Deciphering the mechanisms of action of vaccine adjuvants in human lymphoid tissue

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Declaration

I, Vicki Vasiliki Stylianou, hereby declare that to the best of my knowledge, the content of this thesis is my own work and has not been submitted for the award of any other degree. I certify that the intellectual content of this thesis is the product of my own work and that all the assistance received in preparing this thesis and sources have been acknowledged.

The following publication submissions are contained within this thesis:

- Stylianou, V.V., Bertram, K.M., Dunn, E., Baharlou, H., Terre, D., Elhindi, J., Elder, E., French, J., Meybodi, F., Hsu, J., Temmerman, S., Didierlaurent, A.M., Coccia, M., Sandgren, K.J., Cunningham, A.L., Innate immune cell activation by adjuvant AS01 in human lymph node explants is age-independent. Currently under review with Journal of Clinical Investigation

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Authorship Attribution Statement

Parts of Chapter 3 and 4 of this thesis, indicated with “quotation marks”, have been submitted for publication and are currently in revision as; Stylianou, V.V., Bertram, K.M., Dunn, E., Baharlou, H., Terre, D., Elhindi, J., Elder, E., French, J., Meybodi, F., Hsu, J., Temmerman, S., Didierlaurent, A.M., Coccia, M., Sandgren, K.J., Cunningham, A.L., Innate immune cell activation by adjuvant AS01 in human lymph node explants is age-independent. 2023 *Journal of Clinical Investigation (in revision).*

I conducted all experiments and analysed the data in this manuscript and made the figures for the paper. I also edited the drafts of the MS. In addition, permission to include the material, which may be published prior to the completion of examination of this thesis, has been granted by the corresponding author.

Vicki Vasiliki Stylianou
16 January 2024

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

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Tony Cunningham

16 January 2024

Abstract

Adjuvants are immunostimulatory additives that can enhance the potency of a vaccine by increasing the magnitude, influencing the type, and enhancing the durability of the immune response. Vaccine adjuvants are thought to work by stimulating innate immunity at the site of vaccination and ultimately, at the draining lymph node (LN), where vaccine induced immunity is primarily generated. Different adjuvants are known to have unique innate immune functions that can involve diverse molecular mechanisms, patterns of innate immune cell maturation and activation, and inflammatory cytokine profiles, all of which shape the downstream adaptive immune response. However, these innate immune characteristics have not been proven in human LNs and are largely studied in animal models. To progress vaccine development, it is crucial to understand how vaccine adjuvants work in humans, to improve the success rate of new formulations when transitioning from animal studies to human clinical settings.

To bridge data obtained in animals to humans, we have developed a novel *in situ* human LN explant model for studying innate immune responses and investigate how adjuvants initiate immunity in LNs. Slices of explanted skin-draining LNs were exposed to vaccine adjuvants and revealed responses that were not detectable in LN cell suspensions. We used this model to compare the liposome-based AS01 with TLR ligands R848, MPL and Pam2Cys as well as the saponin QS-21. Liposomes were predominantly taken up by subcapsular sinus-lining macrophages (SMs), monocytes and dendritic cells. AS01 induced dendritic cell maturation and a strong pro-inflammatory cytokine response in intact LN slices but not in dissociated cell cultures, in contrast to R848. This suggests the onset of the immune response to AS01 requires a coordinated activation of LN cells in time and space. Consistent with the robust immune response observed in older adults with AS01-adjuvanted vaccines, the AS01 response in human LNs was independent of age, unlike R848. Additionally, the molecular pathways of AS01 were investigated in part using the explant model. Blocking the NLRP3 inflammasome in whole LN slices produced an inhibitory effect on key AS01-induced pro-inflammatory cytokines. Preliminary assessments on isolated LN macrophages revealed the formation of inflammasome specks in response to AS01 and suggests the involvement of the NLRP3 inflammasome in the AS01 immune response although whether this is the initiating mechanism remains unclear.

This human LN explant model is a novel tool for studying the mechanism of action of adjuvants in humans and for screening new formulations to streamline vaccine development.

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Publications, Presentations and Scholarships

Publications Under Review

- Stylianou, V.V., Bertram, K.M., Dunn, E., Baharlou, H., Terre, D., Elhindi, J., Elder, E., French, J., Meybodi, F., Hsu, J., Temmerman, S., Didierlaurent, A.M., Coccia, M., Sandgren, K.J., Cunningham, A.L., Innate immune cell activation by adjuvant AS01 in human lymph node explants is age-independent. Currently under review with Journal of Clinical Investigation

Conference Presentations

- The Westmead Association Hospital Week Research Symposium: poster presentation (2021, 2023)
- Annual Scientific Meeting of the Australian and New Zealand Society for Immunology, Virtual Meeting: poster presentation (2021)
- Annual Scientific Meeting of the Australian and New Zealand Society for Immunology, Melbourne VIC Australia: oral presentation (2022)
- International Symposium of Dendritic Cells, Cairns QLD Australia: poster presentation (2022)
- Keystone Symposia on Molecular and Cellular Biology, Vaccinology During and After COVID-19, Atlanta GA USA: oral presentation and poster presentation (2023)
- Westmead Research Hub (WRH) Early-Mid Career Researchers (EMCR) Symposium: oral presentation (2023)

Scholarships

- 2020, Australian Government Research Training Program Stipend Scholarship

Table of Contents

List of Abbreviations

Acronym	Definition
Alum	Aluminium salts
ALR	Absent in melanoma-2-Like Receptors
APC	Antigen Presenting Cell
AS	Adjuvant System
ASC	Apoptosis-associated Speck-like protein containing a Caspase recruitment domain
$AS01_B$	Adjuvant System 01_B
$AS01_E$	Adjuvant System 01_E
AS02	Adjuvant System 02
AS03	Adjuvant System 03
AS04	Adjuvant System 04
ATP	Adenosine Triphosphate
BD	Becton Dickson
BFA	Brefeldin A
BSA	Bovine Serum Albumin
β-ME	2-β-mercaptoethanol
CBA	Cytometric Bead Array
CD	Cluster of Differentiation
cDC	Conventional Dendritic Cell
CLR	C-type Lectin Receptors
CpG	Cytosine–Guanine dinucleotides
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DCM	Dendritic Cell Media
DMEM	Dulbecco's Modified Eagle Medium
DNase	Deoxyribonuclease
DPBS	Dulbecco's Phosphate Buffered Saline
DTT	1,4-dithiotreitol
DZ	Dark Zone
EDTA	Ethylenediaminetetraacetic Acid
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FDC	Follicular Dendritic Cells
Flt-3L	Fms-Like Tyrosine kinase 3 Ligand
FMO	Fluorescence Minus One
FRC	Fibroblastic Reticular Cell
FSC	Forward Scatter

Acronym	Definition
FVS	Fixable Viability Stain
gE	Glycoprotein E
γδ	GammaDelta
GSDMD	Gasdermin D
GM-CSF	Granulocyte Macrophage Colony-Stimulating Factor
gMFI	Geometric Mean Fluorescence Intensity
GSK	GlaxoSmithKline
h	Hour/s
hAB	Human Type AB
HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic Acid
HEV	High Endothelial Venules
HIV	Human Immunodeficiency Virus-1
HLA-DR	Human Leukocyte Antigen, Antigen D Related Receptor
HMGB1	High-Mobility Group Protein B1
HSV	Herpes Simplex Virus
ICS	Intracellular Cytokine Staining
IF	immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate Lymphoid Cell
IMC	Imaging Mass Cytometry
IMDM	Iscove's Modified Dulbecco's Medium
IPV	Inactivated Polio Vaccine
IRF	Interferon Regulatory Factor
K+	Potassium
LC	Langerhan Cell
LD	LIVE/DEAD
LN	Lymph Node
LNP	Lipid Nanoparticle
LPS	Lipopolysaccharide
LZ	Light Zone
MACS	Magnetic Activated Cell Sorting
MCM	Medullary Cord Macrophage
MCP-1	Monocyte Chemoattractant Protein-1
M-CSF	Macrophage Colony-Stimulating Factor
MDA5	Melanoma Differentiation-Associated protein 5
MDDC	Monocyte Derived Dendritic Cell
MDM	Monocyte Derived Macrophage

Acronym	Definition
MHC	Major Histocompatibility Complexes
Min	Minutes
MPL	3-deacylated Monophosphoryl Lipid A
MRR	Measles Mumps Rubella
MyD88	Myeloid Differentiation factor 88
MZ	Marginal Zone
NEAA	Non-essential amino acids
NHP	Non-Human Primate
NK	Natural Killer
NLR	Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors
NLRP3	Nucleotide-binding Oligomerization Domain (NOD)-Like Receptors Protein 3
OPV	Oral Polio Vaccine
P2C	Pam2Cys
P3C	Pam3Cys
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PERM	Permeabilisation
PBS	Phosphate Buffered Saline
pDC	Plasmacytoid Dendritic Cell
PFA	Paraformaldehyde
PMA	Paramethoxyamphetamine
Poly(I:C)	Polyinosinic:polycytidylic acid
PRR	Pattern Recognition Receptor
QS-21	*Quillaja Saponaria* Molina: fraction 21
R848	Resiquimod 848
rcf	relative centrifugal force
RF10	Roswell Park Memorial Institute supplemented with 10% Fetal Calf Serum
RIG	Retinoic Acid-Inducible Gene I
RLR	Retinoic acid-Inducible Gene I (RIG-I)-Like Receptors
rpm	Revolutions per Minute
RPMI	Roswell Park Memorial Institute
RSV	Respiratory Syncytial Virus
RT	Room Temperature
RZV	Recombinant Zoster Vaccine
SIGLEC	Sialic Acid-Binding Immunoglobulin-Type Lectins
SIV	Simian Immunodeficiency Virus
SM	Sinus-lining Macrophage
SS	Subcapsular Sinus
SSC	Side Scatter

Acronym	Definition
STING	Stimulator of Interferon Genes
TB	Tuberculosis
TFH	T Follicular Helper
Th	T helper
TLR	Toll-Like Receptor
TLR-L	Toll-Like Receptor Ligand
TNF	Tumour Necrosis Factor
Treg	Regulatory T cell
TRIF	TIR domain-containing adapter-inducing IFNβ
VLP	Virus Like Particle
VSV	Vesicular Stomatitis Virus
VZV	Varicella Zoster Virus
WIMR	Westmead Institute for Medical Research
WSLHD	Western Sydney Local Health District
YOA	Years Of Age

List of Figures

List of Tables

Chapter 1. Literature Review – The Role of Lymph Nodes in Vaccine and Adjuvant Innate Immune Responses in Humans

Introductory Remark

Vaccines are a fundamental foundation in public health as the key preventative measure against various diseases caused by bacterial and viral infections. Vaccines are generally administered intramuscularly, and less often subcutaneously, intradermally or mucosally and these initial tissue locations are important sites of action for vaccines. However regardless of the route of administration, the draining lymph node (LN) is the primary site of action for any vaccine in the generation of immunity. The mechanisms of action of vaccines and vaccine components, such as adjuvants, are however largely unknown in human LNs. This thesis focuses on defining the modes of action of different vaccine adjuvants in human LNs, with particular interest in characterising the initial innate immune responses, which shapes the ensuing adaptive T and B cell responses. Here, the anatomy of LNs is reviewed first, followed by a discussion on the repertoire of innate immune cells present. Finally, the current knowledge of vaccine functions in humans is discussed, with particular focus on vaccine adjuvants and the need to better our understanding of their mechanisms in the context of human LNs.

1.1. Anatomy of Human Lymph Nodes and their Role in the Immune System

The LN is the main organ where immunity is generated against harmful foreign pathogens such as bacteria and viruses. It is therefore important to understand the components of this tissue in humans and the plethora of vital immune cells present, to gain better insights into how vaccines and their components function in this environment. Here, the anatomy and function of LNs are explored and the innate immune cell subsets reviewed in detail.

1.1.1. Lymphatic System

The lymphatic system supports the circulatory system and operates as an essential element in immune functions. It is composed of the lymph fluid, afferent and efferent lymphatic vessels, LNs and a variety of other lymphoid organs such as the tonsils, spleen, and bone marrow (1). Unlike the circulatory system, the lymphatic system is a one-way arrangement that drains fluid, known as lymph, from tissues throughout the body (2). Lymph contains interstitial fluid, derived from leakage out of blood capillary walls into the surrounding tissue, and may also carry cellular debris, immune cells, plasma proteins and pathogens (1). Along the lymphatic route there are clusters of LNs that act as filters of the lymph. They provide immune surveillance for antigens and pathogens but also prevent systemic dissemination of pathogens. These LN clusters are positioned in strategic locations throughout the body including cervical (neck), thoracic (trachea), axillary (armpit), mesentery

(abdomen), inguinal (groin) and iliac (pelvic) regions (Figure 1.1) (3). The LNs within these clusters are responsible for draining the specific areas of the periphery. Lymph fluid enters the LN via afferent lymphatic vessels and drains throughout the sinuses of the lymph node before exiting via the efferent lymphatic vessel. Eventually, the lymph drains back into the circulatory system into the subclavian veins in the upper chest, via the right lymphatic duct and thoracic duct (Figure 1.1) (4).

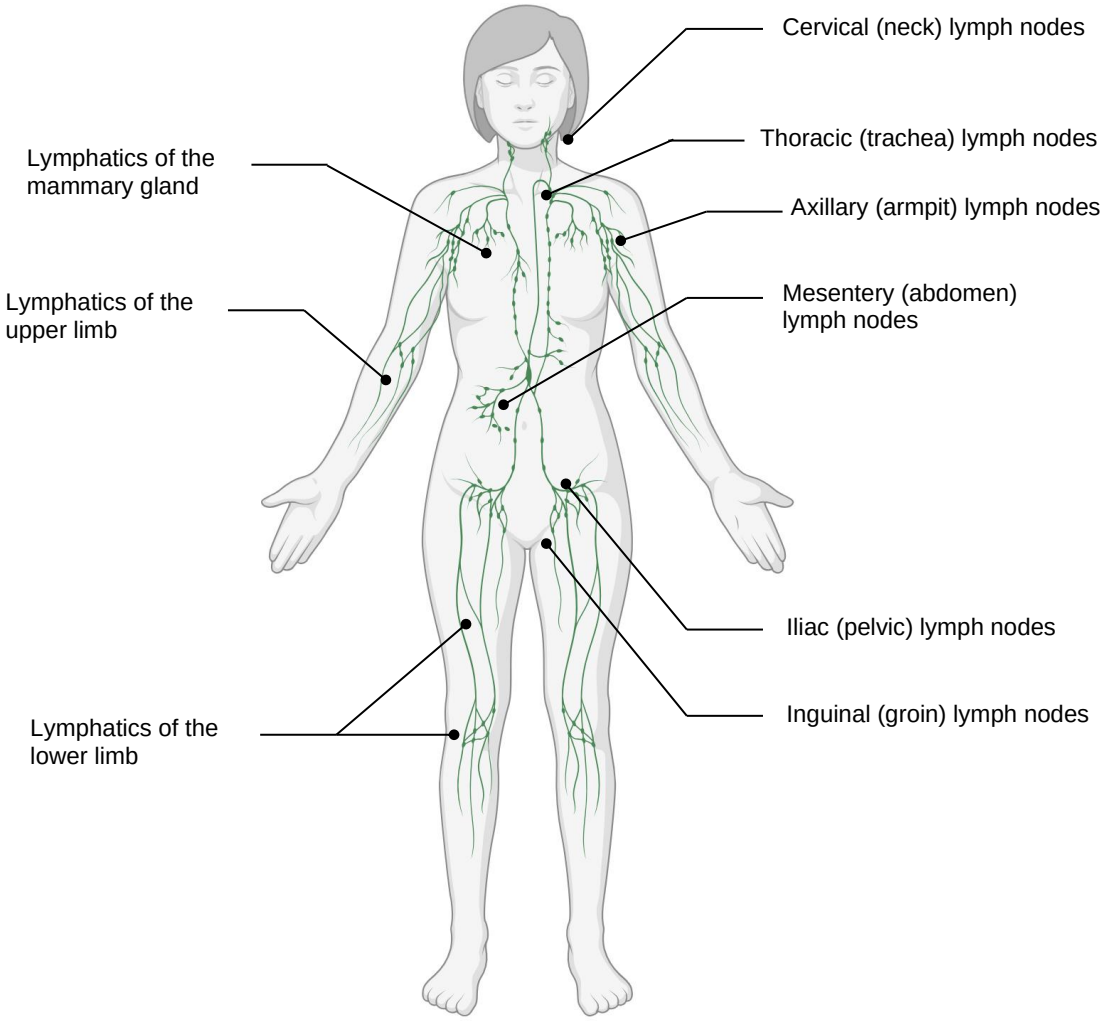


Figure 1.1: The human lymphatic system. Illustration of lymphatic drainage throughout the human body and major LN cluster sites. Image was created with BioRender.com.

1.1.2. Lymph Nodes

1.1.2.1. Lymph Node Structure

The lymph node is a highly structured organ that is separated into distinct compartments for optimal immune functions. These major regions include the cortex which is superficial to the deeper paracortex and medulla. The lymph node is divided into lobules containing these compartments and depending on the lymph node size, varying numbers or lobules can be present (5). A dense fibrous

connective tissue, the LN capsule, forms the outer surface of the LN and from here, the collagen and fibronectin connective tissues concentrate to form sinuses throughout the LN. Each lobule is bathed in lymph that enters via a single afferent lymphatic vessel attached to the capsule surface. The lymph fluid drains into the subcapsular sinus, before it enters the cortex via cortical sinuses, followed by passing through the medullary sinus compartment before finally exiting the LN via the efferent lymphatic vessel positioned at the hilum (4). Blood vessels also enter and exit the LN at the hilum. These lymphatics allow sufficient flow of lymph for the migration of antigen presenting cells (APCs), pathogens, and vaccines into the LN for filtration, as well as providing a structural support network for sufficient supply of blood vessels and nerves to the organ (Figure 1.2) (6, 7). The lobule and its internal compartments, are supported by a range of stromal cells that make up the structural connective tissue within the LN, including blood endothelial cells, lymphatic endothelial cells and a dense reticular network formed by lymphoid fibroblastic reticular cells (FRCs). These structural components not only make up the physical compartments of the LN, but the localised regions they create play a crucial role in the co-ordination and generation of effective immunity towards pathogens and vaccines (8).

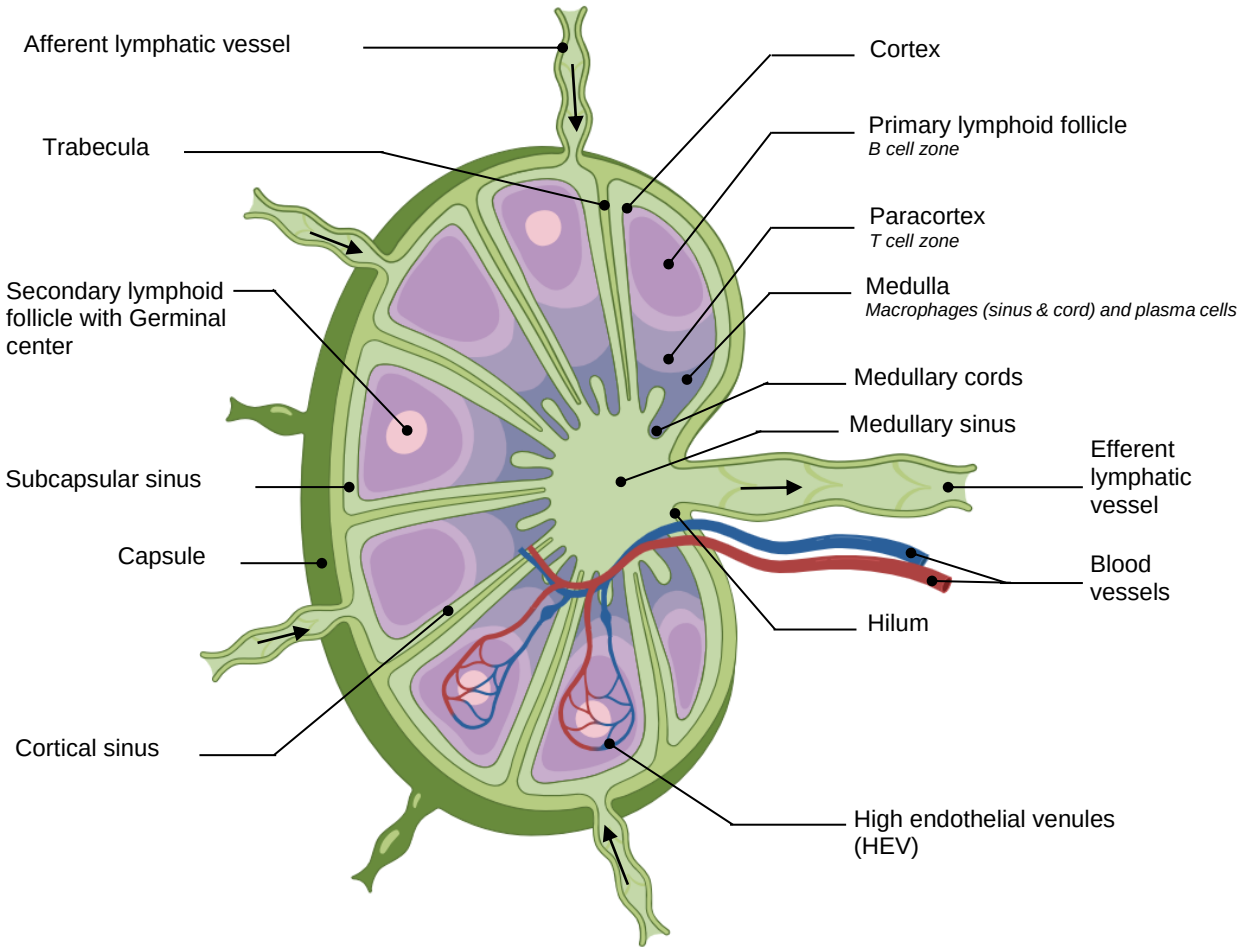


Figure 1.2: Anatomy of lymph nodes. Illustration depicting the structure of the human LN. Black arrows represent the flow direction of lymphatic fluid. Image was created with BioRender.com.

The different compartments in the LN are specifically organised to house certain stromal cells and immune cells for a controlled, co-ordinated immune environment (9, 10). The cortex layer, positioned most superficially beneath the subcapsular sinus, is known as the B cell zone where primary lymphoid follicles are present and can transition into secondary lymphoid follicles containing germinal centres for effector immune functions. Specialised antigen presenting cells (APCs), such as follicular dendritic cells (FDCs), are also present here particularly to promote B cell responses. Beneath the cortex is the paracortex, known as the T cell zone (11). Throughout the LN, fibroblastic reticular cells form micro-conduit networks derived from the sinuses that allows lymph fluid to percolate through the tissue. Large lymph-borne molecules are filtered out by this conduit system, restricted to the major sinuses, and only small molecules <70 kDa can access the B and T cell zones. The latter includes cellular proteins such as cytokines and chemokines produced by cells in other compartments and thus the micro-conduits form a network for efficient immune cell signalling and trafficking, which promotes the coordinated interaction of immune cells (8, 12-15). Additionally, high endothelial venules (HEV) are present throughout the paracortex. These HEVs provide a portal for the entry and exit of immune cells in the LN and play a crucial role in the trafficking of infiltrating naïve lymphocytes from the blood stream into the LN for activation (16). The cortex and paracortex are the key zones where T cell activation and B cell clonal expansion occurs for the generation of cellular and humoral adaptive immune responses. The underlying compartment below the paracortex however is where effector immune responses disseminate out of the LN. This is the medulla region, which is made up of medullary cords and sinuses. Here, effector T cells and predominately antibody producing B cells known as plasma cells, which are generated in germinal centres, are situated in the medullary cords. These effector immune cells can further flow through the medullary sinuses and exit the lymph node via the efferent lymphatic vessel to be distributed systemically (Figure 1.3A) (17).

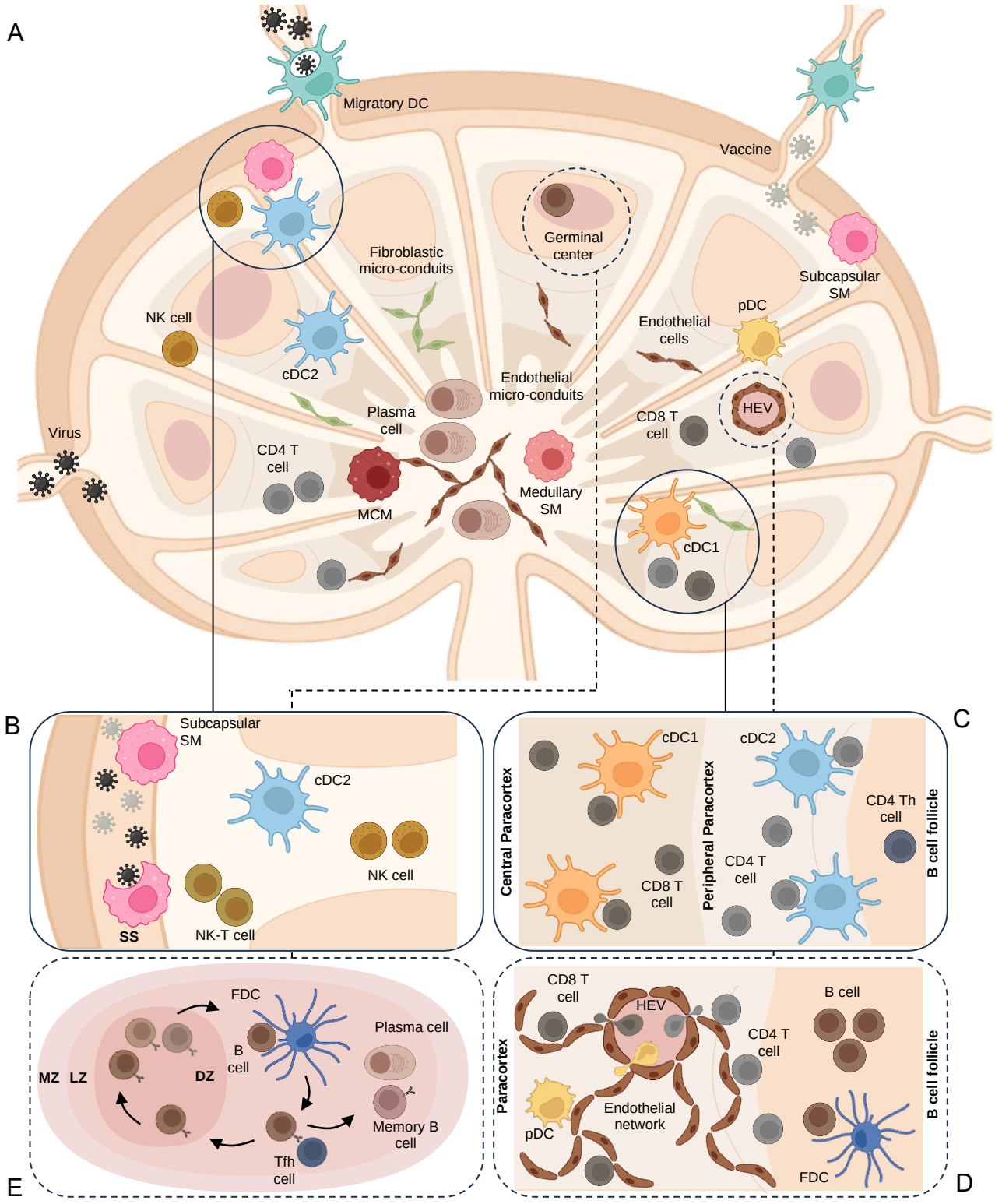


Figure 1.3: Spatial arrangement of immune cell subsets in lymph nodes. SM = sinus-lining macrophages. MCM = medullary cord macrophage. cDC = conventional dendritic cell. pDC = plasmacytoid dendritic cell. NK = natural killer. Th = T helper. FDC = follicular dendritic cell. SS = subcapsular sinus. MZ = marginal zone. LZ = light zone. DZ = dark zone. HEV = high endothelial venule. Image was created by BioRender.com and inspired by Grant et al (11).

1.1.2.2. Lymph Node Function

The generation of pathogen or vaccine induced immunity within this highly structured organ occurs in a series of events beginning with innate immune functions at the site of infection or vaccination. Pathogens and vaccines can flow freely through the lymph or be transported to the LN by activated immune cells, such as dendritic cells (DCs), monocytes and monocyte derived DCs (MDDCs) and neutrophils from the periphery (18). In addition to these infiltrating APCs, LN-resident subcapsular sinus lining macrophages (SMs) and DCs, sample lymph fluid that enters the LN to destroy pathogens, preventing their systemic spread and identify foreign antigens (19). These APCs take up and process pathogenic/antigenic material for downstream presentation to adaptive immune cells (Figure 1.3B) (20). Notably, subcapsular SMs and follicular DCs have been shown to interact with lymphoid follicles from the sinus and directly present antigen to B cells for germinal centre formation (21). Conventional DCs (cDC), which acquired antigen in the periphery or in the LN, present antigen to the $CD4^+$ and $CD8^+$ T cells in the paracortex. In brief, when T and B cells recognise their cognate antigen and receive co-stimulatory signals they differentiate into effector cells – cytotoxic T cells or cytokine-producing helper T cells or antibody-secreting B cells (Figure 1.3C-D). These cells clonally expand and leave the LN to fight off the infection. Some cells differentiate into memory cells, critical for a vaccine induced response to pathogen challenge, which can quickly be activated upon potential re-exposure to a pathogen and prevent future infection from occurring (Figure 1.3E) (11, 17). These immune cell process within the LN are crucial to fight viral and bacterial infections, and hence vaccines aim to mimic these responses to generate immunity for the prevention of future infections.

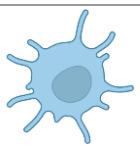
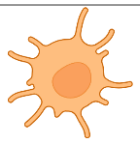
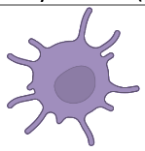
1.2. Innate Immune Cells and their Role in Triggering Adaptive Immunity

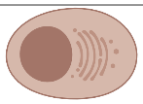
1.2.1. Lymph Node Innate Immune Cells

1.2.1.1. Localisation of cells and function

Although the LN is largely composed of lymphocytes, the limited number of infiltrating and resident innate immune cells are crucial for bridging the gap between the innate and adaptive immune system for optimal immune responses (Table 1.1) (22, 23). Unlike B and T cells which reside in specific localised zones, these innate immune cells reside within various regions of the LN.

Table 1.1: Human lymph node innate immune cell subsets.

Cell subset	Subset	Markers		Location
 Sinus lining and medullary macrophages (SM & MM)	Subcapsular sinus-lining macrophage (SM)	HLA-DR^{+} CD14^{+} CD68^{+} CD169^{+}	CD11c$^{+/-}$ CD11b$^{+/-}$ CD1c^{-}	Subcapsular sinus
	Medullary sinus-lining macrophage (SM)	HLA-DR^{+} CD14^{+} CD68^{+} CD169^{+}	CD11c$^{+/-}$ CD11b$^{+/-}$ CD1c^{-}	Medullary sinus
	Medullary cord macrophages (MCM)	HLA-DR^{+} CD14^{+} CD68^{+} CD169^{-}	CD11c$^{+/-}$ CD11b$^{+/-}$ CD1c^{-}	Medullary cord
 Pre-cursor DC	Pre-cursor DC	HLA-DR^{+} CD11c^{+} CD1c^{-} CD1a^{-}	Langerin^{-} CD141^{-} XCR1^{-}	Paracortex
 Conventional DC2 (cDC2)	Langerin^{+} cDC2	HLA-DR^{+} CD11c^{+} CD1c^{+} CD1a^{+} (skin derived)	Langerin^{+} CD141^{-} XCR1^{-}	Paracortex
	Langerin- cDC2	HLA-DR^{+} CD11c^{+} CD1c^{+} CD1a^{+} (skin derived)	Langerin^{-} CD141^{-} XCR1^{-}	Paracortex
 Conventional DC1 (cDC1)	cDC1	HLA-DR^{+} CD11c^{low} CD1c^{-}	Langerin^{-} CD141^{+} XCR1^{+} Clec9a^{+}	Paracortex
 Plasmacytoid DC (pDC)	pDC	HLA-DR^{+} CD123^{+} CD11c^{-} CD1c^{-}	Langerin^{-} CD141^{-} XCR1^{-} CD303^{+}	Paracortex
 Langerhan cells (LC)	LC (skin derived)	HLA-DR^{+} CD11c^{-} CD1c^{+} CD1a^{++}	Langerin^{+}	
 Natural Killer (NK) cells	NK cells	HLA-DR$^{+/-}$ CD56$_{+}$ CD16$^{+/-}$		Paracortex
 T cells	NK-T cells	CD3^{+} CD56^{+}	CD4^{-} CD8^{-}	Paracortex
	CD4 T cells	CD3^{+} CD56^{-}	CD4^{+} CD8^{-}	Paracortex
	CD8 T cells	CD3^{+} CD56^{-}	CD4^{-} CD8^{+}	Paracortex

 B cells	B cells	$CD19^{+}$ $CD20^{+}$ $HLA\text{-}DR^{+}$	Follicle
 Plasma cells	Plasma cells	$CD45^{+/-}$ $CD20^{+}$ $CD19^{-}$ $CD38^{+}$	Germinal centre Medulla

1.2.1.1.1. Monocytes, Macrophages and Neutrophils

In the periphery, monocytes and neutrophils recognise and take up foreign material such as pathogens or vaccines, and then transport this material to the LN (24-26). Pathogens and vaccines also flow freely to the LN via the afferent lymph and are predominantly taken up by subcapsular SMs or DCs. The subcapsular SMs are strategically positioned within the LN to sample incoming lymph, acting as flypaper to capture foreign material on their surface and present this to downstream resident APCs and T cells , as well as directly to B cells in follicles within close proximity to the subcapsular sinus (19, 27, 28). Macrophages are also present within the medullary regions of the LN. Medullary SMs line the sinuses of the medulla and here, they play a crucial role in phagocytosing foreign particles to prevent systemic spread of pathogens (29). The remaining medullary parenchyma appears as cords where medullary cord macrophages (MCM) are present to support, and phagocytose apoptotic, plasma cells in this region (30). Infiltrating neutrophils also localise to the subcapsular sinus and medullary regions following recruitment at the infection or vaccination sites. Neutrophil migration is mediated by pro-inflammatory cytokine cues including interleukins (IL) IL-1β and IL-18 from subcapsular SMs (31-33) and IL-17 from gammadelta (γδ) T and natural killer (NK)-T innate-lymphoid cells (ILCs) which produce CXCL2 chemokines that promotes recruitment (34-36). Within the LN, neutrophils function as key phagocytosing cells and form clusters to engage in a complex inter-play of interactions with ILCs, particularly NK cells, for mounting a response to foreign material and limiting systemic spread (31, 37). Unlike macrophages, monocytes are rare within steady state LNs and only migrate to this organ following inflammatory chemokine cues from vaccination or infection sites as well as the LN itself. At the primary sites of inflammation, chemokines including monocyte chemoattractant protein-1 (MCP-1) can be produced and drained to the LN via afferent vessels, where it travels from the subcapsular sinus to HEVs via micro conduits (38). These chemokines promote recruitment of monocytes from the blood via HEVs, into the cortical LN region (39) where they differentiate into macrophages or DCs and aid in phagocytosis to eliminate pathogens that have infiltrated the LN and destroy infected cells (40).

Additionally, chemokine signals can also be expressed on HEVs that drain from primary sites to the LN and aid in promoting monocyte recruitment into the LN for effector functions (41).

1.2.1.1.2. Dendritic Cell Subsets

Dendritic cells are also efficient at transporting foreign antigen and pathogens to the LN. However, while monocytes, macrophages and neutrophils can present antigen to memory T cells, only DCs can activate naïve T cell responses and are therefore considered professional APCs. There are three major subsets of DCs within the LN - type I and II cDCs (cDC1 and cDC2) and plasmacytoid DCs (pDCs), which circulate between the blood and lymph via HEVs. Unlike macrophages, both infiltrating and resident DCs are highly mobile and can migrate throughout various compartments in the LN via cortical and medullary sinuses and FRC networks (17). Skin derived dermal cDC2s are noted to migrate towards the paracortex of the LN and remain at the cortical ridge (42, 43). These ridges are regions that connect the T and B cell zones and contain a dense network of HEVs for infiltration of naïve T cells from the blood. Notably, resident cDC2s also reside here (44) as well pDCs that enter the LN via HEVs in a CXCL9 chemokine-dependent manner (45). cDC1s tend to reside in the deep paracortex (46). Additionally, Langerhan cells (LCs) originating in the epidermis of the skin and in mucosal epithelium, migrate to the LN with antigenic material and also reside within the deeper regions of the paracortex (47).

Ultimately, all DC subsets largely localise to the paracortex of the LN for efficient antigen presentation to T cells. Notably, cDCs specifically reside in niche regions of the paracortex for optimal interaction with their cognate lymphocytes (11, 48). The peripheral paracortex, adjacent to the B cell follicle, contains CD4 T cells and this is where cDC2s dwell for efficient activation of CD4 T helper (Th) cells via major histocompatibility complexes (MHC)-II, allowing for Th cells to go on to support B cell differentiation. Subsequently, cDC1s are localised to the central paracortex where CD8 T cells reside for the efficient activation of cytotoxic T cell responses via MHC-I which can effectively disseminate from the LN via the efferent vessel for effector functions at primary sites (49-51). Migratory DCs can activate CD4 T cells within the specific region of the paracortex however LN resident cDC1s are necessary for the cross presentation of antigen to activate CD8 cytotoxic T cells (52). Although pDCs are known to only infiltrate inflamed tissue sites, they are also present within the paracortex of both inflamed and steady state LNs. pDCs can exhibit two functional states. The plasmacytoid cell state is highly antimicrobial, producing large amounts of pro-inflammatory type I interferons (IFN) to clear pathogens. Alternatively, pDCs can differentiate into a more DC

state, where MHC-I and MHC-II is significantly upregulated on the surface of the cells for efficient presentation of antigen to both CD4 and CD8 T cells (53-55). However recent data suggests a new type of (more myeloid) DC, the axl+ siglec6+ myeloid DC (ASDCs), previously grouped with pDCs, may be the cells responsible for this antigen presentation, not pDCs (56). They are found in normal LNs.

Finally, unlike traditional DCs, FDCs are a non-migratory stromal cell subset that reside in the lymphoid follicle (57). FDCs capture, process, and present antibody-bound antigen complexes to B cells. They can recognise small antigens that flow freely through sinuses and micro conduit networks (58, 59) however, larger foreign particles are transported to FDCs by subcapsular SMs (60). FDCs can retain antigen for long periods to continuously support germinal centre formation and B cell activation and survival, class switching and the production of high-affinity antibodies (61). Additionally, FDCs provide support to the surrounding FRC network and produce chemokines, namely CXCL13, which attracts specific T cell subsets to B cell follicles via CXCR5 signalling, to promote B cell immunity (57, 62).

1.2.1.1.3. Innate Lymphoid Cells

ILCs resemble T cells in their phenotypic properties and functions; however, they lack antigen receptors and upon stimulation, do not undergo clonal expansion. The main function of ILCs is to respond to damaged or infected tissue which generates activating stress signals. This allows ILCs to further produce cytokines and function as early effector cells that promote the development of the required Th immune responses (63). The classifications of ILCs include ILC1, ILC2, ILC3 and unconventional T cells. Notably, ILC1, ILC2 and ILC3 are the innate counterparts to, and produce cytokines that drive, Th1, Th2 and Th3 T cell responses respectively. (64) Within the LN, key ILC subsets include NK cells which are characterised as ILC1s, as well as NK-T cells and γδ T cells which are unconventional T cells. NK cells are predominately located within the follicular region and medulla, where they closely interact with medullary SMs and MCMs, however they can rapidly migrate throughout the LN compartments upon cytokine cues (65-67). NK-T cells however are most concentrated in the subcapsular sinus and predominately interact with and become activated by the SMs that reside here (67, 68), whilst naïve γδ T cells are situated within the paracortex and once stimulated, also localise to the cortical and medullary sinus where they interact with SMs (69). Notably, these ILCs are activated by cytokine cues from myeloid cells, particularly IL-18 from macrophages, IL-15 from monocytes and IL-12 from DCs. This leads to their production of cytokines,

primarily the type II IFN, IFN-γ, and this contributes to their cytotoxic responses for clearing infections and damaged cells, as well as the continuing interplay of downstream interactions between innate immune cells and lymphocytes (70-72).

1.2.1.2. Pathogen Recognition Receptors and Signally Pathways for Innate Immunity

Immune cells are equipped with a sophisticated system of pathogen recognition receptors (PRRs) to identify pathogen and danger associated molecular patterns (PAMPs and DAMPs). Signalling via these receptors leads to cellular activation and promotes an innate response that is tailored to the threat. Different types of PRRs include toll like receptors (TLR), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), including RIG-I and MDA-5, C-type lectin receptors (CLRs), stimulator of interferon genes (STING) protein, and absent in melanoma-2-like receptors (ALRs), each functioning via differential signalling pathways (Figure 1.4) (73, 74). Here, we will focus on reviewing TLRs and NLRs.

TLRs recognise various PAMPs depending on their location within the cell. In humans, TLR1, 2, 4, 5 and 6 are expressed as cell surface receptors that form heterodimers (TLR1/2 and TLR2/6) or homodimers and are responsible for recognising membrane components of pathogens such as lipids or proteins (74, 75). The binding of TLR4 to lipopolysaccharides (LPS) on gram-negative bacteria, with the support of the associated MD-2 protein on this receptor, is an example of the sensing capability that enables APCs to recognise foreign pathogens (76). TLR3, 7, 8 and 9 are however expressed in endosomes and recognise nucleic acid material from pathogens that intracellularly infect host cells. Notably, these receptors are differentially expressed on APCs, where monocyte and myeloid derived DCs express TLR1-8, while TLR7 and 9 are expressed in pDCs (74, 75). The signalling pathways initiated by TLRs post binding of ligands, can occur in a myeloid differentiation factor 88 (MyD88) dependent or independent manner (77). MyD88 dependent pathways initiated by TLR1, 2, 3, 5 and 6 leads to the activation of NF-κβ transcription factor in the nucleus and the production of proinflammatory cytokines IL-1β, IL-6, IL-12 and tumour necrosis factor (TNF) which promotes Th1 responses (78-80). TLR7, 8 and 9 also signal via MyD88 for activation of interferon regulatory factor (IRF) 7. In contrast TLR3 utilises a MyD88-independent pathway involving TIR domain-containing adapter-inducing IFNβ (TRIF) signalling for activation of IRF3. These IRFs are key transcription factors for antiviral immunity to produce type I IFNs which also drives a Th1 response in addition to cytotoxic T cell responses (73, 81, 82). Notably, TLR4 can uniquely activate via either

MyD88 dependent or independent signalling pathways for these downstream effector functions (83).

