The Relationship Between Diet, Gut Health and Inflammatory Disease

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Statement of originality

I certify that the intellectual content of this thesis is the product of my own work and that sources and assistance received in preparing this thesis have been acknowledged.

Presented in chapter 2 is the published work of which I am a co-author. This contains an endorsement statement signed by all other authors on this paper.

This thesis has not been submitted for any other degree or other purposes.

Gabriela Veronica Pinget
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**2019**
International Congress of Mucosal Immunology “Effects of Psoriasis on Gut Homeostasis”, Brisbane, Australia

**2019**
Charles Perkins Centre Biology Domain Seminar invited speaker for outstanding presentation: “impact of Psoriasis on Gut Homeostasis”, Sydney, Australia

**2018**
Charles Perkins Centre Biology Domain Seminar: “impact of Psoriasis on Gut Homeostasis”, Sydney, Australia

**2018**
Cell Translational Immunometabolism Conference “Impacts of the Dietary Food Additive Titanium Dioxide (E171) on IL-17 Producing Cells and Psoriasis Outcome”, Basel, Switzerland

**2017**
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Contributing directly to this thesis


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Related to the research but not presented in this Thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Atopic dermatitis</td>
</tr>
<tr>
<td>AMP</td>
<td>Antimicrobial peptide</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's disease</td>
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<td>DC</td>
<td>Dendritic cell</td>
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<tr>
<td>DSS</td>
<td>Dextran sulfate sodium model of colitis</td>
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<td>E171</td>
<td>Food grade titanium dioxide</td>
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<tr>
<td>EAE</td>
<td>Autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EC</td>
<td>Elizabeth collar</td>
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<tr>
<td>GPCR</td>
<td>G-coupled protein receptor</td>
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<tr>
<td>GWAS</td>
<td>Genome-wide association study</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase</td>
</tr>
<tr>
<td>i.v.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IEC</td>
<td>Intestinal epithelial cell</td>
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<tr>
<td>IEL</td>
<td>Intraepithelial lymphocyte</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin 10</td>
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<tr>
<td>IL-17</td>
<td>Interleukin 17</td>
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<tr>
<td>IL-1β</td>
<td>Interleukin 1 beta</td>
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<tr>
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<td>Interleukin 23</td>
</tr>
<tr>
<td>ILC</td>
<td>Innate lymphoid cell</td>
</tr>
<tr>
<td>ILN</td>
<td>Inguinal lymph node</td>
</tr>
<tr>
<td>IMP</td>
<td>Immune modifying particle</td>
</tr>
<tr>
<td>IMQ-pso</td>
<td>Imiquimod-induced psoriasis</td>
</tr>
<tr>
<td>iNKT</td>
<td>Invariant natural killer T cell</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid producing bacteria</td>
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<tr>
<td>LP</td>
<td>Lamina propria</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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</tr>
<tr>
<td>LPS:</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MAMP:</td>
<td>Microbial associated molecular patterns</td>
</tr>
<tr>
<td>MCP-1:</td>
<td>Monocyte chemoattractant protein-1 (CCL2)</td>
</tr>
<tr>
<td>Mφ:</td>
<td>Macrophage</td>
</tr>
<tr>
<td>MFI:</td>
<td>Median fluorescence intensity</td>
</tr>
<tr>
<td>MLN:</td>
<td>Mesenteric lymph node</td>
</tr>
<tr>
<td>NAFLD:</td>
<td>Non alcoholic fatty liver disease</td>
</tr>
<tr>
<td>NK:</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NKT:</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>NMR:</td>
<td>Nuclear magnetic resonance spectroscopy</td>
</tr>
<tr>
<td>PCoA:</td>
<td>Principal coordinate analysis</td>
</tr>
<tr>
<td>PRR:</td>
<td>Pattern recognition receptor</td>
</tr>
<tr>
<td>PsA:</td>
<td>Psoriatic arthritis</td>
</tr>
<tr>
<td>SCFA:</td>
<td>Short chain fatty acid</td>
</tr>
<tr>
<td>TGF-β:</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TiO₂:</td>
<td>Titanium dioxide</td>
</tr>
<tr>
<td>TJ:</td>
<td>Tight junction</td>
</tr>
<tr>
<td>TLR:</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNFα:</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
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<tr>
<td>γδT:</td>
<td>Gamma delta T cells</td>
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Abstract

Gut homoeostasis is central to health outcomes and its disruption is linked to a growing number of inflammatory diseases. Diet plays a major role in shaping gut homeostasis. However, while studies often focused on the effects of macronutrients, the impact of dietary additives, particularly titanium dioxide (E171), is less well defined. Additionally, while many studies focus on how altered gut homeostasis may increase predisposition to inflammatory diseases, few studies have investigated whether inflammatory diseases itself can trigger changes to gut homeostasis. This thesis seeks to understand how the common food additive, E171 and the inflammatory disease, psoriasis each impact key features of gut homeostasis.

Investigations reveal that oral consumption of E171 in mice resulted in changes to the release of bacterial metabolites *in vivo* and promoted biofilm formation by colonic commensal bacteria *in vitro*. This was accompanied by increased transcription of inflammatory cytokines, reduced colonic crypt length and increased production of antimicrobial peptides in the colon, altogether indicating the presence of low-grade colonic inflammation.

Through the use of the imiquimod-induced mouse model of psoriasis we also reveal that this inflammatory skin condition induced proliferation of colonic macrophages, stimulated the transcription of macrophage-associated cytokines and led to microbial dysbiosis. Migrating monocytes were not found to be contributing to increased macrophage populations within the colon of psoriatic mice, although, their migration to the dermis was found to promote disease pathogenesis.

This thesis reveals new insights into the ways that diet and inflammatory diseases affect gut homeostasis. A deepened understanding of the complex interactions between gut homeostasis, diet and inflammatory disease may provide new clinical targets for disease prevention and management.
Chapter 1: Introduction: The relationship Between Diet, Gut Health and Inflammatory Diseases

1.1. The link between diet and inflammatory diseases

Inflammatory and autoimmune diseases such as asthma (Eder, Ege et al. 2006), food allergy (Wang and Sampson 2011) and type 1 diabetes (Patterson, Harjutsalo et al. 2019) have increased in incidence over the last two decades, particularly in the western world at such alarming rates that food allergy is now considered a major public health concern in many countries (Wang and Sampson 2011, Tan, McKenzie et al. 2016). A plethora of research has now linked incidence of these non-communicable, inflammatory diseases to the adoption of the “Western diet” (Maslowski and Mackay 2011, Thorburn, Macia et al. 2014). This diet, which is low in fibre, high in saturated fat and increasingly high in food additives (Laudisi, Stolfi et al. 2019), including emulsifiers and colourants, has been demonstrated to have immense impacts on gut homeostasis (Viennois, Merlin et al. 2016, Bettini, Boutet-Robinet et al. 2017, Laudisi, Stolfi et al. 2019). Disrupted gut homeostasis can lead to dysregulation of the immune system, resulting in increased risk of inflammatory disease development, as has been demonstrated in mouse models of disease and is discussed below. For this reason, research into diet and its impact on gut homeostasis is critical for the understanding of how the western diet may play a causative role in driving inflammatory disease development.

While studies into diet and inflammatory disease in humans are plentiful, these are mostly correlative and so whether dietary changes play a causative role in inflammatory disease outcomes is not well understood. Human epidemiological studies are problematic in that they yield inconsistent results due to a number of complex factors which come into play, including genetic and various environmental influences (Litonjua 2008, Phillips 2013, Manzel, Muller et al. 2014, Minihane, Vinoy et al. 2015). These challenges result in a heavy reliance on animal models as is seen in throughout this thesis.
1.1.1 The impact of food additives

Food additives are commonly used to alter the colour, improve the texture and increase the shelf life of food, particularly on a wide, commercial scale where consistency of food quality and long shelf life are desired. Polysorbate 80 (P80) and carboxymethylcellulose (CMC) are both dietary emulsifiers widely used. These food additives have been demonstrated to alter the microbiome of mice, resulting worsened murine colitis in an IL-10 knock out colitis model, and increase predisposition to metabolic syndrome (Chassaing, Koren et al. 2015).

The food additive titanium dioxide (E171) is commonly used as a whitening and brightening agent and is estimated to be present in over 900 food products (Cao, Han et al. 2020). E171 has been at the centre of media and government attention since its oral intake was found to increase colorectal cancer risk in rats in 2017 (Bettini, Boutet-Robinet et al. 2017). This has resulted in the subsequent ban of the sale of food products containing E171 in France from January 1, 2020 (Boutilliera, Fourmentinb et al. 2020). While strict measures against the sale of foodstuffs containing E171 have been made in some countries, the effect of E171 on gut health, including impacts on the immune system and gut microbiome, remains poorly understood. For this reason, this common food additive is investigated in Chapter 2 with particular focus on how E171 impacts gut homeostasis.

1.2 Gut homeostasis

Gut homeostasis describes complex interactions within the intestine which results in immunological protection from foreign antigen whilst maintaining tolerance towards innocuous antigen such as that from food and certain microbes (Belkaid and Harrison 2017). This delicate balance is the product of millions of years of co-evolution between the host and the trillions of commensal microbes which reside in the gut, the precise mechanisms of which are only just starting to be uncovered (Moeller, Caro-Quintero et al. 2016).

Gut homeostasis is made up of three main components: 1. the intestinal epithelial layer which is the main physical barrier between the host and the outside world (Maloy and Powrie 2011); 2. the
microbial community, most of which reside in the colon known collectively as the microbiome; and 3. the immune system which regulates and is regulated by the bacterial community within the host. These three components are constantly interacting as discussed in more detail below.

1.2.1 Intestinal epithelium

The intestinal epithelium is made up of a monolayer of intestinal epithelial cells (IECs). While it was once thought that these cells were only involved in absorbing luminal contents, it is now accepted that they are highly specialised, structurally organised and are paramount for preserving intestinal homeostasis (Dahan, Roth-Walter et al. 2007, Allaire, Crowley et al. 2018). IECs are subdivided into absorptive enterocytes, secretory cells, including mucus-producing goblet cells and antimicrobial peptide secreting Paneth cells which are almost exclusively present in the crypt of the small intestine and M cells which are present in Payer’s patches of the small intestine and play a key role in antigen sampling (Roth, Franken et al. 2012, Allaire, Crowley et al. 2018). Together, these cells are responsible for key components which maintain homeostasis, including the regulation of paracellular permeability via tight junction proteins, the production of antimicrobial peptides and the maintenance of a mucosal barrier which are explored in more detail below (Pastorelli, De Salvo et al. 2013).

i. Epithelial tight junctions

The epithelium is under constant self-renewal and during this process, cells move in a crypt-to-tip fashion whilst not disrupting the junctions which hold together adjacent cells (Garrett, Gordon et al. 2010). This is a crucial feature for homeostasis and any breach which allows microbial antigen to translocate across the intestinal mucosa triggers huge inflammatory response, as is seen in such conditions as inflammatory bowel disease (IBD) (Capaldo, Powell et al. 2017). The seals which exist between cells are known as tight junctions (TJ) and work to eliminate paracellular space by forming intercellular webs. These webs are made from a number of proteins from the claudin family and each cell expresses multiple claudins, which changes according to location along the crypt (Holmes, Van

ii. Mucosal barrier

Two mucus layers are present within the colon: the outer, moveable, loose mucus layer and the inner, dense layer, which tightly adheres to the surface of the epithelial cells. In a mouse, the outer layer is approximately 100 μm thick and the inner layer is approximately 50 μm thick (Johansson, Larsson et al. 2011). While the outer layer is in contact with the commensal microbes, the inner layer is completely devoid of bacteria, a critical feature of intestinal integrity and homeostasis (Johansson, Phillipson et al. 2008). Without this specialised mucus barrier present, epithelial cells are in close contact with commensal microbes, which can activate TLRs and trigger inflammatory responses, resulting in colitis (van der Post, Jabbar et al. 2019).

Colonic mucus layers are made up of mucins, highly glycosylated proteins produced by goblet cells (Johansson, Larsson et al. 2011). Different tissues express a range of different mucins and the function of mucin is dictated by its structure. Within the colon, MUC2 is the major mucin present both in mice and in humans, coded by Muc2 (Johansson, Larsson et al. 2011). In mice, complete Muc2 knock out models resulted in spontaneous colitis development and Muc2 heterozygous mice develop worse colitis in a dextran sulfate sodium model of colitis (DSS) compared to wild type mice (Van der Sluis, De Koning et al. 2006). In humans, it has been observed that the colonic mucus layer is thinner and that MUC2 synthesis is reduced during active colitis (Tytgat, vanderWal et al. 1996, van der Post, Jabbar et al. 2019). This suggests that dysfunctional mucus barrier may predispose to colitis flare ups, although this has not been demonstrated causatively.
iii. **Antimicrobial peptides**

Antimicrobial peptides (AMPs) are evolutionarily conserved proteins among mammals that act to defend against viral, bacterial and fungal infection, assist in wound repair and induce angiogenesis (Lehrer and Ganz 1999, Nizet, Ohtake et al. 2001, Yang, Biragyn et al. 2002, Koczulla, von Degenfeld et al. 2003, Mangoni, McDermott et al. 2016). Over a thousand AMPs have been identified, including α-defensins, β-defensins and cathelicidins (Wang, Li et al. 2009). AMPs are produced mostly by epithelial cells but also by immune cells in a tissue specific manner (Campbell, Fantacone et al. 2012). These peptides exert their action upon excretion or can act on engulfed microorganisms within phagocytes (Guani-Guerra, Santos-Mendoza et al. 2010). AMPs play a critical role in host defence their reduced expression has been associated with Crohn’s disease and ulcerative colitis (Wehkamp, Harder et al. 2003, Zilbauer, Jenke et al. 2010).

AMPs are expressed both constitutively and upon stimulation. This can be through dietary components, particularly vitamins A and D, as well as certain bacteria (both commensal and pathogenic) and their bi-products including butyrate (Cash, Whitham et al. 2006, Campbell, Fantacone et al. 2012). Certain bacteria can stimulate the production of AMPs via the activation of pattern recognition receptors (PRRs), for instance, commensal *Bacteroides thetaiotaomicron* stimulates angiogenin 4 production, which has strong antimicrobial effects (Hooper, Stappenbeck et al. 2003). Interestingly, this bacteria is less sensitive to the actions of Ang4 compared to other commensals, indicating that this stimulatory action is working in the favour of *B. thetaiotaomicron* by eliminating more sensitive microorganisms (Hooper, Stappenbeck et al. 2003). This example gives a sense of the complexity of the relationship, which exists between the host and microbiome.

1.2.2 **Microbiome, metabolites and homeostasis**

Trillions of microorganisms make up the complex commensal microbiome, which exists within the mammalian gastrointestinal tract (Huttenhower, Gevers et al. 2012, Zhang, Li et al. 2019). Germ-free studies over the last ten years have changed the way the microbiome is viewed in the literature and
these microbes have gone from being classified as passive and tolerated by the host to critical players in regulating intestinal homeostasis and host health. Although this encompasses a range of microorganisms, including fungi, viruses, archaea and bacteria, the focus of this thesis and thus this introductory chapter is the bacterial component (Huffnagle and Noverr 2013, Shreiner, Kao et al. 2015).

The intestinal microbiome impacts the host in a multitude of ways, many of which have not yet been uncovered. Commensals can stimulate IECs to proliferate (Kim and Ho 2010, Thomas, Parker et al. 2018), induce mucus secretion by goblet cells (Birchenough, Nystrom et al. 2016, van der Post, Jabbar et al. 2019), regulate the local immune cells and shape lymphoid structures (Mazmanian, Liu et al. 2005, Belkaid and Hand 2014). The microbiome has also been demonstrated to have a major impact on a range of inflammatory diseases, as shown in mouse models of allergic asthma, food allergy and skin disorders such as psoriasis (Thorburn, McKenzie et al. 2015, Tan, McKenzie et al. 2016, Benhadou, Mintoff et al. 2018, Cait, Hughes et al. 2018, Clemente, Manasson et al. 2018, Sokolowska, Frei et al. 2018).

i. Microbial colonisation

The gastrointestinal tract was, until recently, widely accepted as being sterile until birth, at which point microbes present in the environment were believed to rapidly colonise the gut (Belkaid and Hand 2014). This dogma has been since been challenged and evidence demonstrating that the placenta harbours its own microbiome, which is then transferred to the foetus in utero has resulted in two opposing views regarding the sterility of foetus in utero (Aagaard, Ma et al. 2014, Perez-Munoz, Arrieta et al. 2017). The largest human study to date supports the idea of a sterile placenta and suggests that contamination are the likely cause of conflicting results (de Goffau, Lager et al. 2019). However, as evidence supporting both sides of the debate continues to emerge (Stinson, Boyce et al. 2019), it thus remains unclear when first exposure to microbes occurs.
After birth, environmental factors including mode of birth and breastfeeding shape the microbiota from an early age in both humans and mice (Perez, Dore et al. 2007, Marcobal, Barboza et al. 2010, Shao, Forster et al. 2019).

Early colonisation from the maternal microbiome is thought not only to prevent opportunistic infection but also shape the neonatal immune response towards the non-pathogenic commensal bacteria, with lasting effects into adulthood (Perez, Dore et al. 2007, Marcobal, Barboza et al. 2010, Wampach, Heintz-Buschart et al. 2018, Shao, Forster et al. 2019).

However, the effects of mode of delivery on the microbiome remain controversial. Studies have demonstrated c-section to have significant, long-term effects on microbial composition with implications for allergic disease development (Salminen, Gibson et al. 2004, Mitselou, Hallberg et al. 2018, Reyman, van Houten et al. 2019). However, variables such as maternal obesity and gestational age are both indications for c-section as well as factors shaping infant microbiome, and are thought to confound study outcomes (Stinson, Payne et al. 2018). These factors also play a role in determining breastfeeding behaviour, which again impacts the microbiome. Additionally, the resulting effects on the infant microbiota have more recently demonstrated to be short lived, with major differences no longer present at 9 months of age (Shao, Forster et al. 2019). These conflicting outcomes are partly due to improved technologies but also illustrate the complex interplay between environment and microbiome and highlight limitations to human studies.

ii. Metabolites and short chain fatty acids

The microbiome acts on the host immune system via both direct and indirect stimulation pathways. Direct stimulation of host cells can occur via recognition of microbial associated molecular patterns (MAMPs) by specialised receptors. For example, Toll Like Receptors (TLRs), present on IECs and innate immune cells, recognise ligands such as the endotoxin present on the cell wall of Gram negative bacteria, lipopolysaccharide (LPS) (Belkaid and Hand 2014). Upon stimulating TLR4, LPS can induce a pro-inflammatory immune response by activating NF-kB in innate immune cells, resulting in the
production of inflammatory cytokines and chemokines (Lu, Yeh et al. 2008). Interestingly, IECs from vaginally born mice have been shown to acquire tolerance to LPS after initial exposure at birth, indicating a pathway for acquired tolerance towards commensal bacteria. This was not true for caesarean-born mice, which produced inflammatory cytokines and chemokines upon LPS stimulation in a TLR4-dependant manner (Lotz, Gutle et al. 2006).

Commensal bacteria also stimulate the host indirectly through the production of metabolites. Among these are short chain fatty acids (SCFA), which have been found to have profound effects on the immune system both locally and systemically. SCFAs result from the fermentation of undigestible fibre and include acetate, butyrate, propionate (Daien, Pinget et al. 2017). They act via two main mechanisms, firstly by binding metabolite-sensing G-protein coupled receptors (GPCRs) expressed on both immune cells and non-immune cells (Macia, Tan et al. 2015, Tan, McKenzie et al. 2016). Secondly, SCFA have been shown to inhibit histone deacetylases (HDAC), resulting in the suppression of inflammatory signalling in immune cells (Smith, Howitt et al. 2013, Thorburn, McKenzie et al. 2015). The impacts of microbial metabolites are varied with wide implications for inflammatory diseases. They contribute to the development of local immune system as demonstrated by a recent study in which dendrite protrusion of CX3CR1⁺ immune cells into the intestinal lumen was induced by lactate and propionate in a GPR31-dependant fashion (Morita, Umemoto et al. 2019). These protrusions are necessary for normal functioning of the mucosal immune system and enhanced resistance to a model of Salmonella infection (Morita, Umemoto et al. 2019). Metabolites have also been demonstrated to protect against disease. The SCFAs acetate and butyrate protect against DSS colitis by binding GPR43 and GPR109A on colonic IECs, ultimately resulting in IL-18 release via inflammasome activation, which promoted epithelial repair (Macia, Tan et al. 2015). Butyrate and acetate can also induce regulatory T cell (Treg) differentiation via its actions on CD103⁺DCs, resulting in protection from peanut allergy in mice in a GPR43 and GPR109A dependant way (Tan, McKenzie et al. 2016). Butyrate and acetate can also directly promote Treg differentiation via HDAC inhibition (Smith, Howitt et al. 2013) ultimately protecting mice from allergic airway disease, the mouse model for human asthma (Thorburn,
McKenzie et al. 2015). Together, these studies demonstrate the varied ways bacterial metabolites can exert local and systemic effects on the host immune system.

iii. Factors which shape the microbiome

Many factors dictate the composition of the microbiome and metabolite production, including antibiotic use, mode of birth (Bokulich, Chung et al. 2016), diet (Turnbaugh, Ridaura et al. 2009), exercise (Allen, Berg Miller et al. 2015, Barton, Penney et al. 2018) and genetics (Goodrich, Waters et al. 2014). The consumption of food additives has recently been found to have adverse effects on the microbiome and found to increase risk in mice of colorectal cancer, colitis, and metabolic syndrome (Bettini, Boutet-Robinet et al. 2017, Laudisi, Stolfi et al. 2019). This includes emulsifiers CMC and P80 as mentioned above as well as the food colourant titanium dioxide (E171) (Chassaing, Koren et al. 2015, Chassaing, Van de Wiele et al. 2017, Pinget, Tan et al. 2019). Administration of the emulsifiers CMC and P80 resulted in a shift in the microbiome of mice, increased gut permeability and reduced mucus thickness in the colon, leading to increased inflammatory potential and increased risk of colitis and metabolic syndrome (Chassaing, Koren et al. 2015). These inflammatory outcomes were not seen in germ-free models, indicating the necessary role of the microbiome in the inflammatory pathways initiated by P80 and CMC. Such findings demonstrate the direct impact that food additives can have on the microbiome and further implications for inflammatory disease development, indicating the need for more research in the area. The effects of E171 on the microbiome are explored in chapter 2 of this thesis.

