

Mechanisms of liposomal and squalene
emulsion adjuvants
DPhil Thesis



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Covid-19 statement

I started my DPhil during the spring and summer of 2020, therefore there was a quite significant disruption to the beginning of the experimental work for this thesis. To compensate for restricted and closed-down lab access I performed data-analysis on previously published work during the early phases of the DPhil. This work is shown in figure 5.14 to figure 5.21. Previously published data was used as the source for this work, as I was not able to perform the experiments to collect this data myself. Our lab has now collected similar datasets ourselves, but this was not completed in time for reanalysis and inclusion in this thesis.

In addition, I did Covid-related research in collaboration with Leila Godfrey and Teresa Lambe's group. This work explored single-cell memory T Cell responses pre and post-vaccination in young and aged human participants from the COV2 trial using RNA, surface protein and TCR sequencing. However, this work was not included as it does not fit with the narrative of the thesis.

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List of Abbreviations

3D6AP: 3D-6-acyl-PHAD

AIM2: Absent In Melanoma 2

Acta1: Actin Alpha 1, Skeletal Muscle

ASC: Apoptosis-associated speck-like protein containing a CARD (protein)

APCs: Antigen-Presenting Cells

AS01: Adjuvant System 01

AS04: Adjuvant System 04

AS03: Adjuvant System 03 (An adjuvant containing α -tocopherol and squalene in an oil-in-water emulsion)

axLN: axillary lymph node

BECs: Blood endothelial cells

B220: Protein Tyrosine Phosphatase Receptor Type C

BSU: Biological Services Unit

CCL17: Chemokine (C-C motif) Ligand 17

CCL2: Chemokine (C-C motif) Ligand 2

CCL3: Chemokine (C-C motif) Ligand 3

CCL4: Chemokine (C-C motif) Ligand 4

CCL5: Chemokine (C-C motif) Ligand 5

Ccl7: Chemokine (C-C motif) Ligand 7

CPM: Counts per million

CD169: Cluster of Differentiation 169

CD31: Cluster of Differentiation 31 (Platelet endothelial cell adhesion molecule-1 PECAM-1)

CD3: Cluster of Differentiation 31

CD45: Cluster of Differentiation 45

CLR: C-type Lectin Receptor

CXCL1: Chemokine (C-X-C motif) Ligand 1

Cxcl13: Chemokine (C-X-C motif) Ligand 13

Cxcl10: Chemokine (C-X-C motif) Ligand 10

Cxcr2: Chemokine (C-X-C motif) Receptor 2

cDNA: complementary DNA

Csf3: Granulocyte Colony-Stimulating Factor (Transcript)

DAMPs: Damage-Associated Molecular Patterns

DAPI: 4',6-diamidino-2-phenylindole

DEGs: Differentially Expressed Genes

DiD: 1,1'-Diocadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt

DC: Dendritic Cell

EDTA: Ethylenediaminetetraacetic acid

- F4/80:** EGF-like module-containing mucin-like hormone receptor-like 1 (EMR1)
- FBS:** Fetal Bovine Serum
- FRCs:** Fibroblastic reticular cells
- G-CSF:** Granulocyte Colony-Stimulating Factor (Protein)
- GSMD:** Gasdermin D
- GO:** Gene Ontology
- Gzmb:** Granzyme B
- HMGB1:** High-mobility group box 1
- Hdc:** histidine decarboxylase
- iLN:** Inguinal lymph node
- IFN- γ :** Interferon-gamma (Protein)
- Ifng:** Interferon-gamma (Transcript)
- IL-1 β :** Interleukin 1 beta
- IL-6:** Interleukin 6
- IL-12:** Interleukin 12
- IL-18:** Interleukin 18
- Il-23:** Interleukin 23
- Il-33:** Interleukin 33
- i.m:** Intramuscular
- iLNs:** Immunized lymph nodes

- Itgam:** Integrin alpha M (CD11b transcript)
- IRF3:** Interferon Regulatory Factor 3
- IRF:** Interferon regulatory factor
- ISG:** Interferon-stimulated genes
- IRE1 α :** Inositol-requiring enzyme 1 alpha
- ILC:** Innate Lymphoid Cell
- LDH:** Lactate Dehydrogenase
- LECs:** Lymphatic Endothelial Cells
- LN:** lymph node
- LISI:** Local Simpson's Inverse Index
- Lin-:** Lineage Negative
- Lin+:** Lineage Positive
- Ly6g:** lymphocyte antigen 6 family member G
- Ly6c:** lymphocyte antigen 6 family member C
- MAP kinase:** Mitogen-Activated Protein kinase
- MAST:** Model-based Analysis of Single-cell Transcriptomics
- MD5:** Melanoma Differentiation-Associated Protein 5
- MD-2:** Myeloid Differentiation factor 2
- MoA:** Mechanism of action
- MCC950:** A selective NLRP3 inflammasome inhibitor
- MF59:** Oil-in-Water Emulsion Adjuvant

- MYD88:** Myeloid Differentiation Primary Response 88
- MPLA:** Monophosphoryl-Lipid A
- MHCII:** Major Histocompatibility Complex II
- Myh4:** Myosin Heavy Chain 4
- Nfkb:** Nuclear Factor Kappa B Inhibitor Alpha, mRNA transcript
- NF- κ B:** Nuclear Factor Kappa B, protein
- NLRP3:** NOD-like receptor family pyrin domain containing 3
- NLRs:** NOD-like Receptors
- NK cell:** Natural Killer cell
- pDC:** Plasmacytoid dendritic cells
- PBS:** Phosphate-Buffered Saline
- PDPN:** Podoplanin
- PAMP:** Pathogen-Associated Molecular Patterns
- PEG:** Polyethylene Glycol
- PEGylation:** Polyethylene Glycolylation
- PCA:** Principle Component Analysis
- PRRs:** Pattern Recognition Receptors
- PCR:** Polymerase Chain Reaction
- Pycard:** Apoptosis-associated speck-like protein containing a CARD
(Transcript)
- QC:** Quality Control

QS-21: Quillaja Saponin-21

qPCR: quantitative PCR

RNA-seq: RNA Sequencing

RQ: Relative Quantification

RSV: Respiratory Syncytial Virus

RLRs: RIG-I-like Receptors

RIPK3: Receptor-Interacting Protein Kinase 3

SARS-CoV-2: Severe Acute Respiratory Syndrome Coronavirus 2

scRNAseq: Single-cell RNA sequencing

SCS macrophage: Subcapsular Sinus Macrophage

SEM: Standard error of the Mean

SD: Standard Deviation

STAT1: Signal Transducer And Activator Of Transcription 1

TLR: Toll-like Receptor

TRIF: TIR domain containing adaptor molecule 1

TIRAP: TIR Domain Containing Adaptor Protein

TRAM: Translocation Associated Membrane Protein 1

TLR4: Toll-like Receptor 4

Tnf: Tumor Necrosis Factor Alpha, transcript

TNF- α : Tumor Necrosis Factor Alpha, protein

List of Abbreviations

TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling

UMIs: Unique Molecular Identifiers

UMAP: Uniform Manifold Approximation and Projection

VFI: Vaccine Formulation Institute

Abstract

Adjuvanted protein vaccines can provide a high level of protection against many pathogens. Yet the mechanisms underlying their function are not well understood, a fact that is complicated by the proprietary nature of most potent adjuvants. In this context, the Vaccine Formulation Institute develops liposomal (LQ and LMQ) and squalene emulsion (SQ and SMQ) adjuvants for open-access usage. This thesis addresses the challenge of understanding the mechanisms of action of these adjuvants towards the aim of enabling rational mechanistically informed vaccine design.

In this thesis, adjuvants are mapped across cells and tissues in terms of adjuvant tropism, drainage and transcriptomic modulation. Fluorescently labelled liposomal and squalene emulsion adjuvants were used to identify the cellular targets and drainage patterns of these adjuvants in the muscle spleen and lymph node. Through a comprehensive screening of cell types by flow cytometry it was found that adjuvant drainage was restricted primarily to the muscle and draining lymph nodes with little systemic spread to the spleen. In addition, liposomal adjuvants displayed a higher level of uptake across cell types, particularly with an increased capacity for uptake in resident non-hematopoietic cells in the lymph node in comparison to squalene emulsion adjuvants. Whole transcriptome sequencing approaches were used to generate a map of the transcriptomic inflammatory response to LMQ and SQ in the muscle and draining lymph nodes. It was identified that while both adjuvants induce a core innate immune response, LMQ was characterised by a transcriptomic Type II interferon response driven by ILC1s and NK cells that was not found after immunisation with SQ.

1 | Introduction

1.1 Overview of immunisation.

Vaccines are a highly effective medical technology for public health, exemplified by the eradication of smallpox through vaccination declared in 1980 (1). Vaccination programmes have also led to large reductions in the prevalence of other infectious diseases (2,3). In the United States, the number of cases of diphtheria, measles, paralytic poliomyelitis and rubella have all decreased by more than 99% compared to before national vaccination programmes began (3). Reductions in recorded cases of vaccine-preventable diseases have occurred worldwide not just in high-income countries. For example, globally the number of cases of tetanus has reduced by 88% between 1990 and 2019 (4). Overall, the World Health Organisation (WHO) estimates that 3-5 million fewer deaths occur due the vaccination each year (5) However, many infectious diseases remain poorly controlled by vaccination, or have no available vaccination against them (6). Among those pathogens poorly controlled by immunisation are Malaria, HIV, Influenza and Norovirus.

Adjuvants are substances that enhance the strength and duration of the immune response to vaccination. Additionally, adjuvants can reduce the number of doses of vaccine that must be given to produce effective immunity (7,8). Despite these benefits, historically the development of new vaccine adjuvants has been considered the slowest process in medicine. Only a single adjuvant, aluminium salts, was approved for use in humans from the 1920s to 1997 (9). Since the 1990s this situation has begun to change with the approval of many new adjuvants such as MPL, AS04 and AS01 (10). The SARS-COV-2 pandemic also resulted in the fastest approval of a new adjuvant in the form

of Novavax's Matrix-M in combination with full-length spike protein virus-like particles (11,12).

Low and middle-income countries have challenges accessing vaccines due to a variety of factors, with economic factors being a prominent reason (13,14). During the SARS-COV-2 pandemic, low and middle-income countries received reduced and delayed access to vaccines as well as receiving vaccines with lower efficacy compared to those in high-income countries (15). The majority of novel vaccine adjuvants in use are propriety technologies, and this intellectual property contributes to the lack of equality of access to new vaccines (16). The Vaccine Formulation Institute (VFI) produces open-access vaccine adjuvants to address this problem.

VFI produces vaccine adjuvants based on different combinations of a core set of components, which inform how they are named. The following 4 different adjuvants produced by VFI are investigated or referenced in this thesis: LQ, LMQ, SQ and SMQ. These adjuvants are formulated from either liposomes or a squalene emulsion, indicated in their names with L or S respectively. All 4 of these VFI-produced adjuvants contain the saponin QS-21, which is marked in the names of the adjuvants by Q. Where the adjuvant identifier contains the letter M, it indicates that it contains a synthetic TLR4 ligand. The combinatorial formulation of these four adjuvants has enabled the comparison of the efficacy and mechanisms of related adjuvants (17-19).