Like some TLRs, NLRs are intracellular PRRs, and they are specifically located within the cytosol of cells (84). They recognise bacterial PAMPs but also respond to cytosolic changes such as ATP and potassium (K+). Upon activation, NLRs trigger NF-κβ transcription factors to produce cytokines and additionally recruit proteins to form inflammasome complexes that allow for effective cytokine secretion. These processes are essential for IL-1β, IL-18 and IL-33 production, where the latter two cytokines promote Th2 responses (73, 85). Ultimately, APCs can effectively recognise and categorise foreign threats via their PRRs to further coordinate an appropriate adaptive T cell response. Vaccine technology has developed to incorporate additive components, such as TLR ligands, that can mimic these pathways for increased immune responses, and this will be discussed in more detail below.

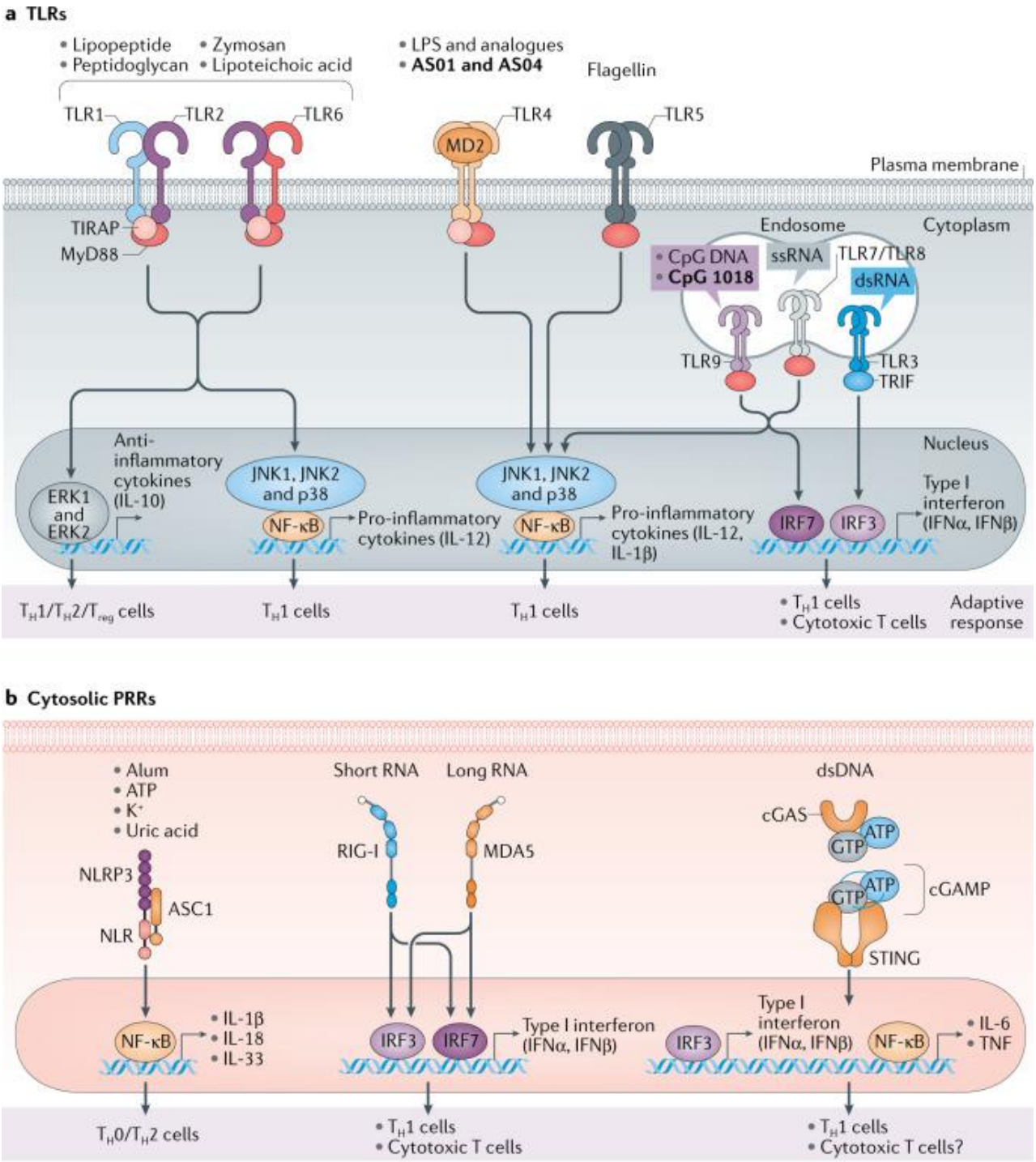


Figure 1.4: Pattern recognition receptors (PRR) for antigen recognition. Illustration of PRRs including (a) Toll-like receptors (TLRs) and (b) cytosolic receptors - nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5) and stimulator of interferon genes (STING). This figure was not prepared by the thesis author and is unaltered from its original publication (73).

1.2.2. Generation of Adaptive Immunity Within the Lymph Node

The purpose of vaccination is to successfully mimic natural immune responses that culminate in B cell effector antibody and effector T cell responses. Ultimately, it is the initial signalling patterns of

APCs that influences the differentiation and activation of particular T cell responses to a foreign antigen, which in turn promotes robust B cell responses (86). This occurs within the paracortex of LNs, where APCs present antigen on MHC to T cells, as well as directly interacting with T cells by binding to CD28 via co-stimulatory molecules CD80/86 which are upregulated on activated APCs (46). These different cell signalling pathways influence the activation of unique T cell responses which can broadly be characterised as CD8 cytotoxic T cells and CD4 Th cell responses (87).

T cells have varying functions that are necessary for generating successful effector immune responses. CD8 T cells are responsible for killing cells infected with intracellular pathogens such as malaria or viruses like herpes simplex virus (HSV) and human immunodeficiency virus (HIV), and these cytotoxic cells are necessary for the clearance of such pathogens (88-92). The activation of CD8 T cells requires direct antigen presentation from an infected DC or cross presentation of exogenously acquired pathogen- or vaccine-derived antigens on MHC class I, and this often occurs via cDC1s (93). These cytotoxic T cell responses are however difficult to elicit by subunit vaccines, due to the difficulty in promoting cross presentation, although there are continuing research efforts to develop better formulations that support this response (94).

In contrast, the primary function of CD4 Th cells is to support adaptive immune responses by activating B cells or CD8 T cells. There are several subsets of Th cells that are stimulated by direct presentation of pathogen or vaccine derived antigen on MHC class II by DCs, and the specific cell signalling pathways of activated DCs drives different Th cell phenotypes. These phenotypes include Th1, Th2, Th3, Th9, Th17, Th22, regulatory T cells (Tregs) and T follicular helper (TFH) cell responses, where each CD4 Th cell subset produces a different profile of cytokines to drive the immune response in a specific manner (95-98). At the interface of T:B cell zones, Th cells activate naïve B cells which go on to form germinal centres for clonal expansion and differentiation of B cells into effector cells, or plasma cells, that secrete antibody. Naïve B cells can also be directly activated by antigen presentation from APCs such as macrophages and FDCs. Once B cells are activated, they go on to initiate germinal centre formation in secondary lymphoid follicles. Germinal centres are split into dark and light zones. B cells undergo clonal expansion in the dark zone and migrate to the light zones, which is proximally located to the T cell zones. Here, with the aid of FDCs and CD4 TFH cells, somatic hypermutation and class switching occurs. This leads to the differentiation of B cells into high affinity, antigen-specific antibody secreting plasma cells, which neutralise infectious pathogen material, or memory B cells, which can quickly become activated into plasma cells if the host is re-

exposed to the same pathogen in the future (99, 100). These differentiated cells exit the germinal centre and migrate towards the medulla for dissemination out of the LN and into circulation.

1.3. Immunosenescence

Immunosenescence is the natural age-related decline of the immune system which begins to occur in individuals around 60-65 years of age (YOA) and has a detrimental effect on vaccine efficacy (101). The hallmarks of immunosenescence include elevated circulating pro-inflammatory cytokines, a reduction in the output of naïve T and B cells and changes to the LN structure (102).

Aging is known to affect innate immune functions however, while age-related differences in the quantity of APCs have not been identified, other features related to the diminished function of antigen presentation contributes to senescence (101, 103). Notably, the expression of TLRs on circulating macrophages, monocytes and DCs as well as signalling pathways decline with age, leading to reduced pro-inflammatory cytokine responses and suboptimal priming and activation of T cells (104-108). TLR4 however is documented to be well conserved within older individuals, where TLR4 ligands such as monophosphoryl lipid A (MPL) present as promising adjuvant candidates for vaccines targeted at the ageing (109, 110). The most prominent innate immune changes with aging however is the presence of pro-inflammatory cytokines which are known to chronically increase in older individuals, a process known as inflammaging (111), where reports of increased levels of circulating IL-1β, IL-18, IL-6, TNF and IFN-γ have been noted in adults >65 YOA compared to young adults <30 YOA (105, 112-115) although the cytokine patterns vary according to the study. These elevated cytokines can contribute to uncontrolled immune responses towards pathogens and vaccines (116).

Decline in adaptive immune features related to aging are also well documented. Notably, within older LNs, lower quantities of naïve CD4 and CD8 T cells have been reported, with increased proportions of memory cells (117) and these observations have also been detected in circulation (118). The reduction of naïve cells may contribute to the limited responses generated in older individuals towards new pathogens and vaccines. Additionally, structural changes to the architecture and organisation of cells within aging LNs contributes to these immune changes and affects overall function. Here, fibrosis of the tissue (119) as well as lipomatosis occurs, where surrounding adipose tissue begins to replace the LN tissue, originating from the hilum and extending into the medullary region (120). This leads to deficiencies in all LN cells and ultimately the reduction in size of LNs in older individuals. Moreover, a disorganisation of the FRC network and subsequently

the different LN zones has been reported, leading to impaired T and B cell activation and functions (102, 119). In combination with the disrupted FRC network, a loss of vasculature and HEVs was also reported, which led to further reduced motility and migration of naïve T cells throughout and into the LN (121, 122).

These changes in the immune system of older individuals are contributing factors to the suboptimal efficacy and immunogenicity of most vaccines in this population (123-126). An example is Pneumovax 23, which is a polysaccharide vaccine covering 23 different serotypes of pneumococcus, aiming to prevent invasive pneumococcal disease in young and old individuals. Here, overall vaccine efficacy decreases with age and is just ~50% for individuals >65 YOA (127). Newer developments of pneumococcal conjugate vaccines incorporating up to 13 variants are however explored as a preferred and cost-effective formulation for individuals >70 YOA, with up to 20-valent formulations incorporating more serotypes currently in clinical trials for future use. Similarly, while the MF59 adjuvanted Fluad Quadrivalent influenza vaccine, developed specifically for those >65 YOA, has a 25% increase in efficacy in preventing influenza related illness, hospitalisations and deaths compared to the unadjuvanted vaccine (128, 129), it still has a relatively low overall efficacy of ~50% in this age group (130). However, vaccine efficacy against influenza is highly variable and dependent on matching the vaccine to the specific circulating viral strain within a given season. New vaccine developments also aim to boost immune responses in older adults. Examples include the recombinant zoster vaccine (RZV), also known as Shingrix, for herpes zoster (shingles), that has a remarkable 91% efficacy in individuals above 70 YOA (131) which persists for more than 10 years (131-133). Additionally, the respiratory syncytial virus (RSV) vaccine Arexvy, is also successful at preventing related acute respiratory illness in adults >60 YOA with 72-88% efficacy (134, 135). Notably, the frail elderly, who are at greater risk of infection-related co-morbidities, respond especially poorly to vaccines (136). Frailty is a condition which is characterised by several factors (137) including declining general, physical, emotional and mental health (depression/anxiety), bodily pain, declining vitality and social function (138), as well as usual activities, mobility and self-care (139). Interestingly, while other vaccines such as Pneumovax 23 and Fluad Quadrivalent, are suboptimal at mounting a strong immune response in the frail elderly (140-143), RZV is proven to be safe and effective even in this high-risk subgroup (144). The reasons for the success of these vaccines in older individuals are however unknown. Ongoing developments to generate effective vaccine candidates for older adults are necessary, with vaccine adjuvants being the potential key for targeting specific immune pathways for overcoming immunosenescence (101).

1.4. Vaccines

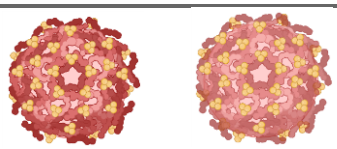
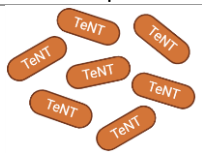
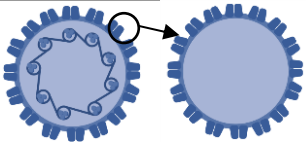
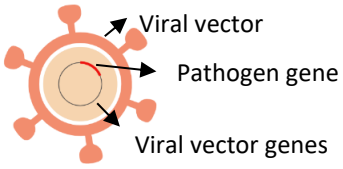
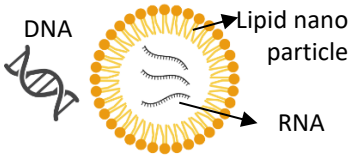
Vaccines are a preventative technology used to protect individuals against infectious diseases and related co-morbidities. Public health has greatly benefited from the introduction of vaccines, with the burden of pathogen related infections, diseases, hospitalisations, and economic costs greatly reduced (145-147). Although vaccines have had great success in the eradication of small pox and near eradication of polio (148), there are many current and emerging pathogens that continue to burden society with limited or no successful preventative methods or cures. The continuing development and improvement of vaccines are therefore necessary for such pathogens including HIV, HSV, cytomegalovirus (CMV), Epstein–Barr virus (EBV) and tuberculosis (TB). Additionally, new advancements are essential to provide better vaccine candidates for high-risk groups such as older adults. Improvements are also needed for current vaccines that do not provide a long period of protection or are highly reactogenic and cause side effects that prevent daily function, including soreness at the vaccination site, painful swollen LNs and fatigue.

1.4.1. Types of Vaccines and their Functions

Vaccines utilise biological products to generate pathogen-specific immunological memory that protects against future infection and is devoid of the risks associated with natural infection. They employ whole organisms or one or more protein antigens that are derived naturally from pathogens or produced synthetically, or nucleic acid sequences from the pathogen. These antigens trigger an immune response in a safe manner to generate protection (149).

Live attenuated vaccines, such as the measles mumps rubella (MMR) and oral polio (OPV) vaccines, include the live pathogen which has undergone virulence attenuation (Table 1.2) (150). Although these vaccines mimic natural infection and hence produce the most effective immune response, it is important to note that there is a risk of the virus reverting to its virulent form to cause mild infection (151). This has been reported to occur with the MMR vaccine where 5-15% of recipients develop symptoms of infection and 2% develop a rash (152). The OPV is also reported to cause paralysis on rare occasions due to genetic alterations of the vaccine-virus that develops into vaccine derived poliovirus disease (153, 154). These instances of vaccine virus reverting to virulence can be disastrous for immune compromised individuals and so restrictions are in place for those with certain immunodeficiencies, HIV infection, or using immunomodulating drugs (155). For this reason, live attenuated viruses are also not suitable for the development of vaccines against lethal and untreatable viruses, including HIV and Ebola, where the risk of reversion to virulence is intolerable (Table 1.2).

Table 1.2: Types of vaccines and their licensed use.

Type of vaccine	Example	Use in current licensed vaccines	First vaccine developed
Live attenuated (inactivated or weakened)		Measles mumps rubella, influenza, yellow fever, oral polio, BCG, rotavirus, Japanese encephalitis, varicella zoster	Smallpox (1798)
Killed whole organism		Pertussis (whole cell), polio, rabies, Japanese encephalitis, hepatitis A	Typhoid (1896)
Toxoid		Tetanus, Diphtheria	Diphtheria (1923)
Subunit (split-purified/ recombinant protein, polysaccharide, peptide)		Influenza, meningococcal, pertussis, hepatitis B, pneumococcal, hepatitis A, herpes zoster, typhoid, respiratory syncytial virus	Anthrax (1970)
Virus like particle (VLP)		Human papillomavirus (HPV)	Hepatitis B (1986)
Viral vectored		Ebola, SARS-CoV-2	Ebola (2019)
Nucleic acid vaccine		SARS-CoV-2	SARS-COV-2 (2020)

[a] This table is inspired from Pollard et al (149).

In comparison to living vaccines, non-living vaccines are a safer option with several current and ongoing developments that provide effective protection against many pathogens. Here, the antigenic component can be present in various forms. Killed whole organisms can be used, such as the three-dose Inactivated Polio Vaccine (IPV) which has replaced the OPV as an effective and safer option (154, 156). Individual antigenic components can also be used such as toxoid vaccines, including the tetanus vaccine, which utilise purified and inactivated pathogenic toxins (157, 158). Subunit vaccines employ pathogen components such as one or more purified proteins, recombinant

proteins or polysaccharides. Examples of purified/split vaccines are the pertussis acellular vaccine and influenza vaccines, and recombinant protein vaccines include RZV and Hepatitis B as well as pneumococcal vaccines which may either be polysaccharide alone or conjugates with protein (130, 149, 159). The development of virus like particles (VLP) has extended the subunit vaccine technology, where viral or synthetic proteins self-assemble into particles that mimic a natural virus particle which can enhance immunogenicity compared to the single soluble protein (Table 1.2) (160). This technology is utilised in the human papillomavirus vaccines and continues to be explored in pre-clinical settings against other pathogens including SARS-COV-2 and influenza (161-164). Another similar technology is the use of pseudotyped viruses, where a replicating or replication deficient non-pathogenic virus is used as a vector to deliver a protein of the target pathogen, with the added ability of generating a natural antiviral immune response (148). This technology was used to develop a recently licensed Ebola vaccine, Erbivo. Here, an attenuated vesicular stomatitis virus (VSV), pseudotyped with the Ebola envelope protein on its surface, is used to deliver the viral subunit protein in a manner that mimics a virus particle (165). Overall, non-living vaccines are safer and often more effective alternatives to living vaccines (Table 1.2).

Non-living vaccine technologies have also made it possible to tailor vaccines specifically to high-risk groups such as older individuals, where there are now multiple, more effective, vaccine options recommended for adults >65 YOA. Particularly, immunostimulatory adjuvant components have been utilised to enhance the immunogenicity of subunit vaccines. Examples include the MF59 adjuvanted Fluad Quadrivalent (Seqirus, subunit), the recombinant zoster vaccine (Shingrix, GSK) and RSV vaccine (Arexvy, GSK) as described in section 1.3. These new innovative technologies however require further elaboration and a better understanding of the mechanisms that lead to their success within humans, to continue to apply them effectively in future vaccine developments.

Nevertheless, vaccine developments have excelled greatly, with the advancement of many new technologies that allow for targeting of appropriate immune cells and the generation of broader immune responses to combat the issues associated with genetic variability in viral strains. Nucleic acid vaccines are a relatively novel development, where DNA or mRNA encoding pathogen antigens can be delivered in recombinant viral vectors or lipid nano-particles (LNPs) respectively. DNA vaccines are often given in a heterologous two-dose regimen, such as naked DNA followed by the recombinant protein or DNA given in two different viral vectors, to boost immunity. For example, the recently licensed Ebola virus DNA vaccine Zabdeno/Mvabea, utilises a recombinant adenovirus 26 vector containing DNA encoding the Ebola virus surface glycoprotein followed by a modified

vaccinia virus Ankara vector, which contains DNA encoding multiple filovirus glycoproteins (166). Although both current Ebola vaccines are safe and effective, the Ervebo protein subunit vaccine has been shown to be the best option, displaying 97.5% efficacy from preliminary clinical studies (165, 167), where great responses are induced in children and adults, as seen in more recent clinical trials (168). Stimulated by the recent COVID-19 pandemic, this viral vector technology continues to be explored for the development of more effective and cheaper vaccines against SARS-CoV-2, hopefully building on the chimpanzee adenovirus vectored vaccine ChAdOx1-nCov-19 (Oxford/Astrazeneca), with many in clinical trials (169-171). Nevertheless, novel developments of effective nucleic acid vaccines against SARS-CoV-2 have emerged during this pandemic, which utilise mRNA encoding the virus' spike protein as the antigenic component, enclosed by LNPs for protection and transport. Here, Pfizer's BNT162b2 and Moderna's mRNA-1273 vaccines are noted as the first ever licensed mRNA based vaccines (172). Although these nucleic acid vaccines have been proven to be highly effective, inducing strong immune responses against many SARS-CoV-2 variants, improvements are required to induce long-lasting immunity within humans (Table 1.2) (172, 173). One interesting development is the self-amplifying RNA vaccines, allowing smaller RNA doses and longer lasting persistence of the immunogen in animal models (174).

Unlike live attenuated or inactivated whole virus vaccines, the individual antigen components in most non-living vaccines, particularly recombinant subunit vaccines, are not inherently immunogenic as they lack PAMPs. These PAMPs are recognised by host cells via PRRs such as TLRs or NLRs, and hence PAMPs are usually responsible for initiating an effective vaccine immune response (175). Therefore, in addition to the antigenic component, these vaccines also require immunostimulatory additives known as adjuvants, which often mimic PAMPs to successfully initiate the immune response by stimulating APCs. Adjuvanted subunit vaccines are therefore not only extremely safe due to their inability to create infectious viral particles, but also highly immunogenic and their success to date is prominent (176). There are however very few adjuvants regularly used in licensed vaccines; however numerous additional formulations are in development and continue to be explored, many of which elicit unique immune profiles. A better understanding of the mechanisms of adjuvant action in humans, of both developing and licensed adjuvants, is still required to allow future vaccine development in a manner where adjuvants can be selected based on their known immune responses profile, for tailoring to specific pathogens or even high-risk individuals.

1.4.2. Types of Adjuvants and their Modes of Action

An adjuvant is an agent which enhances the magnitude, breadth and duration of immunity. Adjuvants can be generally categorised into carrier adjuvants, which function as delivery systems for the vaccine antigen, or immunostimulatory adjuvants, which are specific PAMP-like molecules that trigger immune pathways. However, carrier adjuvants are also recognised to have immunostimulatory capacity as well. A variety of materials can function and be employed as adjuvants including but not limited to, synthetic nanoparticles and molecules, bacterial products and natural organic extracts (177). Although adjuvants have been utilised in vaccines for almost 100 years, the specific modes of action for many adjuvant types are still not completely characterised, especially within humans (Table 1.3).

1.4.2.1. Carrier Adjuvants

The first successfully licensed adjuvant was aluminium hydroxide salts (alum), a carrier adjuvant technology that was exclusively used for decades despite the mechanisms being unknown at the time (26, 73, 178). Recent studies have however revealed that alum does not only adsorb antigens to function as a depot for slow release and delivery to APCs (179), but also specifically activates the immune system via the NLRP3 inflammasome pathway within macrophages, which is a multiprotein complex responsible for IL-18 and IL-1β cytokine production (180). This, combined with the downstream inflammatory responses involving activation of DCs, induces DAMPs for additional APC activation (181-184). Despite alum being the oldest adjuvant, its effectiveness and our recent understandings continue to support its use in many current vaccines such as HPV and hepatitis B (185), as well as developing vaccines for SARS-CoV-2 (186-188).

In addition to alum, oil in water emulsions are also potent delivery systems, such as MF59, which was the next licensed adjuvant following Alum and is commonly used in influenza vaccines (189). MF59 is more potent than alum (190) but has some functional similarities, where NLRP3-independent but MyD88-dependent inflammasome activation occurs (191-193), leading to an inflammatory response governed by cell death and ultimately the generation of a Th1 and Th2 adaptive response (189). It continues to be utilised in the development of new vaccines, such as a CMV and HIV candidate vaccines (194-196).

Recent developments in the generation of SARS-CoV-2 vaccines have also enhanced the progress of liposome and LNP carrier systems that efficiently deliver vaccine to cells of interest, but also have immunostimulatory properties (Table 1.3) (197). They also function as a protective barrier for mRNA

which would normally be degraded by exonucleases (159, 198). LNPs enhance adaptive immune responses to SARS-CoV-2 mRNA vaccines and in pre-clinical settings, influenza subunit vaccines, especially when compared to MF59-like adjuvanted influenza vaccines (199). Being relatively new, LNP mechanisms are not fully understood, however mouse model studies have identified that LNPs alone exert immunostimulatory, adjuvant like functions, which are dependent on IL-6 production (199, 200). Further studies are however required in humans to specifically identify the immune cells targeted by LNPs and the precise immune responses triggered, allowing for their further use in vaccine development against more challenging pathogens.

1.4.2.2. Immunostimulatory Adjuvants

Immunostimulatory adjuvants have been broadly investigated, and some examples include TLR agonists such as Pam2Cys or Pam3Cys, polyinosinic:polycytidylic acid (Poly(I:C)), monophosphoryl lipid A (MPL), flagellin, imiquimods, Resiquimod (R848) and unmethylated cytosine–guanine dinucleotides (CpGs) (Table 1.3). Most of these TLR ligand (TLR-L) adjuvants are however still in experimental development or pre-clinical trials, with MPL and CpG being the only licensed TLR-L adjuvants approved for use in vaccines (185, 201). MPL, a detoxified version of LPS, is included in HPV, hepatitis B, RSV and herpes zoster vaccines and binds to TLR4 on the surface of APCs, a receptor whose expression is well conserved in older individuals, making this TLR an ideal adjuvant target (109). Once bound, MPL activates APCs via NF-κβ signalling to induce a pro-inflammatory cytokine response involving IL-6 and TNF-α, leading to IFN-γ production by CD4 T cells which specifically polarises to a Th1 cellular response (201-204). To date, MPL continues to be studied and structurally adjusted to better apply this adjuvant to potential future vaccines (202, 203). In 2017, CpG 1018 became the second TLR-L approved for use as an adjuvant, in a hepatitis B vaccine (201, 205, 206), providing the option for an effective two-dose vaccine regime compared to the standard three-dose hepatitis B vaccines already available (207). This vaccine generates increased antibody titres and is more effective in older adults compared to the previously approved hepatitis B vaccines utilising alum (208). The CpG 1018 adjuvant used here is one of three classes of CpG ligands which all bind to TLR9 in pDCs and B cells in humans, and many additional TLR9 expressing cells in mice such as macrophages. Many studies have been completed in mouse models to identify the different mechanisms involved for each CpG class and most function by activating pDCs to produce IFN-α which stimulates cross functional CD8 T cell responses (209). Some CpGs also directly bind and activate B cells and overall, the T cell response polarises more towards a Th1 function (73). CpG adjuvants continue to be improved and clinically assessed for their use in SARS-CoV-2, influenza,

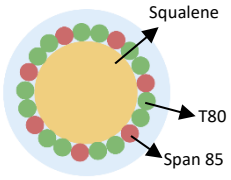
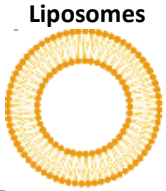
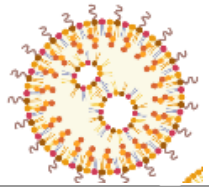
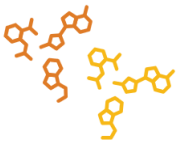
and malaria vaccines (210-213). Although CpG is now a licensed adjuvant, further studies are required to uncover the specific mechanisms of this adjuvant in humans, especially given the clear differences in TLR9 expression compared to mice, and to further identify the factors contributing to its success within older individuals.

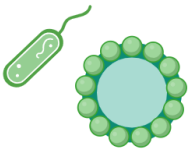
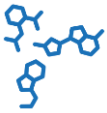
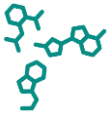
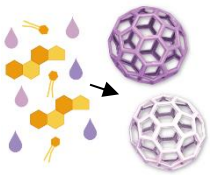
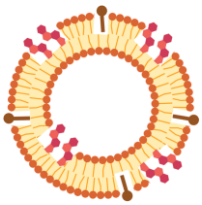
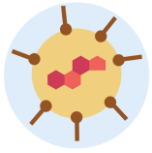
Other developing TLR-L immunostimulatory adjuvants continue to be explored, with some on the cusp of clinical breakthrough, particularly R848, a modified derivative of imiquimod which is licensed for use as a topical antiviral treatment against genital warts caused by HPV. In comparison to imiquimod however, R848 functions as an even more potent stimulator of TLR7 and additionally binds TLR8, but its potency is also accompanied with high levels of toxicity (214). This TLR-L adjuvant is nevertheless assessed in preclinical animal models for use in influenza vaccines and is a potent immunostimulant that induces multiple DCs to generate a strong pro-inflammatory cytokine response that drives a Th1 response (215-218). Toxicity issues are however evident, with many research groups and manufacturers exploring options such as molecular modification, binding to existing adjuvants such as Alum, and packaging within liposomes and nanoparticles to reduce the highly potent effects and increase safety (197, 214, 217, 219). Further research into the understanding and safety developments of R848, whilst maintaining immunogenicity, could allow the use of this potent stimulant as a successful adjuvant in future vaccine developments.

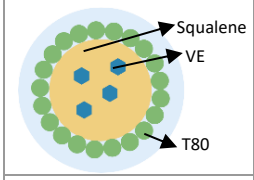
Other materials explored as immunostimulatory adjuvants are saponins, which are naturally derived glycosides from the soap bark tree *Quillaja Saponaria*. Examples of saponin adjuvants are QS-21 (*Quillaja saponaria* Molina: fraction 21) and Matrix M (composed of fractions A and C) which have been explored for vaccine development, with QS-21 licensed for use in herpes zoster, malaria, and RSV vaccines as part of an adjuvant system. Matrix M remains in phase I and II clinical trials for malaria and is a component of the Novavax SARS-CoV-2 vaccine, as well as being in pre-clinical development of Ebola and influenza vaccines (220-223). The mechanisms of these adjuvants have been studied in mouse models and reported to activate APCs inducing a pro-inflammatory response that polarises strong Th1 functions for increased production of downstream antibodies against the target antigen (73, 224-227). In some but not other studies, it has also been identified that lysosomes induce inflammasome activation, functioning as the potential initial mechanism contributing to the development of downstream QS-21 and Matrix M immune functions, although other studies have proposed this pathway to be redundant and additional mechanisms are also involved (228, 229). In isolated human monocytes cholesterol mediated uptake and concentration in lysosomes followed by downstream cytokine production has also been demonstrated. These

mechanisms have been incompletely studied in humans with none at the site of action in LNs. Additional studies are required to understand these adjuvants further for future improvement and development. Particularly, with the known reactogenic effects of QS-21 and the recent ability to chemically synthesise this adjuvant, different chemical congeners are currently being produced and investigated for reduced toxicity and enhanced immunogenicity (230).

Table 1.3: Types of adjuvants and their use in current approved and developing vaccines.

Adjuvant	Composition	Manufacturer	Receptors/Pathway Activated	Vaccine Examples/Status	Ref
Carrier Adjuvants					
Alum 	Aluminium salts	Many	- NLRP3 inflammasome in macrophages and DCs - Th1 and Th2	- Many licensed non-living vaccines - Inactivated SARS-CoV-2: clinical trials, emergency approved	(73, 185, 186)
MF59 	Oil in water emulsion – squalene, tween 80, Span 85	Seqirus	- Cell death inflammatory signalling in DCs - TLR-independent MyD88 activation - NLRP3-independent ASC activation - Th1 and Th2 response	- Fluad (influenza): licensed - HIV vaccine candidates: preclinical	(191-195, 231)
Liposomes 	Lipid bilayer	GSK Crucell	- Activation of APCs - Viral antigen delivery (virosomes) - Other mechanisms not fully identified	- Licensed vaccines containing AS01 (see below) - Inflexal-V (Influenza) - Epaxal (Hepatitis A)	(232)
Lipid nanoparticles (LNP) 	Single outer phospholipid layer	Pfizer Moderna	- Delivery of antigen to and activation of APCs - Other mechanisms not fully identified	- mRNA/LNP SARS-CoV-2: licensed	(197-200)
Immunostimulatory Adjuvants					
Pam2Cys and Pam3Cys 	Synthetic lipoamino acid	No clinical grade available	- TLR2/6 (Pam2Cys) and TLR2/1 (Pam3Cys) - Th1, Th2, CTL responses	- Tuberculous candidate: experimental - SARS-CoV-2 candidate: experimental	(233-238)
Poly-IC 	Synthetic double stranded RNA	No clinical grade available	- TLR3 - Th1 and Th2 responses	- Experimental	(189, 238-241)
MPL 	Modified form of LPS derived from *Salmonella minnesota*	GSK	- TLR4 activation of DCs - Th1 response	- Licensed vaccines containing AS01 (see below)	(109, 185, 201-204)

Flagellin 	Globular protein derived from bacterial flagellum	No clinical grade available	- TLR5 - Th1 and Th2 responses	- Experimental	(242-246)
Imiquimods 	Synthetic compound	Medics Pharmaceutical and generically available	- TLR7 - Th1, CD8 T cell, CTL responses	- Experimental	(214)
Resiquimod 	Synthetic compound	Galderma	- TLR7 (pDCs/B cells) and TLR8 (APCs) - Th1, CD8 T cell, CTL responses	- Influenza vaccines – pre-clinical development	(197, 214, 216-219)
CpG 	Synthetic single-stranded DNA oligonucleotides with unmethylated cytosine-guanine (CpG) motifs	Dynavax	- TLR9 (on pDCs and B cells in humans) - IFN-α pDC response - Th1 and CTL responses - CD8 T cells by CpG-Ag	- Heplisav-B (hepatitis B) – licensed - CoVLP + CpG1018 (SARS-COV-2) – clinical trials	(205-207, 209-212)
ISCOM/Matrix M 	Saponin from soap bark tree *Quillaja Saponaria*. Fraction A/C combined with cholesterol and phospholipids	Novavax	- Lysosomal dependent inflammasome activation on APCs - Th1, Th2, CD8 T cell responses	- Nuvaxovid (SARS-CoV-2) – licensed - R21/Matrix-M (malaria) – clinical trials	(220, 221, 225, 247)
QS-21 	Saponin from soap bark tree *Quillaja Saponaria* combined with cholesterol	GSK Croda Pharma	- Activates NLRP3 inflammasome and caspase 1 in mice - Th1 responses	- Licensed vaccines containing AS01 (see below)	(18, 248, 249)
Adjuvant systems (combination of adjuvants)					
AS01 	MPL and QS-21 formulated in liposomes	GSK[a]	- TLR4 (MPL) - QS-21 activates NLRP3 inflammasome and caspase 1 in mice - Poly-functional CD4 T cell response (IFN-γ, IL-2, TNF)	- Shingrix (herpes zoster): licensed - Mosquirix RTS,S/AS01 (malaria): licensed - Arexvy (respiratory syncytial virus): licensed - M72 (tuberculosis): clinical trials	(131, 132, 250-252)
AS02 	MPL and QS-21 in oil in water emulsion	GSK	- TLR4 (MPL) - Th1 response	- Respiratory syncytial virus candidate: discontinued - Pneumococcal vaccine: clinical trials - RTS,S/AS02 (malaria): discontinued	(253-255)

				- HIV and TB vaccines: discontinued	
AS03 	Oil in water emulsion – vitamin E (immune-enhancer), tween 80	GSK	- Cell death inflammatory signalling in DCs - Th1 and Th2 response	- Arepanrix and Pandemrix (influenza): licensed	(256-259)
AS04 	Alum and MPL	GSK	- TLR4 - Inflammasome **(?)** - Th1 response	- Cervarix (HPV): licensed - Fendrix (hepatitis B): licensed - HSV/AS04 (herpes simplex): discontinued	(260-263)

[a] QS-21 (Quillaja saponaria Molina, fraction 21) (Licensed by GSK from Antigenics LLC, a wholly owned subsidiary of Agenus Inc., a Delaware, USA corporation)

1.4.2.3. Adjuvant Systems (AS)

Overall, distinctive immune responses are now known to be generated by different types of adjuvants. Throughout vaccine development the notion of combining carrier and immunostimulatory adjuvants together has hence been explored to access the unique immune profiles of different adjuvants and generate new, improved responses as well. This is especially helpful against problematic pathogens such as malaria and HIV where standard adjuvants do not suffice. GSK has embraced this approach to develop adjuvant systems (AS), which have had great success in the advancement of new vaccines against pathogens where no vaccine prevented measures were previously available (159, 264), and this has paved the way for many research groups and manufacturers to also innovate using this concept. Some successful combinations include AS01 which is composed of QS-21 and MPL formulated in liposomes, AS02 which again utilises MPL and QS-21 however formulated in an oil-in-water emulsion, AS03 which employs the immunostimulant alpha-tocopherol (Vitamin E) in an oil-in-water emulsion, and finally AS04 where MPL is absorbed onto Alum (73, 159).

AS01 is used at different doses, where $AS01_B$ contains 50 µg MPL and 50 µg QS-21 while $AS01_E$ contains 25 µg MPL and 25 µg QS-21. AS01 is licensed in the RTS,S/$AS01_E$ paediatric malaria vaccine, Mosquirix (250, 251). $AS01_E$ is additionally included in the trial M72 TB vaccine (252) and licensed in the RSV vaccine Arexvy, while $AS01_B$ is licensed for use in the RZV, Shingrix, for herpes zoster shingles infection, the latter two both being highly effective in older individuals >60 YOA. Notably, Shingrix effectively generates long-lasting protective immunity for a minimum of 10 years against herpes zoster infection, even in patients above 80 YOA (131-133, 135, 265). This is notable as

immunosenescence in older individuals >60 YOA usually prevents the ability of vaccines to generate effective immunity. An $AS01_B$ adjuvanted RSV vaccine was also produced and although clinical trials revealed this vaccine to be more immunogenic than an $AS01_E$ formulated version, in individuals >50 YOA, the latter produced less local and general symptoms and hence was favoured for RZV (266). In comparison, AS02 has been relatively unsuccessful with inadequate immunogenicity in phase I and II clinical trials for malaria, TB and HIV vaccines which are now discontinued, as AS01 adjuvanted vaccines are favoured (255). Additionally, although AS02 adjuvanted vaccines appeared promising in pre-clinical trials as a RSV vaccine (253), AS01 was again more effective in clinical settings. AS02 was also trialled in a pneumococcal vaccine (267-269) but it is not currently in clinical development. AS03 on the other hand has also been successfully licensed for use within the Influenza vaccines Arepanrix and Pandemrix to prevent Influenza H5N1 and H1N1 (256, 257). This adjuvant functions similarly to MF59 however, generates greater immunogenicity which may be attributed to the addition of Vitamin E, although the mechanisms involved are unknown. Despite AS03 being more reactogenic than MF59 adjuvanted influenza vaccines, the immune benefits outweigh the risk of acquiring these mild local and fatigue symptoms experienced by individuals (258, 259), although future studies to improve reactogenicity without altering immunogenicity would be ideal. AS04 is also successfully licensed for use within the Cervarix vaccine against HPV as well as the Fendrix vaccine against hepatitis B (260, 261). A vaccine against HSV incorporating AS04 was also developed which was partially protective in clinical trials, however this was ultimately discontinued due to insufficient efficacy (262, 263). The contrast between these adjuvant systems within vaccines highlights the critical impact adjuvants can have on vaccine efficacy and the importance of selecting the right adjuvant for new vaccines against specific pathogens. Understanding their mechanisms of action is crucial to continue the improvement of vaccine candidates.

1.5. Adjuvant System 01

1.5.1. Potential Application of AS01 in Future Vaccine Development

The success of the Arexvy and Shingrix/RZV vaccines have been attributed to AS01, which is notably one of the most potent adjuvants available, especially with its ability to generate strong, long-lasting immunity in older individuals. This immunity is reliant on both strong T cell responses, particularly IFN-γ producing CD4 T cells, and the generation of a potent antibody response (270). RZV has been of particular interest in this context, with several clinical and animal model studies suggesting AS01 as the primary contributor to vaccine efficacy in adults >60 YOA (18, 131, 132). In addition to AS01, the RZV, contains the single recombinant glycoprotein E (gE) from varicella zoster virus (VZV). With

VZV being prone to reactivation in older individuals to induce herpes zoster (commonly known as shingles) infection, the ability of the RZV to generate a strong immune response in this age group with 91% efficacy even in adults >70 YOA, is a remarkable achievement in vaccine development. RZV clinical trials revealed that although AS01/gE is a weak inducer of CD8 T cell responses (271), all participants displayed enhanced CD4 T cell responses one month following vaccination, and this was maintained with minimal plateauing in the 10 years following (131-133, 272). With the additional success of AS01 in the Mosquirix RTS,S/AS01 vaccine, which was predominantly assessed in clinical trials for the prevention of malaria acquisition in children, it is clear that AS01 has the capacity to function as a successful adjuvant in both young and old participants, overcoming the issue of immune senescence. However, the specific mechanisms contributing to the success of AS01 within these vaccines, particularly for infants and older adults, are poorly characterised within humans and this knowledge is needed to effectively utilise this adjuvant in future vaccines targeted at older age groups (262, 263).

1.5.2. Identifying the Immune Mechanisms of AS01

1.5.2.1. AS01 Mode of Action

Several animal studies in mice and non-human primates have been conducted to generate better insights into the mechanisms of action of AS01. Preliminary mouse model studies from pre-clinical assessments of malaria and herpes zoster AS01-adjuvanted vaccines, revealed that adaptive humoral and cellular immune responses were enhanced with AS01 compared to when antigens were administered alone or when other adjuvant systems such as AS02 were utilised instead (87, 273, 274). Further mouse and non-human primate (NHP) studies, discussed below, have attempted to dissect the mode of action of AS01 to identify reasons for its superiority. This knowledge can pave the way for the future application of this adjuvant to other vaccine formulations, as well as provide preliminary information into the potential AS01 mechanisms within humans.

