACLT △ ACLS ▲ ACLR

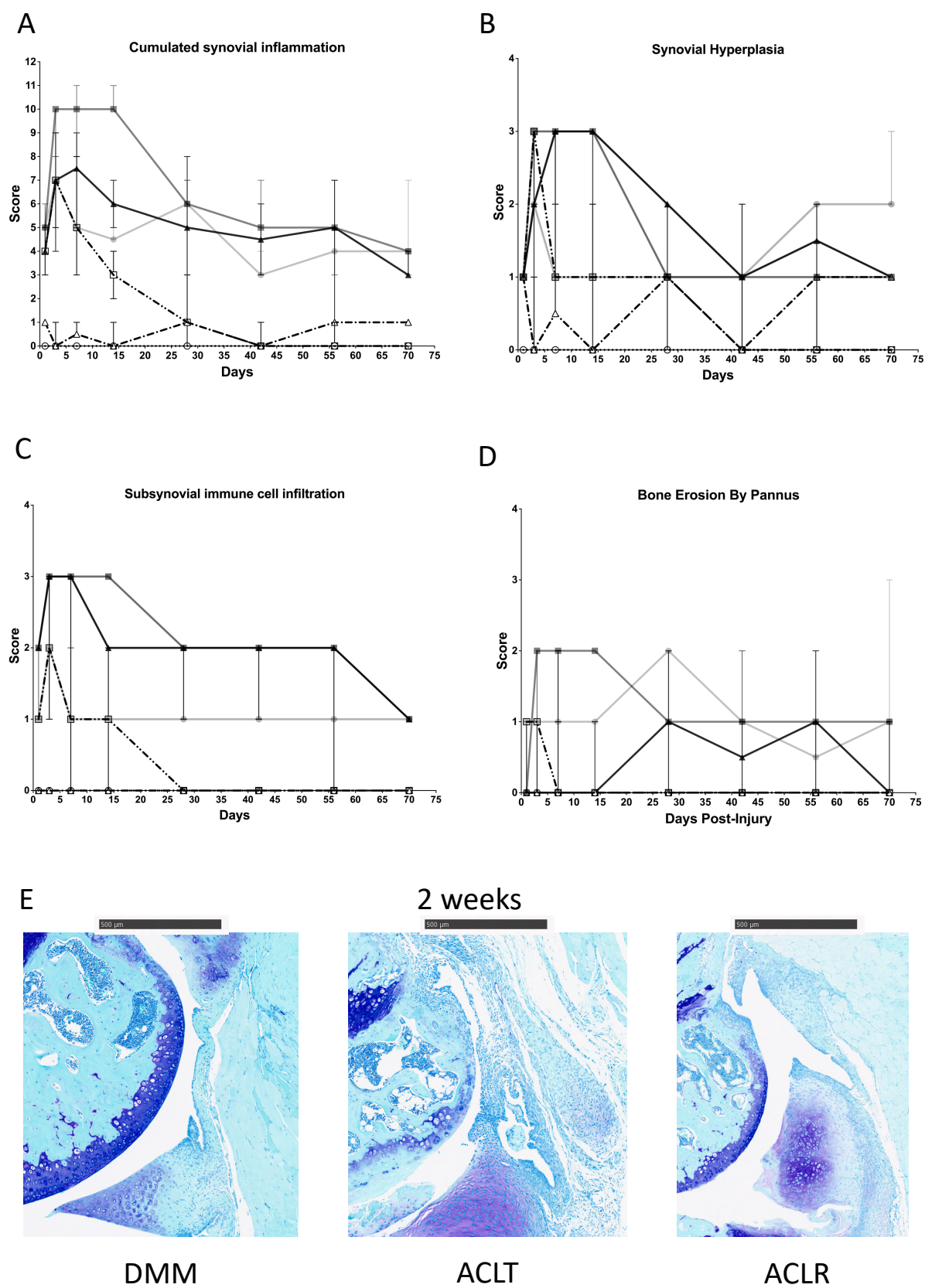


Figure 3:

○ NOC □ SHAM ● DMM ■ ACLT △ ACLS ▲ ACLR

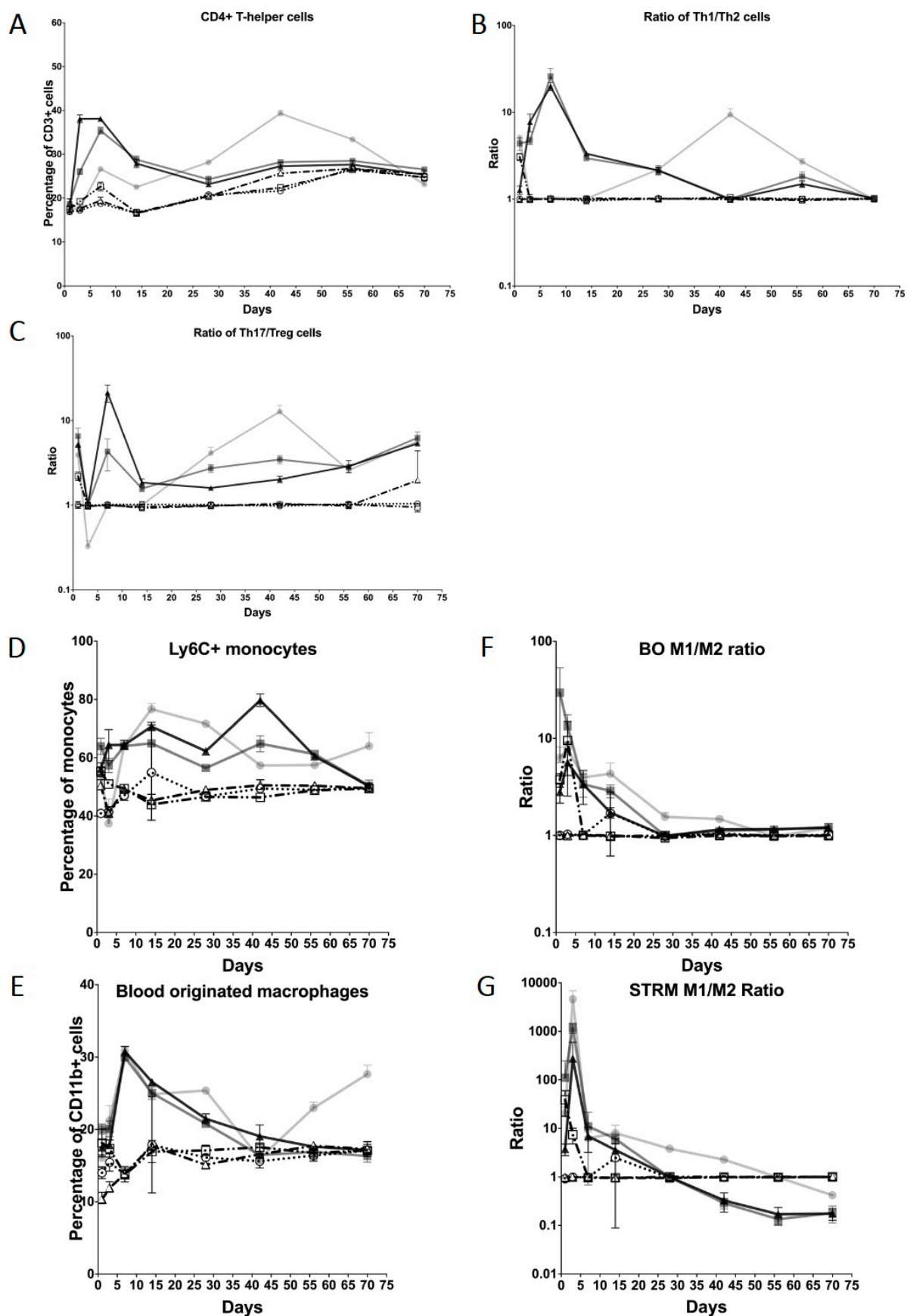


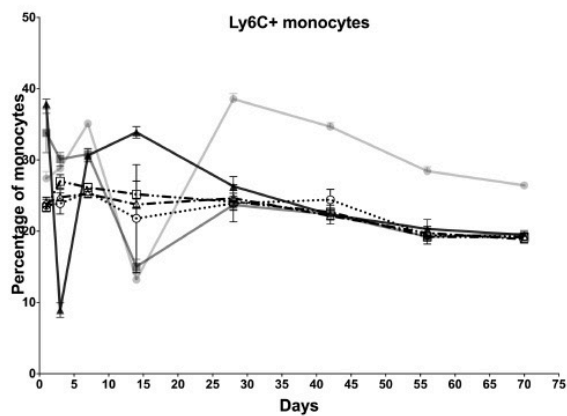
Figure 4:

○ NOC □ SHAM ● DMM ■ ACLT ▲ ACLS ★ ACLR

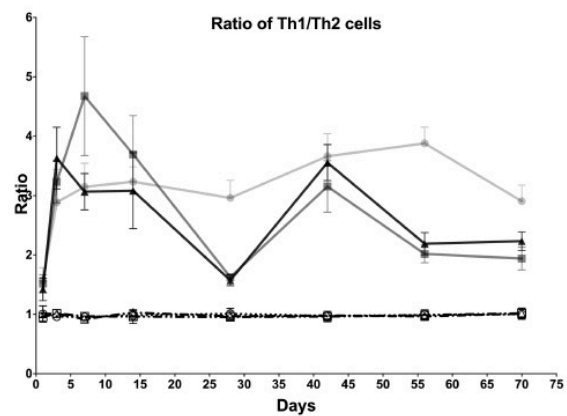
Innate Immune System

Adaptive Immune System

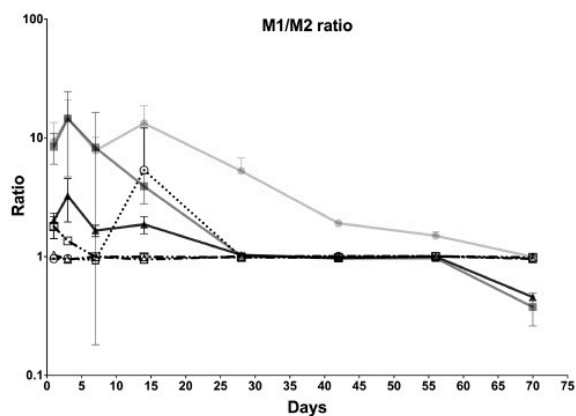
A



C



B



D

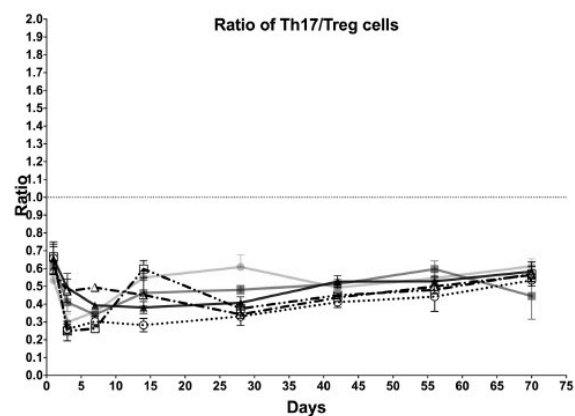
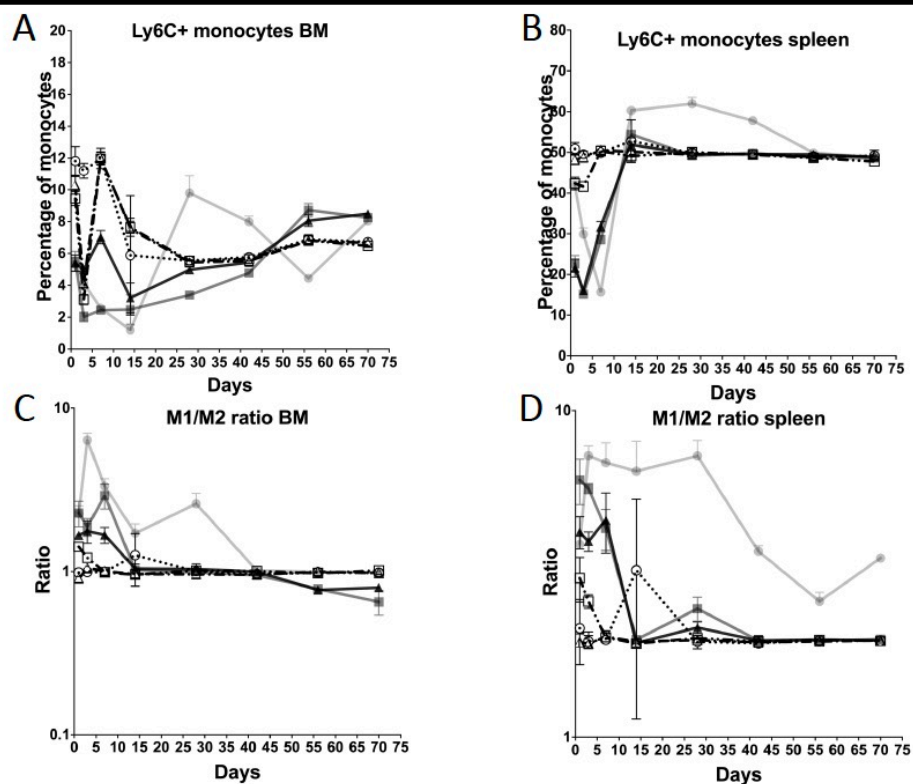


Figure 5:

○ NOC □ SHAM ● DMM ■ ACLT ▲ ACLS ▲ ACLR

Innate Immune System



Adaptive Immune System

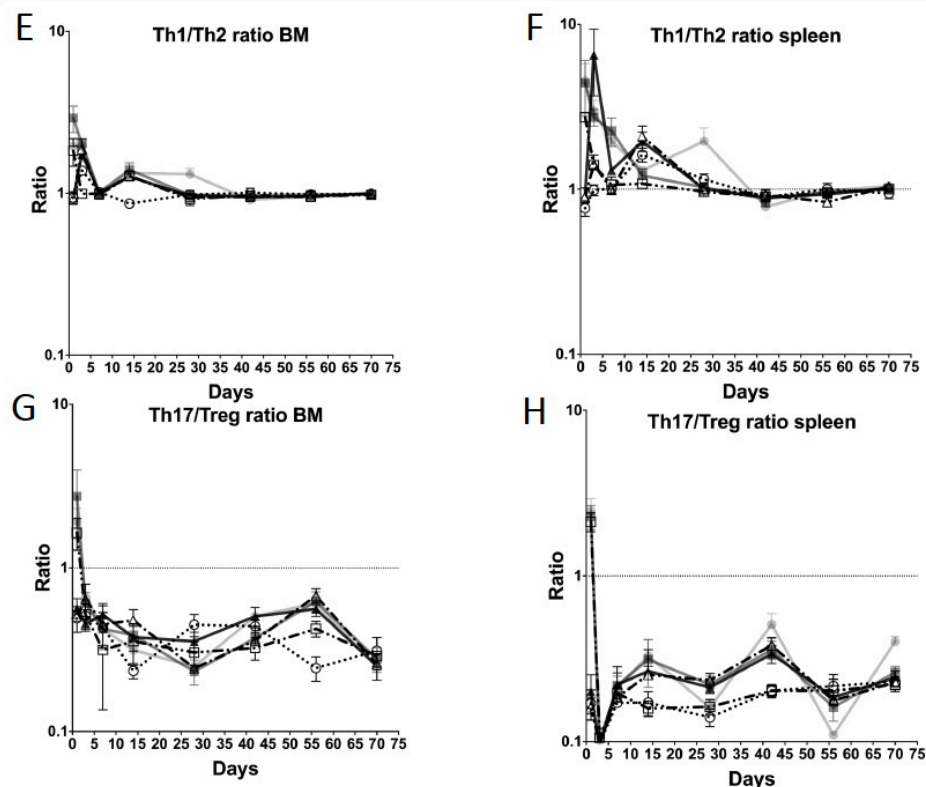
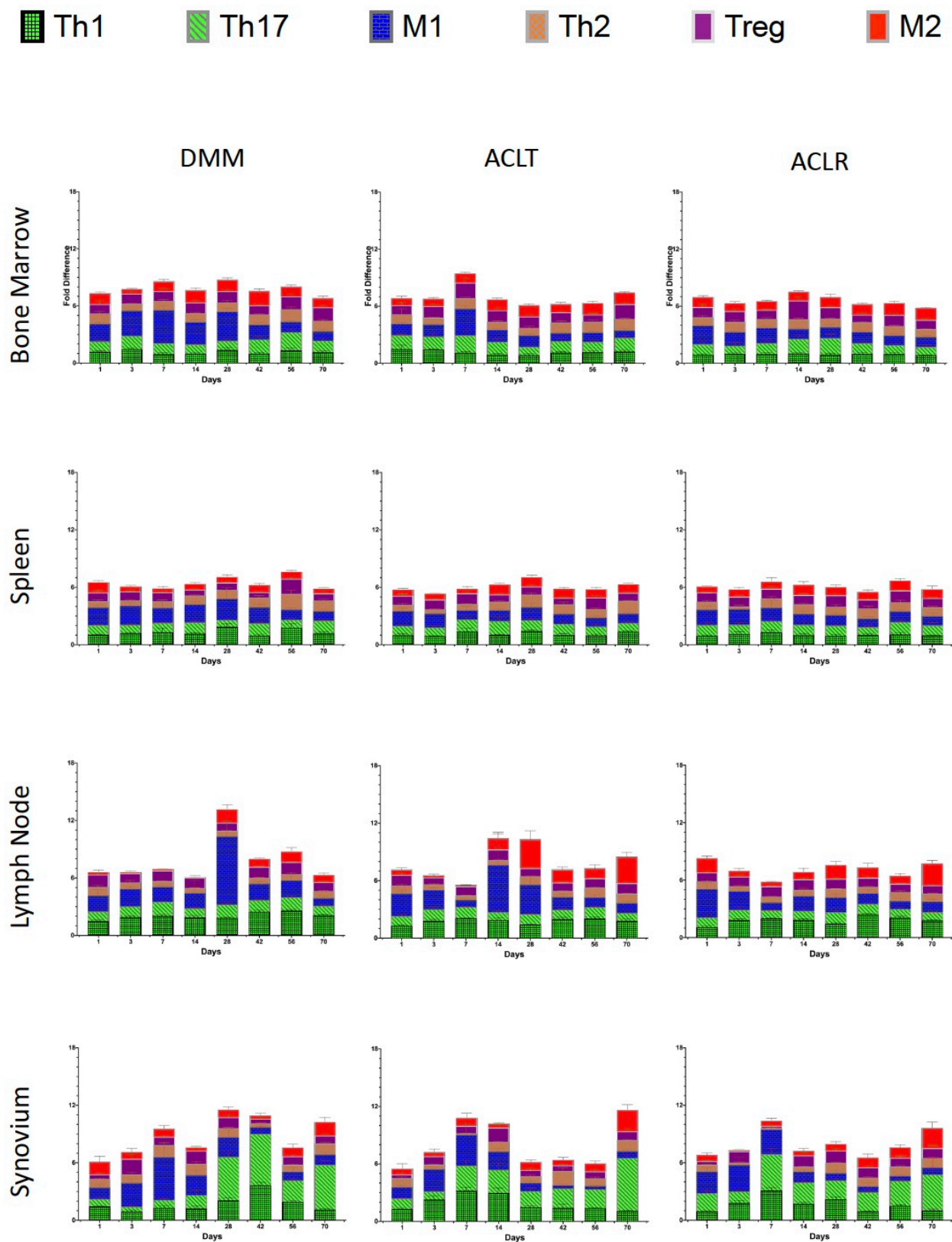
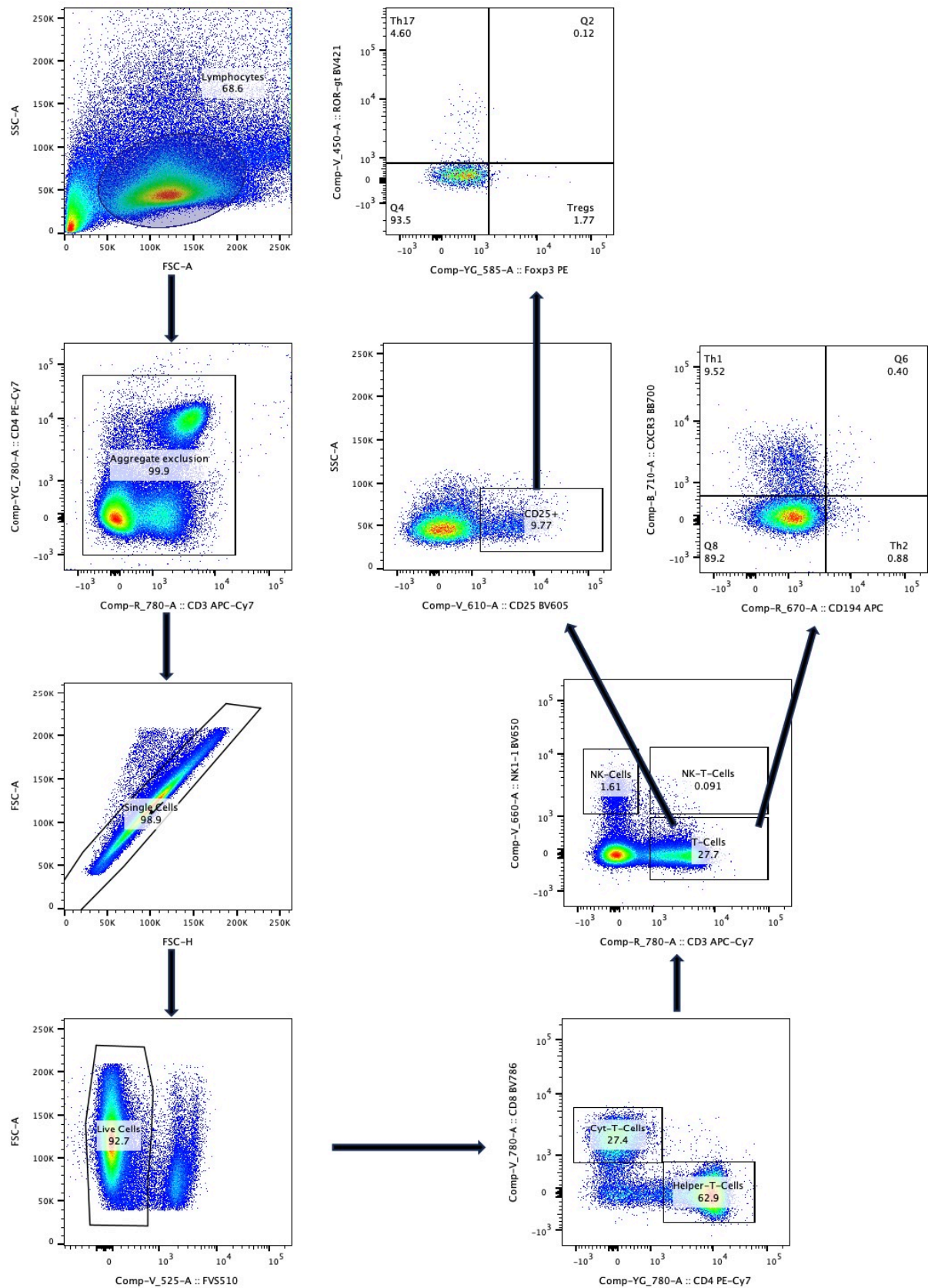


Figure 6:



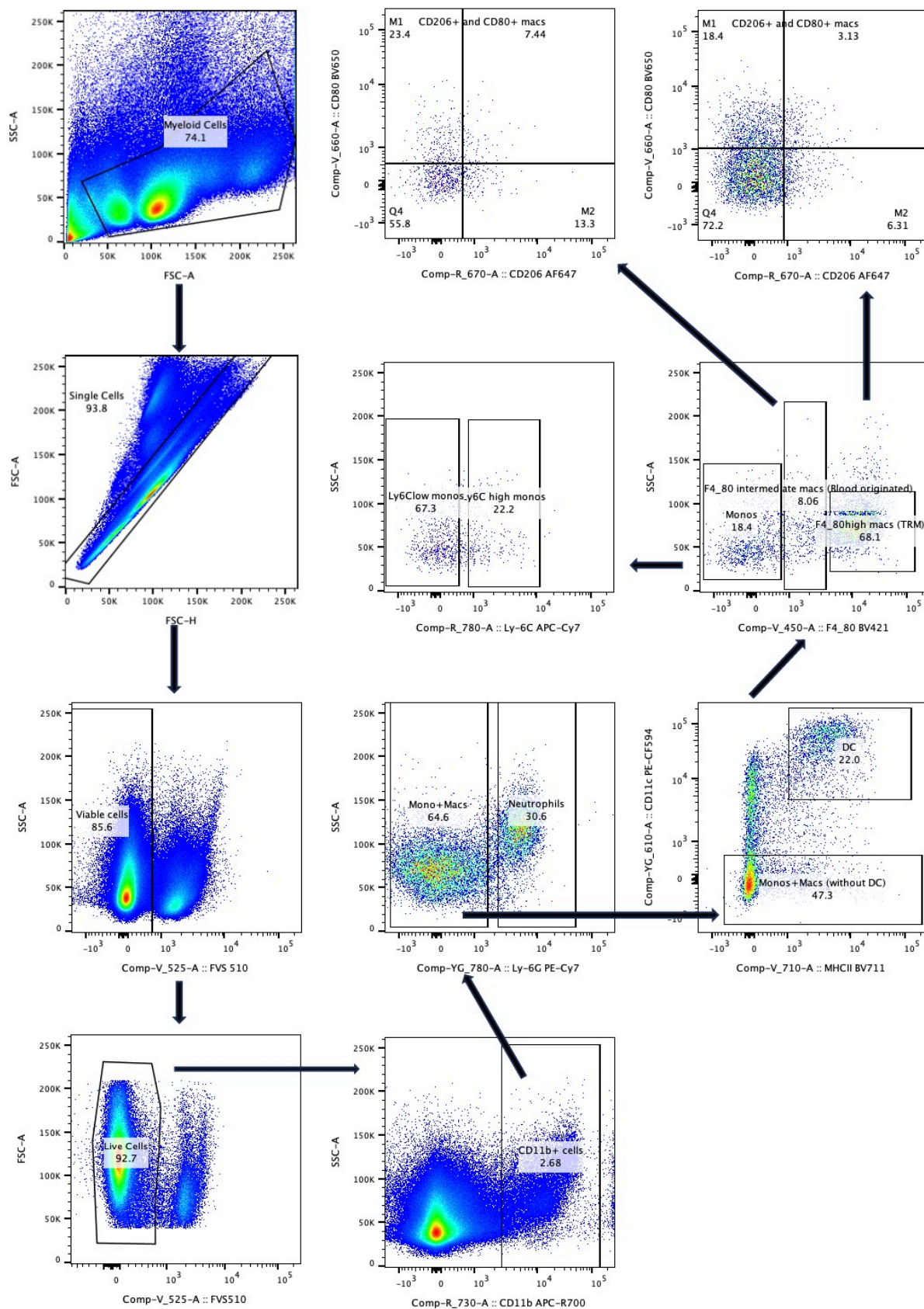
Supplementary Figure 1:

Hierarchical Gating Strategy T-Cell Panel



Supplementary Figure 2:

Hierarchical Gating Strategy Myeloid Panel



Supplementary Table 1:

Details for each antibody used in each of the two panels are given. In addition, information is provided for the relevant buffers used.

Supplementary Tables 2-6:

The detailed p-values for Figures 1-6 are given. Yellow highlighted fields mark a statistically significant difference ($p < 0.05$)

Supplementary Table 1: Used Antibodies for Flow Cytometry

Name of Material/ Equipment	Manufacturer	Catalog Number	Comments/Description
Fixable Viability Stain 510 100ug	BD	564406	T-Cell Panel
Ms CD3e APC-Cy7 145-2C11 100ug	BD	557596	T-Cell Panel
Ms CD8a BV786 53-6.7 50ug	BD	563332	T-Cell Panel
Ms CD4 PE-Cy7 RM4-5 100ug	BD	552775	T-Cell Panel
Ms NK1.1 BV650 PK136 50ug	BD	564143	T-Cell Panel
Ms CD25 BV605 PC61 50ug	BD	563061	T-Cell Panel
Ms CD183 BB700 CXCR3-173 50ug	BD	742274	T-Cell Panel
Ms Foxp3 PE MF23 100ug	BD	560408	T-Cell Panel
Ms ROR Gamma T BV421 Q31-378 50ug	BD	562894	T-Cell Panel
APC anti-mouse CD194 (CCR4)	BioLegend	131212	T-Cell Panel
Fixable Viability Stain 510 100ug	BD	564406	Monocyte Panel
Ms F4/80 BV421 T45-2342 50ug	BD	565411	Monocyte Panel
Ms CD80 BV650 16-10A1 50ug	BD	563687	Monocyte Panel
Ms I-A I-E BV711 M5/114.15.2 50ug	BD	563414	Monocyte Panel
Ms Ly-6G APC-Cy7 1A8 50ug	BD	560600	Monocyte Panel
Ms CD11C PE-CF594 HL3 100ug	BD	562454	Monocyte Panel
Ms Ly-6C PE-Cy7 AL-21 50ug	BD	560593	Monocyte Panel
Ms CD206 Alexa 647 MR5D3 25ug	BD	565250	Monocyte Panel
Ms CD11b APC-R700 M1/70 100ug	BD	564985	Monocyte Panel
Brilliant Stain Buffer Plus 1000Tst	BD	566385	Buffers
Transcription Factor Buffer Set 100Tst	BD	562574	Buffers

Supplementary Table 2: Detailed p-values for Figure 1

Day	ACLs vs NOC	SHAM vs NOC	DMM vs SHAM	ACLT vs SHAM	ACLT vs DMM	ACLT vs ACLR	ACLR vs ACLS	ACLR vs DMM	ACLR vs SHAM
Total Cartilage Damage (A)									
1	>0.9999	0.233	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
3	>0.9999	>0.9999	0.0002	0.0063	>0.9999	>0.9999	0.0233	>0.9999	0.0815
7	>0.9999	>0.9999	>0.9999	0.001	0.2806	>0.9999	>0.9999	0.4986	0.0039
14	>0.9999	>0.9999	0.0019	0.0106	>0.9999	>0.9999	0.002	>0.9999	0.0138
28	>0.9999	0.0289	>0.9999	0.0561	0.0007	>0.9999	<0.0001	0.0002	0.0204
42	>0.9999	>0.9999	0.0014	0.0011	0.6282	>0.9999	0.0012	>0.9999	0.0022
56	>0.9999	>0.9999	0.5112	<0.0001	0.0001	>0.9999	0.0693	0.0032	0.0026
70	0.0003	0.8989	<0.0001	0.0009	>0.9999	>0.9999	0.4404	>0.9999	0.0014
Total Osteochondral Damage (B)									
1	>0.9999	0.513	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
3	>0.9999	>0.9999	0.0002	0.0063	>0.9999	>0.9999	0.0234	>0.9999	0.0816
7	>0.9999	>0.9999	>0.9999	0.0011	0.2828	>0.9999	>0.9999	0.5025	0.0039
14	>0.9999	>0.9999	0.0019	0.0122	>0.9999	>0.9999	0.0024	>0.9999	0.0159
28	>0.9999	0.0289	>0.9999	0.0369	0.0004	>0.9999	<0.0001	0.0002	0.0259
42	>0.9999	>0.9999	0.0014	0.0016	0.7896	>0.9999	0.0012	>0.9999	0.0022
56	>0.9999	>0.9999	0.5112	0.0001	0.0001	>0.9999	0.0543	0.0024	0.0019
70	0.0003	0.8989	<0.0001	0.0006	>0.9999	>0.9999	0.3597	>0.9999	0.001
Total osteo- and enthesophyte development (C)									
1	N/A	N/A	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
3	N/A	N/A	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
7	N/A	N/A	>0.9999	0.0446	>0.9999	>0.9999	0.1072	>0.9999	0.1417
14	N/A	N/A	0.1598	0.0012	0.7516	>0.9999	0.0007	>0.9999	0.0008
28	N/A	N/A	>0.9999	<0.0001	0.0014	>0.9999	0.0001	0.0121	0.0002
42	N/A	N/A	<0.0001	0.0001	>0.9999	>0.9999	<0.0001	0.806	<0.0001
56	N/A	N/A	0.0002	<0.0001	>0.9999	>0.9999	0.0006	>0.9999	0.0006
70	N/A	N/A	<0.0001	<0.0001	>0.9999	>0.9999	<0.0001	>0.9999	<0.0001
Subchondral bone sclerosis (D)									
1	>0.9999	>0.9999	0.1509	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
3	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.1716	>0.9999	0.2536
7	>0.9999	>0.9999	0.2853	0.103	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
14	0.062	>0.9999	0.0659	>0.9999	0.5664	>0.9999	>0.9999	>0.9999	0.8583
28	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
42	>0.9999	>0.9999	0.0213	>0.9999	0.0079	>0.9999	>0.9999	0.0614	>0.9999
56	>0.9999	>0.9999	>0.9999	>0.9999	0.0586	>0.9999	>0.9999	>0.9999	>0.9999
70	>0.9999	>0.9999	0.0022	>0.9999	0.5055	>0.9999	>0.9999	0.2557	>0.9999

Supplementary Table 3: Detailed p-values for Figure 2

Day	ACLS vs NOC	SHAM vs NOC	DMM vs SHAM	ACLT vs SHAM	ACLT vs DMM	ACLT vs ACLR	ACLR vs ACLS	ACLR vs DMM	ACLR vs SHAM
Cumulated Synovial Inflammation (A)									
1	0.7902	0.0002	>0.9999	>0.9999	>0.9999	>0.9999	0.273	>0.9999	>0.9999
3	>0.9999	<0.0001	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	>0.9999	>0.9999
7	0.7324	<0.0001	>0.9999	0.5555	0.0136	>0.9999	<0.0001	0.781	>0.9999
14	>0.9999	0.0024	>0.9999	0.0003	0.0003	0.0332	<0.0001	>0.9999	0.0632
28	0.0419	>0.9999	<0.0001	0.0737	>0.9999	>0.9999	0.0213	>0.9999	0.0167
42	>0.9999	>0.9999	>0.9999	0.1035	>0.9999	>0.9999	0.008	>0.9999	0.0681
56	0.0307	>0.9999	0.1316	0.0467	>0.9999	>0.9999	0.1944	>0.9999	0.0161
70	0.26	>0.9999	0.0306	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
Synovial Hyperplasia (B)									
1	0.7631	0.0083	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
3	>0.9999	<0.0001	>0.9999	>0.9999	>0.9999	>0.9999	0.0007	>0.9999	>0.9999
7	0.849	0.0002	>0.9999	>0.9999	<0.0001	>0.9999	<0.0001	<0.0001	0.7364
14	>0.9999	0.0012	>0.9999	0.119	<0.0001	>0.9999	<0.0001	0.0004	0.2293
28	0.0395	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.8549	>0.9999	0.8448
42	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.8593	>0.9999	>0.9999
56	0.3067	>0.9999	0.0001	>0.9999	0.7931	>0.9999	>0.9999	>0.9999	>0.9999
70	0.2489	>0.9999	<0.0001	0.8337	0.1994	>0.9999	>0.9999	0.0787	>0.9999
Subsynovial immune cell infiltration (C)									
1	N/A	0.0001	>0.9999	>0.9999	0.7154	>0.9999	0.0011	0.0049	>0.9999
3	N/A	<0.0001	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	>0.9999	>0.9999
7	N/A	0.3809	>0.9999	0.0029	0.0034	>0.9999	<0.0001	0.0003	0.0002
14	N/A	0.0155	>0.9999	0.0035	<0.0001	0.0493	0.0005	0.0144	0.5003
28	N/A	>0.9999	0.0162	0.0265	0.3362	>0.9999	0.0106	0.6117	0.0188
42	N/A	>0.9999	0.022	0.0195	0.4835	>0.9999	0.0026	0.0678	0.0018
56	N/A	>0.9999	0.0075	0.0563	>0.9999	>0.9999	0.2211	>0.9999	0.1295
70	N/A	>0.9999	0.0445	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
Bone Erosion By Pannus (D)									
1	N/A	0.0172	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0203	0.5834
3	N/A	<0.0001	>0.9999	>0.9999	>0.9999	0.0024	>0.9999	0.0002	0.0526
7	N/A	0.0578	>0.9999	0.1239	0.0393	0.0001	>0.9999	0.8226	>0.9999
14	N/A	>0.9999	0.0021	<0.0001	0.0159	0.0009	0.5261	>0.9999	>0.9999
28	N/A	>0.9999	<0.0001	0.1851	>0.9999	>0.9999	0.0033	>0.9999	0.1003
42	N/A	>0.9999	0.0146	0.1472	>0.9999	>0.9999	0.1338	>0.9999	>0.9999
56	N/A	>0.9999	>0.9999	0.2269	>0.9999	>0.9999	0.0007	>0.9999	0.0192
70	N/A	>0.9999	0.1117	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999

Supplementary Table 4: Detailed p-values for Figure 3

Day	ACLS vs NOC	SHAM vs NOC	DMM vs SHAM	ACLT vs SHAM	ACLT vs DMM	ACLT vs ACLR	ACLR vs ACLS	ACLR vs DMM	ACLR vs SHAM
CD4+ T-helper cells (A)									
1	0.0853	0.5021	>0.9999	0.0725	0.2911	0.0402	0.2073	0.984	0.9383
3	0.9176	0.0014	0.1664	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
7	0.6388	0.0003	0.0003	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
14	>0.9999	0.9913	<0.0001	<0.0001	<0.0001	0.5452	<0.0001	<0.0001	<0.0001
28	0.8174	0.934	<0.0001	<0.0001	<0.0001	0.0954	0.0002	<0.0001	0.0001
42	<0.0001	0.3198	<0.0001	<0.0001	<0.0001	0.4563	0.0883	<0.0001	0.0002
56	0.9993	0.7334	<0.0001	0.0003	<0.0001	0.5261	0.4663	<0.0001	0.2362
70	0.6125	>0.9999	0.0415	0.0406	0.0007	0.1808	0.9992	0.0069	0.7803
Ratio of Th1/Th2 cells (B)									
1	0.9827	<0.0001	0.0001	0.0423	0.5312	0.0008	0.0281	<0.0001	<0.0001
3	0.9866	>0.9999	0.9998	<0.0001	<0.0001	0.0505	0.0017	0.0016	0.0017
7	0.9354	>0.9999	0.604	<0.0001	<0.0001	0.1356	<0.0001	<0.0001	<0.0001
14	0.5175	0.9749	0.9952	<0.0001	<0.0001	0.0801	<0.0001	<0.0001	<0.0001
28	>0.9999	0.9821	0.0004	0.0007	0.9998	>0.9999	0.0007	0.9968	0.0006
42	>0.9999	0.4968	0.0003	0.2437	0.0003	0.8834	0.9999	0.0003	0.1845
56	0.66	>0.9999	<0.0001	0.0018	0.001	0.1265	0.0044	<0.0001	0.0045
70	0.9979	0.9984	0.6916	0.9436	>0.9999	0.9633	0.9856	0.9311	0.9999
Ratio of Th17/Treg cells (C)									
1	>0.9999	0.0002	0.0443	0.0056	0.0538	0.4116	<0.0001	0.1374	<0.0001
3	0.9966	0.9879	<0.0001	0.8896	<0.0001	0.9988	0.9783	<0.0001	0.7971
7	0.9603	0.7715	>0.9999	0.0044	0.0044	0.0017	0.0012	0.0012	0.0012
14	0.4635	0.7078	0.7669	<0.0001	0.0003	0.1302	0.0002	0.0005	0.0003
28	0.9715	0.953	0.0008	0.0001	0.0247	0.0007	<0.0001	0.002	<0.0001
42	0.4532	0.7153	0.0005	0.0001	0.0012	0.0006	0.0007	0.0007	0.0011
56	0.8625	0.9979	<0.0001	<0.0001	0.3438	0.9997	0.0041	0.7614	0.0046
70	0.9211	0.4975	0.0005	0.0104	0.9358	0.6468	0.1106	0.9775	0.0005
Ly6C+ monocytes (D)									
1	0.0002	<0.0001	0.9999	0.0014	0.3195	0.0034	0.0054	0.9954	0.9055
3	0.9843	<0.0001	0.0661	0.0062	0.0115	0.141	0.0006	0.0012	0.0098
7	0.1457	0.1191	<0.0001	<0.0001	>0.9999	0.9951	<0.0001	>0.9999	<0.0001
14	0.7172	0.6102	<0.0001	<0.0001	<0.0001	0.0004	<0.0001	0.0017	<0.0001
28	0.114	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
42	0.7411	0.0012	<0.0001	<0.0001	0.0052	<0.0001	<0.0001	<0.0001	<0.0001
56	0.0389	0.9989	<0.0001	<0.0001	0.0012	0.7707	<0.0001	0.0005	<0.0001
70	0.8816	0.619	0.0036	0.9173	0.0026	>0.9999	0.6691	0.0042	0.4862
Blood originated macrophages (E)									
1	0.0005	0.0159	0.2499	0.3148	<0.0001	0.0095	<0.0001	0.1281	0.9807
3	0.0024	0.1419	0.0291	0.0192	0.7978	0.0203	<0.0001	0.055	0.9225
7	>0.9999	>0.9999	<0.0001	<0.0001	0.0988	0.1931	<0.0001	>0.9999	<0.0001
14	>0.9999	0.999	<0.0001	<0.0001	0.9997	0.0394	<0.0001	0.0746	<0.0001
28	0.0201	0.151	<0.0001	<0.0001	<0.0001	0.4659	<0.0001	0.0003	<0.0001
42	0.7032	0.0169	0.004	0.3466	0.9238	0.1167	0.1585	0.0577	0.4244
56	0.0537	0.9642	<0.0001	0.9983	<0.0001	0.4014	0.9984	<0.0001	0.3844
70	0.9987	>0.9999	<0.0001	0.6123	<0.0001	0.5372	0.9989	<0.0001	0.9999
BO M1/M2 ratio (F)									
1	0.9999	0.0006	0.0913	0.2385	0.3118	0.2168	0.0076	0.0244	0.1942
3	0.6502	0.0717	0.9672	0.7455	0.139	0.0462	0.0864	0.6385	0.6619
7	0.9979	>0.9999	<0.0001	0.0004	0.5591	>0.9999	0.0412	0.8855	0.0408
14	0.6385	0.6185	0.0089	0.0008	0.216	0.0045	0.0019	0.0244	0.0019
28	0.9776	0.9217	0.0025	>0.9999	0.0013	0.9997	0.9184	0.0021	0.9904
42	0.5439	0.7287	0.0046	0.2297	0.0079	0.9629	0.0816	0.0175	0.1261
56	>0.9999	0.9916	0.93	0.0001	0.0002	0.9994	0.009	0.0076	0.0153
70	0.8314	0.9995	0.1684	0.1191	0.9986	0.9999	0.0786	>0.9999	0.0959
STRM M1/M2 ratio (G)									
1	0.9948	0.0436	0.4156	0.7944	0.628	0.4877	0.0063	0.013	0.0583
3	0.9972	0.0129	0.0263	0.0008	0.0813	0.0033	0.4037	0.0324	0.4231
7	0.9952	0.9865	0.0136	0.1328	0.7541	0.864	0.0667	0.999	0.0652
14	0.6537	0.6509	0.0339	0.0017	0.8164	0.365	0.2565	0.241	0.2551
28	0.9997	0.2687	<0.0001	0.948	<0.0001	0.9982	0.8374	<0.0001	0.9972
42	0.9914	0.9472	0.0042	<0.0001	0.0004	0.9903	0.0017	0.0002	0.0023
56	0.9375	0.9972	>0.9999	<0.0001	<0.0001	0.8589	<0.0001	<0.0001	<0.0001
70	>0.9999	0.9979	<0.0001	0.0002	0.014	>0.9999	<0.0001	0.0049	<0.0001