Previous research on these four adjuvants in combination with the R21 malaria antigen in a murine model revealed a hierarchy of protection against malaria [Fig 1.1] (18). Two adjuvants, LMQ and SQ, showed the highest and equivalent levels of protection against malaria achieving between 81-100% sterile protection, while the other two adjuvants showed a reduced

level of protection, with SMQ showing 63% and LQ showing 44% efficacy (18). Interestingly, protection against Malaria was not associated with any one component of these vaccines [Fig 1.2]. TLR4 agonism increased the efficacy of liposomal-formulated vaccines with LMQ being more protective than LQ. However, for squalene emulsion adjuvants TLR4 agonism had the opposite effect as SMQ showed a lower level of efficacy than SQ. This lack of understanding of efficacy in response to the components of an adjuvant is illustrative of the poor level of knowledge available on the mechanisms of action (MoA) of vaccine adjuvants.

1.2 Innate programming of adaptive immunity

1.2.1 Overview

The understanding of immune mechanisms underlying adjuvant-mediated enhancement of the adaptive immune responses remains incomplete. It is known that the innate immune systems shape and enhance downstream adaptive immune responses, but how this process works in the context of adjuvants is less well known. This section will overview key innate immune mechanisms that have been previously implicated in the mechanism of liposomal and squalene emulsion adjuvants. The details of how innate immune mechanisms are used by specific adjuvants will be covered in the next section.

Upon administration, adjuvants engage innate sensing mechanisms, often through pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (10). These pathways often lead to the production of pro-inflammatory cytokines and can induce localized

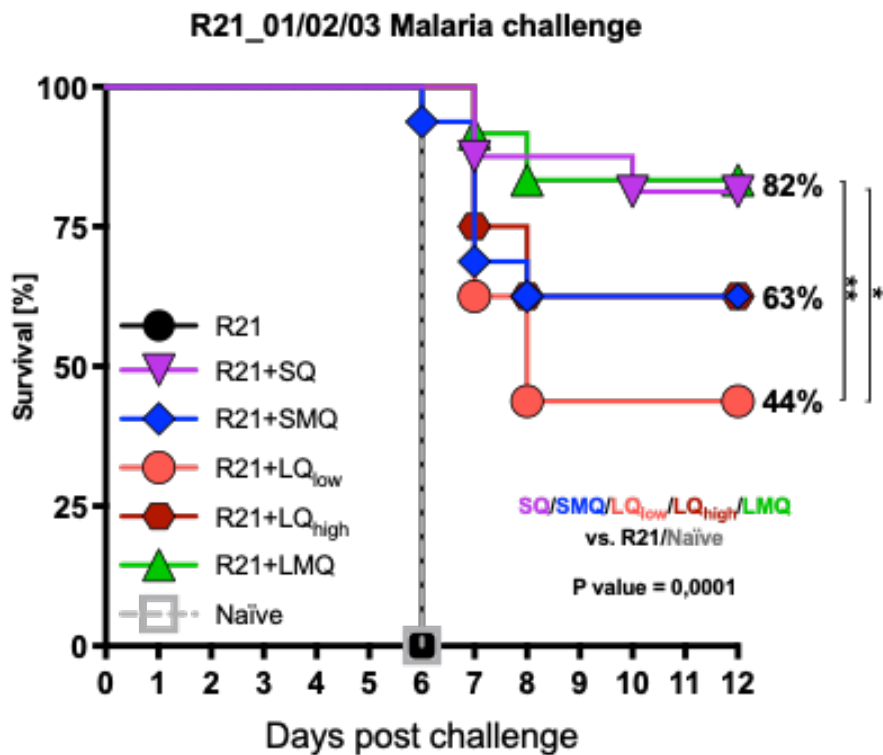


Figure 1.1: LMQ and SQ are highly protective against malaria in a murine challenge model: Reproduced from Reinke et al. Survival post-malaria challenge: BALB/c mice were vaccinated and challenged. Parasitemia was assessed by daily blood smears with 1% parasitemia taken as irreversible malaria infection. * $p < 0.05$, ** $p < 0.01$, log rank (Mantel-Cox) test. Showing pooled data from three independent experiments; $n = 16$ R21, LQ, SQ, SMQ; $n = 24$ naïve, LMQ. * $p < 0.05$, ** $p < 0.01$.

	TLR4 agonism	No TLR4 agonism
Liposomes	LMQ  Liposomes TLR4 agonist QS-21 Highly protective	LQ  Liposomes QS-21 Poorly protective
Squalene emulsion	SMQ  Squalene emulsion TLR4 agonist QS-21 Moderately protective	SQ  Squalene emulsion QS-21 Highly protective

Figure 1.2: No singular component of VFI-produced adjuvants correlates with protection against malaria

cell death, releasing damage-associated molecular patterns (DAMPs) that further stimulate immune activation (10). LMQ is known to induce inflammasome activation, TLR4 and cell death in vivo, while SQ is known to induce cell death in vivo (18). Subsequent to PRR activation, certain adjuvants including AS01 can induce NK cell activation and $\text{INF-}\gamma$ secretion, leading to downstream T cell activation and polarisation (20)

T-cells require multiple signals following immunisation or infection to be activated and differentiate into effector cells (21). Signaling through the T-cell receptor, co-signaling cell, and cytokine signalling are required (22). Signal 1 occurs when T-cell receptors recognise antigens displayed on MHC peptide complexes on the surface of antigen presenting cells. Signal 2 involves activation through co-stimulatory molecules on the surface of antigen presenting cells, such as CD80 and CD86, signalling to T-cells (22). Antigen presenting cells can also signal through co-inhibitory molecules, such as PD-1, to inhibit

T-cell activation and promote immune tolerance (22). Signal 3 is delivered through cytokines, which can be secreted the antigen presenting cell or by other cells distal to the immune synapse (21). Combinations of the three signals lead to polarisation of downstream T-cell responses to produce Th1, Th2 and other types of polarised T helper responses to vaccination (23). In murine models of immunisation to malaria LMQ induces Th1 responses, while SQ induces a Th2 polarised response (18)

B-cell responses after vaccination can also be polarised to produce different immunoglobulin subclasses, which can be controlled by the T-cell polarisation induced by the immunisation (18). Restimulation of LMQ immunised mice in a murine Malaria model showed a broadly Th1 polarised antibody subclass profile, with higher levels of secretion of IgG2b, IgG2c and IgG3 relative to IgG1, while SQ showed a Th2 polarised secretion of antibody subclasses with higher levels of IgG1 (18).

1.2.2 Toll-like receptor 4 signalling

TLRs are examples of PRRs, that recognise conserved structures of pathogens and other microorganisms. There are 10 human and 12 murine functional TLRs, that each recognise different molecules from pathogens (25). Multiple adjuvants utilise TLR signalling, however TLR4 ligand-containing adjuvants are the most commonly used TLR ligand in approved vaccines. TLR4 recognises Lipopolysaccharides (LPS) from gram-negative bacteria, and is also a receptor for ligands found in adjuvants such as Monophosphoryl-Lipid A (MPLA) and 3D-6-acyl-PHAD (3D6AP) (18,25). TLRs are found on a broad range of cell types including innate immune cells, but also on stromal cells including lymphatic endothelial cells (LECs) and fibroblasts (24,25). Muscle cells at the site of injection also respond to TLR4 agonists (26).

TLR4 signalling is enhanced through multiple cofactors such as CD14 and MD-2, but these are not a requirement for signalling (29). After recognition of LPS, TLR signalling is mediated through adaptor proteins including MyD88, TRIF, RIRAP and TRAM (30). TLR4 signalling subsequently results in NF- κ B dimerisation and upregulation of NF- κ B regulated pro-inflammatory genes. The TRIF pathway can also be activated, particularly from TLR4 signalling from endosomes, and lead to IRF3 activation and expression of interferons and other genes.

Innate immune processes, that are downstream of TLR4 signalling from adjuvants, can enhance the response to vaccination (31). TLR4 signalling from MPL is known to induce the secretion of chemokines and cytokines and recruit immune cells through upregulation of CCL2 and CCL3 (32). MPL also enhances antigen uptake in bone marrow derived macrophages, and TLR4 signalling promotes the generation of cytokine producing antigen specific CD8 and CD4 T cells (32,33). TLR4 signalling also contributes to IgG production: TLR4^{-/-} mice show reduced IgG1 and IgG2c titres after immunisation with an MPL containing adjuvant and hepatitis B surface antigens (33).

1.2.3 Type II interferon response

IFN- γ is the sole member of the type II interferon family. Both early innate responses and adaptive responses to vaccination can be mediated through IFN- γ . Signalling through IFN- γ 's receptor results in activation of the JAK-STAT1 pathway and results in the expression of interferon-stimulated genes (34). In human blood, IFN- γ levels are correlated with humoral immunity against SARS-COV-2, in terms of virus-neutralising antibody titres (35). In addition, IFN- γ signalling found in blood samples after RTS,S/AS01 vaccina-

tion is higher in vaccinees that were subsequently protected against malaria (36). While there is no fully accepted correlate of protection against malaria, this suggests an important role of IFN- γ in vaccine immune responses.

Type II interferon signalling is known to enhance Th1 polarisation of CD4+ T-cells, through inducing expression of the key transcription factor T-bet (38). Early IFN- γ produced by NK cells results in Th1 polarisation in adjuvant-draining lymph nodes (37). Th1 cells are known to produce IFN- γ , and both IFN- γ + Th1 cells and IFN- γ independent of T cells induce isotype switching from IgG1 to IgG2a and (40,41). Overall IFN- γ is required for adjuvant-induced Th1 polarised adaptive immune responses.

In addition to its role in humoral immune responses, IFN- γ plays a key role in cellular immunity both in a regulatory function and through induction of effector proteins. IFN- γ aids in the activation of CD8+ T-cells and promotes the development of effector phenotypes (40). In B-cell deficient mice, IFN- γ is required for cell-mediated viral clearance in a SARS-COV-2 model after vaccination (41).

Cell-autonomous immunity is an antimicrobial defence response in which cells act independently of the systemic immune system. IFN- γ is a prominent activator of cell-autonomous immunity and can lead to the production of anti-viral, anti-bacterial and anti-protozoan effectors within both immune and non-immune cells (42). Nitric oxide produced by macrophages, and a variety of other cells, in response to IFN- γ controls intracellular pathogens including viruses and protozoans (40,41). IFN- γ induces Nitric Oxide production in mouse hepatocytes that shows anti-parasitic properties against *Plasmodium yoelii* (43). There are numerous other effector proteins induced by IFN- γ that perform many different functions including inhibition of RNA

reverse transcription by Viruses, blocking RNA transcription and disrupting parasite-containing vacuoles (44). In summary, IFN- γ promotes the development of Th1-biased cellular and humoral immunity and also induces antimicrobial programmes within individual cells.

1.2.4 Inflammasome

Inflammasomes are intracellular multiprotein complexes that trigger the activation of caspases, resulting in the processing of pro-inflammatory cytokines (45). Both canonical, and non-canonical, inflammasomes can be activated by adjuvants. The activation of the inflammasome is triggered by the recognition of pathogen-associated molecular patterns (PAMPs), damage-associated molecular patterns (DAMPs) or loss of cellular homeostasis. Activation of the inflammasome requires two distinct signals as described by the two-signal model (45). An initial priming signal is required that results in the expression of inflammasome component genes and unprocessed cytokines. Following this, an activating signal is required that triggers the assembly and activation of the inflammasome complex.