1.2.3 The intestinal immune system

The intestinal immune system is made up of dendritic cells (DCs), macrophages, B cells, T cells and innate lymphoid cells such as γδT cells and NK cells. These cells work together to develop tolerance towards food antigen and commensal bacteria, while still initiating an appropriate inflammatory response to pathogens. Failure to initiate tolerance is thought to be the basis of inflammatory disorders such as food allergy and inflammatory bowel disease (IBD) (Vickery, Scurlock et al. 2011,
Steinbach and Plevy 2014). These cells exist among an abundance of microbial and food antigen and yet, under normal conditions, do not initiate widespread inflammation. This is due to a number of unique characteristics, which are paramount to maintaining gut homeostasis (Gross, Salame et al. 2015). For example, intestinal macrophages and DCs are both hyporesponsive towards LPS when compared to macrophages and DCs found in other tissues, resulting in a lack of inflammatory signalling when in contact with the common antigen (Smythies, Sellers et al. 2005, Cerovic, Jenkins et al. 2009, Garrett, Gordon et al. 2010, Ueda, Kayama et al. 2010, Bain and Schridde 2018). This hyporesponsive nature is critical to prevent uncontrolled colonic inflammation and is maintained, in part, by continuous high levels of IL-10 production.

IECs facilitate the communication between luminal antigen and the immune system to initiate or attenuate host immune responses (Gross, Salame et al. 2015, Okumura and Takeda 2017). IECs play a particular role in sampling luminal contents and facilitating transcytosis of antigen to immune cells, particularly DCs for processing (Allaire, Crowley et al. 2018). This process is critical for initiating oral tolerance through the induction of Tregs by DCs. Certain subsets of CD103⁺ DCs migrate from the lamina propria into the draining mesenteric lymph node (MLN) where they initiate Treg differentiation as well as induce their expression of the gut homing receptor CCR9 (Bekiaris, Persson et al. 2014, Tan, McKenzie et al. 2016, Shiokawa, Kotaki et al. 2017, Stagg 2018).

**Intestinal macrophages**

Macrophages make up roughly 20% of all leukocytes within the colon and in contrast to DCs are thought to be non-migratory, although this may change during inflammatory processes (Bain and Mowat 2011, Diehl, Longman et al. 2013, Schulthess, Pandey et al. 2019). Macrophages in the gut have varied roles, which support normal gut function, including supporting IEC proliferation (Pull, Doherty et al. 2005), sampling antigen, and clearing debris from cellular apoptosis (Cummings, Barbet et al. 2016, Gordon and Pluddemann 2018).
The origin of intestinal macrophages has been highly debated in the literature. While it was once thought that these cells were exclusively replenished by bone marrow derived monocytes, it has since been demonstrated that populations within the intestine are of heterogenous origin (Bain, Bravo-Blas et al. 2014, Shaw, Houston et al. 2018). While some macrophages are indeed replenished by monocyte progenitors, a distinct population, which arises during embryonic development is locally maintained through proliferation (Shaw, Houston et al. 2018). These populations appear to have distinct functions as demonstrated by varying capacities to produce cytokines and differences in morphological characteristics and transcription profiles. This gives a sense of the highly organised and specialised nature of intestinal macrophages, reflecting their varied roles in maintaining homeostasis. Inconsistencies in the literature are likely due to challenges in accurately identifying these cellular subsets, which is changing with improving technologies.

Macrophages are in close contact with the microbiome and require live bacteria and bacterial metabolites to maintain their populations (Shaw, Houston et al. 2018) as well as develop normal morphology (Morita, Umemoto et al. 2019). CX3CR1+ macrophages extend dendrites from the lamina propria, between adjacent IECs to sample luminal contents and by doing so, also participate in local tolerance through the production of anti-inflammatory IL-10 and the promotion of Treg survival (Hadis, Wahl et al. 2011, Steinbach and Plevy 2014). Additionally, their rapid clearance of apoptotic debris and phagocytosis of neutrophils creates an anti-inflammatory milieu, which promotes inflammation resolution, altogether critical for maintaining gut homeostasis (Na, Stakenborg et al. 2019).

However, if the gut homeostasis is disrupted, intestinal macrophages can be polarised to an inflammatory state and subsequently produce a range of pro-inflammatory cytokines, including TNF-α and IL-23. Theses cytokines have downstream effects on IL-17 production by Th17 cells, leading to a powerful inflammatory cascade (Kamada, Hisamatsu et al. 2008, Na, Stakenborg et al. 2019). For this reason, macrophages are thought to be major players in the pathogenesis of IBD and colorectal cancer and are often targeted in novel therapeutic interventions (Kamada, Hisamatsu et al. 2008, Isidro and
Appleyard 2016, Na, Stakenborg et al. 2019). Together this highlights the central role macrophages play in maintaining gut homeostasis and the damaging impacts that disrupting this delicate balance has.

1.3 Gut homoeostasis plays a central role in health

Gut homeostasis has vast impacts on the immune system and its disruption is implicated in a number of inflammatory diseases as outlined above. A major component of this thesis is understanding whether these effects extend to skin health, more specifically, in the context of psoriasis.

1.3.1 The “gut- skin axis”

The last few years have seen the emergence of the notion of the “gut-skin axis” in which intestinal homeostasis is thought to impact skin health (O’Neill, Monteleone et al. 2016). This concept is supported by the observation that gastrointestinal disorders are often accompanied by presence of skin comorbidities. Examples include the presence of skin ulcers in patients with inflammatory bowel disease (Thrash, Patel et al. 2013), rosacea commonly associated with *Helicobacter pylori* infection (Emiroglu, Cengiz et al. 2018), and dermatitis or psoriasis in patients with coeliac disease (O’Neill, Monteleone et al. 2016). These associations are poorly understood, and emerging theories attribute these skin conditions to dysbiosis of the gut. This has led to investigations into whether common skin conditions can be improved by oral supplementation with probiotics as outlined below.

*Lactobacilli* and *Bifidobacteria* are two species of lactic acid producing bacteria (LAB), which are often the focus of probiotic supplementation studies. *Lactobacillus rhamnosus* GG administration was demonstrated to improve eczema in children with concurrent cow’s milk allergy (Majamaa and Isolauri 1997). Additionally, *Lactobacillus rhamnosus* 19070-2 and *Lactobacillus reuteri* DSM 122460 administered in combination has shown to significantly reduce the severity of atopic dermatitis (AD)
in children (Rosenfeldt, Benfeldt et al. 2003). Some associations have been made between disrupted intestinal homeostasis and psoriasis, which are discussed in greater detail below.

Two small studies looked specifically at the effects of orally supplemented *Lactobacillus paracasei* (*L. paracasei*) on the skin of volunteers. One study found it to improve dandruff (Reygagne, Bastien et al. 2017), while the other found it to reduce skin reactivity to capsaicin challenge and improve skin barrier function after supplementation for 2 months (Gueniche, Philippe et al. 2014). Interestingly, supplementing with *L. paracasei* appeared to reduce colonisation of *Malassezia restricta* and *Malassezia globose* on the skin (Reygagne, Bastien et al. 2017). These are two types of yeast commonly associated with dandruff, indicating that the colonic microbiome may influence skin conditions and therefore alter the skin microbiome. These studies do not explore the mechanisms underlying such outcomes and studies into *L. paracasei* are largely conducted by employees of the cosmetic company L’Oreal. While no conflict of interest is stated, interpretations must be made with caution and more research is needed.

While not yet demonstrated *in vivo*, proposed mechanisms suggest that bacterial products enter the bloodstream via the gut and subsequently reach immune cells within the skin. One study took the supernatant from a co-culture containing the both colonic cell line Caco-2 and peripheral blood mononuclear cells (PBMCs) after stimulation with *L. paracasei*. This supernatant was then applied to an *ex vivo* skin sample with or without the neuropeptide substance P, which is often seen to be elevated in the skin of psoriasis patients (Remrod, Lonne-Rahm et al. 2007, Mashagi, Marmalidou et al. 2016). This resulted in reduced oedema, mast cell degranulation, vasodilation and TNF-α release in skin samples stimulated with the supernatant and substance P than with substance P alone (Gueniche, Benyacoub et al. 2010). However, it is not clear from this study whether it was a product of the Caco-2/ PBMC cells or from the bacteria itself that inhibited inflammation.

Together, these studies highlight a gap in the literature surrounding the impact that the microbiome and gut homeostasis has on skin health and inflammatory skin conditions.
1.4 Psoriasis

1.4.1 Definition of psoriasis

Psoriasis is a chronic auto-inflammatory skin disease, which is estimated to affect 2-4% of the world’s population (Christophers 2001, Chandran and Raychaudhuri 2010, Parisi, Symmons et al. 2013). There are variations in morphological presentations of psoriasis but the most prevalent, accounting for around 90%, is plaque psoriasis, characterised by red, scaly plaques, epidermal hyperplasia, dilatation of dermal blood vessels, accumulation of inflammatory cells in dermis and activation of IL-23 and IL-17 producing cells (Di Cesare, Di Meglio et al. 2009, Cai, Shen et al. 2011, Dogra and Mahajan 2016). Often patients with psoriasis exhibit a relapsing and remitting pattern (Langley, Krueger et al. 2005) and while some treatments are effective, to date there is no cure. There is a strong familial link in patients with psoriasis and genome-wide association (GWAS) studies have identified a number of genes associated with disease susceptibility, including the IL-23 receptor \textit{IL23R} (Chandran 2013, Fu, Lee et al. 2018).

1.4.2 The imiquimod mouse model is similar to the human form of psoriasis

Psoriasis is induced in mice using topical application of the cream Aldara, which contains the TLR7/8 ligand imiquimod (5% w/w) (van der Fits, Mourits et al. 2009). Mice lacking TLR7 are not susceptible to disease development (Pantelyushin, Haak et al. 2012). This established mouse model of disease has been used for the last 10 years (Hawkes, Gudjonsson et al. 2017) and closely resembles the human form of psoriasis.

Aldara is commonly used to treat skin tumours and warts in patients and the induction of scaly, psoriasis-like plaques is a commonly reported side effect (Ermertcan, Ozturk et al. 2011, Griffiths, van der Walt et al. 2017). The TRL7/8 activation by imiquimod triggers a cascade of cytokine production, most importantly IL-23 and IL-1β production by dendritic cells and macrophages, which in turn acts on Th17 and γδT cells to produce IL-17 (Cai, Shen et al. 2011). Both IL-17 and IL-23 are necessary drivers of disease in both humans and in the mouse model and treatment targeting these cytokines is
available with successful outcomes for patients (Reich 2012, Frieder, Kivelevitch et al. 2018). This cytokine profile is necessary for induction of psoriasis and IL-23 or IL-17 receptor deficiency almost completely inhibits disease development (van der Fits, Mourits et al. 2009, Cai, Shen et al. 2011).

IL-6, CXCL-2, TNF-α and IL-22 are also important cytokines and chemokines involved in IMQ-pso, which altogether bears great resemblance to the cytokine profile seen in psoriasis vulgaris in humans (Lowes, Kikuchi et al. 2008, Nograles, Zaba et al. 2009, El Malki, Karbach et al. 2013, Yoshiki, Kabashima et al. 2014, Vinter, Kragballe et al. 2016). These cytokines work to stimulate proliferation of keratinocytes and increased production of chemokines, S100 proteins and beta-defensins, all contributing to disease progression (Lowes, Suarez-Farinas et al. 2014).

Although the use of this model has surged in the last decade, the precise initiators of IMQ-psoriasis are yet to be completely elucidated (Hawkes, Gudjonsson et al. 2017). Early on in disease induction, the affected dermis exhibits increased numbers of monocytes, monocyte-derived DCs and neutrophils (Terhorst, Chelbi et al. 2015). Whether DCs or macrophages are the main drivers of disease is still unclear and contradictory outcomes reported in the literature are likely due in part to new markers available for identifying these cell types (van der Fits, Mourits et al. 2009, Lowes, Suarez-Farinas et al. 2014, Morimura, Oka et al. 2016).

CD103+ DCs migrate from the skin to the draining lymph node and are important for IL-17 induction and antigen presentation (Bedoui, Whitney et al. 2009, Jiao, Bedoui et al. 2014, Tomura, Hata et al. 2014). The major producers of IL-17 in IMQ-pso are the gamma delta T (γδT) cells as demonstrated by lack of disease progression in mice lacking these cells (Cai, Shen et al. 2011). γδT cells are a unique population of T cells which serve both innate and adaptive functions and are thought to be rapid initiators of a number of inflammatory processes (Vantourout and Hayday 2013). These cells are also thought to play a major role in the human form of psoriasis (Cai, Shen et al. 2011, Pantelyushin, Haak et al. 2012). Th17 and some innate cells including NKT cells are also contributors to IL-17 production but to a lesser extent (van der Fits, Mourits et al. 2009, Jaiswal, Sadasivam et al. 2017) .
Two homologs of IL-17, IL-17A and IL-17F are most prominently produced in IMQ-pso (Brembilla, Senra et al. 2018). While IL-17F is thought to be weaker, both act in similar ways and both are critical for forming psoriatic plaques and mice lacking IL-17F in particular are protected from psoriasis formation (Pantelyushin, Haak et al. 2012, Brembilla, Senra et al. 2018). Interestingly, much of the literature to date has been focused on the actions of IL-17A, which been reasonably well defined while the actions of IL-17F are less understood. This is relevant to experiments described in chapter 3 and is explored in greater detail there.

1.4.3 Psoriasis as a systemic disorder linked to the gut

There are a number of comorbidities associated with psoriasis, including psoriatic arthritis (PsA), Crohn’s disease (CD), metabolic syndrome and non-alcoholic fatty liver disease (Cohen, Dreher et al. 2009, Li, Han et al. 2013, van der Voort, Koehler et al. 2014, de Oliveira, Rocha et al. 2015). In the last 30 years, incidence of psoriasis has increased however, this is while incidence of the biggest risk factor for this disease, smoking has declined (Huerta, Rivero et al. 2007, Bo, Thoresen et al. 2008, Parisi, Symmons et al. 2013). This observed rise in psoriasis may be due partly to improved diagnostics but also due to the rising prevalence of other risk factors, particularly obesity, indicating that obesity may be contributing to this inflammatory disease (Parisi, Symmons et al. 2013).

Patients with psoriasis are also noted to have elevated levels of IL-1β, IL-6, TNFα and C-reactive protein in peripheral circulation, some of which are also found to be elevated in obesity (Bastard, Jardel et al. 2000, Balato, Napolitano et al. 2015, Ramirez-Bosca, Navarro-Lopez et al. 2015). As a result, it has been suggested that psoriasis should be upgraded from the category of local to systemic disease.

As explained in more detail in chapter 3, psoriasis is strongly associated with gastrointestinal diseases. Previous studies have demonstrated that patients with psoriasis are more likely to exhibit increased intestinal permeability (Ramirez-Bosca, Navarro-Lopez et al. 2015) and dysbiosis (Codoner, Ramirez-Bosca et al. 2018, Hidalgo-Cantabrana, Gomez et al. 2019), both of which are signs of compromised gut homeostasis. Antibiotic-treated adult mice demonstrate improvement in disease severity,
indicating a pathogenic role of the microbiome (Zanvit, Konkel et al. 2015, Zakostelska, Malkova et al. 2016, Stehlikova, Kostovcikova et al. 2019). However, dysbiosis induced with antibiotics in neonatal mice increases severity of psoriasis later in life (Zanvit, Konkel et al. 2015), suggesting a two-way effect in which psoriasis may lead to dysbiosis but also where dysbiosis may lead to worse psoriasis. In humans, examining the relationship between altered gut homeostasis, comorbidities and psoriasis is much more challenging than mouse models and studies are limited in that they are correlative rather than causative. This creates difficulty in discerning whether psoriasis is the trigger for or a manifestation of dysbiosis and other associated inflammatory diseases. Such challenges emphasise the advantage of using a mouse model like the imiquimod psoriasis (IMQ-pso) model described throughout this thesis. Since psoriasis was induced in these mice, any comorbidities or changes to intestinal homeostasis are secondary to disease, giving a clearer picture of sequence of events.

Diet also appears to have a strong influence on psoriasis outcome. In mice, a high fat diet has been shown to worsen IMQ-pso in an IL-17A dependant manner by increasing IL-17- γδT cells in the dermis (Nakamizo, Honda et al. 2017). This was not true for the obese, leptin-deficient ob/ob mouse model.

1.5 Aims and rational

The research presented in this thesis seeks to understand how gut homeostasis can be altered by diet, particularly the food additive E171 and how changes to gut homeostasis are linked to the inflammatory skin condition, psoriasis. Previous research has demonstrated the central role diet plays in health outcomes however, the impact of food additives such as titanium dioxide remain largely unexplored. Similarly, psoriasis is a common skin condition strongly associated with metabolic disorders as well as inflammatory bowel diseases but how this disease impacts intestinal homoeostasis is yet to be understood.

The insights gained though this thesis will allow for a better understanding of how intestinal homeostasis is linked to systemic immune outcomes and how the inflammatory condition, psoriasis, impacts gut health. Additionally, the pathogenesis of IMQ-pso is analysed in more detail with
particular focus on the role of migratory monocytes. Such investigations are in an attempt to gain a more accurate understanding of the imiquimod-induced mouse model of psoriasis and potentially identify a new treatment for the inflammatory disease in humans.

**Aims**

1. To explore the effects of the food additive TiO2 on gut homeostasis
2. To determine the effect of psoriasis on gut homeostasis
3. To understand the role of migrating monocytes during the course of psoriasis
Chapter 2: Impact of the Food Additive Titanium Dioxide (E171) on Gut Microbiota-Host Interaction

The role that diet plays in shaping the immune system is unequivocal. This chapter explores the effects of the food additive E171 on the gut microbiome and the gut immune system. In the work presented below, we identified altered colonic morphology in E171 fed mice, as well as reduced levels of the bacterially derived short chain fatty acid, acetate in the serum. These E171-fed mice also had changes in cytokine production in the colon, indicative of inflammation as well as an altered colonic immune system. This paper was the first to identify that E171 promoted biofilm formation by commensal bacteria in vitro, creating a possible link between E171 consumption and increased risk of colorectal cancer, as previously described by Bettini et al. (Bettini, Boutet-Robinet et al. 2017). Overall, this chapter provides evidence for the impact that the food additive E171 has on gut homeostasis, particularly on the microbiome and local immune system.
Chapter 2 of this thesis is published as outlined below. GP handled and fed the animals, performed most of the experiments and analysed most of the data which set the research direction for the article. These findings informed future experiments which were co-designed by GP, guided and informed by senior authors. GP wrote most of the manuscript along with JT and LM.

By signing below, I confirm that Gabriela Pinget was the co-first author of and contributed to majority of the experiments, data analysis and manuscript writing of the following article:


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Impact of the Food Additive Titanium Dioxide (E171) on Gut Microbiota-Host Interaction

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The interaction between gut microbiota and host plays a central role in health. Dysbiosis, detrimental changes in gut microbiota and inflammation have been reported in non-communicable diseases. While diet has a profound impact on gut microbiota composition and function, the role of food additives such as titanium dioxide (TiO₂), prevalent in processed food, is less established. In this project, we investigated the impact of food grade TiO₂ on gut microbiota of mice when orally administered via drinking water. While TiO₂ had minimal impact on the composition of the microbiota in the small intestine and colon, we found that TiO₂ treatment could alter the release of bacterial metabolites in vivo and affect the spatial distribution of commensal bacteria in vitro by promoting biofilm formation. We also found reduced expression of the colonic mucin 2 gene, a key component of the intestinal mucus layer, and increased expression of the beta defensin gene, indicating that TiO₂ significantly impacts gut homeostasis. These changes were associated with colonic inflammation, as shown by decreased crypt length, infiltration of CD8+ T cells, increased macrophages as well as increased expression of inflammatory cytokines. These findings collectively show that TiO₂ is not inert, but rather impairs gut homeostasis which may in turn prime the host for disease development.

Keywords: biofilm, gut microbiota, immune cells, inflammation, titanium dioxide

INTRODUCTION

Bacterial species that inhabit the colon interact with the host, promoting the development and function of immune cells locally and systemically. These interactions are mediated by bacterially derived metabolites such as short-chain fatty acids (SCFAs), which have been identified as critical inducers of immune subsets (1–3) key for protecting mice from disease development (2–5), emphasizing the role of the microbiota in gut homeostasis and host health.
The colonic epithelium acts as a physical barrier between the host and the gut microbiota. The secretion of mucus by goblet cells provides a barrier to microbial infiltration. Further, Paneth cells release antimicrobial peptides that protect against pathogen invasion as well as regulate gut microbiota composition (6). Expression of tight junction proteins by enterocytes also limits bacterial penetration. Epithelial function can be regulated by the gut microbiota via SCFAs, by stimulating mucus production (7) and tight junction assembly (8). In contrast, dysbiosis, marked by detrimental changes in gut microbiota composition, triggers increased gut permeability and gut inflammation (9). Alterations in antimicrobial peptide production, mucus layer thickness and/or epithelial permeability have been implicated in the development of a broad range of diseases such as colitis and colorectal cancer (10). These diseases have also been linked to abnormal interactions between the host epithelium and the gut microbiota through the formation of biofilm. Biofilms consist of aggregates of adherent and planktonic bacteria protected by an extracellular matrix and have been observed in the proximal colon of patients diagnosed with such diseases (11). The mechanisms behind the formation and the role of biofilm in the gut are not fully understood, but biofilm formation has been shown to impact both disease development and resolution. Both in a colitis rat model and in humans, biofilm in the colon has been shown to facilitate pathobiont adherence to the epithelium and translocation to the host (12, 13). In human inflammatory bowel disease, biofilm formation at the site of epithelial wound healing has been shown to negatively affect healing by impairing epithelialization and tissue repair (14). Finally, a recent study has shown that inoculation of germ-free mice with biofilm-positive human colon inocula was carcinogenic (15).

The identification of environmental factors that can affect gut homeostasis is thus a critical first step in preventing the development of so-called “western lifestyle diseases,” encompassing autoimmune, allergic and metabolic diseases. A broad range of environmental factors can affect gut homeostasis, with diet composition being the major driver (16). Western-like diets enriched in fat and simple carbohydrates and deficient in dietary fiber have been shown to trigger dysbiosis, increases gut permeability and inflammation (16). While the impact of these macronutrients on gut homeostasis has been extensively studied (17), the role of food additives prevalent in processed food remains poorly defined. Food additives are used to improve the texture, preservation and aesthetics of food. Food grade titanium dioxide (TiO$_2$) or E171, is a whitening agent present in over 900 commonly consumed food products. The average adult consumes between 0.7 and 5.9 mg of TiO$_2$ per kg of body weight (BW) per day throughout their life and children are the most exposed, consuming up to 32.4 mg TiO$_2$/kg BW/day in maximally exposed individuals (18). Despite the fact that regulatory bodies do not define strict guidelines around its consumption, new evidence from animal studies has emerged, highlighting that TiO$_2$ may potentiate cancer development (19) and exacerbate inflammatory bowel disease (20).