Supplementary Table 5: Detailed p-values for Figure 4

Day	ACLS vs NOC	SHAM vs NOC	DMM vs SHAM	ACLT vs SHAM	ACLT vs DMM	ACLT vs ACLR	ACLR vs ACLS	ACLR vs DMM	ACLR vs SHAM
Ly6C ⁺ monocytes (A)									
1	0.9815	0.9529	0.0002	0.0013	0.0121	0.0885	<0.0001	<0.0001	<0.0001
3	0.8243	0.0575	0.3776	0.0022	0.6761	<0.0001	<0.0001	<0.0001	<0.0001
7	0.9878	0.6729	0.0111	0.0087	0.0052	0.9995	0.0897	0.1487	0.1354
14	0.9835	0.8749	<0.0001	<0.0001	0.1544	<0.0001	<0.0001	<0.0001	<0.0001
28	0.9854	>0.9999	<0.0001	0.9671	<0.0001	0.0634	0.336	<0.0001	0.1641
42	0.0777	0.1981	<0.0001	0.9595	<0.0001	>0.9999	0.9996	<0.0001	0.9915
56	0.9935	0.9984	<0.0001	>0.9999	<0.0001	0.5417	0.9343	0.0002	0.5764
70	0.6931	>0.9999	<0.0001	0.6039	<0.0001	0.9896	0.4745	<0.0001	0.9373
M1/M2 ratio (B)									
1	0.5458	0.0167	0.0788	0.0072	0.997	0.0086	0.003	0.0867	0.8677
3	0.9974	<0.0001	0.0222	0.1267	>0.9999	0.2034	0.0463	0.039	0.0942
7	0.8972	0.9309	0.327	0.4608	>0.9999	0.5355	0.244	0.3574	0.2289
14	0.6327	0.6407	0.0927	0.0099	0.1755	0.04	0.0035	0.1112	0.0062
28	0.9603	>0.9999	0.0069	0.7286	0.0072	0.9951	0.8447	0.0073	0.6401
42	>0.9999	0.8947	<0.0001	0.9969	<0.0001	>0.9999	0.3542	<0.0001	0.994
56	>0.9999	>0.9999	0.0006	0.6058	0.0008	0.9628	>0.9999	0.0003	>0.9999
70	>0.9999	0.9958	0.998	0.0037	0.0062	0.7608	<0.0001	<0.0001	<0.0001
Ratio of Th1/Th2 cells (C)									
1	>0.9999	0.9532	0.0054	0.0013	0.994	0.9217	0.0182	0.788	0.0113
3	0.4997	0.6202	0.0028	0.0001	0.7908	0.6443	0.0004	0.2447	0.0004
7	0.2966	0.7802	0.1826	0.0068	0.192	0.132	0.1426	0.9997	0.146
14	0.8128	0.9988	<0.0001	0.0008	0.6181	0.5932	0.0036	0.9919	0.0026
28	0.952	0.7815	<0.0001	<0.0001	0.0005	0.8632	<0.0001	0.0003	<0.0001
42	>0.9999	0.9999	<0.0001	0.0003	0.3286	0.5083	0.0002	0.9937	0.0001
56	0.9908	>0.9999	<0.0001	<0.0001	<0.0001	0.5959	0.0005	<0.0001	0.0003
70	>0.9999	0.999	<0.0001	0.006	0.0016	0.2965	0.0014	0.0098	0.0006
Ratio of Th17/Treg cells (D)									
1	0.6357	>0.9999	0.2038	0.7098	0.4354	0.8463	0.436	0.2552	0.9998
3	0.0018	0.9974	0.5727	0.0039	0.024	0.3756	0.9975	0.0082	0.0019
7	0.0964	0.684	0.1393	0.1852	0.194	0.0183	0.1505	0.059	0.0949
14	0.1585	<0.0001	0.8198	0.0363	0.4669	0.2315	0.8064	0.0262	<0.0001
28	0.995	0.5615	0.0009	0.0022	0.0324	0.0324	0.0566	0.0025	0.6271
42	0.5784	0.1507	0.094	0.0128	0.8408	0.9669	0.0125	0.5379	0.0314
56	0.6412	0.8676	0.0338	0.0059	0.336	0.1084	0.5987	0.925	0.1183
70	0.4756	0.6349	0.5325	0.546	0.3286	0.4862	0.9753	0.9288	0.9969

Supplementary Table 6: Detailed p-values for Figure 5

Day	ACLs vs NOC	SHAM vs NOC	DMM vs SHAM	ACLt vs SHAM	ACLt vs DMM	ACLt vs ACLR	ACLR vs ACLs	ACLR vs DMM	ACLR vs SHAM
Ly6C⁺ monocytes BM (A)									
1	0.0539	0.0052	<0.0001	<0.0001	0.979	0.9984	<0.0001	0.9996	<0.0001
3	<0.0001	<0.0001	0.0888	0.001	0.0021	<0.0001	0.2023	0.1613	<0.0001
7	>0.9999	>0.9999	<0.0001	<0.0001	0.6464	<0.0001	<0.0001	<0.0001	<0.0001
14	0.8712	0.8477	<0.0001	<0.0001	0.1087	0.7551	0.0001	0.0183	<0.0001
28	0.9264	>0.9999	0.0011	<0.0001	0.0002	<0.0001	0.0163	0.0008	0.0086
42	0.7992	0.9729	<0.0001	0.0016	<0.0001	0.0038	0.9958	<0.0001	0.6811
56	0.9966	0.9976	<0.0001	<0.0001	<0.0001	0.1343	0.0027	<0.0001	0.0032
70	0.9923	0.3946	<0.0001	<0.0001	0.291	0.3211	<0.0001	0.0603	<0.0001
Ly6C⁺ monocytes spleen (B)									
1	0.0837	<0.0001	0.9659	<0.0001	<0.0001	0.9282	<0.0001	<0.0001	<0.0001
3	0.8984	<0.0001	<0.0001	<0.0001	<0.0001	0.2231	<0.0001	<0.0001	<0.0001
7	0.5107	0.9802	<0.0001	<0.0001	<0.0001	0.0059	<0.0001	<0.0001	<0.0001
14	0.8259	0.6034	<0.0001	<0.0001	<0.0001	0.0103	0.0251	<0.0001	0.0089
28	0.986	0.9985	<0.0001	0.3805	<0.0001	0.9638	>0.9999	<0.0001	0.9333
42	>0.9999	0.9998	<0.0001	0.9444	<0.0001	0.9169	0.9998	<0.0001	0.9998
56	>0.9999	0.9766	0.1974	>0.9999	0.0812	0.7061	0.8538	0.9761	0.7164
70	>0.9999	0.1422	0.2501	0.6483	0.9964	0.9947	0.9994	>0.9999	0.2893
M1/M2 ratio BM (C)									
1	0.1062	<0.0001	0.0025	0.0208	>0.9999	0.0808	<0.0001	0.0137	0.0032
3	0.6003	<0.0001	<0.0001	0.006	<0.0001	0.971	0.0076	<0.0001	0.0271
7	0.9833	0.9993	0.0001	<0.0001	0.4984	0.0004	0.002	0.0002	0.0017
14	0.6287	0.6056	0.0039	0.3083	0.0057	0.9966	0.4445	0.0057	0.2584
28	0.4485	0.9952	0.0014	0.9507	0.0014	0.8848	0.561	0.0012	0.9969
42	0.9998	0.305	0.9986	0.1088	0.024	0.5866	0.7871	0.9894	0.9994
56	0.9963	0.999	0.8036	<0.0001	0.0001	0.9979	0.0002	0.0013	0.0005
70	>0.9999	0.443	0.8082	0.0279	0.036	0.3276	0.0131	0.0053	0.0025
M1/M2 ratio spleen (D)									
1	0.9062	0.0656	0.0476	0.0017	0.0139	0.0267	0.0003	0.6034	0.0153
3	0.9967	<0.0001	<0.0001	<0.0001	0.0038	<0.0001	<0.0001	<0.0001	<0.0001
7	0.8446	0.5916	0.0006	<0.0001	0.0072	0.9875	0.0079	0.0245	0.008
14	0.684	0.6694	0.0051	0.138	0.0054	0.437	0.9874	0.0052	0.9999
28	0.9879	0.8404	0.0001	0.0148	0.0001	0.0903	0.0399	0.0002	0.085
42	0.9986	0.8713	<0.0001	0.9984	<0.0001	>0.9999	0.9916	<0.0001	0.9994
56	0.9765	>0.9999	0.0011	0.9996	0.001	0.9921	0.8515	0.0016	>0.9999
70	>0.9999	0.9998	<0.0001	0.9208	<0.0001	0.9757	>0.9999	<0.0001	0.9782
Th1/Th2 ratio BM (E)									
1	>0.9999	0.0093	0.9599	0.0232	0.0409	0.0017	>0.9999	<0.0001	0.0095
3	0.0728	0.0001	<0.0001	<0.0001	0.9406	0.009	0.9058	0.0277	<0.0001
7	0.8506	0.7209	>0.9999	0.8714	0.9491	>0.9999	0.896	0.9429	0.8192
14	0.0004	<0.0001	0.9712	0.6283	0.9868	0.4472	0.9997	0.8832	0.9919
28	0.7351	0.6331	0.0007	0.7514	0.0021	>0.9999	0.9568	0.0021	0.8395
42	0.5512	0.8212	0.1574	>0.9999	0.2981	0.9249	>0.9999	0.4113	0.8741
56	0.9246	0.909	>0.9999	0.8934	0.5505	0.8967	>0.9999	>0.9999	>0.9999
70	>0.9999	0.7976	0.5899	0.9977	0.8662	0.9688	0.9999	0.9978	0.7888
Th1/Th2 ratio spleen (F)									
1	0.1233	<0.0001	0.0917	0.2678	>0.9999	0.0217	0.9666	0.0054	<0.0001
3	0.0113	<0.0001	0.0031	0.0007	0.8842	0.1267	0.0437	0.1578	0.0432
7	0.9665	0.8472	0.0021	0.0002	0.6253	0.0014	0.0006	0.0135	0.0288
14	0.1032	0.0016	0.0003	0.2498	0.5519	0.0032	0.9574	0.0098	0.0023
28	0.1007	0.0206	0.0108	0.5524	0.0141	0.9951	>0.9999	0.0113	0.9688
42	0.9891	>0.9999	0.1394	0.9846	0.522	0.9988	0.9402	0.2752	0.9997
56	0.0402	0.8405	0.8943	0.9986	0.9887	0.908	0.1744	0.4226	0.993
70	0.116	0.4589	0.2238	0.9986	0.1984	0.7625	0.867	0.2645	0.937
Th17/Treg ratio BM (G)									
1	0.9991	0.0027	0.7657	0.3901	0.6498	0.0461	0.6605	0.0018	0.0038
3	0.4527	>0.9999	0.3749	0.6204	0.9671	0.0897	0.151	0.0946	0.6539
7	0.9996	0.7506	0.7315	0.7653	>0.9999	0.4903	0.9114	0.4136	0.2506
14	0.0026	0.1882	0.9403	0.9785	0.2322	0.9994	0.249	0.5295	0.9978
28	0.0035	0.0221	0.5499	0.1605	0.9916	0.0182	0.0734	0.0783	0.5535
42	0.2566	0.0131	0.0043	0.485	0.024	0.0461	0.0527	>0.9999	0.0101
56	<0.0001	0.0004	0.002	0.0119	0.9979	0.6981	0.1207	0.7718	0.0203
70	0.5231	0.9326	0.1294	0.994	0.9045	0.9592	>0.9999	>0.9999	0.6425
Th17/Treg ratio spleen (H)									
1	0.9946	<0.0001	0.6026	0.9494	0.9631	0.0002	0.6053	0.0004	<0.0001
3	>0.9999	0.6861	0.9977	0.8966	0.9915	0.9979	0.995	0.9408	0.7788
7	0.9996	0.9929	0.5708	0.5279	0.9966	>0.9999	0.3775	0.9946	0.7149
14	0.5184	0.9186	0.0271	0.0792	0.9996	0.8568	0.9995	0.5477	<0.0001
28	0.0002	0.3024	0.9999	0.0153	0.0124	0.9814	0.4647	0.0029	0.0057
42	0.0003	>0.9999	0.0014	0.0014	0.0345	0.9705	0.5787	0.019	0.0074
56	0.3027	0.9841	0.0074	0.2897	0.0392	0.5132	0.9629	0.0003	0.8266
70	>0.9999	0.9094	<0.0001	0.1426	0.0003	0.8517	0.9611	0.0001	0.6071

3.3 Main Findings of the Study

In this study we compared the loco-regional and systemic innate and adaptive inflammatory cell responses in three of the most commonly used mouse models of ptOA: DMM, ACLT and ACLR. Using these models allowed detailed temporal evaluation and direct comparison with non-OA-inducing injuries (SHAM, ACLS) not possible in human clinical studies, as well as the ability to explore the effect of injury to different joint-tissues (DMM vs ACLT) and surgery itself (ACLT vs ACLR) on the severity and phenotype of the inflammatory response. The key findings of these analyses were that: (1) non-critical/sprain loading injury (ACLS) does not incite a significant loco-regional or systemic immune/inflammatory activation; (2) while surgery alone induced acute synovial (but not lymph-node or systemic) inflammation this resolved rapidly (by day-28 histology; by day-7 innate and by day-3 adaptive response), and there was minimal difference between surgical and non-surgical models (ACLT vs ACLR) in the magnitude or phenotype of the loco-regional and systemic innate and adaptive response; (3) however, there was a distinct temporal difference in the loco-regional and systemic innate and adaptive response depending of the joint-tissue injury (DMM vs ACLT).

The data from this study indicates a distinct spatiotemporal Th1 and Th 17 response that occurs predominantly on a locoregional (synovial tissue block and draining lymph node) level. Furthermore, while the Th1 response subsides gradually once the cartilage damage has reached its maximum, Th17 cells seem to be continuously accumulating in the synovial tissue. Importantly, model-specific temporal differences in both Th1/Th2 and Th17/Treg indicated a major effect of the joint-tissue injured. In contrast to the late-stage decrease of Th1 levels observed in our study, high Th1 levels were found in clinical synovial samples of end-stage osteoarthritic knee joints (Rosshirt, N. et al. 2019). This might be attributed to the fact that the extent of cartilage damage in ptOA mouse model exceeds the extent found in clinical studies and such a “maximum cartilage damage” is only rarely encountered in patients.

On an innate immune level, joint injury led to a distinct pattern of Ly6C^{high} monocytes across tissues. In particular, a short depletion of classical activated monocytes was detected in the bone marrow and spleen whereas an influx was seen in the synovial tissue itself which can be explained by a targeted recruitment of systemic Ly6C^{high} monocytes into the injured joint. As extravasated classically activated monocytes follow the monocyte waterfall when

differentiating into tissue macrophages, this observation is also supported by an initial acute transient BOM influx into the joint of ptOA mice. The lack of this influx in SHAM animals indicates an important role of chemokines (MCP-1/CCL-2) synthesized and released from cells acutely injured in ACL, meniscus or cartilage, that potentially orchestrate the innate immune response subsequent to joint injury and thereafter during progression of ptOA.

Taken together this data indicates a predominant loco-regional and limited systemic response to joint injury, with the magnitude and duration of the initial transient increase in activated-monocyte and M1 macrophages (with a potential role of orchestrating chemokines such as MCP-1), and both acute and long-term Th1 and Th17 T-cell response differentiating pt-OA-inducing from non-ptO-inducing injury. Importantly, what you injure (ACL vs meniscus) rather than how you injure it (surgery vs non-surgical) determines the temporal pattern and systemic involvement of both the innate and adaptive immune response and the structural ptOA progression and severity.

Chapter 4

4 Evaluation of a Distinct Molecular Activation Pathway for the Adaptive Immune Response in Context with ptOA

4.1 Aim of the Study

In **Chapter 3** we observed a skewed pro-inflammatory imbalance dominated by increased levels of Th1 and Th17 cells during pathogenesis of ptOA. A potential pathway contributing towards the co-overexpression of these T-helper cell subsets is the IL12 pathway. IL12 itself is an interleukin that stimulates polarization of Th1 cells, however its IL12B subunit is shared with IL23 which promotes polarization of Th17 cells. Thus, in order to investigate the individual impact of CD4⁺ T-helper cell subsets on the pathogenesis of ptOA we investigated the effects of inhibiting either IL12, or IL12/23 or all CD4⁺ T-helper cells in the immediate peri-injury period (prior to and immediately following joint injury) as a proof-of-principle study. Furthermore, in vitro experiments were included to analyse potential molecular pathways of T-cell activation.

4.2 Drafted Manuscript for Submission: Characterizing the Role of the IL12/23 Pathway during Onset and Progression of ptOA.

Characterising the role of the IL12/23 pathway during onset and progression of post-traumatic osteoarthritis

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Introduction:

Osteoarthritis (OA) of the knee remains common and results in disability and chronic pain for the affected individual as well as substantial healthcare and productivity costs for society [1]. OA contributes substantially to the global burden of disease and was the second highest cause for years lived with disability in 2015 [1]. Importantly knee OA (KOA) contributes to almost 80% of this disease burden [2], and disease following traumatic joint injury (post-traumatic (pt)OA) accounts for at least 12% of all cases of symptomatic KOA [3, 4]. A recent longitudinal cohort study showed that knee injury, such as ACL or meniscus tear, at a young age increased the risk of developing ptOA almost sixfold [5], and restoration of biomechanics alone is insufficient to prevent ptOA development [6, 7]. This highlights the need to investigate other mechanisms associated with ptOA risk, and inflammatory or immunobiological differences in the response to injury could play a role [8-10]. Although evidence has emerged regarding a crucial inflammatory component contributing to ptOA development [8-12], the role of individual molecular and cellular key players needs to be defined in order to identify potential targets for novel and preventative treatment modalities.

Findings from recent studies have shown significant abnormalities in the T-cell profile of OA patients and implicated a crucial role for proinflammatory CD4⁺ T-helper cell subsets, particularly Th1 and Th17, in the disease pathogenesis [13]. In particular, a skewed proinflammatory differentiation towards Th1 cells was observed in the synovial fluid and membrane of early OA patients [14]. Furthermore, not only are Th1 cells predominant in both OA and RA joints [15] but also the number of circulating Th1 cells is significantly higher in OA patients compared to healthy controls [16]. Initially, Th17 cells were mainly implicated in the development of autoimmune disease [13] whereas recent studies have shown that these cells and associated interleukin (IL)17 are elevated in the synovial fluid of OA patients compared with healthy controls and a strong expression of IL17 mRNA was detected in OA

synovial membranes [13]. However, the individual contribution of either Th1 or Th17 cells in ptOA as well as what leads to their activation remains poorly understood.

IL12 is known for its proinflammatory role and the promotion of Th1 differentiation of CD4+ T-helper cells [17]. IL12 itself consists of two subunits, the p40 subunit is shared with IL23, a cytokine that drives Th17 differentiation [17]. Earlier studies have indicated that IL12 plays a crucial role in inducing a shift towards polarisation of rheumatoid T-cells expressing a Th1 cytokine profile [18]. Despite their shared subunit, emerging evidence has proposed a diverging role for IL12 and IL23 in auto-immune joint inflammation [19]. In particular, IL23 deficiency protected mice from the development of joint and bone pathology in the collagen-induced-arthritis RA-model, while IL12 deficient mice developed higher levels of IL17 producing T-cells and a more distinct pro-inflammatory cytokine profile [19]. While compelling evidence exists highlighting the importance of the IL12/23 pathway during the development of RA and auto-immune joint inflammation, to date the role of this pathway in ptOA is unknown. In the current study we sought to decipher the role of the IL12/23 pathway in the onset and development of ptOA, and furthermore investigate if inhibition of individual aspects of this pathway may serve as potential targets for disease modification.

Materials and Methods

Animals and Study Design

All experiments were approved by the Northern Sydney Local Health District Animal Ethics Committee (protocol RESP20/083), with DEPART and ARRIVE guidelines followed for experimental design and reporting [20, 21]. For all experiments a total of 468 10-12-week-old, male C57BL/6 mice were utilized, with mice housed in individually-ventilated cages (up to 5/cage) with commercial bedding, environmental enrichment (perspex housing, wooden sticks, tissues), *ad-libitum* access to standard pelleted mouse feed and acidified water, in a dedicated SPF vivarium maintained at 20-22 °C with 12-hour light/dark cycles, and cared for in accordance with the Australian code [22]. An overview over the study design, sample size per experiment and the allocation of animals is provided in Figure 1. Animals housed in the same cage were randomly assigned to each set of experiments but all animals in the cage were sacrificed at the same time (see below) to avoid isolation stress.

Animal models

In the current study a total of 3 groups were included. A non-surgical ACL rupture (*ACLR*) model [23, 24] was used to induce ptOA without the confounding inflammation of an initial operative trauma. Here, a single compressive load was applied to the tibia of the flexed knee resulting in posterior subluxation of the femur and ACL rupture without additional macroscopic damage to other structures within the joint [24], with subsequent rapid onset and progression of joint-wide ptOA pathology [25] (see also **Chapter 2.2.2.2**). Controls included animals that received a single compressive load to 75% of that required to rupture the ACL. This resulted in an ACL sprain (*ACLS*) that while inducing minor focal osteochondral damage and ACL remodeling does not cause progressive ptOA pathology [24, 26]. The day of injury was defined as day 0 of the experiments with treatments (day -1, 1, and 3) and tissue harvest

(day 1, week-1, -4 or -6) named accordingly. Animals harvested at day-1 only received the treatment prior to injury. Finally, naïve mice were included that served as non-operated age/time-point controls (*NOC*) in a subgroup of experiments.

In vitro experiments

Inguinal, sacral and iliac lymph-nodes were harvested from 10-week-old male C57BL/6 mice and naïve CD4⁺ T-helper cells immediately isolated as per manufacturer instructions (Naïve CD4⁺ T-Cell Isolation Kit, mouse, Miltenyi Biotec, cat #130-104-453). Thereafter, 0.5×10^6 cells were distributed in each well of a 24-well plate and 2ml of standard cell culture media (88.9% RPMI-1640, 10% HI-FBS, 1%PS, 0.1% AmpB, 2mM L-Glutamine, 0.05mM 2-ME) containing particles loaded with antibodies against CD3 and CD28 added (T-Cell Activation/Expansion Kit, mouse, Miltenyi Biotec, cat #130-093-627). A synovial tissue block (as normally harvested for Flow Cytometry [27]) or tibial plateau (tibial epiphysis isolated by dissection through the proximal tibial growth plate with removal of joint capsule, fatty tissue, meniscus and ligament attachments) from mice 1-week post-ACLR or age-matched NOCs were harvested immediately after sacrifice and co-cultured with activated T-cells for 72 hours. Additionally, Apilimod/STA-5326 (Selleck Chemicals, catalogue #S0785; a potent inhibitor of IL12 and IL23 production via direct targeting of PIKfyve [28, 29] [30]), at a final concentration of 10nM was added to half the samples. Four replicates were used for each experiment. Cultures containing either the individual joint tissue or naïve CD4⁺ T-helper cells alone were included as controls. After 72 hours of co-culture, CD4⁺ T-cells and supernatant were harvested and stored at -80°C separately until analysis.

In-vivo experiments

Intraperitoneal injections using 500µg of either anti-CD4 mAB (InVivoMAb anti-mouse CD4, BioXCell, catalogue #BE0003), anti-IL12p40 (InVivoMAb anti-mouse IL12p40, BioXCell, catalogue #BE0051; inhibiting IL12 and IL23 and both Th1/Th17 differentiation), anti-IL12p75 (InVivoMAb anti-mouse IL12p75, BioXCell, catalogue #BE0233; inhibiting IL12 & Th1 differentiation predominantly) were performed at day -1, 1 and 3, while control animals received sterile saline injections with the same volume. ACLR was performed at day 0 and tissues harvested at various time points thereafter as described above.

Flow cytometry

Blood (terminal cardiac puncture under general anaesthesia), contralateral femoral bone marrow, draining lymph-nodes (pooled inguinal and iliac lymph-nodes) and synovial tissue were harvested from all animals included in the proteinic in-vivo experiments (Fig. 1) as described [27]. Tissues were pooled from two animals to increase cell numbers per sample resulting in n=6 biological replicates for each tissue-source at each time point in each animal group. The detailed protocol regarding the harvest of tissues and preparation of single cell solutions and the utilized multi-color antibody panel has been published previously [27] (see Table S1 for list of used antibodies, in addition see also **Chapter 2.2.2.1 and 3.2**).

Gene Expression Analysis

Synovial tissue and tibial plateau were harvested day-1, week-1, -4 and -6 (or equivalent time in NOCs; Fig. 1 “Genomic Experiments”) and immediately snap frozen in liquid nitrogen and stored at -80C until further processing. Following cell culture as described above T-cells were separated from the culture supernatant via centrifugation. Total RNA was isolated (cells - Isolate II RNA Isolation Kit, Bioline cat# BIO-52073; tissue (freeze pulverized): RNeasy Micro Kit, Qiagen cat# 74004; following manufacturer’s protocols), quantified using

Nanodrop and then an equal quantity (1ug) RNA reverse transcribed for all samples (GoScript RT kit, Promega cat# A2791) as previously described [31-34]. Primers were designed using MacVector (see Table S2 for details regarding individual primers) and PCR specificity confirmed by product sequencing and melt-curve analysis [31-34].

Luminex

After separation from cells, culture supernatant was store at -80C until analysis. Concentrations of T-cell specific chemokines and cytokines in culture supernatant was analyzed using a dedicated multiplex bead assay and sample preparation as per manufacturer instructions (Mouse Th17 Magnetic Bead Panel, Milipore, cat #MTH17MAG-47K, #MT17MAG47K-PX25, #MT17MAG47PMX25BK).

Histopathological scoring

After removal of the anterior synovium (for flow cytometry) knee joints were fixed, decalcified, paraffin-embedded, and serial sagittal sections across the medial femoro-tibial joint cut and stained with toluidine blue-fast green as previously described [32, 33, 35]. Utilizing a modified single slide scoring system ([36] and **Chapter 2.2.2.2**) femoral and tibial cartilage and structural damage, tibial and femoral chondrocyte hypertrophy and apoptosis, posterior fibrotic changes to joint capsule, and posterior femoral synovitis, were determined by a single trained observer (PH) blinded to time and treatment (n = 9 mice/group/time point). Finally, median values for each histopathological feature for each group and time point were generated and compared.

Statistics

The individual histology-outcomes were considered as categorical and independent variables, as prior analysis demonstrates they change differentially and with distinct temporal patterns in different OA models [36], and are independently modified in response to treatment/genetic-modification [32-35, 37]. Non-parametric analyses (Kruskal-Wallis and Mann-Whitney-U) were used to assess differences between groups for all histological outcomes at each post-injury timepoint. Histological data is presented as median $\pm$ error. Flow cytometry and PCR data is presented as mean $\pm$ standard error of the mean (SEM). Two-way analysis of variance (ANOVA) with post-hoc Tukey's correction for multiple comparisons was used to compare all individual variables. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). All statistical calculations were performed with GraphPad Prism (Version 9.0.0).

Results

OA inducing joint damage modulates synovial IL12A, IL12B and IL23A expression

Expression of IL12A in the synovial tissue block was significantly lower in ACLR mice compared to naïve controls at all time points, and compared with ACLS from 7 days onwards (Fig 2B; no difference between NOC and ACLS at any time). Synovial expression of IL12B and IL23A were significantly higher in ACLR compared to both ACLS and NOC during the first 7 days. Peak synovial expression levels for both IL12B and IL23A were detected 1 day after injury and then decreased over time, although IL12B was increased again at week-6 in ACLR mice. Despite all three mRNAs being detected in the osteochondral (tibial plateau) tissue, there was no significant difference between groups at any time. Taken together these results indicate a potential role for the IL12/23 pathway associated genes early during ptOA onset and progression, while their point of origin seems to be located in the synovial tissue block rather than the bone or cartilage itself.

Apilimod has an immunomodulatory effect on naïve CD4⁺ T-helper cells co-cultured with osteoarthritic murine joint tissue in vitro

To further evaluate the role of the IL12/23, naïve CD4⁺ T-helper cells were co-cultured with synovium or tibial plateau from NOC or 1-week post-ACLR $\pm$ Apilimod. T-cell activation was evaluated by quantifying mRNA expression of IL12A, IL12B, IL23A, IL17A, RORC and TBX21, and secretion of INF- γ , IL1 β , MIP3-a, IL23 (IL12 was below detection limits) and IL17A into the culture media (Fig 3A). Cultures of joint tissue without T-cells were used as negative control and protein levels observed in these controls subtracted from the co-cultures to account for release of proteins from synovial or osteochondral tissues. Co-culture of joint tissues from either ACLR or NOC and naïve CD4⁺ T-helper cells increased IFN gamma secretion which was significantly decreased via IL12/23 pathway blockage (Fig. 3B). Co-

culture of naïve CD4⁺ T-helper cells and ACLR synovium or osteochondral tissue increased IL1-beta secretion compared with NOC, with this response significantly inhibited using Apilimod with synovium but not tibial plateau. MIP3-alpha and IL23 secretion were also elevated after co-culture with ACLR synovium or tibial plateau, but unlike IL1-beta the response to both joint tissues was significantly inhibited by Apilimod. Secretion of IL17A was significantly increased exclusively when co-culturing ACLR synovium and naïve CD4⁺ T-helper cells, and this response was almost completely inhibited after administration of Apilimod.

Further evaluation of T-cell activation and polarization was done using qPCR analysis, which confirmed a pro-inflammatory effect of OA (ACLR) compared with normal (NOC) joint tissues (Fig. 4). Expression of IL12A mRNA was not detected in T-cells co-cultured with synovium but an increase was induced by OA osteochondral tissue and this was partially inhibited by Apilimod. Increased IL12B and IL23A mRNA were detected in cells co-cultured with both ptOA synovium and cartilage and this was partially reversed by Apilimod. Consistent with IL17A protein levels (Fig 3), IL17A mRNA expression was predominantly increased in CD4⁺ T-helper cells incubated with OA synovium and was inhibited by Apilimod. Expression of RORC (which encodes ROR γ a crucial transcription factor for the polarization of Th17 cells [38]), and Tbx21 (a master regulator of Th1 polarization [39]), were also predominantly increased by co-culture with ptOA synovium and could be inhibited by Apilimod. Taken together, data from the multiplex analysis and T-cell gene expression, indicated that while both OA and naïve joint tissues led to a pro-inflammatory response (increased IFN), only OA (ACLR) joint tissue induced a chemotactic response in T-cells (increased IL1-beta and MIP3-alpha). Expression of IL12/IL23 is increased in T-cells exposed to OA joint tissues, but only ptOA synovium led to a Th17 polarization of naïve CD4⁺ T-helper cells and IL17 secretion. Importantly, all of these effects were regulated by the IL12/23 synthesis, suggesting this

pathway might play a crucial role in the onset and progression of ptOA and could be a viable therapeutic target.

In-vivo inhibition of the IL12/23 pathway results in an anti-inflammatory response and mitigates injury induced ptOA

Based on the temporal pattern of IL12/23 expression in ACLR synovium and the effect of this pathway on T-cell activation/polarization in vitro, we evaluated whether inhibition of IL12/23 at the time of joint injury could modulate the inflammatory response and onset and progression of ptOA pathology in vivo. Polarization of immune cells systemically (blood and bone marrow; Fig 5) and loco-regionally (synovial tissue and draining lymph-node; Fig 6) were evaluated using an established flow cytometry multi-color panel and protocol [27] **see Chapter 2.2.2.1 and 3.2.**

Injection of anti-CD4 antibody led to a profound systemic depletion of T-helper cells lasting 4 weeks before beginning to recover but still less than normal at week-6 (Fig. 5B). In contrast, there was no effect on blood CD4⁺ T-helper cell numbers with either anti-IL12p40 or anti-IL12p75 administration and only a short-lived decrease in the bone marrow CD4⁺ T-helper cells at day-1 (anti-IL12p75) and day-7 (anti-IL12p40). While changes in polarization of systemic T-helper cells after anti-CD4 treatment could not be determined until week-6 due to cell depletion, at this time an anti-inflammatory profile (Th1/Th2 and Th17/Treg <1) was seen compared with saline. Anti-IL12p40 and anti-IL12p75 significantly reduced the systemic Th1/Th2 ratio (<1 and compared with saline), with the effect persisting for 4-6 weeks and largely similar with both antibodies (more rapid return to pro-inflammatory Th1/Th2 ratio in bone-marrow with anti-IL12p40). While both anti-IL12p40 and anti-IL12p75 led to a persistent decrease in Th17/Treg polarization (<1 and compared with saline) the effect was as expected more pronounced with anti-IL12p40 (targeting both IL12 and IL23).

Anti-CD4 antibody administration also led to a loco-regional reduction in T-helper cells, albeit the reduction in the synovium was less than in the draining lymph-node (Fig. 6) or systemically (Fig 5). While neither anti-IL12p40 and anti-IL12p75 led to CD4⁺ T-helper cell depletion in the lymph-node, both induced a transient (day-1) mild reduction in synovium that then rebounded (week-1), and with anti-IL12p40 synovial CD4⁺ T-helper cell numbers were increased relative to saline at week-6 (Fig 6B). Effects of all three antibodies on T-cell polarization in lymph-node was generally similar to that seen systemically, except for a more persistent decrease in Th1/Th2 with both anti-IL12p40 and anti-IL12p75. Persistence of CD4⁺ T-helper cells in synovium allowed the effect on T-cell polarization of anti-CD4 versus anti-IL12p40 and anti-IL12p75 to be compared. A more distinct anti-inflammatory Th1/Th2 polarization in the synovium was observed in the anti-IL12p40 and IL12p75 groups compared to the anti-CD4 group, but significant reduction persisted through week-6 with all antibodies. In contrast, despite ongoing systemic CD4⁺ T-helper cell depletion, anti-CD4 only led to a short-lived anti-inflammatory Th17/Treg polarization, while targeting IL12p40 and IL12p75 in the peri-injury period resulted in a lasting anti-inflammatory response in the synovium. Finally, histopathological evaluation revealed that peri-injury immunomodulatory treatment, regardless of the antibody, significantly slowed/delayed cartilage and bone damage although ultimately reaching similar levels to saline (Fig. 7A). Interestingly, significantly less chondrocyte hypertrophy/apoptosis was observed after inhibition of the IL12/23 pathway compared to saline controls. Histopathological evaluation of synovial inflammation (Fig. 7B) showed a typical pattern of an acute increase and then resolution following ACLR in saline-treated mice. All antibodies significantly abrogated synovial hyperplasia as well as overall synovitis during the first 7 days post injury, while 6 weeks post injury only anti-IL12p40/p75 treatment resulted in continued synovitis reduction (albeit at a non-significant extent). Synovial fibrosis was only decreased by anti-IL12p40/p75 treatment, with a significant reduction lasting

between 7- and 28-days post-injury after which levels increased to be similar to both saline and anti-CD4 (Fig. 7C).

Discussion

For more than two decades IL12/23 has been implicated in the pathogenesis of inflammatory arthritis. In a pre-clinical RA model, systemic suppression of IL12 reduced joint swelling and cell influx into the synovium, reduced IL1 beta and IFN gamma levels and restored chondrocyte function [40]. Clinically, elevated levels of IL12 were detected in patients with RA correlating with their disease activity score [41], and successful treatment of symptoms with disease modifying anti-rheumatoid arthritis drugs (DMARDs) was accompanied by decrease IL12 in synovial fluid and peripheral blood [42]. Ongoing clinical trials are evaluating the DMARD effect of directly targeting IL12 [43], with interest in the role of this pathway increasing. A recent study found a correlation between poorer knee function as well as increased knee pain and higher synovial IL12 levels [44]. In the current study we sought to evaluate the role of the IL12/23 pathway on Th1/Th2 and Th17/Treg CD4+ T-helper cell polarization during the onset and development of ptOA. We used different methods of IL12/23 pathway modulation, *in vitro* and *in-vivo* experimental models, and compared effects in an established non-surgical ptOA mouse model to both naïve and non-OA-inducing joint-loading injury controls. Collectively our data suggests that the IL12/23 pathway plays a significant role in T-cell polarization that is central to ptOA pathogenesis and shows promise as a potential disease-modifying-osteoarthritis-drug (DMOAD) target.

Interestingly, despite expression of IL12A mRNA (the subunit exclusive to IL12) being significantly reduced in the synovium during ptOA development in our study, blockade of IL12p75 (targeting IL12 not IL23) led to an anti-inflammatory T-helper cell subset profile, mitigated cartilage and chondrocyte damage, reduced synovitis and synovial fibrosis during development of ptOA. Thus, our data is corroborating previous findings made in RA [40] by strongly implicating IL12 in ptOA pathophysiology while suggesting regulation may be at the post-transcriptional level. Improved understanding of the IL12 pathway and the underlying

properties of the protein and gene itself has led to further research into the function of IL12B, the shared subunit between IL12 and IL23. A recent study showed that blockade of IL12B using a specific antibody (ustekinumab) resulted in a suppressed synovial inflammation via modulation of MAPK-ERK, Wnt and PI3K-Akt pathways, all implicated in OA pathogenesis [45], whereas higher production of IL12B led to a more distinct polarisation of Th1 and Th17 cells in a reactive arthritis model [46]. Interestingly, dendritic cells were identified as the precise source for IL12B [46], which is consistent with the distinct expression pattern of IL12B exclusively in the synovium in the current study, as dendritic cells are abundantly present in the synovial tissue [47]. Additional data from a study investigating the role of IL12B in inflammation-mediated bone disorders showed that inhibition of IL12B via Apilimod treatment promoted bone formation and protected against aging-related bone loss, whereas this effect was not observed in IL12^{-/-} mice, demonstrating the importance and unique role of IL12B as part of the IL23 pathway in inflammation mediated bone disease [48]. Further studies exploring the role of IL23 in the pathogenesis of inflammatory arthritis have demonstrated that IL23 deficient mice were protected against development of collagenase-induced inflammatory arthritis and lacked functional Th17 but not Th1 T-cell subsets [49]. Furthermore, IL23 has been shown to induce osteoclastogenesis, and subsequently blockade of IL23 not only alleviated inflammation but also bone destruction [49], and IL23 deficient mice were protected from inflammatory arthritis and associated pain [50].

The above data demonstrates the importance of IL23, but whether the therapeutic efficacy of targeting IL23 is associated with its role in activating the IL17 pathway and its critical immune axis role and potential target for DMARDs continues to be investigated [51]. Pre-clinical studies found that cyclooxygenase inhibitors inhibited IL23-mediated neutrophil migration as well as suppressing IL23-induced IL17 production in a murine RA model [52]. Clinically, increased IL23 is associated with bone marrow lesions, however this effect was reserved to

female patients [53]. Despite this compelling evidence, clinical trials targeting IL23-IL17 axis have failed to show any efficacy in treating RA, however only the p19 subunit of IL23 has been targeted to date and not IL12B, and further studies are needed to explore this strategy [49]. In OA, evidence regarding the role of IL23 is limited, however a recent study found that serum levels of IL17A and IL23 were significantly higher in OA patients than healthy controls and a significant correlation between these levels and the WOMAC score was observed indicating a significant role for these cytokines in the development of OA [54]. This study was intended as a proof-of-principle study regarding a critical involvement of the IL12/23 pathway in the pathogenesis of ptOA after ACL injury. A subsequent study is needed to investigate if a post injury blockade of this axis shows a similar efficacy as in the current pre-/peri-injury treatment study. If so, then the IL12/23 is not only a factor driving ptOA pathogenesis but also a promising target for DMOADs. Prior to a potential translation from bench to bedside, beneficial routes of administration, optimal dosage and ideal therapeutical window need to be evaluated. The current study lays important groundwork as a first step in finding a new potential target for future DMOADs.

Limitations

In the current study IL12/23 pathway modulation was conducted prophylactically prior to joint injury as a proof of principle study investigating pathomechanisms and therefore results cannot be translated towards a therapeutical approach without further research. Studies are needed that determine a post-injury therapeutic window for administration as well as investigate if a local intraarticular therapy is sufficient or a systemic administration is needed. The current study purposefully only included a non-surgical rupture model (ACLR) due to previous findings showing joint-injury specific differences in the T-cell response (**see Chapter 3.2**), however future studies are needed that evaluate the influence of the IL12/23 pathway across ptOA

animal models deriving from differing injuries. Furthermore, the current study used only young male mice in order to keep groups homogenous, however, studies are needed that include other variables known to influence OA development (e.g. sex, age, metabolism). Additionally, we were unable to include pain outcome evaluation and comparisons because of the number of animals used across time points and experiments, but the effect of IL12/23 pathway modulation on pain should fall within the scope of a future study. Finally, mice in the current study had ongoing mechanical joint issues and the effect of IL12/23 pathway modulation in combination with restoration of biomechanics warrants detailed investigation.

Conclusions

The current study is the first to provide insights into the role of the IL12/23-IL17 pathway in the pathogenesis of ptOA. We showed that only ptOA inducing injury (ACLR but not ACLS) led to a spatiotemporal overexpression of IL12B and IL23A mRNA which likely is related to dendritic cells in the synovium. Furthermore, co-culture experiments of naïve CD4⁺ T-helper cells with ptOA but not normal/naive joint tissue resulted in a distinct expression of chemotactic proteins and cytokines associated with Th17 cell proliferation. Corroborating data from mRNA expression data demonstrated a distinct IL12B and IL23A overexpression in cells that were in contact with OA synovial tissue, as well as induction of master transcription factors of proinflammatory Th1 and Th17 subsets which were significantly reduced after IL12/23 pathway blockage. Collectively our data demonstrate for the first time that the IL12/23 pathway via acute systemic and loco-regional CD4⁺ T-helper Th1 and particularly Th17 cell polarization, plays a significant role in orchestrating the immune response following joint injury and the pathogenesis of ptOA. Critically, peri-injury inhibition of IL12/23 *in-vivo* led to a persistently decreased synovial inflammatory response and mitigation of histopathological

ptOA development, thereby making the IL12/23 pathway a promising target for future DMOAD development in ptOA.

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Role of the funding source

None of the funding agencies had any input into the conceptualization or implementation of the study or analysis and interpretation of data.

Conflict of interest

The authors declare no competing interests.

References

1. Kloppenburg M, Berenbaum F: **Osteoarthritis year in review 2019: epidemiology and therapy.** *Osteoarthritis Cartilage* 2020, **28**(3):242-248.
2. Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H: **Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies.** *eClinicalMedicine* 2020, **29**.
3. Thomas AC, Hubbard-Turner T, Wikstrom EA, Palmieri-Smith RM: **Epidemiology of Posttraumatic Osteoarthritis.** *J Athl Train* 2017, **52**(6):491-496.
4. Kornah BA, Safwat HM, Abdel-hameed SK, Abdel-Aal M, Abdelaziz M, Abuelesoud MI, Saleem N: **Managing of post-traumatic knee arthritis by total knee arthroplasty: case series of 15 patients and literature review.** *Journal of Orthopaedic Surgery and Research* 2019, **14**(1):168.
5. Snoeker B, Turkiewicz A, Magnusson K, Frobell R, Yu D, Peat G, Englund M: **Risk of knee osteoarthritis after different types of knee injuries in young adults: a population-based cohort study.** *Br J Sports Med* 2020, **54**(12):725-730.
6. Heijink A, Gomoll AH, Madry H, Drobic M, Filardo G, Espregueira-Mendes J, Van Dijk CN: **Biomechanical considerations in the pathogenesis of osteoarthritis of the knee.** *Knee Surg Sports Traumatol Arthrosc* 2012, **20**(3):423-435.
7. Jung H-J, Fisher MB, Woo SLY: **Role of biomechanics in the understanding of normal, injured, and healing ligaments and tendons.** *BMC Sports Science, Medicine and Rehabilitation* 2009, **1**(1):9.
8. Woodell-May JE, Sommerfeld SD: **Role of Inflammation and the Immune System in the Progression of Osteoarthritis.** *J Orthop Res* 2020, **38**(2):253-257.
9. Haubruck P, Pinto MM, Moradi B, Little CB, Gentek R: **Monocytes, Macrophages, and Their Potential Niches in Synovial Joints - Therapeutic Targets in Post-Traumatic Osteoarthritis?** *Frontiers in immunology* 2021, **12**:763702.
10. Vincent TL, Alliston T, Kapoor M, Loeser RF, Troeberg L, Little CB: **Osteoarthritis Pathophysiology: Therapeutic Target Discovery may Require a Multifaceted Approach.** *Clin Geriatr Med* 2022, **38**(2):193-219.
11. Evers BJ, Van Den Bosch MH, Blom AB, van der Kraan PM, Koëter S, Thurlings RM: **Post-traumatic knee osteoarthritis; the role of inflammation and hemarthrosis on disease progression.** *Frontiers in Medicine* 2022, **9**:973870.
12. Lieberthal J, Sambamurthy N, Scanzello CR: **Inflammation in joint injury and post-traumatic osteoarthritis.** *Osteoarthritis and cartilage* 2015, **23**(11):1825-1834.
13. Li Y-s, Luo W, Zhu S-a, Lei G-h: **T Cells in Osteoarthritis: Alterations and Beyond.** *Frontiers in Immunology* 2017, **8**.
14. Rosshirt N, Trauth R, Platzer H, Tripel E, Nees TA, Lorenz H-M, Tretter T, Moradi B: **Proinflammatory T cell polarization is already present in patients with early knee osteoarthritis.** *Arthritis Research & Therapy* 2021, **23**(1):37.
15. Yamada H, Nakashima Y, Okazaki KEN, Mawatari T, Fukushi J-I, Oyamada A, Fujimura K, Iwamoto Y, Yoshikai Y: **Preferential Accumulation of Activated Th1 Cells Not Only in Rheumatoid Arthritis But Also in Osteoarthritis Joints.** *The Journal of Rheumatology* 2011, **38**(8):1569.
16. Zhang L, Li J-m, Liu X-g, Ma D-x, Hu N-w, Li Y-g, Li W, Hu Y, Yu S, Qu X: **Elevated Th22 cells correlated with Th17 cells in patients with rheumatoid arthritis.** *Journal of clinical immunology* 2011, **31**(4):606-614.