Canonical inflammasomes are formed through the association of a variety of different proteins, but all require the Apoptosis-associated speck-like protein containing a CARD (ASC) adaptor protein. NLR family pyrin domain containing 3 (NLRP3) is a PRR that can associate with ASC to form the inflammasome (46). Other members of the NLR family can also form part of the inflammasome, as well as Absent In Melanoma 2 (AIM2) and pyrin. The formation of functional inflammasome complexes leads to the activation of Caspase 1 and the subsequent cleaving of pro-IL-1 β and pro-IL-18 into their functional forms. Inflammasome activation additionally results in the formation of Gasdermin D (GSMD) pores through Caspase 1 cleavage, and

secretion of the processed cytokines. Importantly inflammasome activation can result in a form of cell death called pyroptosis (47).

Inflammasome activation enhances adaptive immunity through the secretion of cytokines and DAMPS, as well as through DAMP release as a consequence of pyroptosis (45). This leads to APC activation and promotes the survival, proliferation and differentiation of T cells. IL-18 secretion, in combination with IL-12 results in IFN- γ production in NK cells and subsequent promotion of Th1 biased immunity (46,47). Additionally, IL-18, and resultant IFN- γ , promotes antigen-specific CD8⁺ T cell responses after immunisation with saponin-containing adjuvants (48). IL-1 β , which is also secreted after inflammasome activation, promotes survival and differentiation of Th17 cells (49,50). IL-1 β also promotes a proinflammatory environment through activation of NF- κ B signalling further enhancing innate immune signalling to programme adaptive immunity (51). APC activation downstream of inflammasome activation occurs through upregulation of co-stimulatory molecules such as CD80 and CD86 as well as increased antigen presentation on MHC II (45).

1.2.5 Cell death and DAMPS and their receptors

Cell death, and the resulting release of DAMPs, have become an emerging area of interest in the mechanism of action of vaccine adjuvants (10). While PRRs recognise exogenous molecules in the form of PAMPs, they can also recognise endogenous products of tissue damage and cell death in the form of DAMPs (54). Proteins, lipids, sugars and nucleic acids are all known to act as endogenous DAMPs. DAMPs are known to be produced in multiple modes of cell death including apoptosis, necroptosis and pyroptosis. DAMPs can also be produced independently of cell death through membrane pores

and exosomes (55). Upon recognition of DAMPs, both immune and non-immune cells can produce cytokines and chemokines to recruit and activate innate immune cells. DAMPs are also known to act on adaptive immune cells (B and T cells) directly, and influence adaptive immunity.

DAMPs are recognised by germline-encoded PRRs including TLRs, C-type Lectin receptors (CLRs), NOD like receptors (NLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) (54). DAMPs are also known to be recognised by non PRR proteins such as ion channels and G-protein coupled receptors. Given the diversity of DAMPs and their receptors, numerous signalling pathways can be activated upon DAMP recognition. CLRs such as Dectin-1 and MINCLE are known to activate MAP kinase signalling through SYK and also induce NF- κ B signalling (54,56,57). TLRs activated by DAMPS signal through the MyD88 adaptor protein and TRIF(58) . In addition, inflammasome activating signals can be induced by DAMP signalling through NLRP3 and AIM2 (59,60).

Receptors for DAMPs are expressed on both innate-immune, adaptive-immune and non-immune cells types (52,60). DAMP signalling results in signalling pathways and inflammasome activation that results in pro-inflammatory cytokine production. This leads to the recruitment and activation of innate immune cells at the site of DAMP release and downstream in the lymph nodes(60,61). DAMPs also impact adaptive immunity by promoting the maturation and antigen-presenting capacity of APCs, including DCs (62). Additionally, through TLR7 expression on B cells, endogenous RNA can directly induce the proliferation and activation of B cells (63).

1.3 Mechanisms of squalene and emulsion-based adjuvants

Adjuvants are thought to improve the immune response to vaccination through activating the innate immune system, and thereby programming the resulting adaptive immune response to be more effective (64). The specific ways through which adjuvants act are numerous and include induction of cytokine and chemokine release, immune cell recruitment, enhancing antigen presentation and increasing antigen drainage to the lymph node (65). Newer concepts in how adjuvants work implicate cell death and damage-associated molecular patterns (DAMPs) as well as changes to metabolism in the MoA of adjuvants (10,61,66,67). This wide range of implicated mechanisms reflects the large diversity of substances used as vaccine adjuvants. In the following section vaccine adjuvants that are particularly relevant to the adjuvants studied in this thesis will be covered in detail, focusing on adjuvants that are formulated from liposomes, squalene emulsions or similar materials.

1.3.1 AS01 and LMQ

AS01 and LMQ are analogous vaccine adjuvants. Both are formed from liposomes combined with the saponin QS-21 and contain a TLR4 ligand (18,68). However, they differ in the respect that the TLR4 ligand of AS01 is Monophosphoryl-Lipid A (MPLA), whereas LMQ contains the synthetic TLR4 ligand 3D-6-acyl-PHAD (3D6AP). Although not yet directly demonstrated, it can be assumed that LMQ acts through similar mechanisms to AS01, given the similar composition.

AS01 is a highly potent vaccine adjuvant, with the potential to induce effec-

tive immune responses in older populations (69,70). Two separate vaccines that are highly protective in older populations have been approved for clinical use that utilise AS01, the Herpes zoster vaccine Shingrix and the Respiratory Syncytial Virus (RSV) vaccine AREXVY. Vaccine efficacy of AS01 adjuvanted vaccines in older adults was 89.8% and 82.6% against Herpes zoster and RSV respectively (69,70). Additionally, AS01 has had success in clinical trials against malaria in children as part of the Mosquirix vaccine, and until the development of the Matrix-M/R21 vaccine was the most successful malaria vaccine developed (71,72). LMQ is currently still in pre-clinical research with GMP product development underway. However, it has shown promising early results, where it generates protection against Malaria in preclinical models comparable with those seen for Matrix-M and AS01 (18).

Both AS01 and LMQ have been shown to induce a Th1-biased immune response in murine models (18,20). The RTS,S/AS01 vaccine induces IFN γ +IL-2+ TNF α + CD4+ T cells characteristic of this Th1 bias, additionally, AS01 induces antibody isotype switching consistent with a Th1 bias (20). Similarly to AS01, LMQ immunisation in mice in combination with the malaria R21 antigen results in increased IgG2 and IgG3 subclass usage consistent with a Th1 bias (18).

AS01, and therefore likely LMQ, is known to require both the TLR4 ligand and the QS-21 component for full activity as immunisation with either component individually produces lower IgG titres (20). The QS-21 component of AS01 is known to activate the inflammasome through the NLRP3 pathway *in vitro* and induce the release of IL1B and IL18 (73). QS-21 is also known to cause cell death in macrophages and dendritic cells *in vitro*, but through an NLRP3, Caspase-1 and ASC independent manner, suggesting a

non-pyroptotic form of cell death (73).

MPL, the TLR4 ligand component of AS01, acts through activation of the TLR4 signalling pathway and triggers NF- κ B through MyD88 and TRIF activation leading to the expression of pro-inflammatory cytokines and chemokines (74). The MPL and QS-21 components of AS01 show a synergistic effect enhancing the immune response to this adjuvant (20). Early release of IFN- γ is required for this synergistic effect and is mediated by IL18 release from SCS macrophages and IL12 (20).

1.3.2 SQ

SQ, one of the other adjuvants explored in this thesis, is relatively less understood than other adjuvants. It is formulated from a squalene emulsion and the QS-21 saponin fraction. As with LMQ, SQ provides protection against malaria and HBV in preclinical models (18,19). Against the malaria antigen R21 it has been shown to induce a Th2 biased immune response in comparison to LMQs Th1 bias, while delivering the same level of protection against infection in a murine model (18). *In vitro* SQ has been shown to induce minimal levels of cytokine release in both murine bone marrow-derived macrophages and in human monocyte-derived dendritic cells (17,18). However SQ does induce cell death in bone marrow-derived macrophages, based on LDH release (18). Interestingly despite the *in vitro* results, *in vivo* significant cytokine release is detected in the muscle site of injection and blood serum after immunisation with SQ (18). Both CXCL1 and TNF α were not detected from *in vitro* stimulation assays but were detected *in vivo* after immunisation. Together this suggests that SQ lacks the capacity to directly activate immune cells, given it's lack of TLR4 agonist, and may act through DAMP release from adjuvant-induced cell death to achieve its adjuvant effect.

1.3.3 MF59 and AS03

Like SQ, MF59 and AS03 are both squalene emulsion adjuvants, however, they do not contain saponins. For MF59 the squalene emulsion is stabilised with Tween 80 and Span 85, while AS03 contains Tween 80 it also contains α -tocopherol which contributes to its adjuvant activity (7,75). MF59 induces the release of cytokines and chemokines at the muscle site of immunisation and draining lymph nodes (76). The release of chemokines such as CCL2, CCL4 and CCL5 recruits monocytes, neutrophils and eosinophils to the injection site. These recruited cells upregulate the costimulatory molecules such CD80 and CD86, and display increased antigen processing and presentation (76). Recruited cells also contribute to trafficking antigens to draining lymph nodes (77). MF59 requires MYD88, but not TLR, signalling for its full adjuvant effect (78). Research on the involvement of the inflammasome in the MoA of MF59 has shown that MF59 activates an ASC dependent, but NLRP3-independent pathway (78,79). Multiple forms of cell death, and subsequent DAMP release, have been implicated in the mechanism of action of squalene emulsion adjuvants. MF59 induces RIPK3-dependent necroptosis in draining lymph nodes, and CD8+ T cell responses are RIPK3-dependent after MF59 immunisation in mice (60). In addition to necroptosis, MF59 induces apoptosis in draining lymph nodes and pan-Caspase inhibition impairs antibody responses to vaccination (60). Release of ATP from muscle cells after immunisation also contributes to the adjuvant effect by MF59, through ATP acting as an endogenous DAMP (61).

1.3.4 Nanoparticles from mRNA vaccines

Lipid nanoparticles are used as adjuvants in mRNA vaccines and are formed of lipids and cholesterol. Lipid nanoparticles have a lamellar outer structure and an internal core of lipids and cholesterol in a liquid crystalline phase (80). Lipid nanoparticles show multiple differences to liposomes and squalene emulsion in terms of their PEGylation of lipids and the presence of ionisable lipids. Interestingly, while lipid nanoparticles are often not described as adjuvants, they are known to induce inflammation after immunisation and enhance the immune response to immunisation (81). Empty lipid nanoparticles produce a stronger humoral immune response to influenza protein antigens than Ad-davax, an MF59 analogue, in a murine model (81). This confirms that lipid nanoparticles show an adjuvant effect independently of their encapsulation of mRNA. In mice, the adjuvant effect of lipid nanoparticles is known to be mediated through IL6 and MYD88 and type I interferon-dependant MDA5 signalling (81,82). Interestingly a variety of TLR knockout mice showed no effect on downstream immune responses including TLR3 $-/-$, TLR4 $-/-$ and TLR9 $-/-$, suggesting TLR signalling is not a mechanism through which LNPs act (82).

1.3.5 Adjuvant size

The particle size of adjuvants is known to impact onto their lymphatic drainage patterns (83). Particles within a size range of 10-200 nm can directly enter lymphatic vessels by diffusing through lymphatic endothelial cell junctions, or alternatively through cellular trafficking into the vessel lumen (83, 84). However, particles with a size of more than 200 nm are less efficiently able to enter lymphatic vessels through diffusion alone, with the

efficacy of entry inversely proportional to particle size (83). The particle size of adjuvants manufactured by VFI are known to be 130-150nm, and stable to at least 300 days of storage at 4 °C (85,86). While these conventions suggest that VFI's adjuvants are able to enter the lumen of lymphatic vessels by diffusion, the lymphatic drainage patterns of these adjuvants has not been previously studied.