Following intra-muscular administration of AS01/gE in mice, AS01 induces a pro-inflammatory response within 30 minutes, where the recruitment of neutrophils and monocytes as well as the activation of resident muscle cells for increased production of cytokines occurs, compared to when the antigen gE is administered alone. AS01 drains rapidly to the LN via the afferent lymph with 30 minutes of intramuscular administration in mice, facilitated by its formulation in liposomes which enhances lymph node targeting. It is further rapidly trafficked to the draining LN within 1-2 hours by migrating DCs (275). A key mechanism of AS01 is the activation of APCs. Notably, the MPL

component of AS01 facilitates activation of DCs from the muscle including monocyte-derived DCs, and their migration to the LN, where the accumulation of many APCs is evident by 24 hours post vaccination (18, 275). Additionally, an increased concentration of cytokines and chemokines related to the IFN-pathway are produced at both the vaccination site and the draining LNs when compared to the antigen alone controls (232, 249). Recent studies have revealed that chemokines fms-like tyrosine kinase 3 (Flt3) and CCR2 play a key role in the recruitment of APCs including cDC1s, cDC2s and notably inflammatory cDC2s, from the muscle to the LN in mice, and in their absence, these cells fail to migrate leading to impaired AS01-induced antibody and T cell responses in the LN (276). With the LN being the primary site for the generation of immunity, following the initial innate immune responses triggered at the site of immunisation, AS01 mechanisms have been further explored here.

Notably, AS01 in mouse LNs initiates a cascade of innate immune events that generates a synergistic response when compared to MPL or QS-21 alone, and this culminates in the early production of IFN-γ which is thought to be crucial for AS01 immune functions. Utilising knock-out mouse models, this synergistic response is noted to be reliant on subcapsular SMs, which are the initial cells that AS01 encounters in the subcapsular sinus upon draining to the LN (249). Following uptake of AS01 by subcapsular SMs, IL-18 is produced which initiates the pro-inflammatory immune cascade. IL-18 from subcapsular SMs, in combination with IL-12 cytokine production by activated migratory and resident DCs, induces the production of IFN-γ which occurs in a synergistic fashion compared to MPL and QS-21 alone, with NK cells and innate CD8 T cells being the highest IFN-γ producers (18). Mature, antigen presenting DCs, in combination with the cytokine cascade induced, leads to the activation of naïve T cells to memory and effector CD4 T cells (232). A recent study in PBMCs derived from RZV vaccinated individuals also identified the vaccine's ability to recruit new naïve CD4 T cells upon gE antigen challenge, in addition to activating pre-existing T cells present, and this correlates with persistent immunity (277). The generation of this Th1 effector response involving polyfunctional CD4+ T cells is driven by IFN-γ (278-280), with the more multifunctional cells (IFN-γ+IL-2+TNF+) producing the highest levels of cytokines (18). These AS01-induced cytokines allow for the recruitment of more migratory DCs into the LN, including MDDCs and pDCs from the blood, for the continuation of the immune cascade. These cytokines further stimulate Tfh cells and B cells in LN follicles for germinal centre formation and antigen-specific antibody production (Figure 1.5) (18, 87). Although these studies have greatly expanded our knowledge of AS01 mechanisms, it is crucial to

investigate whether similar modes of action occur within human LNs to allow for more insightful application of AS01 in future vaccine developments.

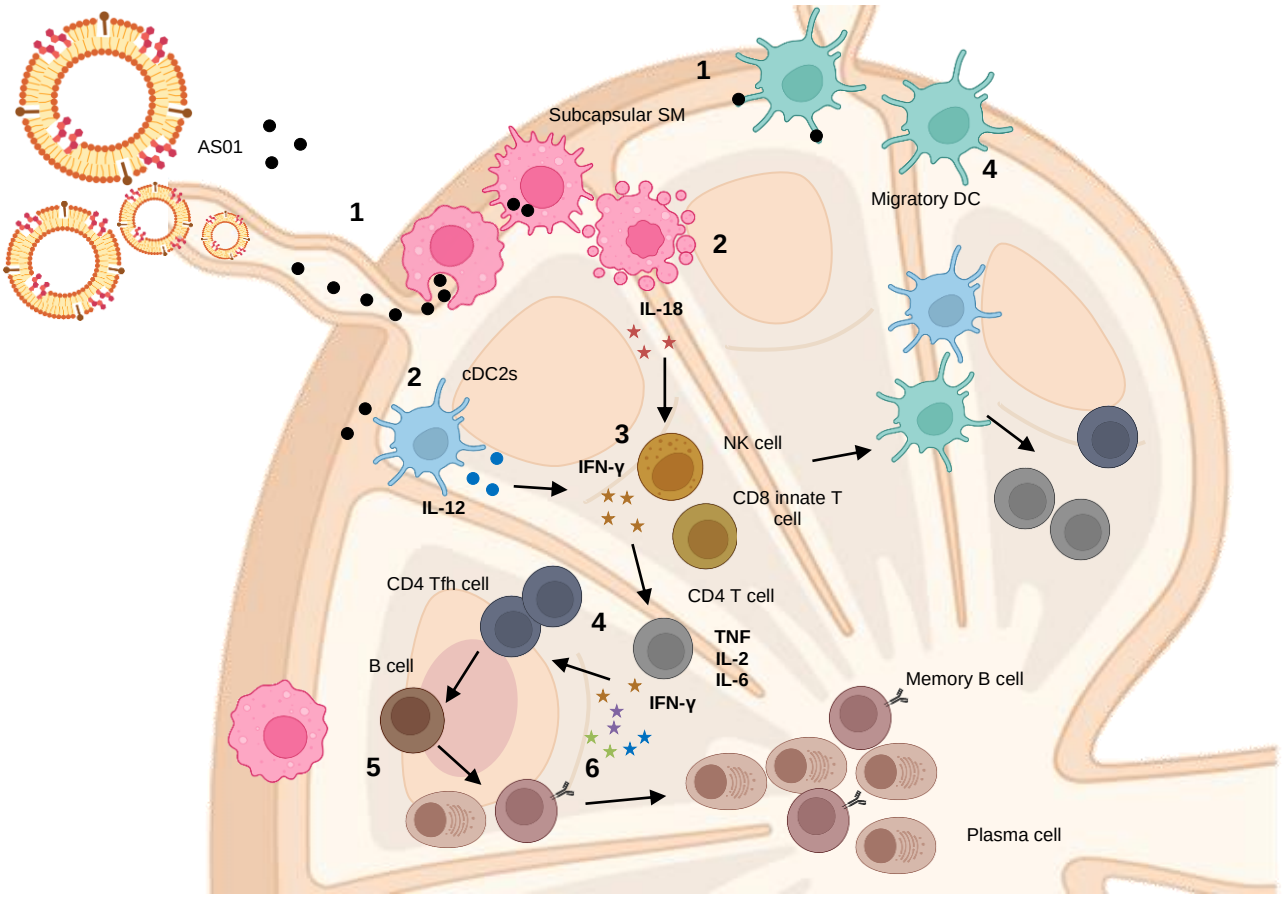


Figure 1.5: AS01 mechanisms of action in mice. AS01 immune cascade in mouse LNs. (1) AS01 drains into the LN via afferent lymphatic vessel and is taken up by subcapsular SMs. (2) Subcapsular SMs are activated and pyroptose, leading to the release of IL-18. AS01 activated cDC2s secrete IL-12. (3) NK and CD8 innate T cells are activated in an IL-18/IL-12 dependent manner to produce IFN-γ. (4) IFN-γ activates CD4 T cells to produce additional cytokines and differentiate into Th cells. The initial innate cytokine cascade leads to additional recruitment of migratory DCs to further promote the CD4 Th cell response. (5) CD4 Th cells support B cells to differentiate antibody secreting plasma cells or memory B cells that disseminate from the LN into circulation for effector function. SM = sinus-lining macrophage. DC = dendritic cell. cDC = conventional dendritic cell. NK = natural killer. Tfh = T follicular helper cell. This image was created using BioRender.com.

1.5.2.2. Potential Molecular Pathways in AS01 Mechanisms

Based on mouse studies, the activation of the inflammasome within macrophages in LNs, particularly subcapsular SMs, has been proposed as the potential key molecular pathway that triggers the AS01 pro-inflammatory immune cascade and IFN-γ production that results in enhanced adaptive immunity (228, 249). Inflammasomes are multi-protein, cytosolic complexes within innate immune cells that play a key role in the initiation of innate immune functions, by recognising foreign

PAMPs and host DAMPs via PRRs (281, 282). There are different types of inflammasomes, each having different sensors for the activation of their immune functions and can be characterised as canonical or non-canonical.

In canonical pathways, NLRs like the NOD-like receptor (NLR) family, pyrin domain-containing protein 3 (NLRP3), are activated by potassium (K^+) efflux caused by PAMPs or DAMPs binding to APCs (283). This triggers the aggregation of a range of inflammasome related proteins to form a complex. These include apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), as well as caspase-1 enzyme which becomes cleaved to its active form by ASC (284). Caspase-1 then cleaves pro-IL-β and pro-IL-18 to their active form, as well as gasdermin D (GSDMD) (281). GSDMD forms pores in the cell membrane which allows the release of IL-1β, IL-18 and other DAMPs, and initiates programmed cell death known as pyroptosis (Figure 1.6) (285). Non-canonical inflammasomes on the other hand do not utilise a protein complex, and instead the unique caspase enzymes, caspase-4/5 (caspase-11 in mice), act as both sensor and effector proteins for the activation of the pathway (282). Caspase 4/5 is activated by LPS and cleaves IL-18 and GSDMD to their active form. This non-canonical pathway can also indirectly activate the NLRP3 inflammasome via the K^+ efflux induced through the GSDMD pores (286).

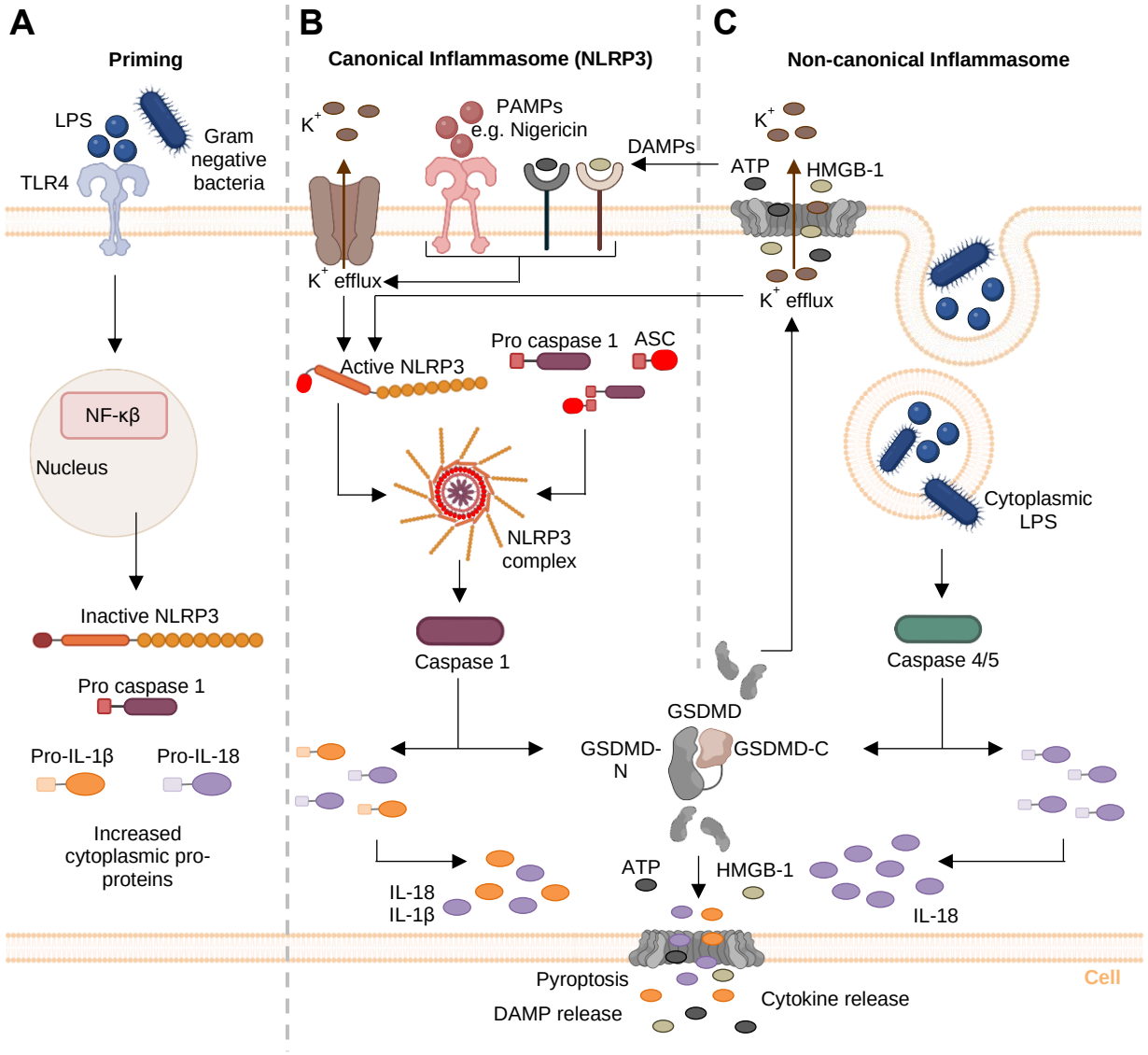


Figure 1.6: NLRP3 inflammasome activation in subcapsular sinus lining macrophages. Illustration of inflammasome activation pathways. (A) LPS and gram negative bacteria are recognised via TLR4 on APCs, and can prime the inflammasomes pathways by inducing NF-κβ signalling for the production of inflammasome related proteins NLRP3, pro-caspase 1, pro-IL-18 and pro-IL-1β. (B) The canonical NLRP3 inflammasome pathway is activated by PAMPs and DAMPs that bind to PRRs and generate a potassium (K^+) efflux. NLRP3 is activated by this efflux and triggers the aggregation of ASC and pro-caspase 1 to form the multi-protein cytosolic inflammasome complex. ASC cleaves pro-caspase 1 to its active form. Caspase 1 within the complex cleaves inactive GSDMD, where GSDMD-N proceeds to form cell membrane pores and induces pyroptosis, as well as pro-IL-18 and pro-IL-1β to active cytokines that are released via GSDMD pores. (C) The non-canonical pathway is triggered by cytosolic LPS and activates caspase 4/5. Caspase 4/5 further activates GSDMD for pore formation and pyroptosis like the canonical pathway, however IL-18 alone is activated here and released through GSDMD pores. The non-canonical pathway can indirectly induce NLRP3 specific IL-18 and IL-1β cytokine production via the activation of the NLRP3 canonical inflammasome pathway by K^+ efflux and the release of DAMPs from non-canonical GSDMD pores.

Evidence that AS01 activates the inflammasome includes IL-18 production by subcapsular SMs (18) in addition to varying levels of IL-1β within different mouse model studies (228, 275), and is also supported by the knowledge that a noted NLRP3 activator, QS-21 (228), is included in this adjuvant

system. However, the role of the inflammasome, in mice or especially in humans, is still unclear. Firstly, in mouse studies, NLRP3 itself was revealed to not be necessary, as NLRP3 knockout mice did not alter the production of AS01-induced IFN-γ and only slightly reduced caspase-1 activation (228). In addition to IL-1β and IL-18 production, inflammasome induced-pyroptosis leads to the release of high-mobility group protein B1 (HMGB1), which has been noted to intersect with the IL-18 signalling pathway and act as a DAMP to activate downstream innate immune responses via MyD88 signalling in targeted APCs (287, 288). The importance of caspase-1 and MyD88 have been individually assessed using knockout mice, where QS-21-induced adaptive T cell immunity was diminished as a result, and downstream HMGB1 signalling was shown to be more important than IL-1β and IL-18 signalling for the production of these T cells responses and adjuvanticity of QS-21 (249). When considering the MPL component of AS01, evidence from mouse model studies also suggests this TLR4-L to play a crucial role in priming the NLRP3 inflammasome via MyD88 signalling for NF-κβ activation and the upregulation of pro-IL-1β and pro-IL-18, in preparation for inflammasome activation and downstream cytokine release (289). However, there are limited studies completed within humans to assess the mechanisms of AS01/QS-21. *In vitro* investigations utilising human blood monocytes have revealed that the QS-21 component of AS01 is trafficked in DCs via a lysosome dependent pathway that leads to activation (227). Additionally, LPS, the TLR-L from which MPL is derived, is known to activate the non-canonical inflammasome via caspase 4/5 within human monocytes (290). Although it remains unknown whether the NLRP3 inflammasome is the sole pathway responsible for QS-21 and AS01 adjuvanticity, the evidence of downstream NLRP3 pathway components, such as caspase-1, IL-18 and HMGB1, being important in respect to QS-21 functions, suggests that at least indirect activation of NLRP3 plays a role in QS-21 adjuvanticity. Therefore, with both AS01 components, in some way, contributing to direct or indirect NLRP3 inflammasome pathway functions, it is important to investigate both canonical and non-canonical inflammasome mechanisms for AS01 adjuvanticity in humans. Completing these studies in primary human LN macrophages will aid in unveiling the reported dichotomies between different mouse model studies as well as human blood monocyte investigations.

1.6. Studying Vaccine and Adjuvant Mechanisms in Humans

The immunogenicity and safety of vaccines and vaccine adjuvants can be assessed in humans via clinical trials, however the investigation of the specific mechanisms generated is not possible in this setting. Instead, animal models are the primary methods used to study these pathways. However, due to interspecies variations, an understanding of these functions within humans is still necessary

to allow for better insights to allow rational adjuvant selection. *In vitro* studies utilising human cells are also able to provide preliminary insights into vaccine functions in humans. This is predominately conducted on isolated peripheral blood mononuclear cells (PBMCs) collected from vaccine trial participants however this does not necessarily reveal the mechanisms that occur within human LNs, which is the primary site for the generation of vaccine immunity. Fine-needle aspiration is an approach that allows for the isolation of LN cells from humans, post vaccination, to characterise the immune responses of vaccines (291-294). However, this is restricted by small cell yields and with predominately only lymphocytes collected, this technique is limited to the assessment of adaptive immune responses. The cell aspirates collected do not contain enough of the innate immune cells to characterise the initiating events. Other approaches focus on specifically isolating innate immune cells, such as APCs, from donated human LN samples to further analyse innate immune functions in an *in vitro* setting (295, 296). Additionally secondary lymphoid organoid systems have been developed *in vitro* from isolated human tonsil immune cells, which have been noted to develop functional germinal centres. Although these organoids can survive for 2 weeks, the complex LN structure including supporting stromal cells is likely not fully recapitulated and they do not have afferent lymphatics or migratory dendritic cells (297). Overall, these study approaches are valuable for broadening our knowledge of immune functions in humans however, there remains a need to develop a physiologically relevant model for studying vaccine mechanisms in intact human LNs with all immune cells and components of this organ intact.

The development of LN explants has been explored for studying pathogen related immune responses within human lymphoid tissues. Instances of culturing viable human LN tissue explants have been reported for studying HIV infections and here, tissue samples are cut into small blocks and cultured on gel foam with media (298). Other similar systems have also been documented however these are predominately utilised to assess adaptive immune functions (299-303). These explants are also sectioned into very small pieces, disturbing the structure of the LN organ where all environmental compartments are likely not present within each slice, nor the full repertoire of innate and adaptive immune cells encapsulated. These options therefore provide limited capacity to study complete sequences of innate immune events that are likely the initiating events of vaccine and adjuvant action in human LNs. Additional methods are required to successfully confirm whether the specific mechanisms identified in animal models, are also similar in intact human LNs, or if differing modes of action occur.

1.7. Aims, Hypothesis and Significance

The aims of this project are therefore:

1. To develop and optimise a human LN explant model for studying innate immune responses to vaccine adjuvants.
2. To compare the innate immune responses to vaccine adjuvants in young and old human LNs, with particular focus on AS01.
3. To dissect the molecular pathways responsible for AS01 adjuvanticity in human LNs.

It is hypothesised that a similar cascade of pro-inflammatory cytokines will be induced in human LNs, as in mice, culminating in IFN-γ production. Older LNs will exhibit a similar innate immune response to AS01 when compared to younger LNs. AS01 will activate the NLRP3 inflammasome in human LNs and be responsible for initiating the pro-inflammatory cytokine cascade.

Chapter 2. Materials and Methods

2.1. Materials and Solutions

Manuscripts incorporated into this section:

Stylianou, V.V., Bertram, K.M., Dunn, E., Baharlou, H., Terre, D., Elhindi, J., Elder, E., French, J., Meybodi, F., Hsu, J., Temmerman, S., Didierlaurent, A.M., Coccia, M., Sandgren, K.J., Cunningham, A.L., Innate immune cell activation by adjuvant AS01 in human lymph node explants is age-independent. Currently under review with Journal of Clinical Investigation.

The manuscript above has been incorporated throughout chapter 2, indicated with "quotation marks". The author of this thesis contributed significantly. Experimental methods here were written by the author of this thesis and guided by Sandgren, K.J., Cunningham A.L. and Bertram, K.M. All experiments were performed by the author of this thesis except the microscopy-based experimental work which was completed by Dunn, E, and the operation of the BD Influx for cell sorting experiments which was operated by Westmead Hub Core Facilities staff.

2.1.1. Materials

All plasticware was purchased from Falcon (Becton Dickson (BD)), Greiner Bio-one (Kremsmünster, Austria) or Eppendorf (Hamburg, Germany) unless otherwise specified.

All washes were performed by centrifuging at 300 relative centrifugal force (rcf) for 5 minutes (min) at room temperature (RT) unless otherwise specified.

All culturing was performed at 37°C in 5% CO_2 unless otherwise specified.

Filter sterilisation was performed using 0.22 µm filters.

Table 2.1: Commercial kits

Commercial Kit	Catalogue #	Source
Dead Cell Removal Kit	130-090-101	Miltenyi
EasySep Dead Cell Removal (Annexin V) Kit	17899	Stemcell Technologies
IFN-γ ELISA	430104	Biolegend
IL-6 ELISA	430501	Biolegend
IL-8 ELISA	431501	Biolegend
LEGENDplex multi-analyte flow assay kit (Human Inflammation Panel 1)	740808	Biolegend
Myeloid Dendritic Cell Isolation Kit	130-094-487	Miltenyi
PanDC Enrichment Kit	130-100-777	Miltenyi
RNeasy Mini Kit	74104	Qiagen

Table 2.2: Reagents

Reagents	Source
2-β-mercaptoethanol (β-ME)	Sigma-Aldrich (Merck)
Accutase	Stemcell Technologies
Adjuvant System 01B ($AS01_B$)	GSK
Arc Amine reactive compensation bead kit	Thermo Fisher Scientific
Adenosine triphosphate (ATP)	Sigma-Aldrich (Merck)
BD CompBead (Anti-mouse Ig)	BD Biosciences
BD CompBead Plus (Anti-mouse Ig)	BD Biosciences
BD Cytofix	BD Biosciences
BD Cytofix/Cytoperm	BD Biosciences
Brefeldin A (BFA)	Sigma-Aldrich (Merck)
Bovine Serum Albumin (BSA)	Sigma-Aldrich (Merck)
Brilliant Stain Buffer Plus	BD Biosciences
Cloning Cylinder	Jiffylite
Collagenase, Type 4	Worthington
Cytokines (IL-4, GMC-SF, M-CSF, Flt-3L, IL-3)	Peprotech
DNase I	Sigma-Aldrich (Merck)
Dulbecco's Modified Eagle Medium (DMEM)	Lonza
Dulbecco's Phosphate Buffered Saline (DPBS)	Lonza
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich (Merck)
Ethanol, absolute	Sigma-Aldrich (Merck)
Ethanolamine	Sigma-Aldrich (Merck)
FcX Fc Receptor block	Biolegend
Fetal calf serum (FCS)	Sigma-Aldrich (Merck)
Ficoll-Paque PLUS	GE Healthcare
Gelfoam	Pfizer
Gentamicin	Gibco (Life Technologies)
HEPES	Gibco (Life Technologies)
Human AB (hAB) serum	Sigma-Aldrich (Merck)
Human CD14 conjugated magnetic MicroBeads	Miltenyi Biotec
Human CD19 conjugated magnetic MicroBeads	Miltenyi Biotec
Human CD3 conjugated magnetic MicroBeads	Miltenyi Biotec
Human HLA-DR conjugated magnetic MicroBeads	Miltenyi Biotec
Iscove's Modified Dulbecco's Medium (IMDM)	Sigma-Aldrich (Merck)
Insulin	Sigma-Aldrich (Merck)
Ionomycin	Sigma-Aldrich (Merck)
Isopentane	Sigma-Aldrich (Merck)
Linoleic acid	Sigma-Aldrich (Merck)
Lipopolysaccharide (LPS)	InvivoGen
Liposomes DiO-labeled (Lipo-DiO; DOPC:Cholesterol 54:45 mol/mol, 1/200 dye:lipid)	T&T Scientific
MCC950	InvivoGen
Monophosphoryl lipid A (MPL)	InvivoGen, GSK
Nigericin	Sigma
Non-essential amino acids (NEAA)	Gibco (Life Technologies)
Oleic acid	Sigma-Aldrich (Merck)
Optical coherence tomography (OCT)	ProSciTech
Palmitic acid	Sigma-Aldrich (Merck)
Pam2Cys	InvivoGen
Paraformaldehyde (PFA)	Electron Microscopy Sciences

Phosphate Buffered Saline (PBS) tablets	Sigma-Aldrich (Merck)
Paramethoxyamphetamine (PMA)	Sigma-Aldrich (Merck)
Poly-L Lysine	Sigma-Aldrich (Merck)
QS-21	GSK
R848	InvivoGen
Red Cell Lysis Buffer (1x)	Biolegend
Roswell Park Memorial Institute (RPMI) 1640 w/ L-Glutamine	Lonza
Saponin	Sigma-Aldrich (Merck)
Sodium azide	Sigma-Aldrich (Merck)
Sodium pyruvate	Gibco (Life Technologies)
Spongostan	SSS Australia
Surgical Glue	B. Braun
Transferrin	Sigma-Aldrich (Merck)
Trypan Blue	Sigma-Aldrich (Merck)
Trypsin-EDTA	Lonza
VX-765	InVivoGen

2.1.2. Buffers, Media and Solutions

Table 2.3: Buffers, media and solutions

Solution	Description
1 X PBS	One PBS tablet was dissolved in 500 mL milliQ water and stored at RT until used. For sterile work, PBS was autoclaved or alternatively, DPBS or commercial PBS from Lonza was used.
FCS	FCS was filter sterilised prior to use and stored at 4°C.
hAB serum	Serum was heat inactivated at 56°C for 30 min and stored at -20°C. Serum was thawed and filter sterilised prior to use.
EDTA	500mM EDTA stock solution at pH8 was prepared by dissolving 73g EDTA in 250 mL milliQ water and pH adjusted to 8. MilliQ water was added to make the solution to a final volume of 500 mL prior to sterilsing by autoclaving, and stored at 4°C.
RF10	RPMI supplemented with 10% (v/v) FCS. Stored at 4°C.
DMEM10	DMEM supplemented with 10%(v/v) FCS. Stored at 4°C.
Dendritic cell media (DCM)	RPMI supplemented with 10% (v/v) FCS, 10 µM (v/v) HEPES, 10 mM (v/v) sodium pyruvate, 1X (v/v) NEAA, 0.05 mM (v/v) gentamicin, 50 µM (v/v) β-ME. Stored at 4°C.
Fluorescence activated cell sorting (FACS) buffer	1X PBS supplemented with 1% (v/v) FCS, 2 mM (v/v) EDTA and 0.1% (w/v) sodium azide (NaN_3). Stored at 4°C.
Magnetic activated cell sorting (MACS) buffer	Sterile PBS supplemented with 1% (v/v) hAB serum and 2 mM (v/v) EDTA and stored at 4°C.
Permeabilisation (PERM) buffer (for flow cytometry)	1X PBS supplemented with 0.1% (w/v) saponin, 1% (v/v) FBS and 1% (w/v) NaN_3 . Stored at 4°C.
Permeabilisation and blocking (PERM/block) buffer (for immunofluorescence microscopy)	1X PBS supplemented with 0.1% (w/v) saponin, 1% (w/v) BSA, 10% human AB serum, 1% (v/v) HEPES, and 1% (w/v) NaN_3. pH 7.4
Blocking buffer	1X PBS supplemented with 1% (w/v) BSA and 10% human AB serum. 1% (v/v) HEPES, 1% (w/v) NaN_3. pH 7.4.
5X Yssel's media stock	IMDM supplemented with 1.25% (w/v) BSA, 1% (v/v) transferrin, 0.25% (v/v) insulin, 0.1% (v/v) linoleic acid, 0.1% (v/v) oleic acid, 0.05% (w/v) palmitic acid, 100uM (v/v) ethanolamine, 0.05 mM (v/v) gentamicin, and 100ng/mL M-CSF or GM-CSF. Stored at -20°C. 1X stock made upon thawing and stored at 4°C for maximum 2 weeks.

2.2. Tissue Culture

2.2.1. Isolation of Peripheral Blood Mononuclear Cells (PBMCs) and Generation of Monocyte Derived Macrophages (MDMs) or Dendritic Cells (MDDCs)

Peripheral blood was acquired from the Australian Red Cross Blood Service. PBMCs were isolated by Ficoll density gradient centrifugation. Briefly, 40 mL of blood was diluted at a 1:1 ratio with PBS, underlaid with 10 mL of Ficoll and centrifuged at 1,800 rpm for 20 min without brakes. PBMCs were collected from the interface between the plasma and Ficoll layers. The cells were washed three times with PBS before being cultured in experiments or used for flow cytometry staining.

In some cases, CD14+ monocytes were isolated from PBMCs by positive selection using Human CD14-conjugated magnetic microbeads as per the manufacturer's instructions.

For generation of monocyte derived macrophages (MDMs), serum-free RPMI was used to seed monocytes in 24 well plates at a density of 0.5 x 10^6 cells/mL with 3 mL/well, or on uncoated glass coverslips in 24 well plates at a density of 0.5 x 10^6 cells/mL with 0.5 ml/well. Plates were centrifuged at 700 rpm for 10 min before incubating for 2-3 h to allow for cell adhesion. Culture media was replenished with RF10 containing 20 ng/mL GM-CSF or 20 ng/mL M-CSF. Cells were cultured for 6 days to differentiate into MDMs. On day 6, MDMs on coverslips were washed with PBS prior to downstream stimulation in experiments. Alternatively, MDMs differentiated on plates without coverslips were treated with Accutase for 15 min to lift the adhered cells for downstream stimulation in assays or flow cytometry staining.

To generate monocyte derived dendritic cells (MDDCs), monocytes were cultured in vented T150 flasks at a density of 0.5 x 10^6 cells/mL in RF10 with 50 µg/mL IL-4 and GM-CSF for 5 days. On day 3, a half media change was completed to replenish cytokines. MDDCs were collected on day 5 and washed twice with PBS prior to stimulation in downstream experiments.

2.2.2. Isolation of Peritoneal Macrophages from Human Ascites Fluid

Informed consent was obtained from patients undergoing paracentesis for their discarded ascites fluid collected from the peritoneal cavity to be used for research purposes (ETH2019/PID02709 LNR/17/WMEAD/570). Upon collection, 1-2 L of ascites fluid was centrifuged at 1800 rpm for 10 min. Cell pellets were combined while washing with PBS/0.02% EDTA. Cells were resuspended in RPMI, and immune cells obtained by Ficoll density gradient centrifugation as described in section 2.2.1. Cells were washed with PBS and stained for flow cytometry. Alternatively, CD14+ peritoneal

macrophages were isolated from peritoneal immune cells as described in section 2.2.1 and prepared for flow cytometry or downstream stimulation experiments.

2.3. Lymph Node Tissue Processing

2.3.1. Ethics and Sample Characteristics

"Human axillary LNs were obtained from clinically node negative breast cancer patients who were undergoing sentinel node biopsies and consented to the removal of an additional LN for this study. Additionally, participants had no relevant comorbidities and were not on immunomodulating drugs such as neoadjuvant therapy, steroids or cytotoxic drugs that could be lymphocytic. The donors ranged from 30 to 96 years of age (YOA) and the LN size ranged from 3-20 mm in the longest dimension. Data was excluded if LN samples had poor viability and/or where insufficient myeloid cell numbers were recovered from a sample. This most often occurred in LNs that were excessively damaged by cauterisation during excision." "This study was approved by the Western Sydney Local Health District (WSLHD) Human Research and Ethics Committee (2019/ETH01894, 2021/ETH12256) and informed, written consent was obtained for all participants prior to the collection of tissue."

2.3.2. Mechanical and Enzymatic Cell Dissociation

Within 60 minutes post-surgery, LNs were collected and trimmed of excess fat under a stereo microscope. Cells were mechanically dissociated from the LN tissue by gently mashing the LN pieces over a nylon mesh cell strainer using PBS/0.02% EDTA (v/v), to ensure a single cell suspension. Where indicated, enzymatic digestion was performed in addition to mechanical dissociation, where cells were isolated from LNs as described above, and the remaining tissue fragments were digested by culturing them in 5 mL of RPMI/0.3% collagenase type IV (w/w)/0.5% DNAse for 1 h at 37°C with agitation every 10 mins. Alternatively, LN tissue was cut into small pieces and subjected to collagenase digestion without prior mechanical dissociation. Dissociated cells were further passed through a cell strainer and washed with PBS/0.2% DNase (v.v).

LN cells isolated using the above methods were stained for flow cytometry or prepared for *in vitro* cultures. Prior to *in vitro* cultures, cells were blocked with FcX Fc Receptor block for 10 mins at 4°C and dead cells were depleted using a Dead Cell Removal Kit as per the manufacturer's instructions.

2.3.3. Enrichment of Monocytes/Macrophages and Dendritic Cells

CD14+ monocytes/macrophages were positively selected from isolated LN cells as described in section 2.2.1. The CD14- fraction was further enriched for DCs by negative magnetic bead selection

using a Myeloid DC Isolation Kit as per the manufacturer's protocol. Alternatively, CD14- cells were depleted of T and B cells using Human CD3 and CD19 conjugated magnetic microbeads as per the manufacturer's instructions. Rather than the above enrichment methods, a PanDC enrichment kit was used on whole isolated LN cells or CD14- cells as per the manufacturer's instructions for depletion of non-PanDCs. As another alternative, HLA-DR+ cells were isolated from whole LN cells using Human HLA-DR-conjugated magnetic microbeads as per the manufacturer's instructions.

Isolated macrophages/monocytes and DC populations from the above methods were stained for fluorescence activated cell sorting (FACS) or prepared for *in vitro* stimulation experiments.

2.3.4. Fluorescence Activated Cell Sorting (FACS)

LN cells were isolated and enriched as described in section 2.3.2 and 2.3.3. Cells were live/dead stained with NIR and a panel of surface antibody markers as described in section 2.5, however all washes were performed with sterile MACS buffer and fixation was not completed. Pure populations of live macrophage and DC subsets were sorted as CD3-CD19-HLA-DR+ and separated into sinus macrophages (CD14+ CD169-), medullary macrophages (CD14+ CD169-), cDC1s (XCR1+ CD1c-), Lang- cDC2s (CD11c+ CD1c+ Lang-), Lang+ cDC2s (CD11c+ CD1c+ Lang+), and pDCs (CD123+ CD11c-). Alternatively, whole live CD3-CD19-HLA-DR+ myeloid cells were sorted separately in addition to CD3+CD19+ lymphocytes. Cells were sorted into sterile collection tubes containing DCM for downstream culturing or centrifuged for storage at -80°C as dry pellets for further RNA extraction. Alternatively, cells were sorted into lysis buffer and lysates stored at -80°C for downstream RNA extraction. All sorts were performed by trained technicians on the BD FACSInflux using a 140 µm nozzle.

2.4. Human Lymph Node Explant Model

"Within 60 minutes post-surgery, LNs were collected and trimmed of excess fat under a stereo microscope and cut longitudinally into two or three 2 mm thick by 5-7 mm wide slices. These were cultured in 48 well plates, cut face down, on gelfoam (Pfizer) pre-soaked with culture medium formulated to support DC viability (DCM; RPMI (Lonza) supplemented with 10 µM HEPES, 1 mM sodium pyruvate, 1X non-essential amino acids, 0.05 mM gentamicin (all Gibco, Thermo Fisher Scientific), 50 µM 2-mercaptoethanol and 10% human serum (both Sigma-Aldrich, Merck), v/v), with or without adjuvant. In some instances, a 6 mm cloning cylinder was sealed to the capsule of the LN slice using surgical glue and the stimulus was applied through this to simulate exposure via the afferent lymphatics. LN slices were cultured for up to 24 h as indicated in individual experiments.

Supernatants were collected and cells were either mechanically dissociated for flow cytometry analysis or tissue slices were fixed or frozen for microscopy. Alternatively to the *in situ* exposure model, cells were dissociated from fresh LN tissue and immediately assessed by flow cytometry or stimulated *in vitro* at 1 x 10^6 cells/mL with adjuvants for 24 h. The adjuvants used for stimulation were 25 µg/mL AS01 (GSK), 25 µg/mL MPL, 10 µg/mL R848 and 1 µg/mL Pam2Cys (InvivoGen). 10 mM DiO or DiD-labeled liposomes (DOPC:Cholesterol 54:45 mol/mol, 1/200 dye:lipid; mean diameter 200 nm) were kindly provided by Dr. Harry Al-Wassiti (Monash University, VIC, Australia). For *in vitro* intracellular cytokine staining (ICS) assays by flow cytometry, cells were stimulated for a total of 24 h at 10 x 10^6 cells/mL with AS01, R848 or 50 ng/mL PMA/1 µg/mL Ionomycin (Sigma-Aldrich (Merck)). 2.5 µg/mL Brefeldin A (BFA, Sigma-Aldrich (Merck)) was added after 2 h or 8-12 h of culture."

2.5. Flow Cytometry

"Cells were stained with Live/Dead Fixable Viability Stain 700 (BD) for 30 min at 4°C. A panel of surface antibodies was then used to stain cells (2.5 x 106 cells/100 mL test), according to standard procedures, in FACSwash (PBS/1% FCS (Sigma-Aldrich (Merck)/5 mM EDTA). Cells were fixed with BD Cytofix prior to acquisition. If intracellular staining was required, cells were permeabilized with BD Cytofix/Cytoperm and stained with antibodies prior to acquisition. For intracellular cytokine staining assays including BFA, all antibody staining was conducted intracellularly. Cells were acquired on the BD Symphony flow cytometer and data analysed by FlowJo V10.8.1 and GraphPad Prism 9. Antibodies included: from BD: CD11c BUV661 (B-ly6), CD14 BUV737 (M5e2), CD1a BV510 (HI149), CD1c BV650 (F10/21A3), CD3 BUV496 (UCHT1), CD45 BUV805 (HI30), CD56 BUV563 (NCAM16.2), CD69 BV480 (FN50), CD8 FITC (5C3), CD80 FITC (L307.4), CD83 PE (HB15e), CD86 BV786 (2331 (FUN-1)), HLA-DR BUV395 (G46-6), IFN-γ PE-Cy7 (B27); from Biolegend: CD11b BV711 (ICRF44), CD123 PE-Cy5 (6H6), CD16 BV570 (3G8), CD19 BV750 (HIB19), CD68 APC-Cy7 (Y1/82A), XCR1 BV421 (ZET); from Thermo Fisher Scientific: CD13 PerCP ef710 (WM15), CD169 PE-ef610 (7-239), IL-1β PE (CRM56), IL-12/23p40 ef660 (HP40), IL-12/IL-23p40 PE (C8.6); from Miltenyi: Langerin PE-Vio770 (MB22-9F5)."