17. Schurich A, Raine C, Morris V, Ciurtin C: **The role of IL-12/23 in T cell–related chronic inflammation: implications of immunodeficiency and therapeutic blockade.** *Rheumatology* 2018, **57**(2):246-254.
18. Kim W, Min S, Cho M, Youn J, Min J, Lee S, Park S, Cho C, Kim H: **The role of IL-12 in inflammatory activity of patients with rheumatoid arthritis (RA).** *Clin Exp Immunol* 2000, **119**(1):175-181.
19. Murphy CA, Langrish CL, Chen Y, Blumenschein W, McClanahan T, Kastelein RA, Sedgwick JD, Cua DJ: **Divergent Pro- and Antiinflammatory Roles for IL-23 and IL-12 in Joint Autoimmune Inflammation.** *Journal of Experimental Medicine* 2003, **198**(12):1951-1957.
20. Smith MM, Clarke EC, Little CB: **Considerations for the design and execution of protocols for animal research and treatment to improve reproducibility and standardization: "DEPART well-prepared and ARRIVE safely".** *Osteoarthritis Cartilage* 2017, **25**(3):354-363.
21. Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M *et al*: **Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0.** *PLoS Biol* 2020, **18**(7):e3000411.
22. NHMRC: **Australian code for the care and use of animals for scientific purposes**, 8th edition edn: National Health and Medical Research Council; Canberra, Australia; 2013.
23. Christiansen BA, Anderson MJ, Lee CA, Williams JC, Yik JH, Haudenschild DR: **Musculoskeletal changes following non-invasive knee injury using a novel mouse model of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2012, **20**(7):773-782.
24. Blaker CL, Little CB, Clarke EC: **Joint loads resulting in ACL rupture: Effects of age, sex, and body mass on injury load and mode of failure in a mouse model.** *Journal of Orthopaedic Research* 2017, **35**(8):1754-1763.
25. Christiansen BA, Guilak F, Lockwood KA, Olson SA, Pitsillides AA, Sandell LJ, Silva MJ, van der Meulen MCH, Haudenschild DR: **Non-invasive mouse models of post-traumatic osteoarthritis.** *Osteoarthritis and cartilage* 2015, **23**(10):1627-1638.
26. Blaker CL, Little CB, Clarke EC: **Joint loads resulting in ACL rupture: Effects of age, sex, and body mass on injury load and mode of failure in a mouse model.** *J Orthop Res* 2017, **35**(8):1754-1763.
27. Haubruck P, Colbath AC, Liu Y, Stoner S, Shu C, Little CB: **Flow Cytometry Analysis of Immune Cell Subsets within the Murine Spleen, Bone Marrow, Lymph Nodes and Synovial Tissue in an Osteoarthritis Model.** *J Vis Exp* 2020(158).
28. Gayle S, Landrette S, Beeharry N, Conrad C, Hernandez M, Beckett P, Ferguson SM, Mandelkern T, Zheng M, Xu T *et al*: **Identification of apilimod as a first-in-class PIKfyve kinase inhibitor for treatment of B-cell non-Hodgkin lymphoma.** *Blood* 2017, **129**(13):1768-1778.
29. Baranov MV, Bianchi F, van den Bogaart G: **The PIKfyve Inhibitor Apilimod: A Double-Edged Sword against COVID-19.** *Cells* 2020, **10**(1).
30. Wada Y, Lu R, Zhou D, Chu J, Przewloka T, Zhang S, Li L, Wu Y, Qin J, Balasubramanyam V *et al*: **Selective abrogation of Th1 response by STA-5326, a potent IL-12/IL-23 inhibitor.** *Blood* 2007, **109**(3):1156-1164.
31. Jackson MT, Moradi B, Smith MM, Jackson CJ, Little CB: **Activation of matrix metalloproteinases 2, 9, and 13 by activated protein C in human osteoarthritic**

- cartilage chondrocytes.** *Arthritis & rheumatology (Hoboken, NJ)* 2014, **66**(6):1525-1536.
32. Jackson MT, Moradi B, Zaki S, Smith MM, McCracken S, Smith SM, Jackson CJ, Little CB: **Depletion of protease-activated receptor 2 but not protease-activated receptor 1 may confer protection against osteoarthritis in mice through extracartilaginous mechanisms.** *Arthritis Rheumatol* 2014, **66**(12):3337-3348.
 33. Shu CC, Jackson MT, Smith MM, Smith SM, Penm S, Lord MS, Whitelock JM, Little CB, Melrose J: **Ablation of Perlecan Domain 1 Heparan Sulfate Reduces Progressive Cartilage Degradation, Synovitis, and Osteophyte Size in a Preclinical Model of Posttraumatic Osteoarthritis.** *Arthritis Rheumatol* 2016, **68**(4):868-879.
 34. Shu CC, Zaki S, Ravi V, Schiavinato A, Smith MM, Little CB: **The relationship between synovial inflammation, structural pathology, and pain in post-traumatic osteoarthritis: differential effect of stem cell and hyaluronan treatment.** *Arthritis Res Ther* 2020, **22**(1):29.
 35. Zaki S, Smith MM, Smith SM, Little CB: **Differential patterns of pathology in and interaction between joint tissues in long-term osteoarthritis with different initiating causes: phenotype matters.** *Osteoarthritis Cartilage* 2020, **28**(7):953-965.
 36. Haubruck P, Heller R, Blaker CL, Clarke EC, Smith SM, Burkhardt D, Liu Y, Stoner S, Zaki S, Shu CC *et al*: **Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models.** *Osteoarthritis and Cartilage* 2023.
 37. Little CB, Hunter DJ: **Post-traumatic osteoarthritis: from mouse models to clinical trials.** *Nat Rev Rheumatol* 2013, **9**(8):485-497.
 38. Capone A, Volpe E: **Transcriptional Regulators of T Helper 17 Cell Differentiation in Health and Autoimmune Diseases.** *Front Immunol* 2020, **11**:348.
 39. Stolarczyk E, Lord GM, Howard JK: **The immune cell transcription factor T-bet: A novel metabolic regulator.** *Adipocyte* 2014, **3**(1):58-62.
 40. Joosten LAB, Helsen MMA, van den Berg WB: **Blockade of endogenous interleukin 12 results in suppression of murine streptococcal cell wall arthritis by enhancement of interleukin 10 and interleukin 1Ra.** *Annals of the Rheumatic Diseases* 2000, **59**(3):196.
 41. Pope RM, Shahrara S: **Possible roles of IL-12-family cytokines in rheumatoid arthritis.** *Nature Reviews Rheumatology* 2013, **9**(4):252-256.
 42. Kim WU, Min SY, Cho ML, Youn J, Min JK, Lee SH, Park SH, Cho CS, Kim HY: **The role of IL-12 in inflammatory activity of patients with rheumatoid arthritis (RA).** *Clinical and Experimental Immunology* 2000, **119**(1):175-181.
 43. Smolen JS, Agarwal SK, Ilivanova E, Xu XL, Miao Y, Zhuang Y, Nnane I, Radziszewski W, Greenspan A, Beutler A *et al*: **A randomised phase II study evaluating the efficacy and safety of subcutaneously administered ustekinumab and guselkumab in patients with active rheumatoid arthritis despite treatment with methotrexate.** *Ann Rheum Dis* 2017, **76**(5):831-839.
 44. Nees TA, Rosshirt N, Zhang JA, Reiner T, Sorbi R, Tripel E, Walker T, Schiltenswolf M, Hagmann S, Moradi B: **Synovial Cytokines Significantly Correlate with Osteoarthritis-Related Knee Pain and Disability: Inflammatory Mediators of Potential Clinical Relevance.** *Journal of Clinical Medicine* 2019, **8**(9).
 45. Fiechter RH, de Jong HM, van Mens LJJ, Fluri IA, Tas SW, Baeten DLP, Yeremenko NG, van de Sande MGH: **IL-12p40/IL-23p40 Blockade With Ustekinumab Decreases the**

- Synovial Inflammatory Infiltrate Through Modulation of Multiple Signaling Pathways Including MAPK-ERK and Wnt.** *Frontiers in Immunology* 2021, **12**.
46. Mayordomo AC, Silva JE, Gorlino CV, Arias JL, Berón W, Di Genaro MS: **IL-12/23p40 overproduction by dendritic cells leads to an increased Th1 and Th17 polarization in a model of Yersinia enterocolitica-induced reactive arthritis in TNFRp55-/- mice.** *PLOS ONE* 2018, **13**(3):e0193573.
 47. Lutzky V, Hannawi S, Thomas R: **Cells of the synovium in rheumatoid arthritis. Dendritic cells.** *Arthritis Research & Therapy* 2007, **9**(4):219.
 48. Xu J, Li J, Hu Y, Dai K, Gan Y, Zhao J, Huang M, Zhang X: **IL-23, but not IL-12, plays a critical role in inflammation-mediated bone disorders.** *Theranostics* 2020, **10**(9):3925-3938.
 49. Najm A, McInnes IB: **IL-23 orchestrating immune cell activation in arthritis.** *Rheumatology (Oxford)* 2021, **60**(Suppl 4):iv4-iv15.
 50. Lee KMC, Sherlock JP, Hamilton JA: **The role of interleukin (IL)-23 in regulating pain in arthritis.** *Arthritis Research & Therapy* 2022, **24**(1):89.
 51. Schinocca C, Rizzo C, Fasano S, Grasso G, La Barbera L, Ciccia F, Guggino G: **Role of the IL-23/IL-17 Pathway in Rheumatic Diseases: An Overview.** *Frontiers in Immunology* 2021, **12**.
 52. Lemos HP, Grespan R, Vieira SM, Cunha TM, Verri WA, Fernandes KSS, Souto FO, McInnes IB, Ferreira SH, Liew FY *et al*: **Prostaglandin mediates IL-23/IL-17-induced neutrophil migration in inflammation by inhibiting IL-12 and IFN γ production.** *Proceedings of the National Academy of Sciences* 2009, **106**(14):5954-5959.
 53. Zhu Z, Otahal P, Wang B, Jin X, Laslett LL, Wluka AE, Antony B, Han W, Wang X, Winzenberg T *et al*: **Cross-sectional and longitudinal associations between serum inflammatory cytokines and knee bone marrow lesions in patients with knee osteoarthritis.** *Osteoarthritis and Cartilage* 2017, **25**(4):499-505.
 54. Askari A, Naghizadeh MM, Homayounfar R, Shahi A, Afsarian MH, Paknahad A, Kennedy D, Ataollahi MR: **Increased Serum Levels of IL-17A and IL-23 Are Associated with Decreased Vitamin D3 and Increased Pain in Osteoarthritis.** *PLoS One* 2016, **11**(11):e0164757.

Figure 1: Visualization of the study design and animal allocation into each study. Experiments were divided into in vitro experiments, genomic in-vivo experiments, proteinic in-vivo experiments. Groups, treatments, time points and outcomes are detailed for each set of experiment.

Figure 2: Results of the genomic in-vivo experiments over time are shown. **A** provides a schematic overview over the tissues utilized and outcome measurements. **B** shows the qPCR results for IL12A, IL12B, and IL23A for the synovial tissue block (left) and tibial plateau (right). Samples from three groups (ACLR, ACLS, NOC) were analyzed. The * symbol indicates results from the statistical comparison of ACLR vs NOC mice, + ACLR vs ACLS mice, and ^ ACLS vs NOC mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as mean $\pm$ SEM and $n = 4$ animals/group/time point/tissue.

Figure 3: Results of the in vitro experiments are shown. **A** provides a schematic overview and what data is presented in figure 3 and figure 4. **B** shows the Luminex results from analysing the cell culture supernatant. In each figure the left side is without Apilimod/STA and the right side shows the same tissues and analytes however with the addition of Apilimod/STA in order to inhibit the IL12/23 pathway. The * symbol indicates results from the statistical comparison of ACLR vs NOC mice, + ACLR vs ACLS mice, and ^ ACLS vs NOC mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as mean $\pm$ SEM and $n = 4$ biological replicates/group/tissue.

Figure 4: qPCR results are shown from the in vitro experiments. In each figure the left side is without Apilimod/STA and the right side shows the same tissues and genes, however with the addition of Apilimod/STA in order to inhibit the IL12/23 pathway. The * symbol indicates results from the statistical comparison of ACLR vs NOC mice, + ACLR vs ACLS mice, and ^ ACLS vs NOC mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as mean $\pm$ SEM and $n = 4$ biological replicates/group/tissue.

Figure 5: Results of the flow cytometry experiments of the systemic adaptive immune response are shown. **A** gives a schematic overview over the tissues included in this figure, while the influence of antibody mediated immune modulation on CD4⁺ helper cells, and the inflammatory status assessed by the Th1/Th2 ratio and Th17/Treg ratio are shown in **B**. Immune cells were harvested from the blood (left) and contralateral bone marrow (right). The * symbol indicates results from the statistical comparison of ACLR vs NOC mice, + ACLR vs ACLS mice, and ^ ACLS vs NOC mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as mean $\pm$ SEM and $n = 6$ biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 6: Results of the flow cytometry experiments of the locoregional adaptive immune response. **A** gives a schematic overview over the tissues included in this figure, while the influence of antibody mediated immune modulation on CD4⁺ helper cells, and the inflammatory status assessed by the Th1/Th2 ratio and Th17/Treg are shown in **B**. Immune

cells were harvested from the synovial tissue block (left) and draining lymph node (right). The * symbol indicates results from the statistical comparison of ACLR vs NOC mice, + ACLR vs ACLS mice, and ^ ACLS vs NOC mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as mean $\pm$ SEM and $n = 6$ biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 7: Results of histopathological scoring of the joint. **A** shows the combined tibiofemoral cartilage damage over time, as well as chondrocyte hypertrophy/apoptosis over time, and osteochondral damage over time. **B** focuses on the synovial inflammation over time, by showing combined total synovitis and the synovial hyperplasia scores. Lastly **C** visualizes the fibrotic changes to the joint. The * symbol indicates results from the statistical comparison of ACLR vs NOC mice, + ACLR vs ACLS mice, and ^ ACLS vs NOC mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as median $\pm$ 95% CI and $n = 9$ animals/group/time point.

Figure 1:

Animal Allocation and Study Design

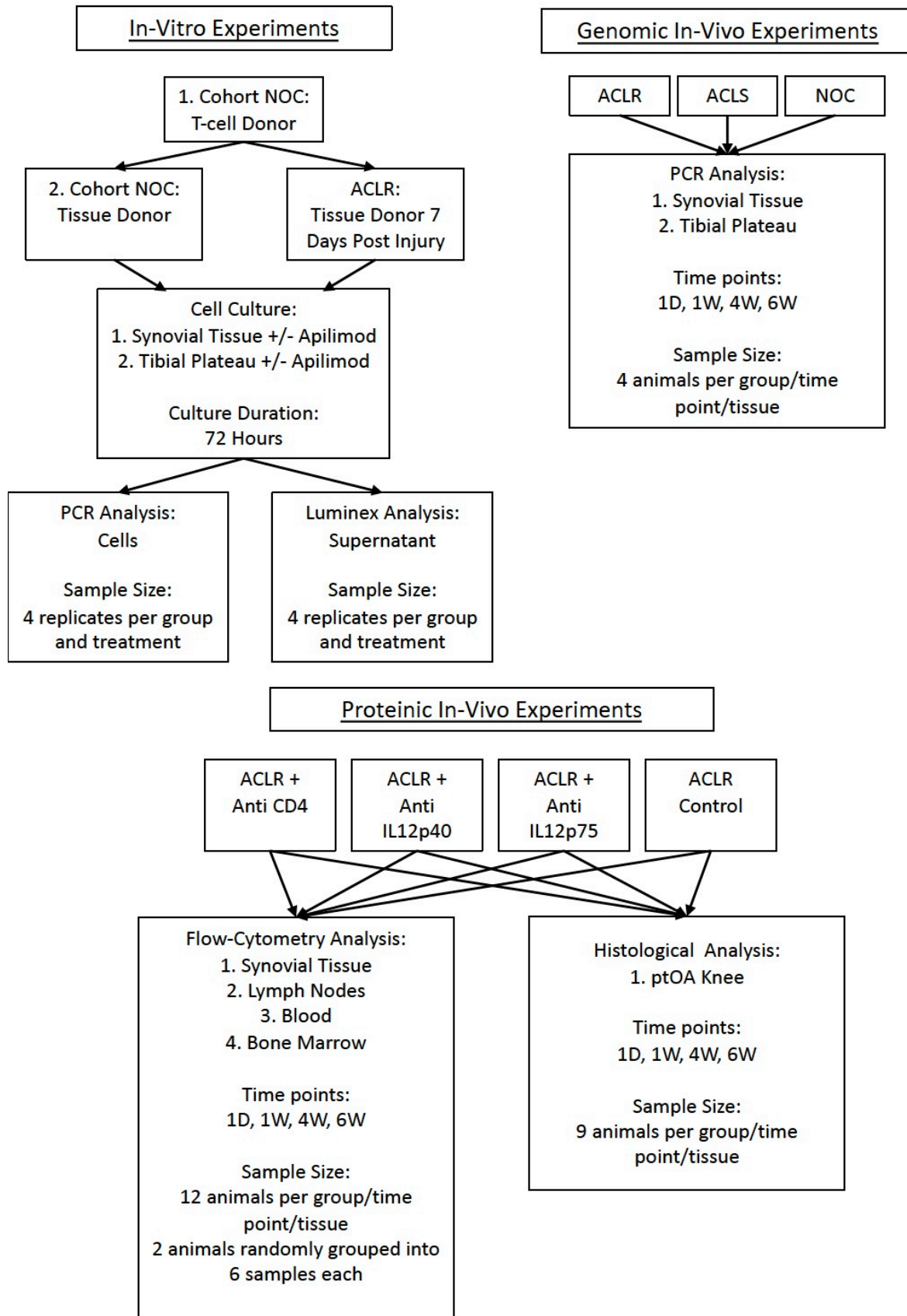
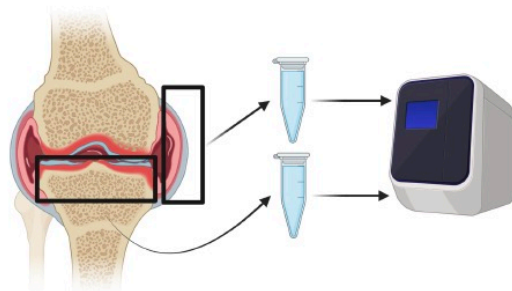


Figure 2:

A)



B)

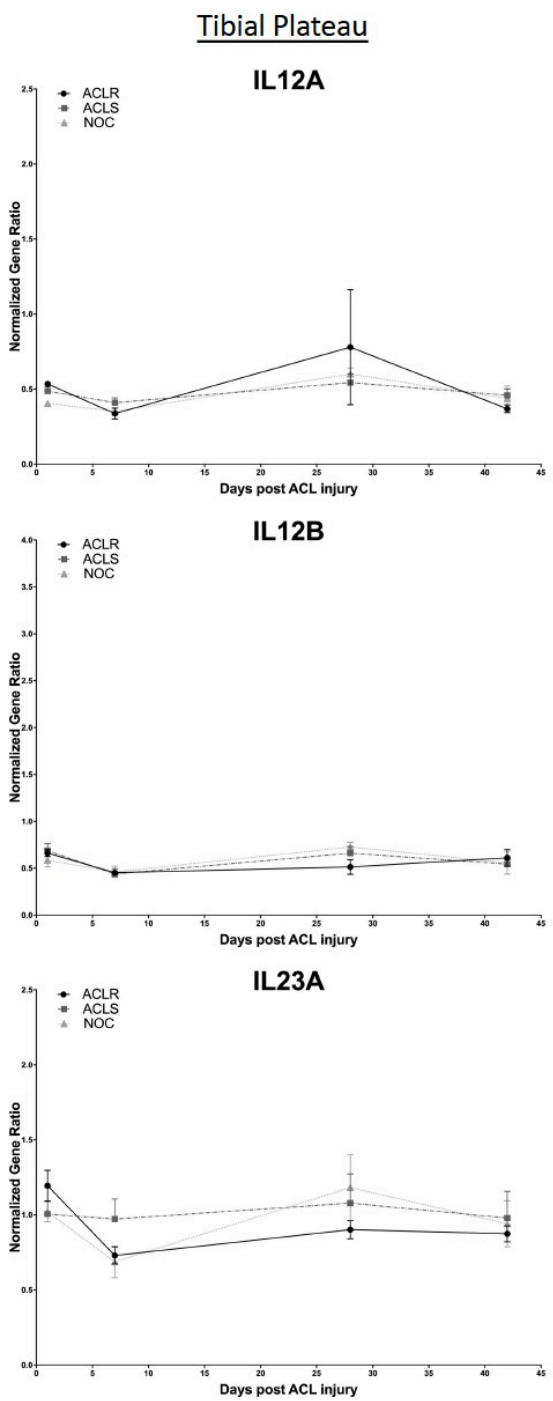
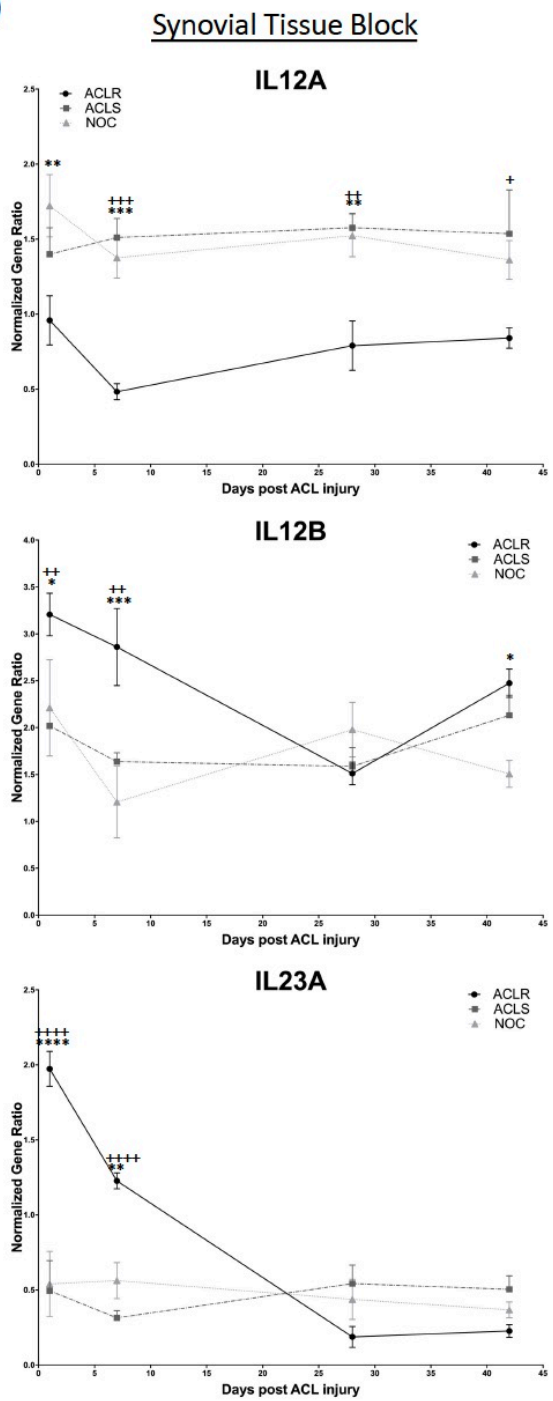


Figure 3:

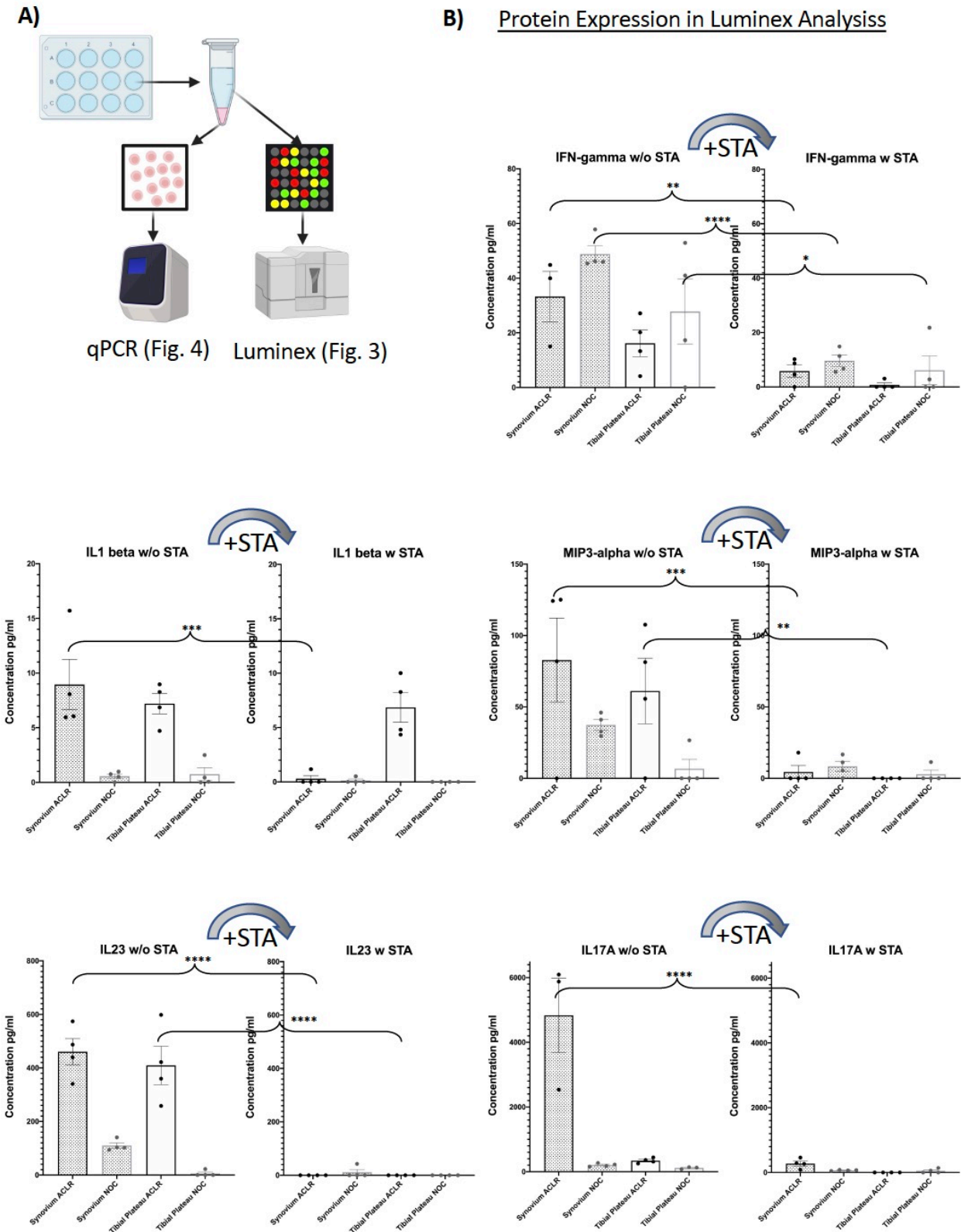
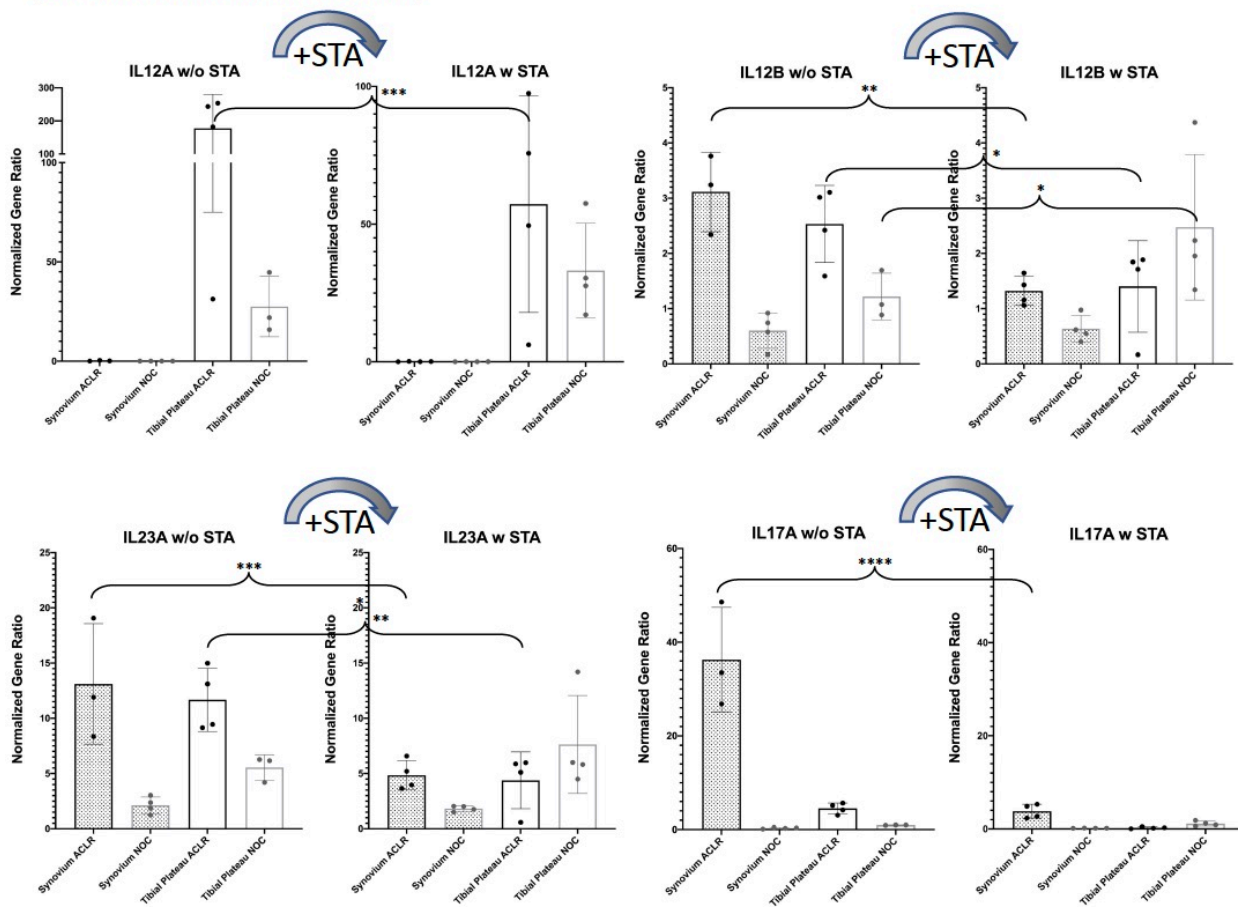


Figure 4:

mRNA Expression in qPCR Analysis

Pro-Inflammatory Interleukins



Transcription Factors

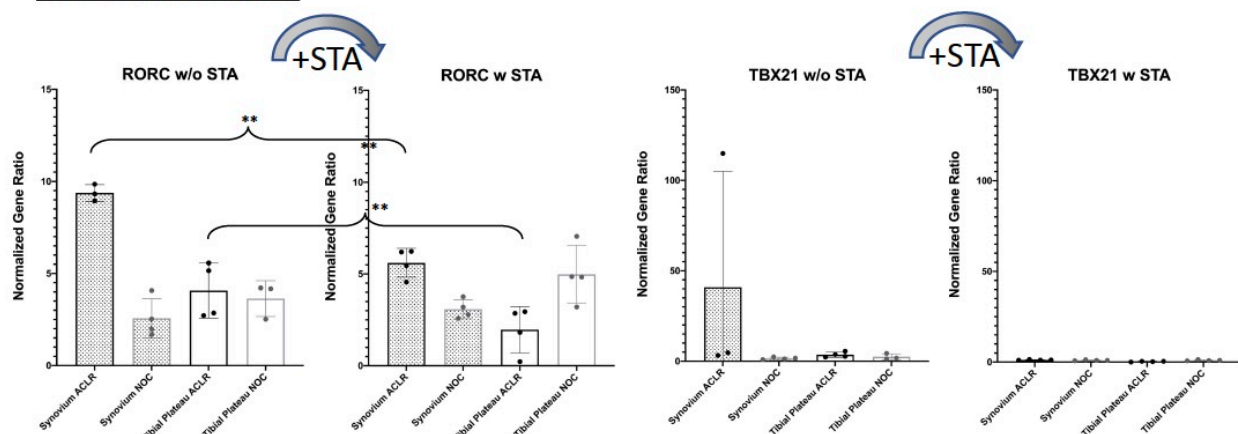


Figure 5:

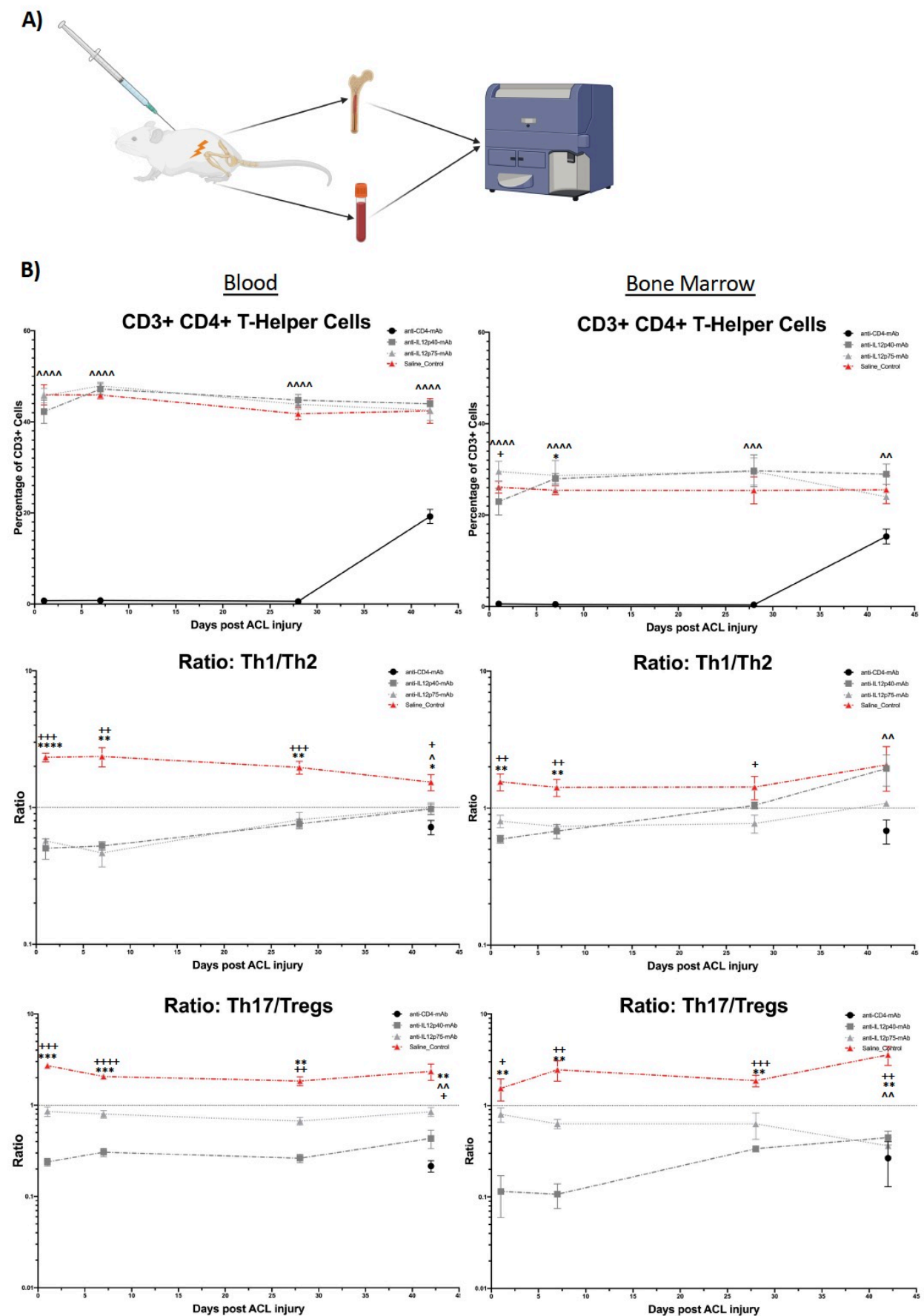


Figure 6:

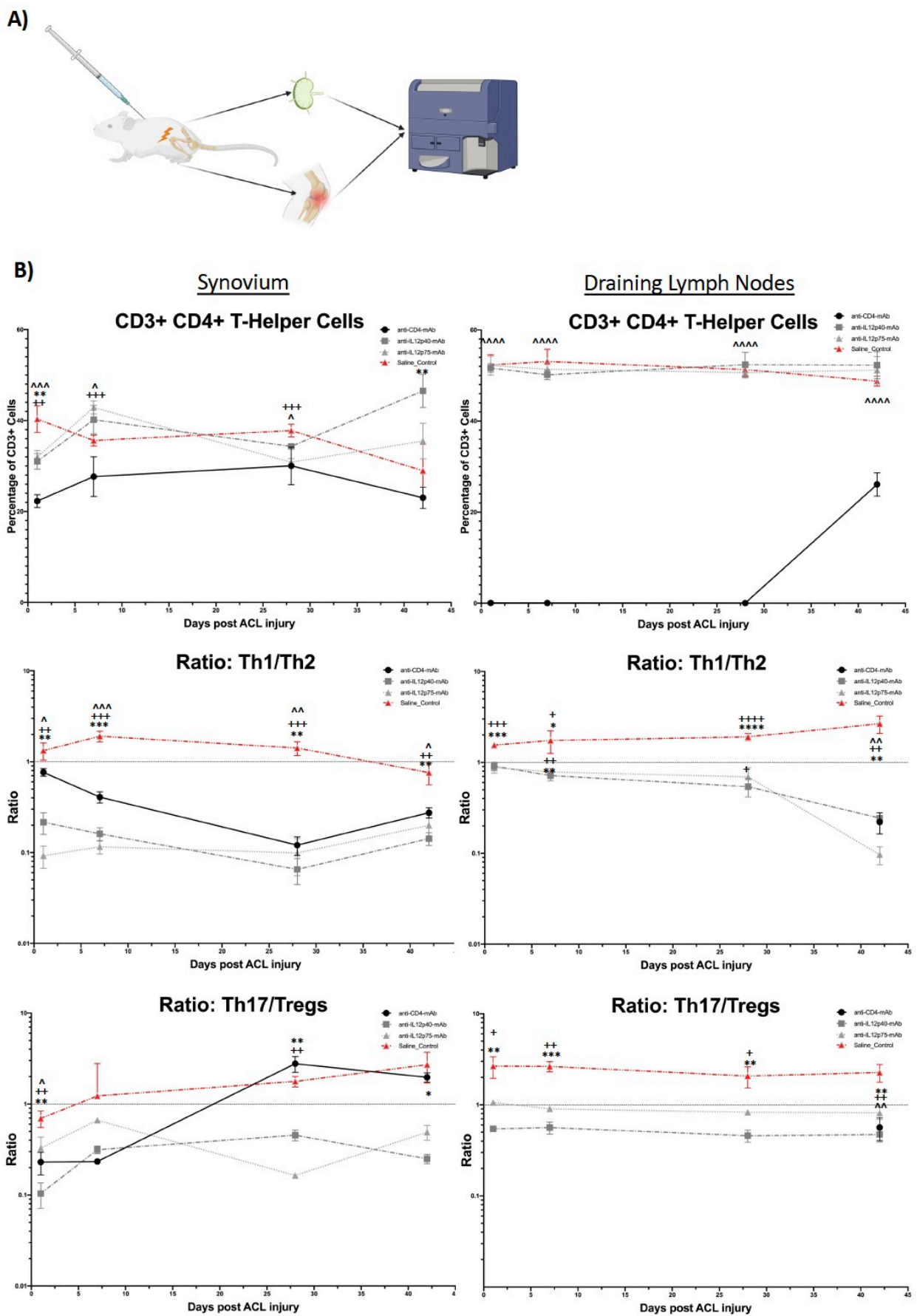
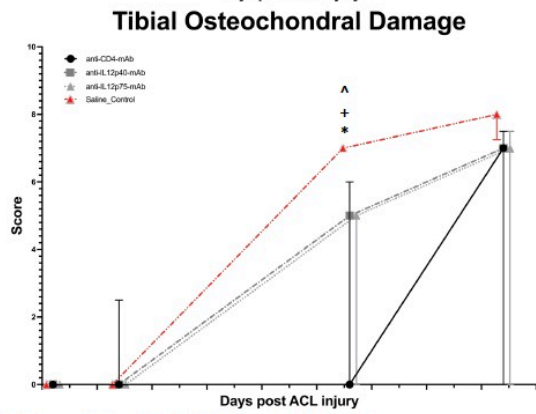
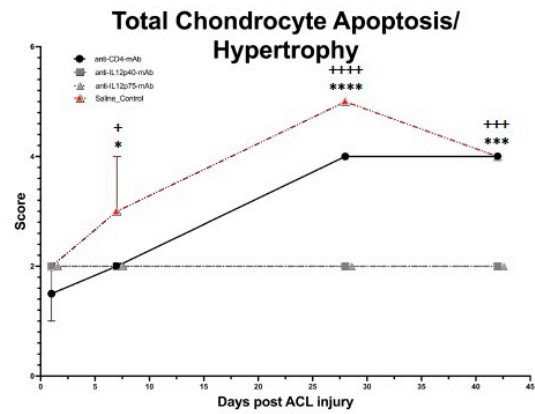
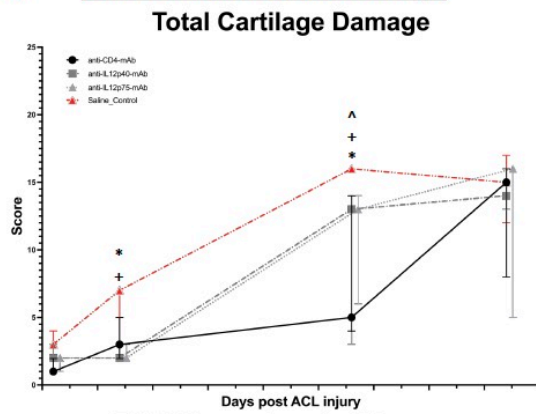
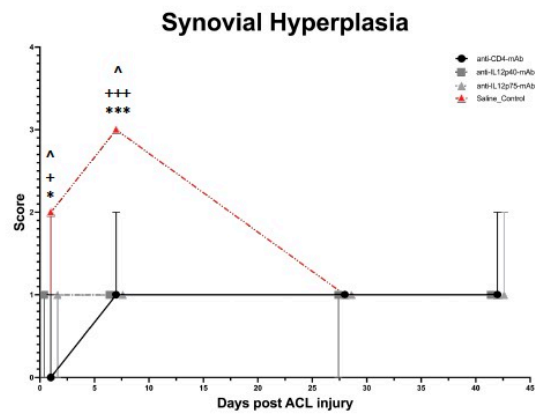
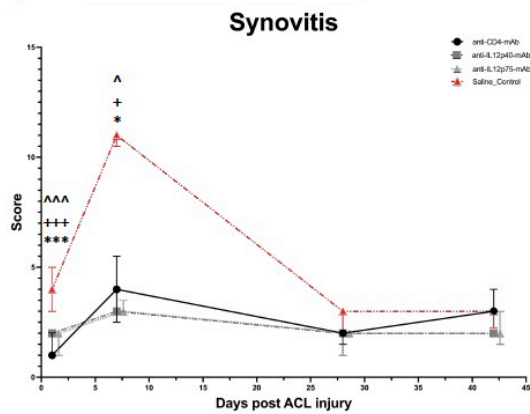


Figure 7:

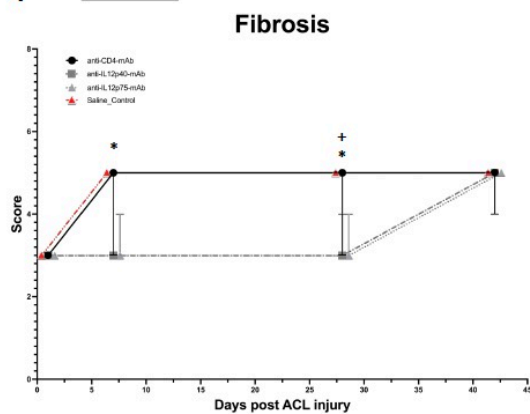
A) Osteochondral Joint Damage



B) Synovial Inflammation



C) Fibrosis



Supplementary Table 1:

Details for each antibody used in the T-cell panel are given. In addition, information is provided for the relevant buffers used.