The effect of TiO$_2$ on gut homeostasis is poorly understood yet evidence suggests that TiO$_2$ interacts with gut epithelial cells. *In vivo* and *in vitro* studies have demonstrated the accumulation of TiO$_2$ in the mucus layer (21) and its uptake by colonic epithelial cells (22, 23). A study in rats has shown that TiO$_2$ affects immune cells in the Peyer’s patches associated with a decreased regulatory T cell proportion (19). However, the impact of TiO$_2$ on colonic immune cells, the site where microbiota is the densest, has never been investigated. While the impact of TiO$_2$ on the colonic microbiota has been previously investigated in a short term study (2.5 mg TiO$_2$/kg BW/day for 1 week) (24) and using a high dose (100 mg TiO$_2$/kg BW/day) for up to 4 weeks (25), the impact of TiO$_2$ on the small intestine microbiota is unknown.

The aim of the present study is to establish the effects of food grade TiO$_2$ on gut homeostasis *in vivo*. We investigated the impact of physiological doses (2 and 10 mg TiO$_2$/kg BW/day) and a high dose of TiO$_2$ (50 mg TiO$_2$/kg BW/day) on mouse colonic and small intestine microbiota composition and function, epithelial function and mucosal inflammation after 3–4 weeks of treatment via drinking water.

**MATERIALS AND METHODS**

**E171 Characterization**

**Size and Morphology**

Food grade TiO$_2$ was purchased from All Color Supplies PTY. Average hydrodynamic diameter, polydispersity index and zeta potential of the TiO$_2$ nanoparticles dispersed in drinking water were determined with a Malvern Zetasizer Nano ZS at 25°C. The dispersion was measured 3 times for both size and zeta potential. The size distribution and shape of the TiO$_2$ nanoparticles dispersed in mice drinking water were determined using a NanoSight NS300 (equipped with a sCMOS camera) at 25°C. The dispersion was measured 5 times (1 min per measurement). The size distribution and shape of the TiO$_2$ nanoparticles dispersed in drinking water were further investigated using a Zeiss Ultra Plus scanning electron microscope operated at an accelerating voltage of 10 kV. A drop of the nanoparticle dispersion was allowed to dry on a stub, after which ~20 Å of platinum metal was sputter coated onto the stub under vacuum to prevent charging.

**Crystal Structure and Elemental Composition**

A D8 Advance Bruker diffractometer was used to conduct the X-ray powder diffraction (XRD) analysis in a flat plate geometry using Ni-filtered Cu K$_\alpha$ radiation and a Bruker Lynx eye detector. The XRD patterns were acquired from 10 to 100° 2θ with a step size of 0.02° and a count time of 0.1 s. Elemental composition was determined using X-ray photoelectron spectroscopy (XPS) with an Al K$_\alpha$ monochromator X-ray source. A survey scan was acquired at 100 eV pass energy between 0 and 1,400 eV. High resolution spectra for individual elements were collected at 100 Ca + 0.05 Ga. Elemental composition was calculated from the high-resolution spectra using CasaXPS with measurements done in triplicate.

**Mice and TiO$_2$ Dosage Information**

Five to six week-old male C57BL/6Ausb mice from Australian Bio Resources were maintained under specific-pathogen-free conditions.
Cells were passed through a 70 µm mesh and lymphocytes were discarded and the remaining tissue was incubated at 37°C for 1 h in HBSS (Gibco) with 6.7 mg/ml collagenase type IV (Gibco), 10% FBS (Gibco) and 15 mM HEPES (Gibco). TiO2 was administered in drinking water at doses of 0, 2, 10, and 50 mg TiO2/kg BW/day, which was calculated based on the water intake measured per cage. At week 4, mice were euthanized using CO2 asphyxiation.

**Colonic Immune Cell Isolation and Flow Cytometry**

Pieces of colon were incubated at 37°C for 40 min in Hank’s Balanced Salt Solution (HBSS; Gibco) with 5 mM EDTA, 5% FBS (Gibco) and 15 mM HEPES (Gibco). Intraepithelial lymphocytes were discarded and the remaining tissue was incubated at 37°C for 1 h in HBSS (Gibco) with 6.7 mg/ml collagenase type IV (Gibco), 10% FBS (Gibco) and 15 mM HEPES (Gibco). Cells were passed through a 70 µm mesh and lymphocytes enriched via percoll gradient of 80% and 40% (GE Life Sciences). The list of antibodies used for flow cytometry is in the Supplementary Methods. Viability was determined using the LIVE/DEAD Fixable Blue Dead Cell stain kit (Invitrogen). Flow cytometry was performed on a LSR II flow cytometer (BD Biosciences) and data analysis with FlowJo software (Treestar Inc., Ashland, OR, USA).

**RNA Extraction and Quantitative Real-Time PCR**

Total tissue RNA was extracted using TRI Reagent (Sigma) and converted into cDNA using iScript RT Supermix (BioRad) according to both manufacturer's instructions. qPCR was performed on a LightCycler 480 (Roche) using SYBR Green (Biorad) with primer sequences listed in Supplementary Table 1.

**Acetate and Trimethylamine (TMA) Quantification**

Quantitative measurements of acetate and TMA in plasma were determined by nuclear magnetic resonance spectroscopy (NMR). Briefly, plasma was filtered through a 3 kDa membrane filter (Merck Millipore) and polar metabolites extracted from the aqueous phase of a water:chloroform:methanol mixture. Samples, containing 4,4-dimethyl-4-silapentane-1-sulfonic acid as an internal standard, were analyzed on a Bruker 600 MHz NMR.

**Plasma Metabolomic Screening**

A hydrophilic interaction chromatography LC-MS/MS method was used for choline detection in plasma as described previously (26). The LC was connected to an AB Sciex Triple Quad 5,500 mass spectrometer run in positive ion mode. Data analysis was done on software Multi-Quant 3.0 for MRM Q1/Q3 peak integration.

**Nanolive Imaging**

*Escherichia coli* K-12 MG1655 (*E. coli*) or *Enterococcus faecalis* NCTC 775 (*E. faecalis*) were incubated for 7 h at 37°C, 5% CO2 with Luria-Bertani (LB) broth containing E171 at indicated concentrations and then fixed in 3% formalin overnight. Cells were resuspended in PBS and visualized using a Nanolive 3D cell explorer. False colors were applied to images based on refractive index using STEVE software.

**Biofilm Visualization**

**Biofilm Formation Assay in vitro on Cultured *E. coli* and *E. faecalis***

The *in vitro* biofilm formation assay was based on a previously published protocol (13). Overnight culture in quadruplicates of *E. coli* (low salt LB broth; Beckton Dickinson), *E. faecalis* (tryptone soya broth supplemented with 0.25% glucose; Sigma Aldrich) or *Staphylococcus epidermidis* NCTC 6512 (LB broth) was adjusted to OD of 0.5 at 600 nm and 100 µl of each bacterial culture was plated on separate round bottom 96-well tissue culture plates. A further 100 µl of appropriate media supplemented with TiO2 was added to achieve the indicated final concentrations. TiO2 at the different final concentrations in media alone was used as background controls. Plates were incubated at 37°C aerobically on a shaker (Ratek, 70 rpm) for either 24, 48, or 72 h.

**Biofilm Formation Assay From Colonic Commensal Bacteria**

Two hundred microliters of colon homogenates were cultured in quadruplicates in flat bottom 96-well-plates containing supplemented tryptic soy broth [sTSY: 30 g/L tryptic soy broth (Oxoid) with 5 g/L yeast extract, 5% L-cysteine, 50 mg/L hemin and 1 mg/L medanione (all from Sigma-Aldrich)] to yield 0.05 µg/µl (w/v) for 24 h, aerobically at 37°C at 70 rpm. Samples were diluted 1:100 in fresh sTSY containing TiO2 at indicated doses and incubated for 5 days. After planktonic cell removal, biofilm was stained with crystal violet (CV). Briefly, plates were washed 3 times with water, air dried and stained with 1% CV (Sigma-Aldrich) for 30 min. After 4 washes in water and air drying, 95% ethanol was added for 15 min. Absorbance was recorded at 595 nm on a microplate reader (Tecan Infinite M1000).

**Resazurin Viability Assay**

Biofilm formation was also quantified based on Resazurin viability assay as previously described (27). Briefly, culture media was removed and wells washed once with phosphate-buffered saline (PBS). Then, media with 10% Resazurin (Sigma-Aldrich) was added to each well. The plates were incubated in the dark at 37°C and fluorescence intensity measured every 15 min (excitation 570 nm, emission 585 nm). TiO2 only controls were used to subtract background.

**Bacteria 16S rRNA Gene Amplicon Sequencing and Bioinformatics**

DNA from fecal samples or entire contents of small intestine lumen were extracted by mechanical disruption using a Fastprep (MP Biomedicals) using autoclaved glass beads (G8772 and G1145; Sigma-Aldrich) in lysis buffer [500 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM EDTA, 4% SDS] followed by 15 min incubation at 95°C. DNA was precipitated in 10M...
**FIGURE 1** | Impact of TiO$_2$ on colonic microbiota composition. (A–D) Diversity of colonic microbiota composition of mice administered 0, 2, 10, or 50 mg TiO$_2$/kg BW/day in drinking water was determined by (A) Shannon index, (B) Inverse Simpson's index, (C) richness, and (D) evenness ($n = 10$ mice per group from 2 cages of 5 mice). (E) Canonical correspondence analysis ordination of Bray-Curtis dissimilarity of colonic microbiota compositions of mice administered 0, 2, 10, or 50 mg TiO$_2$/kg BW/day in drinking water. Ordination was constrained by dose of TiO$_2$ and the arrows represent the doses of TiO$_2$ driving the differences in microbiota composition observed. Composition differences between groups were significant as determined by adonis ($p = 0.0012$ for 0 vs. 2 mg TiO$_2$/kg BW/day, $p = 0.0006$ for 0 vs. 10 mg TiO$_2$/kg BW/day and $p = 0.0105$ for 0 vs. 50 mg TiO$_2$/kg BW/day) ($n = 10$ mice per group from 2 cages of 5 mice). (F–J) Relative abundance of (F) Parabacteroides, (G) Lactobacillus, (H) Allobaculum, (I) Adlercreutzia, and (J) Unclassified Clostridiaceae observed in colonic microbiota of mice administered 0, 2, 10, or 50 mg TiO$_2$/kg BW/day in drinking water. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$ as determined by one-way ANOVA with post-hoc Tukey’s test on Hellinger-transformed data ($n = 10$ mice per group from 2 cages of 5 mice).
ammonium acetate and isopropanol and washed with 80% ethanol. Protein and RNA were removed using the QIAamp DNA stool Minikit (Qiagen) following the manufacturer’s instructions. DNA samples were amplified across the V3-V4 region (Q5 polymerase; New England Biolabs) with these primers F: 5′-ACTCCTACGGGAGGCAGCAG-3′; R: 5′-GGACTACHVGGGTWTCTAAT-3′ and sequenced on an Illumina Miseq (2 × 300 bp). Data analysis was performed using QIIME 1.9.1 (28) using default parameters as described previously (29). Briefly, demultiplexed paired end data were quality filtered and paired using the Fastq-join algorithm with no errors allowed. Operational taxonomic units (OTUs) were picked using 97% similarity with UCLUST, and taxonomy was assigned with Greengenes database. The resulting OTU table was filtered to remove OTUs with <0.01% sequences and those relating to Cyanobacteria or Chloroplast. Further analysis was performed with R software (3.4.2). For statistical analysis, abundance data was transformed using the Hellinger method. Differences between treatment groups were determined by adonis (vegan 2.5-2) with 9999 permutations, alpha 0.05 and with the phyloseq package 1.25.2 (30) and Calypso 8.78 (31).

Statistics
Mann–Whitney U-test was used for analysis of the differences between the mean of groups and Wilcoxon paired test for paired samples. For microbiota data, significant differences in the relative abundance of genus between treatment groups were determined by one-way ANOVA with post-hoc Tukey’s test. Differences in overall microbial community between treatment groups were determined by adonis. p < 0.05 were considered statistically significant.

RESULTS
Characterization of Food Grade TiO2 (E171)
We employed dynamic light scattering (DLS) to determine the hydrodynamic size of the E171 product used in this study. DLS revealed that the TiO2 nanoparticles dispersed in drinking water (5 mg/ml, pH 7.8) have an average hydrodynamic diameter of 367 nm, a polydispersity index of 0.258 and a zeta potential of −23.0 mV (±4.5 mV). We also employed nanoparticle tracking analysis (NTA) and scanning electron microscopy (SEM) to further investigate the size and shape of the TiO2 nanoparticles dispersed in drinking water. NTA (Supplementary Figure 1A) showed that the TiO2 nanoparticles are roughly spherical in shape and range in diameter from 28 to 1,158 nm. On a number basis, the particle size distribution has a mean of 202 nm and a mode of 138 nm and, on a weight basis, the particle size distribution has a mean of 363 nm and a mode of 428 nm. The average particle diameter determined by NTA on a weight basis (363 nm) is in good agreement with that determined by DLS (367 nm). SEM (Supplementary Figure 1B) confirmed that the TiO2 nanoparticles are roughly spherical in shape and revealed that they can be classified into essentially four groups (based on diameter)—300, 150–200, 100, and 30–50 nm—which is consistent with the particle size distribution (on a number basis) obtained by NTA. TiO2 was predominantly in anatase form as per manufacturer’s description. This was verified using X-ray powder diffraction (data not shown).

Impact of Oral Administration of Food Grade TiO2 on Gut Microbiota Composition
We first determined whether exposure to TiO2 over a range of physiologically relevant doses impacted gut bacterial communities in vivo. To achieve this, mice were administered TiO2 via drinking water at doses of either 0, 2, 10, or 50 mg TiO2/kg BW/day for 3 weeks. Sequencing of the 16S rRNA gene from fecal samples revealed that TiO2 had limited effects on bacterial diversity as determined by Inverse Simpson and Shannon analyses (Figures 1A,B) nor bacterial richness (Figure 1C), evenness (Figure 1D) or Faiths diversity (Supplementary Figure 2A) at these doses. However, there

![FIGURE 2](image-url) Impact of TiO2 treatment on gut bacterial metabolites. (A,B) Concentrations of (A) the SCFA acetate and (B) TMA were determined NMR on the serum of mice administered 0, 2, 10, or 50 mg TiO2/kg BW/day in drinking water. Data is represented as median ± interquartile range (IQR). *p < 0.05 as determined by Mann–Whitney U-test (n = 10 mice per group). **p < 0.01 as determined by Mann–Whitney U-test (n = 10 mice per group). ***p < 0.001 as determined by Mann–Whitney U-test (n = 10 mice per group).
TiO$_2$ and Gut Homeostasis

FIGURE 3 | TiO$_2$ triggers biofilm formation by commensal bacteria. (A,B) The clustering effect of TiO$_2$ on (A) E. coli and (B) E. faecalis in vitro was visualized by Nanolive imaging in the presence of 0, 0.5, 1, or 50 µg/ml TiO$_2$ after 24 h incubation. False-coloring was applied to images based on refractive index, where black represents the refractive index of TiO$_2$ and green represents bacteria. (C) Schematic representation of biofilm formation assay and resazurin viability assay to assess biofilm formation in vitro. (D,E) The ability of E. faecalis and E. coli to form biofilm in vitro in the presence of 0, 2, 10, or 50 µg/ml TiO$_2$ was assessed by the resazurin viability assay ($n=8$ replicates). (F) Colonic bacteria were isolated and biofilm formation assessed in the presence of 0, 2, 10, or 50 µg/ml TiO$_2$ in culture after 5 days ($n=6$ mice per group). Data are represented as median ± IQR. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined by Wilcoxon paired test compared to non-treated group.

was still a trend toward decrease in mice treated with physiological doses of TiO$_2$ (2 and 10 mg TiO$_2$/kg BW/day). On the other hand, both weighted (Supplementary Figure 2B) and unweighted UniFrac (Supplementary Figure 2C) principal coordinate analysis (PCoA) showed some clustering of bacterial composition in control vs. TiO$_2$ treated mice. To test this further, we performed canonical correspondence analysis (CCA) constrained to the 4 distinct TiO$_2$ concentrations used, which revealed significant clustering in bacterial composition driven by 2 mg TiO$_2$/kg BW/day ($p=0.0011$) and 50 mg TiO$_2$/kg BW/day ($p=0.0123$) TiO$_2$ treatment (Figure 1E). We also performed CCA with TiO$_2$ as a continuous variable, which reveals a dose dependent effect of TiO$_2$ on microbiota composition (Supplementary Figure 2D). Treatment with TiO$_2$ significantly
affected gut microbiota composition independently of the cage effect (with overall treatment effect: F-value = 8.2407, R² = 0.31644, DF = 3, p < 0.001 and impact of treatment corrected for the cage effect: F-value= 3.8511, R² = 0.2996, DF = 3, p < 0.001 both by adonis). We then determined the impact of TiO₂ at deeper levels and found significant changes at the genus level. *Parabacteroides* were significantly elevated in TiO₂ treated mice, at a dose of 50 mg TiO₂/kg BW/day (Figure 1F) while *Lactobacillus* and *Allobaculum* were significantly elevated at all doses tested (Figures 1G,H). On the other hand, *Adlercreutzia* (Figure 11) and Unclassified *Clostridiaceae* (Figure 11) were significantly decreased in the groups treated with TiO₂ at the doses of 10 and 50 mg TiO₂/kg BW/day relative to the untreated group. These results suggest that TiO₂ had a minor impact on microbiota composition in vivo, while affecting few taxa at the genus level. The gut microbiota composition in the small intestine was also analyzed to determine whether TiO₂ might have a greater effect here than in the colon. Bacterial diversity indices (Richness, evenness, Shannon, Inverse Simpson and Faith’s diversity) were not significantly affected at doses of 10 and 50 mg TiO₂/kg BW/day (Supplementary Figure 2E), although these trended toward decrease with increasing dose of TiO₂. Unlike in the colon, TiO₂ did not significantly alter the small intestine bacterial composition (p > 0.05 by adonis) and weighted and unweighted UniFrac PCoA analysis revealed no obvious clustering (Supplementary Figures 2F,G).

Overall, TiO₂ did not appear to dramatically impact on small intestinal microbiota composition. We also performed co-occurrence analysis by examining microbial interactions from mice treated with either 0, 2, 10, or 50 mg TiO₂/kg BW/day. We found that certain genera are consistently associated with each other regardless of TiO₂ treatment (*Ruminococcus*, *Desulfovibrio*, and *Oscillibrio* are positively connected). Increasing TiO₂ intake, especially at the dose of 10 and 50 mg/kg BW/day resulted in more significant connections within the network, as well as increased number of genera with significant contributions. For example, while Akkermansia was not significantly involved in the microbial network of mice administered 0, 2, or 10 mg TiO₂/kg BW/day, it is involved at a dose of 50 mg/kg involving numerous co-exclusion relationships. These co-occurrence graphs are presented in Supplementary Figures 2H–K. These results were verified using the deblur pipeline (32) which resolves amplicon sequences much more accurately (Supplementary Figures 3A–F).

**Food Grade TiO₂ Modulates Commensal Bacterial Activity**

We and others have shown that gut bacterial metabolites such as SCFAs can have a dramatic impact on host immune function and disease development (1–5, 33, 34). Mice treated with 50 mg TiO₂/kg BW/day had a significant decrease in the SCFAs, acetate, in the plasma, suggesting a possible impact of TiO₂ on host-bacterial interaction (Figure 2A). Such effects on bacterial metabolites were not limited to SCFAs as TMA, a bacterial product associated with development of atherosclerosis (35), was increased at doses of 10 and 50 mg TiO₂/kg BW/day (Figure 2B). TMA is a product of conversion of choline, which was also found to be decreased at 50 mg TiO₂/kg BW/day (Figure 2C), suggesting that increased TMA was not due to a change in the substrate availability but potentially changes in bacterial activity.

**Food Grade TiO₂ Promotes the Cluster of Commensal Bacteria and Biofilm Formation**

Bacteria also communicate with the host via direct interactions. Studies have shown that attachment of biofilm on the colonic epithelium was correlated with colorectal cancer, a disease in which TiO₂ has aggravating effects (36). To explore the possibility that TiO₂ might promote biofilm formation, we incubated two types of commensal bacteria, *E. coli* and *E. faecalis*, in the presence of TiO₂. Nanolive imaging revealed the clustering effect of TiO₂ on both *E. coli* (Figure 3A) and *E. faecalis* (Figure 3B) in vitro in a dose dependent manner. To determine whether the cluster of bacteria was due to biofilm formation, we performed *in vitro* culture of either *E. faecalis* or *E. coli* in the presence of 2, 10, or 50 μg/ml of TiO₂ for 24 or 72 h, respectively. Using the resazurin viability assay (Figure 3C), we found that TiO₂ treatment significantly increased biofilm formation in both subsets of bacteria (Figures 3D,E) but not in *Staphylococcus epidermidis*, a strain known for its
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**Figure 5** | TiO$_2$ promotes colonic inflammation. (A–D) Proportion of the colonic immune subsets (A) neutrophils (B) dendritic cells, (C) macrophages, and (D) monocytes in mice administered 0, 2, 10, or 50 mg TiO$_2$/kg BW/day in drinking water, were determined by flow cytometric analysis ($n=8–10$ mice per group). (E–G) Expression of genes encoding for (E) IL-10, (F) IL-6, and (G) TNF-$\alpha$ was determined by qPCR from colonic tissue of mice treated with 0, 2, 10, or 50 mg TiO$_2$/kg BW/day in drinking water ($n=10$ mice per group). (H) (Left—images) H&E stained colonic tissue section was evaluated for crypt length changes in 0 vs. 50 mg TiO$_2$/kg BW/day. Representative histology images are shown for each group, black lines represent representative crypt length measurements ($n=5$ mice per group). (Right—Graph) Quantification of colonic crypt length in untreated mice vs. mice treated with 50 mg TiO$_2$/kg BW/day. Data are represented as median ± IQR. *$p < 0.05$, **$p < 0.01$ as determined by Mann–Whitney U-test, ***$p < 0.001$. 