Table 2.4: Flow cytometry antibodies

Antibody	Conjugation	Clone	Catalogue #	Source	Volume (µL / 100 uL)
ASC	AF647	B-3	SC-514414	Santa Cruz Biotechnology	1
AXL	PE-Cy7	DS7HAXL	25-1087-42	eBioscience	2

CD11b	BV711	ICRF44	301344	Biolegend	2.5
CD11c	BUV661	B-ly6	565067	BD Biosciences	2
	PE-CF594	B-ly6	5623393	BD Biosciences	1.5
CD123	BV711	9F5	563161	BD Biosciences	1
	PE-Cy5	6H6	306008	Biolegend	0.5
CD13	PerCP-ef710	WM15	46-0138-42	Thermo-Fisher Scientific	5
CD14	BUV737	M5E2	564444	BD Biosciences	2.5
	BV421	M5E2	565283	BD Biosciences	2.5
CD141	BV711	1A4	563155	BD Biosciences	2
CD16	BV570	3G8	302036	Biolegend	2.5
CD169 (Siglec1)	PE	7-239	346004	Biolegend	2.5
	PE-ef610	7-239	61-1699-42	Thermo-Fisher Scientific	5
CD19	APC-Vio770	REA675	130-113-643	Miltenyi Biotech	5
	BV605	J25C1	562653	BD Biosciences	5
	BV750	HIB19	302261	Biolegend	5
CD1a	BV510	HI149	563481	BD Biosciences	3
CD1c	BV650	F10/21A3	331542	BD Biosciences	5
	SB645				7.5
CD3	APC-Vio770	REA613	130-113-136	Miltenyi Biotech	2.5
	BUV496	UCHT1	564809	BD Biosciences	5
	BV605				2.5
CD4	BV570	OKT4		Biolegend	10
CD40	FITC	5C3 (RUO)	555588	BD Biosciences	3
	PE	5C3 (RUO)	555589	BD Biosciences	1
CD45	BUV805	HI30	564914	BD Biosciences	1
CD56	BUV563	NCAM16.2	565704	BD Biosciences	1
CD68	APC-Cy7	Y1/82A	333822	Biolegend	5
CD69	BV480	FN50	747519	BD Biosciences	0.2
CD70	FITC	Ki-24	555834	BD Biosciences	1
CD8	FITC	5C3	555588	BD Biosciences	2.5
CD80	FITC	L307.4	557226	BD Biosciences	2.5
	PE	L307.4	340294	BD Biosciences	3
CD83	PE		556855	BD Biosciences	5
CD86	BV786	2331 (FUN-1)	740990	BD Biosciences	2.5
	BV605	2331 (FUN-1)	562999	BD Biosciences	2.5
GAM IgG	AF488		948490	Thermo-Fisher Scientific	0.05
GAR IgG	AF546		898238	Thermo-Fisher Scientific	0.05
HLA-DR	BUV395	G46-6	564040	BD Pharmingen	1.5
	PerCP	AC122	130-113-404	Miltenyi Biotech	2
IFN-γ	PE-Cy7	B27 (RUO)	557643	BD Pharmingen	2.5
IL-12/23p40	ef660	HP40	50-7235-42	eBioscience	5
	ef660	C8.6	50-7129-42	eBioscience	5
	PE	C8.6	12-7129-81	eBioscience	0.25
IL-12p35	ef660	SNKY35	50-7359-41	eBioscience	5
IL-12p70	APC	REA123	130-103-673	Miltenyi Biotech	5
IL-18	Purified	EPR19954	AB207324	Abcam	2.5
IL-1β	PE	CRM56	12-7018-82	eBioscience	1
IL-6	APC				2.5
Langerin	PE-Vio770	MB22-9F5	130-100-586	Miltenyi Biotec	1.5
	Vioblue	MB22-9F5	130-106-096	Miltenyi Biotec	3
Live/dead	Fixable viability stain (FVS700)		564997	BD Biosciences	0.05

	Near infrared (NIR)		L34976A	Invivogen	0.02
Siglec 6	APC	REA852	130-112-711	Miltenyi Biotec	1
TNF-a	PE-Cy7		557647	BD Biosciences	1
XCR1	APC	S15046E	372606	Biolegend	4
	BV421	S15046E	372610	Biolegend	4
	PE	S15046E	372603	Biolegend	5

2.6. Cytokine Immunoassays

"The LEGENDplex bead-based multi-analyte flow assay kit (Human Inflammation Panel 1, Biolegend) was used to detect a panel of 13 human inflammatory cytokines in culture supernatants as per the manufacturers' protocol. IL-6 and IL-8 were measured by ELISA (both Biolegend) as their concentrations exceeded the range of the LEGENDplex assay. For LEGENDplex experiments, plates were acquired on the BD Canto II and data analysed with LEGENDplex Data Analysis Software (Biolegend). For all ELISA assays, absorbance was measured on the SpectraMax iD5 Plate Reader and data analysed using GraphPad Prism v9.20."

2.7. Statistics

"Statistical analyses were performed using GraphPad Prism 9.0 and R Studio version 4. Data was assumed not to follow a normal distribution based on visual inspection or failure of normality tests. Therefore non-parametric tests were used throughout or in some cases, data was loge transformed to approximate normality and equivalent parametric tests were applied with Bonferroni-Dunn corrections for multiple comparisons, as described in Fig. legends. A GEE model was used to model the immune response as measured by cytokine levels. The model was equipped with a log-normal link function, an exchangeable correlation structure of the multiple treatments received by each donor, and adjustment for the fixed effect of age. When multiple hypotheses were adjusted for, the Bonferroni Dunn method was used. $p< 0.05$ represented statistical significance."

Chapter 3. Optimisation of a Human Lymph Node Explant Model for Studying Vaccine Components

Manuscripts incorporated into this section:

Stylianou, V.V., Bertram, K.M., Dunn, E., Baharlou, H., Terre, D., Elhindi, J., Elder, E., French, J., Meybodi, F., Hsu, J., Temmerman, S., Didierlaurent, A.M., Coccia, M., Sandgren, K.J., Cunningham, A.L., Innate immune cell activation by adjuvant AS01 in human lymph node explants is age-independent. Currently under review with Journal of Clinical Investigation.

This chapter includes data from the above manuscript, indicated with "quotation marks", which has been submitted for publication and is currently under review. Figure numbers have been adjusted to include supplementary material from the manuscript within the main text of this thesis, as indicated by the boxed brackets "[]". The author of this thesis contributed significantly. Sandgren, K.J., Cunningham A.L. and Bertram, K.M. guided all experiments. All experiments were performed by the author of this thesis except the microscopy-based experimental work which was completed by Terre, D and Dunn, E, and the TapeStation RNA quality checks which were performed by Westmead Hub Core Facilities staff. Staff members also aided in the operation of the BD Influx for cell sorting experiments. Analysis was performed by the thesis author and Elhindi, J.

3.1. Introduction

The immunogenicity and safety of vaccines and vaccine adjuvants can be assessed in humans via clinical trials, however the investigation of the specific mechanisms generated is not possible in this setting. Instead, animal models are the primary methods used to study these pathways. However, intrinsic interspecies variations between animals and humans, such as biology of protein receptors, pathophysiology and genetics, create great discrepancies in responses to pathogens, drugs and vaccines (304). This can contribute to unanticipated results during the vaccine development process. There are many instances where vaccine candidates seem promising within pre-clinical animal studies, such as the HIV adenovirus type 5 viral vector vaccine expressing Gag protein in NHPs (305, 306), but fail to generate adequate immune responses in humans (307). Differences in pathophysiology play a large role here, as NHPs cannot be infected by HIV and so the related simian immunodeficiency virus (SIV) specific to infecting NHPs needs to be used throughout pre-clinical assessments (308-310). Other examples include the viral vector vaccine candidate for TB which was effective in mice and NHP studies (311-314) but failed in human clinical trials due to unknown reasons (315, 316). Therefore, an understanding of the functions of vaccines and vaccine components within humans, particularly the innate immune mechanisms of action, are needed prior

to completing expensive pre-clinical animal studies and human clinical trials, to allow for better insights that can enhance the vaccine development process.

Pre-clinical, *in vitro* studies utilising human cells are also able to provide preliminary insights into vaccine functions in humans. This is predominately conducted on isolated PBMCs collected from participants however this does not encompass the mechanisms that occur within human LNs, which is the primary site for the generation of vaccine immunity. Fine-needle aspiration is a relatively new approach that allows for the isolation of LN cells from humans, post vaccination, to characterise the immune responses of vaccines (291-294). However, with LNs being predominately composed of lymphocytes, this technique is limited to the assessment of adaptive immune responses, as the cell aspirates collected do not contain enough of the LN innate immune cells to characterise the initial mechanisms involved in vaccine and adjuvant functions. Additionally, these investigations can also only occur during human clinical trials. Other approaches focus on specifically isolating innate immune cells, such as APCs, from donated human LN samples to further instigate innate immune functions within an *in vitro* setting (295, 296). Overall, these study approaches are valuable for broadening our knowledge of immune functions in humans however, there remains a need to develop a physiologically relevant model for studying vaccine mechanisms in intact human LNs with all immune cells, including innate cells, and structural components, such as reticulin networks, of this organ intact.

The development of explant models has been explored for studying pathogen related immune responses within human lymphoid tissues, however they predominately focus on adaptive immune functions and germinal center formation which typically takes 5-7 days (317). Additional methods are required to assess innate immune responses and determine whether the specific mechanisms identified in animal models, are similar in intact human LNs, or if differing modes of action occur which may lead to vaccine failure. Within this thesis, we attempted to generate a model that would allow for sufficient investigation of innate immune cells to assess the mechanisms and pathways responsible for vaccine and adjuvant function in human LNs. A particular interest in the vaccine adjuvant AS01 is noted, due to its success in the shingles, RSV and malaria vaccines within different age groups, however the reasons for this and underlying mechanisms within human LNs are unknown.

3.2. Methods

3.2.1. Lymph Node Explant Culture Condition Optimisations

LN explants were set up for culture and analysed as described in section 2.4. LN slices were cultured for 8-, 24-, 48-, 72-, or 96-h in the presence of DCM or Yssel's media, with or without the addition of cytokines M-CSF, Flt-3L and GM-CSF at 100 μg/mL.

3.2.2. Lymph Node Cell Dissociation Optimisations

LN cells were isolated as described in section 2.3.2. Both methods, mechanical and enzymatic digestion with collagenase type IV, were conducted in the same donors for direct comparison. Tissue slices were weighed prior to commencing either cell isolation method.

3.2.3. Immunofluorescence Microscopy

"LN slices stimulated *in situ* with DiD-labeled liposomes were frozen in OCT. 7 μm sections were fixed with 2% paraformaldehyde (PFA) then blocked and permeabilised with PBS/0.1% saponin/1% BSA/10% normal donkey serum /1% HEPES. Tissue was incubated with primary antibodies CD11c (clone 3.9, Invitrogen) and CD169 (SP216, Merck) for 1 h at 37°C and secondary antibodies donkey anti-mouse AlexaFluor 555 and donkey anti-rabbit AlexaFluor 647 for 30 min at room temperature (RT). The tissue sections were counter stained with DAPI and mounted with ProLong Diamond (Thermo Fisher Scientific). Images were acquired on the Olympus VS-120 Virtual Slide Microscope at 20X and analysed using Fiji."

3.2.4. Imaging Mass Cytometry

"For imaging mass cytometry (IMC), 5 μm FFPE tissue sections were de-waxed and re-hydrated in xylol for 3 x 5 min, 100% ethanol for 3 x 5 min and 70% ethanol for 5 min then PBS. Antigen retrieval was performed in Dako AR Buffer pH 9.0 at 95°C for 20 min in a Biocare Decloaker NxGen. Slides were blocked with Bloxall (Vector Labs) at RT for 10 minutes then incubated with a metal-conjugated antibody cocktail in TBS-Tris/1% BSA overnight at 4°C. Slides were washed in PBS/0.1% Triton-X for 2 x 8 mins. This was repeated with PBS. Nuclei were stained with a DNA intercalator for 30 min at RT. Images were acquired on a Hyperion Imaging Mass Cytometer (Standard Biotools)." From an optimised panel of 36 metal isotope conjugated antibodies, the antibody cocktail for this experiment contained 3 markers for analysis: CD169-172 Yb, CD68-153Eu and a DNA intercalator on 191Ir.

3.2.5. Fluorescence Activated Cell Sorting Optimisations

LN slices were stimulated with adjuvants, including 25 µg/mL QS-21, for 8-, 12- or 16-h as described in section 2.4. Collagenase IV digestion was used to liberate the cells from the tissue as described in section 2.3.2 and whole LN cells were sorted as described in section 2.3.4.

3.2.6. RNA Extractions

LN cell lysates and dry cell pellets stored at -80°C following FACS of *in situ* cultured LN slices as per section 2.3.4, were partially thawed on ice prior to downstream RNA extractions. The RNeasy Mini Kit was used to extract the RNA from samples as per the manufacturer's instructions. RNA concentration and quality was initially assessed via NanoDrop and further confirmed via TapeStation.

3.3. Results

3.3.1. Human Lymph Node Explant Model

3.3.1.1. Development of Explant Set Up

"To study the mechanisms of action of adjuvants *in situ* in human tissue, we developed a human LN explant model. Human axillary LNs were obtained from clinically node negative female breast cancer patients who were undergoing sentinel node biopsies. Informed consent was obtained for the removal of an additional LN for this study. Donors were aged between 30 and 96 YOA. 53% were ≥ 60 YOA [Figure 3.1]. Longitudinal slices of whole human LNs, approximately 2 mm thick, were cultured on gel foam to provide hydration and structural support, which promoted cell viability [Figure 3.2A]."

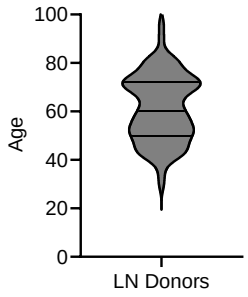


Figure 3.1: "Age distribution of lymph node donors." "Violin plot showing median and interquartile range of LN donor ages."

“We used a high-parameter flow cytometry panel to detect all subsets of myeloid cells, which include resident DC subsets including conventional type 1 and 2 DCs (cDC1, cDC2) and plasmacytoid DCs (pDC), sinus-lining and medullary macrophages (SM and MM) and monocytes (mono), as well as NK, NK-T, B and T cells [Figure 3.2B]. Migratory skin-derived DCs (CD11c+CD1a+Langerin+/-) that were present in the LN at the time of excision were also detected. First, we assessed the viability of different cell populations in *ex vivo* LN cultures. T, B and NK cells survived for 48-72 h but viability of the DCs and macrophages declined in the LN over 24 h culture [Figure 3.2C]. Macrophages in particular exhibited reduced numbers after culture, rather than appearing more strongly stained with the viability marker, indicating that they were lysing [Figure 3.3A]. Importantly, the viability of cells in LN slices cultured for 24 h was similar whether or not the cultures were stimulated with adjuvant AS01. AS01 was well tolerated up to a concentration of 25 μg/mL, *in situ* and *in vitro* but higher concentrations decreased viability [Figure 3.3]. Furthermore, the viability of total dissociated LN cells, which is comprised of > 98% lymphocytes, cultured *in vitro* for 24 h (72.9 ± 17.0%, mean ± SD; [Figure 3.3C]), was only slightly better than the viability of the lymphocytes cultured in LN slices (T cells 69.7 ± 7.6%, B cells 68.8 ± 11.8%; [Figure 3.2C]). As such, we limited *ex vivo* cultures to 24 h and included mock treated controls to account for any effects produced by dying cells.”

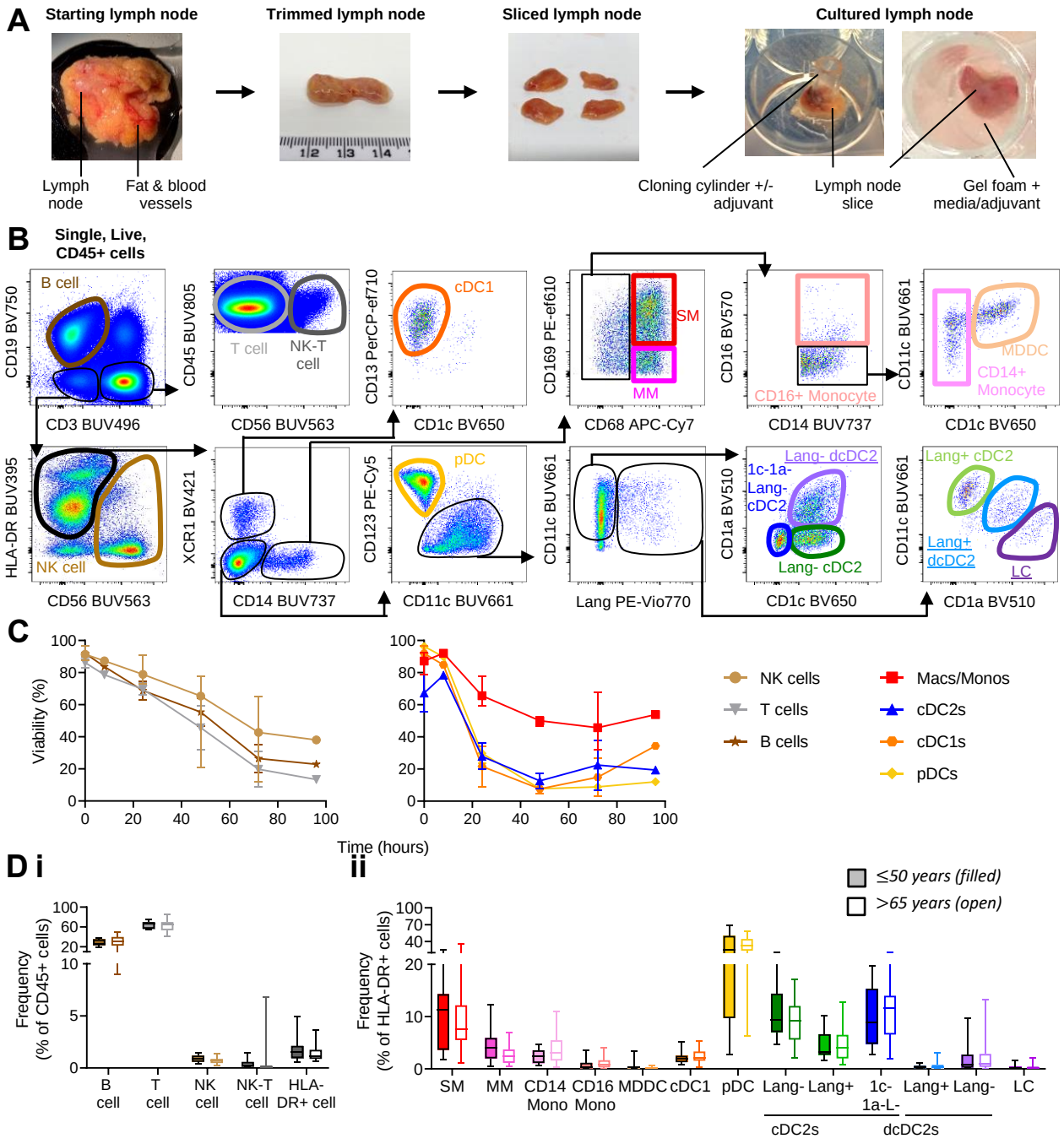


Figure 3.2: "Human lymph node explant model for studying vaccine adjuvants *in situ*." "(A) Surrounding fat is removed from whole human LNs which are then sliced longitudinally and placed cut face down on a piece of gel foam soaked in culture medium. For adjuvant exposure via the most physiological route (i.e., via the afferent lymph), a cloning cylinder is glued to the capsular surface of the lymph node and the adjuvant applied within this. For maximum cellular exposure, LN slices are bathed in the adjuvant. (B) Flow cytometry gating strategy used to identify each LN cell population, including resident and migratory (underlined) dendritic cell populations. (C) Viability of LN lymphocyte and myeloid cell populations following *in situ* culture (n = 4 for 0-, 24-, 48- and 72-hour time points, n = 1 for 8-, and 96-hour time points). Median with interquartile range are shown. (D) Proportion analysis of cell populations within the LN with a comparison between donors aged ≤50 years (filled boxes, n=12) and >65 years (open boxes, n=22). Median and interquartile range are shown. (i) Lymphocyte (B, T, NK, NK-T) and myeloid (HLA-DR+) cell populations as a percent of live, CD45+ immune cells within the lymph node. (ii) Macrophage, monocyte and dendritic cell subsets within the LN as a percentage of live, CD45+, CD19-, CD3-, CD56-, HLA-DR+ myeloid cells. All subset comparisons between young and old LNs were not significant by Mann Whitney test using Bonferroni-Dunn correction method for multiple

comparisons. NK = natural killer. SM = subcapsular sinus-lining macrophage. MM = medullary macrophage. Mono = monocyte. MDDC = monocyte-derived dendritic cell. cDC1/2 = conventional dendritic cell type 1 or 2. dcDC2 = dermal-derived conventional dendritic cell type 2. pDC = plasmacytoid dendritic cell. Lang = langerin. LC = Langerhans cells."

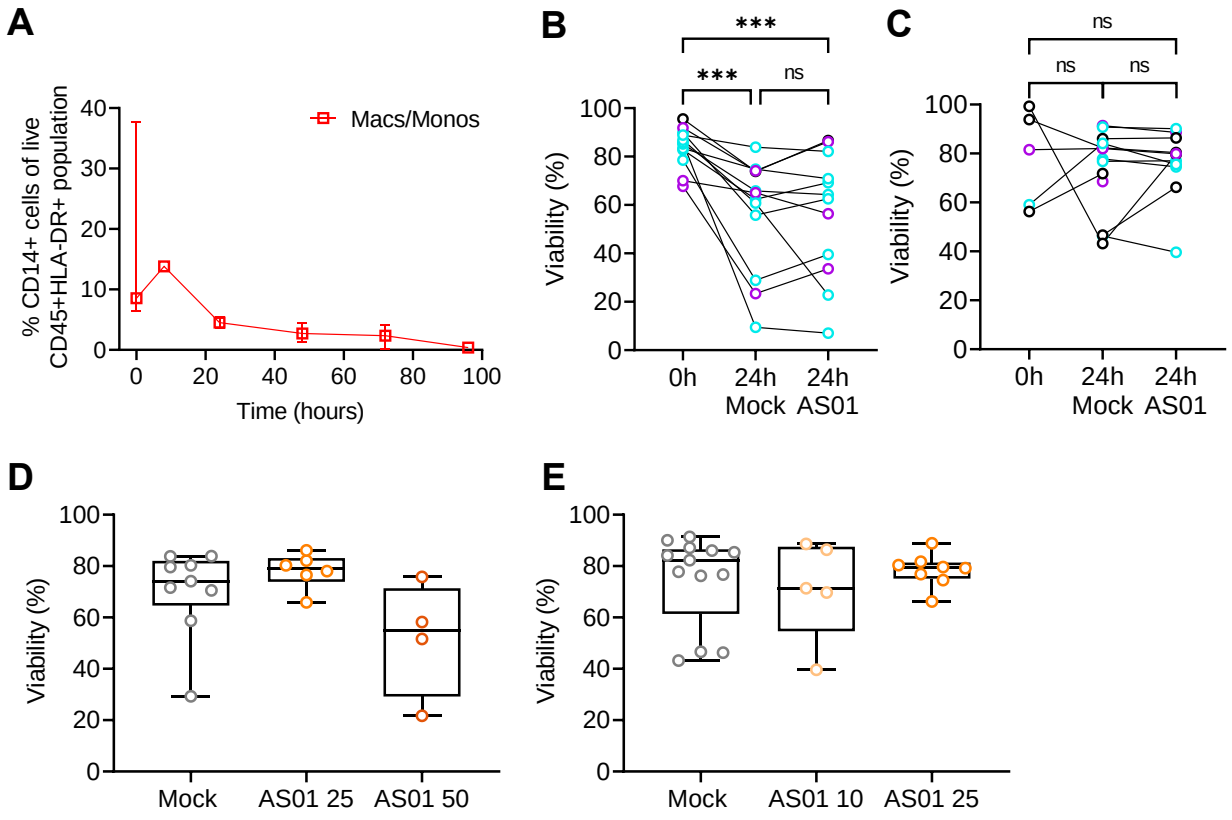


Figure 3.3: "Viability of lymph node immune cells in the *in situ* explant model vs *in vitro* cultured cells." "Slices of human LNs were mock treated or bathed in AS01 for 24 h. Cells were mechanically dissociated from the tissue and immune cell viability was determined by flow cytometry. Alternatively dissociated LN cells were exposed to AS01 *in vitro*. (A) Frequency of CD14+ monocyte/macrophages of the live CD45+HLA-DR+ population over time in LN slices cultured without stimuli (n=2-3). Median with interquartile range plotted. (B) Viability of CD45+ immune cells from LN slices that were AS01 or mock treated *in situ* for 24 h, compared to viability of fresh LN cells (0 h). (C) Viability of isolated LN CD45+ immune cells that were AS01 or mock treated *in vitro* for 24 h, compared to fresh LN cells (0 h). Repeated measures ANOVA with Tukey's multiple comparisons test was performed. *** $p < 0.001$, ns = non-significant. Donor age: purple ≤50 years; black 51-65 years; aqua >65 years. Toxicity study of AS01 (D) in LN slices and (E) in isolated cells up to 50 µg/mL. Viability of CD45+ immune cells is shown, where AS01 was tolerated up to 25µg/mL. Median and interquartile range are shown. All comparisons ns by Wilcoxon matched pairs signed rank test."

"Next, we compared the immune cell constitution of LNs from young (≤50 YOA, n = 12) and older individuals (>65 YOA, n = 22). LNs from young or old donors were remarkably similar [Figure 3.2D, Figure 3.4Ci]. T and B cells represented the bulk of cells, with CD3-CD19-HLA-DR+ antigen presenting cells representing 1.79 ± 1.16% in young and 1.43 ± 0.84% in old LNs (mean ± SD). The constitution of this HLA-DR+ population was also remarkably similar between the two age groups with no

significant differences in the cell subset proportions [Figure 3.2D].” When examining this on a continuous scale, we performed a Spearman rank correlation which has the power to detect non-linear relationships rather than a simple linear regression. We did not observe any noteworthy correlations between age and the proportions of LN cell subsets. There was a significant negative correlation between age and frequency of NK-T cells, NK cells and CD3-CD19-HLA-DR+ antigen-presenting cells, however, it is important to note that the r values are small, indicating a very weak correlation (Figure 3.4A,B,Cii).

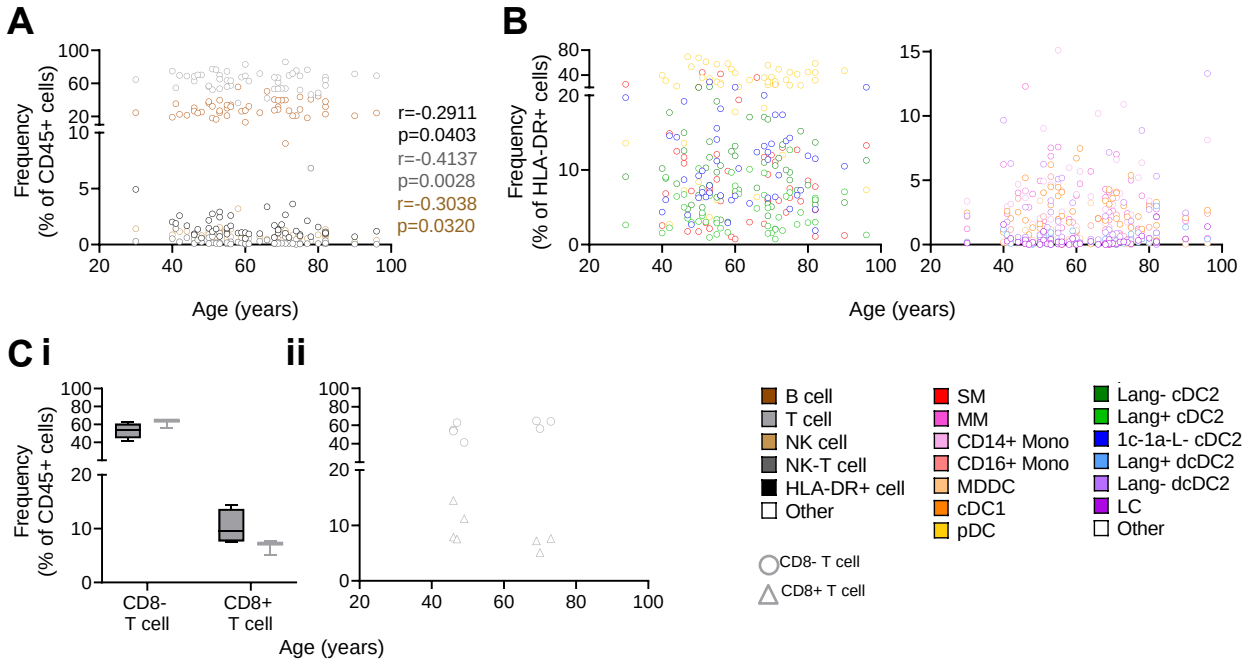


Figure 3.4: Age analysis of lymph node immune cells with age as a continuous variable. Proportion analysis of cell populations within the LN with all ages on a linear scale. Spearman rank correlations were applied with r and p values reported where significant cell population changes across age were seen (n = 34). **(A)** Lymphocyte (B, T, NK, NK-T) and myeloid (HLA-DR+) cell populations as a percent of live, CD45+ immune cells within the LN. **(B)** Macrophage, monocyte and dendritic cell subsets within the LN as a percentage of live, CD45+, CD19-, CD3-, CD56-, HLA-DR+ myeloid cells. **(C) (i)** Proportion analysis of CD8- vs CD8+ T cells within the LN with a comparison between donors aged ≤50 YOA (filled boxes, n = 4) and >65 YOA (open boxes, n = 3). Median and interquartile range are shown. **(ii)** Proportion analysis of CD8- vs CD8+ T cells within the LN with all ages on a linear scale (n = 7). NK = natural killer. SM = subcapsular sinus-lining macrophage. MM = medullary macrophage. Mono = monocyte. MDDC = monocyte-derived dendritic cell. cDC1/2 = conventional dendritic cell type 1 or 2. dcDC2 = dermal-derived conventional dendritic cell type 2. pDC = plasmacytoid dendritic cell. Lang = langerin. LC = Langerhans cells.

3.3.1.2. Optimisation of Explant Culture Conditions

In attempts to enhance the viability of macrophages and DCs in the human LN explant model, an alternate media formulation supplemented with cytokines for support, was assessed. The use of

Yssel's media, with or without the addition of cytokines macrophage colony stimulating factor (M-CSF; for macrophages), FMS-like tyrosine kinase 3 ligand (Flt-3L; for DCs), and granulocyte macrophage colony stimulating factor (GM-CSF; for DCs), did not yield significant enhancements in cell viability for any subsets within the initial 24 h culture period compared to the original DCM used (Figure 3.5). Furthermore, this media formulation did not improve the viability of any LN immune cell subsets at the later time points of 48 and 72 h (Figure 3.5). Alternate methods to improve the viability of rare innate immune cells at early time points, as well as to extend the longevity of the model to assess germinal centre formation and adaptive immune responses, are required.

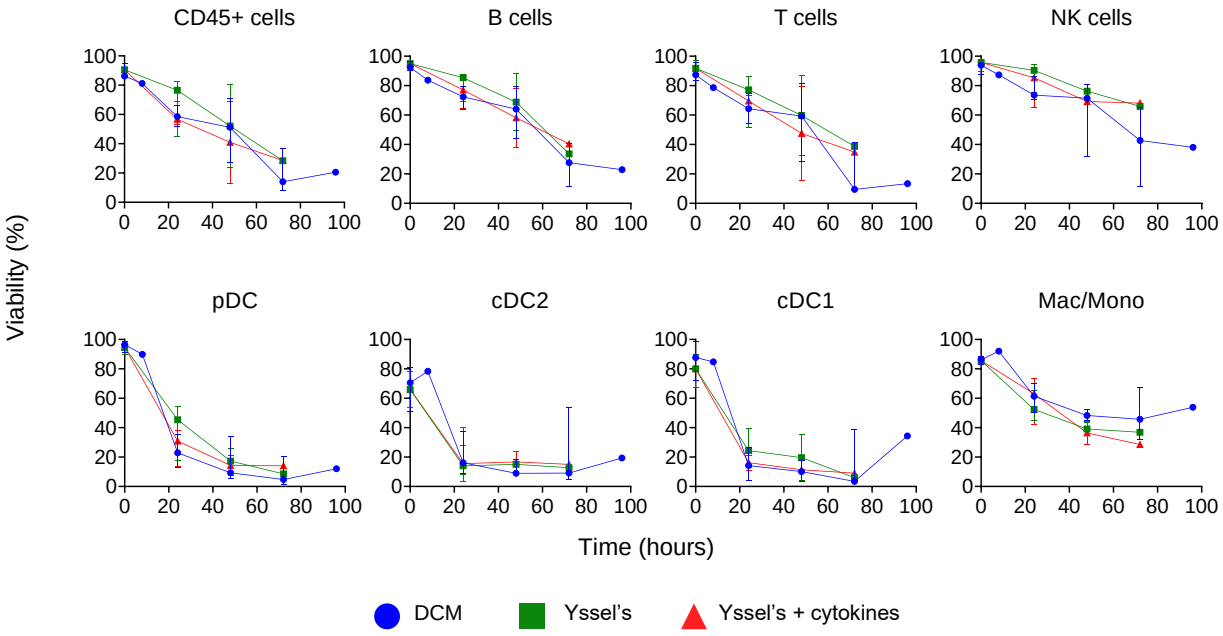


Figure 3.5: Optimisation of culture medium for *in situ* lymph node explant cultures. LN slices were cultured by bathing *in situ* in DCM (circle; blue), Yssel's media (square; green) or Yssel's media plus 100µg/mL Flt-3L, M-CSF and/or GM-CSF (triangle; red). Viability of lymphocyte and myeloid cell populations were assessed on separate slices at serial timepoints up to 96 h. DCM; 0-h (n = 8), 24-h (n = 6), 48-h (n = 5), 72-h (n = 3), 8-, 96-h (n = 1). Yssel's and Yssel's + cytokines; 0-h (n = 4), 24-h (n = 3), 48-h (n = 2), 72-h (n = 1). Medians with interquartile ranges are shown.

3.3.1.3. Optimisation of Cell Dissociation and Enrichment Methods from Explants

To investigate whether the extraction of LN cells could be improved in terms of the cell subsets or numbers isolated and their viability, enzymatic digestion with collagenase type IV was explored as an alternate method to mechanical dissociation. Collagenase digestion, when directly compared to mechanical dissociation, did not yield any significant differences in frequencies of LN cell populations in the total cells isolated (Figure 3.6A-B). In both methods, T and B cells dominated,

although CD3-CD19-HLA-DR+ APCs trended towards increased numbers with collagenase, constituting 1.62 ± 0.87% in mechanical isolation and 2.83 ± 1.90% in collagenase digestion (mean ± SD). Importantly, the composition of the HLA-DR+ population remained reasonably constant irrespective of the isolation method, suggesting that no cell subsets were being left behind by mechanical dissociation (Figure 3.6B). Overall, these investigations confirm that both methods are valid, and neither significantly outperforms the other in isolating the LN immune cell subsets.

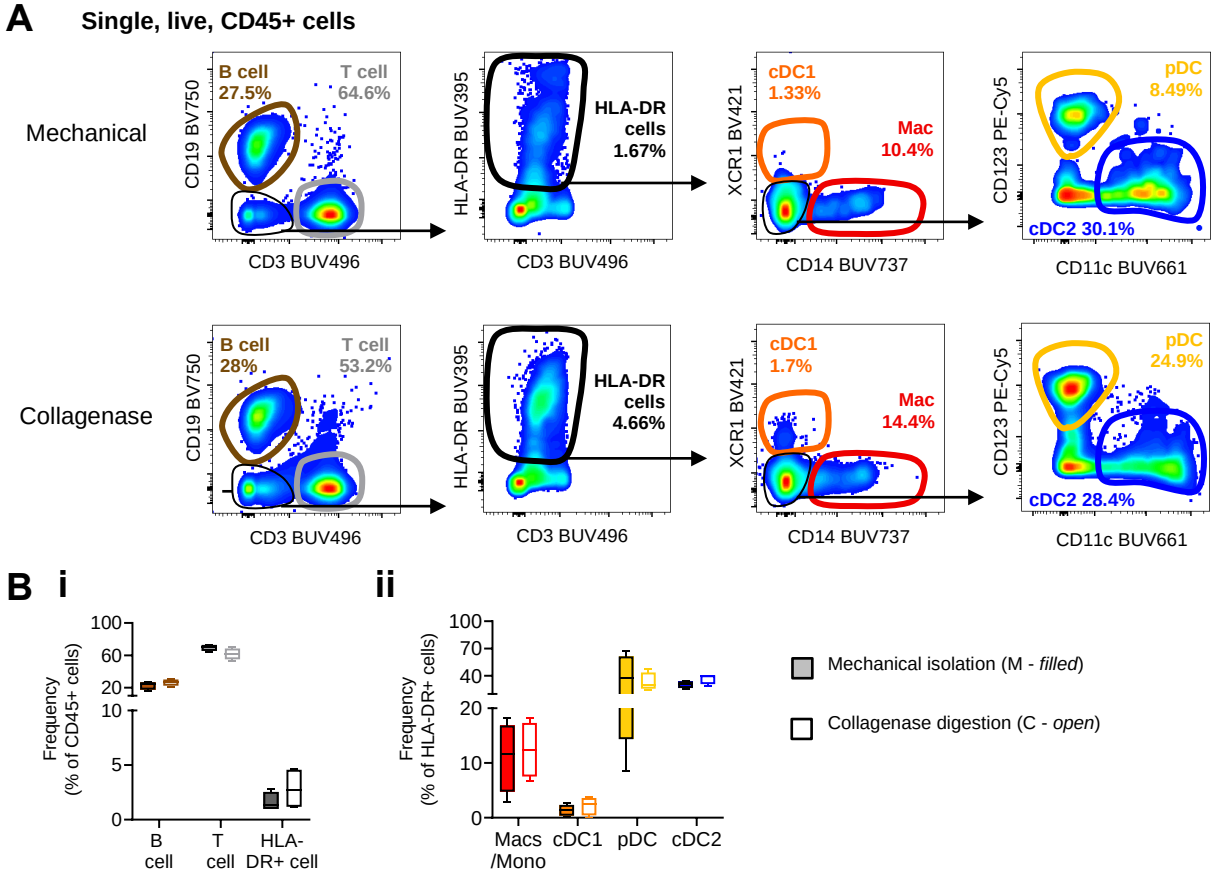


Figure 3.6: Collagenase digestion does not increase the yield of isolated LN immune cells compared to mechanical dissociation. Whole LNs were sliced in half and one side was subjected to mechanical dissociation, while the other collagenase digestion. **(A)** Representative flow cytometry plots comparing LN cell subset frequencies of parent populations between mechanical and collagenase isolation methods. **(B)** Proportion analysis of cell populations within the LN comparing mechanical (M, filled boxes, n=4) and collagenase (C, open boxes, n=4) isolation methods. Median and interquartile range are shown. **(i)** Lymphocyte (B, T, NK, NK-T) and myeloid (HLA-DR+) cell populations as a percent of live, CD45+ immune cells within the LN. **(ii)** Macrophage, monocyte and dendritic cell subsets within the LN as a percentage of live, CD45+, CD19-, CD3-, CD56-, HLA-DR+ myeloid cells. All subset comparisons between mechanical and collagenase were not significant by Mann Whitney test using Bonferroni-Dunn correction method for multiple comparisons. Mac = macrophage. Mono = monocyte. cDC1/2 = conventional dendritic cell type 1 or 2. pDC = plasmacytoid dendritic cell.

3.3.2. Assessment of Vaccine Adjuvant Penetration into the Explant Model

3.3.2.1. Cylinder vs Bathing Exposure Routes

"To assess the effects of adjuvant on LN explants, adjuvant treatments were applied via two routes [Figure 3.2A]: a cloning cylinder glued to the capsule of the LN allowed the adjuvant to enter the LN probably via the afferent lymphatic vessels on the LN surface, as occurs *in vivo* or possibly penetrating through the capsule directly. Alternatively, the cut face of the LN was exposed directly by placing it on gel foam soaked in adjuvant-containing culture medium ('bathing'; [Figure 3.2A]) and this allowed for maximum exposure of LN immune cells, which yielded stronger immune responses." When we did utilise the more physiologically relevant route via the cloning cylinder, immune responses were of a similar profile but not as strong as the bathing method, as thoroughly investigated in the comparisons of these two routes below.

"To model the uptake of AS01 *in situ* in LNs, we used liposomes of equivalent composition and similar size, without MPL and QS-21 but incorporating the lipophilic fluorescent dye DiO or DiD. Slices of human LNs were exposed *in situ* to labeled liposomes via both the bathing and cylinder application methods for 30 min to 24 h to determine the degree of liposome uptake for each immune cell subset by flow cytometry [Figure 3.7A]. Immunofluorescence microscopy confirmed that liposomes penetrated the LN slice within 30 minutes via bathing [Figure 3.7B] and the degree of penetration increased over 24 h [Figure 3.8A]. Correspondingly, uptake of liposomes by each cell type increased over 24 h [Figure 3.7C]. For all subsets, a larger percentage of cells were exposed to the liposomes via bathing compared to cylinder application resulting in a higher degree of uptake, as shown by the percentage of cells that were liposome positive [Figure 3.7D, Figure 3.8B]. However, the distribution of liposome uptake across subsets was proportional [Figure 3.7E, Figure 3.8C], with a strong correlation between the two exposure routes [Figure 3.8D], indicating that the liposomes penetrated the LN effectively via both methods and the bathed route did not introduce a bias on liposome uptake. We therefore conducted all experiments using the bathing exposure method as it a) increased exposure of the cells to the liposomes and therefore presumably the adjuvant and b) did not result in exposure of any cells that would not normally encounter the adjuvant when exposed via the physiological route simulated by the cloning cylinder."