1.4 Knowledge gaps in the mechanism of action of adjuvants

The mechanistic understanding of vaccine adjuvants remains underdeveloped. The adjuvants produced by VFI are illustrative of this, given the unusual relationship between adjuvant components and protection [Fig 1.2]. Additionally, murine models of malaria using these adjuvants have shown that Th1-biased immune responses produced by LMQ can be equally protective as Th2-biased immune responses induced by SQ immunisation. This contradicts conventional thinking that certain Th biases will produce superior efficacy against certain pathogens. It is not known if LMQ and SQ produce entirely different innate immune programs that lead to protection, or if there are shared pathways and mechanisms that converge on protection despite the differences in Th bias. This thesis will explore the mechanisms of LMQ and SQ to identify shared and divergent mechanisms of action.

The cellular targets of liposomal and squalene emulsion adjuvants are little explored. Currently, the only understanding of the target cells of the LMQ like adjuvant AS01 comes from fluorescent microscopy studies of the draining lymph node (87,88). As this fluorescent microscopy employed a limited num-

ber of markers, it is only known that SCS macrophages take up AS01 and not what the other potential cellular targets of liposomal adjuvants are. The uptake of the adjuvant MF59 has been explored in more detail through flow cytometry experiments with fluorescently labelled adjuvant, revealing MF59 uptake in macrophages and DCs (79). However, the uptake of squalene emulsion adjuvants has not been explored by flow cytometry in other cell types. As with AS01, the squalene emulsion adjuvant MF59 was also localised with macrophages in the sinus regions of the lymph node (84). Additionally, there has been minimal exploration of the level of spread of liposomal and squalene emulsion adjuvants from the site of injection, and therefore the location of resulting immune responses cannot be identified. In summary, while there have been attempts to identify target cells of adjuvants, the methods used have lacked resolution and produce an incomplete picture of adjuvant localisation in tissues and cells.

Additionally, there is little resolution on the cellular sources of inflammation produced by adjuvants. Previous research has generally relied on bulk measurements of tissues such as the lymph node or spleen, where information on the cellular sources of inflammation are lost. *in vitro* assays on cultured cells have also often been used to identify responding cells to adjuvants, but these systems ignore the complex interaction of multiple cell types found *in vivo* and suffer from culturing-induced changes in immune cell phenotypes. An *in vivo* understanding of the cellular sources of inflammation is required to gain a greater understanding of the mechanism of action of liposomal and squalene emulsion adjuvants.

1.5 Research questions and hypothesis

The goal of this thesis is to address the previously discussed knowledge gaps in the mechanism of action of squalene emulsion and liposomal adjuvants. I hypothesised that LMQ and SQ have a shared underlying inflammatory mechanism of action. This hypothesis was explored through the following research questions:

- Which lymphoid organs do squalene and liposomal vaccine adjuvants drain to?
- Which cells take up squalene and liposomal vaccine adjuvants?
- What are the transcriptional profiles of inflammation induced by LMQ and SQ across organs?
- What are the cellular sources of the transcriptional inflammation induced by LMQ and SQ?
- Analyse the transcriptomic similarities and differences between cells in the mouse and human lymph node, with a focus on adjuvant sensing receptors.

2 | Methods

2.1 Wet lab methods

2.1.1 Animal care

All mice used were housed in the Kennedy Institute of Rheumatology Biological Services Unit in individually ventilated cages under specific pathogen-free conditions. Mice were fed a standard diet of irradiated mouse feed and water ad libitum and housed under an artificial 12-hour light-dark cycle. Mice were aged between 8-12 weeks. All procedures were performed under a UK home office licence. Mice were of the BALB/cOlaHsd (BALB/c) genotype, and female mice were used in experiments.

2.1.2 Immunisations, adjuvants and dissections

Immunisations of fluorescently labelled LMQ were carried out via intramuscular injections into the gastrocnemius muscle in the calf. Briefly, 50 of adjuvant formulation (25 μ l of adjuvant resuspended in 25 μ l of the corresponding buffer) Is injected at specific time points under isoflurane anaesthesia, mice are then culled, and the organs of interest are collected.

All adjuvants used are formulated by the Vaccine Formulation Institute (VFI) in Geneva. LMQ, SQ, LQ and SMQ have been established to have a particle size of 130-150nm, polydispersity index values below 0.15 and a surface charge Zeta potential of between -10 and -40 mV. All adjuvant formulations used contain 200 μ g/mL of QS-21, while LMQ and SQ adjuvants contain 80 μ g/mL of 3D6AP (85, 86). Liposomal adjuvants (LMQ and LQ) contain 0.8 mg/ml cholesterol and 2 mg/ml DOPC. Squalene emulsion adjuvants (SQ and SMQ)

contain 0.4 mg/ml cholesterol and 40 mg/ml squalene.

To produce fluorescent adjuvants VFI formulated the adjuvants to include the fluorescent lipophilic dye 1,1'-Diocadecyl-3,3,3',3'-Tetramethylindodicarbocyanine, 4-Chlorobenzenesulfonate Salt (DiD). DiD is a far-red fluorophore which fluoresces with an emission maximum of 663 nm, and is detectable in the AF647 or APC channel in flow cytometry and fluorescence microscopy.

2.1.3 Tissue digestions

Different procedures were used to produce single-cell suspensions from different organs. The tissue dissociation procedure developed by Fletcher et al was used for the digestion of lymph nodes (85). Lymph nodes were pierced with two 25 gauge needles and then incubated in an enzymatic digestion mixture (0.8 mg/mL Dispase II, 0.2 mg/mL Collagenase P, 0.1 mg/mL DNase I). Individual LNs were digested for 20 min in 1 ml of digestion mixture at 37 °C, then aspirated with a 1 ml pipette to mechanically disrupt the LN. The supernatant was removed and passed through a 100- μ m cell strainer into 5 ml of quenching buffer (PBS, 10% FBS and 5 mM EDTA). 1 ml of additional digestion mix was then added to the remaining LN fragments and digested at 37 °C for an additional 10 minutes, aspirating with a 1 ml pipette every 5 mins. Digestion and removal of supernatant were repeated at least two times or until no fragments of LN were visible, typically 3 total steps.

Spleens were processed by mincing spleens into small pieces before digesting with 1ml of an enzymatic digestion mixture (0.8 mg/mL Dispase II, 0.2 mg/mL Collagenase P, 0.1 mg/mL DNase I) for 20 mins at. The supernatant was removed and passed through a 100- μ m cell strainer into 5ml of quenching buffer. 1 ml of additional digestion mix was then added to the spleen frag-

ments and digested at 37°C for an additional 10 minutes, aspirating with a 1 ml pipette every 5 mins. The total remaining mixture was passed through a 100- μ m cell strainer and the remaining spleen fragments mashed through the strainer.

For muscles, the tissue was cut into small pieces and incubated in a muscle digestion mixture (0.8mg/ml Dispase II, 0.2mg/ml Collagenase P and 0.5mg/ml DNase I) at 37 °C for 30 minutes and mixed with pipetting every 5 minutes. The supernatant was then removed and passed through a 100 μ m cell strainer into the quenching buffer.

2.1.4 Flow cytometry

For flow cytometry, single cell suspension generated by the digestion step was blocked in FACS buffer (PBS, 2% FBS, 5mM EDTA) with Fc block (BD Biosciences) for 10 min at 4°C. A staining solution containing antibodies and Live/Dead stain was applied for 20 min at 4 °C in the dark. The cells were centrifuged for 10 min at 400g, the supernatant discarded and the cells fixed with fixation buffer (BioLegend) for 20 min at room temperature. Cells were analysed using BD Fortessa or Cytex Aurora cytometers as indicated.

Gating strategies for different cell populations, tissues and staining panels are shown in Figs 2.1-4 To define cells as adjuvant positive a threshold is set using the fluorescence of each of the naïve cell types, those cells above this threshold are defined as adjuvant positive (Fig2.5).

2.1.5 Frozen tissue processing and immunofluorescence

Excised tissue was fixed in PLP buffer (0.075 M lysine, 0.37 M sodium phosphate, 2% formaldehyde, 0.01 M NaIO₄) overnight before freezing in Tissue-

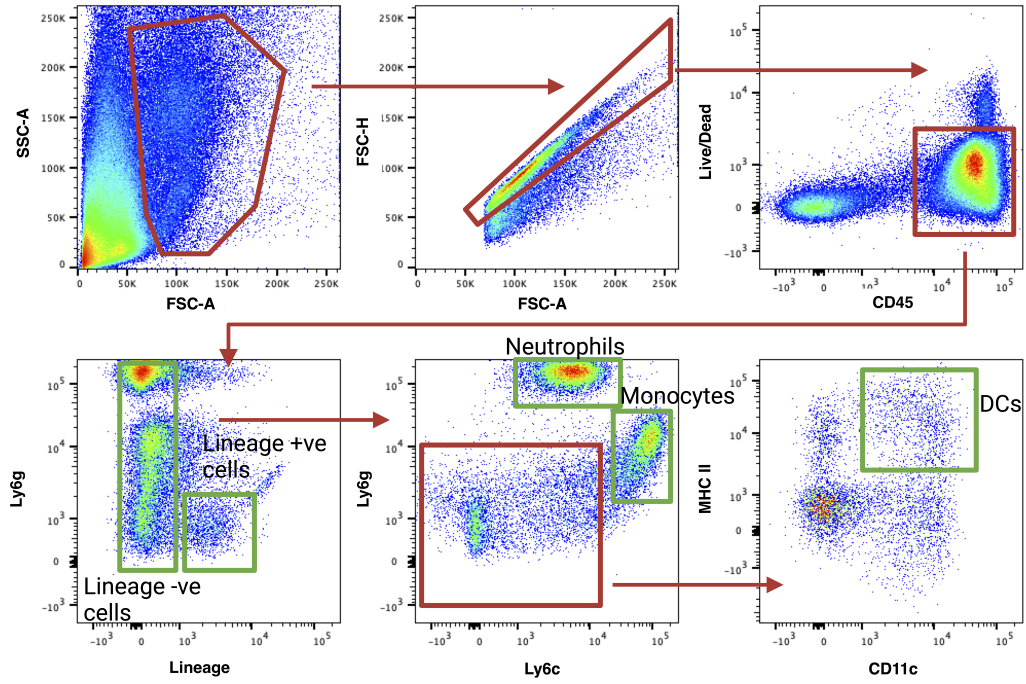


Figure 2.1: Muscle monocyte gating strategy The gating strategy used to identify Lin⁻ cells (CD45⁺, B220⁻, CD3⁻), Lin⁺ cells (CD45⁺, B220/CD3⁺), Neutrophils (CD45⁺, Lin⁻, Ly6g⁺, Ly7c low), monocytes (Cd45⁺, Ly6g low, Ly6c ⁺) and DCs (Cd45⁺, Lin⁻, CD11c⁺, MHCII⁺) cells in the muscle. Red gates show filtering on that population of cells, green gates indicate the final identified populations used in subsequent analysis.