Supplementary Table 2:

Details for the primer sequences used in the study are provided. bp = base pairs.

Supplementary Table 1: Used Antibodies for Flow Cytometry

Name of Material/ Equipment	Manufacturer	Catalog Number	Comments/Description
Fixable Viability Stain 510 100ug	BD	564406	T-Cell Panel
Ms CD3e APC-Cy7 145-2C11 100ug	BD	557596	T-Cell Panel
Ms CD8a BV786 53-6.7 50ug	BD	563332	T-Cell Panel
Ms CD4 PE-Cy7 RM4-5 100ug	BD	552775	T-Cell Panel
Ms NK1.1 BV650 PK136 50ug	BD	564143	T-Cell Panel
Ms CD25 BV605 PC61 50ug	BD	563061	T-Cell Panel
Ms CD183 BB700 CXCR3-173 50ug	BD	742274	T-Cell Panel
Ms Foxp3 PE MF23 100ug	BD	560408	T-Cell Panel
Ms ROR Gamma T BV421 Q31-378 50ug	BD	562894	T-Cell Panel
APC anti-mouse CD194 (CCR4)	BioLegend	131212	T-Cell Panel
Brilliant Stain Buffer Plus 1000Tst	BD	566385	Buffers
Transcription Factor Buffer Set 100Tst	BD	562574	Buffers

Supplementary Table 2: Primer Sequences used for rtPCR

Gene		Sequence (5' -3')	Annealing Temperature (C)	Size (bp)
IL12A	Forward	AACGCAGCACTTCAGAATCA	55.2	223
	Reverse	GATAGCCCATCACCTGTTG		
IL12B	Forward	TCTGGAAAGACCCTGACCAT	52.7	196
	Reverse	GTCCGGAGTAATTTGGTGCT		
IL23A	Forward	ACAAGGACTCAAGGACAACAGC	56.1	221
	Reverse	TAGAACTCAGGCTGGGCATC		
IL17A	Forward	ATCCCTCAAAGCTCAGCGTGTC	56.8	170
	Reverse	GGGTCTTCATTGCGGTGGAGA		
RORC	Forward	CAG CAG CAA CAG GAA CAA GTG G	56	92
	Reverse	TGC CCA TCT GAG AGC CCT AAA G		
TBX21	Forward	AGCAAGGACGGCGAATGTT	57.8	188
	Reverse	TGGGTGGACATATAAGCGGTTT		

4.3 Main Findings of the Study

The current study provides insights into the role of the IL12/23 pathway in the pathogenesis of ptOA. Only ptOA inducing injury led to a spatiotemporal overexpression of IL12B and IL23A mRNA which likely is related to dendritic cells in the synovium. Furthermore, co-culturing experiments of naïve CD4⁺ T-helper cells with osteoarthritic joint tissue resulted in a distinct expression of chemotactic proteins as well as cytokines associated with Th17 cell proliferation, while on a gene level corroborating data was detected indicating a distinct IL12B and IL23A mRNA overexpression in cells that were in contact with osteoarthritic synovial tissue as well as induction of master transcription factors of proinflammatory Th1 and Th17 subsets which was significantly reduced after IL12/23 pathway blockade. Thereby our data indicates that the IL12/23 pathway plays a role in orchestrating the immune response following joint injury and during development of ptOA while contributing towards a production of IL17 producing Th17 cells and thereby perpetuating the inflammatory response initiated by joint disease. Finally, inhibition of IL12/23 *in-vivo* led to a persistently decreased inflammatory response on a cellular level and the mitigation of histopathological changes associated with ptOA development. Importantly, while the current study provided compelling evidence regarding the mechanistic role of the IL12/23 pathway in the development of ptOA the results cannot be translated into a therapeutic concept without further research. Further studies are needed that initiate IL12/23 pathway modulation in a post-injury therapeutic window, include a variety of animals models as well as groups of mice having confounding factors, and combine immunomodulation with restoration of biomechanics in order to confirm the findings made in the current study being that the IL12/23 pathway might be a promising target for future DMOADs in ptOA.

Chapter 5

5 Investigating the Role of Pro-Inflammatory Subsets of the Innate Immune Response and their Molecular Pathway of Activation in the Pathogenesis of ptOA

5.1 Aim of the Study

In **Chapter 3** we observed a distinct M1 dominated pro-inflammatory innate immune response following induction of ptOA. Taken together with findings from the literature review presented in **Chapter 1** two cellular key players of the innate immunity potentially having opposing roles were identified. On the one hand Ly6C^{high} inflammatory monocytes as the main source for ‘blood originated’ M1 macrophages have been identified as a major contributor driving chronic inflammation associated with OA. On the other hand, synovial tissue resident macrophages (STRM) are believed to protect joint functionality by clearing cartilage/bone debris and acting as sentinels for molecular and cellular changes in the joint cavity. However, the exact role of these two cellular key players in the pathogenesis of ptOA and if a targeted depletion of either one has antagonistic effects on the onset and development remains unknown. Thus, this study utilized a specific monoclonal anti-CCR2 antibody which is highly selective for targeting and depleting Ly6C^{high} monocytes (Mack, M. et al. 2001) as well as intraarticular injections of Clodronate effectively depleting STRMs (Moretti, A. et al. 2021) in the synovial tissue in context with a non-surgical murine ptOA model. This study is intended as a second proof-of-principle study (the first one being **Chapter 4**) investigating the contribution of these immune cells subsets towards ptOA development. This is a first step to identify crucial cellular and molecular factors that are driving ptOA development and not intended as a definitive study to investigate a potential therapeutic application. In addition, molecular pathways of activation were analyzed to investigate what drives innate immunity in ptOA.

5.2 Drafted Manuscript for Submission: Targeting different myeloid/innate-immune cell subsets has differential effects on joint inflammation and structural damage associated with post-traumatic osteoarthritis.

Targeting different myeloid/innate-immune cell subsets has differential effects on joint inflammation and structural damage associated with post-traumatic osteoarthritis.

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Introduction:

The global prevalence of osteoarthritis (OA) of the knee has been estimated as 16.0% in individuals over the age of 15 and 22.9% in individuals over the age of 40 corresponding to around 654.1 million affected individuals worldwide [1]. The number of people affected is estimated to substantially increase in the coming years with increased population ageing and obesity [2]. Initially OA was believed to be a purely mechano-degenerative disease whereas in recent years a multifactorial cellular and molecular biological pathogenesis has been widely accepted [2]. OA was identified as the second highest cause for years lived with disability in 2015 [3], but in comparison to the other leading causes our understanding of the pathomechanisms remains limited. The lack of insight is reflected in an ongoing and unaltered need for development of effective and preventative treatments.

Over the last years a plethora of studies have implicated chronic joint inflammation as a crucial part of OA pathogenesis, and activation of the innate immune response was shown to be involved in initiation and perpetuation of this inflammatory reaction [4]. A variety of key cellular players, that are part of the innate immune response, have been identified in playing diverging roles in the onset and development of ptOA [5]. Of interest is an influx into the joint of Ly6C^{high} inflammatory monocytes that occurs following the induction of an inflammatory instability OA model in mice by intra-articular collagenase injection [6]. This Ly6C^{high} monocyte influx is governed by the expression of ‘C-C motif ligand 2’ (CCL2), also known as monocyte chemoattractant protein 1 (MCP-1), which can be derived from a variety of sources, most importantly in the joint from injured chondrocytes and synoviocytes (reviewed in [5]). Following CCL2-induced migration of the ‘C-C motif chemokine receptor 2’ (CCR2)-expressing Ly6C^{high} monocytes from the circulation, they polarize into “blood-originated” (BO) inflammatory M1 macrophages [7] driving chronic inflammation associated with OA [8]. On the other hand, synovial tissue resident macrophages (STRM) which likely have a distinct

embryological derivation are believed to protect joint functionality by clearing cartilage/bone debris and acting as sentinels for molecular and cellular changes in the joint cavity [5]. Their protective function was further supported by a study showing that depletion of STRMs led to an exacerbation of experimental rheumatoid arthritis (RA) in mice [5, 9].

There is clear evidence for involvement of the CCR2/CCL2 signaling pathway in post-traumatic (pt)OA, from both knock-out mice and therapeutic intervention studies [10-13]. However, the exact role of CCR2/CCL2-dependent BO versus STRM-derived macrophages in ptOA pathogenesis and if a targeted depletion of either one has antagonistic effects on disease onset and development following joint injury remains unknown. To improve the understanding of the contribution of these two key innate-immune cell-types to ptOA development, the current study compared effects in a non-surgical anterior cruciate ligament rupture (ACLR) ptOA model in mice of: systemic intraperitoneal (IP) administration of a specific monoclonal anti-CCR2 antibody highly selective for targeting and depleting Ly6C^{high} monocytes [14]; intraarticular (IA) injection of clodronate-liposomes effectively depleting STRMs [15], or intravenous (IV) clodronate-liposomes to deplete mature circulating macrophages. In addition, differential molecular pathway activation in the synovium were analysed to investigate key drivers of innate immunity in ptOA and define potential novel pathways for therapeutic intervention.

Materials and Methods

Animals and Study Design

All experiments were approved by the Northern Sydney Local Health District Animal Ethics Committee (protocol RESP20/082), with DEPART and ARRIVE guidelines followed for experimental design and reporting [16, 17]. For all experiments a total of 336 10-12-week-old, male C57BL/6 mice were utilized, with mice housed in individually-ventilated cages (up to 5/cage) with commercial bedding, environmental enrichment (perspex housing, wooden sticks, tissues), *ad-libitum* access to standard pelleted mouse feed and acidified water, in a dedicated SPF vivarium maintained at 20-22 °C with 12-hour light/dark cycles, and cared for in accordance with the Australian code [18]. An overview of the study design and the allocation of animals is provided in Figure 1.

In the current study a non-surgical anterior cruciate ligament rupture (*ACLR*) model [19, 20] was used to induce ptOA without the confounding inflammation of an initial operative trauma. Briefly, a single compressive load was applied to the tibia of the flexed knee in anaesthetised mice, resulting in posterior subluxation of the femur and ACL rupture without additional macroscopic damage to other structures within the joint [20], with subsequent rapid onset and progression of joint-wide ptOA pathology [21] (see **Chapter 2.2.2.2**). Naïve mice were included, when necessary, serving as non-operated controls (*NOC*) and baseline for all other groups. Animals housed in the same cage were randomly assigned to each set of experiments but all animals in the cage were sacrificed at the same time (see below) to avoid isolation stress.

In-vivo experiments

The day of ACLR was designated as experimental day 0, and mice sacrificed, and tissues harvested 1-day, 1-week, 4-weeks or 6-weeks post-ACLR (n = 12/time/treatment-group). Treatment groups included animals receiving either intravenous (IV) injection of 200µl [22,

23] of clodronate liposomes (Encapsula Nanosciences LLC, catalogue #CLD-8901), intraarticular (IA) injection of 10µl [24] clodronate liposomes (Encapsula Nanosciences LLC, catalogue #CLD-8901), or intraperitoneal (IP) injection of rat anti-mouse CCR2 antibody MC-21 (20µg in 100µl PBS) [14, 25, 26]. Respective controls included injection of control/empty liposomes (Encapsula Nanosciences LLC, catalogue #CLD-8910) using the same volume and route of administration, as well as mice injected with 100µl of PBS intraperitoneally. The treatment schedule of study and control animals varied based on the targeted cell type and previously reported time to optimal target-cell depletion [22, 23] [24] [14, 25, 26]: IV injections were performed on day -1 and day -3; IA injections were performed on day -7 and -3; and IP injections were performed starting on day -1 and thereafter repeated daily for 5 days. As a general rule animals harvested at day-1 only received the treatment prior to injury.

Flow cytometry

Blood (terminal cardiac puncture under general anaesthesia), contralateral femoral bone marrow, draining lymph-nodes (pooled inguinal and iliac lymph-nodes) and synovial tissue were harvested from all animals as previously described [27]. Tissues were pooled from two animals to increase cell numbers per sample resulting in n=6 biological replicates for each tissue-source at each time point in each animal group. The detailed protocol regarding the harvest of tissues and preparation of single cell solutions and the utilized multicolour antibody panel has been published previously [27] (see **Chapter 2.2.2.1** and **Chapter 3.2**) (see Table S1 for detailed information regarding the utilized antibody panel).

RNA sample preparation and Next Generation Sequencing

In a separate set of experiments mice undergoing ACLR were treated with either nothing, local macrophage depletion in the synovium (10µl clodronate liposomes IA [24]) or systemic CCR2⁺

monocyte depletion (20 μ g rat anti-mouse CCR2 antibody MC-21 IP [14, 25, 26]) as described above (n = 6 per time/treatment group). Non-operated controls (NOCs) of the same age not receiving any treatment were included into this set of experiment as well. Animals harvested at day 1 only received the treatment prior to injury, while animals harvested 4 weeks after injury received the complete treatment regime as outlined above. The synovial tissue block was harvested 1-day or 4-weeks post-ACLR, and 6 mice per time/treatment-group randomly pooled into two samples (n=3mice/pool) for each individual time/group. Total RNA was then isolated (freeze pulverized: RNeasy Micro Kit, Qiagen cat# 74004) following manufacturer's protocol, and quantified using Nanodrop. High sample quality was confirmed using the 260/280 absorption ratio (all ~2.0), and samples containing 1250ng of RNA underwent standard whole transcriptome sequencing with 50M PE reads performed by an accredited certified service provider (AGRF Ltd, Melbourne, Australia).

Bioinformatic analysis of RNA sequencing data

The quality of raw sequencing reads was checked, and they were aligned to the reference genome using Genome Workbench 12.0.4 (CLC Bio. Japan). To normalize the read counts for each gene, we used the reads per kilobase per million mapped reads (RPKM = total exon reads/mapped reads in million X exon length in kb). The RPKM values were used to estimate gene expression levels using advanced plugins for RNA-Seq in CLC Workbench 12.0.4. We performed a differential expression test using the native function in CLC 12.0.4, which utilizes multi-factorial statistics based on a negative binomial Generalized Linear Model (GLM). Genes with log2 fold changes >2 or < -2, and FDR-corrected p values <0.05 were considered differentially expressed genes (DEGs). Finally, we visualized the data using the 'ggplot2' package in the statistical language R.

Gene Set Enrichment Analysis (GSEA) was conducted using the fgsea package in R statistical language. The selected genes were annotated with ENTREZ ID through the Genome-wide annotation for Mouse package (org.Mm.eg.db). The enrichment score was computed by matching the ranked genes with the target gene set, which was the Molecular Signatures Database (MsigDB) translated for mouse data by Walter+Eliza Hall Institutes Bioinformatics service. The Gene Ontology gene set (Ms.c5) was used in the analysis and pathways with an adjusted p-value of less than 0.05 was chosen. The dataset used in this study is available for download at <https://bioinf.wehi.edu.au/software/MSigDB/>.

Histopathological scoring

After removal of the anterior synovium (for flow cytometry) knee joints were fixed, decalcified, paraffin-embedded, and serial sagittal sections across the medial femoro-tibial joint cut and stained with toluidine blue-fast green as previously described [28-30]. Utilizing a modified single slide scoring system ([21] and see **Chapter 2.2.2.2**) femoral and tibial cartilage and structural damage, tibial and femoral chondrocyte hypertrophy and apoptosis, osseous neoformation observed as osteophytes and enthesophytes, posterior fibrotic changes to joint capsule, and posterior femoral synovitis, were determined by a single trained observer (PH) blinded to time and treatment. Finally, median values for each histopathological feature for each group and time point were generated and compared.

Statistics

The individual histology-outcomes were considered as categorical and independent variables, as prior analysis demonstrates they change differentially and with distinct temporal patterns in different OA models [31], and are independently modified in response to treatment/genetic-modification [28-30, 32, 33]. Non-parametric analyses (Kruskal-Wallis and Mann-Whitney-

U) were used to assess differences between groups for all histological outcomes at each post-injury timepoint. Histological data is presented as median $\pm$ error. Flow cytometry data is presented as mean $\pm$ standard error of the mean (SEM). Two-way analysis of variance (ANOVA) with post-hoc Tukey's correction for multiple comparisons was used to compare all individual variables. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). All statistical calculations were performed with GraphPad Prism (Version 9.0.0).

Results

Differential systemic effect of targeting CCR2⁺ monocytes and macrophages versus STRM

To determine the systemic effect of the different innate-immune-cell targeting therapeutic strategies, flow cytometric analysis of myeloid cells from blood and contralateral bone marrow was conducted (Fig.2). IP anti-CCR2 decreased total monocyte numbers in blood day-1 to week-4, before an increase at week-6 (Fig 2A). While the percentage of Ly6C⁺ monocytes significantly reduced on day-1 before rebounding to be higher than control at week 1-4, the M1/M2 polarization of circulating monocytes was markedly reduced by anti-CCR2 for the first 4 weeks. Systemic (IV) clodronate-liposomes decreased circulating monocytes for the first week before returning to control levels and then being greater than control at week-6, with more of these being Ly6C⁺ at week-1 and -6 but with a reduced M1/M2 polarization for the first week and then higher M1/M2 than controls at week-6. IA clodronate-liposomes led to a decrease in circulating monocytes at day-1 and week-6, with a reduction in the Ly6C⁺ day-1 and week-1 before increasing at week-6, but unlike systemic treatments the M1/M2 polarization of circulating monocytes was significantly increased at all times.

While some of the effects of the different treatments on myeloid cells in the contra-lateral bone-marrow were similar to those in blood, others were not (Fig 2B). Anti-CCR2 significantly

reduced bone-marrow monocytes at day-1, week-4 and week-6, with a reduction in Ly6C⁺ for the first week before an increase at week-6. Bone marrow macrophages on the other hand were increased by anti-CCR2 at day-1 and week-4 to week-6 but importantly with a marked decrease in M1/M2 for the first 4 weeks. Bone-marrow monocytes were decreased by systemic (IV) clodronate-liposomes at day-1 but then significantly increased at week-1 and -4, with a decrease (albeit not statistically significant) in Ly6C⁺ cells for the first week. Bone-marrow macrophages were initially increased by IV clodronate-liposomes (day-1) before decreasing week-1 to week-4 and importantly with a decreased M1/M2 polarization at all times. IA clodronate-liposomes on the other hand showed little or no effect on bone-marrow monocyte numbers and L6C⁺/L6C⁻ ratio, nor macrophage numbers and M1/M2 polarization.

Differential effect of targeting systemic CCR2⁺ monocytes and macrophage versus STRM on synovial macrophage infiltration and M1/M2 polarization

Systemic depletion of CCR2⁺ monocytes (anti-CCR2) led to reduced synovial BO macrophages for the first week, and while numbers increased at week-4 they were again reduced at week-6 (Fig. 3). Systemic macrophage depletion (IV clodronate-liposomes) also reduced synovial BO macrophages but over the first 4 weeks. In contrast, IA clodronate-liposomes increased synovial BO macrophages for the first week in association with decreasing STRM day-1 to week-4. Systemic CCR2⁺ monocyte depletion did not significantly alter STRM numbers (slightly increased week-1 to -6) while targeting systemic macrophages significantly increased STRM over the first week. Depletion of CCR2⁺ monocytes reduced the Ly6C expression in both BO and STRM at day-1, followed by an increase in Ly6C at week-1 and then lower levels again at week-4 to -6. Systemic macrophage depletion also acutely decreased Ly6C in BO macrophages with the effect persisting for 4 weeks while the acute reduction in STRM Ly6C compared with controls was reversed by 1 week. In contrast STRM depletion

markedly increased BO macrophage Ly6C for the first week. Finally, the synovial BO and STRM M1/M2 ratio was used to evaluate the joint inflammatory state. Systemic depletion of CCR2⁺ monocytes led to an acute and persistent reduction in both BO and STRM M1/M2 polarization (to week-6 or week-4, respectively). Systemic depletion of macrophages had a similar effect although reduction in STRM M1/M2 was more persistent. In marked contrast to the systemic treatments, depletion of STRM resulted in an exacerbated pro-inflammatory M1/M2 polarization in both BO macrophages and residual STRMs.

Targeted depletion of specific myeloid cell subsets mitigates ptOA joint pathology.

Tissues were harvested after 1 day, 1 week, 4 weeks and 6 weeks following ACLR, and ptOA joint pathology scored and compared between treatment groups and their respective controls (Fig. 4A). Only targeted systemic depletion of CCR2⁺ monocytes significantly modified structural ptOA pathology with the decrease in synovial fibrosis and cartilage damage both $p < 0.05$ at week-6 (Fig 4B). The lower cartilage and osteochondral damage scores at earlier time points in both anti-CCR2 and IV clodronate-liposome treatment groups did not reach statistical significance due to inter-mouse variability. Systemic depletion of CCR2⁺ monocytes and macrophages also both significantly decreased chondrocyte hypertrophy/apoptosis, at 4 and 6 weeks in the former but only 4 weeks in the latter. Total osteophyte/enthesophyte severity score was decreased by all active treatments relative to their controls as 4 and 6 weeks but none of the differences were statistically significant. In contrast, histopathologic total synovitis and synovial lining cell hyperplasia were significantly decreased by all treatments but with different temporal patterns (Fig 4C). Systemic CCR2⁺ monocyte depletion induced an acute and persistent reduction in synovitis ($p < 0.05$ day-1 and week-6) and synovial hyperplasia ($p < 0.05$ week-4). Systemic macrophage depletion had a similar early effect on synovitis ($p < 0.05$ week-1 and week-4) and synovial hyperplasia ($p < 0.001$ week-1) but at week 6 scores were similar to

controls. Depletion of STRM also markedly reduced early synovitis ($p < 0.0001$ week-1) and synovial hyperplasia ($p < 0.001$ week-1) but thereafter scores increased to be equal to or greater than controls.

Differential effect of targeting systemic CCR2⁺ monocytes and macrophage versus STRM on synovial molecular inflammatory pathways regulation.

To decipher the underlying molecular mechanisms responsible for and/or associated with the distinct differences observed in the cellular inflammatory response after either systemic CCR2⁺ monocyte or local STRM depletion, we profiled the synovial tissue block transcriptome 1 day and 4 weeks after ACLR (Fig. 5 and 6, respectively). Cluster analysis was performed for the 8 included samples based on the differentially expressed genes (DEGs), with the two samples of each group at each time point clustered together indicating a reproducible differential expression analysis (Fig 5A and 6A). In addition, CCR2⁺ monocyte depletion resulted in clustering closer to untreated NOC synovial tissue rather than untreated ACLR synovial tissue at day-1, while STRM depletion resulted in loss of clustering with all other groups at both 1 day and 4 weeks (Fig. 5A and 6A). At 1 day post-ACLR only 10% of DEGs were the same in STRM-depleted vs untreated and CCR2⁺ monocyte depleted vs untreated mice (Fig. 5B), while 41% of DEGs were the same in STRM depleted vs CCR2⁺ monocyte depleted and STRM depleted vs untreated animals (Fig. 5C). Finally, only 10% of DEGs in CCR2⁺ monocyte depleted vs STRM depleted and CCR2⁺ monocyte depleted vs untreated mice were shared (Fig. 5D). This highlights the very distinct acute synovial molecular regulation of STRM depletion compared with systemic CCR2⁺ monocyte depletion. At 4 weeks post-ACLR the shared DEGs between STRM and CCR2⁺ monocyte depleted vs untreated animals had reduced to 2% (Fig. 6B), and overlapping DEGs between CCR2⁺ monocyte depleted vs either STRM depleted or untreated mice had decreased to 1% (Fig. 6D). In contrast, the number of shared DEGs in

STRM depleted mice *vs* either CCR2⁺ monocyte depleted or untreated animals had remained high (45%; Fig. 6C). Thus, despite the natural temporal resolution of the acute-injury associated synovial molecular response (compare cluster analysis in 5A and 6A), differences in the molecular regulation from peri-injury STRM depletion *vs* systemic CCR2⁺ monocyte depletion remain profound or become even more pronounced.

GSEA was performed to identify up- and downregulated pathways in the different groups identified based on the normalized effect size (NES; Tables 1 and 2, respectively). While the majority of pathways identified at 1 day were not specifically associated with the immune response or ptOA-related tissues, depletion of both STRM or CCR2⁺ Ly6C^{high} monocytes resulted in an upregulation of cytokine activation pathways when compared to untreated controls (Table 1). Interestingly STRM depletion also increased day-1 cytokine receptor binding and neutrophil mediated immunity and immunological synapse (i.e molecules involved in innate/adaptive crosstalk and T-cell activation). Similarly at week-4 STRM depletion was associated with upregulation of pathways involved in monocyte and leukocyte migration as well as IL1 and TNF response when compared to either untreated or CCR2⁺ monocyte depleted samples, as well as chondrocyte and cartilage differentiation pathways. There were common downregulated pathways in both STRM and CCR2⁺ monocyte depleted synovia at 1 day involving cytoskeletal structures, but also differences with STRM depletion decreasing collagen and proteoglycan matrix formation (Table 2). At 4 weeks there were too few down-regulated pathways identified for further evaluation. Deeper analysis of the top 5 individual DEGs at day-1 (based on the log₂ fold change; Fig 5 E-G) identified genes involved in the immune response that were particularly down-regulated by CCR2⁺ monocyte depletion (*Arg1*, *Cx3cr1*) while a decrease in others (*C7*) was more associated with STRM depletion (Table 3). At 4 weeks post-ACLR the top 5 DEGs (Fig. 6 E-G) included genes associated with the innate

(*Arg1*) and adaptive (*Ccl17*) immune response that were upregulated by STRM depletion (Table 3).

Discussion

Ly6C^{high} monocytes are known as potent pro-inflammatory effector cells [5] and the cytokine profile released by Ly6C^{high} monocytes upon activation includes IL-1 β , TNF- α as well as high levels of reactive oxygen species [5]. In OA, it is believed that Ly6C^{high} monocytes directly contribute towards a pro-inflammatory microenvironment but also serve as the main source for BO inflammatory M1 macrophages [34, 35] further promoting inflammation. Soluble inflammatory mediators secreted from Ly6C^{high} monocytes and BO macrophages in the sub-synovium diffuse into the joint space itself and increase secretion of catabolic enzymes by chondrocytes and chondrocyte apoptosis which in turn releases danger-associated molecular patterns (DAMPs) further activating macrophages to release leukocyte chemotaxins [36]. As this pro-inflammatory feedback loop is a crucial contributor to the inflammatory reaction associated with ptOA, Ly6C^{high} monocytes have emerged as potential target of interest in disease modification. In addition, Ly6C^{high} monocyte vascular egress and recruitment into the joint space are governed by CCL2/CCR2 signaling [37] and elevated CCL2 in the synovial fluid has been identified not only subsequent to joint injury [38, 39] but also during established OA [40, 41]. Thus, a crucial immunomodulatory function of the CCL2/CCR2 signaling pathway in context with ptOA has been postulated, with evidence from both knock out mice and therapeutic intervention studies demonstrating a significant role in synovial inflammation and pain but mixed results on structural pathology [10-13]. CCL2^{-/-} and CCR2^{-/-} knockout mice showed significantly reduced cartilage damage 20 weeks after DMM surgery in addition to a marked reduction in activated macrophages in the synovium [13]. In the current study a transient reduction in blood-originated macrophages was observed in addition to a lasting anti-inflammatory polarization of synovial macrophages. However, cartilage damage was not prevented in the current study and only significantly mitigated after 6 weeks. It is possible that the differences between knockout animals and the animals' receiving treatment in our study

derives from the differences in the utilized animal model. ACLR results in a faster and more pronounced onset of cartilage damage when compared to DMM (**Chapter 3**). Due to the ongoing instability, it is likely that restoration of biomechanics in combination with CCR2 blockade is needed to prevent cartilage damage after ACLR. In addition, a recovery of blood-originated macrophages was observed in our study which is prevented in knockout mice. This highlights the importance of finding the right timing and duration of CCR2 inhibition in order to mitigate ptOA development.

In the current study, anti-CCR2 treatment led to a lasting mitigation of chondrocyte hypertrophy and apoptosis, and although the direct role of CCR2 in context with chondrocyte hypertrophy is poorly understood a recent study showed that suppression of CCR2/CCL2 signaling was sufficient to protect type 2 collagen synthesis via a reduction of RUNX2 and IHH thereby improving chondrocyte proliferation and reducing apoptosis [42]. Reduced histopathological synovitis and synovial lining hyperplasia was observed after anti-CCR2 treatment. On a cellular level anti-CCR2 treatment led to a significantly reduced monocyte influx into the joint and an overall anti-inflammatory cellular synovial microenvironment observed via a mitigated and partly reversed Ly6c^{high}/Ly6C^{low} and a persistently reduced BO and STRM M1/M2 macrophage ratio. This is consistent with data showing blockade of CCR2 skews macrophage polarisation towards the anti-inflammatory M2 phenotype and to decrease expression of iNOS and TNF while increasing expression of anti-inflammatory cytokines such as Arg1, Ym1 and TGF- β [43]. Surprisingly, genomic analysis of the synovial tissue block of anti-CCR2 treated animals indicated a decreased Arg1 expression. A potential explanation lies in the fact that both BO macrophages and STRMs are present in the synovial membrane, and although a lasting anti-inflammatory milieu was created in STRMs after CCR2 blockade, immediately after injury M1 macrophages were still the predominant phenotype potentially contributing towards the observed reduction in Arg1 expression. Besides these direct anti-

inflammatory effects of anti-CCR2 treatment sequencing analysis data showed a significantly reduced expression of CX3CR1. CX3CR1 is crucial in the chemotaxis of Ly6C^{low} monocytes and elevated levels have been detected in the synovial fluid of OA patients [44]. Functional analysis indicated that besides its chemo-attractive properties CX3CR1 also stimulates inflammation especially during early stages of OA via a pro-inflammatory activation of recruited Ly6C^{low} monocytes [5]. The observed reduction in CX3CR1 expression adds to the understanding of how CCR2 pathway blockade modulates the inflammatory response subsequent to joint injury and during development of ptOA.

Joint damage was ameliorated after anti-CCR2 treatment and despite the short-term treatment that started prior to injury lasting mitigation of bone and cartilage damage were detected. These findings are supported by a recent study using a similar treatment in the DMM mouse model [45]. Interestingly the protective effects of anti-CCR2 treatment were less when treatment was postponed [45] which was previously demonstrated in mouse models of RA where postponed treatment resulted in a more severe and destructive arthritis [46]. The importance of timing is also highlighted by the results of the first clinical trials that utilized antibody mediated CCR2 inhibition in patients with RA [47, 48]. Despite compelling pre-clinical data indicating the efficacy of CCR2 pathway modulation when using in-vivo models of RA, both clinical trials did not observe any amelioration of inflammation in active RA and clinically a worsened condition occurred in some cases [47, 48]. Authors of both studies concluded that blockade of CCR2 is insufficient for a clinical improvement of RA. A potential explanation could be that both studies included patients with established RA having a disease duration of at least 6 months, whereas pre-clinical studies started the CCR2 blockade during early stages of arthritis development. Taken together it seems that the right timing is crucial for a beneficial effect of anti-CCR2 treatment in context with OA [49]. While this might be difficult to achieve in RA,

ptOA starts with a defined trauma and therefore CCR2 could be a very promising target in the disease modulation.

Although systemic injection of clodronate has been established to induce apoptosis in macrophages, it is a well-known fact that besides the depletion of macrophages, apoptosis is also induced in monocytes and dendritic cells [50]. The simultaneous apoptosis of the large number of immune cells has been shown to cause a temporary inflammatory reaction by itself [51]. In the current study, IV clodronate-liposomes resulted in a number of similar changes as anti-CCR2 in systemic myeloid cells. This can be explained by the overlap in targeted myeloid cells of the two treatments whereas the inflammatory reaction caused by the broad apoptosis of immune cells resulting from IV clodronate-liposomes countermanded some of the anti-inflammatory effects observed when only targeting a specific subset of pro-inflammatory myeloid cells (anti-CCR2). In particular, IV clodronate-liposomes decreased blood and bone-marrow monocytes as well as the bone-marrow M1/M2 polarization while increasing circulating monocyte Ly6C⁺/Ly6C⁻ ratio. Furthermore, this was associated with a different temporal pattern of synovial BO macrophage and STRM Ly6C⁺/Ly6C⁻ ratio and M1/M2 polarization where anti-CCR2 had a much greater early (day-1) anti-inflammatory effect on synovial BO macrophage and STRM activation and M1/M2 polarization than IV clodronate-liposomes while later changes were similar. Thereby the differences observed subsequent to a treatment having both a pro- and anti-inflammatory effect (IV clodronate-liposomes) compared to a targeted depletion of pro-inflammatory monocyte subsets (anti-CCR2) suggest the importance of the immediate post-injury inflammatory response in long-term joint inflammation and ptOA-risk. This may explain the distinct temporal differences in effect of the two treatments on ptOA pathology and lack of sustained or significant disease modification with IV clodronate-liposomes.

STRMs protect the joint space during homeostasis from detrimental stimuli scavenging extracellular matrix molecules that might otherwise act as DAMPs and with inter-cellular tight junctions reducing ingress of serum proteins into the synovium. However, DAMPs released from disrupted joint tissues can activate STRMs and initiate a pro-inflammatory polarisation switch contributing to the migration of blood monocytes as observed in RA [5, 52, 53]. Thus, STRMs have a potential dichotomous function on the one hand as protector of the joint space and on the other hand as an initiator of the pro-inflammatory response. As the exact role of STRMs in the pathogenesis of ptOA is still unknown the current study included an established method to deplete the synovial lining from STRMs. Despite a short-lived reduction in histological synovitis joint damage was unaltered by STRM depletion. On a cellular level depletion of STRM led to a more distinct pro-inflammatory response with a significantly increased synovial BO macrophage Ly6C+/Ly6C- and M1/M2 ratio. Additionally, peri-injury depletion of STRM also had a systemic pro-inflammatory effect significantly increasing circulating and bone-marrow monocyte M1/M2. This suggests that STRM may not only protect the joint from systemic factors but also play a role in containing joint-associated inflammation to decrease the effects of local injury on the other body systems and organs. This is an interesting concept with increasing interest in the role of OA as an active effector of incident co-morbid conditions such as cardiovascular, renal, and neurodegenerative disease [54, 55]. Interestingly, although the depletion of STRM was performed prior to joint injury, lasting pro-inflammatory changes were observed throughout the study period. In addition, GSEA analysis indicated that even after 4 weeks immune cell chemotactic and migratory pathways were increased supporting an ongoing inflammatory response. Again, this indicates that acute peri-injury changes in the joint-inflammatory status are critical for long-term joint inflammation and ptOA risk. This is consistent with data from patients with meniscal injuries where the presence and severity of the initial synovitis is predictive of increased risk of progressive ptOA,

even if the synovitis subsequently resolves [56]. Interestingly, depletion of STRM led to an increased expression of CCL17, a cytokine closely related to the Th17 response, and significant upregulation of the immunological synapse pathway in general, indicating a potential cross activation of not only the innate immune system but also the adaptive immune system as a result of STRM depletion. This prolonged and exacerbated immune response could potentially be explained by the macrophage niche model as depletion of STRMs allowed population of the niche by BO and inflammation primed monocyte subsets [5]. However, further detailed studies are needed decipher the complex underlying pathomechanisms of STRM depletion.

Limitations

This study has a number of limitations that need to be recognised and addressed in future studies. All studies were done on young, otherwise healthy male mice, and confirmation is required if findings would be similar in females, older animals and in the presence of comorbidities such as obesity and type-2 diabetes, factors that can all alter the inflammatory response and ptOA risk [57-60]. Furthermore, our earlier studies (see **Chapter 3.2**) have shown that the phenotype and temporal pattern of inflammatory response differs with different joint injuries, and it is important to confirm whether findings in the current ACLR model are recapitulated with DMM for example. ACLR is a rapid onset ptOA model with a quick progression of joint pathology resulting in a severe structural damage. This model was chosen as it would present the immunological “worst” case in ptOA and therefore an ideal model to test this immunomodulatory approach. However, in the current study only modest mitigation of joint pathology was shown after treatment which could be due to the characteristics of the chosen animal model. Therefore, it is important to test this approach in a subsequent study utilizing the DMM or other ptOA models in order to investigate if a more distinct mitigation of joint pathology can be achieved. In addition to macrophages, CCR2 is expressed on other

non-hematopoietic cells such as fibroblasts, endothelial cells, and mesenchymal stem cells. In the current study anti-CCR2 was administered systemically, and it is possible that the effect on other cells is responsible for masking some effects. Therefore, subsequent studies are needed that use a more targeted route of administration to reduce systemic effects of the treatment. A further potential limitation during the use of RNAseq data and analysis includes the small sample size and significance threshold filtering which can affect the subsequent pathway/network analysis.

Notwithstanding these caveats, the current study has provided valuable insight into the immunobiology of innate immune cell subsets in the context of ptOA associated inflammation and structural pathology. Anti-CCR2 treatment has the potential to be an interesting target for immunomodulation, however further studies are needed to decipher the important temporal aspect of its effect. Additionally, developing strategies to modify STRM depletion and/or pro-inflammatory activation associated with joint injury may have not only local ptOA disease modifying benefit but also in reducing the systemic impact of OA on whole organism health.

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None of the funding agencies had any input into the conceptualization or implementation of the study or analysis and interpretation of data.

Conflict of interest

The authors declare no competing interests.