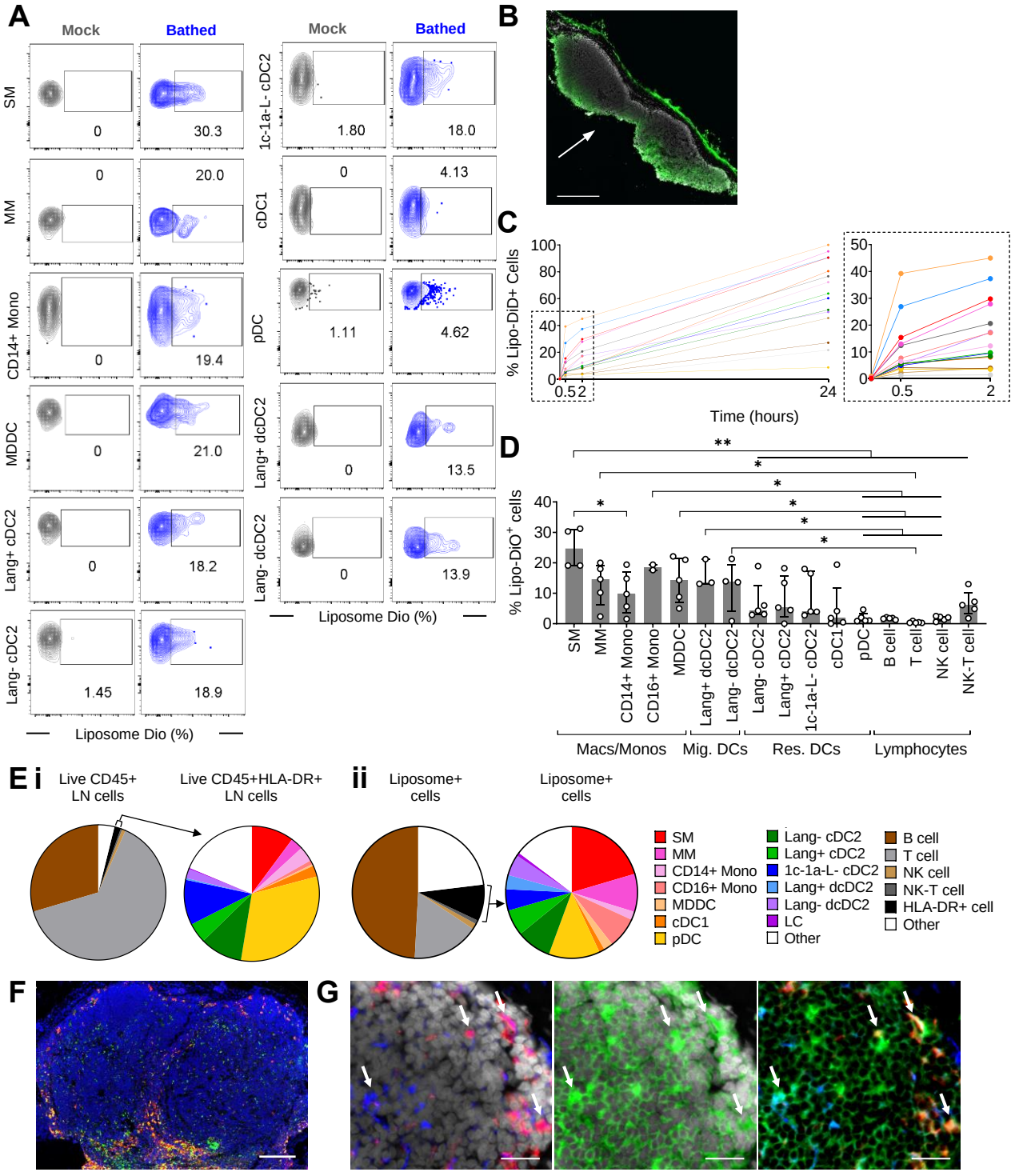


Figure 3.7: "Fluorescent liposomes, a model for AS01, are preferentially taken up by subcapsular sinus lining macrophages in LNs." "Slices of human LNs were exposed to DiO-, or DiD-labeled liposomes for 0.5, 2 or 24 h. Cells were mechanically dissociated from the tissue and the % liposome+ cells was determined for each cell subset by flow cytometry (n = 3-5). For immunofluorescence microscopy analysis, LN slices were frozen and sectioned for staining (n = 3), while for imaging mass cytometry LN slices were fixed. (A) Representative flow cytometry plots showing DiO-liposome uptake after 2 h bathing, by resident myeloid cells and migratory skin-derived cells. (B) Immunofluorescence imaging of LN slice showing DiD-labelled liposomes penetrate the tissue within 30 min exposure. The exposed face of the LN slice is indicated by the arrow. Scale bar = 500µm. (C) Uptake of DiO-labelled liposomes over 24 h measured by flow cytometry (n = 3). Colours in (C) and (E) correspond to cell subset legend. (D) Comparison of 2 h liposome uptake (% positive cells) between LN cell subsets. Cell subsets of the major groups are shown; macrophages/monocytes

(macs/monos), migratory dendritic cells (mig. DCs), resident dendritic cells (res. DCs) and lymphocytes. Median + interquartile range is plotted for each subset. Mixed effects analysis with Tukey's multiple comparisons test was performed. * $p < 0.05$, ** $p < 0.01$. For grouped statistical representation, the highest common p-value is presented but lower values were generated. (E) Proportion analysis of immune cell subsets (i) present in the LN (n = 50) and (ii) making up the total liposome+ fraction in 2 h exposure experiments (n = 5), showing cell subsets as a percentage of total live, CD45+ immune cells and myeloid cell subsets as a percentage of HLA-DR+ cells. (F) Imaging mass cytometry image showing CD169 (red) and CD68 (green) staining in the LN. CD169+CD68+ sinus-lining macrophages appear red-yellow around the capsule and medullary sinuses. DAPI (blue). Scale bar = 200 µm. (G) CD169+ (red) subcapsular sinus macrophages and CD11c+ (blue) dendritic cells take up DiD-labelled liposomes (green) in situ in the LN after 2 h exposure, indicated by arrows. The capsule is visible at the edge of the LN, top right. Scale bar = 25 µm."

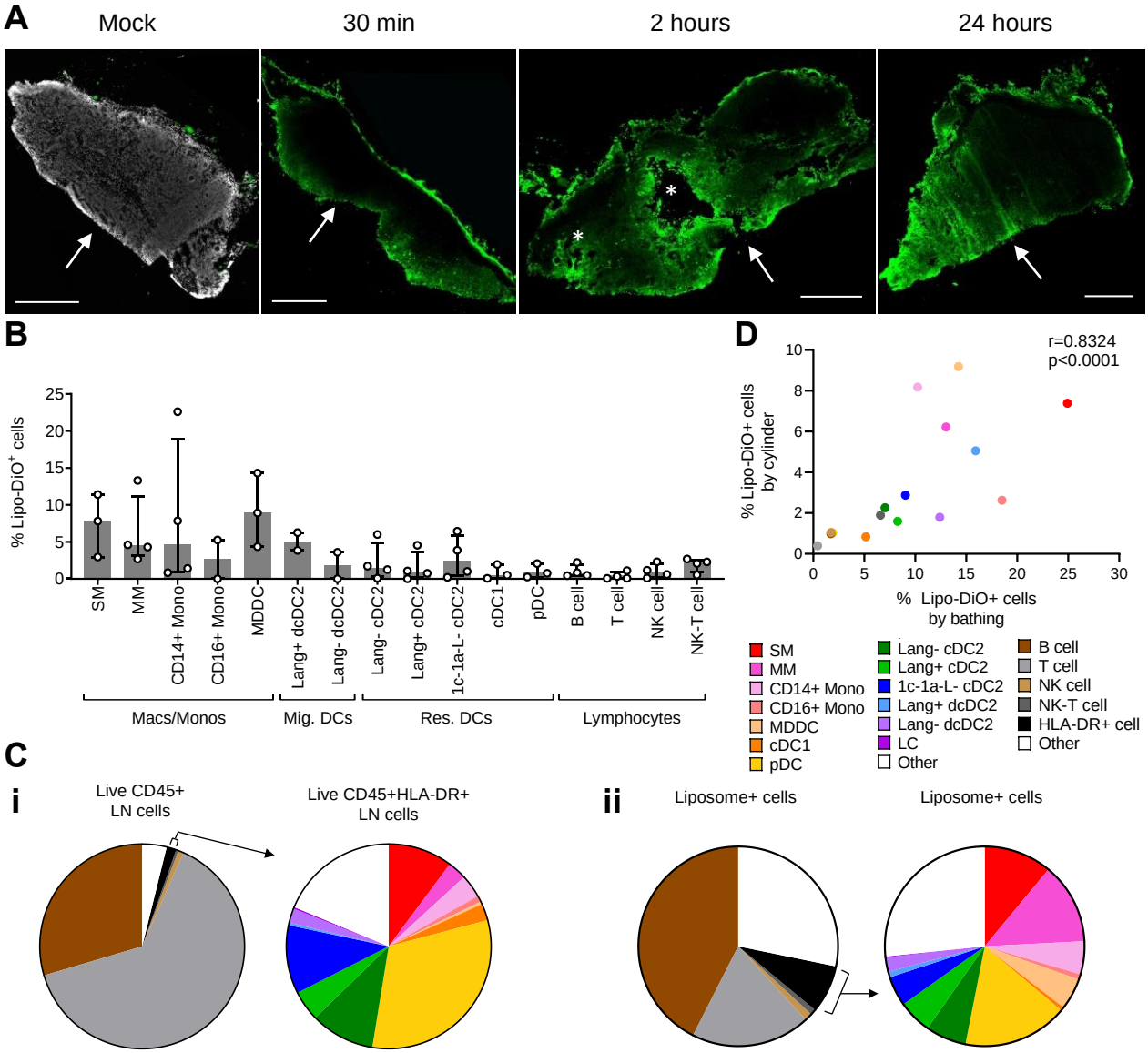


Figure 3.8: "Fluorescent liposomes, a model for AS01, are preferentially taken up by subcapsular sinus lining macrophages following cylinder and bathing exposure *in situ*." "Slices of human LNs were exposed to DiO-labeled liposomes for 2 h via the bathing (A,D) or cylinder application (B-D) methods. (A) Fluorescence microscopy showing liposome (green) penetration of LN slice over time. Bathed face is indicated by white arrow. Cell nuclei are shown in the mock (DAPI, grey). Asterisks indicate fat deposits within the LN which likely facilitated liposome uptake. Scale bar represents 500 µm. (B) Comparison of liposome uptake (%

positive cells) between LN cell subsets after 2 h cylinder exposure, measured by flow cytometry (n = 2-4). Median + interquartile range is shown for cell subsets of the major groups: macrophages/monocytes (macs/monos), migratory dendritic cells (mig. DCs), resident dendritic cells (res. DCs) and lymphocytes. Mixed effects analysis with Tukey's multiple comparisons test was performed. (C) Proportion analysis of immune cell subsets (i) present in the LN (n = 50) and (ii) making up the total liposome+ fraction after 2 h exposure (n = 5), showing cell subsets as a percentage of total live, CD45+ immune cells and myeloid cell subsets as a percentage of HLA-DR+ cells. (D) Spearman's correlation of liposome uptake by various cell subsets via cylinder and bathing exposure."

3.3.2.2. CD169+ Sinus-Lining Macrophages Preferentially Take Up Liposomes Similar to AS01

"The CD169+ SMs, found both in the medullary sinuses and the subcapsular sinus had the highest capacity for liposome uptake at the single cell level [Figure 3.7D], likely due to the superficial position of the subcapsular SMs, lining the large peripheral sinuses of the LN [Figure 3.7F] and also their innate capacity for particle uptake. The accumulation of liposomes in the cytoplasm of CD169+ subcapsular SMs was confirmed by microscopy [Figure 3.7G]. The remaining macrophage and monocyte subsets also had a relatively high capacity for liposome uptake, especially monocyte-derived DCs (MDDCs). Of the DC populations, cDC2s were more efficient at liposome uptake than cDC1s, with migratory dermal cDC2s being better than resident cDC2s [Figure 3.7D]. pDCs took up very little liposome. This hierarchy is consistent with the generally documented phagocytic capacity of these cells (318). These results are also consistent with reports in mice highlighting the role of subcapsular SMs in the initial uptake of AS01 and the critical role of subcapsular SMs and cDC2s in the initiation of the immune response (18, 249)."

"Liposomes were poorly taken up by lymphocytes, with only a modest increase in fluorescence over 24 h [Figure 3.7C] and was likely only surface associated. Of the total liposome positive cells, HLA-DR+ antigen presenting cells (APCs) were over-represented, at 8.93 ± 7.03% (mean ± SD, n=5), despite representing only 1.55 ± 0.90% (mean ± SD, n=49) of live CD45+ cells in the LN [Figure 3.7E]. Myeloid cells, particularly SMs, also preferentially took up liposomes compared to their proportion of the total cell population [Figure 3.7E]."

3.3.3. Optimisations of Cell Enrichment Protocols for Bulk RNA Sequencing

RNA sequencing would be an ideal technique to evaluate innate immune cell responses to vaccine adjuvants in the LN explant model in more depth. However, with innate immune cells accounting for only 1.55 ± 0.90% (mean ± SD, n=49) of live CD45+ cells in the LN (Figure 3.7E), performing RNA sequencing on the entire LN cell population risks overshadowing differences in gene expression related to innate immune responses, with genes related to the bulk lymphocyte population.

As performing fluorescence-activated cell sorting (FACS) of APCs on the whole cell population from fresh LNs was not efficient due to the large proportion of lymphocytes (Table 3.1), we initially considered several magnetic affinity cell sorting (MACS) methods to enrich APCs from fresh LNs prior to FACS. Considering that some macrophage subsets are inherently magnetic (319-322), the sequence in which MACS was performed was vital. Our first approach involved CD14+ selection, which includes macrophages, followed by a combined CD3+ and CD19+ depletion on CD14- cells. However, subjecting cells to multiple column-based bead selections may have been too harsh on rare populations. As a result, the CD14-CD3-CD19- depleted cells lacked the intended DC subsets. Alternatively, DCs may be closely interacting with and conjugated to T cells which may result in their depletion within this protocol (Table 3.2). A single HLA-DR+ bead selection was attempted, but as LN B cells are HLA-DR+, they were also selected out, resulting in insufficient APC enrichment (data not shown). Ultimately, a Myeloid DC Isolation Kit successfully enriched LN APC populations through negative selection, yielding an enhanced population of cDC1s, cDC2s, and pDCs (Table 3.3), despite pDCs not being targeted by the kit (Figure 3.9).

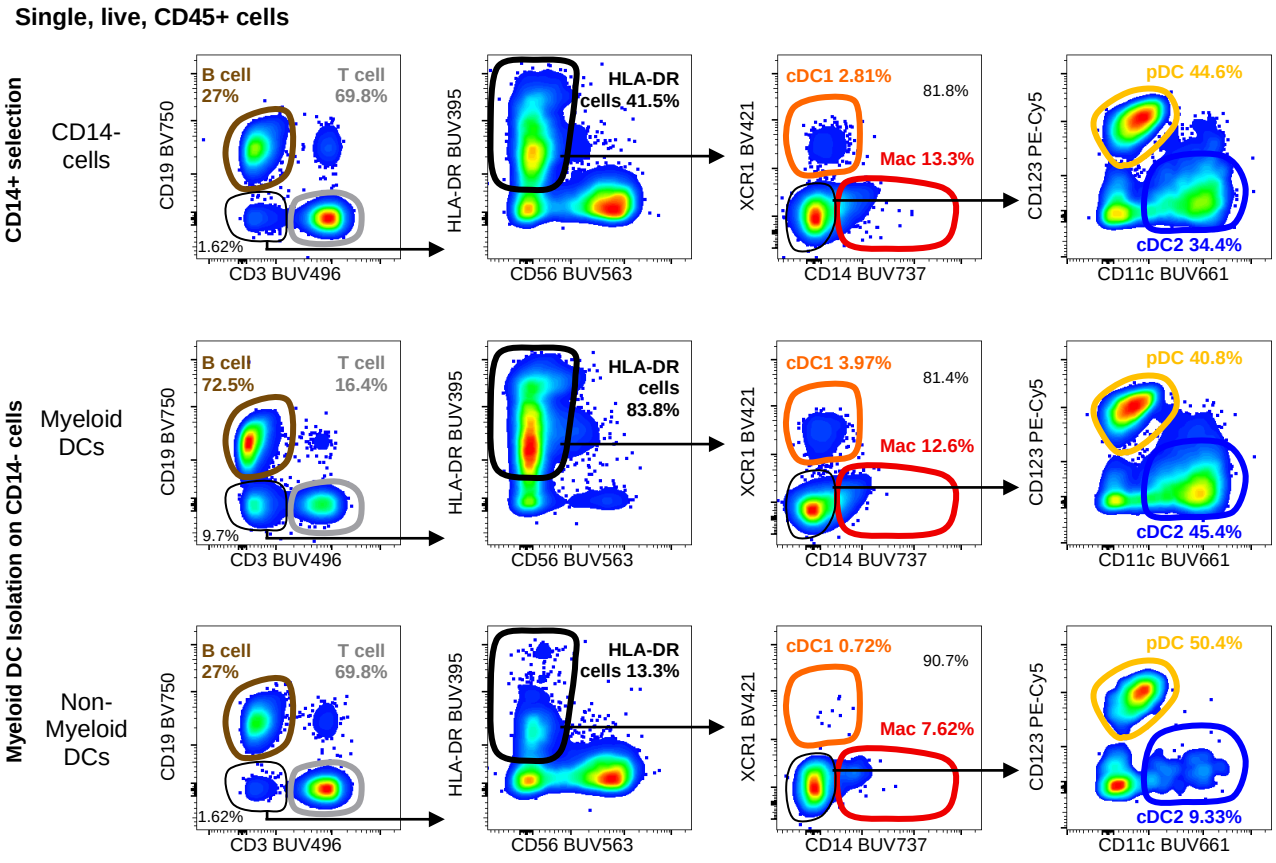


Figure 3.9: Enriching LN macrophage and DCs using a Myeloid DC Isolation Kit. Cells were isolated from whole LNs by mechanical dissociation and a CD14+ selection completed to isolate macrophages and monocytes. A Myeloid DC isolation kit was used to enrich LN DCs from the CD14- cells. Representative flow cytometry plots comparing LN cell subset frequencies of the parent gate is presented at each stage of the enrichment process.

Given that the Myeloid DC Isolation Kit proved to be the most effective method for enriching the LN APCs of interest, we proceeded to attempt this enrichment protocol on cells isolated post *in situ* adjuvant stimulation using the LN explant model. LN slices were stimulated for 8, 12, or 16 hours, with the aim of performing bulk RNA sequencing on isolated cells to identify the optimal timepoint for upregulation of genes related to innate immune functions. However, when the protocol was applied in this context, no viable APCs remained (data not shown).

Due to the relatively small number of cells isolated from LN slices post *in situ* adjuvant stimulation, which likely contributed to the lack of APCs following the MACS protocol, performing pre-sort selections is not ideal, hence direct FACS was further attempted. This led to sufficient numbers of sorted live, CD3-CD19-HLA-DR+ APCs (Table 3.4) and yields of RNA following extraction protocols. To assess RNA quality, the RINe number was used which is calculated on a scale from 1-10 via TapeStation analysis. Here, intact RNA is indicated by a high RINe whereas severely degraded RNA is indicated by a low RINe. Although sufficient RNA yields were obtained, the RNA quality was ultimately too poor to proceed with bulk RNA sequencing without further treatments, as all samples were below the recommended RINe number of 8 (Table 3.4). RINe numbers for cells isolated from *in situ* cultured LN slices without any pre-selections or FACS protocols, were improved however still not ideal for directly performing bulk RNA sequencing (Table 3.5).

Table 3.1: Fluorescence activated cell sorting on cells from fresh LNs with no pre-selections (n = 1).

Starting cell number	Cell subset	Cell yield post sort
55×10^6 Sorted 15×10^6 from the above	CD169+ SM	7,500
	CD169- MM	2,500
	cDC1s	1,300
	pDCs	49,000
	Lang+ cDC2s	1,000
	Lang- cDC2s	1,200

Table 3.2: Fluorescence activated cell sorting on cells from fresh LNs after CD14+ selected, where CD14- cells were subsequently CD3/19 depleted (n = 2).

Starting cell number	Cell subset	**Cell** yield range **post sort**
Cells not counted prior to sorting	CD169+ SM	1,486 - 46,200
	CD169- MM	700 - 66,200
	cDC1s	26 – 429
	pDCs	184 – 733
	Lang+ cDC2s	28 – 358
	Lang- cDC2s	26 – 110

Table 3.3: Fluorescence activated cell sorting on cells from fresh LNs which were Myeloid DC selected (n = 1).

Starting cell number	Cell subset	Cell yield post sort
CD14+ selection	CD14+ cells	500,000
Cells not counted prior to sorting	cDC1s	2,480
	pDCs	16,291
	Lang+ cDC2s	5,057
	Lang- cDC2s	13,077

Table 3.4: Fluorescence activated cell sorting on cells from *in situ* cultured LN slices with no pre-selections (n = 4).

Starting cell number	Cell subset	Cell yield range post sort	RNA conc (pg/uL)	RNA RINe
$1.92 \times 10^6 – 12.24 \times 10^6$	HLA-DR+ cells	1,317 – 32,669	15 - 36	0
	CD3+ T cells	70,000 – 400,000		
	CD19+ B cells	20,000 – 157,360		
	Combined CD3/19+	29,407 – 206,000	18.2 - 854	0 – 4.8

Table 3.5: RNA extractions on cells from *in situ* cultured LN slices with no pre-selections or FACS sorting (n = 4).

Cell number	RNA conc (pg/uL)	RNA RINe
$0.81 \times 10^6 – 2.6 \times 10^6$	4,840 – 11,300	4.6 – 6.6
$0.645 \times 10^6 – 16.62 \times 10^6$	4,030 – 91,400	2.4 – 7.7

3.4. Discussion

Within this study, a successful explant model was generated to culture slices of human LN tissue for analysis of innate immune cells. Our model uses larger LN slices containing all tissue compartments which are intact with the stromal cell network and full repertoire of innate and adaptive immune

cells (Figure 3.10). This allows for the study of initial events to understand how innate immune responses are triggered by vaccines and adjuvants, and subsequently shape the adaptive immune response.

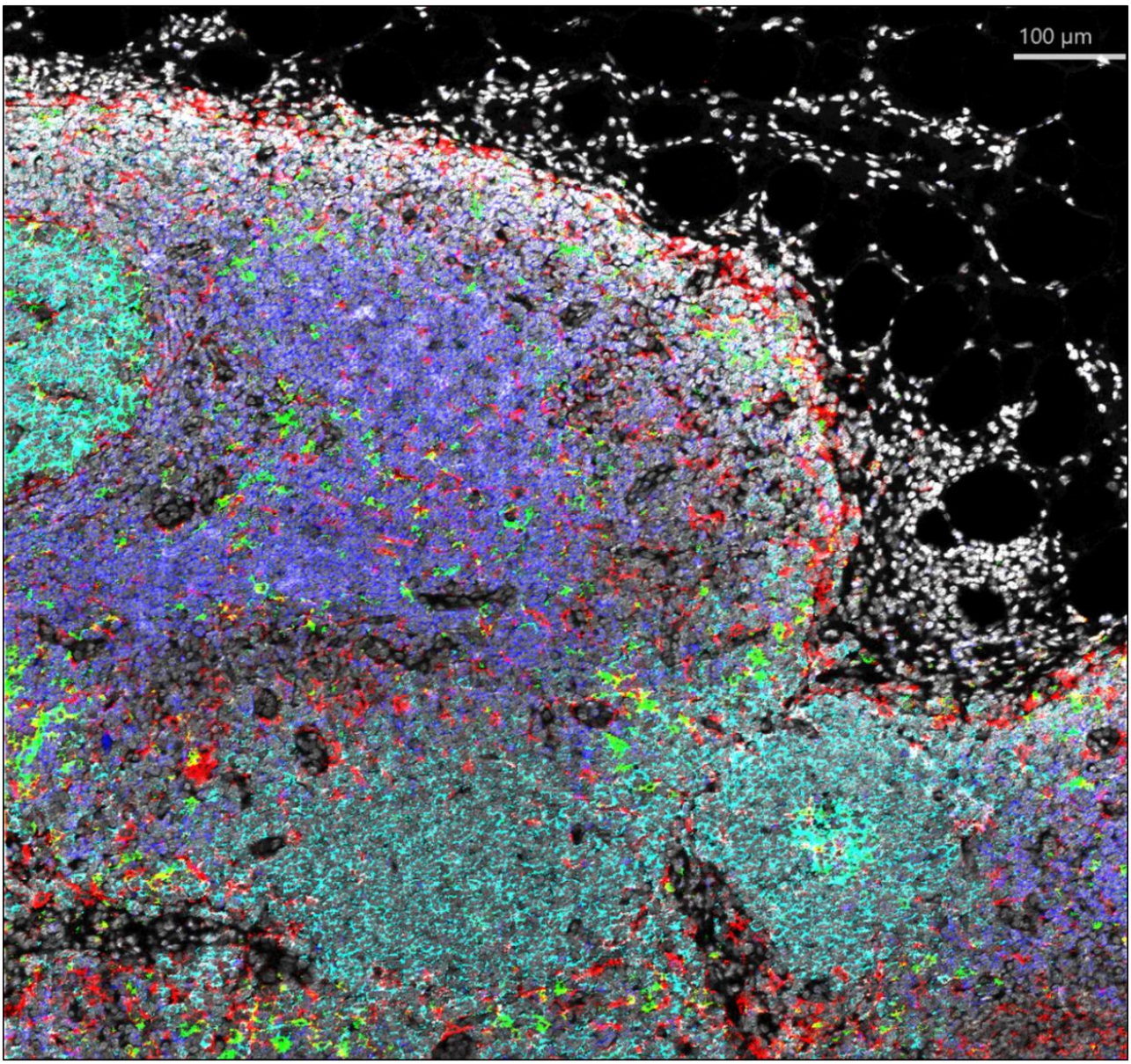


Figure 3.10: All LN compartments and cells are represented within cultured explants. LN slices were cultured in explant models and sections were further assessed by imaging mass cytometry. Clear B cell (CD20, cyan) and T cell (CD3, blue) zones are discernible with infiltrating APCs (CD11c, red and HLA-DR, green) and other myeloid cells, namely macrophages, lining the external capsule and subcapsular sinus (CD11c, red). Cell nuclei are shown in white. This figure was not produced by the thesis author. LN explants were performed by the thesis author however processing and analysis for IMC was conducted by Dunn, E.

Here, we have demonstrated that innate immune cells in our model can successfully be detected and analysed following administration of empty liposomes equivalent to those containing the AS01 adjuvant. “Particles approximately 10 – 100 nm in size can flow freely to the LN via the lymphatics

(323) and AS01 is approximately 100 nm (324). Indeed in mice, QS-21 in liposomes drains to the lymph node via the afferent lymphatics within 30 minutes of administration and is taken up by $CD169^+$ macrophages that line the sinuses of the LN, including the subcapsular sinus, that are ideally positioned to sample lymph-borne antigen (249). These subcapsular SMs play an important role in transferring captured antigen to and activating B cells and produce an array of cytokines to coordinate multiple LN resident immune cells (27). The uptake of our empty liposomes by subcapsular SMs and also DCs is consistent with this and the pattern was the same whether liposomes were applied by a cloning cylinder to the external surface, or by bathing the entire cut surface of the explant. The latter may be explained by the size of the liposomes. Particles > 10 nm are too large to flow through the narrow lymphatic conduits to access the paracortex with its T cells and DCs (325) however, they can access the subcapsular SMs, DCs and other cells in the superficial interfollicular cortex via the wider peripheral sinus and limited percolation into the tissue. AS01, being slightly smaller than our empty liposomes, may penetrate deeper into the cortex and paracortex. Thus, the superior liposome uptake by CD169+ subcapsular SMs is likely to be due to a combination of their advantageous location and inherent endocytic capability."

Our model can also allow for the assessment of innate immune responses between old and young LNs following treatment with vaccines and adjuvants. "Ageing and immunosenescence have a detrimental effect on vaccine responses, with the efficacy of most vaccines being reduced in people over 65 YOA (123-126). AS01-adjuvanted vaccines have overcome this (133, 135) but the reasons why this adjuvant is effective in older adults are unknown. We found the immune constitution of old and young LNs was remarkably similar even with a 15 year age gap buffer. This is consistent with reports that the number and phenotype of circulating DCs is comparable in healthy older adults (326, 327), and young adults, apart from the frail elderly (106)." However, it is well documented that $CD8^+$ and naïve T cell subsets decrease with age, while memory cells increase (117, 328). As blanket T and B cells were assessed in this study, a further categorisation and analysis of the individual subsets within these cell populations could reveal differences otherwise undetected. Nevertheless, "[t]his similarity implies that any differences in adjuvant immune responses observed between young and old donors would likely be due to differences in the functional capacity or interactions of immune cell subsets or the structure of the LN between young and old, rather than differences in the frequency of individual cell populations."

Unlike other culture systems which can be sustained for 4 – 7 days, our model is optimal for shorter culture conditions to primarily focus on the functions of innate immune cells. However, "[t]he

viability of thick tissue explants is difficult to maintain *ex vivo*, with deeper parts of the tissue affected by hypoxia and diminished nutrient supply. The duration of viability of myeloid and lymphoid cells in our 2 mm thick LN slices varied but was consistent with findings in similar models (295, 301, 317) and sufficient to allow determination of their early function in response to various adjuvants. Smaller tissue blocks have been reported to remain viable for three weeks or more (298) but will often not contain the full gamut of sparsely distributed innate and stromal cells and be mostly composed of lymphocytes. Our model will require modification, such as judicious cytokine support or perfusion, to improve its longevity to allow establishment of germinal centres, which generally takes around 4-5 days. Secondary lymphoid organoids, derived from human tonsil have been described that develop functional germinal centres but the complex structure of the LN including the supporting stromal cells is likely not fully recapitulated and they do not have afferent lymphatics or migratory dendritic cells (297). Mechanisms of action of adjuvants on innate cells may be more suited to study in whole LN slices."

Future alterations to the model can be made to support tissue viability for longer culture periods and additionally include the analysis of adaptive immune functions. Although supplemented culture media with M-CSF and Flt-3L was assessed in this thesis, additional supporting cytokines such as IL-7 for T cells and IL-3 for pDCs as well as others such as GM-CSF for myeloid DCs, could be implemented to improve the viability of lymphocytes in the longer cultures of thick LN slices. Other alterations to the LN tissue processing protocol, such as the use of a Vibratome VT1200S, can be explored to assist with LN sectioning and increase reproducibility (329). This equipment has been used in many studies for sectioning ultrathin tissue slices (330, 331) however, functions are also available to generate sections as large as 2 mm. Improvements can be made to our explant system by assessing a range of thicker slices for LN sections, to identify the most optimal for long-term culture as well as preservation of all LN compartments and cells. Additionally, explant viability can be enhanced by using a hydrogel matrix as opposed to gel foam soaked in supplemented media. These gel matrices are predominately used to culture isolated cells in a 3D environment to allow spontaneous arrangement into a functional unit, such as a germinal centre (332). There are many options available such as Matrigel, which is an animal-derived hydrogel, as well as other synthetic options, and they mimic the extracellular matrix and could provide additional support to our LN explants (333). However ultimately, the effect of mechanical isolation compared to collagenase digestion methods was not assessed on cell viability over time. The viability of cells following *in situ* culture in our system at the later timepoints of 48 – 96 h, may be improved if collagenase IV

digestion was instead used as a gentler isolation method for these fragile cells isolated from longer cultures. Although the mechanical isolation method was originally prioritised as it was quicker and collagenase digestion methods are prone to cleaving surface markers, our lab has surveyed a wide range of enzymes and found collagenase IV avoids cleaving most markers (334), hence moving forward, collagenase digestion will be used instead. The combination of all these amendments to our culture model and protocols could provide better conditions for longer cultures to additionally assess adaptive immune responses involving germinal centre functions.

"Another important limitation of the model is the removal of the blood and lymph circulation. Peripheral immune cells can no longer enter the LN via the afferent lymphatics or high endothelial venules. In a vaccination setting, antigens and adjuvants can be transported by APCs, including DCs, monocytes, and neutrophils (190, 275, 335) from the site of administration to the draining LN and migrating monocytes and DCs contribute to the cytokine milieu *in vivo* (276). Our model is suited to studying vaccines and adjuvants that can flow freely to the LN." However, the addition of PBMCs into the explant culture media or matrix can be attempted to model infiltrating migratory cells in response to adjuvant treatments.

Furthermore, it is difficult to study vaccine and adjuvant innate immune cell pathways in our model in more depth, at the gene level. Alternate methods must be investigated to isolate RNA in sufficient quantities and quality for RNA sequencing. The poor RNA quality obtained from FACS sorted APCs maybe be due to the challenges associated with maintaining viability of thick LN explants in the model. Additionally, macrophages are known to harbour high amounts of RNAses for antimicrobial function in breaking down foreign RNA material (336-338). Upon cell lysis during RNA extraction, the high amount of RNAses from the enriched proportion of macrophages following FACS of APCs, may lead to the degradation of the extracted RNA. Although the quality of the RNA from whole LN cell populations isolated post *in situ* stimulation and directly subjected to RNA extraction was better than enriched APC samples via FACS, this was still too poor to perform bulk RNA sequencing. For these existing, poor quality RNA samples, an FFPE library prep kit can be explored prior to bulk RNA sequencing. These are optimised for working with low quality RNA from formalin-fixed, paraffin embedded tissue samples. Moving forward, low-input RNA sequencing protocols that can handle low volume and quality RNA (339), such as SMART-Seq or AmpliSeq (340), should be investigated, where APCs can be sorted directly into the kit's lysis buffer.

“The value of this human LN model is in testing the mechanism of action of vaccines and adjuvants in a human setting. This pre-clinical model holds the potential for comparisons of immunogenicity between different adjuvants and modifications of existing adjuvants by medicinal chemistry, as well as the comparison of modes of action of different vaccine technologies, such as adjuvanted, live attenuated and mRNA-based vaccines.”

Chapter 4. Characterising the Innate Immune Responses of AS01 and Other Adjuvants in Human Lymph Nodes

Manuscripts incorporated into this section:

Stylianou, V.V., Bertram, K.M., Dunn, E., Baharlou, H., Terre, D., Elhindi, J., Elder, E., French, J., Meybodi, F., Hsu, J., Temmerman, S., Didierlaurent, A.M., Coccia, M., Sandgren, K.J., Cunningham, A.L. Innate immune cell activation by adjuvant AS01 in human lymph node explants is age-independent. Currently under review with Journal of Clinical Investigation.

This chapter includes data from the above manuscript, indicated with "quotation marks", which has been submitted for publication and is currently under review. Figure numbers have been adjusted to include supplementary material from the manuscript within the main text of this thesis, as indicated by the boxed brackets "[]". The author of this thesis contributed significantly. All experiments were performed by the author of this thesis and in some cases, with the assistance from co-authors. Sandgren, J.K., Bertram, M.K. and Cunningham, A.L guided all experiments.

4.1. Introduction

"Incorporation of adjuvants into subunit vaccines has markedly increased the long-term immunogenicity and efficacy of these vaccines, particularly in ageing and immune compromised populations. An excellent example is the Recombinant Zoster Vaccine, (Shingrix, GSK) consisting of recombinant varicella zoster virus glycoprotein E (gE) and the adjuvant system $AS01_B$, with high efficacy in older adults (≥50 years of age; YOA), even for those over 80 YOA, for at least ten years (131-133). Phase I/II studies showed that the absence of AS01 reduced gE-specific CD4 T cell responses >10 fold in those >70 YOA (266). AS01 consists of a TLR4 agonist, monophosphoryl lipid A (MPL), and the saponin QS-21 formulated in liposomes. The immunostimulants in AS01 act synergistically in mouse models to enhance CD4 T cell responses (232). However, Shingrix, as shown for other adjuvanted vaccines, presents an increased incidence of local and systemic symptoms occurring shortly after vaccination. 9-11% of Shingrix recipients experience reactions that prevent daily life activities although these only last for 2 to 3 days (341). However, such reactogenicity is not correlated with immunogenicity (342), suggesting that adjuvants can be modified or developed to retain immunogenicity but with lower reactogenicity."

"In order to achieve this, the exact mechanism of action in human subjects needs to be elucidated. For AS01, extensive studies have been conducted in mouse models, showing the rapid transit of AS01 and associated antigens to lymph nodes (LNs) where the onset of the immune response occurs (275). There the immune stimulants are taken up by sinus-lining macrophages, stimulating caspase-1 activation and IL-18 production. Early activation of macrophages initiates a cascade of immune

responses including an early burst of interferon gamma (IFN-γ) from natural killer (NK) and $CD8^+$ T cells in an IL-18 and IL-12 dependent manner. This culminates in dendritic cell (DC) activation and presentation of antigen to T and B cells, measured by a marked increase in antigen-specific antibody and polyfunctional $CD4^+$ T cells (18, 87, 249, 275). Some of these events have been confirmed in non-human primate LNs and human blood (18, 343). However, there are many differences between human and murine immune processes, including in LN (12, 344) and findings in mice should be validated in humans."

"Therefore, we have developed an *ex vivo* human LN explant model to study the mechanism of action of vaccine adjuvants, including AS01, for which abundant data exist in animal models. We found that liposomes of similar composition to AS01 were preferentially taken up by $CD169^+$ sinus-lining macrophages and DCs. AS01 induced maturation of DCs and the production of an array of pro-inflammatory cytokines, including IL-1β, IL-18 and IFN-γ, in intact LN slices but not when LN cells were dissociated from tissue. Unlike the LN response to other adjuvants, the response to AS01 was relatively independent of the adult LN donor's age which may underly the remarkable efficacy of AS01-formulated vaccines in older adults (132, 135)."

4.2. Methods

4.2.1. Adjuvant Stimulation of Isolated Human Lymph Node Cells *In Vitro*

LN DCs were isolated using a PanDC Isolation Kit as described in section 2.3.3 and stimulated *in vitro* with adjuvants as described in section 2.4. Additionally, 25 μg/mL QS-21 was also assessed compared to the original adjuvants tested.

4.2.2. Adjuvant Stimulation Time Course in the Human Lymph Node Explant Model

LN slices were cultured as described in section 2.4. In addition to AS01, MPL (GSK and InvivoGen), R848 and Pam2Cys, QS-21 adjuvant was also tested at 25 μg/mL. For cytokine kinetics time course experiments, 100 μL of culture supernatant was collected from the explant model at each timepoint of 4-, 8-, and 15-hours and the same volume of fresh media was replenished back into the explant. Following the full 24 hours of culture, the remaining culture supernatant was collected. All supernatants were stored at -80°C for future cytokine immunoassays.

4.3. Results

4.3.1. The Optimal Timepoint for Assessing Innate Immune Reponses in the LN Explant Model

Kinetics of Adjuvant-induced Cytokine Release in LN Explant Model

During the development of the LN explant model, cultures were assessed for viability following 24-hour incubations, with or without AS01. Here, we assessed if this timepoint was also optimal in detecting innate immune responses to AS01, MPL, QS-21, R848 and Pam2Cys by measuring the kinetics of production of key pro-inflammatory cytokines. For AS01, pro-inflammatory cytokines including IL-1β, IL-18, IL-8 and TNF were detected as early as 8 h, however production for the majority of cytokines peaked at 24h. Notably, IFN-γ was not detected before 24h (Figure 4.1). The pattern of cytokine release for QS-21 was very similar to AS01. MPL however, barely induced any cytokines, at any timepoint, apart from low levels of IL-1β and IL-18. This was regardless of the source of MPL (Figure 4.1). R848 was the most potent adjuvant, inducing IFN-α and TNF as early as 4 h. Other pro-inflammatory cytokines were strongly detectable by 8 h with most increasing up to 24 h. IL-23 and IFN-α did show signs of peaking at 15 h then dropping off by 24 h however, the nature of sampling has implications here (Figure 4.1). Due to the need to replenish the medium during sampling, each timepoint was more accurately a measure of the amount of cytokine produced in the previous period, rather than the cumulative amount produced over the entire culture. Therefore, for each adjuvant we can conclude the peak concentration of all cytokines would be detected at 24 h, although production of IL-12 and IFN-α may have slowed in response to R848 by this time. 24 h was therefore chosen as the primary timepoints for sampling in future experiments. This is necessary to allow the generation of the complete inflammatory cascade, including IFN-γ, in response to the key adjuvant of interest, AS01.

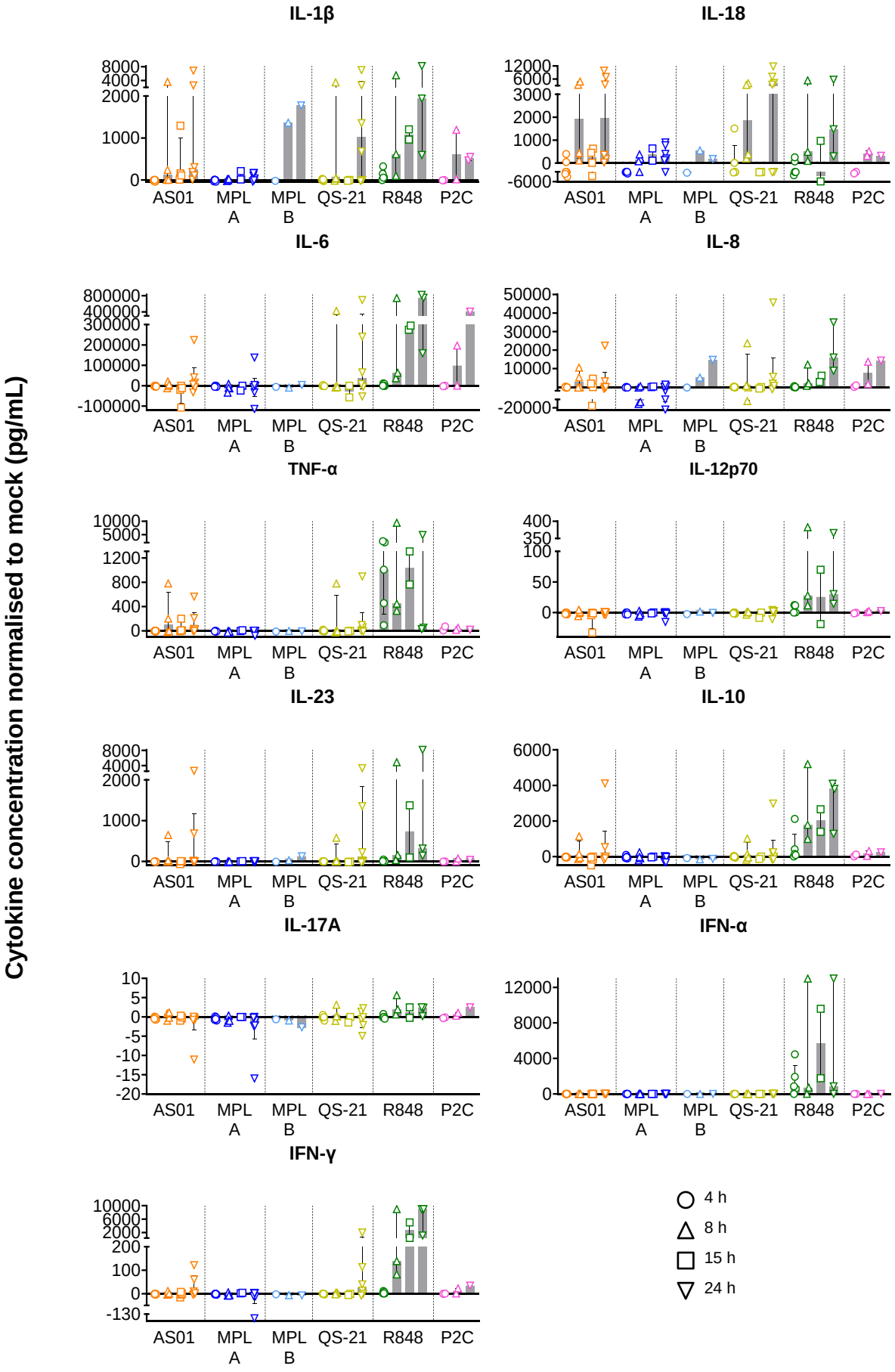

Cytokine concentration normalised to mock (pg/mL)
IL-1β
IL-18
IL-6
IL-8
TNF-α
IL-12p70
IL-23
IL-10
IL-17A
IFN-α
IFN-γ
AS01
MPL A
MPL B
QS-21
R848
P2C
4 h
8 h
15 h
24 h

Figure 4.1: Time course of cytokine kinetics following AS01 stimulation in situ in intact LNs. Slices of human LNs were bathed in adjuvants AS01 (orange, n = 3-7), MPL A (GSK, blue, n = 2-6), MPL B (InvivoGen, light blue, n = 1), QS-21 (yellow, n = 2-6), R848 (green, n = 2-5) or Pam2Cyc (pink, n = 2) for 24 h. Half the volume of culture supernatants were collected at 4, 8, 15 and 24 h and media replenished at each point prior to continuing culture. Cytokine concentrations in culture supernatants were determined by LEGENDplex and data presented as mock subtracted. IL-6 and IL-8 were measured by ELISA.