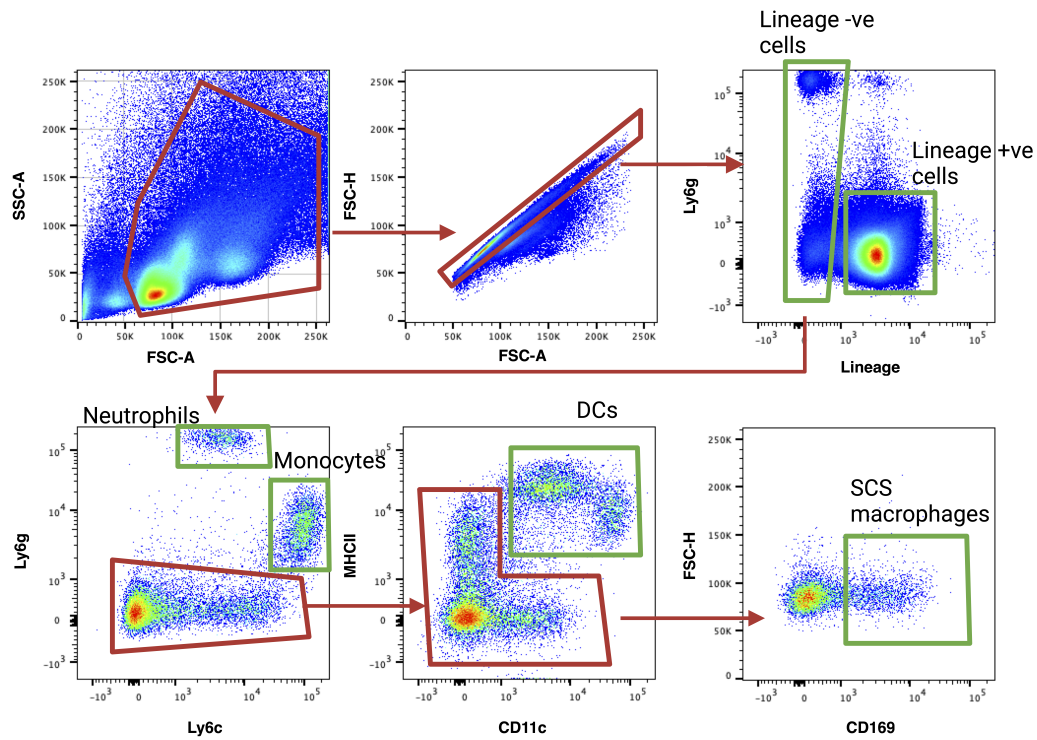


Figure 2.2: iLN monocyte gating strategy The gating strategy used to identify Lin⁻ cells (CD45⁺, B220⁻, CD3⁻), Lin⁺ cells (CD45⁺, B220/CD3⁺), Neutrophils (CD45⁺, Lin⁻, Ly6g⁺, Ly7c low), monocytes (Cd45⁺, Ly6g low, Ly6c ⁺), DCs (Cd45⁺, Lin⁻, CD11c⁺, MHCII⁺) and SCS macrophages (CD45⁺, Lin⁻, CD169⁺) cells in the iLN. Red gates show filtering on that population of cells, green gates indicate the final identified populations used in subsequent analysis.

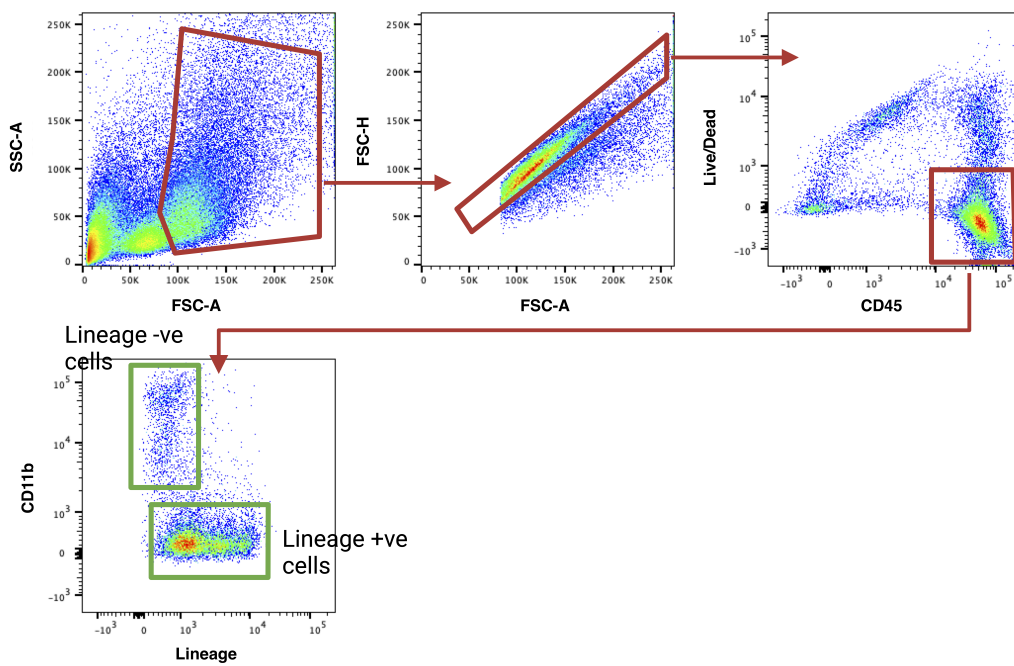


Figure 2.3: Spleen gating strategy The gating strategy used to identify Lin+ (CD3+/B220+) and Lin- (CD45+, CD3-/B220-) cells in the spleen. Red gates show filtering on that population of cells, green gates indicate the final identified populations used in subsequent analysis

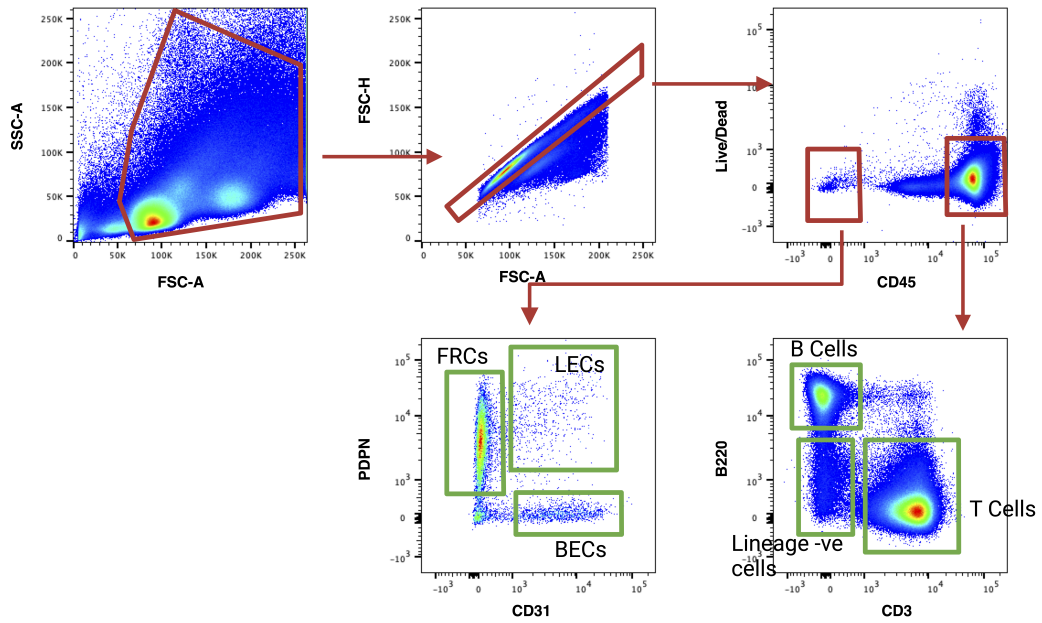


Figure 2.4: iLN stromal gating strategy The gating strategy used to identify FRCs (CD45⁻, PDPN⁺, CD31⁻), LECs (CD45⁻, PDPN⁺, CD31⁺), BECs (CD45⁻, PDPN⁻, CD31⁺), B Cells (CD45⁺, B220⁺), T Cells (CD45⁺, CD3⁺) and Lin⁻ cells (CD45⁺, B220⁻, CD3⁻) cells in the iLN. Red gates show filtering on that population of cells, green gates indicate the final identified populations used in subsequent analysis.

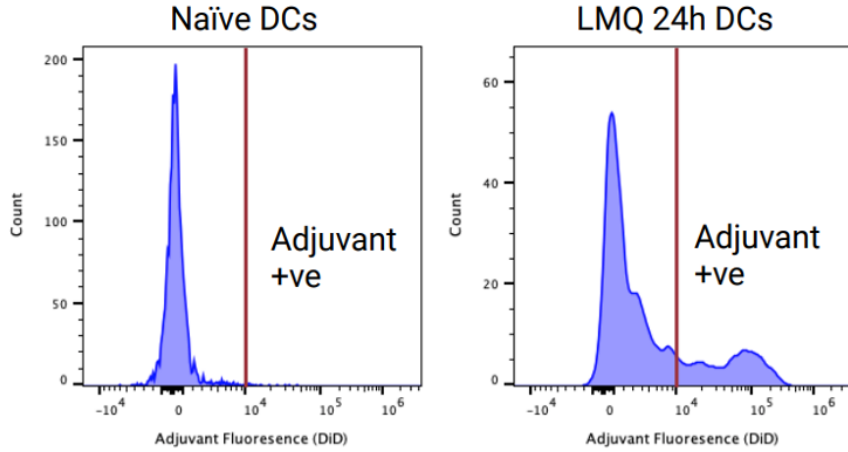


Figure 2.5: Exemplar histograms of the method of defining adjuvant positive cells. For each cell type, a threshold is set on the Naïve populations fluorescence in the AF647 channel that DiD fluoresces in. Cells that are above this threshold are considered adjuvant positive (DiD+).

Tek O.C.T. compound (Sakura) using a slurry of dry ice and isopentane. Tissues were cut to a thickness of 5 μ m using a cryostat before staining. Antibody staining with primary conjugated antibodies (CD45R/B220; BV421; BD Biosciences, CD169; AlexaFluor 594; Biolegend and CD3; AlexaFluor 488; Biolegend) was performed overnight at 4 °C in immunofluorescence blocking buffer (PBS + 20% donkey serum) staining with unconjugated antibodies (F4/80;Biolegend) was performed overnight at 4 °C in immunofluorescence blocking buffer, then stained with secondary antibodies (Donkey anti-rat IgG; AlexaFluor 488; Abcam) for 2 hours at room temperature. Where used nuclei were stained with DAPI for 5 mins at 4°C then coverslips were then added using Prolong Gold antifade reagent as the mounting medium. Images were taken with either a Zeiss LSM 980 confocal microscope or a Zeiss Axioscan 7 as indicated.

2.1.6 RNA extraction

RNA extraction protocols were optimised for muscle and lymph node samples. After tissue isolation mouse muscles were minced with scissors and placed in MACS M tubes (Miltenyi) with 5 ml of RLT lysis buffer (Qiagen). The tissue was then disrupted using a gentleMACS Octo Dissociator (Miltenyi) using RNA programme 2. RNA was then purified using the RNeasy Mini kit (Qiagen) according to manufacturer instructions.

2.1.7 Total cDNA synthesis

RNA concentration was identified with a nanodrop spectrophotometer then samples were diluted to equal concentrations. Synthesis of cDNA was performed using the First Strand cDNA Synthesis Kit (Thermofisher). Briefly, 250-500ng of RNA was mixed with 1 μ L of random hexamer primers and made up to 11 μ L with nuclease-free water. This mixture was run on a thermocycler with the following conditions: 5 mins at 65°C then 5 mins at 4°C. Subsequently, the following reagents were added to the mixture: 4 μ L of 5X Reaction Buffer, 1 μ L RiboLock RNase Inhibitor, 2 μ L dNTP Mix (10 mM) and 2 μ L M-MuLV Reverse Transcriptase. The reverse transcription reaction is then performed on a thermocycler using the following programme: 5 min at 25°C, 60 min at 37°, 5 min at 70°C then stored at -20°C.