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FIGURE 6 | TiO₂ treatment results in adaptive immune cell infiltration into the colon. (A) Proportion of colonic CD8⁺ T cells was quantified by flow cytometric analysis on mice treated with 0, 2, 10, or 50 mg TiO₂/kg BW/day in drinking water (left); representative gating is shown (right) (n = 5 mice per group). (B) Expression of the gene encoding for IFN-γ was determined by qPCR on colon tissue of mice treated with 0, 2, 10, or 50 mg TiO₂/kg BW/day in drinking water (n = 6-8 mice per group). (C) Proportion of IL-17A producing CD4⁺ T cells from the colon was determined by flow cytometric analysis on mice treated with 0, 2, 10, or 50 mg TiO₂/kg BW/day in drinking water (left); representative gating is shown (right) (n = 5 mice per group). (D) Expression of the gene encoding for IL-17A was determined by qPCR on colon tissue of mice treated with 0, 2, 10, or 50 mg TiO₂/kg BW/day in drinking water (n = 6-8 mice per group). (E) Proportion of colonic regulatory T cells was determined by flow cytometric analysis on mice treated with 0, 2, 10, or 50 mg TiO₂/kg BW/day in drinking water (left); representative gating is shown (right) (n = 8 mice per group). (F) Expression of the gene encoding for TGF-beta was determined by qPCR on colon tissue of mice treated with 0, 2, 10 or 50 mg TiO₂/kg BW/day in drinking water (n = 6-8 mice per group). Data are represented as median ± IQR. **p < 0.01, ****p < 0.0001 as determined by Mann–Whitney U-test.
inability to form biofilm (Supplementary Figure 4). We confirmed by confocal microscopy that TiO$_2$ treatment increased biofilm formation in both *E. coli* and *E. faecalis* (Supplementary Figure 5). To determine whether such effects were applicable to bacteria in the complex environment of the gut microbiota, we incubated commensal bacteria derived from mouse colons anaerobically for 5 days with doses of 2, 10, and 50 µg/ml of TiO$_2$. Both the doses of 10 and 50 µg/ml TiO$_2$ significantly promoted biofilm formation by commensal bacteria (Figure 3F). These data highlight that TiO$_2$ can affect the spatial organization of the gut microbiota and thus its potential interaction with the host.

**TiO$_2$ Affects Colonic Epithelial Function**

While the impact of biofilm formation on the host is unclear, impaired mucus production has been correlated with the presence of bacterial biofilms (11). To determine whether TiO$_2$ might impact the mucus layer, we examined colonic *Muc2* gene expression in the colon. We found that both 10 and 50 mg TiO$_2$/kg BW/day decreased *Muc2* expression, suggesting a detrimental impact of TiO$_2$ on the mucus layer (Figure 4A). While biofilm formation has been reported in colitis and colorectal cancer (3%), these diseases have also been linked to increased gut permeability (37). To test whether TiO$_2$ affects gut permeability, we measured the expression of *Tjp1* (encoding for zonula occludens 1), which was unchanged by TiO$_2$ treatment (Figure 4B), suggesting no impact of TiO$_2$ on gut permeability. The other major mechanism of bacterial exclusion is through the release of antimicrobial peptides. Beta defensin is expressed predominantly in the colon and we found that Defb3 (encoding for beta-defensin-3) was elevated by treatment at doses of both 10 and 50 mg TiO$_2$/kg BW/day (Figure 4C). Expressions of other antimicrobial peptides such as granzyme B (Figure 4D), cathelin-related antimicrobial peptide (CRAMP), regenerating islet-derived protein 3 gamma (REG3 gamma) and p-lysozyme (PLYz) (Supplementary Figure 6) were unchanged. Therefore, TiO$_2$ treatment impairs the expression of key colonic epithelial factors involved in gut homeostasis.

**TiO$_2$ Contributes to Increased Colonic Macrophages and Associated Cytokines**

Decreased *Muc2* has been correlated with inflammation and *MUC2* deficiency leads to spontaneous colitis (38). To test whether TiO$_2$ might affect innate immune cells in the colon, we studied myeloid immune cell populations by flow cytometry. While neutrophils (CD45$^+$Ly6g$^+$CD11b$^+$) (Figure 5A) and dendritic cells (CD45$^+$I-ab$^+$Ly6g$^+$F4/80$^-$CD11c$^+$) were unchanged (Figure 5B), macrophages (CD45$^+$F4/80$^+$CD8$^-$Ly6g$^-$I-ab$^+$CD11b$^+$CD103$^-$) were significantly increased by TiO$_2$ at 10 and 50 mg TiO$_2$/kg BW/day (Figure 5C). This change was not due to an increased recruitment of total monocytes (CD45$^+$CD8$^-$Ly6G$^-$Ly6C$^+$CD11b$^+$I-ab$^+$) (Figure 5D), suggesting a potential *in situ* proliferation of macrophages (gating strategies shown in Supplementary Figure 7). Colonic macrophages are a major source of IL-6, TNF-alpha and IL-10, cytokines, which were also upregulated in the colon of TiO$_2$ treated mice (Figures 5E–G). We also observed a significant reduction in colonic crypt length by histological analysis of mice treated with 50 mg TiO$_2$/kg BW/day (Figure 5H) while colon length was unchanged (data not shown). Thus, TiO$_2$ treatment triggers changes in the colonic myeloid compartment as well as structural changes in the colon.

**TiO$_2$ Promotes Increased CD8$^+$ T Cell Infiltration in the Colon and Increased Inflammatory Cytokines**

Other cell subsets can produce TNF-alpha, particularly CD8$^+$ T cells (39). By flow cytometry analysis, we found that CD8$^+$ T cells were significantly increased from 10 mg TiO$_2$/kg BW/day treatment (Figure 6A), as was expression of interferon-gamma in this cell subset (Figure 6B). Increased proportions of both macrophages and CD8$^+$ T cells suggest a state of colonic inflammation in TiO$_2$ treated mice which is consistent with the increased proportion of colonic Th17 cells ($p = 0.0556$) (Figure 6C) as well as significantly increased expression of IL-17A (Figure 6D). On the other hand, neither regulatory T cells (Figure 6E) nor TGF-beta (Figure 6F) were affected by TiO$_2$ treatment. Gating strategies for flow cytometry analysis are shown in Supplementary Figure 8. These findings show that TiO$_2$ treatment impairs immune homeostasis in the colon and promotes an inflammatory environment.

**DISCUSSION**

The ubiquitous use and daily consumption of TiO$_2$ by the general population warrants investigation into its potential impact on health. After only a few weeks of daily TiO$_2$ consumption, we observed that colonic homeostasis was significantly impaired in mice. While TiO$_2$ impaired bacterial homeostasis by causing changes in bacterial metabolites (acetate and TMA) and by promoting biofilm formation by commensal bacteria, TiO$_2$ had minimal impact on gut microbiota composition. One of the major mechanisms of physical separation between host and gut bacteria was impaired by TiO$_2$, as shown by decreased *Muc2* expression and increased Defb3 expression in colonic epithelial cells. We also observed increased macrophages, CD8$^+$ T cells and Th17 T cells as well as increased inflammatory cytokines in the colon. This increased inflammation was associated with decreased colonic crypt length, as reported in inflammatory bowel diseases (40). Disruption of gut homeostasis due to chronic exposure to TiO$_2$ may thus prime the host for conditions such as inflammatory bowel diseases or colorectal cancer.

Consumption of TiO$_2$ had no impact on microbiota diversity in either the small intestine or colon. Using a constrained analysis, we found that microbiota composition in the small intestine was unchanged while some colonic microbiota changes were driven by 2 and 50 mg TiO$_2$/kg BW/day. However, only a few taxa at the genus level were significantly altered in the colon, suggesting that TiO$_2$ consumption is associated with minor changes in bacterial communities. Similarly, TiO$_2$ might not dramatically reshape the human microbiota *in vivo* which would confirm previous *in vitro* findings in a model...
of simplified human microbiota (41, 42). However, treatment with TiO₂ over a longer period of time, as previously done by treating mice for 12 weeks with emulsifiers polysorbate-80 (P80) and carboxymethyl cellulose (CMC), might have a more dramatic impact (43).

The impact of TiO₂ on gut microbiota at the genus level shared some similarities with this study on emulsifiers in which mice treated with CMC had a significant increase in Lactobacillus and Allobaculum (43). The increase in Lactobacillus is particularly interesting as these bacteria are a major biofilm producer, suggesting that TiO₂ might favor the growth of biofilm producing bacteria. Another study suggests that TiO₂ may enhance the growth of Lactobacillus (44). Previous studies have shown that TiO₂ could either bind onto the surface of bacteria or bacteria could uptake TiO₂ (45), which might trigger a defense mechanism contributing to biofilm formation as we observed in vitro. We also found that TiO₂ mediated changes in the gut environment, such as decreased Muc2 expression, which have been shown to favor biofilm formation. Since bacterially derived SCFAs have been shown to promote mucus layer thickness, decreased acetate at the dose of TiO₂ of 50 mg TiO₂/kg BW/day could partially explained changes in mucus gene expression in mice treated at this dose of TiO₂. TiO₂ might also directly affect the function of mucus-producing goblet cells, as a previous report suggests an efficient uptake of TiO₂ by goblet cells in vitro (46). The mucus layer is an efficient physical barrier preventing bacterial attachment to the epithelium and so its impairment by TiO₂ might thus favor bacterial attachment and biofilm formation in the gut. Similarly, emulsifiers have been shown to decrease the mucus layer leading to closer contact between commensal bacteria and the epithelium (43). However, whether emulsifiers might favor biofilm formation is unknown. While we did not observe any impact of TiO₂ on gut permeability related genes, Defb3 was upregulated which might be a compensatory mechanism to control the interaction with the commensal bacteria. In the colonic lamina propria, we observed a significant impact of TiO₂ on both innate and adaptive immune cells with increased macrophages, Th17 and CD8⁺ T cells. This proinflammatory effect of TiO₂ is confirmed by changes in the cytokine environment with increased IL-6, IL-17, and TNF-alpha gene expression as well as decreased colonic crypt length. The later has also been reported in rats treated for 100 days with 10 mg TiO₂/kg BW/day (19). Our findings suggest that some of the changes induced by TiO₂ occur after as little as 30 days of daily TiO₂ treatment.

In summary, our findings demonstrate that TiO₂ profoundly affects gut homeostasis in mice and that such changes can occur over a period of time significantly shorter than the exposure typical for the human population. These changes were most significant at the highest dose of 50 mg TiO₂/kg BW/day, but still significant at the physiological doses of 2 and 10 mg TiO₂/kg BW/day. The pro-inflammatory environment and biofilm formation induced by TiO₂ predispose the host to conditions such as inflammatory bowel diseases and colorectal cancer, both of which have been shown to be aggravated by TiO₂ (19, 20). The reduced SCFA production at the highest dose of TiO₂ has profound health implications as acetate has been shown to provide protection from colitis, colorectal cancer, food allergy, asthma and type 1 diabetes (2–5).

Finally, this work highlights the need for further research into how TiO₂, on its own and in combination with other food additives, affects human health. Such research would better inform the regulation of food additives such TiO₂ and thus potentially reduce the incidence of non-communicable diseases associated with the western lifestyle.

ETHICS STATEMENT

All experimental procedures involving animals were approved by the University of Sydney Animal Ethics Committee under protocol number 2014/696.

AUTHOR CONTRIBUTIONS

GP and JT performed most of the experiments and analysis and wrote the manuscript. BJ and NK did the experiments related to biofilm and contributed to the manuscript writing. AA did the NMR and associated analysis and did the NTA for the TiO₂ characterization. JO and YK did the mass spectrometry and associated analysis. FS contributed to the animal work, immune analysis, and TiO₂ characterization and provided input throughout the project. JD did the SEM for the TiO₂ characterization. SD did the CLSM. DK helped with the nanolive imaging. RM did the microbiota sequencing. DS did the microbiota sequencing and bioinformatic analysis. WC conceived the idea to study the impact of TiO₂ on gut microbiota composition, and assisted with the TiO₂ characterization. LM is the chief investigator in all the funding that supported this project, conceived the idea to study the impact of TiO₂ on gut homeostasis and biofilm formation, designed and supervised the project, participated in the experiments and wrote the manuscript. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnut.2019.00057/full#supplementary-material
12. Ellermann M, Sartor RB. Intestinal bacterial biofilms modulate...
11. Dejea CM, Wick EC, Hechenbleikner EM, White JR, Mark Welch...


**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Chapter 3: Psoriasis alters the microbiome and induces proliferation of colonic CX3CR1+ macrophages in the imiquimod-induced mouse model

3.1 Abstract

Psoriasis is a common inflammatory skin disorder associated with a number of comorbidities including obesity and diabetes. In particular, patients with psoriasis are more likely to have altered gut homeostasis and intestinal inflammatory diseases such as Crohn’s disease. However, such observations are correlative and the impact of psoriasis on gut homeostasis remains poorly understood. The skin-gut axis is an area of growing research with immense therapeutic implications. In order to explore how psoriasis impacts gut health, we utilised the imiquimod-induced mouse model of psoriasis (IMQ-pso) and analysed different parameters of gut homeostasis during disease state. Similar to previous studies, we found that psoriasis resulted in shifts in intestinal bacterial communities. We also demonstrate for the first time that IMQ-pso resulted in altered bacterially derived metabolites succinate and pyruvate within the caecum and increased proliferation of CX3CR1+ macrophages and production of their associated cytokines within the colon. These profound changes to gut homeostasis provide a possible explanation for the strong association between psoriasis and gastrointestinal dysfunction. New insights into the relationship between intestinal homeostasis and disease pathogenesis are valuable and potentially provides new targets for treatment for this poorly understood, chronic disease, which currently has no cure.
3.2 Introduction

Psoriasis is a chronic auto-inflammatory disorder, which results in red, scaly plaques on the skin (von Csiky-Sessoms and Lebwohl 2019). IL-17 and IL-23 are necessary cytokines, which drive disease and inactivating the actions of either of these cytokines, either by neutralising antibodies or receptor deficiency, completely ameliorates disease pathogenesis in mouse models and is a powerful therapeutic in humans (Di Cesare, Di Meglio et al. 2009, Fotiadou, Lazaridou et al. 2018, Frieder, Kivelevitch et al. 2018, Hawkes, Yan et al. 2018, Takahashi, Koga et al. 2018). While its exact prevalence is not clear, psoriasis is currently estimated to affect up to 2-3% of the world’s population, with greater incidence among particular ethnicities (Christophers 2001, Chandran and Raychaudhuri 2010). This indicates a familial link and indeed, pathogenesis is thought to arise from a combination of both a genetic predisposition and environmental factors (Chandran 2013, Capon 2017).

Psoriasis is associated with a number of comorbidities, including metabolic syndrome, obesity, non-alcoholic fatty liver disease (NAFLD), cardiovascular disease and arthritis and as such, is often referred to as a systemic disease, rather than one which is limited to the site of skin lesion (Neumann, Shin et al. 2006, Kimball, Gladman et al. 2008, van der Voort, Koehler et al. 2014, Balato, Napolitano et al. 2015, Dogra and Mahajan 2016). Gut inflammatory disorders are particularly common among patients with psoriasis, including ulcerative colitis and Crohn’s disease, the risk of which is 7-fold higher in people with psoriasis than in the general population (Christophers 2001, Li, Han et al. 2013, de Oliveira, Rocha et al. 2015). This adds to the growing evidence for the existence of a gut-skin axis in which gut homeostasis is tightly linked to skin health and gut pathologies often present with skin comorbidities (O’Neill, Monteleone et al. 2016).

Gut homeostasis refers to three main pillars: the microbiome, and microbial metabolites; the intestinal epithelial layer; and finally the intestinal immune system, including T cells, dendritic cells (DCs) and macrophages, the latter of which are highly specialised to have an anergic phenotype to certain bacterial products compared to macrophages found in other tissues (Smythies, Sellers et al. 2005, Garrett, Gordon et al. 2010, Bain and Schridde 2018).
Patients with psoriasis are more likely to suffer from disrupted intestinal barrier integrity (Sikora, Chrabaszczy et al. 2018), inflammatory intestinal illnesses (de Oliveira, Rocha et al. 2015), and altered gut microbiome or dysbiosis (Codoner, Ramírez-Bosca et al. 2018, Visser, Kell et al. 2019), together pointing to disrupted homeostasis within the gut. However, these links are mostly correlative and whether psoriasis triggers gut disturbances or this occurs in the reverse order is not yet known, highlighting the value of mouse models.

Dysbiosis has been causally linked to IL-17-driven disease (Ivanov, Atarashi et al. 2009, Belkaid and Hand 2014, Calcinotto, Brevi et al. 2018) and while some evidence for altered microbiome in psoriasis exists, studies are few and contradictory, highlighting a need for more research. Patients with psoriasis have an altered gut microbiome (Codoner, Ramírez-Bosca et al. 2018, Tan, Zhao et al. 2018) and reduced microbial diversity, a sign of dysbiosis when compared to healthy controls (Scher, Ubeda et al. 2015). Additionally, they have been shown to have DNA from intestinal bacteria present in the peripheral blood, indicating altered epithelial integrity and a potential role of intestinal bacteria to act as a trigger for flare-ups (Ramírez-Bosca, Navarro-Lopez et al. 2015).

Limited research into the potential benefits of probiotics has revealed that mice supplemented with Lactobacillus pentosus had a reduction in key cytokines driving psoriasis, including TNF-α, IL-17A and IL-23 as well as attenuated imiquimod-induced psoriasis (IMQ-pso) (Gueniche, Philippe et al. 2014, Chen, Wu et al. 2017). Similarly, a human trial found that participants who were orally administered Lactobacillus paracasei had less skin reactivity than placebo upon skin challenge (Gueniche, Philippe et al. 2014), although this was not specific to patients with psoriasis. In another study, patients with psoriasis were given Bifidobacterium infantis, and their recorded serum levels of TNF-α was reduced but no changes to psoriasis were reported as having been recorded (Groeger, O’Mahony et al. 2013). Importantly, these are just two of a multitude of species, which exist in the colon, many of which are yet to be identified and whose therapeutic potential remain to be explored.

Antibiotic use in psoriasis has also been examined to a limited extent. Both germ-free and antibiotic-treated adult-mice are protected from developing psoriasis, indicating the microbiome may be playing
a pathogenetic role in disease progression (Zanvit, Konkel et al. 2015, Zakostelska, Malkova et al. 2016). In contrast, neonatal mice treated with antibiotics exhibited the opposite effect and developed exacerbated psoriasis (Zanvit, Konkel et al. 2015). Authors attributed this to an inability of a “healthy” microbiome to colonise the gut of mice, hence disrupting the critical process of immunological threshold establishment (Zanvit, Konkel et al. 2015). This can lead to disproportionate inflammatory response to innocuous antigen as is often seen in allergies. Moreover, these mice did not develop a stable “healthy” microbiota even when analysed 8 weeks post antibiotic treatment.

The reported effects of antibiotic use in human psoriasis is much more inconsistent than findings from mouse studies. Very few studies have analysed the role of antibiotics in psoriasis other than small, questionnaire-based or case-studies from the 1980s finding links between antibiotic use and increased disease severity (Katz, Seidenbaum et al. 1987, Tsankov, Botevzlatkov et al. 1988). More recently, a study concluded antibiotic use had little or no effect of on psoriasis (Horton, Scott et al. 2016). Further insight may be gained from atopic dermatitis (AD), a Th2- driven, inflammatory skin disorder that has been shown to be worse in patients with history of antibiotic use, particularly early in life (Flohr and Mann 2014). Importantly, these conditions are not the same and there are key differences in environmental triggers for AD and psoriasis.

Contradicting outcomes demonstrate not only the complexities of individual microbiome, but also the poorly understood nature of psoriasis. Notably, while antibiotics impact the gut flora, they also affect bacteria living on the skin, some of which have been strongly associated with psoriasis, particularly *Staphylococcus aureus* and *Streptococcus pyogenes* (Tomi, Kranke et al. 2005, Raza, Usman et al. 2007, Alekseyenko, Perez-Perez et al. 2013, Fry, Baker et al. 2013). Previous throat infection with *Streptococcus* is also thought to trigger psoriasis later in life (Telfer, Chalmers et al. 1992). In these instances, antibiotic treatment appears to have therapeutic benefits, particularly in non-infant patients, further complicating matters.

Importantly, none of these studies analysed the effects of antibiotic use in the first 1000 days of life in the context of long-term risk of psoriasis development. This time frame is critical for shaping the
microbiome and has long-term effects on immune function, with similar possible implications for psoriasis as seen in the aforementioned neonatal mouse study by Zanvit et al. (Zeissig and Blumberg 2014, Zanvit, Konkel et al. 2015, Gensollen, Iyer et al. 2016, Selma-Royo, Tarrazo et al. 2019).

Altogether, links between psoriasis and gut homeostasis remain poorly understood at best and certainly nothing has been written on the topic of bacterially derived metabolites in relation to psoriasis, either in humans or mice. This is with the exception of projections made in a study in which the microbiome of 52 patients with plaque psoriasis was analysed and speculations were made regarding changes to butyrate and propionate-producing microbes (Codoner, Ramirez-Bosca et al. 2018). These speculations were not verified by data and there was no adequate control group for the sequencing. Nevertheless, this focus on bacterial-metabolites and psoriasis highlights a critical gap in our knowledge.

Current literature is only just beginning to uncover the multitudes of ways in which microbially-derived metabolites shape the host epithelium and immune system, the most well-known of which are short-chain fatty acids (SCFA) (Tan, McKenzie et al. 2014). SCFA signal to the host in two major ways: via activation of G-protein coupled receptors (GPCRs) and via inhibition of histone deacetylase (HDACs). SCFA are also the primary energy source for epithelial cells, promoting their function and turnover (den Besten, van Eunen et al. 2013, Kaiko, Ryu et al. 2016). SCFA and other bacterial metabolites, including succinate and lactate, can regulate the expansion and alter the function of colonic immune cells including type 3 innate lymphoid cells (ILC3s) (Kim, Cho et al. 2017), regulatory T cells (Tregs) (Furusawa, Obata et al. 2013, Bhaskaran, Quigley et al. 2018), and DCs (Nastasi, Candela et al. 2015, Tan, McKenzie et al. 2016). They can also impact cytokine and chemokine production and alter cellular morphology as seen in the induction of dendrite protrusion of CX3CR1+ cells by lactate and pyruvate in a GPR31-dependant manner (Morita, Umemoto et al. 2019).