References

1. Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H: **Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies.** *eClinicalMedicine* 2020, **29**.
2. Mora JC, Przkora R, Cruz-Almeida Y: **Knee osteoarthritis: pathophysiology and current treatment modalities.** *J Pain Res* 2018, **11**:2189-2196.
3. Kloppenburg M, Berenbaum F: **Osteoarthritis year in review 2019: epidemiology and therapy.** *Osteoarthritis Cartilage* 2020, **28**(3):242-248.
4. Orlowsky EW, Kraus VB: **The role of innate immunity in osteoarthritis: when our first line of defense goes on the offensive.** *J Rheumatol* 2015, **42**(3):363-371.
5. Haubruck P, Pinto MM, Moradi B, Little CB, Gentek R: **Monocytes, Macrophages, and Their Potential Niches in Synovial Joints – Therapeutic Targets in Post-Traumatic Osteoarthritis?** *Frontiers in Immunology* 2021, **12**.
6. Cremers NAJ, van den Bosch MHJ, van Dalen S, Di Ceglie I, Ascone G, van de Loo F, Koenders M, van der Kraan P, Sloetjes A, Vogl T *et al*: **S100A8/A9 increases the mobilization of pro-inflammatory Ly6Chigh monocytes to the synovium during experimental osteoarthritis.** *Arthritis Research & Therapy* 2017, **19**(1):217.
7. Italiani P, Boraschi D: **From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation.** *Frontiers in Immunology* 2014, **5**.
8. Sun Y, Zuo Z, Kuang Y: **An Emerging Target in the Battle against Osteoarthritis: Macrophage Polarization.** *Int J Mol Sci* 2020, **21**(22).
9. Culemann S, Gruneboom A, Nicolas-Avila JA, Weidner D, Lammle KF, Rothe T, Quintana JA, Kirchner P, Krljanac B, Eberhardt M *et al*: **Locally renewing resident synovial macrophages provide a protective barrier for the joint.** *Nature* 2019, **572**(7771):670-675.
10. Jablonski CL, Leonard C, Salo P, Krawetz RJ: **CCL2 But Not CCR2 Is Required for Spontaneous Articular Cartilage Regeneration Post-Injury.** *J Orthop Res* 2019, **37**(12):2561-2574.
11. Miller RE, Tran PB, Das R, Ghoreishi-Haack N, Ren D, Miller RJ, Malfait AM: **CCR2 chemokine receptor signaling mediates pain in experimental osteoarthritis.** *Proc Natl Acad Sci U S A* 2012, **109**(50):20602-20607.
12. Miotla Zarebska J, Chanalaris A, Driscoll C, Burleigh A, Miller RE, Malfait AM, Stott B, Vincent TL: **CCL2 and CCR2 regulate pain-related behaviour and early gene expression in post-traumatic murine osteoarthritis but contribute little to chondropathy.** *Osteoarthritis Cartilage* 2017, **25**(3):406-412.
13. Raghu H, Lepus CM, Wang Q, Wong HH, Lingampalli N, Oliviero F, Punzi L, Giori NJ, Goodman SB, Chu CR *et al*: **CCL2/CCR2, but not CCL5/CCR5, mediates monocyte recruitment, inflammation and cartilage destruction in osteoarthritis.** *Ann Rheum Dis* 2016.
14. Mack M, Cihak J, Simonis C, Luckow B, Proudfoot AE, Plachy J, Bruhl H, Frink M, Anders HJ, Vielhauer V *et al*: **Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice.** *J Immunol* 2001, **166**(7):4697-4704.
15. Moretti A, Paoletta M, Liguori S, Ilardi W, Snichelotto F, Toro G, Gimigliano F, Iolascon G: **The Rationale for the Intra-Articular Administration of Clodronate in Osteoarthritis.** *Int J Mol Sci* 2021, **22**(5).
16. Smith MM, Clarke EC, Little CB: **Considerations for the design and execution of protocols for animal research and treatment to improve reproducibility and**

- standardization: "DEPART well-prepared and ARRIVE safely". *Osteoarthritis Cartilage* 2017, **25**(3):354-363.
17. Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M *et al*: **Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0.** *PLoS Biol* 2020, **18**(7):e3000411.
 18. NHMRC: **Australian code for the care and use of animals for scientific purposes**, 8th edition edn: National Health and Medical Research Council; Canberra, Australia; 2013.
 19. Christiansen BA, Anderson MJ, Lee CA, Williams JC, Yik JH, Haudenschild DR: **Musculoskeletal changes following non-invasive knee injury using a novel mouse model of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2012, **20**(7):773-782.
 20. Blaker CL, Little CB, Clarke EC: **Joint loads resulting in ACL rupture: Effects of age, sex, and body mass on injury load and mode of failure in a mouse model.** *Journal of Orthopaedic Research* 2017, **35**(8):1754-1763.
 21. Haubruck P, Heller R, Blaker CL, Clarke EC, Smith SM, Burkhardt D, Liu Y, Stoner S, Zaki S, Shu CC *et al*: **Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models.** *Osteoarthritis and Cartilage* 2023, **Accepted for publication**.
 22. Dickson BC, Yang H, Savelkoul HF, Rowden G, van Rooijen N, Wright JR, Jr.: **Islet transplantation in the discordant tilapia-to-mouse model: a novel application of alginate microencapsulation in the study of xenograft rejection.** *Transplantation* 2003, **75**(5):599-606.
 23. Louten J, van Rooijen N, Biron CA: **Type 1 IFN deficiency in the absence of normal splenic architecture during lymphocytic choriomeningitis virus infection.** *J Immunol* 2006, **177**(5):3266-3272.
 24. Lawlor KE, Wong PK, Campbell IK, van Rooijen N, Wicks IP: **Acute CD4+ T lymphocyte-dependent interleukin-1-driven arthritis selectively requires interleukin-2 and interleukin-4, joint macrophages, granulocyte-macrophage colony-stimulating factor, interleukin-6, and leukemia inhibitory factor.** *Arthritis Rheum* 2005, **52**(12):3749-3754.
 25. Hammond MD, Taylor RA, Mullen MT, Ai Y, Aguila HL, Mack M, Kasner SE, McCullough LD, Sansing LH: **CCR2+ Ly6C(hi) inflammatory monocyte recruitment exacerbates acute disability following intracerebral hemorrhage.** *J Neurosci* 2014, **34**(11):3901-3909.
 26. Mildner A, Schmidt H, Nitsche M, Merkler D, Hanisch UK, Mack M, Heikenwalder M, Bruck W, Priller J, Prinz M: **Microglia in the adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host conditions.** *Nat Neurosci* 2007, **10**(12):1544-1553.
 27. Haubruck P, Colbath AC, Liu Y, Stoner S, Shu C, Little CB: **Flow Cytometry Analysis of Immune Cell Subsets within the Murine Spleen, Bone Marrow, Lymph Nodes and Synovial Tissue in an Osteoarthritis Model.** *J Vis Exp* 2020(158).
 28. Jackson MT, Moradi B, Zaki S, Smith MM, McCracken S, Smith SM, Jackson CJ, Little CB: **Depletion of protease-activated receptor 2 but not protease-activated receptor 1 may confer protection against osteoarthritis in mice through extracartilaginous mechanisms.** *Arthritis Rheumatol* 2014, **66**(12):3337-3348.
 29. Shu CC, Jackson MT, Smith MM, Smith SM, Penm S, Lord MS, Whitelock JM, Little CB, Melrose J: **Ablation of Perlecan Domain 1 Heparan Sulfate Reduces Progressive**

- Cartilage Degradation, Synovitis, and Osteophyte Size in a Preclinical Model of Posttraumatic Osteoarthritis.** *Arthritis Rheumatol* 2016, **68**(4):868-879.
30. Zaki S, Smith MM, Smith SM, Little CB: **Differential patterns of pathology in and interaction between joint tissues in long-term osteoarthritis with different initiating causes: phenotype matters.** *Osteoarthritis Cartilage* 2020, **28**(7):953-965.
 31. Haubruck P, Heller R, Blaker CL, Clarke EC, Smith SM, Burkhardt D, Liu Y, Stoner S, Zaki S, Shu CC *et al*: **Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models.** *Osteoarthritis and Cartilage* 2023.
 32. Shu CC, Zaki S, Ravi V, Schiavinato A, Smith MM, Little CB: **The relationship between synovial inflammation, structural pathology, and pain in post-traumatic osteoarthritis: differential effect of stem cell and hyaluronan treatment.** *Arthritis Res Ther* 2020, **22**(1):29.
 33. Little CB, Hunter DJ: **Post-traumatic osteoarthritis: from mouse models to clinical trials.** *Nat Rev Rheumatol* 2013, **9**(8):485-497.
 34. Goldring MB, Otero M: **Inflammation in osteoarthritis.** *Curr Opin Rheumatol* 2011, **23**(5):471-478.
 35. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM: **M-1/M-2 macrophages and the Th1/Th2 paradigm.** *J Immunol* 2000, **164**(12):6166-6173.
 36. Loukov D, Karampatos S, Maly MR, Bowdish DME: **Monocyte activation is elevated in women with knee-osteoarthritis and associated with inflammation, BMI and pain.** *Osteoarthritis and Cartilage* 2018, **26**(2):255-263.
 37. Conti P, DiGioacchino M: **MCP-1 and RANTES are mediators of acute and chronic inflammation.** In: *Allergy and asthma proceedings: 2001*: OceanSide Publications; 2001: 133.
 38. Watt FE, Paterson E, Freidin A, Kenny M, Judge A, Saklatvala J, Williams A, Vincent TL: **Acute Molecular Changes in Synovial Fluid Following Human Knee Injury: Association With Early Clinical Outcomes.** *Arthritis & rheumatology (Hoboken, NJ)* 2016, **68**(9):2129-2140.
 39. Scanzello CR, McKeon B, Swaim BH, Dicarlo E, Asomugha EU, Kanda V, Nair A, Lee DM, Richmond JC, Katz JN *et al*: **Synovial inflammation in patients undergoing arthroscopic meniscectomy: Molecular characterization and relationship to symptoms.** *Arthritis Rheum* 2011, **63**(2):391-400.
 40. Li L, Jiang BE: **Serum and synovial fluid chemokine ligand 2/monocyte chemoattractant protein 1 concentrations correlates with symptomatic severity in patients with knee osteoarthritis.** *Ann Clin Biochem* 2015, **52**(Pt 2):276-282.
 41. Endres M, Andreas K, Kalwitz G, Freymann U, Neumann K, Ringe J, Sittlinger M, Haupl T, Kaps C: **Chemokine profile of synovial fluid from normal, osteoarthritis and rheumatoid arthritis patients: CCL25, CXCL10 and XCL1 recruit human subchondral mesenchymal progenitor cells.** *Osteoarthritis Cartilage* 2010, **18**(11):1458-1466.
 42. Wang Z, Wang B, Zhang J, Wu Z, Yu L, Sun Z: **Chemokine (C-C Motif) Ligand 2/Chemokine Receptor 2 (CCR2) Axis Blockade to Delay Chondrocyte Hypertrophy as a Therapeutic Strategy for Osteoarthritis.** *Med Sci Monit* 2021, **27**:e930053.
 43. Gschwandtner M, Derler R, Midwood KS: **More Than Just Attractive: How CCL2 Influences Myeloid Cell Behavior Beyond Chemotaxis.** *Frontiers in Immunology* 2019, **10**.

44. Endres M, Andreas K, Kalwitz G, Freymann U, Neumann K, Ringe J, Sittlinger M, Häupl T, Kaps C: **Chemokine profile of synovial fluid from normal, osteoarthritis and rheumatoid arthritis patients: CCL25, CXCL10 and XCL1 recruit human subchondral mesenchymal progenitor cells.** *Osteoarthritis and cartilage* 2010, **18**(11):1458-1466.
45. Longobardi L, Temple JD, Tagliafierro L, Willcockson H, Esposito A, D'Onofrio N, Stein E, Li T, Myers TJ, Ozkan H *et al*: **Role of the C-C chemokine receptor-2 in a murine model of injury-induced osteoarthritis.** *Osteoarthritis and Cartilage* 2017, **25**(6):914-925.
46. Vergunst CE, van de Sande MGH, Lebre MC, Tak PP: **The role of chemokines in rheumatoid arthritis and osteoarthritis.** *Scandinavian Journal of Rheumatology* 2005, **34**(6):415-425.
47. Haringman JJ, Gerlag DM, Smeets TJM, Baeten D, van den Bosch F, Bresnihan B, Breedveld FC, Dinant HJ, Legay F, Gram H *et al*: **A randomized controlled trial with an anti-CCL2 (anti-monocyte chemotactic protein 1) monoclonal antibody in patients with rheumatoid arthritis.** *Arthritis & Rheumatism* 2006, **54**(8):2387-2392.
48. Vergunst CE, Gerlag DM, Lopatinskaya L, Klareskog L, Smith MD, van den Bosch F, Dinant HJ, Lee Y, Wyant T, Jacobson EW *et al*: **Modulation of CCR2 in rheumatoid arthritis: A double-blind, randomized, placebo-controlled clinical trial.** *Arthritis & Rheumatism* 2008, **58**(7):1931-1939.
49. Miller RE, Malfait AM: **Can we target CCR2 to treat osteoarthritis? The trick is in the timing!** *Osteoarthritis and Cartilage* 2017, **25**(6):799-801.
50. Mass E: **Delineating the origins, developmental programs and homeostatic functions of tissue-resident macrophages.** *International Immunology* 2018, **30**(11):493-501.
51. Mass E: **The stunning clodronate.** *Journal of Experimental Medicine* 2023, **220**(6):e20230339.
52. Asquith DL, Ballantine LE, Nijjar JS, Makdasy MK, Patel S, Wright PB, Reilly JH, Kerr S, Kurowska-Stolarska M, Gracie JA *et al*: **The liver X receptor pathway is highly upregulated in rheumatoid arthritis synovial macrophages and potentiates TLR-driven cytokine release.** *Ann Rheum Dis* 2013, **72**(12):2024-2031.
53. Alivernini S, Tolusso B, Ferraccioli G, Gremese E, Kurowska-Stolarska M, McInnes IB: **Driving chronicity in rheumatoid arthritis: perpetuating role of myeloid cells.** *Clin Exp Immunol* 2018, **193**(1):13-23.
54. Swain S, Coupland C, Mallen C, Kuo CF, Sarmanova A, Bierma-Zeinstra SMA, Englund M, Prieto-Alhambra D, Doherty M, Zhang W: **Temporal relationship between osteoarthritis and comorbidities: a combined case control and cohort study in the UK primary care setting.** *Rheumatology (Oxford)* 2021, **60**(9):4327-4339.
55. Soheli MJ, Aiken D, Katie T, Alexandra KOD, Cindy S, Susan S, Meena S, Aaron S, Natasha MR, Christopher BL: **Disease-modifying interactions between chronic kidney disease and osteoarthritis: a new comorbid mouse model.** *RMD Open* 2023, **9**(3):e003109.
56. MacFarlane LA, Yang H, Collins JE, Jarraya M, Gueremazi A, Mandl LA, Martin SD, Wright J, Losina E, Katz JN: **Association of Changes in Effusion-Synovitis With Progression of Cartilage Damage Over Eighteen Months in Patients With Osteoarthritis and Meniscal Tear.** *Arthritis & rheumatology (Hoboken, NJ)* 2019, **71**(1):73-81.
57. Blaker CL, Clarke EC, Little CB: **Using mouse models to investigate the pathophysiology, treatment, and prevention of post-traumatic osteoarthritis.** *J Orthop Res* 2017, **35**(3):424-439.

58. Blaker CL, Clarke EC, Little CB: **Adding insult to injury: synergistic effect of combining risk-factors in models of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2019, **27**(12):1731-1734.
59. Little CB, Hunter DJ: **Post-traumatic osteoarthritis: from mouse models to clinical trials.** *Nat Rev Rheumatol* 2013, **9**(8):485-497.
60. Zaki S, Blaker CL, Little CB: **OA foundations - experimental models of osteoarthritis.** *Osteoarthritis Cartilage* 2022, **30**(3):357-380.

Figure 1: Visualization of the study design and animal allocation into each study. Experiments were divided into cellular and structural in-vivo experiments and transcriptional in-vivo experiments. Groups, treatments, time points and outcomes are detailed for each set of experiment.

Figure 2: Results of the flow cytometry experiments of the systemic innate immune response are shown. The influence of the immunomodulation on the expression of monocytes, their inflammatory state (data derived from cells harvested from the blood (**A**) and contralateral bone marrow (**B**) is shown) as well as macrophages and their M1/M2 ratio (**B**) in the bone marrow is visualized. The * symbol indicates results from the statistical comparison of ‘ACLR+anti-CCR2 treatment’ vs ‘ACLR+saline control’ mice, the + symbol is used for the comparison of ‘ACLR+iV Clododronate treated’ vs ‘ACLR+iV Liposome control’ mice, and the ^ symbol is used for the comparison of ‘ACLR+iA Clodronate treated’ vs ‘ACLR+iA Liposome treated’ mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as mean $\pm$ SEM and $n = 6$ biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 3: Results of the flow cytometry experiments of the locoregional innate immune response. The influence of different treatments on the expression of blood originated (BO) and synovial tissue resident macrophages in the synovium as well as their inflammatory polarization assessed via the Ly6C expression and M1/M2 ratio is shown. The * symbol indicates results from the statistical comparison of ‘ACLR+anti-CCR2 treatment’ vs ‘ACLR+saline control’ mice, the + symbol is used for the comparison of ‘ACLR+iV Clododronate treated’ vs ‘ACLR+iV Liposome control’ mice, and the ^ symbol is used for the

comparison of ‘ACLR+iA Clodronate treated’ vs ‘ACLR+iA Liposome treated’ mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Data is presented as mean $\pm$ SEM and $n = 6$ biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 4: Results of histopathological evaluation of the joint. **A** provides a schematic overview over the tissues utilized and outcome measurements. **B** visualizes the fibrotic changes to the joint. Overlaying groups were nudged in order make all groups clearly visual. **C** shows the combined tibiofemoral cartilage damage over time, as well as chondrocyte hypertrophy/apoptosis over time, and osteochondral hypertrophy/apoptosis over time as well as the total remodeling assessed as the combined osteophyte and enthesophyte severity adjacent to the joint. Lastly, **D** focuses on the synovial inflammation over time, by showing combined total synovitis and the synovial hyperplasia scores. Overlaying groups were nudged in order make all groups clearly visual. The * symbol indicates results from the statistical comparison of ‘ACLR+anti-CCR2 treatment’ vs ‘ACLR+saline control’ mice, the + symbol is used for the comparison of ‘ACLR+iV Clododronate treated’ vs ‘ACLR+iV Liposome control’ mice, and the ^ symbol is used for the comparison of ‘ACLR+iA Clodronate treated’ vs ‘ACLR+iA Liposome treated’ mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+,^), $p < 0.01$ (**,++,^^), $p < 0.001$ (***,+++,^^^), and $p < 0.0001$ (****,++++,^^^^). Error bars are not shown in groups/time points where all values were the same. Data is presented as median $\pm$ 95% CI and $n = 9$ animals/group/time point.

Figure 5: Results from the transcriptomic analysis 1 day after ACL injury are shown. **A** heat map of the transcriptional pattern of mice treated systemically with anti-CCR2 **mAB** or intraarticularly with **Clodronate** as well as wild-type animals including **ACLR**, and non-

operated controls (**NOC**) is shown. **B-D** Venn diagrams showing the individual number of DEGs and their individual distribution between groups are shown. **E-F** Volcano plot. The log₂ fold change indicates the mean expression level for each gene while the false discovery rate (FDR) p-value was used to detect significant differences. Each dot represents one gene. Black dots represent no significant DEGs between groups, the blue dots represent down-regulated genes and red dots represent up-regulated genes. The 5 DEGs with the largest differences are numbered and details given in Table 3. N = 2 biological replicates (pool of tissues from 3 mice)/group/time point.

Figure 6: Results from the transcriptomic analysis 4 weeks after ACL injury are shown. **A** heat map of the transcriptional pattern of mice treated systemically with anti-CCR2 **mAB** or intraarticularly with **Clodronate** as well as wild-type animals including **ACLR**, and non-operated controls (**NOC**) is shown. **B-D** Venn diagrams showing the individual number of DEGs and their individual distribution between groups are shown. **E-F** Volcano plot. The log₂ fold change indicates the mean expression level for each gene while the false discovery rate (FDR) p-value was used to detect significant differences. Each dot represents one gene. Black dots represent no significant DEGs between groups, the blue dots represent down-regulated genes and red dots represent up-regulated genes. The 5 DEGs with the largest differences are numbered and details given in Table 3. N = 2 biological replicates (pool of tissues from 3 mice)/group/time point.

Figure 1:

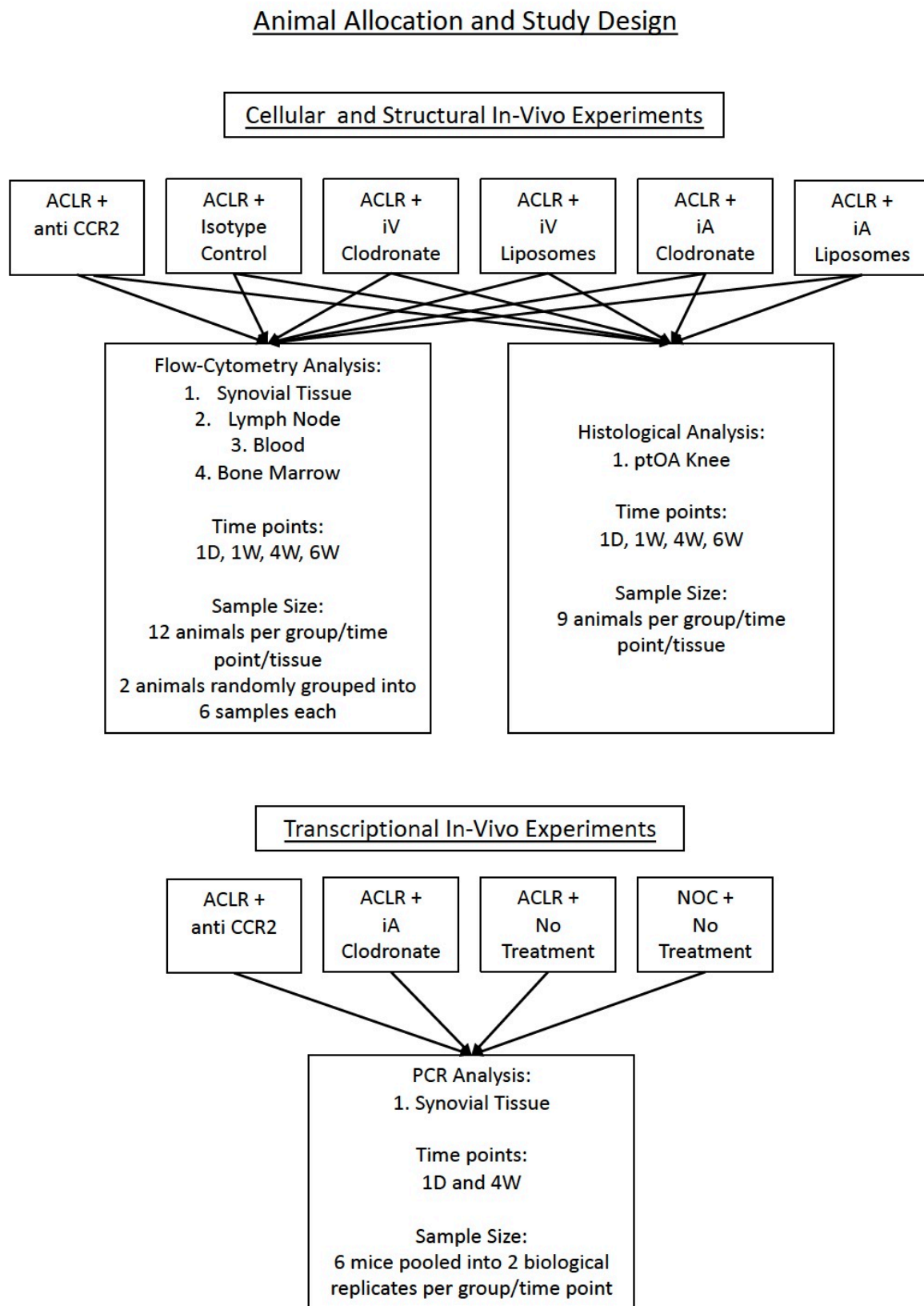
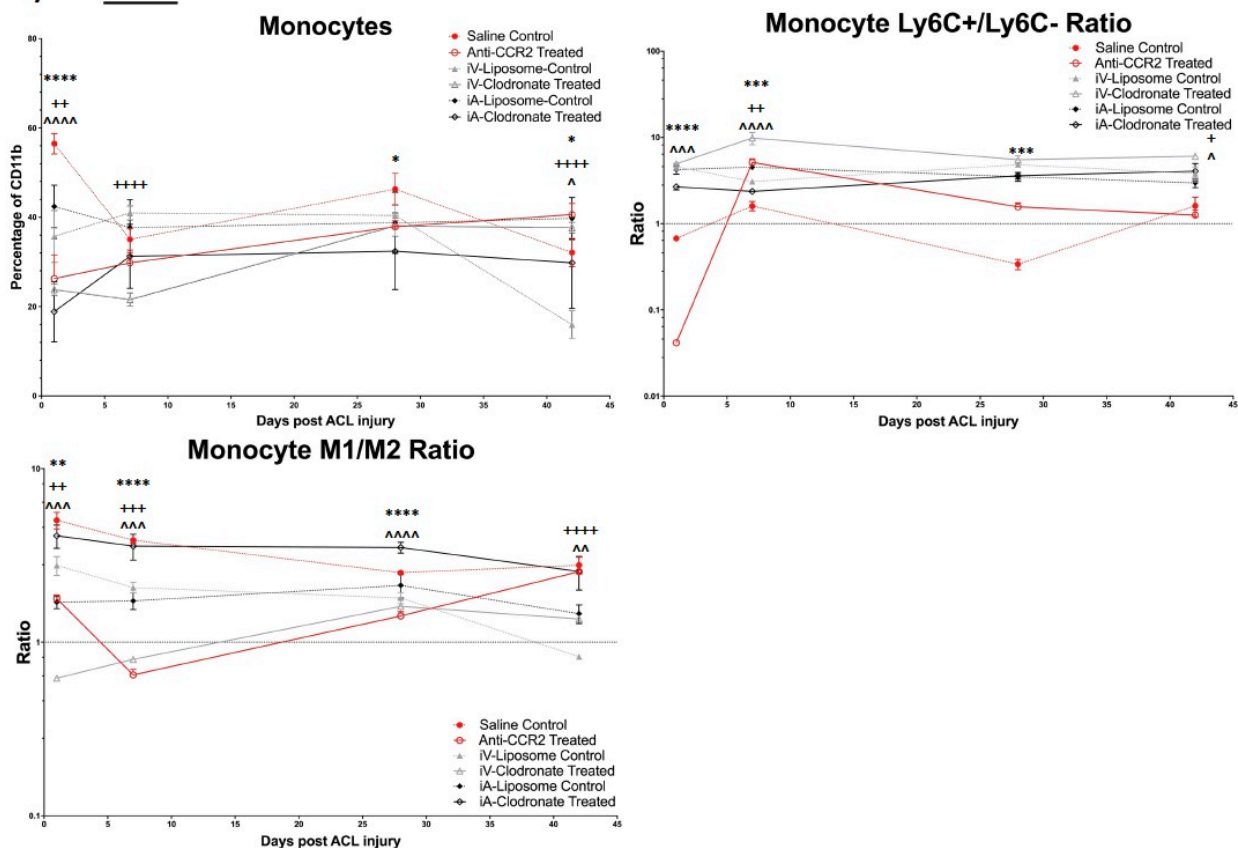


Figure 2:

A) Blood



B) Bone Marrow

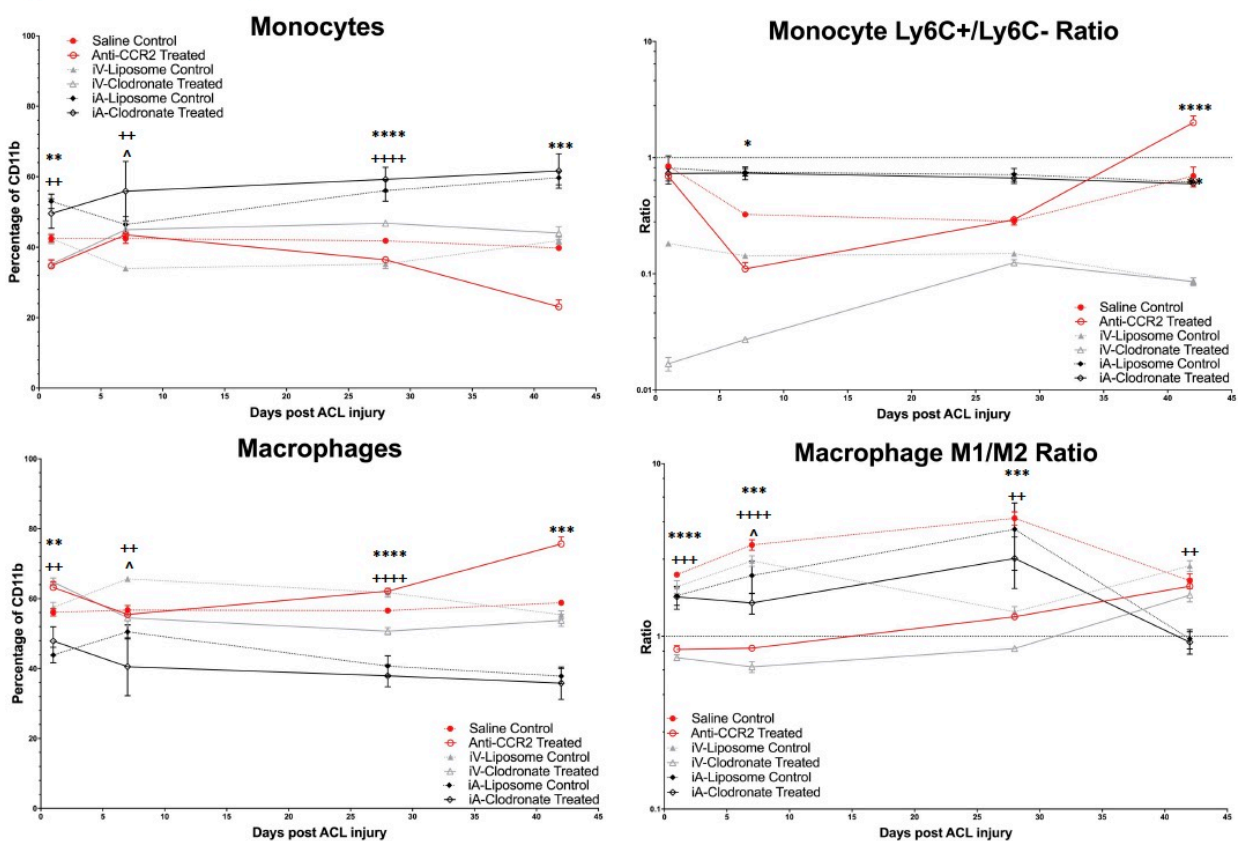


Figure 3:

Synovium

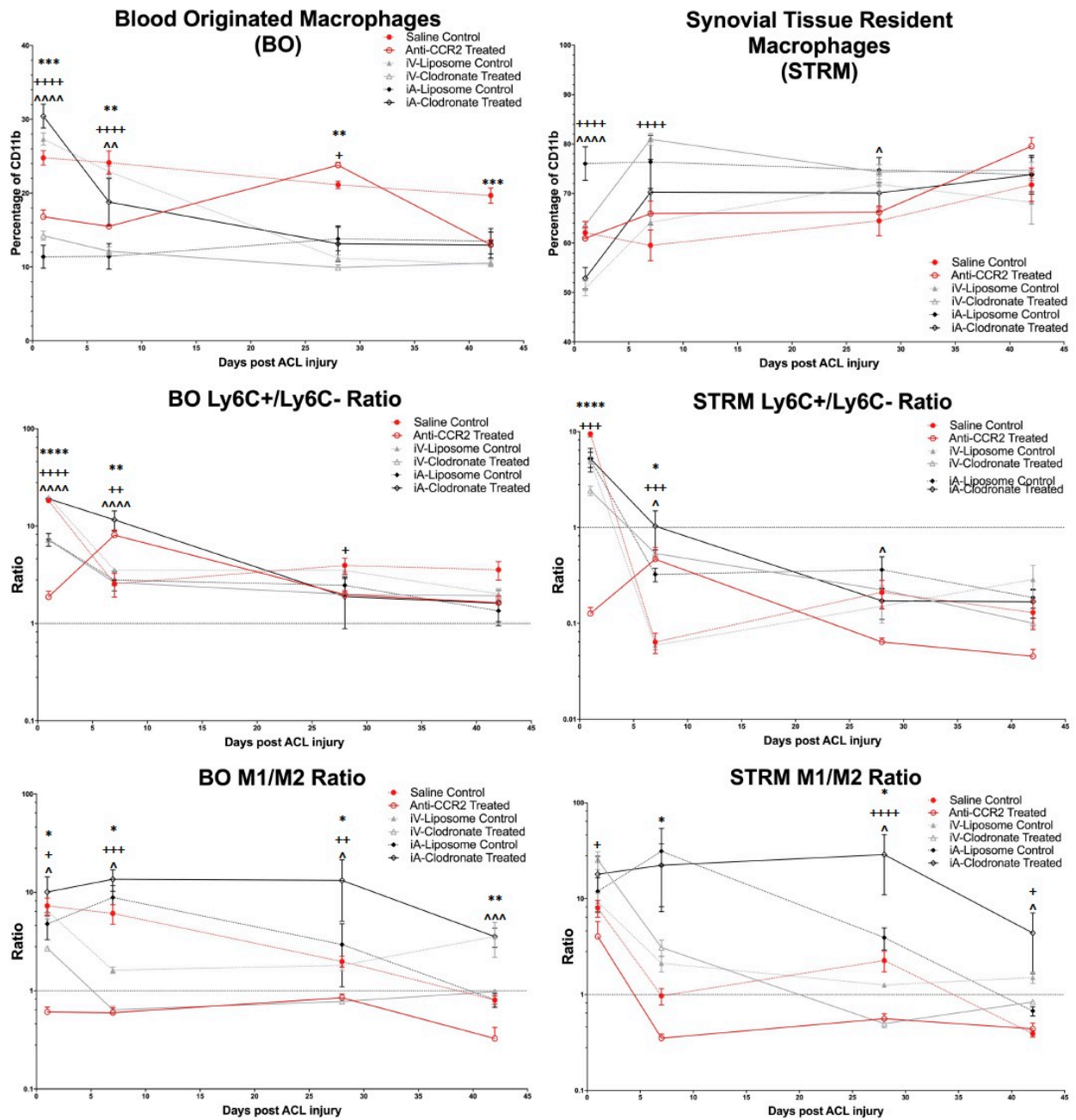
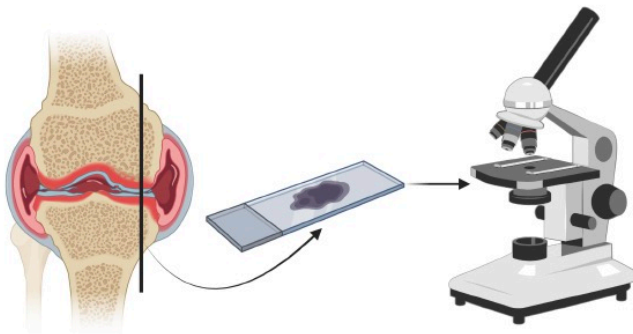


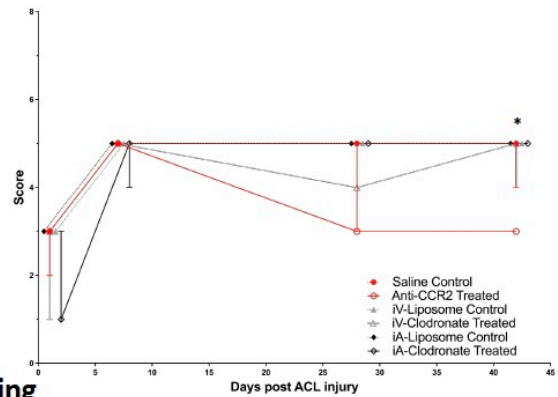
Figure 4:

A)



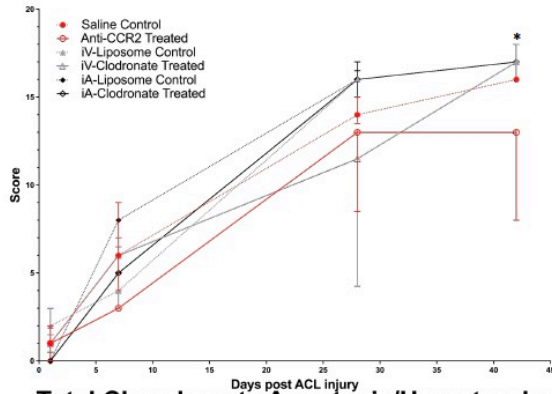
B) Fibrosis

Maximum Fibrosis

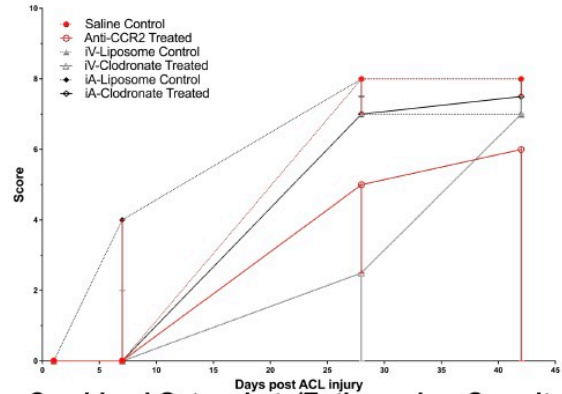


C) Osteochondral Joint Damage and Bone Remodeling

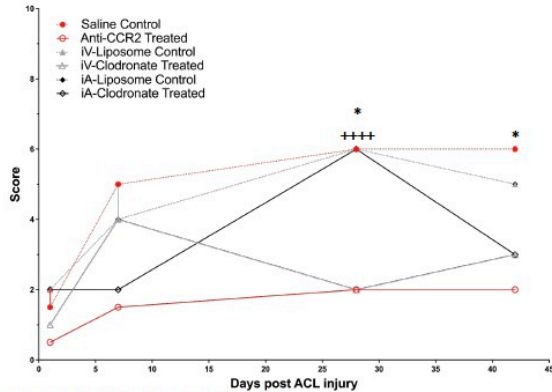
Total Cartilage Damage



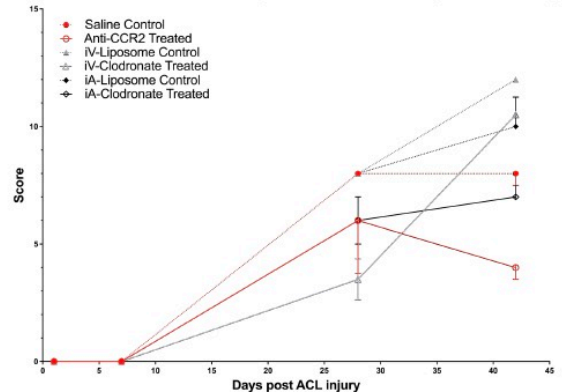
Tibial Osteochondral Damage



Total Chondrocyte Apoptosis/Hypertrophy

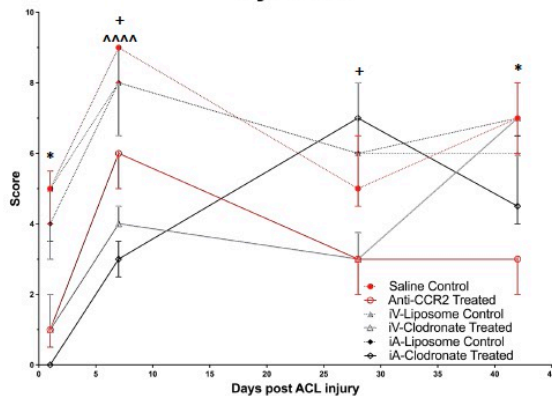


Combined Osteophyte/Enthesophyte Severity



D) Synovial Inflammation

Synovitis



Synovial Hyperplasia

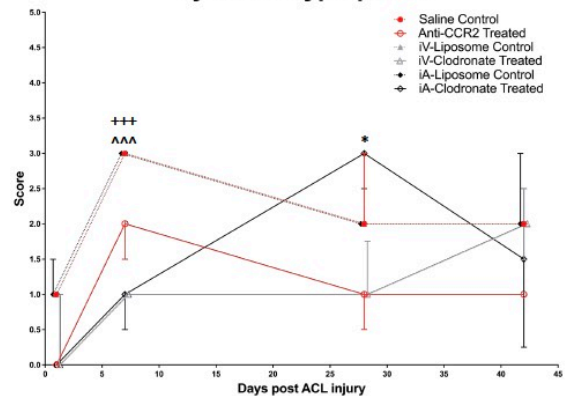


Figure 5:

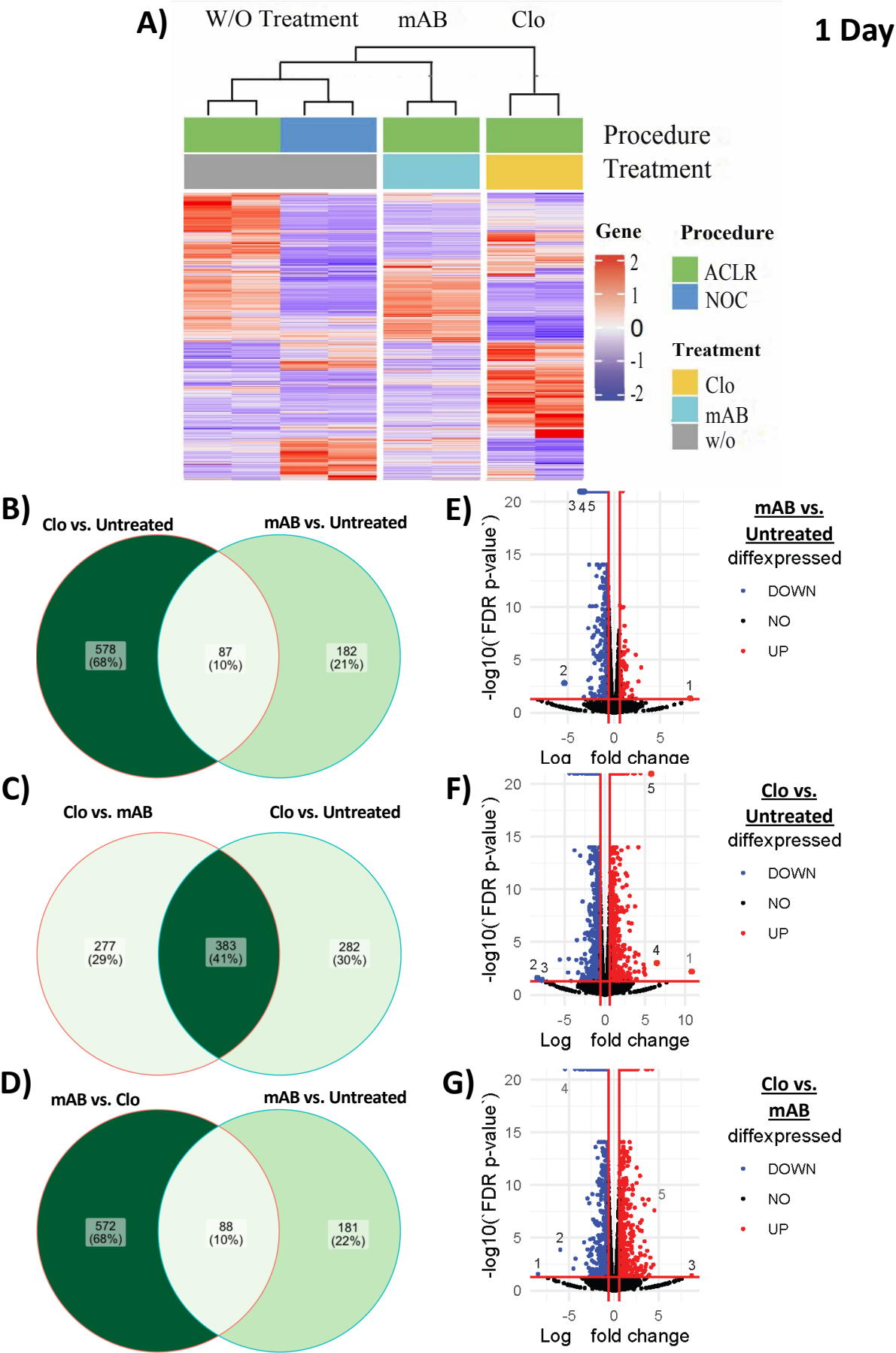


Figure 6:

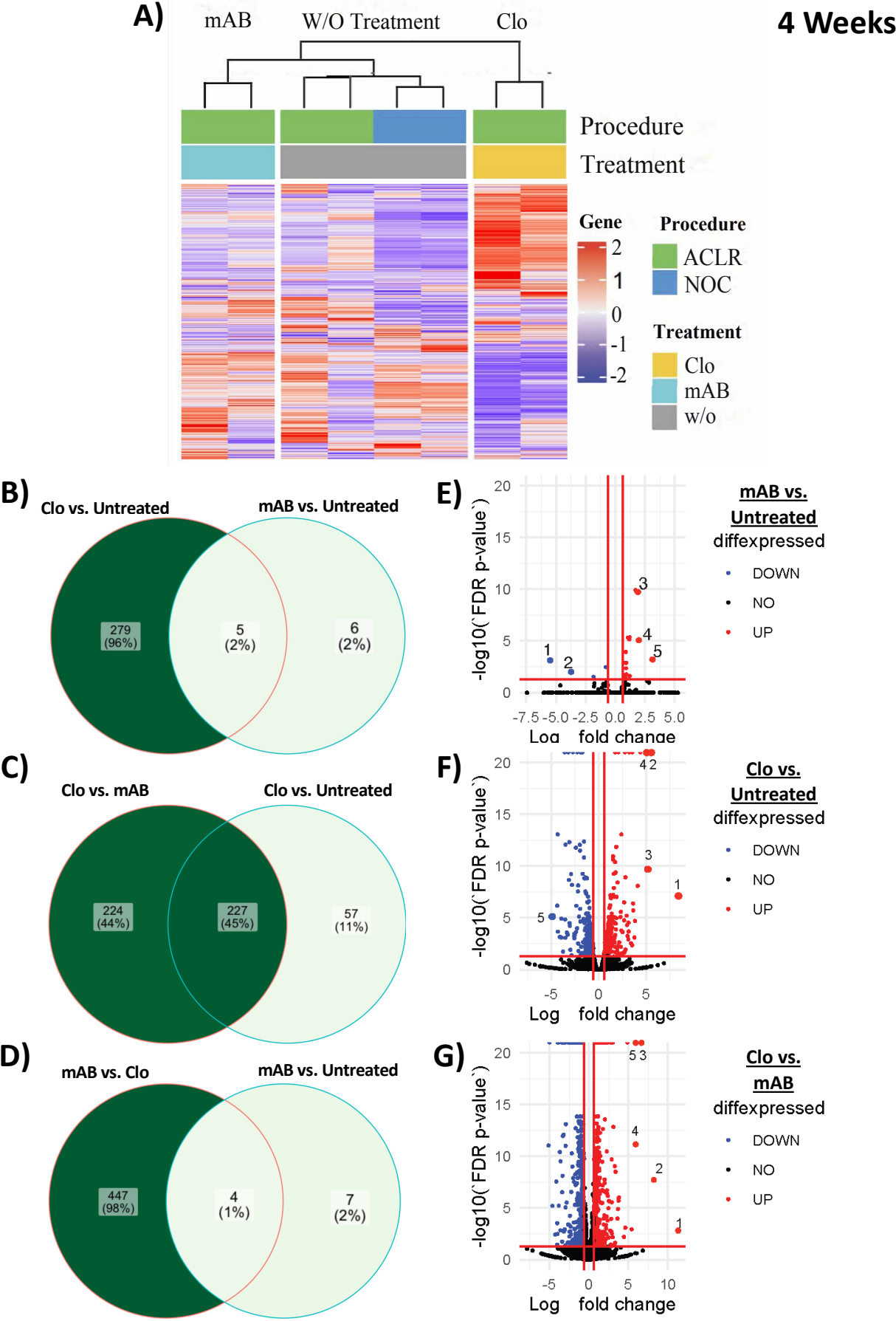


Table 1: Top 10 upregulated GSEA pathways per comparison and time point.