2.1.8 Quantitative PCR

The qPCR reaction was composed of 5 μ L of TaqMan Universal PCR Master Mix, 1 μ L of endogenous control primer, 1 μ L of test primer, 10ng of cDNA and nuclease-free to make the volume up to 10 μ L. Each sample was run in triplicate on a ViiA 7 Real-Time PCR System for 40 cycles. All primers

were ordered from Thermofisher with the endogenous primer using the VIC dye and the tested primer using the FAM-MGB dye. Relative quantification analysis was performed using the applied biosystems Design and Analysis software using the $\Delta\Delta C_t$ method (86).

2.1.9 Sample multiplexing for single-cell sequencing

Single-cell suspensions of lymph nodes were counted and adjusted to 1×10^6 cells per ml. Samples were then stained with either TotalSeq-B anti-mouse hashtag antibodies (M1/42,30-F11; BioLegend) if processed for 10X chromium or antibodies from the Ms Single Cell Sample Multiplexing Kit (BD biosciences) if processed for BD rhapsody. Samples were washed twice with 1 ml of PBS to remove excess hashing antibodies to enhance empty droplet detection.

2.1.10 Depletion of B and T cells

Multiplexed single cell suspensions of lymph nodes were pooled then sorted using a SH800S chip sorter (Sony) with the 100 μm Sony Sorting Chip using the following antibodies CD45 (BV785; 30-f11; Biolegend), CD3 (APC; 17A2; Biolegend) B220 (FITC; RA3-6B2; eBioscience) and LIVE/DEAD Fixable Far Red Dead Cell stain (Invitrogen). The gating strategy for the depletion of B and T cells is shown in Figure 5.1A. Each pooled sample was sorted with the gating set such that 95% of the sample came from the gate that was CD3- and B220-. The remaining 5% of the cells sorted were from the B220+ and CD3+ positive cells.

2.1.11 10X chromium Single cell sample preparation

Single cell suspensions that were depleted of B and T cells, and samples multiplexed with antibodies, were checked for cell count and viability with Acridine orange using LUNA 8-Channel Cell Counting Slides on the LUNA-FX7 automated cell counter. Cells were run using the Chromium Next GEM v3.1 3 system to produce single-cell libraries. For each cartridge 20,000 cells were loaded as the use of multiple hashing antibodies enables overloading the cartridge to increase the number of cells captured.

2.1.12 BD Rhapsody Single cell sample preparation and reverse transcription

Single cell suspensions that were depleted of B and T cells, and samples multiplexed with antibodies, were checked for cell count and viability with Acridine orange using LUNA 8-Channel Cell Counting Slides on the LUNA-FX7 automated cell counter. Reagents from the BD Rhapsody Enhanced Cartridge Reagent Kit were used to load the cartridge with single cells. For each cartridge, 20,000 cells were resuspended in a cold Sample Buffer and 575 μ L of cell suspension was loaded onto a BD Rhapsody Cartridge and incubated at room temperature for 15 mins. Next, 630 μ L of BD Rhapsody Enhanced Cell Capture Beads is added to the cartridge and incubated for 15 mins at room temperature then for 15 mins at room temperature shaking at 1,00rpm on an Eppendorph ThermoMixer. The cartridge is then washed twice with cold Sample buffer before the cells are lysed by adding 550 μ L Lysis buffer and incubating at room temperature for 2 mins. Beads are then retrieved from the cartridge using the BD Rhapsody express instruments magnetic retrieval mode, adding 4,950 μ L of lysis buffer to the cartridge and

collecting the eluted beads in a 5ml FACS tube. Using a magnetic rack the supernatant of lysis buffer is removed from the beads and washed with 1ml of bead wash buffer twice.

Using the BD Rhapsody cDNA Kit a reverse transcription mix is made from the following components: 48 μ L RT buffer, 24 μ L dNTPs, 12 μ L RT 0.1 M Dithiothreitol, 14.4 μ L Bead RT/PCR Enhancer, 12 μ L RNase Inhibitor, 12 μ L Reverse Transcriptase and 117.6 μ L Nuclease-Free Water. Any supernatant is removed from the cell capture beads on a magnet, the tube is removed from the magnet and 200 μ L of the reverse transcription mix is added to the beads before mixing. The mixture is incubated at 37°C with mixing at 1,200rpm for 20 mins then the supernatant is removed from the beads on a magnetic stand. Following reverse transcription, 200uL of Exonuclease I mix (24 μ L 10X Exonuclease I Buffer, 12 μ L Exonuclease I, 240 μ L Nuclease-Free Water) is added to the beads, then incubated at 37°C with mixing at 1,200rpm for 30 mins then incubated at 80°C for 20 mins without mixing. The supernatant is then removed from the sample and beads are resuspended in 200 μ L of cold bead resuspension buffer.

2.1.13 Next-generation sequencing

Samples were sequenced through Novogene using a paired-end 150 base pair reads on a Novoseq 6000 instrument. For each single-cell sample, the amount of sequencing was requested to be approximately 50,000 reads per cell. Sequencing QC metrics were analysed to ensure sample quality was sufficient for further analysis.

2.1.14 FFPE tissue processing

The excised tissue was fixed in 4% PFA overnight before being moved into 70% ethanol. Tissues are then dehydrated, cleared and infiltrated with paraffin in an automated tissue processing system before being oriented and embedded in paraffin. Tissues were sectioned to a thickness of 5 μ m with a Leica HistoCore BIOCUT microtome.

2.1.15 TUNEL assay

All steps in the TUNEL assay were performed using the Click-iT Plus TUNEL Assay Kits for In Situ Apoptosis Detection (Thermofischer). Slides are first deparaffinised by washing with the following steps: Xylenes for 5 mins twice, 50:50 Xylenes: Ethanol for 3 mins, 100% ethanol for 5 mins, 100% ethanol for 3 mins, 95% ethanol for 3 mins, 85% ethanol for 3 mins, 75% ethanol for 3 mins, 50% ethanol for 3 mins, 50% sodium chloride for 5 mins and PBS for 5 mins. Slides are then fixed with 4% PFA for 15 mins at 37°C then washed with PBS for 5 mins. The tissue sections were permeabilised with a 1X proteinase K solution for 15 mins at room temperature, washed with PBS for 5 mins, fixed with 4% PFA at 37°C for 5 mins then washed again with PBS for 5 mins. To perform the TdT reaction, 100 μ L of TdT reaction buffer is added to each slide and incubated at 37°C for 15 mins, the buffer removed from the slides, 50 μ L TdT reaction mix added (per slide: 47 μ L TdT Reaction Buffer, 1 μ L EdUTP, 2 μ L TdT enzyme) followed by incubation at 37°C for 60 mins, rinsed with deionised water, washed for 5 mins in 3% BSA and 0.1% Triton X-100 in PBS for 5 mins and washed with PBS for 5 mins. For the Click-iT reaction, 50 μ L of Click-iT Plus TUNEL reaction mix (45 μ L Click-iT Plus TUNEL Supermix, 5 μ L 10X Click-iT Plus TUNEL Reaction

Buffer Additive) is added to the tissue sections and incubated at 37°C for 30 mins while protected from exposure to light, washed with 3% BSA in PBS for 5 mins and washed with PBS for 5 mins. Slides are then stained with DAPI for 5 mins at room temperature, washed twice with PBS and coverslips added using Prolong Gold antifade reagent as the mounting medium.

TUNEL-stained slides were analysed with QuPath (v0.5) to quantify nuclei that stained positive with DAPI, TUNEL or both. Damaged tissue sections were removed from the analysis. Following DAPI-positive nuclei and TUNEL-positive nuclei were detected across the tissue section and the percentage of TUNEL-positive nuclei was calculated.

2.2 Computational methods

2.2.1 Bulk RNA pre-processing and normalisation

Transcripts were aligned to the mouse genome (GRCm39, Mm39) and quantified using the fast pseudo-alignment algorithm Kalisto (87). Genes with low expression were filtered and the remaining genes normalised. For visualisation genes were transformed using variance stabilising transformation from the DeSeq2 package and scaled based on their Z-score (88).

2.2.2 Bulk differential expression testing with DEseq2

Gene expression changes were identified from the bulk RNA count data using the DeSeq2 package (88). Genes were identified as differentially expressed if they passed the cutoff of the Bonferroni adjusted P-value from DESeq2 result < 0.05 and the absolute value of $\text{Log}_2\text{FC} > 1$.

2.2.3 Single-cell transcriptome data pre-processing and quality control

Raw gene counts were obtained by aligning reads to the mouse genome (GCRm39, Mm39) using Cellranger (v7.0.0) or BD Genomics Rhapsody Analysis pipeline (v2.0) for transcriptomes collected using 10X Chromium or BD Rhapsody respectively. For both approaches reads were aligned to both intron and exon regions of transcripts.

Two approaches were used for quality control. The first approach was gene and cell quality control. To remove low-quality transcriptomes a simple threshold was applied to both cells and genes. Cells that contained above 20% mitochondrial RNA content or below 200 total RNAs were filtered out. Genes that were detected in less than 10 cells in the dataset were also removed. The second approach was doublet removal. To remove doublets from single-cell transcriptomic data the Python package single-cell remover of doublets (Scrublet) was applied (89). Doublets occur when two cells are encapsulated in the same droplet or well depending on sequencing technology, leading to two cells being recorded as a single transcriptome. Scrublet simulates doublets based on the scRNA-seq transcriptome provided by combining single-cell transcriptomes randomly from the data, based on the assumption that most transcriptomes are singlets. Transcriptomes in the single data are scored based on their proximity to the simulated doublet data on a K nearest neighbour graph. Cells are then identified using a doublet-score threshold.

2.2.4 Batch correction with Harmony

To correct for batch effects from a variety of sources both BBKNN and Harmony were explored, and Harmony performed better for batch correcting

[data not shown] (90,91). Harmony performs batch correction by utilising input principal components to create corrected principal components for batch effects by utilising fuzzy clustering and a form of linear model, called a mixture of expert models, to remove the batch effects.

2.2.5 Batch effect metrics (LISI)

To identify the magnitude of batch effects the Local Simpsons Inverse Index (LISI) was used(91). LISI scores each cell on the number of different batches or other categories in its local neighbourhood, with the score representing the different number of these categories. Therefore, a LISI score of 1 represents samples from different batches that are completely separate and unmixed, scores higher than this show the amount of mixing across batches.

2.2.6 UMAP visualisation

Due to the multidimensional nature of scRNA-seq data, it is not possible to have useful representations of the data in two dimensions without using dimensionality reduction techniques. Uniform Manifold Approximation and Projection (UMAP) is a computationally efficient method that can be used to display the global structure of complex transcriptomic data (92). UMAP visualisations were produced using the Scanpy implementation of UMAP using 25-50 principal components of normalised scRNA-seq data as the input (93).

2.2.7 Clustering

Cells are clustered using the Leiden algorithm using the top 25 Harmony batch-corrected principal components (94). Clustering quality is evaluated

using the clustering tree visualisation method to ensure cluster stability (95).

2.2.8 scRNA-seq normalisation and log transformation

Library size normalisation was performed on raw scRNA-seq count data to normalise for the total amount of RNA in each cell. Cell RNA counts were normalised to produce data in the counts per million (CPM) format using the following equation.

$$CPM = \frac{\text{gene count in cell} \times 10^6}{\text{total gene counts in cell}}$$

Following CPM normalisation the data was natural logarithm transformed to aid in visualisation and reduce the effect of extremely large values.

$$Y = \text{Log}(X + 1)$$

Where Y is the log transformed counts, X is the CPM normalised RNA counts and +1 is used as a pseudo-count to prevent negative values.