These effects can have huge implications for inflammatory disorders and SCFA have been demonstrated to prevent the development of food allergy (Tan, McKenzie et al. 2016), asthma
Macrophages are similarly shaped by bacterial metabolites. While they account for about 20% of total leukocytes in the colon, much remains unknown about their regulation and activation, particularly by bacterial products (Bain and Mowat 2011, Schulthess, Pandey et al. 2019). Intestinal lamina propria macrophages switch to an anti-inflammatory state when treated with butyrate via HDAC inhibition, as measured by reduced IL-6 and IL-12 cytokine production upon LPS stimulation (Chang, Hao et al. 2014). This was demonstrated ex vivo in a population of mostly CX3CR1hi macrophages, as well as in vivo in antibiotic treated mice. Seemingly in contrast, butyrate can also bolster the antimicrobial action of macrophages and promote the differentiation of human peripheral blood monocytes into macrophages in vitro (Schulthess, Pandey et al. 2019). This is not via the induction of inflammatory cytokines but instead through a shift in metabolism and increased antimicrobial peptide production via HDAC inhibition. Similarly, colonic macrophages from mice administered butyrate displayed increased antimicrobial activity and thus, these mice were protected from Citrobacter rodentium infection (Schulthess, Pandey et al. 2019). Such a compelling finding demonstrates the powerful impact bacterial metabolites have on colonic immune cells and their wider implications in pathology. Succinate and pyruvate are also metabolic products of both immune cells and bacteria and have profound effects on macrophages. As mentioned above, pyruvate in the presence of lactate stimulates dendrite protrusion in intestinal CX3CR1+ macrophages (Morita, Umemoto et al. 2019). Succinate is elevated in the luminal contents of patients with inflammatory bowel disease (IBD) (Connors, Dawe et al. 2019) and has been shown to activate macrophages via GPCR91 (SUCNR1) (He, Miao et al. 2004), resulting in increased inflammatory cytokine production (Macias-Ceja, Ortiz-Masia et al. 2019) as well as their mobilisation into other tissues (van Diepen, Robben et al. 2017).

Macrophages are of particular interest as they play a key role in controlling gut homeostasis. These cells are highly phagocytic but must eliminate bacteria in a way that is effective but does not evoke intestinal inflammation (Bain and Mowat 2011). This is via the production of antimicrobial peptides and as well as maintaining the function of anti-inflammatory Tregs via the secretion of IL-10. This
attenuation of inflammation is shown to be critical for survival after infection with \textit{Citrobacter rodentium} infection (Krause, Morris et al. 2015).

Colonic macrophages can either arise from local proliferation or from monocyte recruitment and differentiation (Bain and Schridde 2018, Schulthess, Pandey et al. 2019). Most colonic macrophages within the lamina propria express high levels of CX3CR1 and mice deficient in CX3CR1 exhibit more bacterial translocation to the mesenteric lymph nodes, highlighting their role in microbial control (Medina-Contreras, Geem et al. 2011).

Macrophages also recruit and maintain effector immune cells within the colon and initiate inflammation via the production of cytokines and chemokines including TNF-\(\alpha\), IL-4, IL-1, IL-6 and IL-23 (Medina-Contreras, Geem et al. 2011). For this reason, they are implicated in a number of diseases, including IBD and are considered as a therapeutic target (Na, Stakenborg et al. 2019). Such contrasting functions of macrophages emphasise their central role in maintaining the delicate balance within the colon known as homeostasis. This stresses the importance of understanding their actions during pathology in which gut homeostasis is compromised, as is indicated in psoriasis.

The work presented in this chapter explores the impact of psoriasis on gut homeostasis. This is achieved by examining changes to the microbiome, including bacterially derived metabolites, as well as their downstream effects on key players of intestinal immunity, the macrophages during in mice with IMQ-pso. This mouse model involves inducing psoriasis by daily topical application of Aldara, which contains the TLR7/8 ligand, imiquimod. This signals the production of IL-22, IL-23, IL-1\(\beta\) and subsequent induction of IL-17 by Th17 and \(\delta\gamma\)T cells, resulting in red, scaly plaques similar to that seen in the human form and with a very similar cytokine profile (van der Fits, Mourits et al. 2009, Cai, Shen et al. 2011). By utilising this approach, we aim to provide some much-needed insight into the microbe-host interaction in the context of psoriasis.
3.3 Methods

Mice

Five to six-week-old male C57BL/6J Ausb mice from Australian Bio Resources were maintained under specific-pathogen-free conditions. All experimental procedures involving animals were approved by the University of Sydney Animal Ethics Committee under protocol number 2014/696. Mice were single housed with water and food (AIN93G; Specialty Feeds) access ad libitum. Experiment repeated 4 times at day 6 endpoint and 3 times at day 3 endpoint.

Imiquimod-induced psoriasis induction

Dorsal hair was shaved using clippers and depilatory cream (Nair). Psoriasis was induced by daily applications of 41.7mg Aldara (5% imiquimod) for 3 or 6 days as previously described (van der Fits, Mourits et al. 2009). Erythema and scaling were assessed daily and each given a score between 0 and 4: 0, none; 1, slight; 2, moderate; 3, marked; 4, very marked (van der Fits, Mourits et al. 2009). Cumulative disease severity score was obtained by adding the erythema and scaling scores. Electronic callipers were used to measure skin thickness daily (van der Fits, Mourits et al. 2009).

Administration of Immune Modifying Particles

4.26 × 10⁹ particles of poly(lactic-co-glycolic acid) (PLGA) carboxylated IMP with a diameter of 500 nm (Phosphorex Inc, Hopkinton, MA, USA) was administered daily in 200ul sterile PBS via intravenous injection of the tail vein. PBS alone was used for untreated mice. Administration was done daily at the time the Adara was administered.

BRDU staining for flow cytometry

Mice were administered intraperitoneal injections with BrdU (BD Biosciences) four hours before sacrifice. Stain was performed using kit containing APC conjugated antibody according to manufacturer’s instructions (BD Biosciences).
IL-17 administration

IL-17A/F recombinant antibody (Biolegend) was injected intravenously in a total volume of 200ul saline daily for 3 days. Control mice received saline alone. 24h after the final treatment, mice were sacrificed using CO₂ asphyxiation and organs harvested for flow cytometry and qPCR.

Flow cytometry

Spleens and lymphoid organs were mechanically disrupted. Treated hair-free skin samples were cut into small pieces and digested using RPMI 1640 (Gibco) + 2mg/ml collagenase IV (Gibco) in an orbital shaking incubator for 60 minutes at 200 rpm, 37°C. After brief vortex, supernatant was pushed through a 70um filter and cells centrifuged and stained for flow. Colon was washed in PBS, cut into small pieces and intraepithelial cells removed by incubating in HBSS solution containing 100 U/Ml penicillin-streptomycin (Sigma), 5% FCS, 15mM HEPES and 5mM EDTA for 40 minutes at 200 rpm, 37°C. Whole tissue was then digested in HBSS containing 100 U/Ml penicillin-streptomycin (Sigma), 10% FCS, 15mM HEPES + 6.7mg/ml Collagenase type IV (GIBCO) for 40 minutes at 200 rpm, 37°C. After brief vortex, supernatant was pushed through a 70um filter and isolated using 40% and 80% percol gradient. Lamina propria leukocytes were isolated from the middle ring of percol gradient, washed and pelleted in complete media (RPMI+ 100 U/Ml penicillin-streptomycin (Sigma), 10% FBS, 2mM L-Glutamine (Sigma) and 0.01M HEPES (Sigma)). Cells were first incubated with FcR blocker (Biolegend) and Live/dead stain (ThermoFisher) in PBS for 30 minutes on ice. For intracellular cytokine staining, cells were stimulated with PMA, ionomycin and brefeldin A in complete media for 4 hours. After surface staining, cells were fixed and stained using a BD Cytofix/Cytoperm kit (BD Biosciences) as per manufacturer’s instructions. Antibodies as indicated in supplementary table 1. Data was acquired using a BD LSR II (5 laser) flow cytometer and analysed using FlowJo software. Automatic compensations were performed on the flow cytometer before running samples.
**Histology and dermal measurements**

Paraffin-embedded skin samples were sectioned and stained with haematoxylin and eosin. Slides were visualised using light microscopy (Zeiss Axioscope) and ImageJ (NIH) used for measuring dermal thickness. A minimum of 4 fields of view at 10x magnification were taken per mouse and 20 measurements per field.

**Bacteria 16S rRNA Gene Amplicon Sequencing and Bioinformatics**

DNA from fecal samples or entire contents of small intestine lumen were extracted by mechanical disruption using a Fastprep (MP Biomedicals) using autoclaved glass beads (G8772 and G1145; Sigma-Aldrich) in lysis buffer [500 mM NaCl, 50 mM Tris-HCl (pH 8), 50 mM EDTA, 4% SDS] followed by 15 min incubation at 95°C. DNA was precipitated in 10M ammonium acetate and isopropanol and washed with 80% ethanol. Protein and RNA were removed using the QIAamp DNA stool Minikit (Qiagen) following the manufacturer's instructions. DNA samples were amplified across the V3-V4 region (Q5 polymerase; New England Biolabs) with these primers F: 5′-ACTCTACGGGAGGCAGCAG-3′; R: 5′-GGACTACHVGGGTWTCTAAT-3′ and sequenced on an Illumina Miseq (2 × 300 bp). Data analysis was performed using QIIME 1.9.1 (28) using default parameters as described previously (29). Briefly, demultiplexed paired end data were quality filtered and paired using the Fastq-join algorithm with no errors allowed. Operational taxonomic units (OTUs) were picked using 97% similarity with UCLUST, and taxonomy was assigned with Greengenes database. The resulting OTU table was filtered by removing OTUs with <0.01% sequences and those relating to Cyanobacteria or Chloroplast. Further analysis was performed with R software (3.4.2). For statistical analysis, abundance data was transformed using the Hellinger method. Differences between treatment groups were determined by adonis (vegan 2.5-2) with 9999 permutations, alpha 0.05 and with the phyloseq package 1.25.2 (30) and Calypso 8.78 (31).
**Protein analysis**

Protein was extracted from colon using mechanical disruption on dry ice and processed in RIPA buffer with the addition of Complete, EDTA-free Protease Inhibitor Cocktail (Sigma-Aldrich) and phosphatase inhibitor (Roche). Samples were sonicated and centrifuged to remove large particulates.

Multiplex (Biorad) was performed according to manufacturer’s instructions and samples were normalised to the total protein as determined using Bicinchoninic Acid Kit (Sigma-Aldrich) according to manufacturer’s instructions.

**Bacterial metabolite analysis using nuclear magnetic resonance (NMR) spectroscopy**

Quantitative measurements of metabolites in caecum contents were determined by nuclear magnetic resonance (NMR) spectroscopy. Briefly, caecum contents were homogenized in deuterium oxide, the homogenate was filtered through a 3 kDa membrane and then metabolites in the filtrate were extracted from the aqueous phase of a deuterium methanol/deuterium chloroform mixture. The samples, containing 4,4-dimethyl-4-silapentane-1-sulfonic acid as an internal standard, were analyzed on a Bruker 600 MHz NMR.

**RNA extraction and Real time PCR**

Skin samples were snap-frozen in liquid nitrogen and stored in -80°C until analysis. Samples were homogenised with a Qiagen TissueLyser LT in TRI Reagent and a metal bead to extract RNA according to manufacturer’s instructions (Sigma-Aldrich). RNA was converted into cDNA using iScript RT Supermix (BioRad) according to manufacturer’s instructions. qPCR was performed on a LightCycler 480 (Roche) using SYBR Green (Biorad) with primer sequences listed in Supplementary Table 2.

**Colon ex vivo cell culture**

Colonic lamina propria cells were isolated as indicated above for flow cytometry. Cells were then counted using trypan blue and placed into 24-well tissue culture plates (corning) at a density of 1 million per well in 2ml complete media (DMEM + 100 U/MI penicillin-streptomycin (Sigma), 10% FBS,
2mM L-Glutamine (Sigma) and 0.01M HEPES (Sigma) +/- sodium pyruvate (1mM) (sigma) and sodium succinate (500uM) (sigma) as indicated. Cells were stained for flow cytometry after 3 days.

**RAW cell culture**

RAW264.7 cells at a passage less than 20 were seeded in 6 well plates at a density of 0.25 x 10^6 cells per well in 2ml final volume in complete media (DMEM + 100 U/MI penicillin-streptomycin (Sigma), 10% FBS, 2mM L-Glutamine (Sigma) and 0.01M HEPES (Sigma)) +/- sodium pyruvate (2mM) (sigma) and sodium succinate (1mM) (sigma) as indicated. After 2 days, supernatant was removed and replaced with fresh media. After 3 days, supernatant was removed, and adherent cells were resuspended in Trizol (Sigma) and RNA extraction was performed as indicated.

**Statistics**

Statistics were performed using Prism GraphPad. Normal distribution within sample groups was first determined using the D'Agostino & Pearson normality test. Subsequently, non-parametric Kruskal-Wallis test (non-normal distribution) or parametric one-way ANOVA (normal distribution) statistical analysis was utilised when comparing three or more groups with one independent variable. A non-parametric Mann Whitney U-test (non-normal distribution) was utilised when comparing two groups. Two-way ANOVA was used when comparing more than 2 groups and/or more than two independent variables, such as time and treatment. P<0.05 considered statistically significant. P < 0.05 was considered statistically significant.
3.4 Results

1. **Psoriasis leads to increased CX3CR1+ macrophages in the colon of mice.**

The imiquimod-induced mouse model of psoriasis, (IMQ-pso) is characterised by increased epidermal thickness, redness and scales (van der Fits, Mourits et al. 2009). We confirmed the development of epidermal thickness in IMQ-pso mice using histology (Fig 1A and 1B) and callipers (Fig S1) and the development of red, scaly plaques were scored for disease severity (Fig S1). The inflammatory process in IMQ-pso is largely driven by the cytokine IL-17, produced primarily by δγT cells (Cai, Shen et al. 2011), which we found to be increased in the affected dermis (Fig 1C), the skin-draining lymph nodes (ILN) (Fig 1D, Fig 1G) and the spleen (Fig 1E) but not in the gut-draining mesenteric lymph nodes (Fig 1F). In line with previous reports (van der Fits, Mourits et al. 2009), Th17 cells were also found to contribute to IL-17 production (Figure S2). Myeloid cells, particularly CX3CRC1+ macrophages, have been shown to play a key role in psoriasis induction (Clark and Kupper 2006, Morimura, Oka et al. 2016). We found that indeed IMQ-pso mice had increased CX3CR1+ macrophages in the skin draining ILN (Fig 1J), spleen (Fig 1I) and surprisingly in the MLN (Fig 1J) and colon (Fig 1K and Fig 1L). This novel finding in the colon and MLN has not been previously reported and was limited to CX3CR1+ macrophages. We did not observe changes to any other type of myeloid cells (Fig 1M), including, total monocytes, Ly6C high, Ly6C intermediate or Ly6C low monocytes, dendritic cells or neutrophils. Lymphocytes also remained unaltered in the colon of mice with psoriasis (Fig 1N and 1O), including CD4 T, CD8 T, δγT, NKT and NK cells.

2. **Increased CX3CR1+ macrophages in the colon of mice with psoriasis is not due to oral ingestion of imiquimod.**

It has been reported that systemic effects of psoriasis observed in mice are induced by oral ingestion of imiquimod (Grine, Steeland et al. 2016). To test if this was the cause of the changes in macrophages observed in the colon, mice wore plastic, truncated-cone shaped collars around
their necks, termed Elizabethan collars (EC) to prevent access to the site of psoriasis lesion. These mice once again remained single housed during disease progression. Redness and scales, characteristic of psoriasis were given a combined score of 8 (Fig 1A) and skin thickness was measured by callipers (Fig 2B). The skin- draining ILN exhibited an increase in IL-17 producing δyT cells in mice wearing EC to the same degree as mice without collars (Fig 2C, Fig 2F). These mice similarly exhibited increased CX3CR1+ macrophages in the colon when compared to their mock counterparts (Fig 2E). This finding demonstrates that the increased CX3CR1+ macrophages observed in the colon of IMQ-pso mice were not as a result of oral ingestion of imiquimod as previously suggested.

3. IMQ-pso induces colonic CX3CR1+ macrophage proliferation

Macrophages in the colon are heterogeneous and of two distinct origins: either recruitment of Ly6C high monocytes, which subsequently differentiate into macrophages or in situ proliferation (Jenkins, Ruckerl et al. 2013, Bain and Schridde 2018, Shaw, Houston et al. 2018). To understand the origin of the enriched macrophage population observed in the colon of IMQ-pso mice, we blocked monocyte recruitment using immune modifying particles (IMP). IMP are taken up by circulating Ly6C high monocytes, thus preventing their migration to the colon (Getts, Terry et al. 2014). We found IMP to have no impact on colonic CX3CR1+ macrophages (Fig 3A) suggesting observed changes to macrophages are not as a result of increased monocyte migration. Further, monocyte chemoattractant protein-1 (MCP-1), a monocyte recruitment chemokine (Deshmane, Kremlev et al. 2009) was not increased in the colon of IMQ-pso mice as analysed by qPCR (Fig 3B) and multiplex (Fig 3C). The chemokine CX3CL1, which is released predominantly by endothelial cells attracts leukocytes expressing CX3CR1 (Kim, Vallon-Eberhard et al. 2011). Gene expression of CX3CL1 remained unchanged between mock and psoriatic groups when analysed by qPCR (Fig 3D) suggesting that the enriched macrophage population is not as a result of recruitment via CX3CR1/ CX3CL1 chemotaxis (Becker, Holthoff et al. 2017). Since we did not find that the enriched
population of CX3CR1+ macrophages in the colon of IMQ-pso mice were as a result of recruitment, we next investigated whether these cells were a consequence of increased proliferation. Bromodeoxyuridine (BrdU) is taken up by proliferating cells and we found its uptake in colonic CX3CR1+ macrophages to be higher in IMQ-pso mice at day 6 compared to mock mice (Fig 3E) indicating higher proliferation rate. Surface expression of CD4 and T cell immunoglobulin mucin receptor 4 (Tim4) can be used to separate subsets of colonic macrophages into long-lived, tissue resident macrophages and macrophages replenished from blood monocytes at slow and fast turnover rates (Shaw, Houston et al. 2018). Utilising this method, we found macrophages quickly replenished from blood monocytes to be increased in proportion at day 6, compared to mock mice and to have increased uptake of BrdU at both day 3 and 6 (Fig 3F). Macrophages, which were slowly turned over from blood monocytes were also increased by day 6 and BrdU was non-significantly increased at day 6 (Fig 3G). Locally maintained macrophages did not appear to change in either proportion or their uptake of BrdU at either day 3 or 6 (Fig 3H).

4. Psoriatic mice have increased expression of cytokines associated with colonic macrophages.

IL-17A and IL-23 are produced as a result of TLR7 stimulation by the ligand imiquimod (van der Fits, Mourits et al. 2009). To ensure that changes in the colon were not due to direct, systemic ligand action, both these cytokines were analysed in the colon and IL-17A was not found to be increased either by qPCR (Fig 4A) or multiplex (Fig 4B). Similarly, IL-23 was not increased in the colon of mice with IMQ-pso but a decrease was noted (Fig 4C). Macrophages are major producers of key cytokines, which signal either inflammation or repair in the colon, including IL-10 and TNF-α (Na, Stakenborg et al. 2019). IL-4 can signal macrophages to proliferate above homeostatic levels without the need for monocyte recruitment (Jenkins, Ruckerl et al. 2013) and transforming growth factor-β (TGF-β) is important for macrophage maturation (Schridde, Bain et al. 2017). In order to gain insight into the effects of IMQ-pso on the colon, these cytokines were analysed via qPCR. At day 6 but not day 3, IL-4 and TGFβ expression were significantly increased (Fig 4D, Fig 4E), while TNFα and IL-10 were already increased at day 3 and still by day 6 (Fig 4F, Fig 4G). This increased
expression profile of pro-inflammatory and pro-repair signals indicate changes to colonic homeostasis, which are in line with increased CX3CR1+ macrophages. Simultaneously, the lack of IL-23 and IL-17A observed in the colon of IMQ-pso mice indicates that such changes are unlikely the result of systemic TLR stimulation by imiquimod.

5. **Psoriatic mice have reduced microbial richness.**

The microbiome is largely shaped by the colonic immune system, particularly CX3CR1+ macrophages (Medina-Contreras, Geem et al. 2011, Mowat 2011, Bain and Mowat 2014). Conversely, macrophages are regulated by the microbiome (Ueda, Kayama et al. 2010, Chang, Hao et al. 2014) and require live microbiota to sustain their populations (Shaw, Houston et al. 2018), demonstrating their complicated and interconnected relationship. To understand whether the microbiome is affected by IMQ-pso as macrophages are, faeces was analysed at days 0, 3 and 6 of disease. Greater microbial richness, evenness and diversity of the microbiome are correlated with reduced inflammatory and metabolic disease, and with remission of particular inflammatory disorders, such as inflammatory bowel disease (IBD) (Le Chatelier, Nielsen et al. 2013, Gong, Gong et al. 2016, Slingerland, Schwabkey et al. 2017, Mirsepasi-Lauridsen, Vrankx et al. 2018, Kieler, Osto et al. 2019). By day 3, IMQ-pso mice exhibited reduced microbial richness at the OTU level, which was further reduced by day 6 (Fig 5A). There was a trending reduction in evenness at day 6 but not day 3 (Fig 5B). Shannon diversity takes into account the numbers of species and their relative evenness and is strongly associated with gut physiology (Reese and Dunn 2018). We did not find Shannon diversity to be significantly changed with IMQ-pso (Fig 5C). Weighted and unweighted UniFrac are both commonly used to compare microbial communities and determine similarities or differences between samples. Unweighted UniFrac is qualitative and takes in to account phylogenetic similarities between communities, while weighted UniFrac is quantitative and takes into account relative abundance of these communities in addition to phylogenetic similarities (Lozupone, Hamady et al. 2007). Weighted and unweighted principal coordinate
analysis of UniFrac distances (PCoA) revealed clustering of samples from IMQ-pso mice at days 0, 3 and 6 (Fig 5D). This indicates that IMQ-pso has a consistent effect on the microbiome of each mouse throughout disease progression. Analysis at the genus level reflects the loss of richness as mentioned above, with increased Bacteroides and loss of Allobaculum the greatest impact (Fig 5E, Fig 5A). IMQ-pso mice treated with IMP similarly demonstrated a loss of richness (Fig 5F), trending loss in evenness (Fig 5G), as well as a drop in Shannon’s diversity index (Fig 5H) in a similar manner to untreated mice. Clustering on weighted and unweighted UniFrac PCoA plots from samples at disease day 6 indicates that psoriasis has a far greater impact on the microbiome than IMP or saline treatment (Fig 5I). This is demonstrated by the clustering of groups according to mock and psoriasis but not saline or IMP treatment. These results again indicate that the impact IMQ-pso has on the colon is independent of monocyte recruitment and may be directly linked to the proliferation of CX3CR1+ macrophages and their relationship to the microbiome.