1 Day Post Injury				4 Weeks Post Injury			
mAB vs. Untreated		NES	P-value	mAB vs. Untreated		NES	P-value
1	GO_CYTOKINE_ACTIVITY	1.607398082	0.043878094	N/A			
2	GO_PROTEINACEOUS_EXTRA CELLULAR_MATRIX	1.447842905	0.045137619				
Clo vs. Untreated				Clo vs. Untreated			
1	GO_CORNIFIED_ENVELOPE	1.898568647	0.025568219	1	GO_MYELOID_LEUKOCYTE_MI GRATION	1.952275543	0.002553624
2	GO_CYTOKINE_ACTIVITY	1.78230508	0.006045744	2	GO_GRANULOCYTE_MIGRATI ON	1.948114957	0.01300742
3	GO_STRIATED_MUSCLE_CON TRACTION	1.688550154	0.021656297	3	GO_MONOCYTE_CHEMOTAXI S	1.943827509	0.049959862
4	GO_HEART_PROCESS	1.664139555	0.021656297	4	GO_CELLULAR_RESPONSE_TO INTERLEUKIN_1	1.90521406	0.006741856
5	GO_CONTRACTILE_FIBER	1.656291023	0.008763047	5	GO_CHONDROCYTE_DIFFERE NTIATION	1.881796864	0.032355971
6	GO_MUSCLE_CELL_DEVELOP MENT	1.647902722	0.033817998	6	GO_RESPONSE_TO_INTERLEU KIN_1	1.827358763	0.01300742
7	GO_CYTOKINE_RECEPTOR_BI NDING	1.589857924	0.0148951	7	GO_CYTOKINE_ACTIVITY	1.690895856	0.028632026
8	GO_MUSCLE_CELL_DIFFEREN TIATION	1.575771409	0.0148951	8	GO_CARTILAGE_DEVELOPME NT	1.674590984	0.042261723
9	GO_MUSCLE_SYSTEM_PROCE SS	1.557375904	0.014305273	9	GO_POSITIVE_REGULATION OF_EPITHELIAL_CELL_PROLIFE RATION	1.625627972	0.049959862
10	GO_MUSCLE_ORGAN_DEVEL OPMENT	1.554141566	0.0148951	10	GO_SKELETAL_SYSTEM_MOR PHOGENESIS	1.601571485	0.032355971
Clo vs. mAB				Clo vs. mAB			
1	GO_CORNIFIED_ENVELOPE	2.001483264	0.000779995	1	GO_CHONDROCYTE_DIFFERE NTIATION	1.891976723	0.022653629
2	GO_DISRUPTION_OF_CELLS_ OF_OTHER_ORGANISM	1.992298424	0.000779995	2	GO_GRANULOCYTE_MIGRATI ON	1.859586621	0.022653629
3	GO_KILLING_OF_CELLS_OF_O THER_ORGANISM	1.992298424	0.000779995	3	GO_MYELOID_LEUKOCYTE_MI GRATION	1.85148507	0.011292766
4	GO_IMMUNOLOGICAL_SYNAP SE	1.95090184	0.000959132	4	GO_LEUKOCYTE_CHEMOTAXIS	1.750215553	0.022653629
5	GO_NEGATIVE_REGULATION OF_VIRAL_GENOME_REPLIC ATION	1.946322695	0.000584765	5	GO_RESPONSE_TO_TUMOR_ NECROSIS_FACTOR	1.600890809	0.021421286
6	GO_NEUTROPHIL_MEDIATED_ IMMUNITY	1.923325306	0.002399371	6	GO_CYTOKINE_RECEPTOR_BI NDING	1.589533926	0.022653629
7	GO_DISRUPTION_OF_CELLS_ OF_OTHER_ORGANISM_INVOL VED_IN_SYMBIOTIC_INTERA CTION	1.880100293	0.008809664				
8	GO_KILLING_OF_CELLS_IN_O THER_ORGANISM_INVOLVED _IN_SYMBIOTIC_INTERACTIO N	1.880100293	0.008809664				
9	GO_POSITIVE_REGULATION OF_PROTEIN_INSERTION_INT O_MITOCHONDRIAL_MEMBR ANE_INVOLVED_IN_APOPTOT IC_SIGNALING_PATHWAY	1.873025175	0.01218886				
10	GO_REGULATION_OF_PROTEI N_INSERTION_INTO_MITOCH ONDRIAL_MEMBRANE_INVOL VED_IN_APOPTOTIC_SIGNALI NG_PATHWAY	1.873025175	0.01218886				

Table 2: Top 10 downregulated GSEA pathways per comparison and time point.

1 Day Post Injury			4 Weeks Post Injury		
mAB vs. Untreated	NES	P-value	mAB vs. Untreated	NES	P-value
1 GO_INTERMEDIATE_FILAMENT	-2.385193534	4.94268E-24	N/A		
2 GO_INTERMEDIATE_FILAMENT_CYTOSKELETON	-2.344234708	1.06422E-25			
3 GO_KERATIN_FILAMENT	-2.274918096	1.29267E-14			
4 GO_HAIR_CYCLE	-2.1808461	1.49074E-11			
5 GO_MOLTING_CYCLE	-2.1808461	1.49074E-11			
6 GO_EPIDERMIS_MORPHOGENESIS	-1.922385959	0.0009427			
7 GO_NEGATIVE_REGULATION_OF_VIRAL_GENOME_REPLICATION	-1.913181551	0.000931448			
8 GO_NEGATIVE_REGULATION_OF_B_CELL_PROLIFERATION	-1.911350777	0.002473688			
9 GO_SKIN_EPIDERMIS_DEVELOPMENT	-1.883308182	0.000599719			
10 GO_INTERMEDIATE_FILAMENT_BINDING	-1.871059627	0.000370091			
Clo vs. Untreated			Clo vs. Untreated		
1 GO_INTERMEDIATE_FILAMENT_CYTOSKELETON	-2.727025318	7.27818E-28	1 GO_U2_TYPE_SPLICEOSOMAL_COMPLEX	-2.163127081	1.02157E-07
2 GO_INTERMEDIATE_FILAMENT	-2.717612907	4.25144E-23	2 GO_CATALYTIC_STEP_2_SPLICEOSOME	-1.989032689	0.000132267
3 GO_KERATIN_FILAMENT	-2.631455897	5.31072E-15	3 GO_SLEEP	-1.83785914	0.042261723
4 GO_HAIR_CYCLE	-2.202489003	6.47113E-08			
5 GO_MOLTING_CYCLE	-2.202489003	6.47113E-08			
6 GO_INTRACELLULAR_ESTROGEN_RECEPTOR_SIGNALING_PATHWAY	-2.029167154	0.0148951			
7 GO_VIRAL_GENOME_REPLICATION	-2.01578286	0.0148951			
8 GO_AGING	-1.704991888	7.77891E-06			
9 GO_RIBOSOME_BIOGENESIS	-1.612722597	0.006045744			
10 GO_NCRNA_PROCESSING	-1.477590603	0.02363793			
Clo vs. mAB			Clo vs. mAB		
1 GO_COLLAGEN_FIBRIL_ORGANIZATION	-1.957373976	0.002801814	1 GO_U2_TYPE_SPLICEOSOMAL_COMPLEX	-2.117067584	2.33768E-06
2 GO_ENDOPLASMIC_RETICULUM_GOLGI_INTERMEDIATE_COMPARTMENT_MEMBRANE	-1.854787304	0.002399371	2 GO_CATALYTIC_STEP_2_SPLICEOSOME	-1.770259919	0.043527664
3 GO_ENDOPLASMIC_RETICULUM_GOLGI_INTERMEDIATE_COMPARTMENT	-1.839835739	0.000574822			
4 GO_PROTEOGLYCAN_BINDING	-1.790549998	0.039545347			
5 GO_COLLAGEN_BINDING	-1.712524266	0.021983756			
6 GO_COLLAGEN_TRIMER	-1.709083485	0.032748081			
7 GO_POSITIVE_REGULATION_OF_CELL_SUBSTRATE_ADHESION	-1.702632812	0.008809664			
8 GO_PROTEINACEOUS_EXTRACELLULAR_MATRIX	-1.60389123	0.000779995			
9 GO_EXTRACELLULAR_MATRIX_COMPONENT	-1.596687305	0.032748081			
10 GO_CELL_MATRIX_ADHESION	-1.538971334	0.035159525			

Table 3: Top 5 DEGS per comparison and time point.

1 Day Post Injury				4 Weeks Post Injury			
	mAB vs. Untreated	Log ₂ fold change	P-value		mAB vs. Untreated	Log ₂ fold change	P-value
1	Fam177a2	8.251031595	0.004477848	1	Gm49388	-5.498258871	3.69366E-07
2	Dppa3	-5.386982549	8.93158E-05	2	H2ac19	-3.73970665	7.53176E-06
3	Arg1	-3.702545494	0	3	Angptl4	1.067243429	1.05094E-09
4	Apoc2	-3.655969572	0	4	Has1	1.15769597	1.93317E-09
5	Cx3cr1	-3.375912108	0	5	Tppp	1.195402653	7.45516E-10
	Clo vs. Untreated				Clo vs. Untreated		
1	Fam177a2	10.79583422	0.000677368	1	Rnase2a	8.320759409	2.45155E-10
2	Krt71	-8.355108751	0.004662645	2	Fam177a2	5.350918404	0
3	1810049J17Rik	-7.947567043	0.007123406	3	Arg1	5.182133764	4.07008E-13
4	Fads2b	6.420589889	7.71131E-05	4	Ccl17	5.036669441	0
5	Prnd	5.739585817	0	5	Npy2r	-4.934468709	3.28629E-08
	Clo vs. mAB				Clo vs. mAB		
1	Gm49388	-8.514262905	0.003597088	1	Fam177a2	11.24955483	7.50431E-05
2	Nyap2	-6.01272387	8.00449E-06	2	Rnase2a	8.233645967	3.25946E-10
3	Fads2b	8.717890533	0.00553324	3	Ccl17	6.627442333	0
4	C7	-5.473909379	0	4	Arg1	5.887521623	7.80487E-14
5	Arg1	4.533672454	7.20828E-10	5	H2ac19	5.853943482	0

Fam177a2: Family With Sequence Similarity 177, Member A2; Dppa3: Developmental Pluripotency Associated 3; Arg1: Arginase 1; Apoc2: Apolipoprotein C2; Cx3cr1: C-X3-C Motif Chemokine Receptor 1; Krt71: Keratin 71; 1810049J17Rik: RIKEN cDNA 1810049J17 gene; Fads2b: Fatty Acid Desaturase 2B; Prnd: Prion Like Protein Doppel; Gm49388: predicted gene, 49388; Nyap2: neuronal tyrosine-phosphorylated phosphoinositide 3-kinase adaptor 2; C7: complement component 7; H2ac19: H2A Clustered Histone 19; Angptl4: Angiotensin-like 4; Has1: Hyaluronan synthase 1; Tppp: Tubulin Polymerization Promoting Protein; Rnase2a: Ribonuclease A Family Member 2; Ccl17: C-C Motif Chemokine Ligand 17; Npy2r: Neuropeptide Y Receptor Y2;

Supplementary Table 1:

Details for each antibody used in the T-cell panel are given. In addition, information is provided for the relevant buffers used.

Supplementary Table 1: Used Antibodies for Flow Cytometry

Name of Material/ Equipment	Manufacturer	Catalog Number	Comments/Description
Fixable Viability Stain 510 100ug	BD	564406	Monocyte Panel
Ms F4/80 BV421 T45-2342 50ug	BD	565411	Monocyte Panel
Ms CD80 BV650 16-10A1 50ug	BD	563687	Monocyte Panel
Ms I-A I-E BV711 M5/114.15.2 50ug	BD	563414	Monocyte Panel
Ms Ly-6G APC-Cy7 1A8 50ug	BD	560600	Monocyte Panel
Ms CD11C PE-CF594 HL3 100ug	BD	562454	Monocyte Panel
Ms Ly-6C PE-Cy7 AL-21 50ug	BD	560593	Monocyte Panel
Ms CD206 Alexa 647 MR5D3 25ug	BD	565250	Monocyte Panel
Ms CD11b APC-R700 M1/70 100ug	BD	564985	Monocyte Panel
Brilliant Stain Buffer Plus 1000Tst	BD	566385	Buffers

5.3 Main Findings of the Study

Depletion of Ly6C^{high} monocytes via systemic anti-CCR2 treatment in the immediate peri-injury time period led to a lasting mitigation of chondrocyte hypertrophy and apoptosis as well as a reduced synovitis and synovial lining hyperplasia on a histopathological level. On a cellular level anti-CCR2 treatment led reduced monocyte influx into the joint and an overall anti-inflammatory cellular microenvironment. Joint damage was ameliorated after anti-CCR2 treatment and despite the short-term treatment that started prior to injury, lasting mitigation of bone and cartilage damage were detected. Treatment with IV clodronate-liposomes resulted in a number of similar changes as anti-CCR2 in systemic myeloid cells. This can be explained by the overlap in targeted myeloid cells of the two treatments whereas the inflammatory reaction caused by the broad apoptosis of immune cells resulting from IV clodronate-liposomes counteract some of the anti-inflammatory effects observed when only targeting a specific subset of pro-inflammatory myeloid cells (anti-CCR2). Thereby the differences observed subsequent to a treatment having both a pro- and anti-inflammatory effect (IV clodronate-liposomes) compared to a targeted depletion of pro-inflammatory monocyte subsets (anti-CCR2) suggest the importance of the immediate post-injury inflammatory response in long-term joint inflammation and ptOA-risk. This may explain the distinct temporal differences in effect of the two treatments on ptOA pathology and lack of sustained or significant disease modification with IV clodronate-liposomes. Despite a short-lived reduction in histological synovitis joint damage was unaltered by STRM depletion. On a cellular level depletion of STRM led to a more distinct pro-inflammatory response with a significantly increased synovial BO macrophage Ly6C⁺/Ly6C⁻ and M1/M2 ratio. Interestingly, although the depletion of STRM was performed prior to joint injury, lasting pro-inflammatory changes were observed throughout the study period. In addition, GSEA analysis indicated that even after 4 weeks immune cell chemotactic and migratory pathways were increased supporting an ongoing inflammatory response. Again, this indicates that acute peri-injury changes in the joint-inflammatory status are critical for long-term joint inflammation and ptOA risk.

Chapter 6

6 Evaluating CD40:CD40L as a Potential Early Target in the Treatment of ptOA

6.1 Aim of the Study

Data from **Chapter 4** and **Chapter 5**, acquired in addition to the main immune cell focus of the respective studies (and thus not presented previously), indicated that a depletion of immune cell subsets of either the adaptive or innate immune system prior to joint injury had a broader anti-inflammatory effect beyond the targeted cells on pro-inflammatory cells from the other (non-targeted) arm of the immune response. This data indicated a potentially crucial role for factors orchestrating the interplay between key players of the innate and adaptive immune response which could serve as a promising target for an early immunomodulatory treatment of ptOA. The CD40/CD40L axis has been established as a prominent orchestrating factor and thus became the focus of this subsequent study.

CD40L, also known as CD154, is a type II transmembrane protein almost exclusively expressed on the surface of activated CD4⁺ T-cells (Elgueta, R. et al. 2009; Graf, D. et al. 1995), whereas its receptor CD40 is expressed primarily on antigen presenting cells (APCs) and B-cells (Bretscher, P. A. 1999; Elgueta, R. et al. 2009). CD40/CD40L interactions stimulate a broad pro-inflammatory response (Zirlik, A. et al. 2007) including macrophage activation and promotion of a Th1 immune response (Soong, R. S. et al. 2014) with the potential to initiate a pro-inflammatory amplification loop. Thus, this study was designed to inhibit the CD40:CD40L pathway to investigate the therapeutic potential of an early inhibition of factors orchestrating the interplay between innate and adaptive inflammation post joint injury.

6.2 Drafted Manuscript for Submission: In-vivo evaluation of CD40 ligand as an early potential target in the treatment of post-traumatic osteoarthritis

In-vivo evaluation of CD40 ligand as an early potential target in the treatment of post-traumatic osteoarthritis

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Introduction:

Osteoarthritis (OA) is a multi-factorial and slowly progressing disease of synovial joints [1] ending in structural damage to all joint tissue, pain and disability affecting patients on an individual level and, due to the high global prevalence, society as a whole on a socioeconomic level [2]. Despite increased research efforts over the last decades to further improve understanding of the underlying mechanisms driving OA, to date the most effective treatment option to improve the quality of life of patients remains joint replacement which due to cost and implant life is largely only done at end-stage disease [1]. Therapies that can be implemented earlier to prevent structural damage and functional consequences are urgently needed [3]. In recent years a variety of treatments have entered the clinical trial stage targeting different aspects of OA (such as cartilage destruction, inflammation, or associated pain [1, 4]) albeit with limited success. One potential explanation could be that to date clinical studies have included patients with already established structural and symptomatic OA which might prove to be too late for a preventative or reversative treatment approach.

Substantial evidence indicates a crucial role for both the adaptive and innate immune system in the pathogenesis of OA [5], and immunomodulation has been proposed as a promising early treatment modality [6]. Adaptive immune targets have been identified as proinflammatory CD4⁺ T-helper (Th) cell subsets Th1 and Th17 that promote OA development [7]. In particular, a skewed proinflammatory differentiation towards Th1 cells has been demonstrated in the synovial fluid and membrane of early OA patients [8]. On the innate immune side, monocyte-derived macrophages [9, 10] polarizing into their pro-inflammatory M1 state [11, 12] have been shown to promote and sustain inflammation during OA development while contributing directly towards cartilage destruction via secretion of IL-1 β and TNF- α as well as catabolic enzymes such as ADAMTS-4 and MMP-13 [13-15]. While the effects of a targeted depletion of individual cellular components has been explored in previous studies of our group [16] (see

Chapter 4.2 and **Chapter 5.2**), an intricate interplay between cells of the adaptive and innate immune system exists that might compensate immunomodulatory effects originating from an individual/selective approach [17]. Targeting molecular mechanisms orchestrating this innate/adaptive interplay pose a promising potential treatment approach.

The ligand for CD40 (CD40L; also known as CD154) is a type II transmembrane protein of the TNF superfamily almost exclusively expressed on the surface of activated CD4⁺ T-cells [18, 19]. The receptor, CD40, is expressed primarily on antigen presenting cells (APCs) and B-cells [18, 20]. CD40/CD40L interactions stimulate a broad pro-inflammatory response [21] including macrophage activation and promotion of a Th1 immune response [22] with the potential to initiate a pro-inflammatory amplification loop. While an overexpression of both CD40L [23-25] and CD40 [26] has been shown in rheumatoid arthritis, the role of the CD40/CD40L axis in OA is presently unknown.

In the current paper we report on the “cross-immune-system” effects from previous interventional studies independently targeting specific aspects of either the adaptive (**see Chapter 4.2**) or innate (**see Chapter 5.2**) immune system in a non-surgical murine post-traumatic (pt)OA model (i.e. innate immune cell changes when targeting T-cells, and vice-versa). We then describe the effects on the inflammatory/immune response and the onset and development of ptOA, of directly targeting the innate-adaptive immune system interaction by inhibiting CD40L.

Materials and Methods

Animals and ptOA model

All experiments were approved by the Northern Sydney Local Health District Animal Ethics Committee (protocols RESP/19/067, RESP20/082, RESP20/083 and RESP21/014), with DEPART and ARRIVE guidelines followed for experimental design and reporting [27, 28]. The present manuscript includes presentation of previously unpublished data from earlier studies inhibiting IL12/p40 (affecting development of Th1 and Th17 T-cells; **see Chapter 4.2**) and CCR2 (depleting of Ly6C^{high} monocytes; **see Chapter 5.2**) in mouse ptOA. In this study new experiments investigating the effect of targeting CD40L in ptOA used a total of 240 10-12-week-old, male C57BL/6 mice, with animals housed as described in earlier studies (see **Chapter 4.2** and **Chapter 5.2**) in individually-ventilated cages (up to 5/cage) with commercial bedding, environmental enrichment (perspex housing, wooden sticks, tissues), *ad-libitum* access to standard pelleted mouse feed and acidified water, in a dedicated SPF vivarium maintained at 20-22 °C with 12-hour light/dark cycles, and cared for in accordance with the Australian code [29]. An overview over the study design, sample size per experiment and the allocation of animals is provided in Figure 1. Animals housed in the same cage were randomly assigned to each set of experiments but all animals in the cage were sacrificed at the same time (see below) to avoid isolation stress.

The non-surgical Anterior Cruciate Ligament Rupture (ACLR) model [30, 31] was used to induce ptOA. Briefly, a single compressive load was applied to the tibia of the flexed knee in mice anesthetized with isoflurane in oxygen, resulting in posterior subluxation of the femur and ACL rupture without additional macroscopic damage to other structures within the joint [31]. ACLR results in rapid onset and progression of joint-wide ptOA structural pathology accompanied by acute post-injury synovitis and loco-regional innate and adaptive immune response (see **Chapter 2.2.2.2**, **Chapter 3.2**, **Chapter 4.2**, and **Chapter 5.2**). Age- and sex

matched naïve non-operated control (NOC) mice were included in a subset of experiments as indicated in the respective results section.

In-vivo experiments

Details regarding IL12/p40 and CCR2 inhibition with specific antibodies, along with their respective controls, have been published previously (see **Chapter 4.2** and **Chapter 5.2**). Two different approaches to target CD40L (with separate controls) were utilised, resulting in four treatment groups for this arm of the study as detailed below. An established and commercially available anti-CD40L antibody (InVivoMAb anti-mouse CD40L, BioXCell, catalogue #BE0017) or the respective isotope control, as advised by the manufacturer (InVivoMAb polyclonal Armenian hamster IgG, BioXCell, catalogue #BE0091), was administered 3 times by intraperitoneal injection (500µg 6, 24 and 72 hours after ACLR for each animal). A novel small-molecule inhibitor (SMI) of the CD40/CD40L axis (DRI-C21045; MedChemExpress, catalogue #HY-120323) [32] that can be injected subcutaneously was used to explore an alternative targeting pathway. A 10mM solution of DRI-C21045 in 30% 2-Hydroxypropyl- β -cyclodextrin (HP- β -CD) was prepared fresh before administration as published previously [32]. Animals received 250µl subcutaneous injections 6, 24, 48 hours after ACLR of either DRI-C21045 (1.6mg/mouse/injection) or carrier solution (as control). As a general rule for all treatments animals harvested 1-day post-ACLR only received the first injection. As in the previous IL12p40 and CCR2 inhibition experiments, day 0 in the current CD40L studies was defined as the day of ACLR, with tissues harvested day-1, week-1, -4 or -6 (n = 12 mice/group/time).

Flow cytometry experiments

Blood (terminal cardiac puncture under general anaesthesia), contralateral femoral bone marrow, and synovial tissue were harvested from all animals as described previously [33] (see **Chapter 2.2.2.1**). In the current CD40L inhibition studies tissues were pooled randomly within groups from three animals to increase cell numbers per sample resulting in n=3 replicates for each tissue-source at each time point in each animal group (9 mice/group/time point resulted in n = 3 biological replicates/group/time). The detailed protocol that was followed regarding the harvest of tissues and preparation of single cell solutions and the utilized multi-color antibody panel has been validated and published previously [33] (see **Chapter 2.2.2.1** and **Chapter 3.2, Chapter 4.2 and Chapter 5.2**). All gating and flow cytometry were done by a single researcher (PH), batched by timepoint but with randomization and concealment of intervention group in each batch. Additional information regarding the utilized antibodies in the current study can be found in Table S1.

Gene Expression Analysis

As outlined in Fig. 1 two cohorts were used for gene expression analysis. Total RNA was isolated from the synovial tissue and lymph node of:

- 1) Untreated ACLR and NOC mice (n = 6/group/time point) as baseline levels without intervention.
- 2) Animals receiving treatment as described in ‘In-vivo experiments’ (n = 3/group/time point) to assess the influence of the treatment on the genomic expression of CD40 and CD40L.

In particular, tissues were harvested and immediately snap frozen in liquid nitrogen and stored at -80C until further processing. Total RNA was isolated (tissue (freeze pulverized): RNeasy Micro Kit, Qiagen cat# 74004; following manufacturer’s protocols), quantified using Nanodrop and then an equal quantity (1ug) RNA reverse transcribed for all samples (GoScript

RT kit, Promega cat# A2791) as previously described [34-37]. Primers were designed using MacVector (see Table S2 for individual primer sequences) and PCR specificity confirmed by product sequencing and melt-curve analysis [34-37].

Histopathological scoring

After removal of the anterior synovium (for flow cytometry) knee joints were fixed, decalcified, paraffin-embedded, and serial sagittal sections across the medial femoro-tibial joint cut and stained with toluidine blue-fast green as previously described (n = 9 mice/group/time point) [35, 36, 38]. Utilizing a modified single slide scoring system [39] (see **Chapter 2.2.2.2**) femoral and tibial cartilage and structural damage, tibial and femoral chondrocyte hypertrophy and apoptosis, anterior/posterior tibial/femoral osteophyte/enthesophyte size and maturity, fibrotic changes in the joint capsule and posterior femoral synovitis, were determined by a single trained observer (PH) blinded to time and treatment. Finally, median values for each histopathological feature for each group and time point were generated and compared as described below.

Statistics

The individual histology-outcomes were considered as categorical and independent variables, as prior analysis demonstrates they change differentially and with distinct temporal patterns in different OA models [39], and are independently modified in response to treatment/genetic-modification [35-38, 40]. Non-parametric analyses (Kruskal-Wallis and Mann-Whitney-U) were used to assess differences between groups for all histological-outcomes at each post-injury timepoint. Histological data is presented as median $\pm$ error. Flow cytometry and PCR data is presented as mean $\pm$ standard error of the mean (SEM). Two-way analysis of variance (ANOVA) with post-hoc Tukey's correction for multiple comparisons was used to compare all

individual variables. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***) and $p < 0.0001$ (****). All statistical calculations were performed with GraphPad Prism (Version 9.0.0).

Results

Co-dependent effects of selective innate or adaptive immune system inhibition during onset and development of ptOA

In a previous study conducted in our group using the same ACLR-ptOA model, we targeted the IL12p40 pathway affecting development of both Th1 and Th17 cells in response to joint injury and investigated the effects on the onset and development of ptOA (Fig. 2A) (see **Chapter 4.2**). Despite peri-injury inhibition of IL12p40 being specifically directed towards and significantly decreasing synovial Th1 and Th17 T-cell polarization (see **Chapter 4.2**), this treatment also led to a significantly lower synovial M1/M2 macrophage ratio not only in blood originated (BO) macrophages but also in synovial tissue resident macrophages (STRM) between 1- and 4-weeks post injury (Fig. 2B). Similarly, peri-injury anti-CCR2 administration targeting Ly6C^{high} monocytes (Fig. 2A) not only depleted synovial BO and STRM macrophage numbers and their M1/M2 polarization (see **Chapter 5.2**), but also led to a reversed inflammatory state of synovial CD4⁺ T-helper cells observed by a ratio <1 of Th1/Th2 and Th17/Treg polarization for the 6-week duration of the study (Fig. 2C). Taken together these results indicate that specific depletion of immune cell subsets of either the adaptive or innate immune system in the peri-injury period has a broader anti-inflammatory effect significantly modulating cells in the other arm of the immune response. These results confirm the importance of crosstalk between innate and adaptive immune cell subsets during pathogenesis of ptOA.

Expression of CD40:CD40L is modulated in ACLR.

To evaluate if the CD40/CD40L axis may be involved in the pathogenesis of ptOA we compared the mRNA expression of both genes in ACLR compared with NOC (Fig. 3). Levels of both CD40L and CD40 mRNA were significantly transiently (day 1) increased in synovium but unchanged in draining lymph-nodes after joint injury (Fig. 3A and B, upper panels). Over time levels of both CD40 and CD40L mRNA decreased and were comparable to NOCs after 4 weeks. Anti-CD40L treatments induced very little change in CD40L mRNA in synovium or draining lymph-node, with only mAB treated mice at 1-day showing an increase in expression in lymph-node relative to controls (Fig 3A and B lower panels). Similarly, effects on CD40 mRNA expression were also limited, again the mAB induced transient (day-1) increase in in draining lymph-node with both agents decreasing CD40 mRNA in lymph-node at week 6 relative to controls.

Anti-CD40L treatment modulates synovial and systemic innate and adaptive immune response.

Despite the lack of effect on CD40/CD40L mRNA expression, peri-injury anti-CD40L treatments had a significant impact on the synovial innate and adaptive cell influx following ACLR (Fig. 4). The anti-CD40L mAB significantly decreased synovial CD4⁺ T-helper cells between 1-and 4-weeks, while the effect of the SMI was less and not evident until 4-weeks (Fig 4A). By 6 weeks there was no difference in synovial CD4⁺ T-helper cell expression between either anti-CD40L treatment and their controls. However, both anti-CD40L treatments had a significant anti-inflammatory immunomodulatory effect decreasing the synovial Th1/Th2 and Th17/Treg polarization over the entire study period (Fig. 4A). While not significantly different to each other there was some suggestion that the mAB had a more pronounced effect on Th1/Th2, while the SMI tended to be more active in reducing Th17/Treg. Analysis of synovial innate immune cell subsets indicated that the influx of blood originated

(BO) macrophages into synovium was unchanged until 6-weeks when numbers were decreased by both the mAB and SMI relative to their controls (Fig. 4B). In contrast, synovial BO macrophages had a clear anti-inflammatory M2 dominated polarization from week 1-4 with both anti-CD40L treatments. The number of STRM was also minimally affected by anti-CD40L, with the SMI actually increasing these cells acutely post-ACLR (day-1) while the mAB decreased STRM at 4-weeks after joint injury. Despite this, as with BO there were major effects on STRM M1/M2 polarization, with SMI-treated mice having an anti-inflammatory polarization ratio (<1) at 1-week while in mAB treated mice this did not occur until 4-weeks. To further evaluate the extent of the anti-CD40L treatment and analyse if a systemic effect was present, cells from blood and contralateral bone marrow were analysed using flow cytometry (Fig. 5). The anti-CD40L mAb induced a marked anti-inflammatory polarization in blood innate (M1/M2 day-1 to week-4; Ly6C^{high/low} day-1 to week-1) and adaptive (Th1/Th2 day-1 to week-4; Th17/Treg day-1 to week-1) immune cell subsets. The SMI on the other hand had less effect on blood M1/M2 and Th1/Th2 but similar suppression of Th17/Treg polarization. Surprisingly, treatment with SMI led to an increased Ly6C^{high/low} monocyte ratio in blood (Fig. 5A). While both anti-CD40L treatments had only minor influence on the overall percentages of macrophages in the contralateral bone marrow (Fig. 5B), they led to an anti-inflammatory M1/M2 polarization and as in blood, these changes were more pronounced with the mAB in comparison to SMI. Interestingly, analysis of adaptive immune cell subsets showed that a pro-inflammatory Th1/Th2 ratio (i.e. >1) persisted in all groups over the entire study period. However, mAB treatment led to a significantly lower ratio 1-week post injury onwards, whereas SMI treatment only led to a significant decrease acutely (day-1; Fig. 5B). In contrast with peripheral blood, anti-CD40L treatment had limited effect on bone-marrow Th17/Treg polarization.

Targeting anti-CD40L mitigated ptOA joint pathology and synovitis.

Tissues were harvested after 1 day, 1 week, 4 weeks and 6 weeks following joint injury and joint pathology compared between treatment groups and their respective controls (Fig. 6A). Evaluation of the fibrotic changes in the joint capsule showed that only the mAB significantly reduced synovial fibrosis (week-4) (Fig. 6B). Although treatment with the mAB reduced both the cartilage and osteochondral damage over time, differences were only significant at 6-weeks (total cartilage damage). While similar effects on cartilage and osteochondral damage were seen with the SMI at 4-weeks this did not persist at 6 weeks (Fig. 6C). The mAB also had a much more pronounced and persistent effect in reducing chondrocyte hypertrophy and apoptosis compared with the SMI. This same pattern was evident in osteophyte/enthesophyte severity, with the mAB having lower scores 1-week post injury onwards while SMI treatment only reduced week-4 scores (albeit at a non-significant extent). Histopathological assessment of total synovitis and the synovial hyperplasia sub-scores revealed a significant anti-inflammatory response after the mAB treatment (Fig. 6D).

Discussion

The findings from this study indicate that blockade of anti-CD40L has an immunomodulatory effect in context with ptOA. On a cellular level, it seems to lead to an anti-inflammatory microenvironment in the synovial tissue block by reducing the Th1/17 dominated adaptive immune response observed in the control group. Although the expression level of macrophages originating from peripheral blood did not decrease during early and intermediate stages of ptOA development anti-CD40L treatment resulted in an anti-inflammatory polarization of not only migrating macrophages but also STRM. Furthermore, we observed an immunomodulatory effect of the anti-CD40L treatment and a mitigation in joint pathology.

However, anti-CD40L treatment was not able to prevent degeneration and joint damage which might be explained by an ongoing biomechanical instability in the utilized murine ptOA model.

In recent years studies have investigated the role of the CD40:CD40L pathway in the pathogenesis of RA [41] [42]. Upregulation of CD40 was determined as a cause rather than a consequence during chronic inflammation [42], and a recent study that utilised a specific high-throughput drug screen of the CD40 signaling pathway in RA patients predicted that drugs attenuating the CD40 mediated inflammatory pathway will either protect from RA or mitigate symptoms during active disease [41]. Furthermore, in diseased tissues of RA patients the expression of CD40L and full-length CD40 was increased compared to healthy controls, and downstream mediators of the CD40L signaling pathway were identified as GPR120 and KDM6B [43]. Stimulation of CD40L led to a downregulation of GPR120 and upregulation of KDM6B [43]. Interestingly, GPR120 was later identified as an important regulator of the inflammatory response during development of OA, and GPR120 knockout mice had higher inflammation and cartilage damage compared to WT controls [44]. Additionally, KDM6B expression was increased in damaged cartilage compared to undamaged controls, implicating a role for KDM6B in OA articular cartilage damage and loss [45-47]. Drivers for an induced KDM6B expression have been identified as IL-1 β and TGF- β , both of which are a known promoters of OA development [45]. While the role of the CD40:CD40L is mostly unexplored in context with ptOA, dysregulation of CD40L downstream signaling in OA joint tissues suggest this pathway may play a role.

A variety of CD40L antagonists have entered the early clinical trial phase for RA [48], with reduction in inflammatory response bone resorption markers [49], as well as clinical efficacy reported [48, 50]. To the best of our knowledge the current study is the first to directly investigate the efficacy of anti-CD40L treatment on the development of ptOA albeit on a pre-

clinical level. Our findings indicate that acute post-injury blockade of CD40L has an immunomodulatory effect. As anticipated based on the role of CD40/CD40L in crosstalk between the innate and adaptive immune response, CD40L blockade modulated the number and polarization of cells from both arms of the inflammatory response in the synovial tissue. In particular, the acute and persistent reduction in both BO and STRM macrophage M1/M2 was temporally consistent with reduced synovial CD4⁺ Th1/Th2 and Th17/Treg polarization. As with targeting either innate (see **Chapter 5.2**) or adaptive (see **Chapter 4.2**) pathways alone, trans-immune-system modulation by blocking CD40L decreased the progression and severity of structural osteochondral ptOA pathology and synovial inflammation and fibrosis. Importantly the CD40L-targeting strategy, unlike the previous individual innate/adaptive system inhibition studies was initiated post-injury (albeit only 6 hours), providing evidence that a clinically relevant therapeutic approach may be possible. Future studies with greater delays in initiating anti-CD40L treatment are needed to better define the therapeutic window. In contrast to RA, ptOA is associated with biomechanical instability and a recent review highlighted the importance of the mechanical status of the joint when considering immunomodulatory therapies for the early treatment of OA/ptOA [1]. Without restoration of the joint's biomechanical status, immunomodulatory treatment by itself may be unlikely to fully prevent structural damage. Anti-CD40L treatment while mitigating was not able to prevent progression of joint structural, and future studies should look to combine restoration of the biomechanics and adjunct immunomodulatory treatment utilizing anti-CD40L treatment. In addition, studies in females and older mice are needed to confirm the broader efficacy of anti-CD40L. Although both treatments used in the current study targeted CD40L we detected a more distinct and longer lasting effect after treatment with mAB compared to the SMI. A potential reason lies in the different pharmacokinetic profiles of each treatment modality. DRI-C21045 is metabolised rapidly after sub-cutaneous injection with an elimination half-life of 2

hours in plasma [32] while a clinical study utilizing a similar concentration of mAB treatment (1.7mg/kg) showed an elimination half-life of 9.79-16.1 days [51]. Future studies with more frequent administration and/or developing and using a more sustained release formulation of DRI-C21045 would be beneficial.

Limitations

In the current study CD40 pathway modulation was conducted 6 hours post joint injury. This time point was chosen as 6 hours would allow enough time for an injured patient to seek medical advice and confirm the ACL rupture with imaging modalities. However, this study is intended as a pilot study focusing only on the immunomodulatory effect of anti-CD40L treatment subsequent to joint injury and ptOA development. Studies are needed that determine the optimal therapeutic window for administration as well as investigate if a local intraarticular therapy is possible or a systemic administration is needed.

The current study purposefully only included a non-surgical rupture model (ACLR), however future studies are needed that evaluate the influence of this treatment across ptOA animal models deriving from differing injuries. Furthermore, the current study used only young male mice in order to keep groups homogenous, however, studies are needed that include other variables known to influence OA development (e.g. sex, age, metabolism). Additionally, we were unable to include pain outcome evaluation and comparisons because of the number of animals used across time points and experiments, but the effect of CD40 pathway modulation on pain should fall within the scope of a future study. Finally, mice in the current study had ongoing mechanical joint issues and the effect of CD40 pathway modulation in combination with restoration of biomechanics warrants detailed investigation.

Conclusion

The current study provides first evidence regarding a potential benefit of anti-CD40L treatment via an immunomodulatory effect during onset and progression of ptOA. Further studies are needed that investigate the effect of this treatment in combination with restoration of biomechanics as well as optimising ways of administration, dosage, and duration of treatment.

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None of the funding agencies had any input into the conceptualization or implementation of the study or analysis and interpretation of data.

Conflict of interest

The authors declare no competing interests.

References

1. Grassel S, Muschter D: **Recent advances in the treatment of osteoarthritis.** *F1000Res* 2020, **9**.
2. Cui A, Li H, Wang D, Zhong J, Chen Y, Lu H: **Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies.** *eClinicalMedicine* 2020, **29**.
3. Goldring SR, Goldring MB: **Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk.** *Nat Rev Rheumatol* 2016, **12**(11):632-644.
4. Oo WM, Little C, Duong V, Hunter DJ: **The Development of Disease-Modifying Therapies for Osteoarthritis (DMOADs): The Evidence to Date.** *Drug Des Devel Ther* 2021, **15**:2921-2945.
5. Woodell-May JE, Sommerfeld SD: **Role of Inflammation and the Immune System in the Progression of Osteoarthritis.** *Journal of Orthopaedic Research* 2020, **38**(2):253-257.
6. Zhao X, Zhao Y, Sun X, Xing Y, Wang X, Yang Q: **Immunomodulation of MSCs and MSC-Derived Extracellular Vesicles in Osteoarthritis.** *Front Bioeng Biotechnol* 2020, **8**:575057.
7. Li Y-s, Luo W, Zhu S-a, Lei G-h: **T Cells in Osteoarthritis: Alterations and Beyond.** *Frontiers in Immunology* 2017, **8**.
8. Rosshirt N, Trauth R, Platzer H, Tripel E, Nees TA, Lorenz H-M, Tretter T, Moradi B: **Proinflammatory T cell polarization is already present in patients with early knee osteoarthritis.** *Arthritis Research & Therapy* 2021, **23**(1):37.
9. Kurowska-Stolarska M, Alivernini S: **Synovial tissue macrophages: friend or foe?** *RMD open* 2017, **3**(2):e000527.
10. Shi C, Pamer EG: **Monocyte recruitment during infection and inflammation.** *Nat Rev Immunol* 2011, **11**(11):762-774.
11. Goldring MB, Otero M: **Inflammation in osteoarthritis.** *Curr Opin Rheumatol* 2011, **23**(5):471-478.
12. Mills CD, Kincaid K, Alt JM, Heilman MJ, Hill AM: **M-1/M-2 macrophages and the Th1/Th2 paradigm.** *J Immunol* 2000, **164**(12):6166-6173.
13. Fernandes JC, Martel-Pelletier J, Pelletier JP: **The role of cytokines in osteoarthritis pathophysiology.** *Biorheology* 2002, **39**(1-2):237-246.
14. Schlaak JF, Pfers I, Meyer Zum Buschenfelde KH, Marker-Hermann E: **Different cytokine profiles in the synovial fluid of patients with osteoarthritis, rheumatoid arthritis and seronegative spondylarthropathies.** *Clin Exp Rheumatol* 1996, **14**(2):155-162.
15. Blom AB, van der Kraan PM, van den Berg WB: **Cytokine targeting in osteoarthritis.** *Curr Drug Targets* 2007, **8**(2):283-292.
16. Moradi B, Jackson MT, Shu C, Smith SM, Smith MM, Zaki S, Platzer H, Rosshirt N, Giangreco MD, Scanzello CR *et al*: **The role of synovial T-cell infiltration following knee joint injury in symptoms and progression to osteoarthritis.** *medRxiv* <https://doi.org/10.1101/19013227> 2019.
17. Haubruck P, Pinto MM, Moradi B, Little CB, Gentek R: **Monocytes, Macrophages, and Their Potential Niches in Synovial Joints – Therapeutic Targets in Post-Traumatic Osteoarthritis?** *Frontiers in Immunology* 2021, **12**.