2.2.9 Overrepresentation analysis

Overrepresentation analysis was performed on DEGs for each cell cluster using the GSEAPy package (96). P-values for the overrepresentation analysis were defined by the hypergeometric distribution and significant Gene ontology (GO) terms were defined as those with an adjusted p-values <0.01.

2.2.10 Enrichment analysis

For bulk RNA sequencing, enrichment analysis was performed using the fGSEA (v1.29.1) was used to identify enriched GO terms. Log fold changes between conditions and the p-value of the changes from DeSeq2 was used as an input to fGSEA. Enriched GO terms were considered significant where the Benjamini-Hochberg adjusted p-value < 0.01 .

2.2.11 Differential expression testing with MAST

Model-based Analysis of Single-cell Transcriptomics (MAST) (v1.01) was used for differential expression testing for the scRNA-seq experiment. For each immunisation, every cell type was tested against the corresponding PBS control cell type. MAST was run using default parameters. P values after testing were adjusted for multiple comparisons using the Bonferroni adjustment. Genes were considered significant if both the Bonferroni adjusted p-value was less than 0.05 and the absolute value of the Log base 2 fold change (Log2FC) was larger than 1 (*p<0.05 and Log2FC>1).

2.2.12 Cell-Cell communication analysis

NicheNet was implemented to model intercellular communication between NK and ILC1 cells (senders) and lymph node cells (receivers) (97). The analysis was performed using the default NicheNet parameters. Differentially expressed comparing LMQ or SQ immunisation to the PBS control were used as the input gene set.

2.2.13 Differential abundance with SCCODA

Statistics on proportional composition of cells in scRNA-seq data were calculated using scCODA (version 0.1.9) (<https://github.com/theislab/scCODA>) using the Hamiltonian Monte Carlo sampling method. The model formula used was based on the immunisation only for each cell type. The bayesian model use does not support traditional p-values, and statistical significance, or credibility, is determined using spike and slab priors.

2.3 Materials

Table 2.2: Antibodies used

Antibody	Clone	Dye	Manufacturer
TotalSeq-B anti-mouse hashtag antibodies	M1/42, 30- F11	None	BioLegend
Ms Single Cell Sample Multiplexing Kit	M1/42	None	BD
CD45	30-f11	BV785	Biolegend
CD3	17A2	APC	Biolegend
B220	RA3-6B2	FITC	eBioscience
B220	RA3-6B2	BV421	BD
CD169	3D6.112	AlexaFluor 594	Biolegend
CD3	17A2	AlexaFluor 488	Biolegend
F4/80	BM8	None	Biolegend
Donkey anti-rat IgG	Polyclonal	AlexaFluor 488	Abcam
PDPN	8.1.1	PE	Biolegend
Continued on next page			

Table 2.2 – continued from previous page

Antibody	Clone	Dye	Manufacturer
CD31	390	PE-Cy7	Biolegend
Ly6g (Gr1)	RB6-8C5	Pacific Blue	Biolegend
Ly6c	HK1.4	PE	Biolegend
CD11b	M1/70	BV711	Biolegend
CD11c	N418	BV605	Biolegend
MHCII	M5/114.15.2	PerCP/Cy5.5	Biolegend

Table 2.1: Materials used

Product	Cat #	Manufacturer
Dispase II	04942078001	Roche
Collagenase P	COLLP-RO	Roche
DNase 1	10104159001	Roche
Fixation buffer	420801	Biolegend
Tissue-Tek O.C.T. compound	SA62550-01	Sakura
Prolong Gold antifade reagent	P36930	Invitrogen
MACS M tubes	130-093-236	Miltenyi
RLT lysis buffer	79216	Qiagen
First Strand cDNA Synthesis Kit	K1612	Thermofischer
TaqMan [®] Universal PCR Master Mix	4304437	Applied Biosystems
Sony Sorting Chip-100um	LE-C3210	Sony
Chromium Next GEM Single Cell 3' Kit v3.1	1000268	10X Genomics
Chromium Next GEM Chip G Single Cell Kit	1000127	10X Genomics
Dual Index Kit TT	1000215	10X Genomics
BD Rhapsody [™] Enhanced Cartridge Reagent Kit	664887	BD
BD Rhapsody [™] Cartridge Kit	633733	BD
BD Rhapsody [™] cDNA Kit	633773	BD
Click-iT Plus TUNEL Assay for In Situ Apoptosis Detection, AF647	C10619	Invitrogen
LIVE/DEAD [™] Fixable Far Red Dead Cell stain	L10120	Invitrogen

3 | Distribution of liposomal and emulsion adjuvants

3.1 Introduction

The innate immune response to vaccination happens both at the site of injection, but also in lymphoid organs including lymph nodes (65,92,104). Primate studies have shown that vaccine priming is restricted to lymph nodes that drain the vaccination site, suggesting that drainage patterns to secondary lymphoid organs are important to understand where productive vaccine immune responses are generated (103). Some adjuvants are known to drain directly to lymph nodes, while others form a depot at the site of injection (71,83,105).

The post-immunisation distribution of 4 different adjuvants produced by VFI: LQ, LMQ, SQ and SMQ, are currently unknown. These adjuvants are formulated from either liposomes or a squalene emulsion, indicated in their names with L or S respectively. The 4 adjuvants all contain the saponin fraction QS-21, and can contain a synthetic TLR4 ligand. Adjuvants formulated with a TLR4 ligand are indicated with an M in the name. Notably the LMQ adjuvant is analogous to the highly effective AS01 vaccine, as both are liposomal adjuvants that contain both QS-21 and a TLR4 agonist (72). Importantly for some liposomal and squalene-based adjuvants, it has been shown that antigens are not physically associated with the adjuvant (106,107). Therefore, the distribution of adjuvant and antigen are not necessarily the same, and studies of antigen distribution after vaccination cannot be used to infer adjuvant distribution. While the distribution of these adjuvants after

immunisation has not been studied directly, there have been explorations of the distribution of alternate liposomal and squalene emulsion adjuvants.

By radiolabelling components of the squalene-based adjuvant AS03, it has been shown that after i.m. injection it drains from the muscle into adjacent lymph nodes including the iliac and inguinal lymph nodes but not the distal axillary lymph nodes or spleen (108). Liposome biodistribution appears to be impacted by size, chemical modification and fluidity of the lipid bilayer components (108-1110). Cationic liposomes with reduced fluidity of their bilayers show more of a depot effect with more adjuvant being retained at the injection site, whereas cationic liposomes with increased fluidity drain more rapidly from the injection site to draining lymph nodes (105).

The location of adjuvants within tissues can provide information on prospective mechanisms of action due to the composition of cells in particular tissue microenvironments. Immunofluorescent microscopy localises AS01 to the subcapsular sinus region of the lymph node due to the overlap of the signal from fluorescently labelled QS-21 component with that of CD169, a marker of subcapsular sinus macrophages (87,88). The squalene emulsion adjuvant MF59 also shows distribution to the subcapsular sinus based on using the same method of measuring colocalisation of fluorescently labelled adjuvant with CD169 (89). In both cases drainage to the lymph nodes is rapid with AS01 being detectable by 30 minutes and MF59 being detectable by 1 hour post immunisation (87-89).

Cell targeting of adjuvants is also important to understand the key cells of interest in their mechanisms of action. Immunofluorescence colocalisation experiments in mice suggest that there is uptake of the squalene emulsion and liposomal adjuvants, MF59 and AS01, in subcapsular sinus macrophages

(87-89). MF59 has additionally been shown to be detected in macrophages and dendritic cells in the lymph node after immunisation, as assessed by flow cytometry (79). However, a lack of markers used in these experiments leads to little cellular resolution, therefore it is not known which if any other

Cell uptake of mRNA vaccines that are encapsulated in lipid nanoparticles has also been explored. Lipid nanoparticles share certain similarities with liposomes in that they are both comprised of lipids and cholesterol, but lipid nanoparticles are typically smaller due to including PEG (polyethylene glycol) and ionisable lipids (83). In primates, uptake of the mRNA vaccine was detected through flow cytometry in multiple antigen presenting cells including neutrophils, monocytes and dendritic cells (112). Overall, the cell tropism of the vaccine adjuvants of interest in this study is poorly understood which leads to a lack of understanding of their mechanisms of action on a cellular basis.

3.1.1 Aims

- Identify organs to which LMQ and SQ drain after intramuscular immunisation in mice.
- Identify cells that take up LMQ and SQ after immunisation.
- Identify the spatial localisation of LMQ and SQ within the draining lymph nodes.

3.2 Results

3.2.1 LMQ and SQ localise to the muscle and draining lymph nodes, but not the spleen.

The analysis of the localisation of LMQ and SQ was begun by investigating the drainage patterns of both adjuvants across organs following intramuscular injection in the gastrocnemius in mice. Fluorescent liposomes and emulsions were generated by VFI through labelling with the lipophilic dye DiD, fluorescent labelling of adjuvants with lipophilic dyes has been previously used to study the distribution and cell uptake of adjuvants (104,107). Mice were culled at 3 hours and 24 hours post-injection and compared to a baseline control to identify adjuvant positive cells as described in the methods section. Flow cytometry was used to detect the uptake of fluorescently labelled adjuvants partitioning to cells that were lineage positive (Lin+) (CD45+,CD3+,B220+) or lineage negative (Lin-) (CD45+,CD3-,B220-) cells (Methods). The Lin+ cells comprise B and T cells, whereas Lin- cells are the remaining CD45+ fraction of the lymph node and therefore primarily myeloid cells.

Higher levels of adjuvant uptake was seen in the muscle for both adjuvants compared to the lymph node after immunisation (Fig 3.1 A, B, C, D). After immunisation on average 77% and 33% of Lin- cells in the muscle of cells took up LMQ and SQ respectively at 24 hours (Fig 3.1A). While in the lymph node 27% of Lin- cells stained positive for LMQ uptake, while only 7% of Lin- cells were positive for SQ (Fig 3.1B). In the spleen there was almost no adjuvant uptake detected at 3, 24 or 48 hours with only 2% uptake of LMQ and 0.05% uptake of SQ in Lin- cells (Fig 3.1C). In addition, both adjuvants showed

3: Distribution of liposomal and emulsion adjuvants

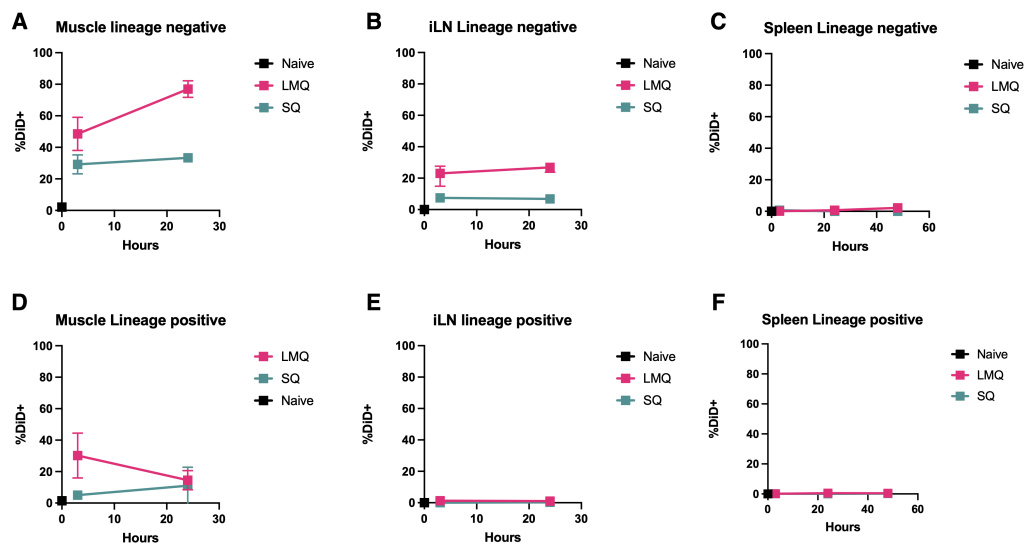


Figure 3.1: Adjuvant drainage is restricted to muscle and draining lymph node, with minimal adjuvant detected in the spleen. Mice were immunised i.m. with LMQ or SQ (n=3/group) and the muscle iLN and spleen were analysed by flow cytometry. All organs were analysed at 3- and 24-hours post-immunisation or in naïve mice, the spleen was also analysed at 48 hours to confirm no delayed drainage to the spleen. The percentage of cells that stain positive for each adjuvant (%DiD+) at 3 hours and 24 hours post immunisation for Lin- and Lin+ cells in the (A, D) muscle, (B, E) lymph nodes and (C, F) spleen is shown (methods). Mean $\pm$ SEM is shown.