6. Psoriasis increases production of the bacterial metabolite succinate in the colon, which increases macrophage proliferation in vitro

Gut bacteria can exert its actions on host immune system indirectly via the production of metabolites, for example, short chain fatty acids (SCFA) can induce antimicrobial activity by altering gene-expression of macrophages via histone deacetylase (HDAC) inhibition and alter immune function via activation of G-protein-coupled receptors (GPCRs) (Chang, Hao et al. 2014, Singh, Gurav et al. 2014, Tan, McKenzie et al. 2016, Schulthess, Pandey et al. 2019). To examine whether changes to the microbiota and macrophage populations were associated with altered metabolite profile, we analysed metabolites present in the caecum of mice and found significantly increased levels of succinate in IMQ-pso mice (Fig 6A). No changes to butyrate, acetate, propionate, or lactate were seen (Fig 6A). Elevated levels of succinate have previously been reported in the colon content of patients with IBD, as well as in mouse colitis models (Ariake, Ohkusa et al. 2000, Hallert, Bjorck et al. 2003, Connors, Dawe et al. 2019, Macias-Ceja, Ortiz-Masia et al. 2019). Interestingly, macrophages lacking the G-protein coupled receptor for succinate, Succinate receptor 1 (SUCNR1) showed impaired expression of inflammatory cytokines, including
TNF-α (Macias-Ceja, Ortiz-Masia et al. 2019). Tuft cells within the small intestine have high expression of SUCNR1 and proliferate in the presence of succinate, resulting in downstream modulation of the local immune system but this has not been demonstrated in macrophages (Nadjsombati, McGinty et al. 2018). Having observed increased expression of IL-10, TNF-α, TGF-β and IL-4 within the colon of IMQ-pso mice and increased proliferation of macrophages, our next step was to understand whether these changes were as a result of the increased succinate and pyruvate. To do this, we tested the effects of these metabolites on the production of TNF-α (Fig 6B) and IL-10 (Fig 6C) and found a trending elevation in TNF-α gene expression in RAW264 cells treated with succinate but no change to IL-10 gene expression. IL-4 and TGF-β remained undetectable. To understand whether these metabolites drove the heightened CX3CR1+ macrophage proliferation seen in the colon of IMQ-pso mice, colon lamina propria cells were stimulated ex-vivo with succinate and pyruvate and stained with the proliferation marker Ki67. Ex-vivo CX3CR1+ macrophages demonstrated elevated expression of Ki67 after 3 days in culture with succinate but not pyruvate (Fig 6D). In order to investigate whether macrophage subsets were affected differently by succinate and pyruvate, these cells were separated into their subsets using surface cell markers Tim4 and CD4 as done above. Interestingly, similar to seen in vivo, we found greater proliferation in the Tim4-CD4+ quickly replenished population of macrophages with succinate stimulation (Fig 6E). However, the slow turn over Tim4-CD4+ (Fig 6F) and locally maintained Tim4+CD4+ macrophages (Fig 6G) macrophages were not stimulated to proliferate with succinate or pyruvate, again, reflecting that which was seen in vivo. These results suggest that the increased levels of succinate observed in the caecum of the IMQ-pso mice may be stimulating the CX3CR1+ macrophages to proliferate, leading to the increased proportion and proliferation rate of macrophages observed in the colon of these mice. Succinate may also be directly stimulating macrophages to produce TNF-α within the colon, but not IL-10, IL-4 or TGF-β.
7. Intravenous injections of IL-17A/F did not result in increased CX3CR1\(^+\) macrophages or associated cytokines in the colon.

Increased levels of circulating IL-17A is commonly reported in patients with psoriasis (Brembilla, Senra et al. 2018, Fitz, Zhang et al. 2018) as well as IMQ-pso mice (Yang, Zhou et al. 2013, Chen, Han et al. 2017, Takuathung, Wongnoppavich et al. 2018, Xu, Duan et al. 2018). Both IL-17A and IL-17F are reported to increase in psoriasis and while IL-17A is primarily drives disease, IL-17F is often co-expressed and appears to play a role, which is not yet well defined (Brembilla, Senra et al. 2018). We next wanted to determine whether IL-17A/F could affect microbial metabolites pyruvate and succinate but also lactate, butyrate, acetate and propionate and increase colonic macrophage proliferation as seen in IMQ-pso. Since we observed an increase in CX3CR1\(^+\) macrophages by day 3 of disease, as well as altered cytokine profile as measured by qPCR, mice were administered 3 daily intravenous (i.v.) injections of IL-17A/F at two different doses to give a final serum concentration estimate comparable to that reported in IMQ-pso mice (Chen, Han et al. 2017, Takuathung, Wongnoppavich et al. 2018, Xu, Duan et al. 2018). We then analysed CX3CR1\(^+\) macrophages in the colon (Fig 7A) and spleen (Fig 7B) and found 3 doses of IL-17A/F did not increase the proportion of CX3CR1\(^+\) macrophages as was seen in the colon of IMQ-pso mice. Gene expression of cytokines IL-4 (Fig 7C), TNF-\(\alpha\) (Fig 7D), IL-10 (Fig 7E) and TGF-\(\beta\) (Fig 7F) similarly were unchanged in IL-17A/F treated mice. We also analysed the bacterial metabolites within the caecum and did not find IL-17A/F i.v. injections to have any impact on the presence of lactate, succinate, butyrate, acetate or propionate (Fig 7G) and pyruvate was undetectable in all samples. These findings altogether indicate that such a time course of IL-17A/F administration is not sufficient to alter colonic CX3CR1\(^+\) macrophage proliferation or cytokine profile within the colon.
3.5 Figures

A. Average Epidermal Thickness (μm)
B. Mock vs Psoriasis

C. Dermis
D. ILN
E. Spleen
F. MLN

G. δy17 ILN

H. ILN
I. Spleen
J. MLN

K. Colon

L. Flow Cytometry Analysis

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64
IMQ-pso leads to increased CX3CR1+ macrophages in the colon of mice. Mice were treated daily for 6 days with either 62.5mg Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock). A Epidermal thickness (um) measured with Image J from H&E stained sections from paraffin-embedded skin isolated at day 6. B representative pictures of H&E stained sections at day 6 from mock (top left) or psoriasis (top right) mice. γδ IL-17+ T cells as determined by flow cytometry after 4 hours of stimulation ex vivo with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml). Proportion of γδ IL-17+ T cells of all CD45+ leukocytes present in the colon. D Gating strategy for colon CX3CR1+ macrophages. M Proportion of monocytes, Ly6C high monocytes, Ly6C low monocytes, Ly6C intermediate monocytes, dendritic cells (DCs) and neutrophils expressed as a percentage of CD45+ leukocytes in the colon. N Proportion of CD4 T, CD8 T, γδ T, NK and NKT cells expressed as a percentage of total CD45+ leukocytes in the colon. O Gating strategy for T cells in the colon. Results are presented as mean ± SEM with n=5-20 mice per group and results of 2 experiments presented, representative of 5 repeats. *p<0.05, **p<0.005, ***p<0.001 by Mann-Whitney U test.
Mice wearing Elizabeth collars (EC) developed psoriasis and exhibited increased CX3CR1+ macrophages in the colon. Erythema and scaling were monitored in mice with and without Elizabeth collars (EC) treated daily with either 62.5mg of Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock) with and given. A Cumulative disease severity score. B Skin thickness was also monitored daily. C γδ IL-17+ T cells were assessed in inguinal lymph node (ILN) by flow cytometry after 4 hours of stimulation ex vivo with PMA (10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml). D CX3CR1+ macrophages in the colon expressed as a proportion of total CD45+ leukocytes. E Representative plots of γδ IL-17+ T cells in the ILN. Results are shown as mean +/- SEM with n=5-6 mice per group. *p<0.05, **p<0.005, ***p<0.001 by two-way ANOVA and Kruskal-Wallis test.
**Figure 1:**

A. Colon CX3CR1+ macrophage (% of CD45+ leukocytes)

B. MCP-1 gene expression relative to RPL13A (% of CD45+ leukocytes)

C. MCP-1 (% of total protein)

D. CX3CL1 gene expression relative to β-2 microglobulin

E. BRDU+ CX3CR1 macrophage (% of CD45+ leukocytes)

F. Quickly replenished Mo-derived macro (CX3CR1+ Tim4-CD4-)

G. Slow turnover Mo-derived macro (CX3CR1+ Tim4-CD4+)

H. Locally maintained macro (CX3CR1+ Tim4+CD4+)

I. Subsets of CD45+ leukocytes (% of CD45+ leukocytes)

J. BRDU+ subset (% of CD45+ leukocytes)

**Legend:**

- **Mock**
- **Psoriasis**

**Notes:**

- Blood vessel
- Lamina propria
- Blood monocytes
- Locally maintained macro (CX3CR1+ Tim4+CD4+)
- Slow turnover Mo-derived macro (CX3CR1+ Tim4-CD4+)
- Quickly replenished Mo-derived macro (CX3CR1+ Tim4-CD4-)

**Significance:**

- *p < 0.05
- **p < 0.01
- ns = not significant
**Figure 3**

**IMQ-psl induces colonic CX3CR1+ macrophage proliferation.** Mice were treated daily for 6 days with either 62.5mg Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock) combined with daily i.v. injection of either sterile PBS (saline) or 4.26 x 10⁹ IMP particles dispersed in sterile PBS (IMP). B Proportion of CX3CR1+ macrophages in the colon as a percentage of all CD45+ leukocytes. C Expression of MCP-1 was assessed in the colon by qPCR using Tri-reagent at day 6. C MCP-1 protein as a percentage of total protein in the colon was assessed by multiplex analysis at day 6. D Expression of CX3CL1 was assessed in the colon by qPCR at day 6. BrdU was injected IP 4h before sacrifice and samples stained ex vivo with anti-BrdU, APC conjugated antibody. E BrdU+ CX3CR1+ macrophages expressed as a percentage of total CD45+ leukocytes at psoriasis days 3 and 6. F Schematic representation of macrophage subsets in the colon, including quickly replenished monocyte-derived macrophages (CX3CR1+ Tim4+CD4+), slow turnover monocyte-derived macrophages (CX3CR1+ Tim4+CD4+) and locally maintained macrophages (CX3CR1+ Tim4+CD4+). Plots (above) show macrophage subsets expressed as a percentage of total CD45+ leukocytes and (below), BrdU+ subsets as a percentage of total CD45+ leukocytes. Results are presented as mean +/- SEM with n=5-15 mice per group and experiments were performed 1-2 times. *p<0.05, **p<0.005, ***p<0.001 by Mann-Whitney U test or Kruskal-Wallis test.
Psoriatic mice have increased expression of cytokines associated with colonic macrophages. Mice were treated daily for 6 days with either 62.5mg Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock). IL-17A production was assessed in the colon by A qPCR using Tri-reagent and B total protein lysate using multiplex at day 6. C Expression of IL-23 was assessed in the colon by qPCR at day 6. Colonic expression of D IL-4, E TGF-β, F IL-10 and G TNF-α was determined by qPCR at days 3 and 6. Results are presented as mean +/- SEM with n=5-20 mice per group and experiments repeated 2-4 times. *p<0.05, **p<0.005, ***p<0.001 by Mann-Whitney U test.
A weighted unifrac

B Unweighted unifrac

C

D

E

Genus
Figure 5:

Psoriatic mice have reduced microbial richness. Mice were treated daily for 6 days with either 62.5mg Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock). Impact of IMQ-pso on the diversity of Observed Species in faecal samples was determined by A richness, B evenness and C Shannon diversity index at days 0, 3 and 6. D Weighted and unweighted UniFrac principle coordinate analysis (PCoA) plot of individual single house mice at day 6 and E relative abundance at the Genus level at day 6. IMQ-pso and mock mice were treated with daily i.v. injection of either sterile PBS (saline) or $4.26 \times 10^9$ IMP particles dispersed in sterile PBS (IMP). Impact of IMQ-pso and IMP on the diversity of Observed Species in faecal samples was determined by F richness, G evenness and H Shannon diversity index at days 0, 3 and 6. D Weighted and unweighted UniFrac principle coordinate analysis (PCoA) plot of individual single house mice at day 6 and I relative abundance at the Genus level at day 6. The same mice are colour coded by either disease state: mock red and psoriasis blue (left panel); or divided by both treatment and disease group: mock saline light blue, mock IMP dark blue, psoriasis saline light orange, psoriasis IMP dark orange (right panel) to visually demonstrate impact of psoriasis on microbiome is unchanged by IMP. Results are shown as mean +/- SEM with n=5-6 mice per group. *p<0.05, **p<0.005, ***p<0.001 by Mann-Whitney U test or Kruskal-Wallis test.
Psoriasis increases production of bacterial metabolites, succinate and pyruvate in the colon, which increases macrophage proliferation in vitro. Mice were treated daily for 6 days with either 62.5mg Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock). A Concentration of bacterial metabolites lactate, succinate pyruvate, butyrate, acetate and propionate in caecal content at day 6 was determined using nuclear magnetic resonance (NMR) spectroscopy (n= 5-6 mice per group). RAW cells were grown in vitro +/- sodium pyruvate (2mM) and sodium succinate (1mM) for 72h. qPCR was used to determine expression of B TNFα and C IL-10 in RNA extracted from RAW cells using Tri-reagent (n=4-6 per group, experiment conducted 2 times). Colonic lamina propria cells were isolated and grown ex vivo +/- sodium pyruvate (1mM) and sodium succinate (500μM) for 72h. Flow cytometry was used to determine Ki67 median fluorescence intensity (MFI) in D all macrophages, E quickly replenished subset of macrophages, F slow turnover subset of macrophages and G locally maintained macrophages (n=4-6 per group, experiment performed 3 times). All results are shown as mean +/- SEM. *p<0.05, **p<0.005, ***p<0.001 by Mann-Whitney U test and one-way ANOVA.
Psoriasis increases production of the bacterial metabolite succinate in the colon, which increases macrophage proliferation in vitro. Mice were treated daily for 6 days with either 62.5mg Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock). A Concentration of bacterial metabolites lactate, succinate pyruvate, butyrate, acetate and propionate in caecal content at day 6 was determined using nuclear magnetic resonance (NMR) spectroscopy (n=5-6 mice per group). RAW cells were grown in vitro +/- sodium pyruvate (2mM) and sodium succinate (1mM) for 72h. qPCR was used to determine expression of B TNFα and C IL-10 in RNA extracted from RAW cells using Tri-reagent (n=4-6 per group, experiment conducted 2 times). Colonic lamina propria cells were isolated and grown ex vivo +/- sodium pyruvate (1mM) and sodium succinate (500uM) for 72h. Flow cytometry was used to determine Ki67 median fluorescence intensity (MFI) in D all macrophages, E quickly replenished subset of macrophages, F slow turnover subset of macrophages and G locally maintained macrophages (n=4-6 per group, experiment performed 3 times). All results are shown as mean +/- SEM. *p<0.05, **p<0.005, ***p<0.001 by Mann-Whitney U test and one-way ANOVA.
3.6 Discussion

Psoriasis is strongly linked with a number of comorbidities, particularly gastrointestinal disorders such as Crohn’s disease and inflammatory bowel disease (Christophers 2001, Li, Han et al. 2013, de Oliveira, Rocha et al. 2015). However, studies are mostly correlative, making it difficult to determine whether psoriasis results from loss of gut homeostasis or whether psoriasis itself triggers changes in the gut. For the first time, we demonstrate here that psoriasis does indeed disrupt colonic homeostasis as marked by increased macrophage proliferation, increased inflammatory cytokine transcription, altered gut microbiome and increased succinate and pyruvate production within the caecum. Altogether, these findings support the idea that psoriasis it is a systemic inflammatory condition rather than one which is localised to the skin lesion and in particular lending support to the concept of a “gut-skin axis” (O’Neill, Monteleone et al. 2016).

The microbiome within the colon has immense impact on the immune system and has been shown to shape morphology, function and activation of local immune cells and impact systemic inflammatory disease both directly and indirectly through the production of metabolites (den Besten, van Eunen et al. 2013, Tan, McKenzie et al. 2014, Nastasi, Candela et al. 2015, Kaiko, Ryu et al. 2016, Tan, McKenzie et al. 2016, Morita, Umemoto et al. 2019). Microbial shifts in both mice and patients with psoriasis have previously been reported in the literature, indicating a state of dysbiosis (Scher, Ubeda et al. 2015, Zanvit, Konkel et al. 2015, Codoner, Ramirez-Bosca et al. 2018, Tan, Zhao et al. 2018). In line with these findings, we demonstrate a shift in microbial communities, particularly a loss of richness and diversity in IMQ-pso mice, indicating dysbiosis. Further to this, we investigated the bacterially-derived metabolites present in the caecum at day 6 and found that dysbiosis was accompanied by an increased in succinate in IMQ-pso mice while no changes to SCFA were observed.

Interestingly, succinate has previously been reported to be elevated in the colon of patients and mice with IBD and as previously stated, IBD has a higher incidence among patients with psoriasis (Ariake, Ohkusa et al. 2000, Hallert, Bjorck et al. 2003, Fu, Lee et al. 2018, Connors, Dawe et al. 2019, Macias-Ceja, Ortiz-Masia et al. 2019). In addition, the increased transcription of inflammatory cytokines TNF-
α and TGF-β, which are also elevated in IBD (Muzes, Molnar et al. 2012) as well as IL-10, which can signal repair in the gut (Quiros, Nishio et al. 2017), indicate a possible inflammation and repair process may already be taking place in the intestine of IMQ-pso mice. Further to this, blocking macrophage migration has been shown to have therapeutic effects in colitis (Kuboi, Nishimura et al. 2019), indicating an imbalance of these cells in the colon may have a pathogenic role during inflammation. While macrophages are implicated in IBD, their exact role is not yet well defined and is likely dictated by their activation state and cytokine profile (Na, Stakenborg et al. 2019). These findings together indicate that IMQ-pso mice may have an increased predisposition to developing IBD. However, further investigation into the susceptibility and severity of IBD development in mice with IMQ-pso is required. Unfortunately, this was beyond the timeframe and scope of this study and is something to consider for future studies.

A critical piece of the overall picture is whether increased CX3CR1+ macrophage proliferation and altered colonic cytokine profile was induced through changes to bacterial metabolites in IMQ-pso mice. In vitro experiments revealed that succinate alone was able to induce proliferation of lamina propria macrophages ex vivo. Interestingly, it was the macrophages that are quickly replenished from the blood monocytes (Shaw, Houston et al. 2018), which we found to be proliferating the most, reflecting the in vivo results. This suggests that succinate may be directly acting on CX3CR1+ macrophages to induce proliferation during psoriasis. While we did find that these cells were proliferating, since these are derived from newly arrived monocytes, we expected to successfully block their recruitment by blocking monocytes in vivo and at least in part reduce the macrophage proportion. We used immune modifying particles (IMP), which redirect migrating Ly6Chi monocytes to the spleen where they undergo apoptosis and hence no longer become macrophages at the site of inflammation (Getts, Terry et al. 2014). While we did find reduced migration to the dermis and as a result less macrophages at this site and improved disease (data shown in chapter 4), we were surprised to find that the colonic macrophages were not at all affected. This indicates that increased populations are possibly due exclusively to proliferation of recently arrived monocytes and not to new recruitment
of monocytes. However, this would have to be further investigated in other models in which Ly6C<sup>hi</sup> monocyte migration is blocked, such as in CCR2 knock-out mice.

IL-4 transcription was also found to be increased within the colon of IMQ-pso mice, which can induce macrophage proliferation (Jenkins, Ruckerl et al. 2013). Since IL-4 is produced by a number of cells including macrophages (La Flamme, Kharkrang et al. 2012), T-helper 2 CD4 cells (Chu, Mohammed-Ali et al. 2014), γδT cells (Hayday 2009) and NKT cells (Bendelac, Savage et al. 2007), the cells which may be contributing to IL-4 production are not immediately clear. Unfortunately, we were not able to determine if we had greater production of IL-4 in the ex vivo experiment in which succinate induced macrophage proliferation. IL-4 can also signal macrophages to differentiate into M2 anti-inflammatory type, which promotes wound repair, again indicating an inflammation-repair process may be taking place within the colon of IMQ-pso mice (Isidro and Appleyard 2016). Further investigation into the source of IL-4 in the colon and whether macrophage proliferation is stimulated by this cytokine in the colon of IMQ-pso mice would provide valuable insight into the mechanisms of altered homeostasis. This is something for further investigation in vitro and in vivo.

Succinate also induced a trending increase in TNF-α transcription in RAW cells, although this was not significant and IL-10 transcription was not greatly affected. While RAW cells are often used to complement animal experiments, differences in our findings highlight limitations to using cell lines to model observations made in vivo. Of note, RAW cells were not stimulated with LPS, which has previously been shown to induce IL-10 production (Pengal, Ganesan et al. 2006). LPS stimulation is physiologically relevant as colonic macrophages are in contact with LPS and other TLR-ligands and so is something to consider for future experiments.

To understand what may have prompted changes to gut homeostasis during psoriasis, IL-17A/F was administered intravenously. Unfortunately, this did not result in changes in gut homeostasis, which mirrored that seen in IMQ-pso. In fact, we found no changes to colonic cytokine transcription, macrophage populations or succinate and pyruvate concentrations after 3 daily doses. This time point was chosen because by day 3 in the psoriasis model, we already began to see increased expression of
IL-10 and TNF-α, as well as some increase in CX3CR1+ macrophages within the colon, indicating that altered gut homeostasis begins to occur quite early on in disease. However, a 3-day model may be too short to see effects, and the same investigation should be repeated in a 6-day model, confirming whether or not systemic IL-17A/F is as the cause for altered gut homeostasis in IMQ-pso mice.

Additionally, doses of IL-17A/F administered were based on previous reports of this circulating cytokine in IMQ-pso mice (Chen, Han et al. 2017, Takuathung, Wongnoppavich et al. 2018, Xu, Duan et al. 2018). This exact concentration was not confirmed in our own IMQ-pso model, providing room for error. Further, IL-17A/F concentration is likely to drop in the hours post injection as it is used up or broken down due to protein instability. Because there is no continuous cytokine source resembling that seen in IMQ-pso, future experiments could circumvent these fluctuations by utilising a drug administration pump or multiple daily injections. Another important consideration is the activity of the commercial cytokine compared to that within the IMQ-pso mice. While cytokine concentration is a useful parameter to indicate disease severity, activity (measured in units) gives a far more accurate prediction of cytokine potency. This is difficult to measure and so our study was limited by this factor.

Finally, changes to gut homeostasis in IMQ-pso may not have been mirrored in this cohort of mice administered IL-17A/F because this may not in fact be the driving factor. Other circulating cytokines such as IL-23, TNF-α and IL-6, also reported to be increased systemically in IMQ-pso mice may be driving this change (Chen, Lu et al. 2017, Takuathung, Wongnoppavich et al. 2018). It may be valuable to analyse effects of systemic administration of IL-23, TNF-α and IL-6 in a similar way to that done using IL-17A/F. Systemic effects of TLR7/8 ligand are also a possibility considering the relatively large dose that IMQ-pso mice receive relative to body size. However, less than 0.9% of imiquimod applied to the skin is absorbed into the body as reported by manufacturer pharmacokinetics studies (imiquimod insert, 2002). Further to this, the lack of IL-23 and IL-17 transcription in the colon suggests that colonic effects are not TLR7/8 stimulation driven.