18. Elgueta R, Benson MJ, de Vries VC, Wasiuk A, Guo Y, Noelle RJ: **Molecular mechanism and function of CD40/CD40L engagement in the immune system.** *Immunol Rev* 2009, **229**(1):152-172.
19. Graf D, Muller S, Korthauer U, van Kooten C, Weise C, Kroczeck RA: **A soluble form of TRAP (CD40 ligand) is rapidly released after T cell activation.** *Eur J Immunol* 1995, **25**(6):1749-1754.
20. Bretscher PA: **A two-step, two-signal model for the primary activation of precursor helper T cells.** *Proc Natl Acad Sci U S A* 1999, **96**(1):185-190.
21. Zirlik A, Maier C, Gerdes N, MacFarlane L, Soosairajah J, Bavendiek U, Ahrens I, Ernst S, Bassler N, Missiou A *et al*: **CD40 ligand mediates inflammation independently of CD40 by interaction with Mac-1.** *Circulation* 2007, **115**(12):1571-1580.
22. Soong RS, Song L, Trieu J, Lee SY, He L, Tsai YC, Wu TC, Hung CF: **Direct T cell activation via CD40 ligand generates high avidity CD8+ T cells capable of breaking immunological tolerance for the control of tumors.** *PLoS One* 2014, **9**(3):e93162.
23. MacDonald KP, Nishioka Y, Lipsky PE, Thomas R: **Functional CD40 ligand is expressed by T cells in rheumatoid arthritis.** *J Clin Invest* 1997, **100**(9):2404-2414.
24. Roman-Fernandez IV, Garcia-Chagollan M, Cerpa-Cruz S, Jave-Suarez LF, Palafox-Sanchez CA, Garcia-Arellano S, Sanchez-Zuno GA, Munoz-Valle JF: **Assessment of CD40 and CD40L expression in rheumatoid arthritis patients, association with clinical features and DAS28.** *Clin Exp Med* 2019, **19**(4):427-437.
25. Liu MF, Chao SC, Wang CR, Lei HY: **Expression of CD40 and CD40 ligand among cell populations within rheumatoid synovial compartment.** *Autoimmunity* 2001, **34**(2):107-113.
26. Guo Y, Walsh AM, Fearon U, Smith MD, Wechalekar MD, Yin X, Cole S, Orr C, McGarry T, Canavan M *et al*: **CD40L-Dependent Pathway Is Active at Various Stages of Rheumatoid Arthritis Disease Progression.** *J Immunol* 2017, **198**(11):4490-4501.
27. Smith MM, Clarke EC, Little CB: **Considerations for the design and execution of protocols for animal research and treatment to improve reproducibility and standardization: "DEPART well-prepared and ARRIVE safely".** *Osteoarthritis Cartilage* 2017, **25**(3):354-363.
28. Percie du Sert N, Ahluwalia A, Alam S, Avey MT, Baker M, Browne WJ, Clark A, Cuthill IC, Dirnagl U, Emerson M *et al*: **Reporting animal research: Explanation and elaboration for the ARRIVE guidelines 2.0.** *PLoS Biol* 2020, **18**(7):e3000411.
29. NHMRC: **Australian code for the care and use of animals for scientific purposes**, 8th edition edn: National Health and Medical Research Council; Canberra, Australia; 2013.
30. Christiansen BA, Anderson MJ, Lee CA, Williams JC, Yik JH, Haudenschild DR: **Musculoskeletal changes following non-invasive knee injury using a novel mouse model of post-traumatic osteoarthritis.** *Osteoarthritis Cartilage* 2012, **20**(7):773-782.
31. Blaker CL, Little CB, Clarke EC: **Joint loads resulting in ACL rupture: Effects of age, sex, and body mass on injury load and mode of failure in a mouse model.** *Journal of Orthopaedic Research* 2017, **35**(8):1754-1763.
32. Chen J, Song Y, Bojadzic D, Tamayo-Garcia A, Landin AM, Blomberg BB, Buchwald P: **Small-Molecule Inhibitors of the CD40-CD40L Costimulatory Protein-Protein Interaction.** *J Med Chem* 2017, **60**(21):8906-8922.
33. Haubruck P, Colbath AC, Liu Y, Stoner S, Shu C, Little CB: **Flow Cytometry Analysis of Immune Cell Subsets within the Murine Spleen, Bone Marrow, Lymph Nodes and**

- Synovial Tissue in an Osteoarthritis Model.** *Journal of visualized experiments : JoVE* 2020(158).
34. Jackson MT, Moradi B, Smith MM, Jackson CJ, Little CB: **Activation of matrix metalloproteinases 2, 9, and 13 by activated protein C in human osteoarthritic cartilage chondrocytes.** *Arthritis & rheumatology (Hoboken, NJ)* 2014, **66**(6):1525-1536.
 35. Jackson MT, Moradi B, Zaki S, Smith MM, McCracken S, Smith SM, Jackson CJ, Little CB: **Depletion of protease-activated receptor 2 but not protease-activated receptor 1 may confer protection against osteoarthritis in mice through extracartilaginous mechanisms.** *Arthritis & rheumatology (Hoboken, NJ)* 2014, **66**(12):3337-3348.
 36. Shu CC, Jackson MT, Smith MM, Smith SM, Penm S, Lord MS, Whitelock JM, Little CB, Melrose J: **Ablation of Perlecan Domain 1 Heparan Sulfate Reduces Progressive Cartilage Degradation, Synovitis, and Osteophyte Size in a Preclinical Model of Posttraumatic Osteoarthritis.** *Arthritis & rheumatology (Hoboken, NJ)* 2016, **68**(4):868-879.
 37. Shu CC, Zaki S, Ravi V, Schiavinato A, Smith MM, Little CB: **The relationship between synovial inflammation, structural pathology, and pain in post-traumatic osteoarthritis: differential effect of stem cell and hyaluronan treatment.** *Arthritis Res Ther* 2020, **22**(1):29.
 38. Zaki S, Smith MM, Smith SM, Little CB: **Differential patterns of pathology in and interaction between joint tissues in long-term osteoarthritis with different initiating causes: phenotype matters.** *Osteoarthritis Cartilage* 2020, **28**(7):953-965.
 39. Haubruck P, Heller R, Blaker CL, Clarke EC, Smith SM, Burkhardt D, Liu Y, Stoner S, Zaki S, Shu CC *et al*: **Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models.** *Osteoarthritis and Cartilage* 2023.
 40. Little CB, Hunter DJ: **Post-traumatic osteoarthritis: from mouse models to clinical trials.** *Nat Rev Rheumatol* 2013, **9**(8):485-497.
 41. Li G, Diogo D, Wu D, Spoonamore J, Dancik V, Franke L, Kurreeman F, Rossin EJ, Duclos G, Hartland C *et al*: **Human Genetics in Rheumatoid Arthritis Guides a High-Throughput Drug Screen of the CD40 Signaling Pathway.** *PLOS Genetics* 2013, **9**(5):e1003487.
 42. Raychaudhuri S, Remmers EF, Lee AT, Hackett R, Guiducci C, Burt NP, Gianniny L, Korman BD, Padyukov L, Kurreeman FA: **Common variants at CD40 and other loci confer risk of rheumatoid arthritis.** *Nature genetics* 2008, **40**(10):1216-1223.
 43. Guo Y, Walsh AM, Fearon U, Smith MD, Wechalekar MD, Yin X, Cole S, Orr C, McGarry T, Canavan M *et al*: **CD40L-Dependent Pathway Is Active at Various Stages of Rheumatoid Arthritis Disease Progression.** *The Journal of Immunology* 2017, **198**(11):4490.
 44. Chen Y, Zhang D, Ho KW, Lin S, Suen WC, Zhang H, Zha Z, Li G, Leung PS: **GPR120 is an important inflammatory regulator in the development of osteoarthritis.** *Arthritis Res Ther* 2018, **20**(1):163.
 45. Abu-Hanna J, Patel JA, Anastasakis E, Cohen R, Clapp LH, Loizidou M, Eddama MMR: **Therapeutic potential of inhibiting histone 3 lysine 27 demethylases: a review of the literature.** *Clin Epigenetics* 2022, **14**(1):98.

46. Jun Z, Xinmeng J, Yue L, Zhi W, Yan Z, Tieyi Y, Jiangan T: **Jumonji domain containing-3 (JMJD3) inhibition attenuates IL-1 β -induced chondrocytes damage in vitro and protects osteoarthritis cartilage in vivo.** *Inflamm Res* 2020, **69**(7):657-666.
47. Yapp C, Carr AJ, Price A, Oppermann U, Snelling SJ: **H3K27me3 demethylases regulate in vitro chondrogenesis and chondrocyte activity in osteoarthritis.** *Arthritis Res Ther* 2016, **18**(1):158.
48. Lai JH, Luo SF, Ho LJ: **Targeting the CD40-CD154 Signaling Pathway for Treatment of Autoimmune Arthritis.** *Cells* 2019, **8**(8).
49. Visvanathan S, Daniluk S, Ptaszynski R, Muller-Ladner U, Ramanujam M, Rosenstock B, Eleftheraki AG, Vinisko R, Petrikova A, Kellner H *et al*: **Effects of BI 655064, an antagonistic anti-CD40 antibody, on clinical and biomarker variables in patients with active rheumatoid arthritis: a randomised, double-blind, placebo-controlled, phase IIa study.** *Ann Rheum Dis* 2019, **78**(6):754-760.
50. Karnell JL, Albulescu M, Drabic S, Wang L, Moate R, Baca M, Oganessian V, Gonsior M, Thisted T, Yan L *et al*: **A CD40L-targeting protein reduces autoantibodies and improves disease activity in patients with autoimmunity.** *Sci Transl Med* 2019, **11**(489).
51. Tocoian A, Buchan P, Kirby H, Soranson J, Zamacona M, Walley R, Mitchell N, Esfandiari E, Wagner F, Oliver R: **First-in-human trial of the safety, pharmacokinetics and immunogenicity of a PEGylated anti-CD40L antibody fragment (CDP7657) in healthy individuals and patients with systemic lupus erythematosus.** *Lupus* 2015, **24**(10):1045-1056.

Figure 1: Visualization of the study design and animal allocation into each study. Experiments were divided into genomic in-vivo experiments and therapeutic in-vivo experiments. Groups, treatments, time points, sample size and outcomes are detailed for each set of experiment.

Figure 2: Results of flow cytometry data is shown that was acquired during previous studies using the same animal model (ACLR) and outcome parameters. **A** provides a schematic overview over the underlying treatments for data shown in **B** and **C**. In **B** antibody mediated IL12/23 pathway inhibition was used to modulate the adaptive immune response. Here the indirect influence of this treatment on innate immunity in the synovium is shown. In **C** antibody mediated Ly6C^{high} monocyte depletion was performed to modulate the innate immune response. Here we show the indirect influence of this treatment on the adaptive immune response in the synovium. The + symbol indicates results from the statistical comparison of anti-IL12p40 treated vs saline control, * anti-CCR2 treated vs saline control. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (*,+), $p < 0.01$ (**,++), $p < 0.001$ (***,+++), and $p < 0.0001$ (****,++++). Data is presented as mean $\pm$ SEM and $n = 6$ biological replicates (pool of tissues from 2 mice)/group/time point.

Figure 3: Results of the genomic in-vivo experiments over time are shown. The qPCR results for CD40L (**A**) and CD40 (**B**) for the draining lymph node (left) and synovial tissue block (right) are shown. The upper two panels of both **A** and **B** show the expression pattern in either ACLR or NOC mice over time without any pathway modulation or treatment. The lower two panels of both **A** and **B** show the expression pattern in the interventional groups of the study as well as their controls (1:ACLR+anti-CD40L treatment; 2:ACLR+small molecule anti-CD40L; 3:ACLR+isotype antibody $\rightarrow$ control for group 1; 4:ACLR+carrier solution $\rightarrow$ control for group 2). The + symbol indicates results from the statistical comparison of ‘ACLR+small

molecule anti-CD40L' vs 'ACLR+carrier solution' mice, while the ^ symbol is used for the comparison of 'ACLR+anti-CD40L treatment' vs 'ACLR+isotype antibody' mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (+, ^), $p < 0.01$ (++, ^^), $p < 0.001$ (+++, ^^) and $p < 0.0001$ (++++, ^^). Data is presented as mean $\pm$ SEM and n = 6 animals/group/time point.

Figure 4: Results of the flow cytometry experiments of the locoregional adaptive and innate immune response. **A** shows the influence of antibody mediated and small molecule mediate pathway modulation on CD4+ helper cells, and the inflammatory status assessed by the Th1/Th2 ratio and Th17/Treg. **B** shows the influence of antibody mediated and small molecule mediate pathway modulation on blood originated as well as synovial tissue resident macrophages and their inflammatory polarization status assessed with the M1/M2 ratio. The + symbol indicates results from the statistical comparison of 'ACLR+small molecule anti-CD40L' vs 'ACLR+carrier solution' mice, while the ^ symbol is used for the comparison of 'ACLR+anti-CD40L treatment' vs 'ACLR+isotype antibody' mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (+, ^), $p < 0.01$ (++, ^^), $p < 0.001$ (+++, ^^) and $p < 0.0001$ (++++, ^^). Data is presented as mean $\pm$ SEM and n = 3 biological replicates (pool of tissues from 3 mice)/group/time point.

Figure 5: Results of the flow cytometry experiments of the systemic adaptive and innate immune response are shown. **A** gives an overview over the innate (upper two panels) and adaptive (lower two panels) immune response in blood. **B** gives an overview over the innate (upper two panels) and adaptive (lower two panels) immune response in the contralateral bone marrow. The + symbol indicates results from the statistical comparison of 'ACLR+small molecule anti-CD40L' vs 'ACLR+carrier solution' mice, while the ^ symbol is used for the

comparison of ‘ACLR+anti-CD40L treatment’ vs ‘ACLR+isotype antibody’ mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (+, ^), $p < 0.01$ (++, ^^), $p < 0.001$ (+++, ^^) and $p < 0.0001$ (++++, ^^). Data is presented as mean $\pm$ SEM and $n = 3$ biological replicates (pool of tissues from 3 mice)/group/time point.

Figure 6: Results of histopathological evaluation of the joint. **A** provides a schematic overview over the tissues utilized and outcome measurements. **B** visualizes the fibrotic changes to the joint. **C** shows the combined tibiofemoral cartilage damage over time, as well as chondrocyte hypertrophy/apoptosis over time, and osteochondral damage over time as well as the total remodeling assessed as the combined osteophyte and enthesophyte severity adjacent to the joint. Lastly, **D** focuses on the synovial inflammation over time, by showing combined total synovitis and the synovial hyperplasia scores. The + symbol indicates results from the statistical comparison of ‘ACLR+small molecule anti-CD40L’ vs ‘ACLR+carrier solution’ mice, while the ^ symbol is used for the comparison of ‘ACLR+anti-CD40L treatment’ vs ‘ACLR+isotype antibody’ mice. All statistical tests used an α -level of 0.05, and differences are marked as follows: $p < 0.05$ (+, ^), $p < 0.01$ (++, ^^), $p < 0.001$ (+++, ^^) and $p < 0.0001$ (++++, ^^). Data is presented as median $\pm$ 95% CI and $n = 9$ animals/group/time point.

Figure 1:

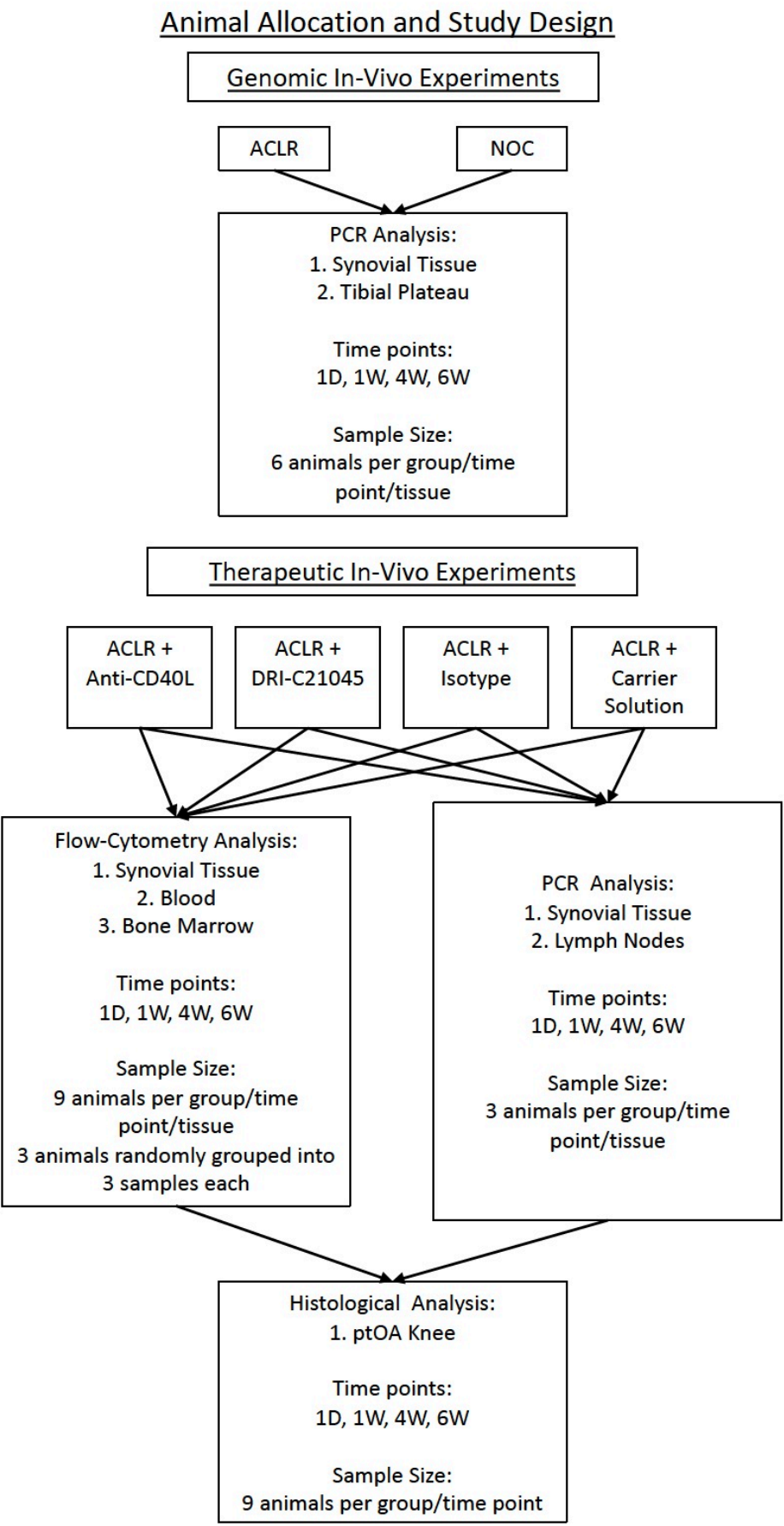


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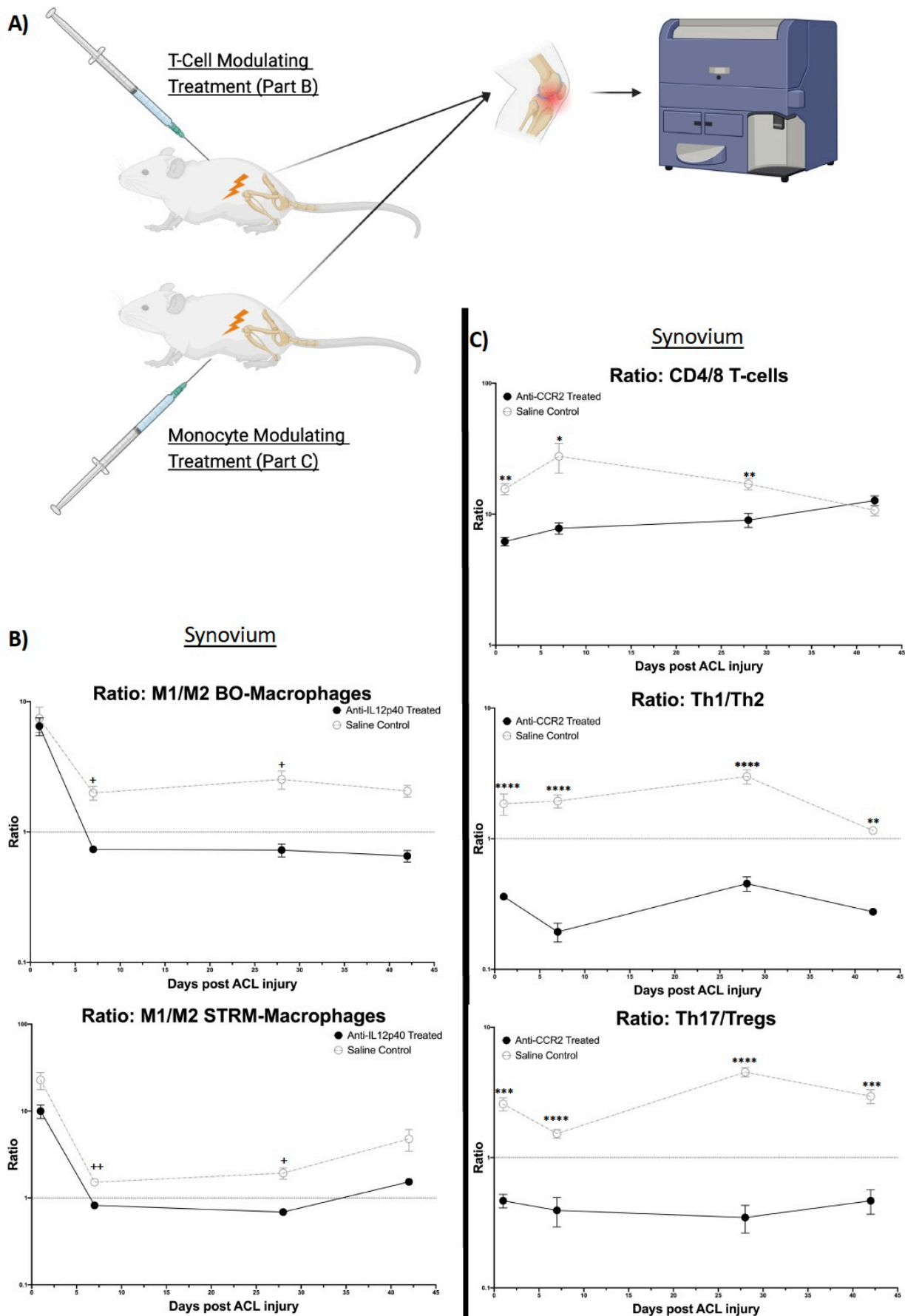
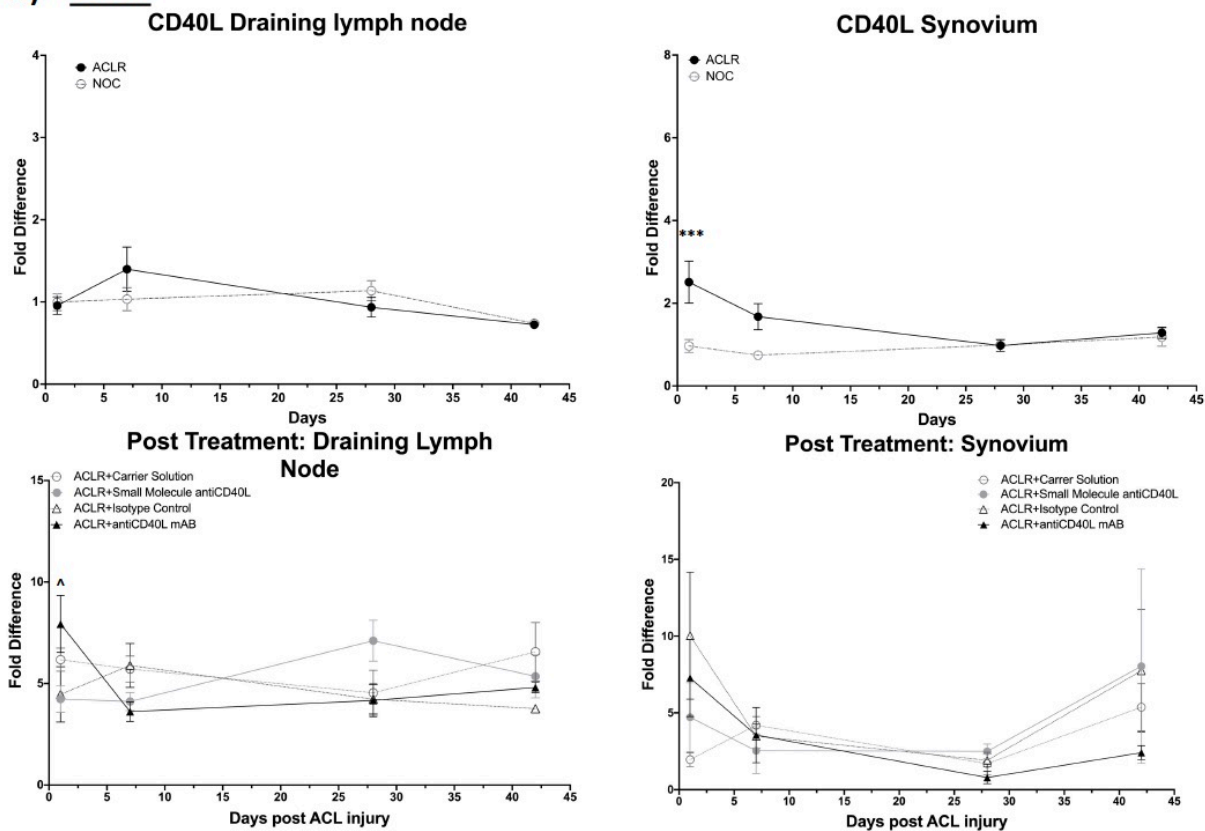


Figure 3:

mRNA Expression in qPCR Analysis

A) CD40L



B) CD40

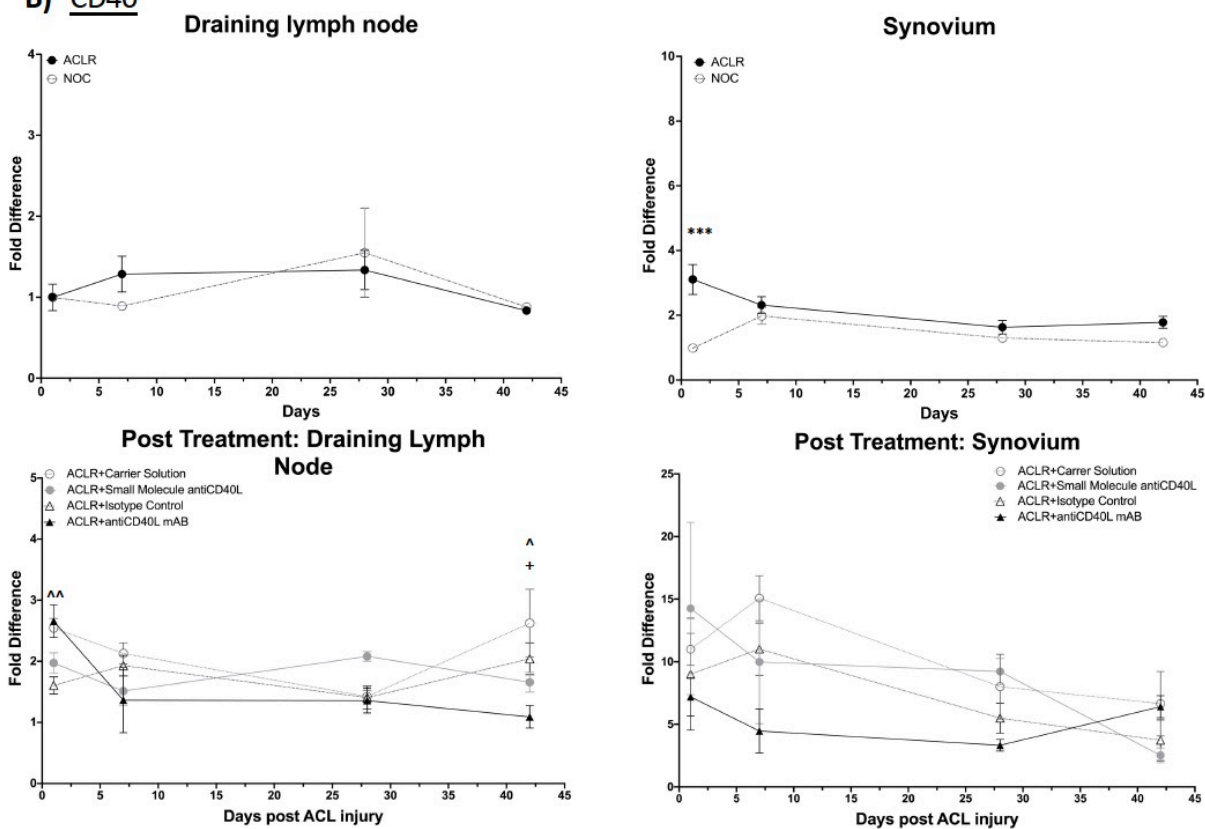
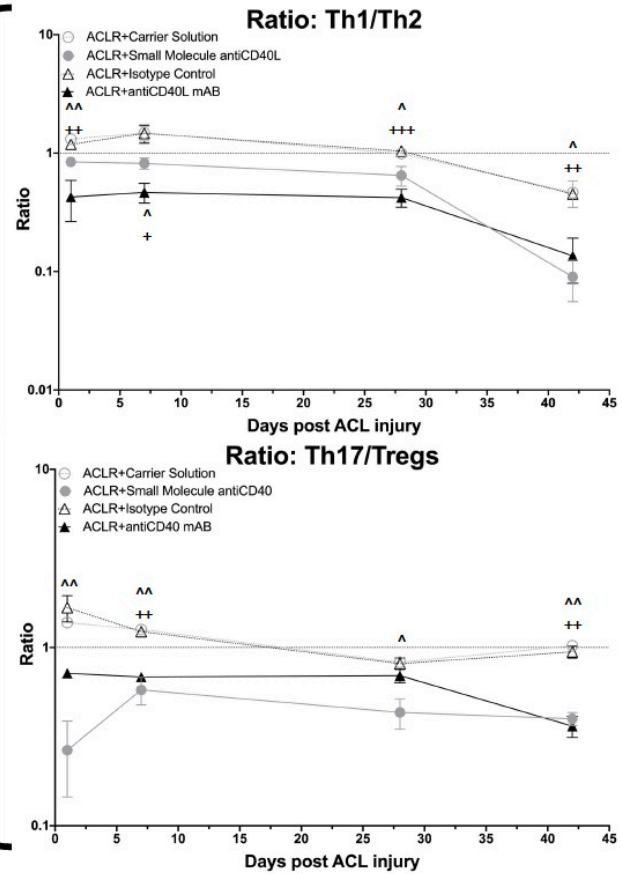
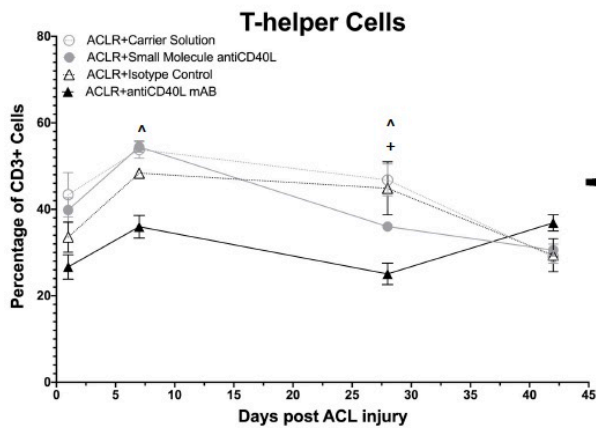


Figure 4:

A) T-cell Response



B) Macrophage Response

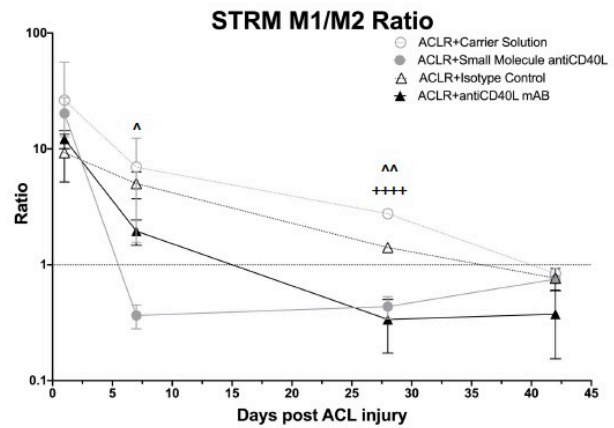
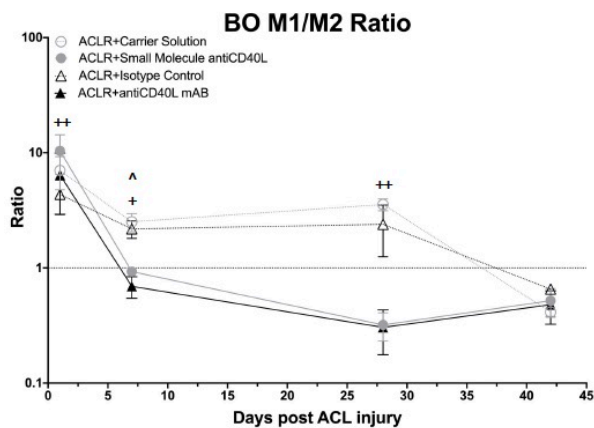
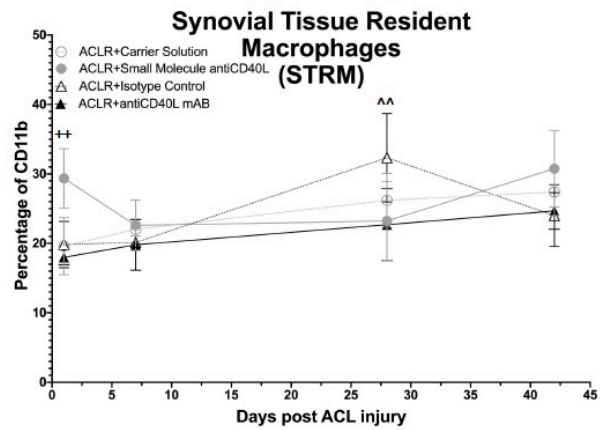
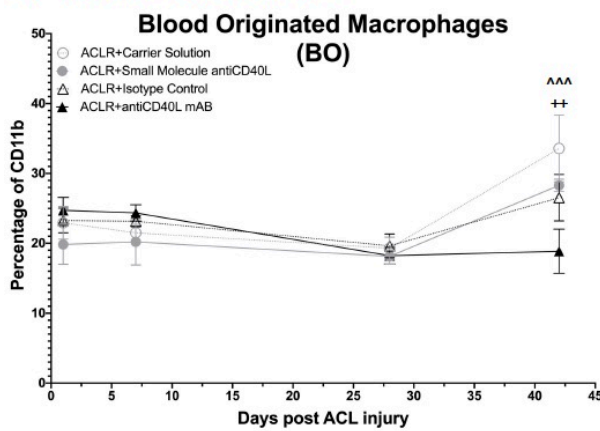
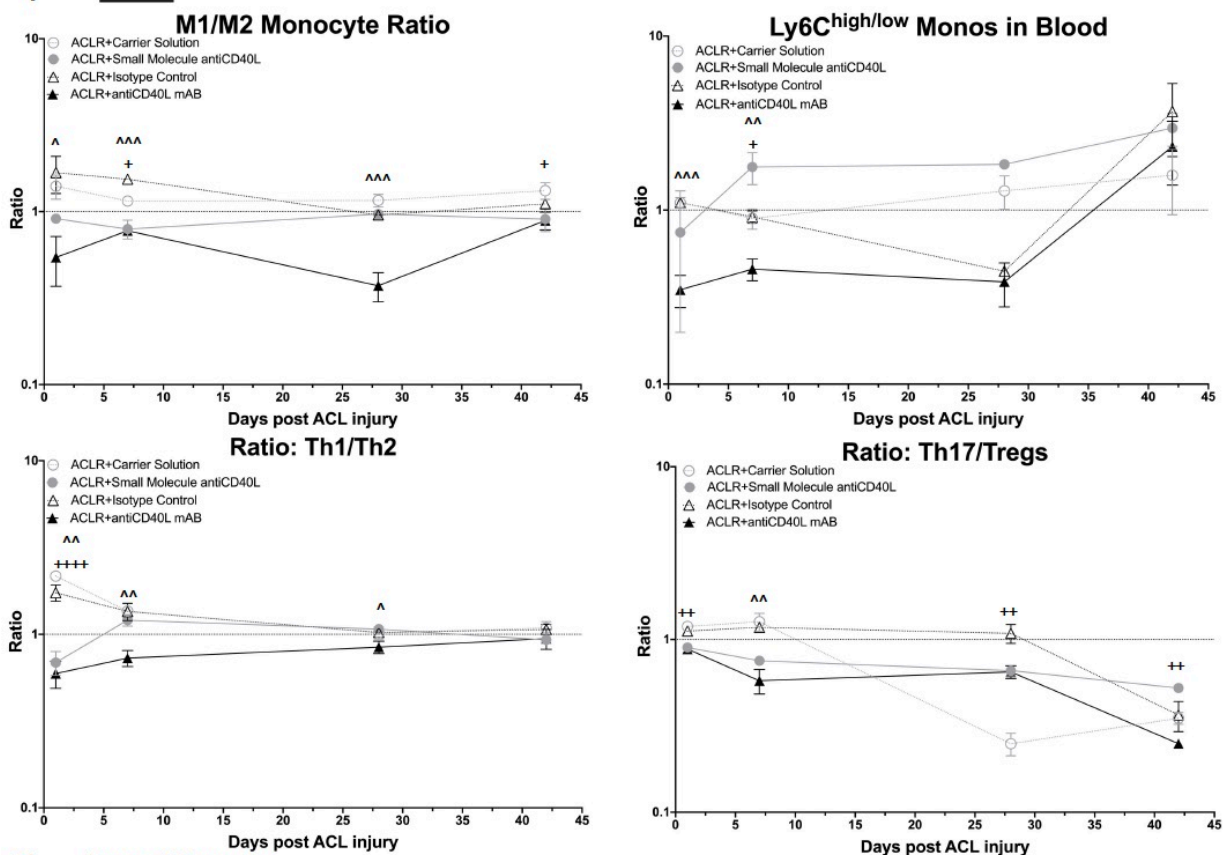


Figure 5:

A) Blood



B) Bone Marrow

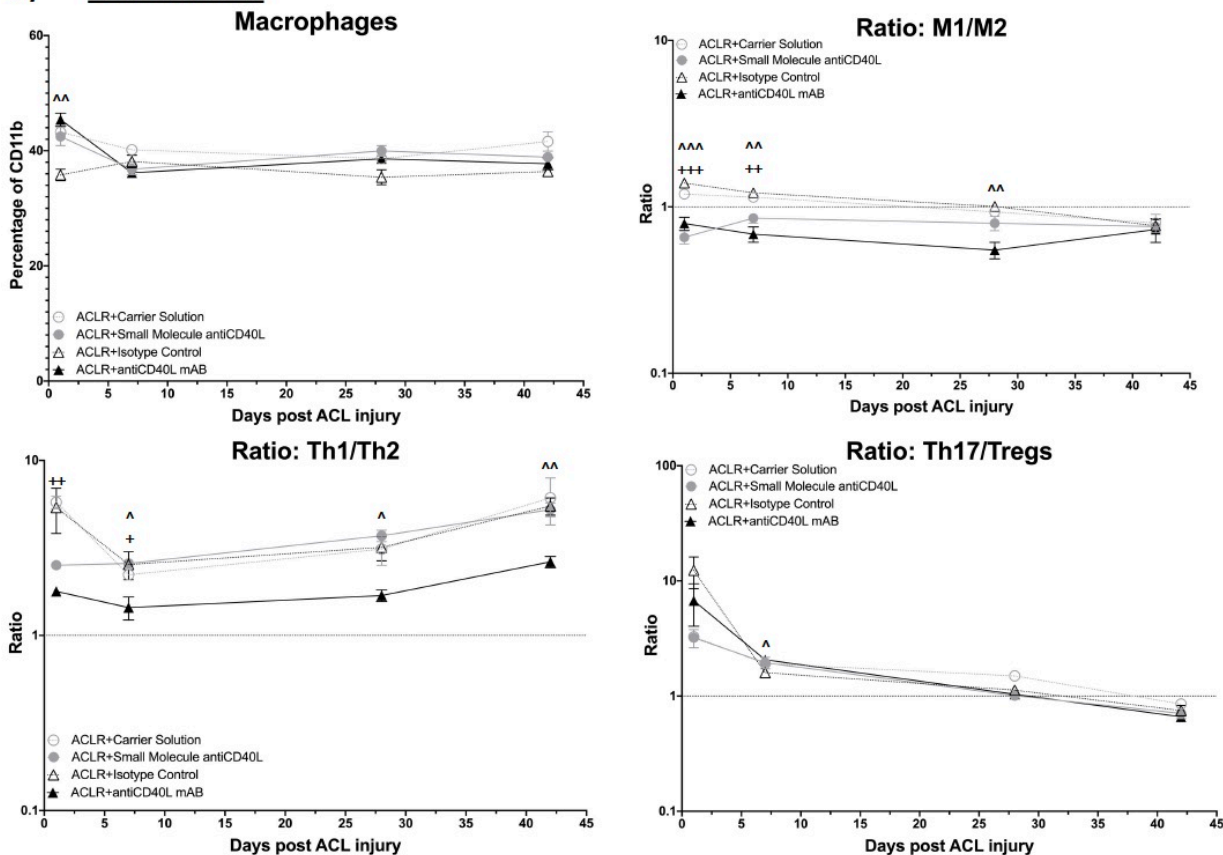
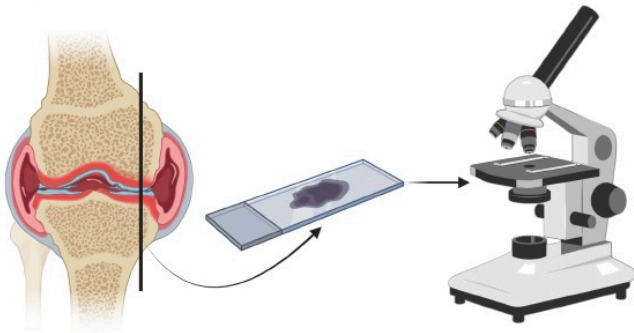
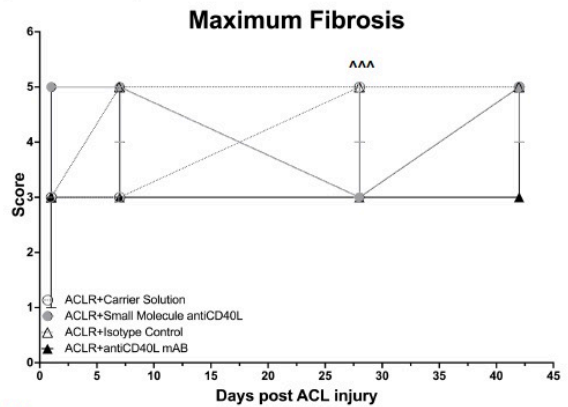


Figure 6:

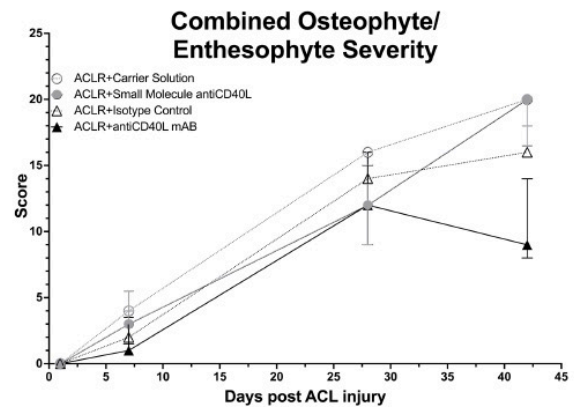
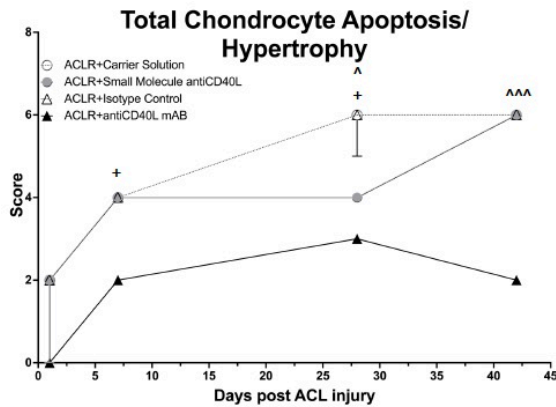
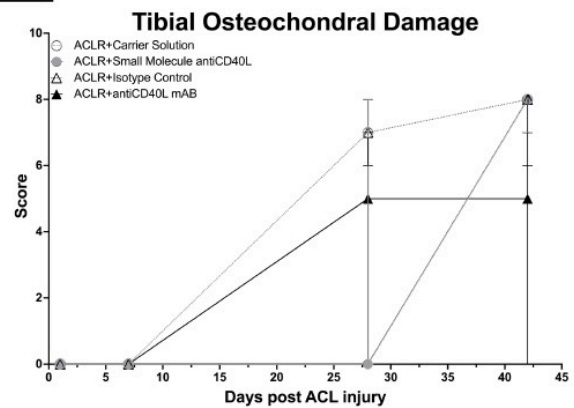
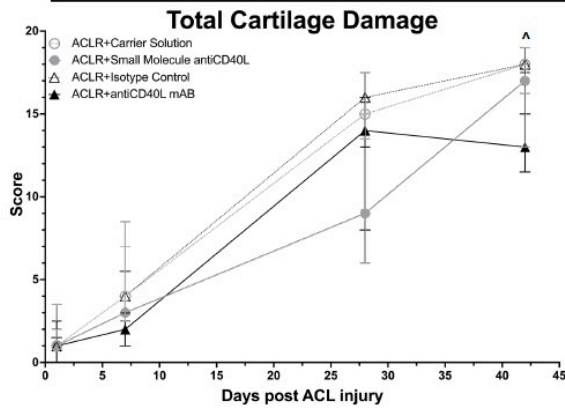
A)



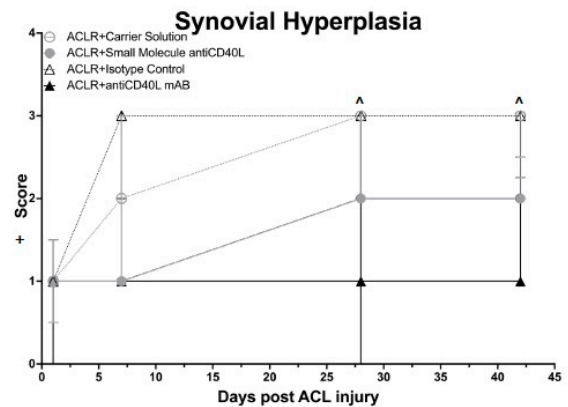
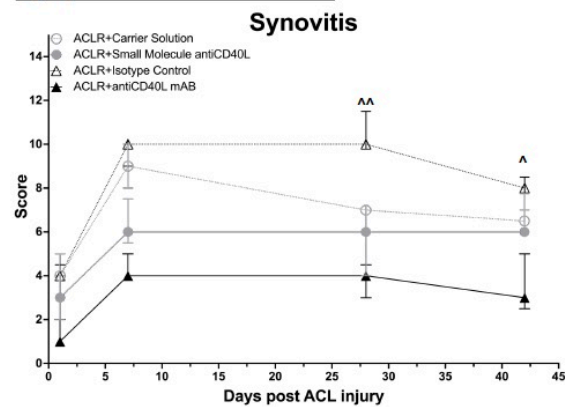
B) Fibrosis



C) Osteochondral Joint Damage and Bone Remodelling



D) Synovial Inflammation



Supplementary Table 1:

Details for each antibody used in each of the two panels are given. In addition, information is provided for the relevant buffers used.