4.3.2. AS01 Induces Maturation of Dendritic Cells, but Only in Intact Human Lymph Nodes

"A key property of an adjuvant is the capacity to enhance activation of APCs. We assessed whether AS01 or other Toll-like receptor (TLR) ligand adjuvants – R848 (TLR7/8), MPL (a component of AS01; TLR4) and Pam2Cys (TLR2) – induced maturation of LN myeloid cells and activation of NK cells and lymphocytes. Initially, to clarify the direct effect of AS01 on immune cells, cells were mechanically dissociated from LN tissue and stimulated as a mixed population with AS01 *in vitro* for 24 h. MPL and R848 were included as comparators." A range of concentrations were tested for each adjuvant (data not shown) however even at the highest concentration of 25 µg/mL, "AS01 induced no or very weak upregulation of the maturation markers CD80 and CD86 on dissociated DCs compared to donor matched control samples. AS01 also did not activate lymphocytes and NK cells, measured by CD69 upregulation [Figure 4.2]. MPL was similarly non-stimulatory *in vitro* at an equivalent concentration to the MPL component of AS01. In contrast, R848 was potent at inducing upregulation of CD86 on macrophages, cDC2s ($CD14^{-}CD11c^{+}$ cells) and pDCs, and in 4/6 cDC1 donors, as well as CD69 upregulation on NK, NK-T, T and B cells [Figure 4.2]." Enzymatic digestion using collagenase type IV was also attempted to ensure all LN cell subsets were being isolated for *in vitro* stimulations, however no differences were observed in the proportions of isolated cells and AS01 responses remained undiscernible *in vitro* (data not shown). Furthermore, cells were also cultured at higher concentrations, up to 10 x 10^6/mL, to mimic the high cell density within the LN, however this also did not result in AS01- or MPL-induced cellular activation (data not shown).

To confirm that AS01 does not directly activate DCs, we isolated DCs from the LN using a PanDC Enrichment Kit to directly stimulate these cells to adjuvants *in vitro* (Figure 4.3A). Dead cells were first removed using a non-column-based magnetic kit prior to the enrichment of DCs, to ensure the isolated cells were healthy and eliminate this as a possible confounder in the DCs ability to mature in response to adjuvant stimulation. Here, the viability of the LN cells significantly increased to 79.58 ± 5.56% (mean + SD) compared to the initial viability of 64.08 ± 11.57% (mean + SD) upon mechanical isolation from the LN tissue (Figure 4.3B). Following enrichment, the proportion of CD3-CD19-HLA-DR+ APCs was enhanced to 42.73 ± 26.23% (mean + SD) compared to the normal constitution of just

1.07 ± 0.26% (mean + SD) in the LN. Here, cDC2s and pDCs constituted the majority of the APC population, however cDC1s were also enriched. Macrophages/monocytes were depleted out by the PanDC enrichment (Figure 4.3C). Despite directly exposing healthy, enriched DCs to AS01, no up-regulation of co-stimulatory molecules CD80, CD83 and CD86 was observed. Individual adjuvants, MPL and QS-21, were also directly compared to AS01 and neither appeared to induce direct maturation of DC subsets. On the other hand, R848 directly upregulated CD80 and CD86 on pDCs *in vitro* (data not shown).

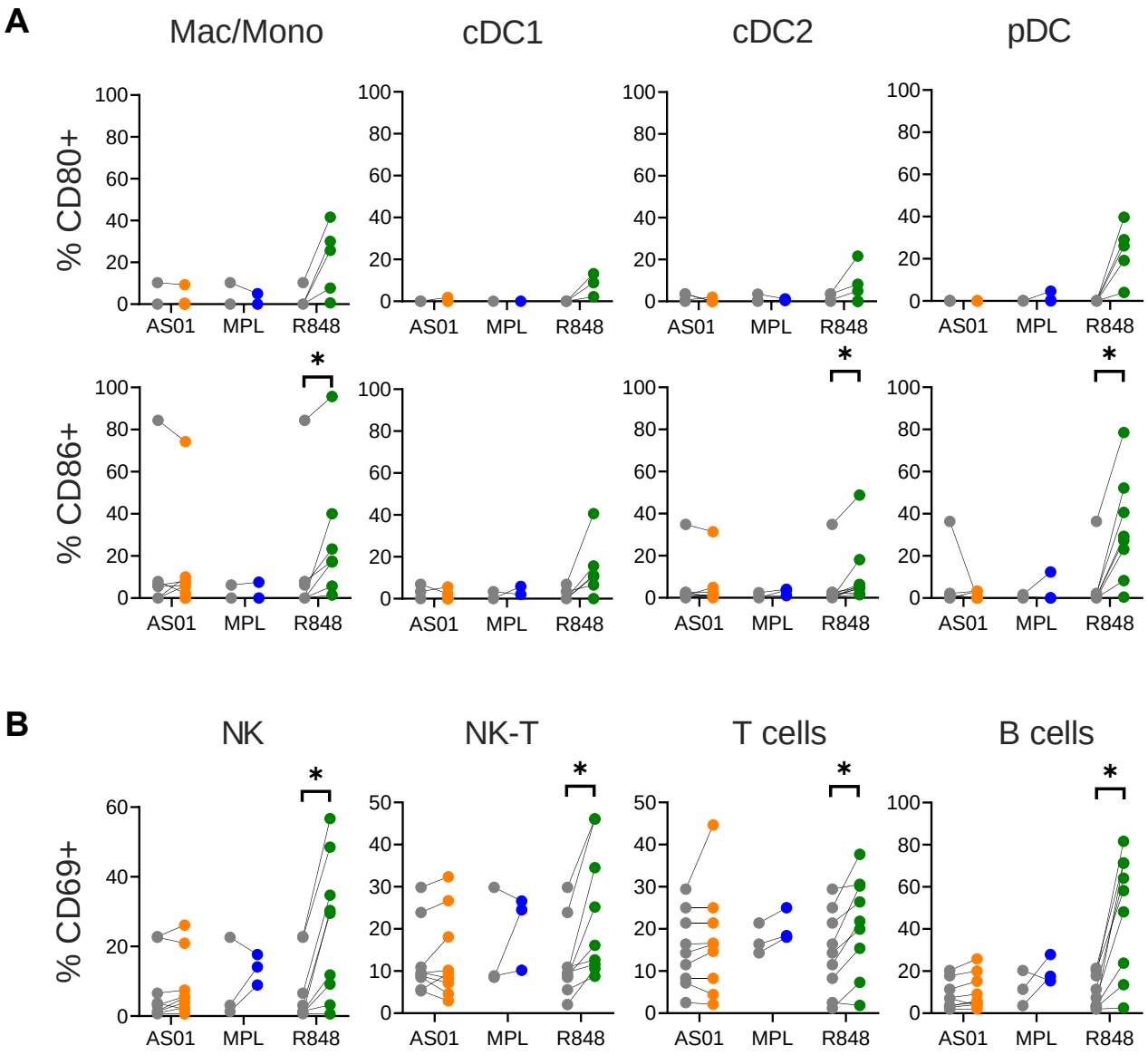


Figure 4.2: "AS01 does not induce maturation of macrophages and dendritic cells or activation of lymphocytes *in vitro* in dissociated human LN cells." "Cells from whole human LNs were mechanically dissociated and stimulated *in vitro* for 24 h with adjuvants AS01 (orange), MPL (blue) and R848 (green) or mock treated (grey). The percent of **(A)** macrophage and dendritic cell populations expressing maturation markers CD86 (AS01 n = 6-9, MPL n = 2-3, R848 n = 6-9) and CD80 (AS01 n = 3-6, MPL n = 1-3, R848 n = 3-6), and **(B)** NK cells and lymphocytes expressing the early activation marker CD69 (AS01 n = 9, MPL n = 3, R848

n = 9) were assessed by flow cytometry. Wilcoxon matched-pairs signed rank tests were applied with Bonferroni-Dunn correction for multiple comparisons. * p < 0.05."

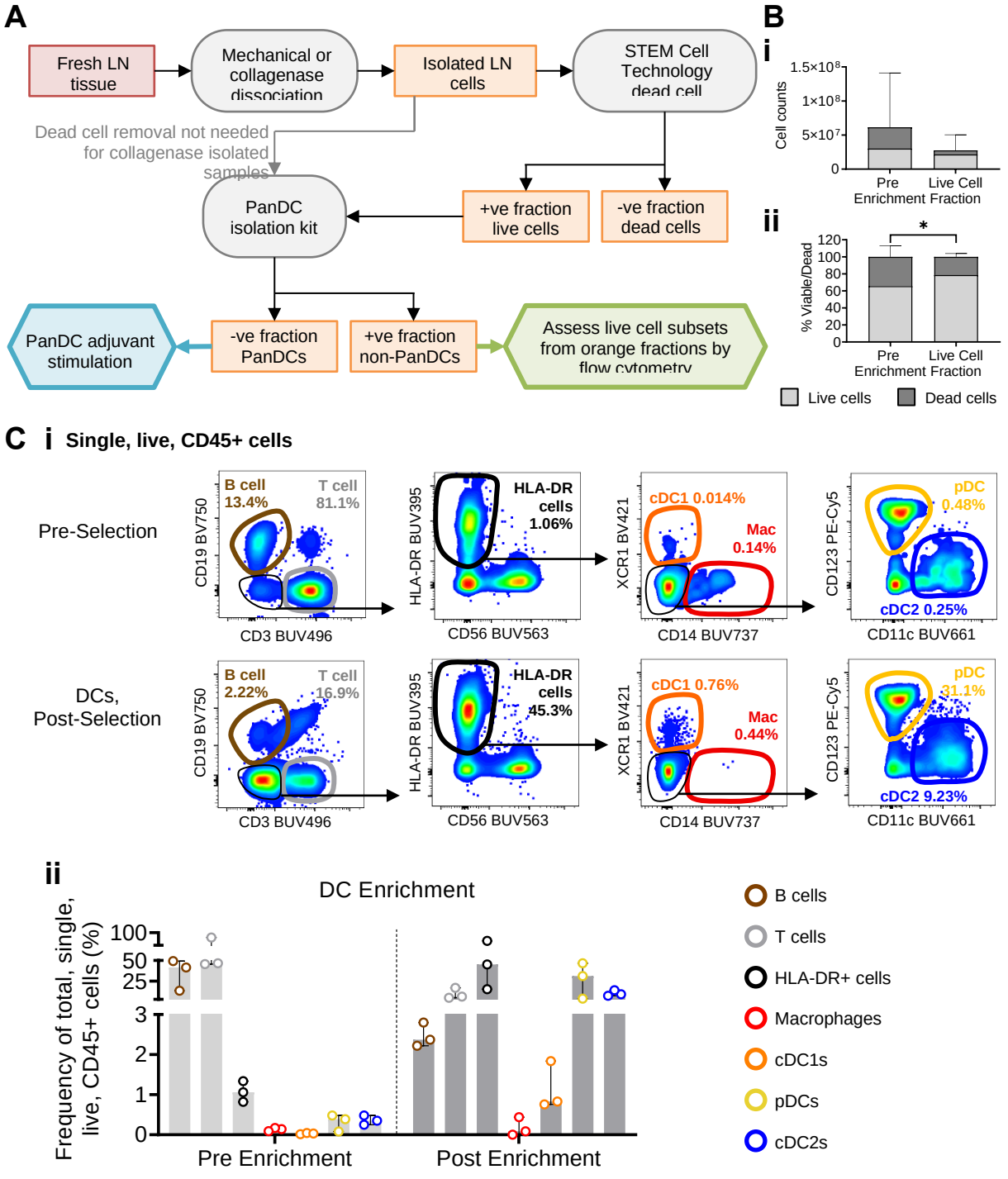


Figure 4.3: AS01 stimulation of isolated LN DCs in vitro does not induce maturation of DCs. (A) Flow diagram of the methods used for isolation and culture of LN DCs. **(B) (i)** Raw cell counts of live and dead cells within cell fractions pre-, vs the live cell fraction post-, dead cell removal. **(ii)** Cell counts of live and dead cells in the dead cell removal fractions, presented as % viability of total cells in each fraction. Stats done. Two-way ANOVA with multiple comparison using the Bonferroni method were applied where * p < 0.05. **(C) (i)** Representative flow cytometry plots of the cell subset frequencies of total, live, CD45+ cells within each

fraction for the dead cell removal and PanDC enrichment protocols. **(ii)** Cell subset frequencies of total, live CD45+ cells, comparing cell compartments pre- and post PanDC enrichment.

"In contrast to the *in vitro* results, AS01 did induce maturation of cDCs when intact LN slices were exposed *in situ* for 24 h. We observed significant upregulation of CD83 and CD86 on cDC1s and CD80 and CD83 on cDC2s [Figure 4.4, Figure 4.5A]. CD86 and CD83 were significantly upregulated when resident cDC2s were analysed by subsets, including langerin^{+} and langerin^{-} subsets [Figure 4.6A]. In some donors, rare DC subsets, such as cDC1s, could not be detected. Higher concentrations of AS01 tended to reduce cell viability [Figure 3.3D] as well as the maturation response [Figure 4.6B]. Unlike AS01, MPL, R848 and Pam2Cys did not consistently induce maturation of cDCs *in situ*, although R848 did mature pDCs, increasing their expression of CD86 [Figure 4.5A], and activated both NK and B cells [Figure 4.5B]. The results of these two experiments show that AS01 induces maturation of cDCs but only when the LN structure is intact. This suggests that rather than directly activating DCs, AS01 induces this effect via an amplifying immune cascade that requires not only the presence of multiple cell types, but critically, also the native structural organisation of the LN. It also shows that lymphocytes are not directly activated by AS01. Conversely R848 was more effective in directly activating TLR7/8-expressing cells *in vitro* than in the tissue, while MPL and P2C did not induce cellular maturation or activation."

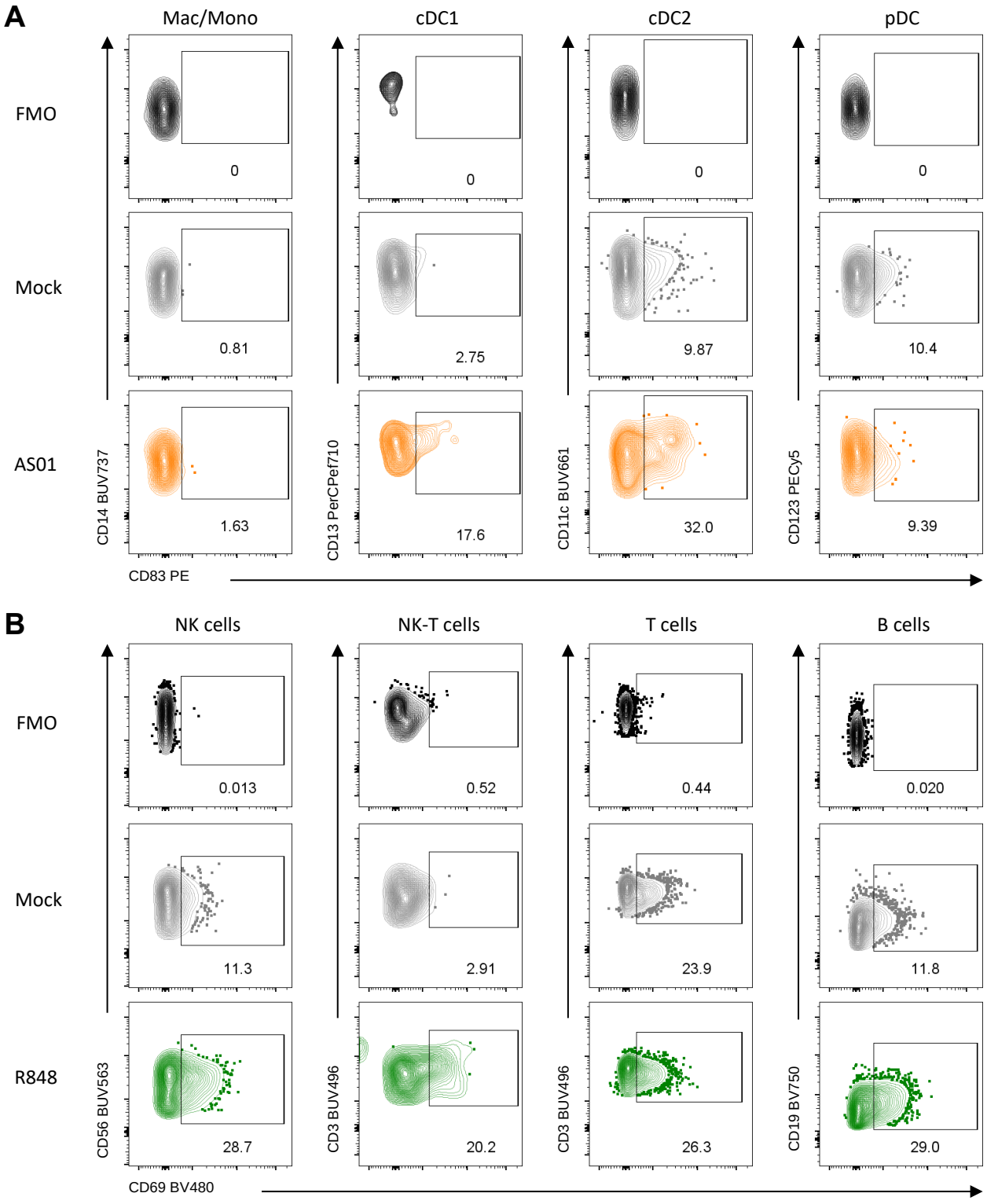


Figure 4.4: Effect of AS01 and R848 on activation of lymph node immune cells *in situ* Slices of human LNs were bathed in adjuvants AS01 (orange) and R848 (green) or mock treated (grey) for 24 h. Cells were then mechanically dissociated from the LN tissue and the percent of maturation assessed compared to an FMO control (black). Representative flow cytometry plots are presented for maturation marker gating for each cell subset. (A) CD83 maturation marker gating following AS01 stimulation and based on Mock and FMO samples. (B) CD69 activation marker gating following R848 stimulation and based on Mock and FMO samples.

Representative plots in (A) and (B) are from two different donors. FMO = fluorescence minus one control samples.

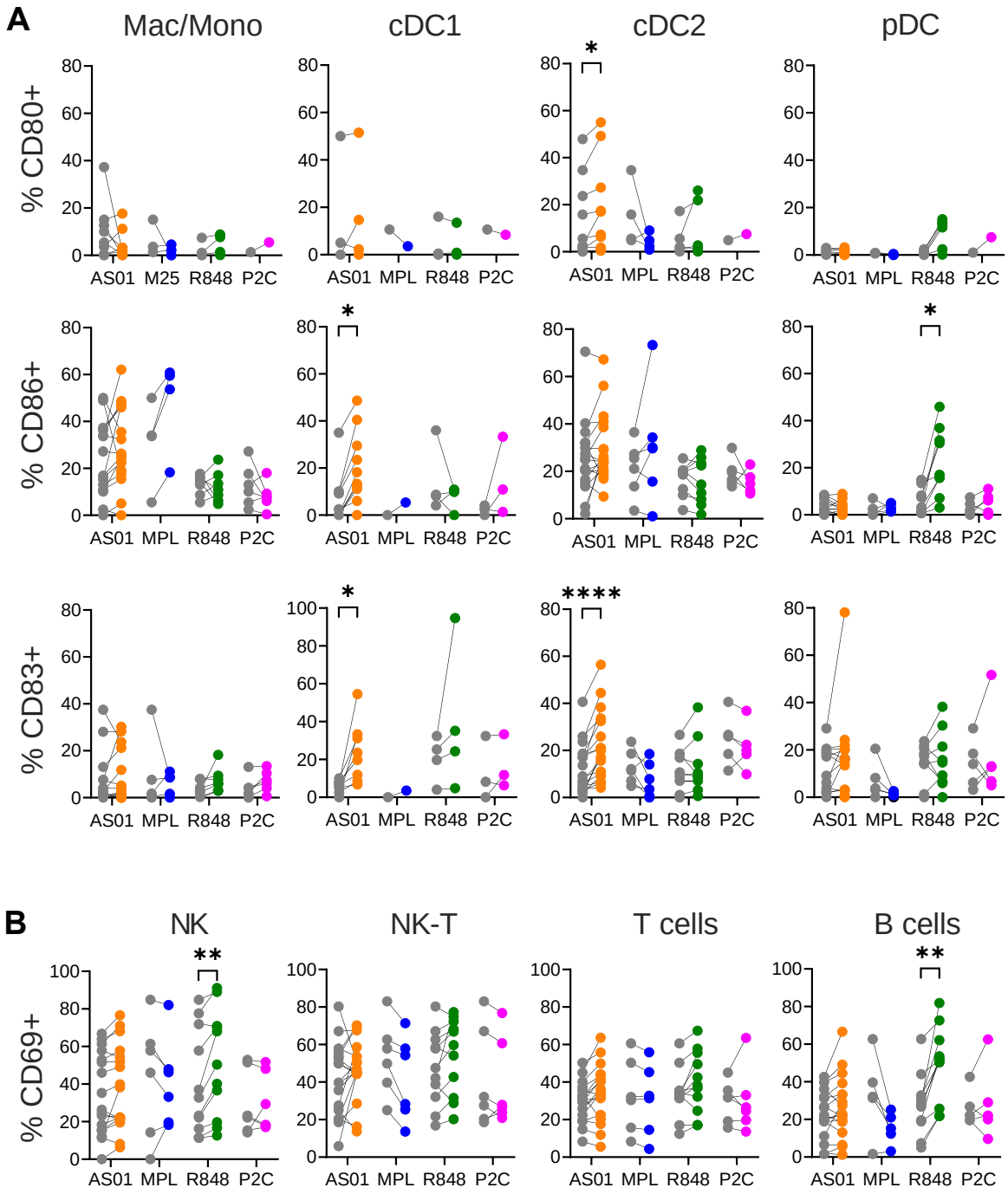


Figure 4.5: "AS01 induces maturation of both type I and type II conventional dendritic cells *in situ* in intact human LNs." "Slices of human LNs were bathed in adjuvants AS01 (orange), MPL (blue), R848 (green), Pam2Cys (pink, P2C) or mock treated (grey) for 24 h. Cells were then mechanically dissociated from the LN tissue and the percent of **(A)** macrophage/monocyte and dendritic cell populations expressing maturation markers CD80 (AS01 n = 3-10, MPL n = 1-4, R848 n = 3-6, Pam2Cys n = 1), CD83 (AS01 n = 8-17, MPL n = 1-6, R848 n = 4-9, Pam2Cys n = 3-6) and CD86 (AS01 n = 10-17, MPL n = 1-6, R848 n = 4-9, Pam2Cys n = 3-6), and **(B)** NK cells and lymphocytes expressing the early activation marker CD69 (AS01 n = 17, MPL n = 6, R848 n =

11, Pam2Cys n = 6) were assessed by flow cytometry. Wilcoxon matched-pairs signed rank tests were applied with Bonferroni-Dunn correction for multiple comparisons. * p < 0.05, ** p < 0.01, **** p < 0.0001."

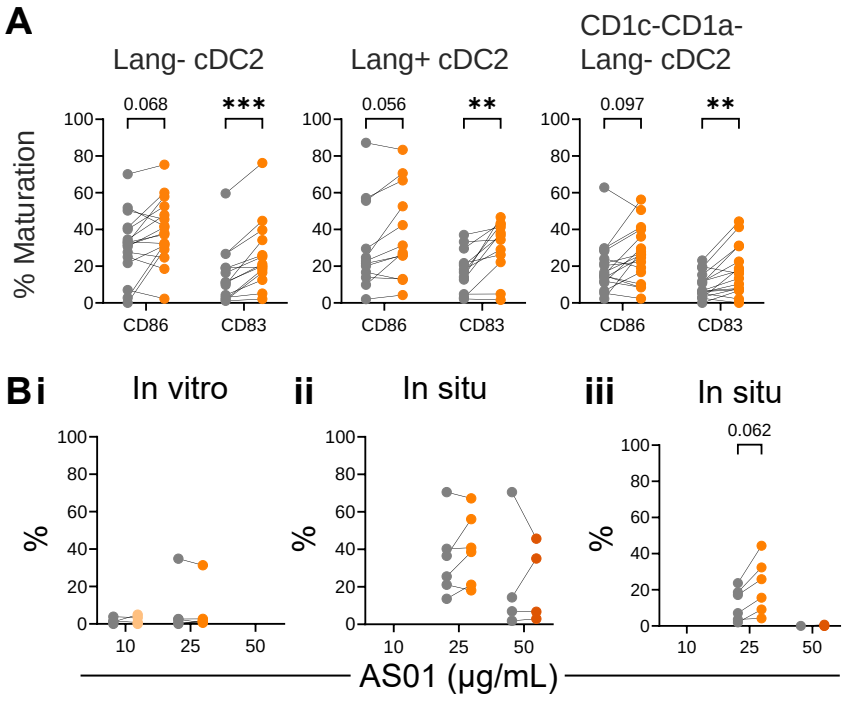


Figure 4.6: "AS01 induces maturation of subsets of resident type II conventional dendritic cells, only *in situ* at the optimal concentration." "Slices of human LNs were bathed in adjuvants AS01 (orange) or mock treated (grey). Cells were then mechanically dissociated from the LN tissue and the percent of different dendritic cell populations expressing maturation markers CD83 and CD86 was assessed via flow cytometry. **(A)** Maturation in cDC2 subsets after *in situ* exposure to 25 µg/mL AS01 (n = 8-17). Analyses by Wilcoxon matched-pairs signed rank tests were applied with Bonferroni-Dunn correction for multiple comparisons. * p < 0.05, ** p < 0.01, *** p < 0.001. **(B)** Titration of AS01 showing expression of **(i)** CD86 *in vitro* (10 µg/mL n = 4; 25 µg/mL n = 6); **(ii)** CD86 *in situ* (25 µg/mL n = 6; 50 µg/mL n = 4); **(iii)** CD83 *in situ* (25 µg/mL n = 6; 50 µg/mL n = 3) on all CD11c+ cDC2s."

4.3.3. AS01 Induces Pro-Inflammatory Cytokines, but Only in Intact Human Lymph Nodes

"We assessed the production of pro-inflammatory cytokines in response to the four adjuvants AS01, R848, MPL and Pam2Cys, when dissociated LN cells were exposed *in vitro* and when whole LN slices were exposed *in situ*. As with maturation, no pro-inflammatory cytokines could be consistently detected after AS01 stimulation of the total dissociated cell population *in vitro* [Figure 4.7]. IL-1β was detected in 4/10 donors. Conversely, and consistent with its *in vitro* effect on maturation, R848 did induce inflammatory cytokines IFN-α, IL-1β, -18, -6, -8, and TNF as well as anti-inflammatory IL-10 and showed a trend for the induction of IFN-γ in 5/8 donors. MPL was less inflammatory, significantly inducing IL-6, and -8 and inducing an increase in IL-1β and -18 in 5/7 donors [Figure 4.7]." These findings were consistent across a range of adjuvant concentrations, increased cell

densities, and whether mechanical or collagenase digestion methods were used (data not shown). Cytokine profiles of supernatants from enriched DC cultures stimulated with these adjuvants were consistent with total cell cultures (data not shown).

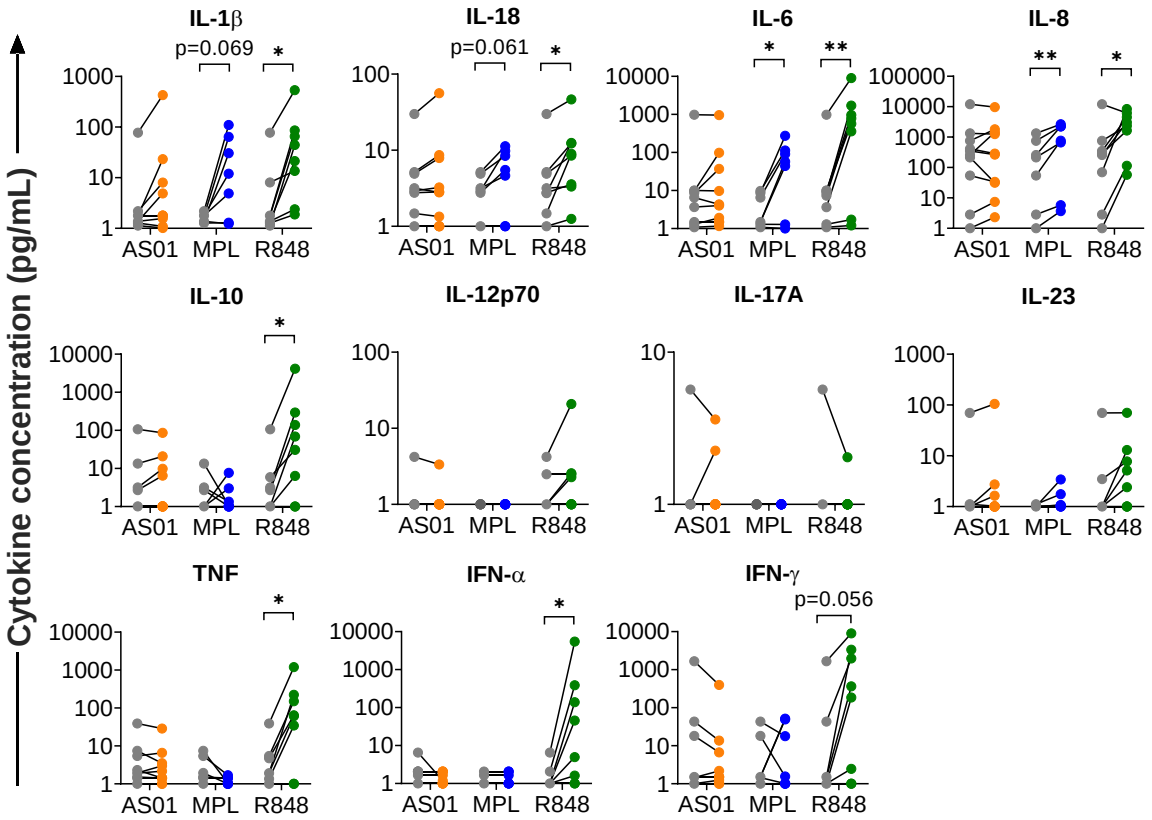


Figure 4.7: "AS01 does not induce the production of key pro-inflammatory cytokines from total lymph node immune cells *in vitro*." "Cells were mechanically dissociated from whole human LNs and exposed to adjuvants AS01 (orange, n = 10), MPL (blue, n = 7) and R848 (green, n = 8) in culture for 24 h. Cytokine concentrations in culture supernatants were determined by LEGENDplex and compared to their donor matched mock (grey) samples. Data were $\log_e$ transformed to approximate normality then paired t-tests corrected for multiple comparisons using the Bonferroni-Dunn method were applied. * $p < 0.05$, ** $p < 0.01$."

"Although AS01 was rather inert in isolated cells, as with myeloid cell activation, we saw a much greater immune response in the more physiological *in situ* exposure model in terms of pro-inflammatory cytokine induction. We therefore focused on the *in situ* model. *In situ*, AS01 induced a range of pro-inflammatory cytokines, including IL-1β, -18, -6, -23, as well as TNF and IFN-γ [Figure 4.8A]. There was a trend towards increased IL-12p70 (p=0.062) but IL-17A, IL-10 and IFN-α were not detected in response to AS01. Higher concentrations of AS01 did not increase the level of cytokine production (data not shown), consistent with the previously demonstrated decreased viability and maturation. R848 induced an even broader range of cytokines *in situ* than *in vitro,* adding IL-12p70,

IL-23, and clear induction of IFN-γ to its *in vitro* profile, although IL-18 was not significantly induced *in situ*. MPL was again less inflammatory, only inducing IL-1β and downregulating IL-10. Pam2Cys was tested *in situ* and although it did not induce cellular maturation or activation it did induce a broad inflammatory response with IL-1β, -6, -8, TNF and IFN-γ detected [Figure 4.8A]."

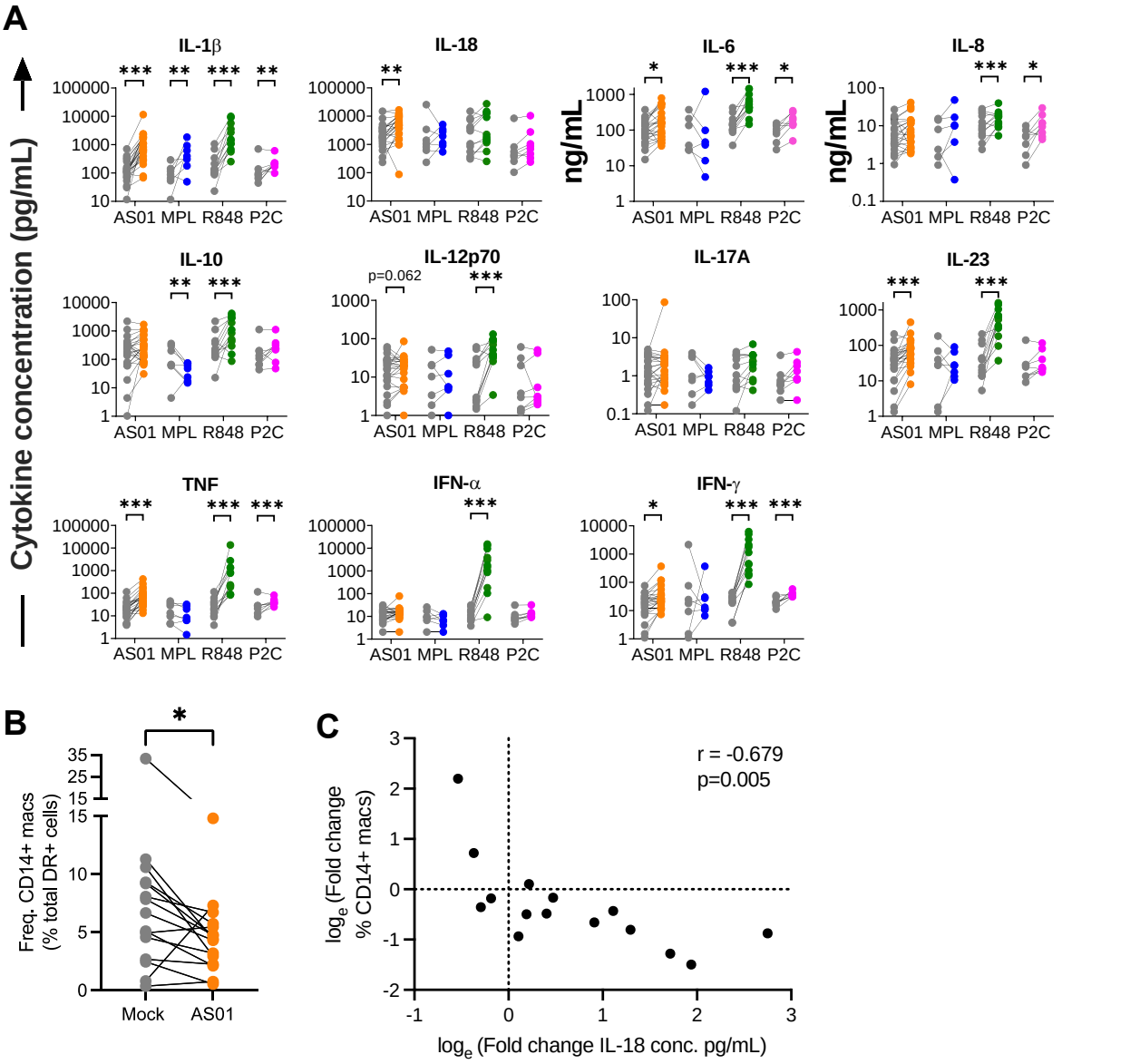


Figure 4.8: "AS01 induces the production of pro-inflammatory cytokines in LN cells *in situ*." "Slices of human LNs were bathed in adjuvants AS01 (orange), MPL (blue), R848 (green) or Pam2Cys (pink) for 24 h. **(A)** Cytokine concentrations in culture supernatants were determined by LEGENDplex and compared to their donor matched mock (grey) samples AS01 (n = 25), MPL (n = 7), R848 (n = 12) or Pam2Cys (n = 8). IL-6 and IL-8 were measured by ELISA AS01 (n = 24), MPL (n = 6), R848 (n = 12) or Pam2Cys (n = 8). Data were $\log_e$ transformed to approximate normality and GEE models were performed with a Bonferroni correction for multiple comparisons to compare donor-paired data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(B)** Frequency of CD14+ cells in AS01 compared to mock treated LN slices (n = 15). * $p < 0.05$. **(C)** The fold-change in CD14+ cell frequency negatively correlated with the production of IL-18 (n = 15). Pearson's correlation was applied to $\log_e$ transformed data."

"To summarise, in keeping with the maturation data, apart from IL-1β in some dissociated cell donors, AS01 only induced pro-inflammatory cytokines in intact LN tissue, again suggesting the requirement of the LN structure for the transmission of signals to multiple cell types upon exposure to AS01. At the concentrations tested, R848 was more immunostimulatory, activating several cell subsets directly, while MPL and Pam2Cys were less immunostimulatory with moderate activation of the immune system both *in vitro* and *in situ*."

"In mice, it has been shown that AS01 triggers an immune cascade, beginning with the activation of subcapsular SMs that produce IL-18. In synergy with IL-12, IL-18 rapidly enhances early IFN-γ production from NK and $CD8^+$ T cells (18, 249). The production of IL-18 is linked to pyroptosis of the cell (345). In this study, after *in situ* AS01 exposure, the frequency of $CD14^+$ cells, including macrophages, was significantly reduced [Figure 4.8B] and IL-18 production inversely correlated with the size of the $CD14^+$ population (Pearson's correlations r=-0.679, p=0.005) [Figure 4.8C]. Samples that had a strong upregulation of IL-18 in the supernatant, had very depleted macrophage populations, with very few $CD14^+$ cells and no discernible $CD169^+$ SM population. Correlation of IL-18 production with the SM population was therefore weaker (r=-0.641, p=0.010). Samples that had weak or no induction of IL-18 had much more robust populations, still smaller than the original population when the tissue was fresh, but distinct $CD14^+$ and $CD169^+$ populations remained. Therefore, it is likely that macrophages produce IL-18 in response to AS01 *in situ*, although they are dying in the process, as seen for QS-21 in mice (249). Increased IL-18 production however did not correlate with increased DC maturation (data not shown)."

"To confirm the discrepancy between our *in vitro* and *in situ* results, we directly assessed a range of cytokines by intracellular cytokine staining (ICS) in dissociated human LN cells *in vitro*. IL-18 production is difficult to detect by ICS due to the induction of pyroptosis, as mentioned above. IL-1β, clearly induced *in situ* by AS01 in human LNs although only detected in very low amounts in mice upon AS01 administration (275) , is produced by a common activation pathway to IL-18 but we could detect this by ICS. R848, included as a comparator, induced IL-1β production by $CD14^+$ cells, which included macrophages, and IL-12/23p40 production by $CD1c^+$ cDC2s. NK and T cells could produce IFN-γ in response to PMA/ionomycin stimulation [Figure 4.9]. In contrast, AS01 induced IL-1β in macrophages from 5/5 donors but did not induce IL-12/23p40 in cDCs or IFN-γ in NK or T cells from any donors [Figure 4.9]. These results were the same regardless of whether brefeldin A was added early (2 h into the culture, potentially blocking the early release of cytokines and their downstream

effects such as IFN-γ induction) or late (8-12 h into the culture, allowing more time for the full cytokine cascade before its addition) and therefore the combined data is shown [Figure 4.9].”