In conclusion, our findings demonstrate that psoriasis is not a disease localised to the skin but one which also affects intestinal homeostasis. These results begin to form a picture of increased
inflammatory potential in the colon providing an explanation for higher incidence of gastrointestinal co-morbidities in patients with psoriasis. We show that IMQ-pso mice exhibit changes to microbial communities, resulting in increased succinate production, which in turn stimulate lamina propria macrophages to proliferate. While this was found to be quite convincing, further experiments in germ free models may allow us to see whether such changes to gut homeostasis are microbially dependant or occur through other mechanisms. As previously stated, patients with psoriasis are more prone to developing gut disturbances such as IBD. Further experiments could examine whether a predisposition to IBD exists in IMQ-pso mice, providing valuable insight into this link in humans. Further, understanding whether such changes to gut homeostasis exacerbate psoriasis is a critical piece of the puzzle. Such valuable insight will not only aid in understanding pathogenesis of psoriasis but may also result in changes to clinical approaches to treating disease, which currently fails to address gut symptoms.
3.7 Supplementary

Supplementary figures

Figure S1 (relating to figure 1).

Mice were treated daily for 6 days with either 62.5mg Aldara (containing 5% IMQ w/w) (psoriasis) or mock cream (mock). Skin erythema and scaling were monitored daily and given a score of 4. Scores were added to give a cumulative disease severity score of 8 (left panel). Skin thickness was measured daily (right panel). Results are presented as mean +/- SEM with n=5-20 mice per group and results of 2 experiments presented, representative of 5 repeats. *p<0.05, **p<0.005, ***p<0.001 by two way ANOVA.
Table 1: list of antibodies used for flow cytometry

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### Table 2: list of primers used of qPCR

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<th>Primer sequence</th>
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<tr>
<td>Il23</td>
<td>Pre-designed SYBR green primer (Sigma)</td>
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<td>Ccl2 (MCP1)</td>
<td>Pre-designed SYBR green primer (Sigma)</td>
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<tr>
<td>Il17a</td>
<td>Pre-designed SYBR green primer (Sigma)</td>
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| Il10        | F’ 5-AAGGGTTACTTGGGTTGCCA-3  
              | R’ 5-AAATCGATGACAGCGCCTCAG-3 |
| Tgfβ        | Pre-designed SYBR green primer (Sigma) |
| Tnfα        | F’ 5-ATGGCCTCCCTCTCATCAGT-3  
              | R’ 5-GTTTGCTACGACGTGGGCTA-3 |
| Il4         | Pre-designed SYBR green primer (Sigma) |
Chapter 4: Immune Modulation of Monocytes Dampens IL-17+ γδT cell Response and Associated Psoriasis Pathology in Mice

IL-17 producing CD4 and γδ T cells are critical drivers of IMQ-pso, however, the exact mechanisms driving this commonly used mouse model remain unclear. In this chapter, immune modifying particles (IMP) are utilised to understand the involvement of migrating Ly6Chigh monocytes in IMQ-pso pathology and offer a potential new therapeutic target for treatment of psoriasis in humans. This chapter outlines work which was undertaken to identify key drivers of the murine mouse model of psoriasis used throughout this thesis.
**Author contribution statement**

Chapter 4 of this thesis is published as outlined below. GP handled and fed the animals, performed most of the experiments and analysed the most of the data which set the research direction for the article. These findings informed future experiments which were co-designed by GP, guided and informed by senior authors. GP wrote most of the manuscript along with JT, LM and NK.

By signing below, I confirm that Gabriela Pinget was the first author of and contributed to majority of the experiments, data analysis and manuscript writing of the following article:


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Immune Modulation of Monocytes Dampens the IL-17⁺ γδ T Cell Response and Associated Psoriasis Pathology in Mice

Gabriela V. Pinget¹,², Jian Tan¹,²,³, Paula Niewold¹,², Eugenia Mazur¹,², Alexandra S. Angelatos¹,², Nicholas J.C. King²,²,⁴,⁵ and Laurence Macia¹,²,²

Psoriasis is a chronic inflammatory autoimmune skin condition that affects millions of people worldwide. It is driven by IL-17⁺-producing CD4⁺ and γδ T cells and targeted by current anti-IL-17 or anti-IL-23 mAb therapies. These treatments are expensive, increase the risk of opportunistic infections, and do not specifically target the inflammatory cascade. Other cells, including inflammatory monocytes, have been shown to migrate to psoriatic plaques in both human disease and the imiquimod-induced mouse model and could thus constitute potential alternative therapeutic targets. In the mouse, immune modifying particles (IMPs) specifically target Ly6C⁺ inflammatory monocytes migrating to the site of inflammation, sequestering them in the spleen. In this project, we determined whether IMPs could mitigate the development of imiquimod-induced psoriasis in mice. IMP treatment significantly reduced imiquimod-induced psoriasis severity, decreasing dermal infiltration of Ly6C⁺ monocytes as well as early-stage monocyte-derived macrophages. This was associated with reduced levels of hallmark cytokines IL-23 and IL-1β as well as associated IL-17⁺-producing γδ T cells. Our work highlights the crucial importance of inflammatory monocytes in the development of this disease as well as a therapeutic potential for IMP in psoriasis.

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INTRODUCTION

Psoriasis is an autoimmune skin disorder that is auto-immune in nature and is characterized by erythematous scaly plaques (Lee et al., 1990; Wilson et al., 2009). Currently, there is no cure for psoriasis and first-line treatments include topical corticosteroids, which are short-lived, nonspecific, and have side effects (Menter et al., 2008; Rustin, 2012). Anti-IL-17 and IL-23 antibody treatments are effective but expensive and halt disease only late in the inflammatory cascade and after lengthy treatment (Frieder et al., 2018; Reich, 2012). These treatments can have immunosuppressive effects, which may increase the risk of opportunistic infections, highlighting the need for alternative therapeutic approaches (Hawkes et al., 2017a).

The imiquimod (IMQ) model of psoriasis (IMQ-ps) in the mouse is elicited by topical epidermal application of the cream Aldara, which contains the toll-like receptor 7/8 ligand IMQ (van der Fits et al., 2009). Aldara is widely used to treat skin tumors and warts in humans, commonly resulting in scaly, psoriasis-like plaques (Patel et al., 2011). In mice, IMQ application triggers potent IL-23 and IL-1β production by dendritic cells (DCs) and macrophages, promoting IL-17 production mostly by γδ T cells (γδ T-17) and also by IL-17--producing T helper type 17 (Th17) cells (Cai et al., 2011). Just as in the human form of the disease, this cytokine profile is necessary for the induction of psoriasis-like skin inflammation in mice, and absence of IL-23 or IL-17 receptors protects mice from disease development (Cai et al., 2011; van der Fits et al., 2009). IL-6 and IL-22 are also important cytokines involved in IMQ-ps and stimulate keratinocyte proliferation. Altogether, the cytokine profile seen in IMQ-ps is very similar to psoriasis vulgaris in humans (El Malki et al., 2013; Lowes et al., 2014, 2008; Nogales et al., 2009; Vinter et al., 2016; Yoshiki et al., 2014).

Furthermore, whereas Th17 cells play a major role in the human form of psoriasis, γδ T cells are also a major source of IL-17; other innate cells, including NKT cells, also contribute but to a lesser extent (Blaueul and Chiricozzi, 2018; Cai et al., 2011; Cruz et al., 2018; Jaiswal et al., 2017; Pantelyushin et al., 2012; van der Fits et al., 2009).

Although the use of this model has surged in the last 10 years, the precise initiators of IMQ-ps have yet to be completely elucidated (Hawkes et al., 2017b). Under basal conditions, Ly6C⁺ (inflammatory) blood monocytes enter the dermis and acquire MHC-II and CD64 expression and lose Ly6C expression. These cells can differentiate into dermal...
DCs or macrophages (Tamoutounour et al., 2013). During the early stages of IMQ-pso induction, the affected dermis is characterized by increased infiltration and activation of Ly6C<sup>hi</sup>-inflammatory monocytes, monocyte-derived DCs (moDCs), neutrophils, and macrophages (Deng et al., 2019; Terhorst et al., 2013). However, the main driver of the disease is still unclear, in part due to the challenge of accurately identifying these cell types (Lowes et al., 2014; Morimura et al., 2016; van der Fils et al., 2009).

The CCR2-CCL2 chemokine-receptor pair is a crucial axis for CCR2<sup>hi</sup>Ly6C<sup>hi</sup> inflammatory monocyte egress from the bone marrow. To elucidate the role of Ly6C<sup>hi</sup> monocytes in psoriasis, studies have targeted CCR2 with conflicting results. Whereas one study found no difference in IMQ-pso disease development between wild-type and Ccr2 knockout mice (Ccr2<sup>−/−</sup>) (Costa et al., 2017), another found that Ccr2<sup>−/−</sup> mice were protected (Singh et al., 2016). The authors attributed the protective effect to the lack of monocytes migrating to the affected dermis. However, CCR2 is also expressed on a number of other cells, including Th17 and γδ T cells, highlighting the point that this gene knockout model does not solely impact monocytes (Kara et al., 2015; McKenzie et al., 2017). One study in which monocytes were antibody-depleted showed reduced dermal DC accumulation and improved psoriasis, although simultaneous neutrophil depletion was an unavoidable outcome of this treatment (Singh et al., 2016). Thus, the potential role of migrating Ly6C<sup>hi</sup> monocytes in IMQ-pso remains unclear.

Infiltration of Ly6C<sup>hi</sup> monocytes at the site of inflammation can be reduced using immune-modifying particles (IMPs). IMPs are negatively-charged particles manufactured from poly(lactic-co-glycolic acid). When administered intravenously, IMPs are taken up by the class A scavenger receptor MARCO (or SCARA2), which is present at high levels on circulating Ly6C<sup>hi</sup> monocytes, and are phagocytosed. IMP-containing monocytes become sequestered and undergo apoptosis in the spleen (Gotts et al., 2014; Niewold et al., 2018). Studies reveal the powerful therapeutic potential of IMP treatment. In a mouse model of multiple sclerosis (MS), which, similar to psoriasis, is driven by IL-17, IMP treatment reduced Ly6C<sup>hi</sup> monocyte migration to the CNS and ameliorated clinical disease (Gotts et al., 2014).

In this study, IMPs were administered to IMQ-pso mice to determine whether reduced Ly6C<sup>hi</sup> monocyte migration to the dermis could reduce the disease severity. We demonstrate that therapeutic reduction in monocyte migration during psoriasis significantly improves the disease severity, highlighting the crucial role of Ly6C<sup>hi</sup> monocyte migration in the pathogenesis of this disease.

**RESULTS**

**Treatment with IMPs reduce IMQ-induced psoriasis severity** To determine the role of Ly6C<sup>hi</sup> monocytes in the pathogenesis of psoriasis, we used the previously described IMQ-pso mouse model (van der Fils et al., 2009) and concurrent daily IMP treatment to reduce Ly6C<sup>hi</sup> monocyte migration to the dermis.

Mice treated daily with IMQ developed psoriasis characterized by erythema (Figure 1a and d), skin scaling (Figure 1b and d), and skin thickening (Figure 1c). Intravenous administration of IMPs significantly decreased psoriasis severity as shown by reduced erythema (Figure 1a and d), scaling (Figure 1b and d), and skin thickening (Figure 1c). Psoriasis is characterized by keratinocyte hyperproliferation, which is assessed by measuring epidermal layer thickness on histological sections. After 6 days of treatment, IMQ-pso mice developed significant acanthosis (Figure 1e and f). Administration of IMPs in the IMQ-pso mice reduced this epidermal thickness by 25% (P < 0.01) compared with untreated IMQ-pso mice (Figure 1e and f). Under basal conditions, IMP treatment did not affect mouse epidermal thickness (Figure 1e and f). These results show that IMP treatment significantly reduces psoriasis severity.

IMPs reduce IL-17-producing γδ T lymphocytes in the skin-draining inguinal lymph nodes of mice with psoriasis IL-17 is necessary for psoriasis pathogenesis (Cai et al., 2011; van der Fils et al., 2009). γδ T cells expand rapidly in skin-draining inguinal lymph nodes (ILNs) on skin exposure to IMQ and are the main source of IL-17 (Ramirez-Valle et al., 2015). Naive CD4<sup>+</sup> T cells are similarly polarized toward Th17 cells in the ILNs. To determine whether the beneficial effects of IMPS on psoriasis were linked to changes in IL-17-producing cells, we studied these populations in ILNs by flow cytometry.

IMQ treatment increased both the proportion and the absolute number of γδ T-17 cells (Figure 2a and b) and Th17 cells (Figure 2c and d) by day 6. We observed a similar effect of IMQ treatment on these populations in the spleen (Figure 2e). IMP administration significantly reduced both the proportion and number of γδ T-17 cells in the ILNs (Figure 2a and b) but did not affect Th17 cells (Figure 2c and d) in IMQ-pso mice. In the spleen, neither γδ T-17 cells nor Th17 cells were affected by IMP treatment (Figure 2e). IMPs did not affect these populations in the ILN or spleen in mock mice (Figure 2a–e). These results show that IMPS impair the typical immune response associated with IMP treatment by reducing the generation of γδ T-17 cells in ILN.

IMPs reduce dermal γδ IL-17-producing T cells and IL-1ß and IL-23 expression in the skin of mice with psoriasis Psoriatic skin lesions are characterized by a high level of IL-17 production in the dermis primarily because of Th17 cells in humans and γδ T-17 cells in the IMQ-pso mouse model (Bouckaert and Chincozzi, 2018; Cai et al., 2011). To determine whether a decrease in the number of γδ T-17 cells in the ILN of IMP-treated mice reflected changes in the dermis, we assessed the prevalence of γδ T-17 cells by flow cytometry. As reported by other groups, IMQ treatment resulted in an increased proportion and number of γδ T-17 lymphocytes in the dermis on day 6 (Figure 3a and b). However, as observed in the ILN, the proportion of γδ T-17 cells was significantly reduced in psoriatic mice treated with IMP (Figure 3a and b), whereas Th17 cells were not affected (data not shown). In addition, we enumerated IL-17-producing cells in the skin, which we identified immunohistochemically by the expression of the transcription factor RORγt (Barros-Martins et al., 2016). Total counts of these cells per mm<sup>2</sup> confirmed that IMP treatment significantly reduced this population of RORγt<sup>+</sup> cells (Figure 3c and d). Most cells in the skin that express RORγt are γδ TCR-positive (Lochner et al., 2009). These cells are also responsible for the majority of the local IL-17 production, particularly during IMQ-pso pathogenesis (Cai et al., 2011). The reduction in RORγt-expressing cells,
as visualized using immunohistochemistry, is therefore likely to reflect changes in γδ T cells.

Stimulation of γδ T-17 cells is highly dependent on the local cytokine milieu, especially IL-23 and IL-1β, produced mainly by macrophages and DCs (Cai et al., 2011). To determine whether IMPs impaired IL-17 production through alteration of the psoriatic cytokine profile in the skin, we analyzed cytokine production by flow cytometry and qPCR. By flow cytometry, we assessed the production of IL-23 by CD45+CD3− cells by median fluorescence intensity analysis in the skin-draining ILN. IMQ-psoriasis mice had a significant increase in IL-23 production on day 3 and a trend toward increased expression on day 6. IMP treatment significantly reduced the production of IL-23 on day 3 but did not have a significant impact on day 6 (Figure 3e). Using qPCR, we found increased IL-23 and IL-1β mRNA expression on both days 3 (Figure 3f) and 6 (Figure 3g) in psoriatic mice, whereas IMP treatment resulted in a trend toward decreased IL-23 and IL-1β expression on day 3 (Figure 3f).
Figure 2. IMP treatment leads to reduced proportion and number of IL-17+ γδ T cells in the inguinal LNs of mice with psoriasis. (a) Proportion and the absolute number of γδ IL-17+ T cells and (b) representative flow cytometry plots of cell subsets. (c) Proportion of IL-17+ CD4+ T cells and the absolute number of Th17 T cells and (d) representative flow cytometry plots. (e) Proportion of IL-17+ cells of γδ or CD4+ T cells assessed in the spleen by flow cytometry. Results are shown as mean ± SEM with n = 9 mock, n = 9 pso (α, β) two combined experiments and n = 5 mock, n = 10 pso (α) one experiment representative of two, *P < 0.05, **P < 0.01, ***P < 0.001 by Mann–Whitney U test. FCS, forward scatter; IMP, immune modifying particle; LN, lymph node; pso, psoriasis; Th17, T helper type 17.
Figure 3. IMP treatment leads to reduced infiltration of IL-17-producing cells and reduced cytokine expression in the dermis of psoriatic mice. (a) Proportion of IL-17+ γδ T cells assessed by flow cytometry. (b) Representative FACS plots. (c) Proportion of RORγt+ cells per mm² of skin assessed by IHC and counterstained with hematoxylin. Each dot represents one mouse. (d) Representative pictures of skin IHC. Red arrows indicate RORγt+ cells. Bar = 50 μm. (e) IL-23 MFI in CD3+CD45+ cells isolated from ILN on days 3 and 6. (f, g) Expression of IL-1β and IL-23 in the skin by qPCR at (f) day 3 and (g) day 6. Results are shown as mean ± SEM with n = 5 mock, n = 10 pso (a, e, f) one experiment representative of two and n = 9 mock, n = 19 pso (f, g) two combined experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by Mann–Whitney U test. FCS, forward scatter; IHC, immunohistochemistry; ILN, inguinal lymph node; IMP, immune modifying particle; MFI, median fluorescence index; pso, psoriasis.
Together, these data show that the beneficial effects of IMP on psoriasis are characterized by decreased numbers of γδ T cells producing IL-17 in the dermis, associated with reduced expression of key cytokines involved in their generation.

IMP treatment leads to Ly6C<sup>hi</sup> monocyte sequestration in the spleen, reducing early lineage monocyte-derived macrophages in the dermis

Circulating Ly6C<sup>hi</sup> monocytes regularly migrate to the dermis where they replenish DCs and macrophage populations (Tamoutounour et al., 2013). In psoriasis, these cells produce cytokines IL-1β and IL-23, promoting IL-17 production. Circulating Ly6C<sup>hi</sup> monocytes that take up IMPs become phosphatidylserine-positive and thus are sequestered in the spleen, preventing their migration to the site of inflammation (Gerts et al., 2014; Niewold et al., 2018). To determine whether IMP-mediated modulation of Ly6C<sup>hi</sup> monocyte migration underlies the improved clinical outcome, we first analyzed the changes in the numbers of splenic Ly6C<sup>hi</sup> monocytes 3 and 6 days after treatment with IMPs. From day
3, the numbers of splenic Ly6C<sup>hi</sup> monocytes were increased in the IMP-treated mice, with a further increase in their numbers by day 6 (Figure 4a and b).

Second, we detailed changes in monocyte recruitment to the dermis. To do this, we categorized dermal cells isolated from psoriatic skin according to lineage order using CCR2 and CD64 as previously described (Tamoutounour et al., 2013). Newly migrating dermal monocytes (P1) and dermal monocyte-derived DCs (P2 and P3) were separated from monocyte-derived macrophages (P4 and P5) (Figure 4c and d). As expected, the relative proportion of P1 monocytes was increased significantly in the dermis of IMQ-psoriasis mice on day 3 and day 6 (Figure 4e). Whereas IMP treatment did not affect the relative number of P1 monocytes in the dermis at day 3 (Figure 4e), by day 6, their proportion was significantly reduced (Figure 4e).

Psoriatic mice also had increased proportions of P2 moDCs on day 6, with a trend toward increased P3 moDCs. Neither of these populations were affected by IMP treatment on day 3 or 6 (Figure 4f). No changes were observed in the proportions of monocyte-derived macrophages P4 or P5 on day 3 or day 6 in IMQ-psoriasis mice (Figure 4g). However, IMP treatment resulted in a reduced P4 macrophage proportion at day 6 (Figure 4g), whereas P5 monocyte-derived macrophages (Figure 4g) were not affected by IMP treatment at either time points. Together, these results suggest that IMP treatment reduces the recruitment of Ly6C<sup>hi</sup> monocytes to the dermis in psoriasis by diversion to the spleen, thereby reducing relative numbers of early differentiated monocyte-derived dermal myeloid populations. Importantly, the marked reduction in the P4 subset by day 6 supports the notion that these cells are replaced by Ly6C<sup>hi</sup> cells from the blood (Tamoutounour et al., 2013). This argues that under homeostatic conditions, these cells are almost completely turned over within this time, in contrast to the P5 population,
which is either independently maintained in situ or much more slowly maintained by immigrant Ly6C^{hi} monocytes.

DISCUSSION

Current treatment approaches for psoriasis mostly address the symptoms, as such, they are nonspecific and may be strongly immunosuppressive. Our data show a beneficial effect of IMP treatment in the IMQ-pso mouse model in reducing keratinocyte hyperproliferation, skin thickness, redness, and scaling. This was associated with IMP-mediated sequestration and accumulation of Ly6C^{hi} cells in the spleen, with a parallel reduction of immigrant Ly6C^{hi} inflammatory monocytes in the dermis of psoriatic mice. As a result, we observed reduced early lineage dermal monocyte-derived populations as well as evidence of decreased expression of myeloid-derived IL-1β and IL-23 in the skin. These cytokines are major inducers of IL-17 expression by γδ T cells, which decreased after IMP treatment. These results illustrate the critical importance of the inflammatory monocyte lineage in the pathogenesis of psoriasis and are consistent with studies showing that both Ccr2 knockout mice and mice depleted of Ly6C^{hi} monocytes have reduced IMQ-pso (Costa et al., 2017; Singh et al., 2016). We demonstrate that IMPs mediate disruption of inflammatory monocyte recruitment to psoriatic lesions, potentially offering a safe alternative to current treatments.

Both P1 monocytes and P4 monocyte-derived macrophages were reduced in the dermis of IMP-treated psoriatic mice, whereas only the P4 subset was reduced by IMP treatment in mock mice. This strongly argues that the P4 cell subset is the most frequently renewed in the skin under homeostatic conditions, with almost the entire population turning over within 6 days. In contrast, normal turnover of the P1 subset is evidently slower but significantly increased by proinflammatory stimuli. Only the P1 dermal monocyte subset is increased substantially in IMQ-pso and significantly decreased by IMP treatment, thereby ameliorating psoriasis. This argues for a critical role of the P1 subset in the initial pathogenesis of psoriasis and is consistent with the importance in psoriasis of the P2 and P3 moDC populations that are putatively different from P1 dermal monocytes.