Supplementary Table 2:

Details for the primer sequences used in the study are provided. bp= base pairs.

Supplementary Table 1: Used Antibodies for Flow Cytometry

Name of Material/ Equipment	Manufacturer	Catalog Number	Comments/Description
Fixable Viability Stain 510 100ug	BD	564406	T-Cell Panel
Ms CD3e APC-Cy7 145-2C11 100ug	BD	557596	T-Cell Panel
Ms CD8a BV786 53-6.7 50ug	BD	563332	T-Cell Panel
Ms CD4 PE-Cy7 RM4-5 100ug	BD	552775	T-Cell Panel
Ms NK1.1 BV650 PK136 50ug	BD	564143	T-Cell Panel
Ms CD25 BV605 PC61 50ug	BD	563061	T-Cell Panel
Ms CD183 BB700 CXCR3-173 50ug	BD	742274	T-Cell Panel
Ms Foxp3 PE MF23 100ug	BD	560408	T-Cell Panel
Ms ROR Gamma T BV421 Q31-378 50ug	BD	562894	T-Cell Panel
APC anti-mouse CD194 (CCR4)	BioLegend	131212	T-Cell Panel
Fixable Viability Stain 510 100ug	BD	564406	Monocyte Panel
Ms F4/80 BV421 T45-2342 50ug	BD	565411	Monocyte Panel
Ms CD80 BV650 16-10A1 50ug	BD	563687	Monocyte Panel
Ms I-A I-E BV711 M5/114.15.2 50ug	BD	563414	Monocyte Panel
Ms Ly-6G APC-Cy7 1A8 50ug	BD	560600	Monocyte Panel
Ms CD11C PE-CF594 HL3 100ug	BD	562454	Monocyte Panel
Ms Ly-6C PE-Cy7 AL-21 50ug	BD	560593	Monocyte Panel
Ms CD206 Alexa 647 MR5D3 25ug	BD	565250	Monocyte Panel
Ms CD11b APC-R700 M1/70 100ug	BD	564985	Monocyte Panel
Brilliant Stain Buffer Plus 1000Tst	BD	566385	Buffers
Transcription Factor Buffer Set 100Tst	BD	562574	Buffers

Supplementary Table 2: Primer Sequences used for rtPCR

Gene	Sequence (5' -3')		Annealing Temperature (C)	Size (bp)
CD40	Forward	GGGTTAAGAAGGAGGGCACC	55.3	243
	Reverse	TCTTATCCTCACAGCTTGTCCA		
CD40L	Forward	CGAAGCCAACAGTAATGCAG	53.8	247
	Reverse	ATTGCGCGCTTGAGTAAGA		

6.3 Main Findings of the Study

The findings from this study indicate that blockade of anti-CD40L has an immunomodulatory effect in context with ptOA. On a cellular level, it seems to lead to an anti-inflammatory microenvironment in the synovial tissue block by reducing the Th1/17 dominated adaptive immune response observed in the control group. Although the expression level of macrophages originating from peripheral blood did not decrease during early and intermediate stages of ptOA development anti-CD40L treatment resulted in an anti-inflammatory polarization of not only migrating macrophages but also STRM. Furthermore, we observed an immunomodulatory effect of the anti-CD40L treatment and a mitigation in joint pathology. However, anti-CD40L treatment was not able to prevent degeneration and joint damage which might be explained by an ongoing biomechanical instability in the utilized murine ptOA model. As stated in the review by Grassel et al. (Grassel, S. et al. 2020) it is likely that immunomodulatory treatment itself is not sufficient to prevent joint pathology thus a combination of the restoration of the biomechanics and adjunct immunomodulatory treatment utilizing anti-CD40L treatment seems promising and warrants further investigation.

Chapter 7

7 Overarching Discussions and Conclusion

In this thesis I sought to evaluate the influence of the innate and adaptive immune response on the pathogenesis of ptOA. Prior to starting this research project an extensive literature review was conducted to identify important questions and knowledge gaps in the existing literature. Furthermore, existing laboratory techniques were evaluated for their suitability for the planned research projects and as a result additional techniques were established, and an established technique improved. This preparatory work and method development as part of the PhD has been published in international journals (Haubruck, Patrick et al. 2020; Haubruck, Patrick et al. 2023; Haubruck, Patrick et al. 2021). In particular, flow cytometry methods were established to allow simultaneous analysis of a multitude of immune cell subsets in a variety of primary and secondary immunological tissues in mice (**Chapter 2.2.2.1**). In addition, we streamlined an established histopathological scoring method to facilitate application of this system which allows evaluation of not only cartilage and osteochondral pathology, but also synovitis, and meniscal pathology and remodelling. In the future this streamlined scoring method will reduce histopathological processing time and costs and make our scoring method more accessible in a wider range of international laboratories and research groups (**Chapter 2.2.2.2**). Both published methods reduce animal numbers necessary to undertake research without limiting the robustness of the methods, and thus have a direct ethical impact in addressing the 3-R's. During the initial review of the literature knowledge gaps regarding the role of the inflammatory response during pathogenesis of ptOA were identified (**Chapter 1.3.5**). While to date a plethora of studies have investigated aspects of the immune response during onset and development of OA, no complete characterisation and direct comparison of the cellular immune response following induction of ptOA via a variety of joint injuries has been conducted. Thus, in a first step we used a comprehensive flow cytometry analysis and rapid- as well as slow-onset surgical and non-surgical murine ptOA models as well as their respective controls over 9 time-points, covering early, intermediate, and advanced stages of ptOA, to

characterise the innate and adaptive immune response (**Chapter 3.2**). Here we found a distinct spatiotemporal Th1 and Th17 response. While elevated Th1 levels have been shown in osteoarthritic synovial tissue on a pre-clinical and clinical level (Li, Y. S. et al. 2017) and thus support the findings of our study, Th17 cells were mostly recognized for their role in autoimmune disease, such as RA (Waite, Janelle C. et al. 2012). Only recently evidence emerged suggesting a potential role for Th17 cells in OA. The findings from our study further corroborate these initial results and indicate a crucial role for Th17 cells in the pathogenesis of ptOA.

Based on these findings we intended to further investigate the role of inflammatory T-helper cell subsets and thus inhibited either the IL12p40 or IL12p75 pathway, or depleted CD4⁺ T-helper cell as a whole and compared the immunological and histopathological effects to respective controls (**Chapter 4.2**). Here, we found that blockade of IL12p40, affecting both polarization of Th1 and Th17 cells, had a considerably higher impact on immunomodulation and mitigation of histopathological changes. A potential explanation was found in in vitro experiments conducted as part of the same study. Naïve CD4⁺ T-helper cells produced chemotactic proteins such as MIP3A, while OA synovial tissue in contact with naïve CD4⁺ T-helper cells produced significantly higher amounts of IL1 beta, a known promotor of ptOA. Importantly, these molecular processes were governed by IL12/23 and application of Apilimod resulted in a significant reduction. Taken together the results of this study indicated that the IL12p40 pathway plays a crucial role in promoting and perpetuating the inflammatory response during pathogenesis of ptOA via promotion of a pro-inflammatory polarization of T-helper cells into Th1 and Th17 cells as well as induction of a direct expression of damaging cytokines, such as IL1 beta. Limited studies have been conducted that found an anti-inflammatory effect of antibody mediated IL12p40 blockade on Crohns Disease (Castro-Mejia, J. et al. 2016; Wang, X. et al. 2015) whereas our study is the first study to show an anti-inflammatory effect in context with ptOA introducing IL12p40 as a potential target for disease prevention.

Characterisation of the innate immune response during development of ptOA showed an influx of Ly6C^{high} monocytes into the injured joint (**Chapter 3.2**). Furthermore, induction of ptOA led to a pro-inflammatory polarization of both BO macrophages as well as STRMs, whereas a polarization switch from a pro-inflammatory M1 subset domination towards an anti-inflammatory M2 subset domination was observed exclusively in STRMs simultaneous with the Th1/Th2 ratio dropping beneath 2.7. Although previous studies have shown that induction of CiOA leads to influx of Ly6C^{high} monocytes into the joint (Cremers, Niels A. J. et al. 2017) our study is the first study that shows a similar pathomechanisms for ptOA.

To further investigate the role of Ly6C^{high} monocytes as well as STRMs we conducted a subsequent study selectively depleting either subset and comparing the results on a cellular, molecular, and histopathological level with respective controls (**Chapter 5.2**). Histopathological findings from this study indicated that depletion of Ly6C^{high} monocytes on a systemic level led to a mitigated joint pathology and decreased local inflammatory response in the joint while preventing fibrosis during later stages of the disease. Surprisingly, depletion of STRMs did not adversely affect joint pathology. On a cellular level only depletion of Ly6C^{high} monocytes led to a persistent anti-inflammatory response both in BO macrophages as well as STRM while systemic depletion of macrophages had a similar yet less distinct response. Importantly, depletion of STRM resulted in an exacerbated pro-inflammatory response both in BO macrophages as well as STRMs. On a systemic level, the ratio of M1-like and M2-like monocytes was significantly decreased in central blood after Ly6C^{high} monocyte depletion, while STRM depletion had the opposite effect. Thereby, results from this study supported the importance of Ly6C^{high} monocytes in the pathogenesis of ptOA while a protective function of STRMs can be assumed by our data but was not conclusively shown.

Data from **Chapter 4** and **Chapter 5**, acquired in addition to the main focus of the respective studies, indicated that targeted depletion of immune cell subsets of either the adaptive or innate immune system prior to joint injury had a broader anti-inflammatory effect on pro-inflammatory cells from the other non-targeted arm of the immune response. Thereby this data indicated a potentially crucial role for factors orchestrating the interplay between the innate and adaptive immune response which could serve as a promising target for an early immunomodulatory treatment of ptOA. This led us to design a study inhibiting CD40L, a prominent orchestrating factor, via either a monoclonal antibody or small molecule inhibitor. The findings from this study indicate that blockade of anti-CD40L has an immunomodulatory effect in context with ptOA. On a cellular level, it seems to lead to an anti-inflammatory microenvironment in the synovial tissue block by reducing the Th1/17 dominated adaptive immune response observed in the control group. Although the expression level of macrophages originating from peripheral blood did not decrease during early and intermediate stages of ptOA development, anti-CD40L treatment resulted in an anti-inflammatory polarization of not only migrating macrophages but also STRM. Furthermore, we observed an immunomodulatory effect of the anti-CD40L treatment and a mitigation in joint pathology. Initial studies investigated the effects of targeting the CD40:CD40L pathway via monoclonal antibodies for the treatment of autoimmune disease (Karnell, Jodi L. et al. 2019). Although an immunomodulatory effect was observed, early clinical trials encountered unforeseen

thromboembolic events resulting in the discontinuation of treatment (Karnell, Jodi L. et al. 2019). Recent advances in engineering novel anti-CD40L domain antibodies resulted in an uncoupling of immunosuppression and thromboembolism (Xie, Jenny H. et al. 2014) and later clinical trials using modified anti-CD40L antibodies in the treatment of autoimmune diseases such as RA are ongoing (Karnell, Jodi L. et al. 2019). Our study is the first study that provides data regarding the role of the CD40:CD40L pathway in ptOA as well as the potential benefit of an early blockade.

Taken together the results from this thesis show that anti-CCR2, anti-IL12p40 and anti-CD40L treatment show promise as biologicals for ptOA disease modification. However, it is important to note that both anti-CCR2 and anti-IL12p40 treatment was started prior to ptOA induction. Thus, while results from these studies support a pathophysiological role for these pathways in the development of ptOA, evaluation of a therapeutic effect post-injury was beyond the scope of this thesis and requires further evaluation. Furthermore, despite immunomodulation and mitigation of histopathological joint injuries, degeneration and damage to the knee occurred over time in each study and was not prevented. A potential explanation lies in the unique combination of biomechanical and biological underlying factors in the development of ptOA. It is likely that immunomodulatory treatment itself is not sufficient on its own to prevent joint pathology, and thus a combination of the restoration of the biomechanics and adjunct immunomodulatory treatment seems promising and warrants further investigation (Grassel, S. et al. 2020).

When assessing the translatability of the findings made during the studies included into this thesis it is critical to highlight that **Chapter 4-5** used the immunomodulatory agents were administered prior to joint injury. Therefore, these studies were labelled proof-of-principle studies to analyze the contribution of each respective cell type and pathway towards the pathogenesis of ptOA. When focusing on a clinical application of these treatments it is not feasible to start a treatment prior to injury as the time of injury is unknown. These studies were intended to identify key cellular players in the development of ptOA and act as a first step in the drug development pipeline. However, it is mandatory that subsequent studies will be conducted that evaluate if a treatment post injury has the same anti-inflammatory effect. Furthermore, dosage finding, treatment duration, and route of administration need to be optimized and tested prior to labelling any of the tested agents a potential candidate for DMOADs. In addition, systemic depletion of crucial immune cells might not be feasible in a clinical setting as side effects to this treatment are likely. In the animals included into the project in this thesis no side effects were observed, and all animals tolerated the treatment well,

however safety studies in a larger animal model will be necessary to confirm these preliminary observations.

Chapter 6 utilized a transient inhibition of the CD40 pathway starting 6 hours post injury. While this time point was chosen to allow an affected patient to seek medical advice and receive imaging diagnostics it is an arbitrary time point and further work is necessary to potentially optimize the time frame of treatment. The studies presented in this thesis focus on the anti-inflammatory effect of each agent as well as the structural impact during development of ptOA. However, these studies used a rapid onset ptOA model resulting in a severe and fast progressing ptOA phenotype. Confirmatory studies are needed including a variety of ptOA models, as well as female animals. Importantly, pain alleviation has become a major hallmark when assessing the efficacy of novel DMOADs. In the studies presented in this thesis pain was not assessed as an outcome parameter. Further studies are needed that assess the impact on pain after treatment besides the anti-inflammatory and structural outcome parameters. Evidence regarding the use of immunomodulation for reducing pain in a variety of disease is starting to emerge. For example, IL-1 receptor antagonists were successfully used to treat chronic bladder pain (Wullt, Björn et al. 2021). Whether any of the pathway modulations investigated in this thesis promote alleviation of pain will fall under the scope of future studies.

Despite the promising findings of studies included in this thesis, each study is subject to its own set of limitations. While flow cytometry has been established as a powerful tool allowing simultaneous analysis of a plethora of immune cell subsets it is limited by the set of markers used. Although both antibody panels were optimized and tested prior to utilization accuracy of cell subset determination is limited. To minimize false identification of subsets a hierarchical gating strategy in combination with a multicolor antibody panel was used. Furthermore, harvesting of the synovial tissue itself is complex and results in a low cell yield, thus in the presented studies the harvested synovial tissue block from multiple mice was pooled to ensure sufficient numbers as previously established by our group (Shu, Cindy C. et al. 2020). This limits the ability for within-animal correlation of immune-cell changes and pathology outcomes. Finally, inhibition of the respective molecular pathways was done via monoclonal antibodies and small-molecule inhibitors administered systemically. While this does not limit the conclusions on the pathophysiological role of the different pathways in ptOA, it does limit the direct clinical applicability, and future research is needed to investigate potential therapeutic agents administered and acting on a locoregional level.

The studies included in this thesis improved the understanding of the role of the innate and adaptive immune system in the pathogenesis of ptOA and provided molecular pathways of

activation for each identified cellular key play. Furthermore, three distinct targets were identified that have an immunomodulatory effect on the onset and development of ptOA. While this adds important knowledge to the literature in the field it also lays the groundwork for future studies to further the evaluation of pathway modulation as potential DMOADs. In particular, the direction of future studies needs to focus on these important aspects:

- 1) Evaluate the efficacy of anti-CCR2 and anti-IL12p40 treatment at different times after induction of ptOA to define a clinically relevant therapeutic window.
- 2) Evaluate more localized therapeutic approaches with the existing and/or different pathway modulating agents.
- 3) Evaluate the efficacy of immune-modulation in combination with a restoration of the biomechanics.
- 4) Evaluate the influence of the surgical repair approach in #3 above on the loco-regional and systemic inflammatory response (as we have shown in chapter 3 that surgery itself leads to pro-inflammatory effects) and thus whether the same or additional immunomodulatory therapy is needed at the time of restorative surgery (e.g. ACL repair) to improve outcomes.
- 5) Optimize dosage, duration and pharmacokinetics of each treatment.
- 6) Utilize larger animal models and test the efficacy in different models prior to advancing a potential treatment into the clinical trial phase for ptOA.

8 Bibliography

Ackerman, I. N., Bohensky, M. A., Pratt, C., Gorelik, A. and Liew, D. (2016). "Counting the cost. Part 1 Healthcare costs: The current and future burden of arthritis."

Ackerman, I. N., Kemp, J. L., Crossley, K. M., Culvenor, A. G. and Hinman, R. S. (2017). "Hip and Knee Osteoarthritis Affects Younger People, Too." *Journal of Orthopaedic & Sports Physical Therapy* 47(2): 67-79.

AIHW (2020). "Osteoarthritis." Canberra: Australian Institute of Health and Welfare.

Anderson, D. D., Chubinskaya, S., Guilak, F., Martin, J. A., Oegema, T. R., Olson, S. A. and Buckwalter, J. A. (2011). "Post-traumatic osteoarthritis: improved understanding and opportunities for early intervention." *J Orthop Res* 29(6): 802-809.

Aubourg, G., Rice, S. J., Bruce-Wootton, P. and Loughlin, J. (2022). "Genetics of osteoarthritis." *Osteoarthritis and Cartilage* 30(5): 636-649.

Blaker, C. L., Ashton, D. M., Doran, N., Little, C. B. and Clarke, E. C. (2021). "Sex- and injury-based differences in knee biomechanics in mouse models of post-traumatic osteoarthritis." *Journal of Biomechanics* 114: 110152.

Blaker, C. L., Clarke, E. C. and Little, C. B. (2016). "Using mouse models to investigate the pathophysiology, treatment, and prevention of post-traumatic osteoarthritis." *J Orthop Res*.

Blaker, C. L., Clarke, E. C. and Little, C. B. (2017). "Using mouse models to investigate the pathophysiology, treatment, and prevention of post-traumatic osteoarthritis." *J Orthop Res* 35(3): 424-439.

Blaker, C. L., Little, C. B. and Clarke, E. C. (2016). "Joint loads resulting in ACL rupture: Effects of age, sex, and body mass on injury load and mode of failure in a mouse model." *J Orthop Res*.

Bliddal, H., Leeds, A. R. and Christensen, R. (2014). "Osteoarthritis, obesity and weight loss: evidence, hypotheses and horizons - a scoping review." *Obes Rev* 15(7): 578-586.

Bretscher, P. A. (1999). "A two-step, two-signal model for the primary activation of precursor helper T cells." *Proc Natl Acad Sci U S A* 96(1): 185-190.

Buckley, C. D. (2003). "Why does chronic inflammatory joint disease persist?" *Clin Med (Lond)* 3(4): 361-366.

Burman, A., Haworth, O., Bradfield, P., Parsonage, G., Filer, A., Thomas, A. M., Amft, N., Salmon, M. and Buckley, C. D. (2005). "The role of leukocyte-stromal interactions in chronic inflammatory joint disease." *Joint Bone Spine* 72(1): 10-16.

Castro-Mejia, J., Jaksevic, M., Krych, L., Nielsen, D. S., Hansen, L. H., Sondergaard, B. C., Kvist, P. H., Hansen, A. K. and Holm, T. L. (2016). "Treatment with a Monoclonal Anti-IL-12p40 Antibody Induces Substantial Gut Microbiota Changes in an Experimental Colitis Model." *Gastroenterol Res Pract* 2016: 4953120.

Chen, D., Shen, J., Zhao, W., Wang, T., Han, L., Hamilton, J. L. and Im, H. J. (2017). "Osteoarthritis: toward a comprehensive understanding of pathological mechanism." *Bone Res* 5: 16044.

Chen, L., Zheng, J. J. Y., Li, G., Yuan, J., Ebert, J. R., Li, H., Papadimitriou, J., Wang, Q., Wood, D., Jones, C. W. and Zheng, M. (2020). "Pathogenesis and clinical management of obesity-related knee osteoarthritis: Impact of mechanical loading." *J Orthop Translat* 24: 66-75.

Christiansen, B. A., Anderson, M. J., Lee, C. A., Williams, J. C., Yik, J. H. and Haudenschild, D. R. (2012). "Musculoskeletal changes following non-invasive knee injury using a novel mouse model of post-traumatic osteoarthritis." *Osteoarthritis Cartilage* 20(7): 773-782.

Chua, J. R., Jamal, S., Riad, M., Castrejon, I., Malfait, A. M., Block, J. A. and Pincus, T. (2019). "Disease Burden in Osteoarthritis Is Similar to That of Rheumatoid Arthritis at Initial Rheumatology Visit and Significantly Greater Six Months Later." *Arthritis Rheumatol* 71(8): 1276-1284.

Cremers, N. A. J., van den Bosch, M. H. J., van Dalen, S., Di Ceglie, I., Ascone, G., van de Loo, F., Koenders, M., van der Kraan, P., Sloetjes, A., Vogl, T., Roth, J., Geven, E. J. W., Blom, A. B. and van Lent, P. L. E. M. (2017). "S100A8/A9 increases the mobilization of pro-inflammatory Ly6Chigh monocytes to the synovium during experimental osteoarthritis." *Arthritis Research & Therapy* 19(1): 217.

Cui, A., Li, H., Wang, D., Zhong, J., Chen, Y. and Lu, H. (2020). "Global, regional prevalence, incidence and risk factors of knee osteoarthritis in population-based studies." *eClinicalMedicine* 29.

Dell'Isola, A., Allan, R., Smith, S. L., Marreiros, S. S. P. and Steultjens, M. (2016). "Identification of clinical phenotypes in knee osteoarthritis: a systematic review of the literature." *BMC Musculoskeletal Disorders* 17(1): 425.

Ditton, E., Johnson, S., Hodyl, N., Flynn, T., Pollack, M., Ribbons, K., Walker, F. R. and Nilsson, M. (2020). "Improving Patient Outcomes Following Total Knee Arthroplasty: Identifying Rehabilitation Pathways Based on Modifiable Psychological Risk and Resilience Factors." *Frontiers in Psychology* 11.

Dolganiuc, A., Stăvaru, C., Anghel, M., Georgescu, E., Chichoş, B. and Olinescu, A. (1999). "Shift toward T lymphocytes with Th1 and Tc1 cytokine-secterion profile in the joints of patients with osteoarthritis." *Roum Arch Microbiol Immunol* 58(3-4): 249-258.

- Elgueta, R., Benson, M. J., de Vries, V. C., Wasiuk, A., Guo, Y. and Noelle, R. J. (2009). "Molecular mechanism and function of CD40/CD40L engagement in the immune system." *Immunol Rev* 229(1): 152-172.
- Faust, H. J., Zhang, H., Han, J., Wolf, M. T., Jeon, O. H., Sadtler, K., Peña, A. N., Chung, L., Maestas, D. R., Jr., Tam, A. J., Pardoll, D. M., Campisi, J., Housseau, F., Zhou, D., Bingham, C. O., III and Elisseff, J. H. (2020). "IL-17 and immunologically induced senescence regulate response to injury in osteoarthritis." *The Journal of Clinical Investigation* 130(10): 5493-5507.
- Glasson, S. S., Blanchet, T. J. and Morris, E. A. (2007). "The surgical destabilization of the medial meniscus (DMM) model of osteoarthritis in the 129/SvEv mouse." *Osteoarthritis Cartilage* 15(9): 1061-1069.
- Gordon, S. and Martinez, F. O. (2010). "Alternative activation of macrophages: mechanism and functions." *Immunity* 32(5): 593-604.
- Graf, D., Muller, S., Korthauer, U., van Kooten, C., Weise, C. and Kroczeck, R. A. (1995). "A soluble form of TRAP (CD40 ligand) is rapidly released after T cell activation." *Eur J Immunol* 25(6): 1749-1754.
- Grassel, S. and Muschter, D. (2020). "Recent advances in the treatment of osteoarthritis." *F1000Res* 9.
- Guo, S. Y., Ding, Y. J., Li, L., Zhang, T., Zhang, Z. Z. and Zhang, E. S. (2015). "Correlation of CD(4)(+) CD(2)(5)(+) Foxp(3)(+) Treg with the recovery of joint function after total knee replacement in rats with osteoarthritis." *Genet Mol Res* 14(3): 7290-7296.
- Haubruck, P., Colbath, A. C., Liu, Y., Stoner, S., Shu, C. and Little, C. B. (2020). "Flow Cytometry Analysis of Immune Cell Subsets within the Murine Spleen, Bone Marrow, Lymph Nodes and Synovial Tissue in an Osteoarthritis Model." *JoVE*(158): e61008.
- Haubruck, P., Heller, R., Blaker, C. L., Clarke, E. C., Smith, S. M., Burkhardt, D., Liu, Y., Stoner, S., Zaki, S., Shu, C. C. and Little, C. B. (2023). "Streamlining quantitative joint-wide medial femoro-tibial histopathological scoring of mouse post-traumatic knee osteoarthritis models." *Osteoarthritis and Cartilage*.
- Haubruck, P., Pinto, M. M., Moradi, B., Little, C. B. and Gentek, R. (2021). "Monocytes, Macrophages, and Their Potential Niches in Synovial Joints – Therapeutic Targets in Post-Traumatic Osteoarthritis?" *Frontiers in Immunology* 12.
- Haynes, M. K., Hume, E. L. and Smith, J. B. (2002). "Phenotypic characterization of inflammatory cells from osteoarthritic synovium and synovial fluids." *Clin Immunol* 105(3): 315-325.

Higuchi, H., Shirakura, K., Kimura, M., Terauchi, M., Shinozaki, T., Watanabe, H. and Takagishi, K. (2006). "Changes in biochemical parameters after anterior cruciate ligament injury." *International orthopaedics* 30: 43-47.

Hsu, H. and Siwiec, R. M. (2022). *Knee Osteoarthritis*. StatPearls. Treasure Island (FL).

Hume, D. A. (2015). "The Many Alternative Faces of Macrophage Activation." *Front Immunol* 6: 370.

Hussein, M. R., Fathi, N. A., El-Din, A. M., Hassan, H. I., Abdullah, F., Al-Hakeem, E. and Backer, E. A. (2008). "Alterations of the CD4(+), CD8 (+) T cell subsets, interleukins-1beta, IL-10, IL-17, tumor necrosis factor-alpha and soluble intercellular adhesion molecule-1 in rheumatoid arthritis and osteoarthritis: preliminary observations." *Pathol Oncol Res* 14(3): 321-328.

Irie, K., Uchiyama, E. and Iwaso, H. (2003). "Intraarticular inflammatory cytokines in acute anterior cruciate ligament injured knee." *The knee* 10(1): 93-96.

Ishii, H., Tanaka, H., Katoh, K., Nakamura, H., Nagashima, M. and Yoshino, S. (2002). "Characterization of infiltrating T cells and Th1/Th2-type cytokines in the synovium of patients with osteoarthritis." *Osteoarthritis Cartilage* 10(4): 277-281.

Jackie, L. W., Adam, G. C., Carsten Bogh, J., Bjørnar, B., Alessio, B., Stephanie Rose, F., Pætur, H., Erin, M., Anouk, P. U., Clare, L. A., Andrea, M. B., Garrett, S. B., Allison, M. E., Michael, G., Melissa, H., Mick, H., Lina Holm, I., Karim, M. K., Christina, Y. L., Justin, M. L., Matilde, L., Maxi, M., Britt Elin, Ø., Brooke, P., Anu, M. R., Søren, T. S., Jonas Bloch, T., Clodagh, T., Linda, K. T., Belle, L. v. M., Thomas James, W., James Justin, Y., Lohmander, L. S., Carolyn, E., May Arna, R., Marienke van, M., Ewa, M. R. and Kay, M. C. (2022). "OPTIKNEE 2022: consensus recommendations to optimise knee health after traumatic knee injury to prevent osteoarthritis." *British Journal of Sports Medicine* 56(24): 1393.

Jackson, M. T., Moradi, B., Zaki, S., Smith, M. M., McCracken, S., Smith, S. M., Jackson, C. J. and Little, C. B. (2014). "Depletion of protease-activated receptor 2 but not protease-activated receptor 1 may confer protection against osteoarthritis in mice through extracartilaginous mechanisms." *Arthritis Rheumatol* 66(12): 3337-3348.

Judge, A., Cooper, C., Williams, S., Dreinhoefer, K. and Dieppe, P. (2010). "Patient-reported outcomes one year after primary hip replacement in a European Collaborative Cohort." *Arthritis Care & Research* 62(4): 480-488.

Kamekura, S., Hoshi, K., Shimoaka, T., Chung, U., Chikuda, H., Yamada, T., Uchida, M., Ogata, N., Seichi, A., Nakamura, K. and Kawaguchi, H. (2005). "Osteoarthritis development in novel experimental mouse models induced by knee joint instability." *Osteoarthritis Cartilage* 13(7): 632-641.

- Kandahari, A. M., Yang, X., Dighe, A. S., Pan, D. and Cui, Q. (2015). "Recognition of Immune Response for the Early Diagnosis and Treatment of Osteoarthritis." *J Immunol Res* 2015: 192415.
- Karnell, J. L., Rieder, S. A., Ettinger, R. and Kolbeck, R. (2019). "Targeting the CD40-CD40L pathway in autoimmune diseases: Humoral immunity and beyond." *Advanced Drug Delivery Reviews* 141: 92-103.
- Kittelson, A. J., Schmiede, S. J., Maluf, K., George, S. Z. and Stevens-Lapsley, J. E. (2021). "Determination of Pain Phenotypes in Knee Osteoarthritis Using Latent Profile Analysis." *Pain Medicine* 22(3): 653-662.
- Kloppenborg, M. and Berenbaum, F. (2020). "Osteoarthritis year in review 2019: epidemiology and therapy." *Osteoarthritis Cartilage* 28(3): 242-248.
- Li, Y. S., Luo, W., Zhu, S. A. and Lei, G. H. (2017). "T Cells in Osteoarthritis: Alterations and Beyond." *Front Immunol* 8: 356.
- Lieberthal, J., Sambamurthy, N. and Scanzello, C. R. (2015). "Inflammation in joint injury and post-traumatic osteoarthritis." *Osteoarthritis Cartilage* 23(11): 1825-1834.
- Little, C. B. and Hunter, D. J. (2013). "Post-traumatic osteoarthritis: from mouse models to clinical trials." *Nat Rev Rheumatol* 9(8): 485-497.
- Little, C. B. and Zaki, S. (2012). "What constitutes an "animal model of osteoarthritis"--the need for consensus?" *Osteoarthritis Cartilage* 20(4): 261-267.
- Loeser, R. F., Goldring, S. R., Scanzello, C. R. and Goldring, M. B. (2012). "Osteoarthritis: a disease of the joint as an organ." *Arthritis Rheum* 64(6): 1697-1707.
- Lohmander, L. S., Englund, P. M., Dahl, L. L. and Roos, E. M. (2007). "The long-term consequence of anterior cruciate ligament and meniscus injuries: osteoarthritis." *Am J Sports Med* 35(10): 1756-1769.
- Lohmander, L. S., Englund, P. M., Dahl, L. L. and Roos, E. M. (2007). "The Long-term Consequence of Anterior Cruciate Ligament and Meniscus Injuries: Osteoarthritis." *The American Journal of Sports Medicine* 35(10): 1756-1769.
- Lorenz, J. and Grassel, S. (2014). "Experimental osteoarthritis models in mice." *Methods Mol Biol* 1194: 401-419.
- Lotz, M. K. and Kraus, V. B. (2010). "New developments in osteoarthritis. Posttraumatic osteoarthritis: pathogenesis and pharmacological treatment options." *Arthritis Res Ther* 12(3): 211.

- Ma, H. L., Blanchet, T. J., Peluso, D., Hopkins, B., Morris, E. A. and Glasson, S. S. (2007). "Osteoarthritis severity is sex dependent in a surgical mouse model." *Osteoarthritis and Cartilage* 15(6): 695-700.
- Mack, M., Cihak, J., Simonis, C., Luckow, B., Proudfoot, A. E., Plachy, J., Bruhl, H., Frink, M., Anders, H. J., Vielhauer, V., Pfirstinger, J., Stangassinger, M. and Schlondorff, D. (2001). "Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice." *J Immunol* 166(7): 4697-4704.
- March, L., Smith, E. U. R., Hoy, D. G., Cross, M. J., Sanchez-Riera, L., Blyth, F., Buchbinder, R., Vos, T. and Woolf, A. D. (2014). "Burden of disability due to musculoskeletal (MSK) disorders." *Best Practice & Research Clinical Rheumatology* 28(3): 353-366.
- Martinez, F. O. and Gordon, S. (2014). "The M1 and M2 paradigm of macrophage activation: time for reassessment." *F1000Prime Rep* 6: 13.
- Maruyama, M., Rhee, C., Utsunomiya, T., Zhang, N., Ueno, M., Yao, Z. and Goodman, S. B. (2020). "Modulation of the inflammatory response and bone healing." *Frontiers in endocrinology* 11: 386.
- Mathiessen, A. and Conaghan, P. G. (2017). "Synovitis in osteoarthritis: current understanding with therapeutic implications." *Arthritis Res Ther* 19(1): 18.
- Mobasheri, A. and Batt, M. (2016). "An update on the pathophysiology of osteoarthritis." *Annals of Physical and Rehabilitation Medicine* 59(5): 333-339.
- Mobasheri, A., Saarakkala, S., Finnilä, M., Karsdal, M. A., Bay-Jensen, A. C. and van Spil, W. E. (2019). "Recent advances in understanding the phenotypes of osteoarthritis." *F1000Res* 8.
- Moretti, A., Paoletta, M., Liguori, S., Ilardi, W., Snichelotto, F., Toro, G., Gimigliano, F. and Iolascon, G. (2021). "The Rationale for the Intra-Articular Administration of Clodronate in Osteoarthritis." *Int J Mol Sci* 22(5).
- Murray, P. J. (2017). "Macrophage Polarization." *Annu Rev Physiol* 79: 541-566.
- Muthuri, S. G., McWilliams, D. F., Doherty, M. and Zhang, W. (2011). "History of knee injuries and knee osteoarthritis: a meta-analysis of observational studies." *Osteoarthritis Cartilage* 19(11): 1286-1293.
- Nelson, A. E., Allen, K. D., Golightly, Y. M., Goode, A. P. and Jordan, J. M. (2014). "A systematic review of recommendations and guidelines for the management of osteoarthritis: The Chronic Osteoarthritis Management Initiative of the U.S. Bone and Joint Initiative." *Seminars in Arthritis and Rheumatism* 43(6): 701-712.
- Oo, W. M., Little, C., Duong, V. and Hunter, D. J. (2021). "The Development of Disease-Modifying Therapies for Osteoarthritis (DMOADs): The Evidence to Date." *Drug Des Devel Ther* 15: 2921-2945.

Pereira, D., Peleteiro, B., Araújo, J., Branco, J., Santos, R. A. and Ramos, E. (2011). "The effect of osteoarthritis definition on prevalence and incidence estimates: a systematic review." *Osteoarthritis and Cartilage* 19(11): 1270-1285.

Punzi, L., Galozzi, P., Luisetto, R., Favero, M., Ramonda, R., Oliviero, F. and Scanu, A. (2016). "Post-traumatic arthritis: overview on pathogenic mechanisms and role of inflammation." *RMD Open* 2(2): e000279.

Qi, C., Shan, Y., Wang, J., Ding, F., Zhao, D., Yang, T. and Jiang, Y. (2016). "Circulating T helper 9 cells and increased serum interleukin-9 levels in patients with knee osteoarthritis." *Clin Exp Pharmacol Physiol* 43(5): 528-534.

Riordan, E. A., Little, C. and Hunter, D. (2014). "Pathogenesis of post-traumatic OA with a view to intervention." *Best Pract Res Clin Rheumatol* 28(1): 17-30.

Robinson, W. H., Lepus, C. M., Wang, Q., Raghu, H., Mao, R., Lindstrom, T. M. and Sokolove, J. (2016). "Low-grade inflammation as a key mediator of the pathogenesis of osteoarthritis." *Nat Rev Rheumatol* 12(10): 580-592.

Roemer, F. W., Jarraya, M., Collins, J. E., Kwoh, C. K., Hayashi, D., Hunter, D. J. and Guermazi, A. (2023). "Structural phenotypes of knee osteoarthritis: potential clinical and research relevance." *Skeletal Radiology* 52(11): 2021-2030.

Rosshirt, N., Hagmann, S., Tripel, E., Gotterbarm, T., Kirsch, J., Zeifang, F., Lorenz, H. M., Tretter, T. and Moradi, B. (2019). "A predominant Th1 polarization is present in synovial fluid of end-stage osteoarthritic knee joints: analysis of peripheral blood, synovial fluid and synovial membrane." *Clinical & Experimental Immunology* 195(3): 395-406.

Scanzello, C. R. (2017). "Chemokines and inflammation in osteoarthritis: Insights from patients and animal models." *J Orthop Res* 35(4): 735-739.

Shu, C. C., Zaki, S., Ravi, V., Schiavinato, A., Smith, M. M. and Little, C. B. (2020). "The relationship between synovial inflammation, structural pathology, and pain in post-traumatic osteoarthritis: differential effect of stem cell and hyaluronan treatment." *Arthritis Research & Therapy* 22(1): 29.

Sica, A. and Mantovani, A. (2012). "Macrophage plasticity and polarization: in vivo veritas." *J Clin Invest* 122(3): 787-795.

Soong, R. S., Song, L., Trieu, J., Lee, S. Y., He, L., Tsai, Y. C., Wu, T. C. and Hung, C. F. (2014). "Direct T cell activation via CD40 ligand generates high avidity CD8⁺ T cells capable of breaking immunological tolerance for the control of tumors." *PLoS One* 9(3): e93162.

Struglics, A., Larsson, S., Kumahashi, N., Frobell, R. and Lohmander, L. S. (2015). "Changes in Cytokines and Aggrecan ARGS Neoepitope in Synovial Fluid and Serum and in C-Terminal Crosslinking Telopeptide of Type II Collagen and N-Terminal Crosslinking Telopeptide of

Type I Collagen in Urine Over Five Years After Anterior Cruciate Ligament Rupture: An Exploratory Analysis in the Knee Anterior Cruciate Ligament, Nonsurgical Versus Surgical Treatment Trial." *Arthritis & Rheumatology* 67(7): 1816-1825.

Sun, B. H., Wu, C. W. and Kalunian, K. C. (2007). "New Developments in Osteoarthritis." *Rheumatic Disease Clinics of North America* 33(1): 135-148.

Swain, S., Sarmanova, A., Mallen, C., Kuo, C. F., Coupland, C., Doherty, M. and Zhang, W. (2020). "Trends in incidence and prevalence of osteoarthritis in the United Kingdom: findings from the Clinical Practice Research Datalink (CPRD)." *Osteoarthritis and Cartilage* 28(6): 792-801.

Tran, C. N., Lundy, S. K. and Fox, D. A. (2005). "Synovial biology and T cells in rheumatoid arthritis." *Pathophysiology* 12(3): 183-189.

Tsark, E. C., Wang, W., Teng, Y. C., Arkfeld, D., Dodge, G. R. and Kovats, S. (2002). "Differential MHC class II-mediated presentation of rheumatoid arthritis autoantigens by human dendritic cells and macrophages." *J Immunol* 169(11): 6625-6633.

Wagner, U., Pierer, M., Wahle, M., Moritz, F., Kaltenhauser, S. and Hantzschel, H. (2004). "Ex vivo homeostatic proliferation of CD4+ T cells in rheumatoid arthritis is dysregulated and driven by membrane-anchored TNF α ." *J Immunol* 173(4): 2825-2833.

Waite, J. C. and Skokos, D. (2012). "Th17 Response and Inflammatory Autoimmune Diseases." *International Journal of Inflammation* 2012: 819467.

Wallace, I. J., Worthington, S., Felson, D. T., Jurmain, R. D., Wren, K. T., Maijanen, H., Woods, R. J. and Lieberman, D. E. (2017). "Knee osteoarthritis has doubled in prevalence since the mid-20th century." *Proceedings of the National Academy of Sciences* 114(35): 9332-9336.

Wang, X., Wu, T., Zhou, F., Liu, S., Zhou, R., Zhu, S., Song, L., Zhu, F., Wang, G. and Xia, B. (2015). "IL12p40 regulates functional development of human CD4+ T cells: enlightenment by the elevated expressions of IL12p40 in patients with inflammatory bowel diseases." *Medicine (Baltimore)* 94(10): e613.

Watt, F. E. (2021). "Posttraumatic osteoarthritis: what have we learned to advance osteoarthritis?" *Current Opinion in Rheumatology* 33(1).

Woodell-May, J. E. and Sommerfeld, S. D. (2020). "Role of Inflammation and the Immune System in the Progression of Osteoarthritis." *Journal of Orthopaedic Research* 38(2): 253-257.

Wullt, B., Butler, D. S. C., Ambite, I., Kinsolving, J., Krintel, C. and Svanborg, C. (2021). "Immunomodulation—A Molecular Solution to Treating Patients with Severe Bladder Pain Syndrome?" *European Urology Open Science* 31: 49-58.

Xie, J. H., Yamniuk, A. P., Borowski, V., Kuhn, R., Susulic, V., Rex-Rabe, S., Yang, X., Zhou, X., Zhang, Y., Gillooly, K., Brosius, R., Ravishankar, R., Waggle, K., Mink, K., Price, L., Rehfuess, R., Tamura, J., An, Y., Cheng, L., Abramczyk, B., Ignatovich, O., Drew, P., Grant, S., Bryson, J. W., Suchard, S., Salter-Cid, L., Nadler, S. and Suri, A. (2014). "Engineering of a Novel Anti-CD40L Domain Antibody for Treatment of Autoimmune Diseases." *The Journal of Immunology* 192(9): 4083.

Yamada, H., Nakashima, Y., Okazaki, K., Mawatari, T., Fukushi, J., Oyamada, A., Fujimura, K., Iwamoto, Y. and Yoshikai, Y. (2011). "Preferential accumulation of activated Th1 cells not only in rheumatoid arthritis but also in osteoarthritis joints." *J Rheumatol* 38(8): 1569-1575.

Zhang, L., Li, Y. G., Li, Y. H., Qi, L., Liu, X. G., Yuan, C. Z., Hu, N. W., Ma, D. X., Li, Z. F., Yang, Q., Li, W. and Li, J. M. (2012). "Increased frequencies of Th22 cells as well as Th17 cells in the peripheral blood of patients with ankylosing spondylitis and rheumatoid arthritis." *PLoS One* 7(4): e31000.

Zirlik, A., Maier, C., Gerdes, N., MacFarlane, L., Soosairajah, J., Bavendiek, U., Ahrens, I., Ernst, S., Bassler, N., Missiou, A., Patko, Z., Aikawa, M., Schonbeck, U., Bode, C., Libby, P. and Peter, K. (2007). "CD40 ligand mediates inflammation independently of CD40 by interaction with Mac-1." *Circulation* 115(12): 1571-1580.

Ziyi, J., Dandan, W., Huayong, Z., Jun, L., Xuebing, F., Jinkou, Z. and Lingyun, S. (2020). "Incidence trend of five common musculoskeletal disorders from 1990 to 2017 at the global, regional and national level: results from the global burden of disease study 2017." *Annals of the Rheumatic Diseases* 79(8): 1014.