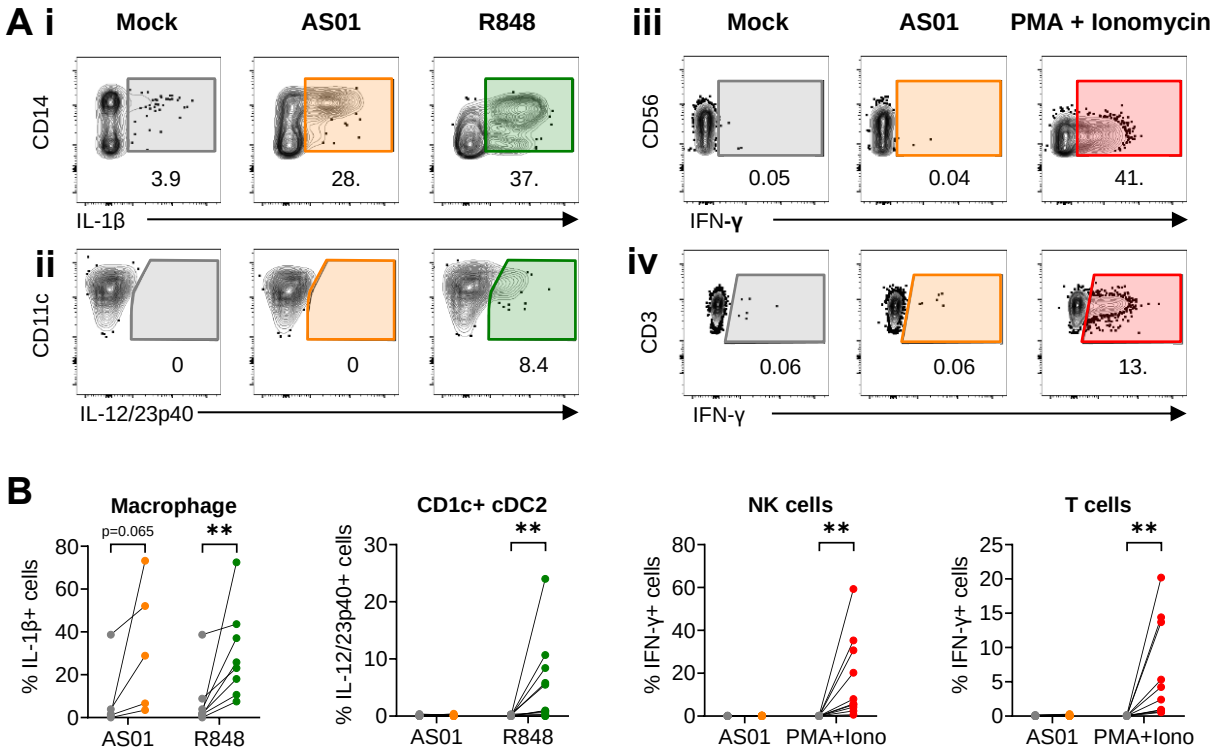


Figure 4.9: “Macrophages produce IL-1β in response to AS01 in vitro but downstream cytokines are not detected.” “Cells were dissociated from the LN and exposed *in vitro* to AS01, R848 or PMA/Ionomycin in the presence of brefeldin A. Production of IL-1β, IL-12/23p40 and IFN-γ was measured by flow cytometry. **(A)** Representative data for **(i)** IL-1β and **(ii)** IL-12/23p40 expression by total macrophages and CD1c+ cDC2s respectively, as well as IFN-γ expression by NK **(iii)** and T **(iv)** cells, in response to mock or adjuvant treatments. **(B)** Percentage of macrophages expressing IL-1β, DCs expressing IL-12/23p40 and, NK and T/NK-T cells expressing IFN-γ**,** in response to AS01 (IL-1β n = 5, IL-12/23p40 n = 10, IFN-γ n = 13), R848 (IL-1β n = 8, IL-12/23p40 n = 12), and PMA + Ionomycin (IFN-γ n = 10). Wilcoxon matched-pairs signed rank tests were applied. * $p < 0.05$, ** $p < 0.01$.”

“The induction of IL-1β and IL-18 *in situ* and at least IL-1β *in vitro*, is consistent with the early AS01/QS-21 cytokine cascade shown in mice (18, 228, 249, 275), however, in humans when cells are dissociated from the LN, the downstream parts of the AS01 cytokine cascade are lost, highlighting an important role for this LN explant system in preserving the cell-cell contact required for the native immune responses.”

4.3.4. Comparison of AS01 Innate Immune Responses to Its Individual Components, MPL and QS-21

Mouse and non-human primate model studies have shown the immunostimulants in AS01 act synergistically to enhance CD4 T cell responses (232). Hence, comparative analysis of AS01 to its individual components, MPL and QS-21, both formulated in liposomes, were conducted in our LN explant model to investigate if synergistic effects were also evident within the innate immune pathways in humans. Pro-inflammatory cytokines were assessed following stimulation with AS01, MPL and QS-21 within donor-matched LNs to produce accurate data for direct comparisons. Here, AS01 induced the same profile of cytokines as described above, including a significant upregulation of IL-1β and IL-18. Other cytokines did not reach significance however there was a trend towards the production of TNF (seen in 6/9 donors) and IL-6, -23 and IFN-γ (seen in 7/10 donors) (Figure 4.10). QS-21 behaved most similarly to AS01 and had the same cytokine profile, whereas both sources of MPL tested were again less inflammatory, only inducing IL-1β and IL-18 and downregulating IL-10 and IL-17A in 6/9 donors (Figure 4.10). Additional data is required to confirm these trends. Therefore, there was no clear evidence from these *ex vivo* experiments of a synergistic effect between MPL and QS-21 in humans, however *in vivo* studies may reveal more, as in the animal models.

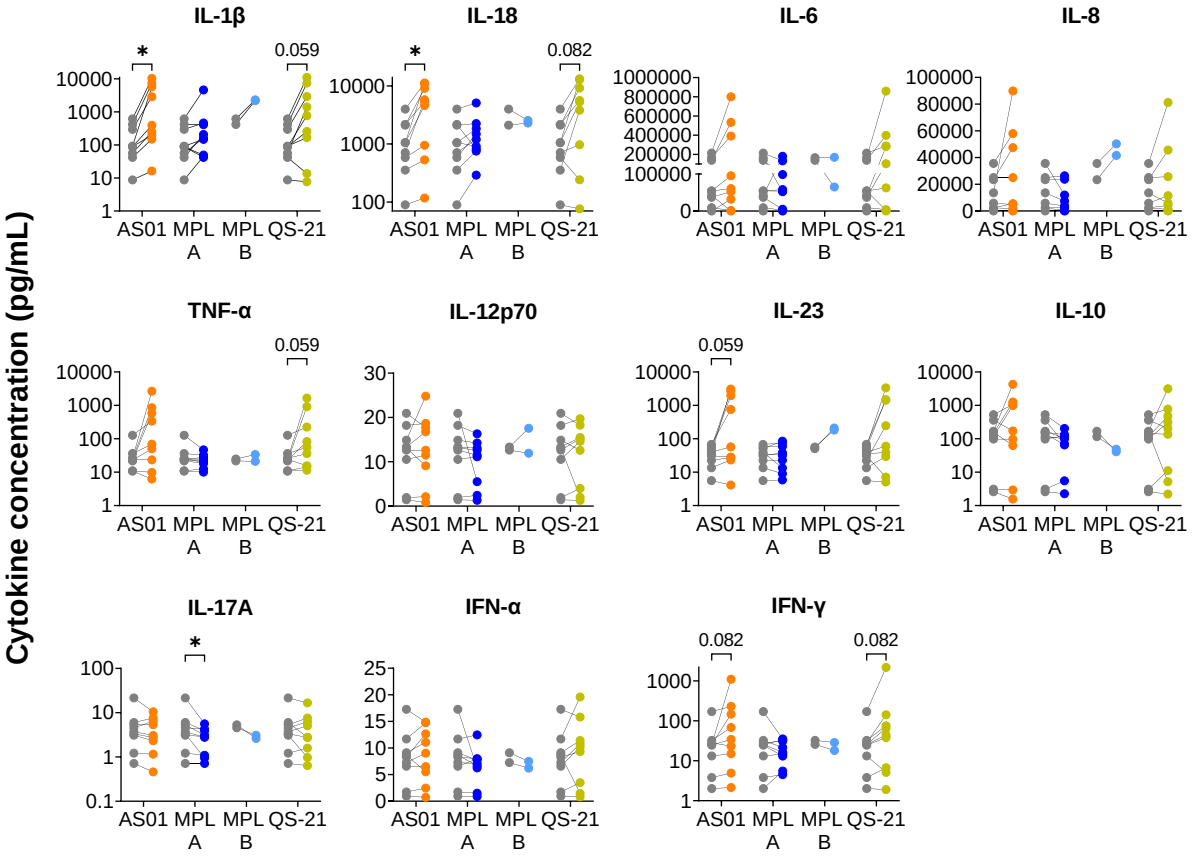


Figure 4.10: Comparison of pro-inflammatory cytokine production of LN cells *in situ* between AS01, MPL and QS-21. Slices of human LNs were bathed in adjuvants AS01 (orange, n = 9), MPL GSK (blue, n = 9), MPL InvivoGen (light blue, n = 2) or QS-21 (yellow, n = 9) for 24 h. Cytokine concentrations in culture supernatants were determined by LEGENDplex and compared to their donor matched mock (grey) samples. IL-6 and IL-8 were measured by ELISA. Wilcoxon matched-pairs signed rank tests were applied with Bonferroni-Dunn correction for multiple comparisons. * $p < 0.05$.

4.3.5. Age Did Not Influence Basal Levels of Dendritic Cell Maturation and Cytokine Release in the Lymph Node but did Influence the Dendritic Cell Response to Adjuvants

"Ageing results in disturbances in the structure of LNs, disorganization of the internal zones and impaired intercellular interactions and cytokine responses (101, 102). Together with a reduced thymic output of naïve T cells, these changes result in impaired immune responses to pathogens and vaccines."

"In terms of functional effects, we did not observe a difference in the basal expression of co-stimulatory molecules CD83 or CD86 on cDC1, cDC2 or pDCs from older (≥60 YOA) compared to younger adults (<60 YOA) [Figure 4.11A]. Furthermore, cDC2s from younger donors upregulated CD83 more than older donors in response to AS01 but otherwise there was no difference in DC capacity to mature in response to AS01 or R848 *in situ* [Figure 4.11B]." When this analysis was repeated with age as a continuous variable, similar observations were made. No correlation (Spearman's correlation) with age was identified for basal expression of co-stimulatory molecules or the ability of DCs to mature in response to adjuvants (data not shown).

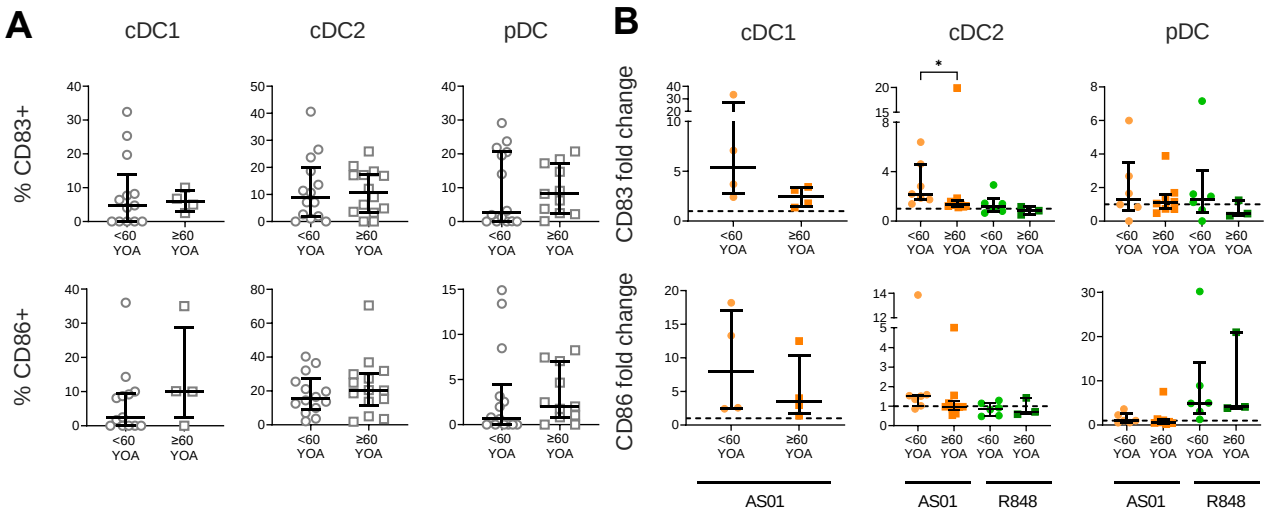


Figure 4.11: "Expression of co-stimulatory molecules on DCs at baseline and in response to AS01 and R848 are similar between younger and older LN donors." "Slices of human LNs were stimulated *in situ* for 24 h with AS01 (orange) and R848 (green) or left unstimulated (grey). Cells were mechanically dissociated from the LN tissue and expression of CD83 and CD86 was assessed by flow cytometry. Comparisons were made

between young (<60 YOA, circles) and old (≥60 YOA, squares) donors. **(A)** Percentage of CD83 and CD86 expression (young n = 12-14; old n = 4-14). **(B)** Fold change in expression of CD83 and CD86 in response to AS01 (young n = 4-7; old n = 4-10) or R848 (young n = 3-6, old n = 0-3) compared to donor-matched mock samples. Medians with interquartile range are indicated throughout. Mann Whitney tests were applied for each treatment. * $p < 0.05$."

"Whilst increased basal circulating levels of TNF, IL-1β and IL-6 have been reported in ageing adults >65 YOA compared to young adults <30 YOA (105), we did not observe this in LNs in our cohort. Initially, in a univariate analysis, we found no difference in the basal level of these or other pro-inflammatory cytokines in the supernatants of cultured LNs from older or younger donors [Figure 4.12A]. As with maturation, we also did not find a significant difference in the capacity of older LNs to respond with pro-inflammatory cytokines to any of the adjuvants when comparing the median fold-change [Figure 4.12B, Figure 4.13]. To further investigate the impact of ageing, we used a robust general estimating equation (GEE model) to cluster readings by donor, considering age as a continuous variable. Here, an interaction between age and adjuvant was identified for IFN-γ and IL-18 [Figure 4.12C, Table 4.1], indicating that adjuvants have different effects on cytokine production depending on the age of the subjects. A natural increase in IFN-γ production and IL-18 production was observed with age at baseline, consistent with inflammaging (114) and previous reports (112, 113), e.g. each YOA confers an additional immune response of 0.027 log-IFN-γ units [Table 4.1]. IFN-γ production in response to R848 strongly and significantly increased with age, although the opposite has been reported in blood (105). IFN-γ production in response to AS01 only increased slightly, and was not significantly different to the natural increase observed with age alone. These two adjuvants differed from MPL and Pam2Cys, where there was no age relationship for the IFN-γ response. The IL-18 response to R848 and Pam2Cys increased with age at a similar rate but only the R848 response was significantly greater than the natural increase. The response to MPL also only increased in line with the natural increase. In contrast, the age-related increase in IL-18 in response to AS01 was slower than the natural incline with age although their confidence intervals slightly overlapped [Table 4.1]. Thus, we observed differential responses to TLR ligands with age but a consistent AS01-induced pro-inflammatory response was maintained in LNs from younger and older adults."

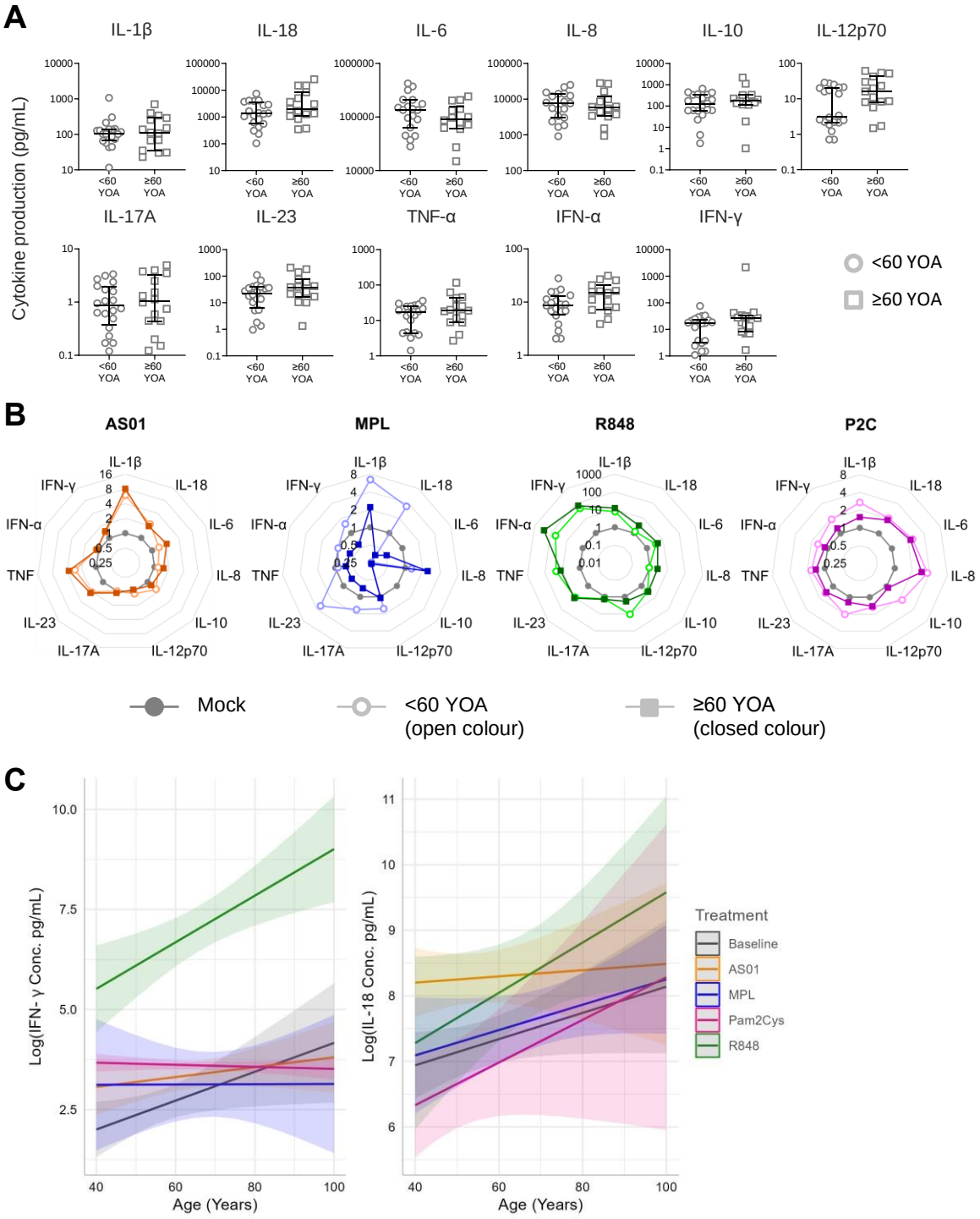


Figure 4.12: "The cytokine response to distinct adjuvants differs with respect to donor age." "Slices of human LNs were stimulated *in situ* for 24 h with AS01 (orange), MPL (blue), R848 (green) or Pam2Cys (purple) or left unstimulated (grey) and comparisons made between young (<60 YOA, circles) and old (≥60 YOA, squares) donors. **(A)** Basal level of cytokines (young n = 19-20; old n = 14-15). Medians with interquartile range are indicated. **(B)** Median fold-change in cytokine production in response to adjuvants (coloured) compared to donor-matched mock samples (grey), is plotted. AS01 (<60 n = 11, ≥60 n = 12-13), MPL (<60 n = 5, ≥60 n = 1-2), R848 (<60 n = 6-7, ≥60 n = 4), Pam2Cys (<60 n = 5, ≥60 n = 3). Mann Whitney tests corrected for multiple comparisons using the Bonferroni-Dunn method were applied. **(C)** IFN-γ and IL-18 had a significant interaction with age in a GEE model. The amount of cytokine produced in response to each adjuvant is plotted with respect to the age of the LN donor. Baseline (n = 34-36), AS01 (n = 24-25), MPL (n = 6-7), R848 (n = 12) or Pam2Cys (n = 8)."

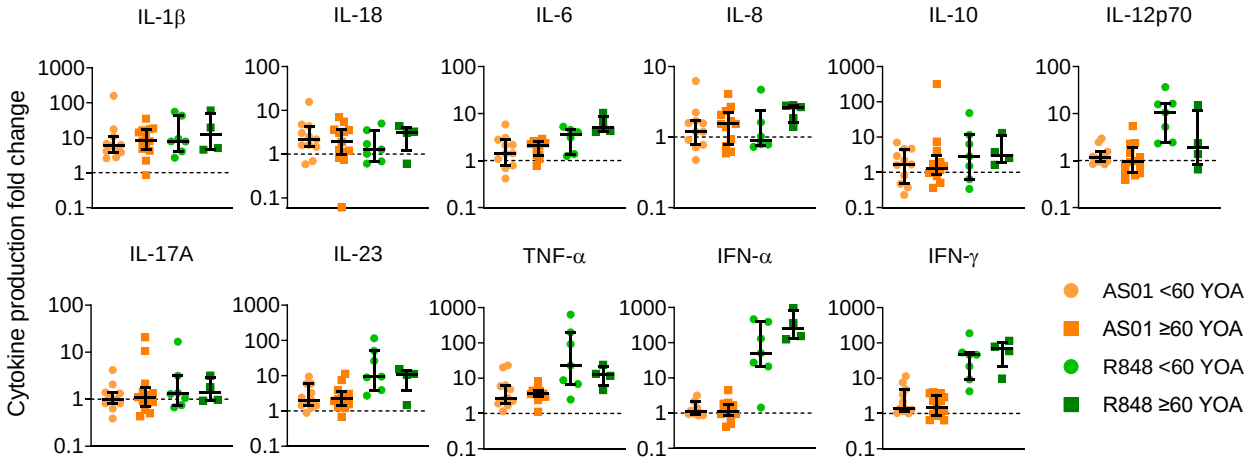


Figure 4.13: "Fold change in cytokine levels in responses to AS01 and R848 in young (< 60 YOA) and old (> 60 YOA) LN donors." "Slices of human LNs were stimulated *in situ* for 24 h with AS01 (orange) and R848 (green) and comparisons made between young (<60 YOA, circles) and old (≥60 YOA, squares) donors. Fold change in cytokine production compared to donor-matched mock samples is plotted. (AS01; young n = 10-12, old n = 9-10. R848; young n = 5-6, old n = 3-4). Mann Whitney tests corrected for multiple comparisons using the Bonferroni-Dunn method were applied. Medians with interquartile range are indicated throughout."

Table 4.1: Point estimates per year increase in age, derived from the GEE model, with 95% confidence intervals.

	IFN		IL-18	
Adjuvant	**Estimated Coefficient**	**95% CI**	**Estimated Coefficient**	**95% CI**
Baseline	0.027	0.014, 0.040	0.023	0.012, 0.029
AS01	0.016	0.013, 0.019	0.005	-0.007, 0.017
MPL	-0.001	-0.013, 0.011	0.019	0.018, 0.020
Pam2Cys	0.001	-0.002, 0.003	0.033	0.007, 0.058
R848	0.059	0.054, 0.065	0.038	0.036, 0.041

4.4. Discussion

"Predictive pre-clinical models to define the immunogenicity and mechanisms of action of vaccines and adjuvants in humans could be instrumental in the iterative development of new vaccines. Here we have demonstrated the utility of a human LN explant model for investigating *in situ* innate immune responses to vaccine adjuvants. In this model, whole tissue slices were used to preserve the complex internal structure of the LN including the capsule, at a thickness designed to maximise representation of all compartments and the number of rare APC subsets that may be lost in small tissue blocks or thin slices. Cascading immune responses were preserved that were otherwise lost in dissociated cells, demonstrating the physiological relevance of the model and the importance of

maintaining the spatial organization of cells and extracellular matrix structures within the organ. This model can be used as an additional tool to *in vivo* mouse and non-human primate models for testing mechanisms of action of existing and novel vaccines and adjuvants, and their immunostimulatory properties at the very site where they work *in vivo*, after intramuscular injection. With this human model, we describe the key LN cells that are targeted and stimulated by AS01. Liposomes with similar composition to those found in AS01 were preferentially taken up by subcapsular SMs and DCs. AS01 induced maturation of multiple subsets of DCs, as well as the production of pro-inflammatory cytokines from multiple cell types, in *in situ* exposed LN slices but not in dissociated LN cell cultures. The age of the adult LN donor did not influence the production of cytokines in response to AS01, unlike other adjuvants. This may be one factor underlying the efficacy of AS01-formulated vaccines, e.g. for herpes zoster and respiratory syncytial virus in older adults (132, 135)."

There are many factors at play within the intact LN environment that are crucial to preserve cascading immune responses, especially for adjuvants such as AS01. When assessing immune functions in response to vaccines, adjuvants or pathogens *in vitro*, previous studies have highlighted the importance of ensuring all immune cells are adequately isolated from the tissue sample of interest, as when suboptimal isolation protocols are used, inaccurate findings are reported (334, 346, 347). Even though mechanical and enzymatic digestion methods were both assessed in this thesis, where all LN stromal and immune cells were present, and with the highly dense network of closely interacting cells in the LN being important for cell interactions (17, 32, 46), this is not enough in an *in vitro* setting to support sufficient immune responses. While stromal cells were not directly assessed in this study, notable populations of CD45- LN cells were isolated and included within *in vitro* stimulations. This implies that the structural organisation and spatial arrangement of all cells in the specific LN compartments, particularly the fibroblastic reticular and endothelial micro-conduit networks and their facilitation of cellular interactions along reticulin fibres, are essential for cell signaling to generate immune responses.

"We have made several findings that demonstrate there are strong similarities between the mode of action of AS01 in mice and humans. 1. Pattern of uptake in LNs, primarily by $CD169^{+}$ subcapsular SMs but also DCs; 2. Activation of APCs - macrophages and DC; 3. Initiation of a cytokine cascade that culminates in the early production of IFN-γ. The latter is likely produced by NK or CD8+ T cells."

"The adjuvanticity of AS01 in mice is in part due to the activation of DCs (275, 276). AS01 activated macrophages and cDCs *in situ* in the human LN model, inducing upregulation of co-stimulatory molecules. R848, a TLR7/8 ligand, did not mature cDC2s *in situ*, even though they express TLR8 and they were activated *in vitro*. R848 did mature pDCs, which express high amounts of TLR7, as well as NK and B cells (both $TLR7^+$). R848, a small molecule immune potentiator, would be able to penetrate the LN thoroughly but may have a stronger affinity for TLR7 than TLR8, or the lack of cDC2 activation may be a dose effect with R848 being diluted in the LN explants."

"A key cytokine axis in the AS01 response in mice is the IL-18/IL-12-dependent induction of IFN-γ (18). QS-21 has been shown to activate the NLRP3 inflammasome, resulting in IL-1β and IL-18 production (228, 249), although in response to AS01, IL-1β has only been detected at very low levels in mice (275) and whether AS01 activates the inflammasome is still unclear. Our findings in human LNs that AS01 induces IL-1β, IL-18, IL-12 detectable in some donors, and IFN-γ, supports a similar cytokine cascade in humans that likely begins with inflammasome activation in macrophages and culminates in the production of IFN-γ."

"As we found, stronger and broader cytokine responses in intact lymphoid tissue slices compared to dissociated cell cultures have also been observed before (300, 302, 317). The bioavailability, and therefore potency of an adjuvant used at similar doses should be higher in dissociated cultures than *in situ* cultures so the phenomenon likely relates to the need for complex cell-cell interactions along the reticulin framework (15, 17) as well as cytokine signaling. 3D cytokine gradients will be established much more effectively when the producing cells are fixed in place or utilizing the LN architecture to move in a deliberate direction, as in intact tissue, rather than floating freely. IFN-γ-producing NK and $CD8^+$ T cells may also need to be in close proximity to IL-12 and IL-18 production which suggests a compartmentalisation of the AS01 response to the subcapsular region of the LN."

The MPL-QS-21 synergy observed in mice was not as clear in this human model however there were trends detected. Although the MPL effects on IL-1β, -18, -6, -8 and -10 varied in some experiments across *in vitro* and *in situ* set ups, the combination of MPL and QS-21 in AS01 showed a more significant effect than QS-21 for the production of IL-1β and IL-18 *in situ* (Figure 4.10), implying the addition of MPL is required for the enhanced AS01 responses. Further experiments and replicates are needed to determine whether the synergy is as strong and of the same mechanism as reported in mice, possibly with MPL upregulating production of pro-IL-1β and pro-IL-18, as described in Figure 1.6.

“Although inflammaging, the age-related increase in inflammation, is characterised by an increase in the circulating levels of pro-inflammatory cytokines IL-1β, IL-6 and TNF and reduced levels of anti-inflammatory cytokines such as IL-10 (348, 349), we did not observe these changes in the LNs from donors older or younger than 60 YOA. The increase in circulating pro-inflammatory cytokines in older adults may be driven by altered gut integrity or microbiota, or adipocytes, which increase with age and are another source of pro-inflammatory cytokines (350, 351). Our GEE model for testing the effect of age on adjuvant-induced cytokine production was influenced by a paucity of donors at the far ends of the age spectrum as well as the expected wide human donor variability of cytokine production and thus had wide confidence intervals. Nonetheless, IL-18 and IFN-γ pathways were found to be particularly conserved across the adult age spectrum, compared to other adjuvants, which may contribute to the efficacy of AS01 in older adults.”

While a range of key macrophage and DC phenotypes were assessed for AS01 innate immune functions within our explant model, other important innate immune cells, particularly FDCs, were not evaluated. Although FDCs do not have a large capacity for APC functions in terms of recognising antigen or other foreign material, they have been noted to receive these antigens from other LN cells in close proximity such as subcapsular SMs (29, 352, 353). This may be occurring post AS01 stimulation and as FDCs are the key cells that present antigen to B cells and support their activation (354), they may play a crucial role in promoting AS01 immune responses to shape adaptive immunity. Future analyses can incorporate these cells, particularly in our high parameter flow cytometry technique which allows for the ability to analyse 40-50 markers at once. However, with fluorescence spillover being a limitation of flow cytometry, and the intact LN environment crucial for preserving cascading AS01 responses, IMC techniques are being explored. This is a high parameter technology that can allow for the study of a plethora of cellular markers in the absence of fluorescence spillover due to the use of metal conjugated antibodies. Additionally, localisation patterns in the intact LN can be assessed, which is crucial when referring to AS01, where the compartmentalisation of cell-cell interactions and cytokine signally within the intact LN structure appears to be necessary. This technique can further be applied to dissecting the synergistic responses of AS01 compared to its individual compartments, QS-21 and MPL.

Chapter 5. Dissecting the Molecular Pathway of Cellular Activation by AS01 in Human Lymph Nodes

5.1. Introduction

The mode of action of AS01 has been partially deciphered in mouse models and NLRP3 inflammasome activation has been proposed but the role of this pathway is not confirmed in mice (228, 249). Here we considered whether the activation of the NLRP3 inflammasome within human LN subcapsular SMs, is the potential key molecular pathway that initiates the innate response to AS01. As reviewed in Chapter 1, inflammasomes are characterised as canonical or non-canonical, where canonical pathways are reliant on NLR sensor proteins, such as NLRP3, and form a multi-protein, cytosolic complex. The activation of the NLRP3 canonical inflammasome complex occurs via potassium (K+) efflux following PRR contact with stimuli. The NLRP3 sensor is triggered to recruit inflammasome proteins including the ASC adaptor protein and pro-caspase-1 effector protein, which are all individually present within the cytoplasm of cells (284). Within the complex, ASC cleaves pro-caspase-1 to its active form which goes on to cleave pro forms of IL-1β and IL-18 to active proteins. Caspase-1 additionally activates gasdermin D (GSDMD) which is responsible for creating pores within the cells membrane, leading to DAMP signalling via pyroptosis and subsequent cytokine release for downstream signalling (Figure 1.6) (285). The non-canonical pathway does not recruit a protein complex but involves activation of caspase 4/5, usually by LPS, activation of GSDMD and cleavage and release of IL-18. Additionally, the formation of pores via this pathway induces K^+ efflux which then triggers the NLRP3 inflammasome (Figure 1.6).

In mice, an important feature of the AS01 cytokine response profile is the IL-18/IL-12-dependent induction of IFN-γ (18). QS-21, the saponin component of AS01, has been reported to activate the NLRP3 inflammasome in mice leading to IL-18 production (228, 249) and variable levels of IL-1β (275). QuilA, a mixture of saponin adjuvants, induces both IL-18 and IL-1β in human monocytes (355).The role of the inflammasome response, however, remains unclear as *in vivo* studies utilising NLRP3 knockout mice revealed an increase in QS-21-induced IFN-γ as well as antigen specific T cell and antibody responses to HIV antigen, gp120 (228). A clearer understanding of whether the NLRP3 inflammasome is activated in humans is necessary. As a similar AS01 cytokine cascade to mice was identified in human LNs (Chapter 4), where IL-1β, IL-18, IL-12 in some donors, and IFN-γ were induced, activation of the NLRP3 inflammasome was further explored here. Our human LN explant model, as well as isolated LN macrophages, along with specific NLRP3 and caspase inhibitors, have been applied to assess the AS01 activation pathway in greater detail within human cells. Determining the initial mechanisms that may be responsible for the success of AS01, particularly within older adults, could create new insights for the future application of this adjuvant.

5.2. Methods

5.2.1. *In Situ* NLRP3 Blocking in Human Lymph Node Explant Model

LN tissue explants were processed and analysed as described in section 2.4 and 2.6. Prior to stimulation with AS01, some LN slices were pre-treated with 1-10 uM MCC950 – a specific NLRP3 inhibitor (356) – for 1 h. Culture supernatants were analysed by Legendplex as described in section 2.6, and IFN-γ additionally assessed by ELISA (Biolegend) as per the manufacturer's instructions.

5.2.2. Inflammasome Activation of Human Macrophages

Initially human monocyte-derived macrophages (MDMs) were generated and harvested as described in section 2.2.1, or human peritoneal macrophages were isolated from ascites fluid as per section 2.2.2. Cells were placed in suspension (1 x 10^6 cells/mL) in poly-HEMA coated 24-well plates or 15 mL falcon tubes for the assay. Alternatively, for assays completed on adhered cells, MDMs were generated directly on coverslips as described in section 2.2.1, or freshly isolated LN macrophages, as per section 2.3.3, were adhered to coverslips in 24 well plates in DCM or Yssel's media with 100 µg/mL M-CSF or GM-CSF (0.1 – 0.25 x 10^6 cells/0.5 mL/well). LN macrophages were cultured for 2-3 days prior to inflammasome activation.

Macrophages were exposed to 25 µg/mL AS01 for 2-5 h or, as a positive control for inflammasome activation, cells were primed with 1000 ng/mL LPS for 2-4 h then stimulated with 10 µM Nigericin or 1.25 µM ATP for 1 h. In some samples, the specific NLRP3 inhibitor 10 µM MCC950 was added for 30 min prior to the addition of inflammasome stimuli (AS01, Nigericin or ATP). Additionally, the pan-caspase inhibitor 20 µM VX765 (357, 358) was also added to treatments in some experiments, 30 min prior to the inflammasome stimuli or at the beginning of the assay, to prevent the release of the inflammasome complex via GSDMD pores in the cell membrane and pyroptosis of the macrophages. Following all culture treatments, the cells were fixed with 2% PFA for 20 mins at RT. Alternatively, for flow cytometry, experiments were completed using MDMs and peritoneal macrophages in suspension, fixed with 100 % ethanol for 20 min at RT (359). For all experiments, cells were washed with PBS and stored at 4°C overnight prior to downstream flow cytometry analysis or immunofluorescence (IF) microscopy staining on cells adhered to coverslips.

5.2.3. Flow Cytometry on Inflammasome Activated Human Macrophages

Cells from inflammasome stimulation assays as per section 5.2.2, were blocked with FcX Fc Receptor block for 10 mins followed by additional blocking with PBS supplemented with 3 % FCS, 0.1 % BSA and 0.1 % NaN_3, prior to the completion of any flow cytometry staining. These cells were pelleted

and stained with AF647-conjugated anti-ASC antibody for 90 mins at RT, then washed twice with FACS buffer prior to acquisition. Cells were acquired on the BD FACSymphony flow cytometer and compensated with FACS Diva before analysing in FlowJo Version 10.6.

5.2.4. Immunofluorescence Microscopy on Inflammasome Activated Human Macrophages

MDMs and LN macrophages on coverslips were inflammasome activated as per section 5.2.2. Coverslips were removed from the plate for staining. Prior to staining, cells were permeabilized and blocked for 30 mins at RT. Cells were stained with AF647-conjugated anti-ASC antibody for 1 h at RT in the dark followed by DAPI for 5 mins at RT in the dark. Coverslips were mounted onto slides with Slowfade or Prolong Gold mounting media.

Alternatively, MDMs and peritoneal macrophages were inflammasome stimulated in suspension as described in section 5.2.2 and fluorescently stained with ASC-AF647 as described in section 5.2.3. Remaining cells were washed twice with PBS, cytocentrifuged onto slides and coverslips mounted with Prolong DAPI or Slowfade DAPI.

Slides were imaged on the Olympus VS-120 Virtual Slide Microscope at 20X and images were analysed using VS Olyvia and Fiji ImageJ.

5.2.5. Quantification of ASC Inflammasome Specks

Quantification of ASC specks within IF microscopy images from inflammasome assay experiments, was determined using Fiji ImageJ. Brightness and contrast of the DAPI and AF647 channels were optimally set to sufficiently visualise cell nuclei and ASC inflammasome specks. Cells were quantified in a square region of interest (ROI) that extended to the edges of the round image. Cell counts were first performed by quantifying the DAPI channel for the number of nuclei present. The Gaussian Blur function with radius set to 1.5 was applied to the DAPI channel images. Assessment of Auto Local Threshold algorithms was performed on a small ROI to determine the most optimal method. Depending on the experiment and cell types, Otsu, Bernsen or Niblack algorithms were most optimal with the radius set at 30-100. Optimal methods were applied to the large ROI to create a mask. The Find Edges function was further used to create the final mask which was overlayed with the original image to confirm cell nuclei were accurately represented. The Analyze Particles tool was used on the final mask to identify total cell numbers by generating a count of all nuclei. To quantify ASC specks in the AF647 channel, the Find Maxima function was used with the pixel size set to 150-infinity. Inflammasome activation in macrophages was calculated by dividing the number of ASC specks by the total nuclei counts and assessing this as a percentage.

5.3. Results

5.3.1. NLRP3 Blocking in Intact Lymph Nodes Generates a Reduction in AS01-Induced Cytokines

To assess whether NLRP3 inflammasome activation in LN macrophages is the key mechanism responsible for the success of downstream AS01 immune responses, LN explants were pre-treated with the NLRP3 inhibitor MCC950 prior to AS01 stimulation. Pan-caspase inhibitor VX765 was not used in these experiments. Consistent with our previous findings, AS01 stimulation alone induced the production of key cytokines IL-1β, -18, -6, -23, TNF and IFN-γ *in situ* when compared to mock samples (Figure 5.1A). When the NLRP3 inflammasome was blocked, AS01-induced IFN-γ and TNF production was significantly inhibited and there was a trend towards inhibition of IL-1β (Figure 5.1A). In some instances, trends in cytokine production were inconsistent and instead increased in response to NLRP3 blocking, particularly for IL-18 in 4/9 donors and IL-23 in 3/9 donors. When looking at the % maximum reduction of AS01-induced cytokines due to NLRP3 inhibition, IL-1β and IFN-γ showed a significant reduction however cytokines were often not completely reduced back to basal levels of production (Figure 5.1B). Even for IFN-γ where a significant blocking effect was observed, there was a cluster of donors in whom IFN-γ was not blocked by MCC950. The same split response was also observed for IL-18 and IL-23. Therefore, although NLRP3 appears to be contributing to AS01 function, other mechanisms, including other inflammasomes, could be at play to support the generation of AS01-induced pro-inflammatory responses.

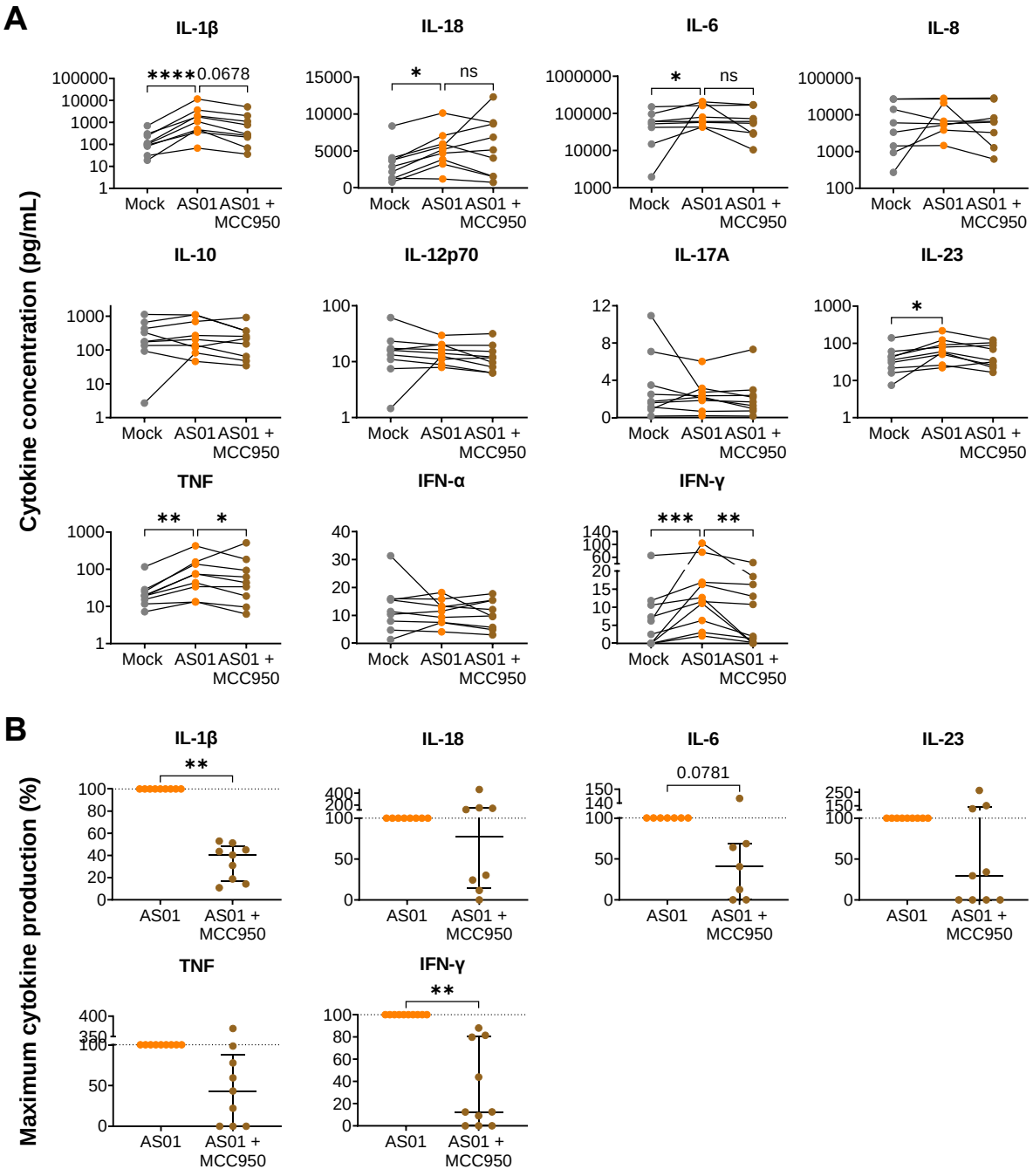


Figure 5.1: Blocking of the NLRP3 inflammasome in situ in intact LN slices, leads to a reduction of AS01-induced cytokines. Slices of human LNs were bathed in AS01 for 24 h (orange) or pre-treated with MCC950 for 1 h prior the addition of AS01 for 24 h (brown). **(A)** Cytokine concentrations in culture supernatants were determined by LEGENDplex and compared to their donor matched mock (grey) samples (n = 9). IL-6, IL-8 (n = 8) and IFN-γ (n = 10) were measured by ELISA. A Friedmans One-Way ANOVA with Dunn correction for multiple comparisons where AS01 was set as the control, was used to compare donor-paired data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(B)** Percentage of maximum cytokine production in AS01 + MCC950 treatments normalised to AS01 alone treatments for donors where AS01 induced cytokines (n = 8-9, IL-6 n = 7, IFN-γ n = 10). Wilcoxon matched-pairs signed rank tests were applied. * $p < 0.05$, ** $p < 0.01$.