In contrast to previous findings, we did not see a significant reduction in the DC population (Singh et al., 2016). IMP administration over a longer period of time may affect dermal moDCs that are similarly renewed by extravasated Ly6C^{hi} monocytes (Tamoutoumour et al., 2013) and P5 macrophages, which are renewed over a longer period of time. This is the case in IMP treatment of autoimmune encephalomyelitis (EAE), where downstream DCs in the CNS are depleted over 10 days of treatment (Getts et al., 2014). Unfortunately, the effects of long-term treatment with IMP could not be tested owing to the self-resolving nature of IMQ-pso.

Monocytes and macrophages are among the major producers of IL-23 and IL-1β, which are necessary for IMQ-pso development (Morimura et al., 2016). In IMQ-pso mice, these cytokines increased in the skin from day 3, which coincided with the increased numbers of P1 monocytes. IMP treatment decreased the proportions of both P1 and P4 monocytes from day 3 and significantly on day 6. IMP treatment also reduced dermal IL-23 and IL-1β expression from day 3 as well as γδ T-17 cell generation in the dermis on day 6. This reduction in skin cytokines in IMP treatment is most likely explained by reduced cytokine expression by leukocytes combined with reduced recruitment of monocytes into the dermis. The fact that IMP decreased γδ T-17 and not Th17 also suggests a specific role of macrophages in inducing γδ T-17 cells, whereas Th17 cells may preferentially be stimulated by DCs. Previous studies have demonstrated that DCs migrating from psoriatic skin to draining lymph nodes polarize naïve CD4^{+} cells to Th17 cells (Elibso et al., 2012; El Malik et al., 2013). Because DCs were not affected over the short term by IMP treatment, this may also explain why complete disease resolution was not achieved with IMP treatment.

IMP treatment had a strong therapeutic effect in our animal model, but it remains to be determined whether it would be effective in humans. Similarities between human psoriasis and IMQ-pso in mice include monocyte infiltration and macrophage activation in psoriatic lesions (Wang et al., 2019). In addition, IL-23R polymorphisms in humans have a strong association with psoriasis development (Zhu et al., 2012) and IL-23 blockade is an effective form of treatment. This supports the idea that IMPs may be therapeutic in humans through the reduction of monocyte infiltration and IL-23 production.

IL-17 is also the driver of a number of autoimmune diseases, including MS. Similar to what we observe in psoriasis, IMPs improved the pathogenesis of the mouse model of MS, EAE (Getts et al., 2014). In this model, the differentiation of Ly6C^{hi} monocytes into DCs drives Th17 differentiation (Bailey et al., 2007; King et al., 2009). An important distinction between IMQ-pso and EAE is the nature and timing of the immune response. In IMQ-pso, IL-17 production is largely driven through rapid innate toll-like receptor–stimulated pathways, whereas in EAE, it involves an adaptive antigen-specific response with symptoms visible 10 days after immunization (Getts et al., 2014). This difference in timeframe of disease development may explain why in our own study, IMP treatment diminished dermal macrophage and γδ T-17 cells, whereas in EAE, DCs were diminished by IMP. Importantly, this suggests that IMP treatment could be a strategy to improve a broad range of IL-17–mediated diseases by targeting both moDCs and macrophages.

The mice administered with IMPs showed no signs of distress during the course of IMP treatment and did not lose weight (data not shown). In humans, a polylactic-co-glycolic acid particle formulation of tolerizing IMPs for celiac disease incorporating gladin, with the Food and Drug Administration–approved biodegradable polymer used in our study, has shown promising human biocompatibility both in vitro and in completed Food and Drug Administration–monitored phase 1 (NCT01486990) and phase 2 (NCT03738475) trials, in which no toxicity was observed (Freitag et al., 2020). However, the long-term safety of IMP treatment has yet to be determined.

There are potential drawbacks to IMP treatment in humans. In chronic diseases, it is not clear how long treatment would be required to enable a lasting remission and what the effects of continued inflammatory monocyte modulation are. However, because IMP reduces immigrant cell numbers rather than ablating them, this treatment is unlikely to interfere with locally maintained populations. In addition, in models of flavivirus, alphavirus, and malaria (Getts et al., 2014; Niewold et al., 2018; Zaid et al., 2020), IMP treatment did not interfere with the development of an ongoing adaptive
immune response. Interestingly, in relapsing–remitting EAE mouse models, reduced clinical signs persist for some 10 days after IMI treatment cessation (Getts et al., 2014), suggesting IMI-mediated disease remission. Efficacy could further be potentiated if used as part of a combination therapy. This would potentially offer quicker benefits in patients and possibly lower the dose required for individual drugs, minimizing the side effects. This type of combination therapy approach was extremely effective in the treatment of cerebral malaria in mice at the onset of clinical signs (Niewold et al., 2018). However, owing to the self-resolving nature of the IMQ-psm mouse model, whether IMI may serve as an effective treatment if administered at the onset of disease cannot be determined in this model.

In conclusion, IMIs are a promising treatment for psoriasis and more broadly, autoimmune diseases, in which inflammatory monocytes are an exacerbating feature. Although safe for human use, the efficacy of IMI treatment has still to be tested. If effective, IMI would represent a fast and cost-effective therapy.

MATERIALS AND METHODS

Mice
Five-to-six-week-old male C57BL/6J/AUSB mice from Australian BioResources (Moss Vale, NSW, Australia) were maintained under specific pathogen-free conditions. The mice were singly housed with water and food (AIN93G; Specialty Feeds) access ad libitum. All experiments were approved by the University of Sydney Animal Ethics Committee under protocol number 2014/696.

IMQ-induced psoriasis induction
Dorsal hair was shaved using clippers and depilatory cream (Nair, Church & Dwight, Ewing, NJ, USA). Psoriasis was induced by daily applications of 41.7 mg IMQ (5% IMQ) for 3 or 6 days as previously described (van der Fits et al., 2009). Erythema and scaling were assessed daily and each given a score between 0 and 4: 0, none; 1, slight; 2, moderate; 3, marked; and 4, very marked (van der Fits et al., 2009). To normalize the results between cohorts, the highest score per cohort was assigned max and all scores in that cohort were assigned a percentage of max. Electronic calipers were used to measure skin thickness daily (van der Fits et al., 2009).

Administration of IMIs
Poly(lactic-co-glycolic acid)-carboxylated IMIs with a diameter of 500 nm (Phosphorex, Hopkinton, MA) was infused daily from the time Aldara was administered on wards at 4.26 × 10⁹ particles in 200 μl sterile saline through intravenous tail vein injection (Niewold et al., 2018).

Flow cytometry
Lymphoid organs were mechanically disrupted. The skin was digested in RPMI 1640 (Gibco, Australia) 10% fetal bovine serum with 2 mg/ml collagenase type IV (Gibco) and filtered through a 70 μm filter. All samples were incubated with FcR blocker (Biolegend, San Diego, CA) and live/dead stain (Thermo Fisher Scientific, Waltham, MA). CD2 staining was conducted at 37 °C for 30 minutes (PBS, 2% fetal bovine serum) before other surface staining where indicated (Jalbert et al., 2013). All other surface staining was performed on ice for 30 minutes (PBS, 2% fetal bovine serum). For intracellular cytokine staining, cells were stimulated with phorbol 12-myristate 13-acetate, ionomycin, and brefeldin A for 4 hours. After surface staining, cells were fixed and stained using a kit (BD Biosciences, Franklin Lakes, NJ). Cells were analysed on the LSRII (BD Biosciences) and FlowJo v10 software was used to analyse data according to gating strategies presented in Supplementary Table S1. The antibodies used are presented in Supplementary Table S2.

Histology
Paraffin-embedded skin samples were sectioned and stained with H&E and visualized using light microscopy (Zeiss Axioscope, Zeiss, Oberkochen, Germany), and ImageJ was used for measuring epidermal thickness (four fields of view at ×10 magnification per mouse, 20 measurements per field).

Immunohistochemistry
Heat-induced antigen retrieval was performed in Tris-EDTA buffer (pH of 9.0). Slides were blocked in 10% serum and 1% fetal bovine serum and incubated overnight in primary antibody at 4 °C rabbit anti-mouse RORγ antibody, Abcam, Cambridge, United Kingdom. Rabbit-specific horseradish peroxidase (3,3'-diaminobenzidine detection immunohistochemistry kit (Abcam) was used according to the manufacturer’s instructions. Slides were counterstained using Harris’ modified hematoxylin (Sigma-Aldrich, St. Louis, MO). Four sections per sample and four fields of view per mouse were measured by three blinded participants.

RNA extraction and qPCR
RNA was extracted from skin samples using TRI Reagent (Sigma-Aldrich)). RNA was converted to cDNA using Script RT Supermix (Bio-Rad, Hercules, CA). qPCR was performed on a LightCycler 480 (Roche, Basel, Switzerland) using SYBR green (Bio-Rad). Primer sequences are listed in Supplementary Table S3.

Statistical analysis
The D’Agostino–Pearson normality test was used to determine whether the distribution of the samples was normal, and subsequently, parametric (normal distribution) or nonparametric Mann–Whitney U test (non-normal distribution) statistical analysis was utilized. Two-way ANOVA was used when comparing more than two groups and/or more than two independent variables such as time and treatment. P < 0.05 was considered statistically significant.

Data availability statement
No data sets were generated or analyzed during this study.

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CONFLICT OF INTEREST
NJCK is a coauthor on a patent application for immune-modifying nanoparticles (PWO2012065153A3) and owns stock in Coum Pharmaceutical Development Company, the licensor-hold of the patent application. The remaining authors state no conflicts of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: NJCK, LM; Funding Acquisition: LM, NJCK; Investigation: GVP, IT, PT, EM, ASA; Visualization: GVP, LM, IT, PN, NJCK; Writing – Original Draft Preparation: GVP, LM; Writing—Review and Editing: GVP, LM, IT, NJCK.
Supplementary Material

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.03.973.

References


Topical application of imiquimod on the skin resulted in increased local IL-23 and IL-1β production by dermal macrophages, stimulating IL-17 production by γδ T-17⁺ cells and resulting in keratinocyte hyperproliferation, epidermal thickening, and scaling. Immune modifying particles administered intravenously in the tail were phagocytosed by Ly6C⁺ monocytes, which resulted in their sequestration to the spleen, no longer replenishing the dermal macrophages. This reduced the overall IL-23 and IL-1β production, leading to fewer γδ T-17⁺ cells, less keratinocyte hyperplasia, reduced epidermal thickening, and improved psoriasis score.
Chapter 5: Integrated Discussion and Future Directions

The central aims of this thesis were to investigate how diet and inflammatory diseases can each affect intestinal homeostasis. The vast impacts that diet has on gut homeostasis have been studied extensively (Conlon and Bird 2015), however the impact of food additives remains less well known. The food additive E171 was found to significantly alter gut homeostasis though changes to cytokine expression, altered colon morphology, and reduced bacterial metabolite production. Similarly, the inflammatory skin disorder, psoriasis, led to shifts in microbial communities consistent with dysbiosis and stimulated macrophage proliferation and associated cytokine production within the colon. These results demonstrate the complex nature of gut homeostasis and rejects the idea that inflammatory diseases, such as psoriasis, are localised to the site of the lesion. Also, these findings highlight the need for further research into the effects of dietary additives, which were once thought of as inert and how they can impact intestinal homeostasis and systemic health.

Oral administration of E171 was found to increase the expression of TNF-α, IL-6 and IL-17A within the colon of mice, resulting in a cytokine profile resembling that found within IBD patients (Atreya and Neurath 2005, Kobayashi, Okamoto et al. 2008, Sakuraba, Sato et al. 2009, Koelink, Bloemendaal et al. 2019). This was accompanied by reduced colonic crypt length, increased transcription of the antimicrobial peptide beta-defensin-3 (analogous to human beta-defensin-2 (Burd, Furrer et al. 2002)) and reduced bacterial metabolite production. E171 also promoted biofilm formation in vitro by colonic commensal bacteria (Pinget, Tan et al. 2019). Reduced colonic crypt length is symptomatic of colonic inflammation and is similarly a hallmark of IBD (Hendrickson, Gokhale et al. 2002, Fujino, Andoh et al. 2003, Yen, Cheung et al. 2006). Further, biofilm formation has been associated with both colorectal cancer and IBD (Srivastava, Gupta et al. 2017, Ellermann and Sartor 2018, Tomkovich, Dejea et al. 2019). Together, these results strongly suggest that E171 consumption triggers low grade inflammation within the colon and may predispose to the development of cancer and IBD. This finding is in line with a recent study demonstrating that E171 fed rats had worse colorectal cancer in a
chemically-induced model and developed spontaneous preneoplastic lesions after 100 days of daily exposure (Bettini, Boutet-Robinet et al. 2017).

The role of biofilms in intestinal disease pathogenesis is not well understood and whether they are a symptom, or a driver of disease is unclear. Biofilms allow bacteria to evade antimicrobial substances such as AMPs released by the host (Cole and Nizet 2016). As a result, they can facilitate adhesion and hence bypass mucosal adaptations within the host which normally attenuate bacterial growth. This allows previously harmless commensal bacteria to become potential pathobionts by triggering inflammatory responses within the colon (Buret, Motta et al. 2019). Indeed, biofilms from patients with IBD grown ex vivo have been shown to have increased virulence as indicated by their ability to invade intestinal epithelia and stimulate chemokine production (Motta, Allain et al. 2018). Biofilms are 4-fold more common in patients with colorectal cancer than in the general population (Tomkovich, Dejea et al. 2019). Further, homogenates from human colonic biofilm samples were found to induce tumour formation in three different mouse models of colon cancer (Tomkovich, Dejea et al. 2019). Together, this indicates that biofilms may play a pathogenic role in cancer development, and suggests that the E171-induced carcinogenesis, reported by Bettini et al., may involve biofilm formation (Bettini, Boutet-Robinet et al. 2017). However, the study by Bettini et al. did not investigate the possible involvement of the microbiome or of biofilm. Future research utilising germ free or antibiotic-treated mice would provide further insights into whether E171-induced low grade inflammation and increased cancer risk is dependent upon microbiome and biofilm formation. Combining E171 feeding with a colitis model, such as DSS colitis, would also confirm whether this food additive predisposes to the development of more severe colitis.

E171 is widely used in food products and it is estimated that adults consume an average of up to 3.9mg and children a maximum of up to 32.4mg TiO2/kg BW/day (Aguilar, Crebelli et al. 2016, Pinget, Tan et al. 2019). This alarming rate of exposure to an additive whose safety cannot be guaranteed has resulted in action by some countries such as France where the addition of E171 in food will be banned from 2020 according to the National Agency for Food, Environmental and Occupational Health Safety.
One limitation to our study is that in the general population, E171 is consumed in foodstuffs, as coating on medicine or in confectionary rather than suspended in water as was administered to mice in chapter 2. Nanoparticles can aggregate and form secondary structures with proteins and other molecules known as coronas (Walkey and Chan 2012, Aguilar, Crebelli et al. 2016). While particle aggregation has also been observed in water suspension, corona formation is thought to have an impact on the way that particles interact with the environment, which potentially changes their ability to be absorbed by the body. Further studies in which food containing E171 is administered to mice would more closely resemble the mode of exposure in humans and thus would provide valuable insight into how E171 affects intestinal homeostasis.

Gastrointestinal disorders and microbial dysbiosis have previously been reported in patients with inflammatory diseases, particularly psoriasis (Christophers 2001, Li, Han et al. 2013, de Oliveira, Rocha et al. 2015). However, whether this plays a causative role in triggering psoriasis or is an outcome of this inflammatory disease is unclear. Chapter 3 of this thesis revealed that the imiquimod-induced mouse model of psoriasis (IMQ-pso) resulted in significant changes to markers of gut homeostasis. An increase in CX3CR1+ macrophage proliferation was observed, accompanied by increased transcription of macrophage-associated cytokines within the colon, including TNF-α, IL-10 and IL-4. We also observed shifts in microbial communities consistent with dysbiosis, particularly a loss in microbial richness and evenness. This was accompanied by increased presence of the bacterial metabolites pyruvate and succinate. Since bacterial metabolites can stimulate the activation and proliferation of leukocytes, it was hypothesised that pyruvate and succinate may have played a role in promoting macrophage proliferation and cytokine production in the colon of IMQ-pso mice. Indeed, succinate was found to stimulate the proliferation of macrophages and induce TNF-α production in vitro, suggesting that altered metabolites in the colon of IMQ-pso mice may be directly stimulating macrophage proliferation and cytokine production. Further investigations in which IMQ-pso is induced in germ free mice may provide in vivo confirmation that CX3CR1+ macrophage proliferation and cytokine production is induced by bacterially-derived metabolites, as was observed in vitro. Together, these findings provide strong evidence that IMQ-pso induces loss of gut homeostasis, lending support
to the concept of the “skin-gut-axis”. Although, whether this loss of gut homeostasis is contributing to disease pathogenesis remains to be seen.

Limited research suggests that the microbiome may play a pathogenic role in psoriasis (Zakostelska, Malkova et al. 2016). Mice treated neonatally with antibiotics develop persistent dysbiosis and are predisposed to more severe psoriasis compared to non-antibiotic treated mice, indicating that dysbiosis plays a role in disease progression (Zanvit, Konkel et al. 2015, Zakostelska, Malkova et al. 2016). Contrary to this is the finding that germ-free mice develop less severe psoriasis and antibiotic treatment in adult mice completely ameliorates disease (Zanvit, Konkel et al. 2015, Zakostelska, Malkova et al. 2016). This could be for a number of reasons. Firstly, the antibiotics in these studies were not specific to the gut microbiome and also target skin bacteria. Previous studies have demonstrated that the skin microbiome may have a pathogenic role in psoriasis (Chang, Yan et al. 2018, Wang and Jin 2018) and so eliminating skin bacteria may have had an unintended therapeutic effect in these studies. Indeed Zanvit et al. demonstrated that antibiotic treatment significantly reduced skin microbiota but whether this improved disease was not investigated (Zanvit, Konkel et al. 2015). In light of the finding presented in chapter 3 demonstrating that psoriasis triggers dysbiosis, it is also possible that the psoriasis-induced dysbiosis may be playing a role in disease pathogenesis. This may be the reason for disease improvement upon antibiotic treatment as reported by Zanvit et al. Whether changes to the microbiome provoked worse psoriasis was outside of the scope of thesis. However further studies in which psoriasis is induced in germ free mice colonised with the microbiome of healthy or IMQ-pso mice may provide valuable insight into whether dysbiosis induced by IMQ-pso is contributing to disease severity.

The sequence of events leading to the loss of gut homeostasis observed in IMQ-pso mice are still not clear from our studies. As discussed above, changes to the microbiome can alter mucosal immunity. Mice colonised with the intestinal microbiome from IMQ-pso mice may answer the question of whether macrophage proliferation and cytokine production are triggered by the microbiome directly or whether these immune changes occurred first and subsequently shaped the microbiome. Another possibility is that elevated serum cytokine levels observed during IMQ-pso are impacting immune cells
within the colon, in turn inducing dysbiosis. However, findings in chapter 3 reveal that IL-17A/F is unlikely to be involved. Other cytokines such as IL-23 have also been shown to be systemically increased in IMQ-pso mice and patients with psoriasis, opening up avenues for further investigation (Chen, Lu et al. 2017, Takuathung, Wongnoppavich et al. 2018). Understanding the close relationship between the microbiome and the mucosal immune system will provide valuable insight and inform future studies into intestinal homeostasis.

It was initially hypothesised that increased macrophages in the colon of IMQ-pso mice were monocyte-derived. To investigate whether this was contributing to disease progression, IMP was administered to IMQ-pso mice to prevent monocyte migration to the colon. Although this did not alter populations of macrophages in the colon, nor did it prevent dysbiosis, psoriasis was improved in these mice due to reduced monocyte migration to the dermis. This reveals the role that monocyte-derived macrophages have in disease progression and potentially indicates a new therapeutic target in treating psoriasis. These findings together provide strong evidence supporting the idea that CX3CR1+ macrophages are proliferating in the colon rather than migrating as monocytes. Future experiments utilising other methods of blocking monocyte influx, such as though CCR2 antagonist, may further confirm these findings (Brodmerkel, Huber et al. 2005).

Systemic effects of imiquimod have previously been suggested to occur due to absorption through the skin (Nerurkar, McColl et al. 2017) and while absorption is minimal, this may still be enough to impact the immune system. While this is not something that can be disproven without drug tracing studies, the finding that neither IL-17 or IL-23 were increased in the colon of IMQ-pso mice suggests that it is unlikely that systemic TLR7/8 activation is taking place. To further explore this, future studies may utilise another commonly used psoriasis mouse model, which is induced via IL-23 injections into the skin (Rizzo, Kagami et al. 2011). This bypasses the involvement of macrophages and DCs in disease pathogenesis and directly stimulates Th17 cells to produce IL-17. While this does not emulate the cytokine profile found in the human form of psoriasis as well as the IMQ-pso model, IL-23 induced psoriasis may provide further insights into how psoriasis alters gut homeostasis.
Finally, while the microbiome encompasses a range of microorganisms, including fungi, viruses, archaea and bacteria, the focus of this thesis was only the bacterial component (Huffnagle and Noverr 2013, Shreiner, Kao et al. 2015). Although this is common practice in many studies due to cost and technological challenges, this underlines a major limitation in our understanding of how the microbiome, mucosal immune system and intestinal epithelium interact. As more sophisticated methods of analysis are developed, the complete microbiome and its metabolites can be analysed with greater ease and accuracy, providing a more detailed picture of the effects that dietary components and inflammatory diseases have on intestinal homeostasis.

The research presented in this thesis reveal gaps in the literature surrounding the interactions between gut homeostasis, diet and inflammatory diseases. The consumption of food additives and incidence of inflammatory diseases have steadily increased over time (Eder, Ege et al. 2006, Wang and Sampson 2011, Tan, McKenzie et al. 2016, Laudisi, Stolfi et al. 2019, Patterson, Harjutsalo et al. 2019). As this trend continues, a deep understanding of the complex relationship between gut homeostasis, inflammation and diet is paramount to the prevention and treatment of inflammatory diseases.

In summary, findings in this thesis contribute to the vast research which demonstrates undeniably that a westernised diet promotes disrupted gut homeostasis, leading to increased predisposition to inflammatory diseases, including colorectal cancer and IBD. This was demonstrated in chapter 2 which revealed that the common food additive E171 significantly altered mucosal immunity, colonic morphology and impacted the microbiome. It was also demonstrated that the skin inflammatory disease, IMQ-pso, had profound impacts on intestinal mucosal immunity and microbiota. While mechanisms behind this are yet to be fully elucidated, together these findings demonstrate the interconnected relationship between intestinal homeostasis and systemic health. Further research will not only provide a greater understanding of psoriasis and its comorbidities but may also inform clinical practice, which currently lacks a wholistic approach to treating inflammatory diseases.
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