5.3.2. Inflammasome Activation of Human Macrophages

5.3.2.1. Optimisation of NLRP3 ASC Inflammasome Activation Assay in MDMs

Due to the limiting cell numbers of primary LN macrophages, alternate sources were explored to optimise inflammasome assay culture conditions. MDMs and peritoneal macrophages were assessed as comparable options (data not shown) and used alongside LN macrophages for optimisations. Positive controls were tested to confirm the induction and detection of canonical NLRP3 inflammasome activation within macrophages by IF. Length of culture stimulations as well as titrations of LPS priming stimulations and inflammasome activation with Nigericin or ATP were completed. Optimal concentrations of 1000 ng/mL LPS for 3 h and 10 µM Nigericin for 1 h were deemed ideal (Figure 5.2). The pan-caspase inhibitor VX765 was also introduced to prevent the formation of GSDMD pores and pyroptosis of macrophages. In the case of the canonical NLRP3 inflammasome, this should retain activated inflammasomes within the cells for detection. Here, the addition of 20 µM VX765 increased the number of ASC inflammasome specks detected within LPS + Nigericin treatments, compared to stimulations in the absence of VX765 (Figure 5.2). The NLRP3 specific inhibitor MCC950 was also used and resulted in a reduction in ASC specks in response to positive controls (Figure 5.2).

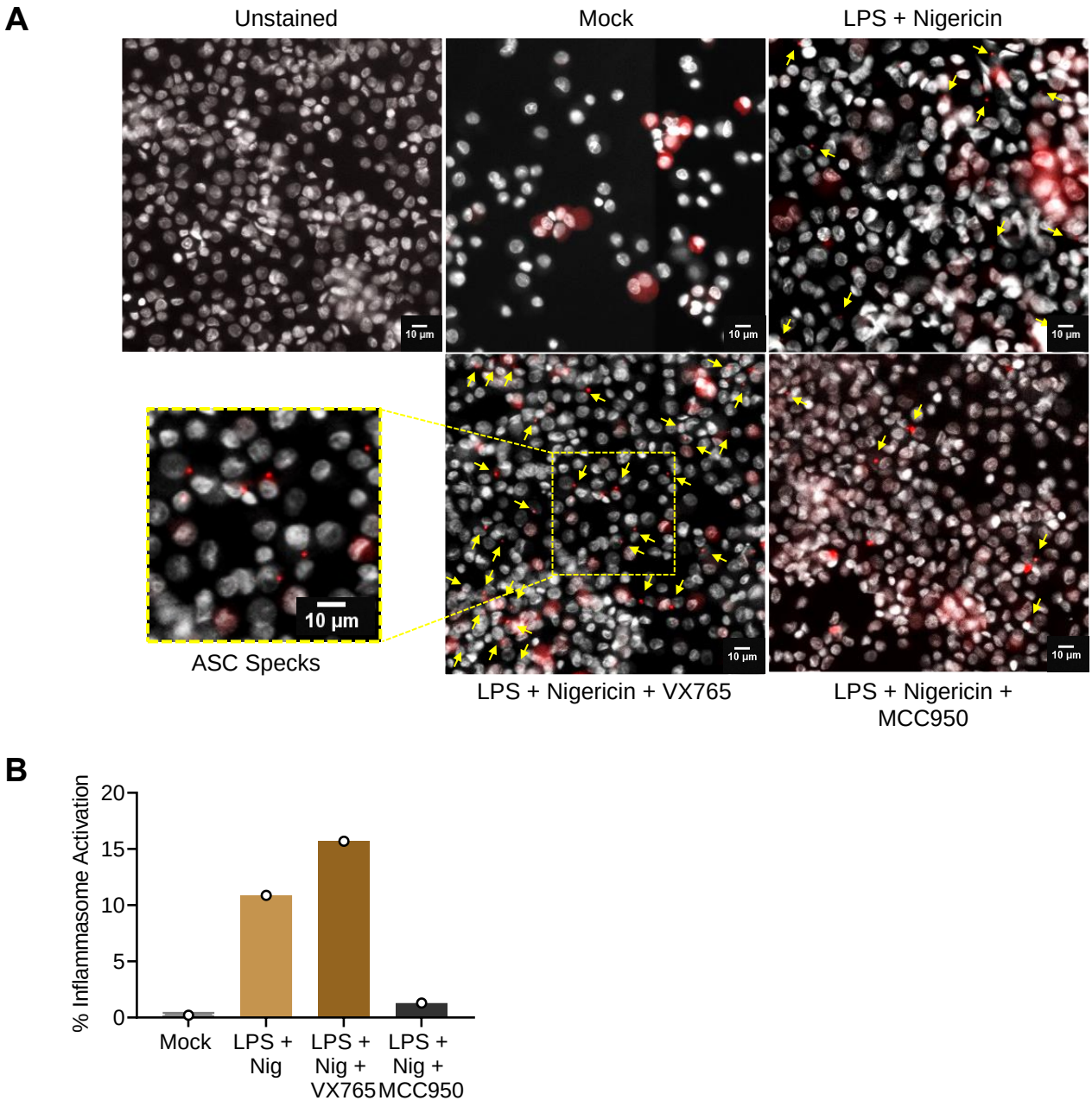


Figure 5.2: Optimisation of NLRP3 inflammasome ASC activation assay in MDMs. MDMs were inflammasome activated with LPS + Nigericin or left untreated. In some instances, cells were pretreated with either 20 µM VX765 or 1 µM MCC950. Cells were further assessed by IF microscopy. **(A)** Representative composite images of inflammasome activation where staining for nuclei on DAPI (grey) and ASC-AF647 (red) was performed. Yellow arrows indicate ASC specks. **(B)** Quantification of ASC specks presented as a percentage of total nuclei.

Different techniques were explored to investigate inflammasome activation however flow cytometry methods (359) were not sensitive enough (data not shown) and instead IF microscopy was superior. A range of phenotypic markers were attempted for IF microscopy staining. Initially, CD169 was considered ideal to identify the SMs, however additional optimisations are required, specifically within LN macrophages (data not shown). The ability to identify the cell membrane of

macrophages would be informative as they are particularly large cells and ASC inflammasome specks can be difficult to associate with the cell nuclei. Cell mask orange stain was attempted however this was incompatible with permeabilisation which is required for ASC staining (data not shown). Alternatively, brightfield images could be incorporated to identify macrophage cell membranes although these cannot be automatically overlaid on the imaging system used here. Instead, increasing the brightness of the AF647 channel post-acquisition revealed the extent of the cytoplasm (Figure 5.4).

5.3.2.2. Optimal Isolation and Culture of Viable Lymph Node Macrophages for Inflammasome Stimulation

An optimised protocol for the isolation and culture of human LN macrophages was developed to study AS01 immune mechanisms in primary tissue resident human macrophages, the cells thought to initially encounter AS01 in the LN (Figure 5.3A). Although enzymatic digestion methods were not found to significantly increase LN cell yields compared to mechanical isolation (Figure 3.6B), this was re-visited here as a gentler isolation technique to improve LN macrophage viability and prevent the cell losses experienced through dead cell removal kits (Figure 5.3B). In some donors, the viability of isolated CD14+ cells were higher when collagenase digestion was used for initial isolation of LN cells, instead of mechanical dissociation (Figure 5.3C). Therefore, the final optimal isolation protocol was initial enzymatic digestion of LN tissue using collagenase type IV to attain the most viable LN cells, followed by a CD14+ magnetic bead-based selection for enrichment of LN macrophages/monocytes. Through lengthy experimentation it was also determined that a recovery period post macrophage isolation of 3-4 days in culture, in Yssel's media with GM-CSF (Figure 5.3Di), further improved macrophage viability to the point where the inflammasome activation assay could be attempted with these fragile cells. GM-CSF promoted the differentiation of fried-egg shaped macrophages associated with an inflammatory phenotype, as opposed to M-CSF which induced spindle-like macrophages that are associated with angiogenesis (Figure 5.3Dii) (360).

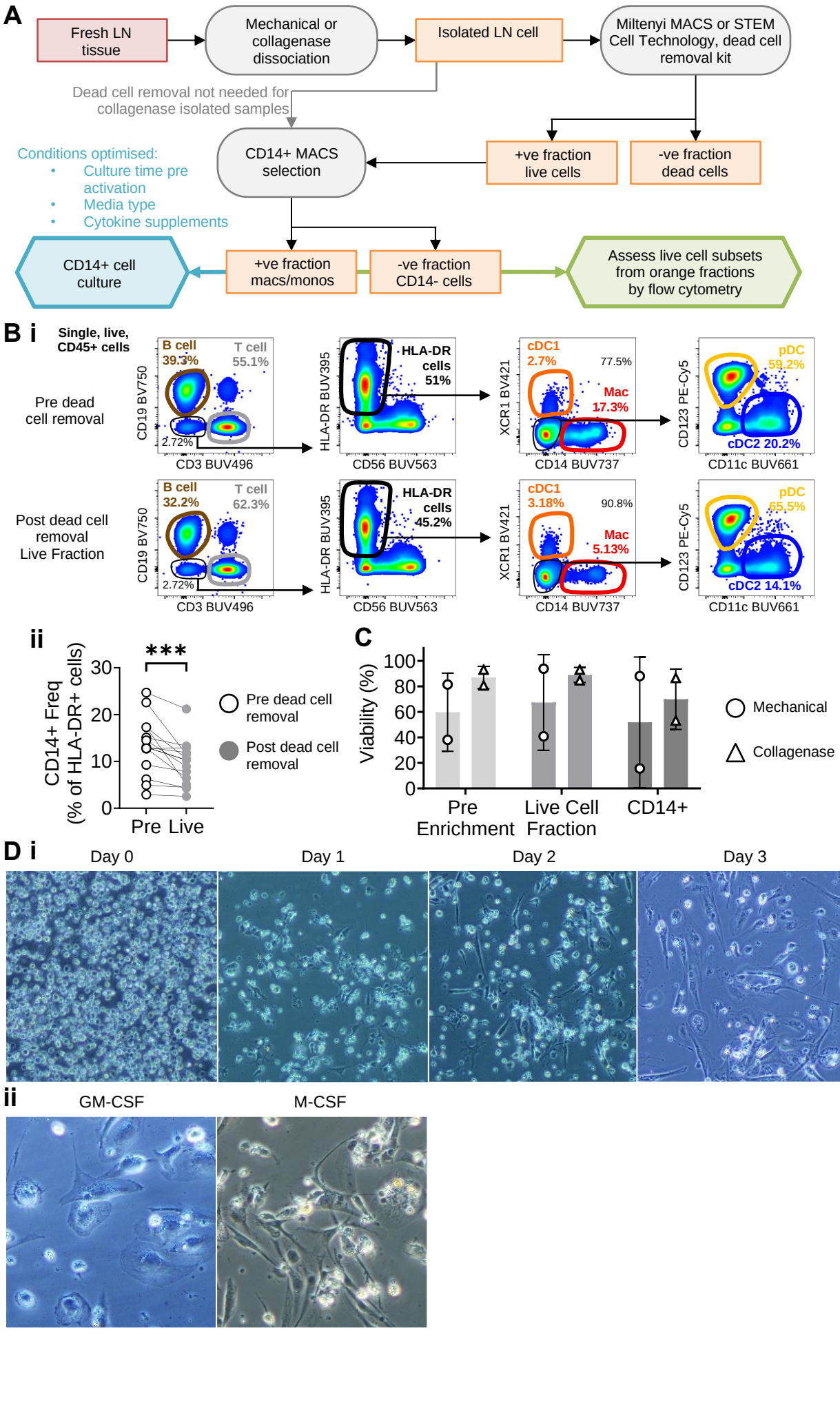

A
Fresh LN tissue
Mechanical or collagenase dissociation
Isolated LN cell
Miltenyi MACS or STEM Cell Technology, dead cell removal kit
Dead cell removal not needed for collagenase isolated samples
Conditions optimised:
Culture time pre activation
Media type
Cytokine supplements
CD14+ MACS selection
+ve fraction live cells
-ve fraction dead cells
CD14+ cell culture
+ve fraction macs/monos
-ve fraction CD14- cells
Assess live cell subsets from orange fractions by flow cytometry
B i
Single, live, CD45+ cells
Pre dead cell removal
B cell 39.3%
T cell 55.1%
2.72%
CD19 BV750
CD3 BUV496
HLA-DR cells 51%
HLA-DR BUV395
CD56 BUV563
cDC1 2.7%
77.5%
Mac 17.3%
XCR1 BV421
CD14 BUV737
pDC 59.2%
cDC2 20.2%
CD123 PE-Cy5
CD11c BUV661
Post dead cell removal Live Fraction
B cell 32.2%
T cell 62.3%
HLA-DR cells 45.2%
cDC1 3.18%
90.8%
Mac 5.13%
pDC 65.5%
cDC2 14.1%
ii
***
CD14+ Freq (% of HLA-DR+ cells)
Pre
Live
Pre dead cell removal
Post dead cell removal
C
Viability (%)
Pre Enrichment
Live Cell Fraction
CD14+
Mechanical
Collagenase
D i
Day 0
Day 1
Day 2
Day 3
ii
GM-CSF
M-CSF

Figure 5.3: Optimisation of lymph node macrophage isolation and culture. (A) Flow diagram highlighting the methods attempted for optimisation. **(B) (i)** Representative flow plots comparing cell subset frequencies of parent gates between pre vs post dead cell removal. **(ii)** Frequency of CD14+ macrophages of total CD3-CD19-HLA-DR+ APCs comparing pre vs post dead cell removal. Wilcoxon tests are applied. *** $p < 0.001$. **(C)** Viability of total CD45+ LN cells in each cell fraction following the isolation protocol, comparing initial mechanical isolation (circle) to collagenase digestion (triangle) (n = 2). **(D) (i)** Representative images of cultured macrophages in Yssel's media containing GM-CSF. **(ii)** Representative images on Day 3 macrophages cultures in Yssel's media comparing GM-CSF to M-CSF.

5.3.2.3. Activation of NLRP3 Inflammasomes by AS01 in Human Lymph Node Macrophages

To assess the activation of the NLRP3 inflammasome in human LN macrophages by AS01, LN macrophages were isolated and cultured as above, then stimulated *in vitro* for 3 h. Following AS01 stimulation, in the presence of VX765, the formation of ASC specks was observed within macrophages, compared to unstimulated treatments (Figure 5.4Ai). In most cases, ASC specks were relatively distant from nuclei and hence difficult to identify as intracellular. Observations of macrophages within cultures revealed the extent of how largely spread the cells can become upon settling (Figure 5.3D). By increasing the brightness of the AF647 channel in the IF images, the extent of the cytoplasm could be observed. Here, the ASC specks were recognized to be within the macrophages' expansive or elongated cytoplasm (Figure 5.4Aii) and the specks were quantified. In an initial experiment, ASC speck formation was noted to be greatly increased in the positive control LPS + Nigericin, as well as AS01 treatments with VX765, compared to mock (Figure 5.4B), suggesting canonical activation of the NLRP3 inflammasome. Now, further experiments are required to confirm this finding. In addition, the inclusion of the NLRP3 specific inhibitor MCC950 and the pan-caspase inhibitor VX765 will determine if the canonical NLRP3 inflammasome pathway is solely responsible or if non-canonical pathways are also involved, as suggested by the cytokine data (Figure 5.1).

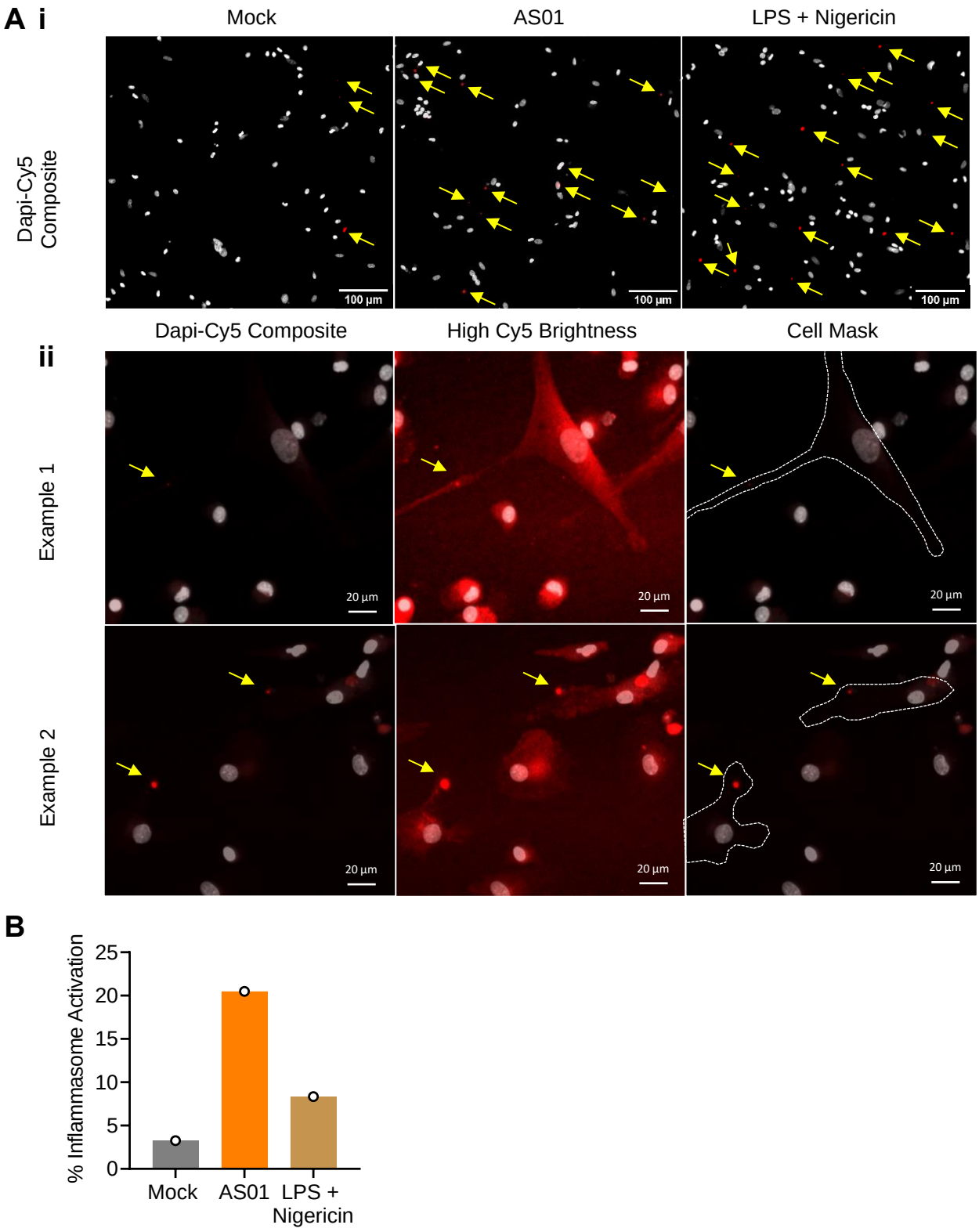


Figure 5.4: The NLRP3 inflammasome in LN macrophages is directly activated by AS01. LN macrophages were stimulated with AS01 or LPS + Nigericin and further assessed by IF microscopy for NLRP3 inflammasome activation via staining for ASC specks (ASC-AF647, red) and DAPI (grey). Yellow arrows indicate the presence of an ASC speck. **(A) (i)** Representative images visualizing ASC specks. **(ii)** Zoomed in representative examples of ASC specks associated with a cell body but distant from the nucleus. **(B)** Quantification of inflammasome activation (n = 1).

5.4. Discussion

AS01 induced inflammasome complexes within LN macrophages, and NLRP3 was specifically necessary for maximum production of key AS01-induced cytokines, as shown by inhibition of IL-1β and downstream IFN-γ. However, this effect was incomplete. Therefore, while it is clear the NLRP3 inflammasome is involved in the AS01 response, it remains unclear whether it is the initiating mechanism responsible for AS01 functions in humans. Instead, it is possible that an upstream non-canonical inflammasome pathway may also be at play. QS-21 was responsible for the major effect of AS01 so presumably is responsible for the NLRP3 inflammasome activation. However, the non-canonical inflammasome is known to be activated by LPS and therefore it is possible that MPL, a detoxified version of LPS and component of AS01, is a trigger here, as well as priming NLRP3 (Figure 1.6).

A key clue that the non-canonical caspase 4/5 inflammasome may be triggered by AS01 in LN macrophages is the differential effect on IL-1β and IL-18 production by NLRP3-specific inhibitor, MCC950. While IL-1β production was blocked, IL-18 was only partially reduced. Specifically, IL-18 was blocked in some donors but not others. We hypothesise that caspase 4/5, which is not affected by MCC950, is triggered by AS01, at least in some people, causing the release of IL-18. Subsequent indirect activation of the NLRP3 inflammasome, leading to IL-1β and further IL-18, is then able to be blocked by MCC950. The inclusion of VX765, a pan-caspase inhibitor, to assess its impact on cytokine release could confirm this hypothesis. If the hypothesis is true, VX765 should block caspase 4/5 and the production of IL-1β and IL-18 and lead to a decrease in specks, as the NLRP3 inflammasome will not be activated via the K^+ efflux. If the canonical pathway is dominant, the number of speck positive cells should increase with VX765 as the specks will not be able to leak out of pyroptosing cells (Figure 5.5).

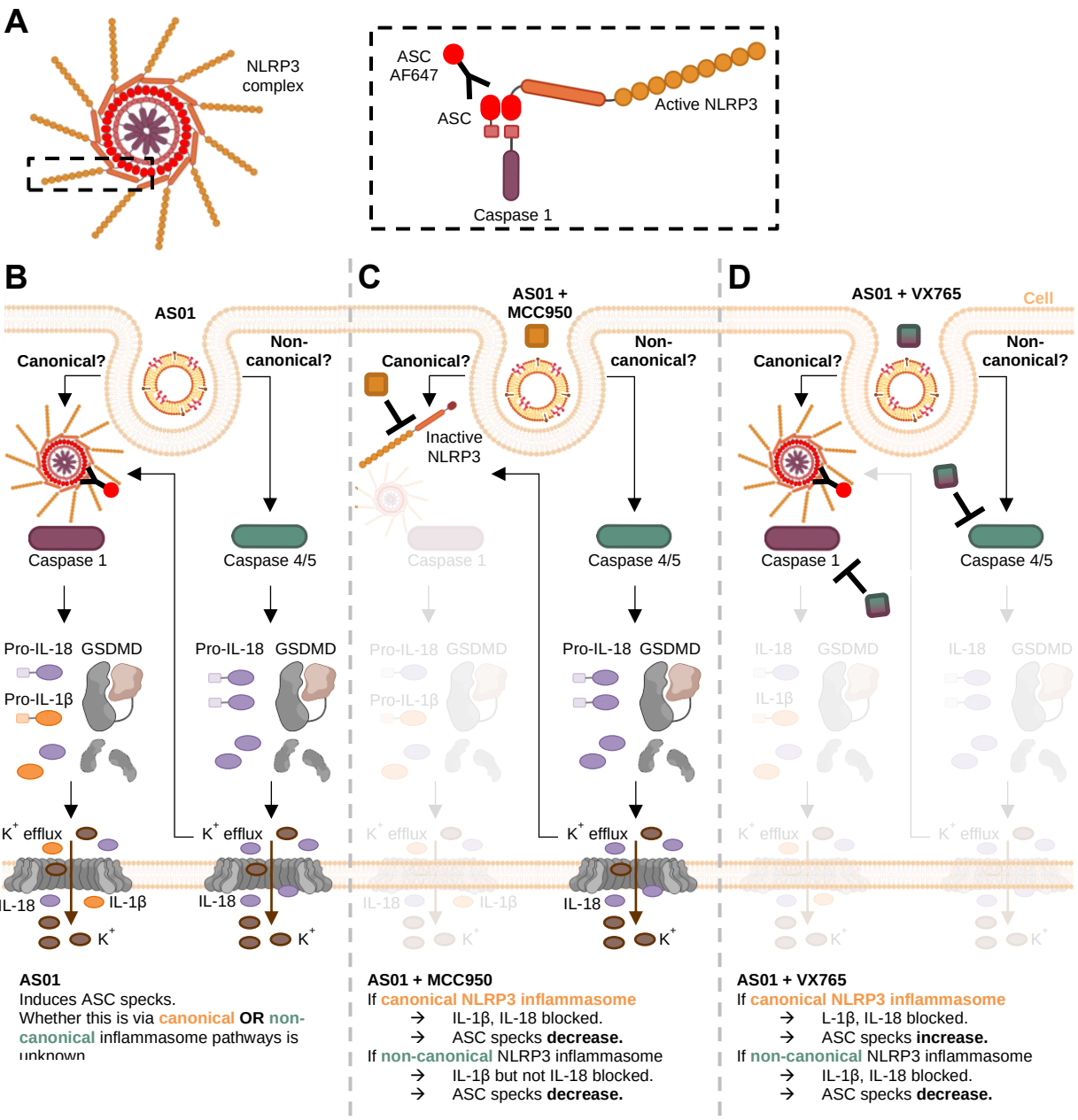


Figure 5.5: Proposed experiments to identify whether the canonical or non-canonical inflammasomes are responsible for AS01 responses.NLRP3 inflammasome complex. ASC-AF647 antibody (Ab) detects inflammasome specks by IF microscopy. (B) Potential pathways of AS01 stimulation via canonical and non-canonical inflammasomes. (C) Potential outcomes for AS01 stimulation in the presence of MCC950, a specific NLRP3 inflammasome inhibitor. (D) Potential outcomes for AS01 stimulation in the presence of VX765, a pan-caspase inhibitor.

In some donors, an increase in AS01-associated pro-inflammatory cytokines was observed in the presence of NLRP3 blocking MCC950. This is consistent with findings in NLRP3-knock out mice with QS-21 which suggest the adjuvant effect of QS-21 is not entirely due to NLRP3 activation. When these mice were immunised with QS-21 and a HIV antigen, gp120, a significant increase in antigen-specific antibody and T cell effector cytokines IFN-g and IL-2, as well as IL-4 and IL-6, was observed (228). Hence, additionally assessing the importance of caspase 1 and caspase 4/5 activation in

response to AS01, will allow for identifying which enzyme has the highest activity to further dissect the molecular pathways contributing the most to AS01 cytokine responses. Recent mouse studies have implied caspase 1 to be more crucial (287, 288). If caspase 1 is also revealed to be more important for AS01-induced cytokines within our explant model, this would suggest that the canonical pathway is the dominant one.

Furthermore, while AS01 stimulation visibly induced the formation of inflammasome complexes within LN macrophages, assessment using the specific NLRP3 inhibitor MCC950 and the pan-caspase inhibitor VX765, would allow for determination of whether the canonical NLRP3 inflammasome is the driver or if the (upstream) non-canonical caspase 4/5 inflammasomes is the initiator with subsequent downstream activation of NLRP3. This requires further experiments, as outlined above and in Figure 5.5, which could be improved with small area chamber slides (361), as we attempted. If AS01 initiates activation via the canonical pathway, VX765 should increase the number of specks detected by preventing pyroptosis of cells, as was seen in MDMs (Figure 5.5), and MCC950 should inhibit ASC speck formation. If the non-canonical pathway is initiated by AS01, VX765 should directly inhibit caspase 4/5 and prevent the K^+ efflux that triggers the NLRP3 inflammasome and thus decrease the number of specks formed. MCC950 by itself should reduce the number of specks formed by the NLRP3 inflammasome but inhibition of cytokines would be incomplete without the addition of VX765 which acts upstream, on caspase 4/5.

Peritoneal macrophages offered an abundant and alternate solution for optimisations as they are yolk-sac derived human tissue resident macrophages, similar to LN macrophages. Patients, from which peritoneal fluid was extracted for the isolation of macrophages, can however suffer from spontaneous bacterial peritonitis (SBP), and immune cells from these inflamed samples may display a higher degree of activation than normal (362-364). The fluid can also often be contaminated with the patients' blood. It is therefore important to perform these assays on LN macrophages which are more relevant in the context of AS01 adjuvanticity.

Moving forward, collagenase digestion over mechanical dissociation will be used for the isolation of LN cells prior to enrichment of macrophages for culture and inflammasome activation assays. Here, cells are more viable and do not require dead cell removal protocols which notably remove macrophages irrespective of the use of FcR blocking reagents to reduce non-specific antibody binding during the manufactures protocol. Therefore, it is unclear if this selective removal is because the macrophages are indeed non-viable and hence are accurately removed from the population, or

if their inherent magnetic nature (321, 322) is selectively eliminating them incorrectly. Subsequently, the selected optimised protocol using enzymatic digestion, is gentler and requires less handling of the cells, contributing an improved macrophage yield. Additionally, LN macrophage cultures and inflammasome activation assays will utilise a cytokine supplement for increased support. Morphology differences are however promoted by different cytokines and this has been reported to occur within monocytes, where GM-CSF and M-CSF lead to differentiation into a classically-activated phenotype or an alternatively-activated phenotype respectively (360). As all CD14+ cells were isolated, the potential presence of monocytes within the LN macrophage cultures also raised the question of whether the macrophages post recovery, were in fact LN monocytes that had undergone differentiation. These polarising LN macrophage morphologies are however evident 1 day post culture and hence unlikely to be monocytes undergoing differentiation, which typically require 4-6 days. It is therefore concluded that these are true tissue resident LN macrophages polarising to alternate phenotypes in response to the cytokine present. CD169+ SMs from LNs have been reported to exhibit functions similar to classically-activated macrophages which are known to be pro-inflammatory, while M-CSF polarised macrophages are typically anti-inflammatory (360, 365, 366). For this reason, GM-CSF should be used as the supporting cytokine for all future inflammasome activation assays.

While the involvement of the inflammasomes has been partially defined here within human LNs, further studies are required to definitively identify the interplay between the canonical and noncanonical inflammasomes and their role in the initial mechanisms for triggering AS01 immune responses. A better understanding of these initial mechanisms can shed light on the key factors responsible for the efficacy of AS01 and aid in the future applications of this potent adjuvant.

Chapter 6. Concluding Remarks and Future Directions

The mechanisms of action of vaccines and adjuvants are commonly studied in animal models. However, with interspecies differences being a key factor that may lead to the failure of vaccine formulations when transitioning into human clinical trials (304), an early understanding of immune functions in humans is necessary. With LNs being the primary immune organ where vaccines initiate immunity, a human LN explant model was successfully developed in this study to characterise innate immune functions following stimulation with vaccines or adjuvants (Chapter 3). Other developed human models focus on generating *in vitro* lymphoid organoids or culturing small explants of lymphoid tissue (295, 297, 298, 301, 317), and hence can only investigate adaptive immune responses. We know that the initial innate immune response however shapes the ensuing adaptive response and understanding the profile of the innate immune response can help address problems with the adaptive response. Our model addresses this gap by successfully culturing larger explants which allows for all LN compartments and rare cell populations to be present to study innate immune responses. Here, our model importantly allows for a snapshot of innate immune events within the LN. While other immune sites are important, such as the muscle or skin where vaccines are administered and where initial innate immune responses occur, followed by migration to the draining LN (367), our model focuses on studying immune events of vaccine rapidly free-flowing from these sites via lymph into LNs. Indeed, vaccine components, including QS-21, are detected in the draining LN in mice within 30 min of intramuscular injection, prior to the arrival of immigrating cells (275). In future studies, infiltrating cells can be incorporated *in vitro* to mimic migration into the LN explant. Additionally, future studies can be performed on explanted human muscle, to assess AS01 functions and local reactogenicity at this initial vaccination site. Co-culture with PBMCs may also be able to mimic infiltration of immune cells from the blood.

Adjuvants are known to trigger the immune system in distinct ways (73) and using our LN explant model, we identified unique innate immune profiles for the adjuvants assessed here (Chapter 4). Additionally, investigations in intact LNs via the explant model revealed immune responses that were not discernible in isolated cells, as observed in previous studies (300, 302, 317). This was particularly important for AS01 innate immune functions, where the specific structured compartments allowing for cell-cell interactions and cytokine signalling along micro-conduit networks, appears to be necessary to preserve the innate immune cascade (17, 32, 46). Within our explant model, similar AS01 innate immune profiles to those reported in mouse model studies were also identified, although there were also differences. The similarities include the pattern of AS01 uptake by APCs, notably subcapsular SMs which are the initial cells that encounter AS01 in the LN

and necessary for downstream functions (249), as well as the activation of DC subsets including cDC1s and cDC2s, which were reported to be important in shaping the AS01 adaptive immune response (275, 276). The cytokine profile of AS01 in humans was also relatively similar to mouse studies (18), with IL-18, as well as IL-12 in some donors, and most importantly IFN-γ, being detected. This highlights a clear pro-inflammatory cytokine cascade which appears to be necessary for AS01 immune functions and downstream activation of adaptive immune responses. Although similarities were observed in this AS01 cascade, a key dichotomy between responses in the human LN explant model to mouse model studies was importantly noted. In addition to IL-18, IL-1β was strongly induced by AS01 in human LNs, however a weak production of this cytokine was reported in mouse studies (275). The combination of these two initial cytokines, IL-18 and IL-1β, point towards specific molecular pathways that may be responsible for initiating this cascade in human LNs and were further assessed in this thesis. However most interestingly, these AS01 functions were consistent irrespective of the LN donors age, and especially when compared to other TLR-L adjuvants assessed such as R848, where differential pro-inflammatory cytokine responses were noted. This may contribute to the remarkable efficacy of AS01-adjuvanted vaccines across all age groups, and continuing investigations into the mechanisms of this adjuvant in humans will aid in unveiling the reasons underlying its effectiveness within older individuals.

Inflammasome pathways within LN macrophages, leading to the production of IL-18 and IL-1β, are thought to play a key role in initiating AS01 responses and the induction of an early wave of IFN-γ. These molecular pathways were explored in studies in mouse models and isolated human blood monocytes with different results. In the human monocytes QS-21 was transferred from liposomal to cell membrane cholesterol, then concentrated in lysosomes, causing pore formation and direct expression of cytokines including pro-IL-1β. However, in mice, although inflammasome activation was suspected, NLRP3 knockouts did not prevent AS01 activity (227, 228). Welsby et al also suggested there may be cell type specific effects of QS-21. Thus, the initiating events of AS01/QS-21 in human LNs remain undefined. In our study we showed a clear pattern of IL-1β/IL-18 stimulation by AS01, suggesting inflammasome activation (Chapter 5). Indeed, blocking of NLRP3 by the specific inhibitor MCC950, did partially reduce IL-1β and downstream cytokines but not completely. The pattern of efficient inhibition of IL-1β compared to IL-18 suggests upstream release of IL-18 by the non-canonical pathway, perhaps via initial AS01 stimulation of caspase 4 (281). We will study this further in the LN explant model in collaboration with the expert Schroder lab. Firstly, we will compare inhibition of IL-1β/IL-18 and IFN-γ by MCC950 and VX765. The latter inhibits

caspase 1 and 4. Then, assessing the activity and cleavage of caspase 1 compared to caspase 4/5, as well as downstream effector responses including IL-18, IL-1β and GSDMD cleavage in addition to pyroptosis, on isolated LN macrophages and MDMs will further enlighten the key AS01 molecular pathways. Additionally, future collaborations with external researchers who utilise caspase 1 and caspase 4 silenced MDMs compared to wild type MDMs, will be informative for assessing the role of these inflammasome pathways in response to AS01. Dissecting the AS01 molecular pathways in more depth can reveal the mechanisms responsible for the success of AS01 adjuvanticity in humans.

While this study extensively characterises cellular activation and pro-inflammatory cytokine profiles of adjuvants, as well as partially dissects the molecular pathways of AS01, this is all assessed as protein expression and a deeper understanding of these responses at the gene level could yield much richer information. Inroads were made during this thesis to optimise a protocol for conducting RNA sequencing on LN explants following adjuvant stimulations (Chapter 3) although this was technically challenging and will continue to be optimised. With the intact LN environment being crucial for AS01 immune functions, future studies aim to employ *in situ* RNA sequencing as the ideal technique to quantify gene expression while also mapping these genes to specific locations within the LN (368). Unlike bulk RNA sequencing where a full genome analysis is conducted, *in situ* sequencing can only be performed on a panel of genes (369) and hence pre-optimisations using bulk RNA sequencing are required to identify the most important genes of interest related to AS01 immune functions and their temporal expression, with particular focus on innate immune responses. However, as the LN is predominantly composed of lymphocytes, there is a risk of overshadowing differences in gene expression related to innate immune responses, with genes related to the lymphocyte population. Nevertheless, bulk RNA sequencing can provide a guide for the key gene differences that may be detected, and this can further be confirmed by performing single cell RNA sequencing on isolated APCs following adjuvant stimulation in the LN explant model. Another key factor that requires optimisation is the ideal timepoint of *in situ* stimulations to detect the peak expression of genes related to innate immune responses. These timepoints will likely be earlier than what was identified in this thesis for the analysis of secreted protein cytokines however may also differ between each cytokine of interest. For example, IL-18 in response to AS01 is produced as early as 8 h and peaks at 24 h, like most AS01-induced cytokines, while cytokines such as TNF in response to R848 peaks at 4 h and is diminished by 24 h. Although timepoints in these experiments can only be directly compared to donor matched samples, as differing timepoint collections of supernatants are completed within each donor and all times observed were from the

same LN slice, they nevertheless provide preliminary guidelines for future culture times to assess for RNA sequencing. Ultimately, *in situ* sequencing will confirm our current studies on protein expression and further provide an in-depth analysis of the potential molecular pathways related to AS01 innate immune functions within intact human LNs and their anatomical cellular relationships at sequential times. Additionally, completing these gene sequencing investigations on AS01 compared to its individual components, QS-21 and MPL, will shed further light on the synergistic responses generated which may underly its remarkable efficacy within humans.

While *in vitro* and animal model studies are effective in providing preliminary knowledge of immune responses to vaccine formulations and adjuvants, additional pre-clinical screening technologies are needed to better understand these functions within human LNs prior to commencing clinical trials. Knowledge of the specific innate and adaptive mechanisms, particularly for vaccine adjuvants, can allow for a more accurate application of these technologies in future vaccine developments to target related problematic pathogens especially in high-risk groups such as older individuals. For adjuvants such as AS01, where the reasons for its effectiveness in older adults are still unknown, further studies could facilitate tailoring of vaccines for this high-risk group to specifically overcome immunosenescence. Additionally, a library of vaccine adjuvants with their known mechanisms identified in humans, could allow an informed selection of these components to tailor developing vaccine candidates for targeting specific pathogens of interest (370). Our human LN explant model can therefore provide a test bed for new adjuvants or vaccine formulation series alongside other pre-clinical models and animal studies.

In addition to understanding vaccine and adjuvant mechanisms, the LN explant model is particularly important for the assessment of adjuvants such as AS01, in endeavouring to separate factors that initiate immunity compared to reactogenicity. AS01 induces a high frequency of reactogenic responses (131, 133-135, 144). Local reactogenicity is initiated in the muscle or site where the vaccine is administered, and these include responses such as redness and injection-site pain. However, systemic reactogenicity probably occurs once the vaccine/adjuvant has drained to the LN where it persists for only 2-3 days but the effects are disseminated throughout the body, and these likely induce responses such as fevers and headaches (371). With QS-21 known to be highly reactogenic (228), although these responses are partly dampened when encapsulated by the liposomes within AS01, it is likely that QS-21 is a main contributor to AS01's reactogenicity. As QS-21 can now be chemically synthesized (230, 372), there are opportunities to produce different congeners to minimise reactogenicity without altering immunogenicity, as studies have implied the

former is not required for the latter (342). These slightly modified versions of QS-21 can be further examined within the LN explant model to determine which options are best for retained immunogenicity but minimal reactogenicity within humans and then applied in future vaccine improvements. Moreover, the new technologies of RNA-lipid nanoparticle (LNP) vaccines which are also highly reactogenic, can continue to be developed in a similar way. Innovative adjustments can also be made to LNP formulations for the specific targeting of particular cells required for generating certain immune responses. For example, some pathogens such as HSV require CD8 T cell responses for effective clearing and hence LNPs targeting vaccines to cDC1s to induce CD8 T cell responses could enhance these responses (373). Our LN explant model can be a useful tool to initially investigate these new adjuvant formulations within humans prior to, or alongside, other pre-clinical models.

With the rapid development of new vaccine technologies against emerging pathogens, such as mRNA vaccines against COVID-19, investigations into the specific immune mechanisms within humans are necessary as this knowledge is limited. Our explant model can act as a platform to further unveil these immune responses and additionally compare mRNA vaccines to protein-adjuvant vaccine formulations, which likely function via completely different mechanisms. Notably, mRNA vaccines are highly potent however when compared to subunit vaccines, long-lasting immunity and longevity has not been achieved (374-377). This new vaccine technology hence requires further improvements and generating a better understanding of the differences compared to current formulations, could provide guidance on future developments.

Overall, our novel LN explant model can act as a pre-clinical screening platform for vaccine developers to assess the initial innate immune responses as well as mechanisms of new vaccine formulations and adjuvant components, prior to expensive large animal model studies and guiding development of human clinical trial studies. Here, a better understanding of these immune responses, in combination with other pre-clinical assessment methods, can aid in the seamless development of vaccines. This can allow for improvements in immunogenicity and durability and for specifically generating tailored responses for targeting pathogens or high-risk groups such as older individuals, as well as reducing reactogenicity.

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