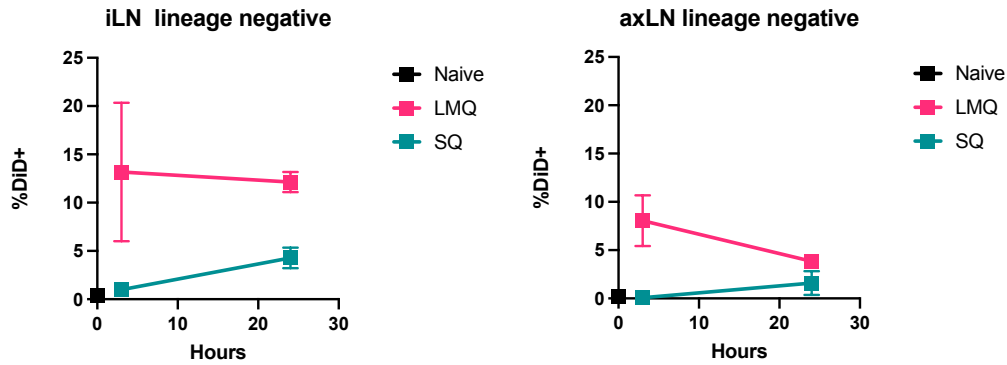


Figure 3.2: Reduced adjuvant drainage detected at lymph nodes distal to injection site. Mice were immunised i.m. with LMQ or SQ (n=3/group) and the (A) iLN which is proximal to the injection site and (B) the axLN which is distal to the injection site were both analysed by flow cytometry. The percentage of Lin⁻ cells that stain positive for each adjuvant (%DiD⁺) at 3 hours and 24 hours post immunisation is shown along with the baseline level from naïve mice. Mean $\pm$ SEM is shown.

an increased tropism towards Lin⁻ cells, which are comprised of myeloid cells and other B220⁻ and CD3E⁻ cells, compared to Lin⁺ (T and B) cells (Fig 3.1A-F). In the lymph node less than 1% of Lin⁺ cells took up adjuvant for both LMQ and SQ (Fig 3.1E). While there was uptake of LMQ and SQ into Lin⁺ cells in the muscle, this may be in part due to the small number of these cells present in the muscle before immunisation and minimal additional recruitment of these cells post immunisation with LMQ or SQ (Fig3.1D, Fig 3.3).

To investigate the extent of drainage of adjuvants through the lymphatic system, uptake in the axillary lymph nodes (axLN) were analysed. These lymph nodes are known to be downstream of lymphatic drainage from the inguinal lymph nodes (113). While adjuvants could be detected in the axLN, there was an overall reduction in the percentage of Lin⁻ cells that were adjuvant positive in the axLN compared to the inguinal lymph node (Fig 3.2).

3.2.2 The naïve muscle contains few lymphocytes before immunisation, in contrast to the lymph node.

The immune cell composition of the gastrocnemius muscle and inguinal lymph node was analysed in the naïve state and 24h after adjuvant immunisation. At baseline the lymph node has a large number of immune cells, both Lin⁻ and Lin⁺ (Fig 3.3A, C). However, the muscle has very few Lin⁻ cells (mean 678 cells) and Lin⁺ cells at baseline despite its larger size (Fig 3.3B, D). In the muscle, a large increase in the Lin⁻ cells 24h post immunisation for both adjuvants is observed. Both at baseline and post immunisation the lymph node contains an order of magnitude or more Lin⁻ and Lin⁺ cells.

3.2.3 LMQ is taken up by a broader range of myeloid cells in the lymph node in comparison to SQ.

To gain additional information on the cell-level distribution of LMQ and SQ, the myeloid compartment was analysed by flow cytometry in the muscle and inguinal lymph node. Four main myeloid populations were examined: dendritic cells (MHCII⁺, CD11c⁺), monocytes (CD11b⁺, Ly6g⁺, Ly6c⁺), neutrophils (CD11b⁺, Ly6g⁺, Ly6c low) and subcapsular sinus macrophages (CD11b⁺, CD169⁺) a lymph node specific cell type (Methods). Cells were defined as adjuvant positive by comparing the fluorescence of specific cell populations after immunisation with the same cell populations at baseline (Methods).

Within the muscle, uptake was detected for LMQ and SQ in dendritic cells, monocytes and neutrophils at levels significantly above PBS (Fig 3.4B-D). Within the lymph nodes, LMQ is significantly higher than PBS in all cell

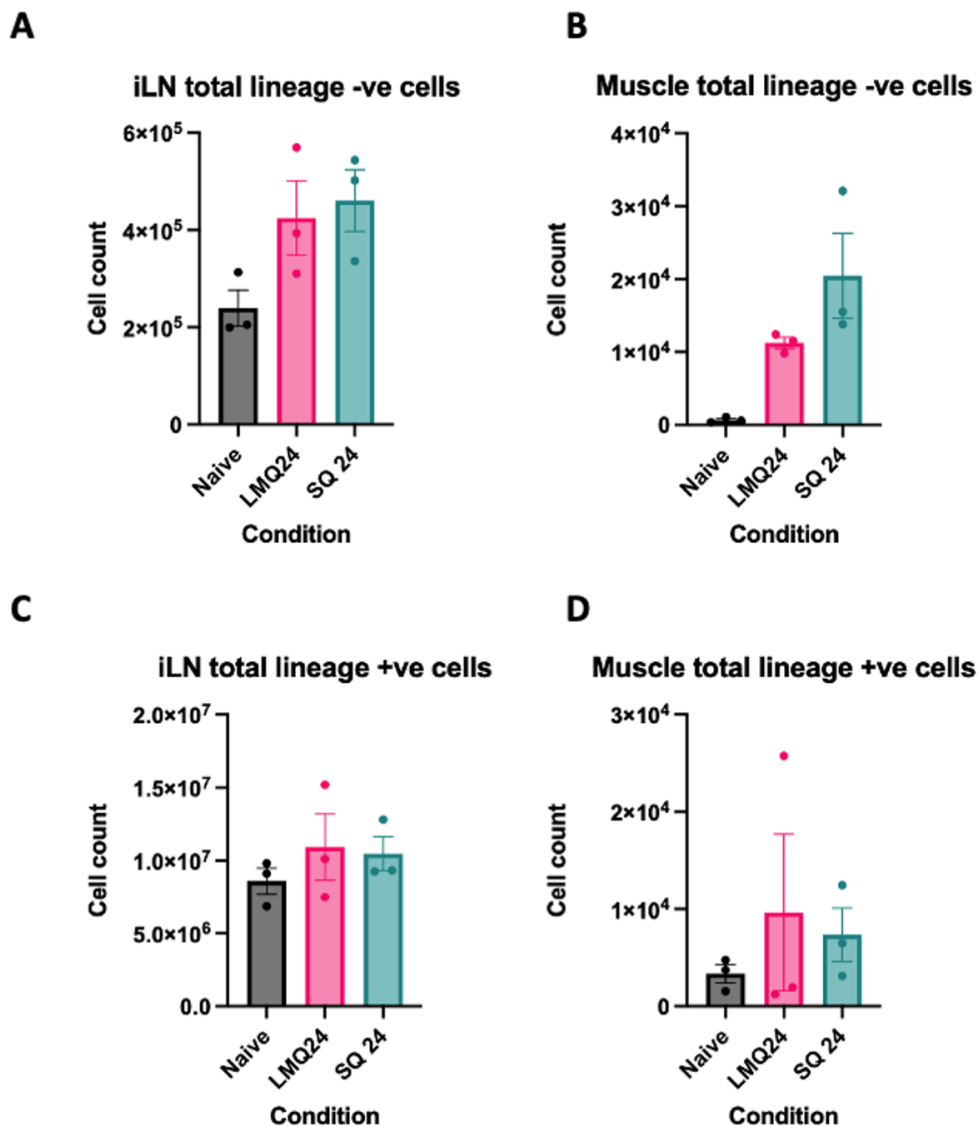


Figure 3.3: Differences in total number of haematopoietic cells between muscle and lymph nodes before and 24h after immunisation. Mice were immunised i.m. with LMQ or SQ (n=3/group) and the muscle and iLN were analysed by flow cytometry. The muscle and iLN were analysed 24 hours after immunisation or in naïve mice. Total cells are counted from digested lymph nodes or muscles using counting beads [methods]. (A, B) Lin⁻ cells are counted in the iLN and Muscle, (C, D) Lin⁺ cells are counted in the iLN and muscle. Mean $\pm$ SEM is shown with individual data points, n=3/group.

types, whereas SQ is significantly above PBS only within neutrophils and in monocytes at 24 hours (fig 3.5B-E). In addition, the proportion of adjuvant positive cells is significantly higher for LMQ compared to SQ in dendritic cells, subcapsular sinus macrophages, monocytes and neutrophils in at least one-time point (Fig 3.5B-E). In summary, LMQ shows higher uptake in almost all cell types than SQ within both the lymph node and the muscle, and the distribution of SQ within the lymph node is particularly low with uptake detected at significant levels only within neutrophils.

3.2.4 LMQ, but not SQ, shows uptake into lymph node resident stromal cells.

Given LMQs high level of uptake in resident SCS macrophages after immunisation, the uptake of LMQ, LQ, SMQ and SQ within resident stromal cell types in the lymph node was investigated [Fig 3.5E] (114). LQ and SMQ, which were less protective in a malaria model, were used to see if there was any correlation between vaccine efficacy and uptake in resident cell types. Flow cytometry was used to analyse uptake in the lymph node at 24 hours post immunisation, which confirmed that neither B-cells or T-cells took up significant levels of adjuvant at that time point (Fig 3.6E, F). It was found that LMQ and LQ showed high levels of uptake in CD45- cells including fibroblasts (CD45-, PDPN+, CD31-), lymphatic endothelial cells (CD45-, PDPN+, CD31+) and blood endothelial cells (CD45-, PDPN-, CD31+) (Fig 3.6A-C). Almost no uptake was detected for SQ in any stromal cell type. The other squalene emulsion adjuvant, SMQ, showed an intermediate amount of uptake in resident stromal cells, higher than SQ but lower than LMQ. Overall SQ was the only adjuvant tested that showed no level of uptake in resident lymph node cells.

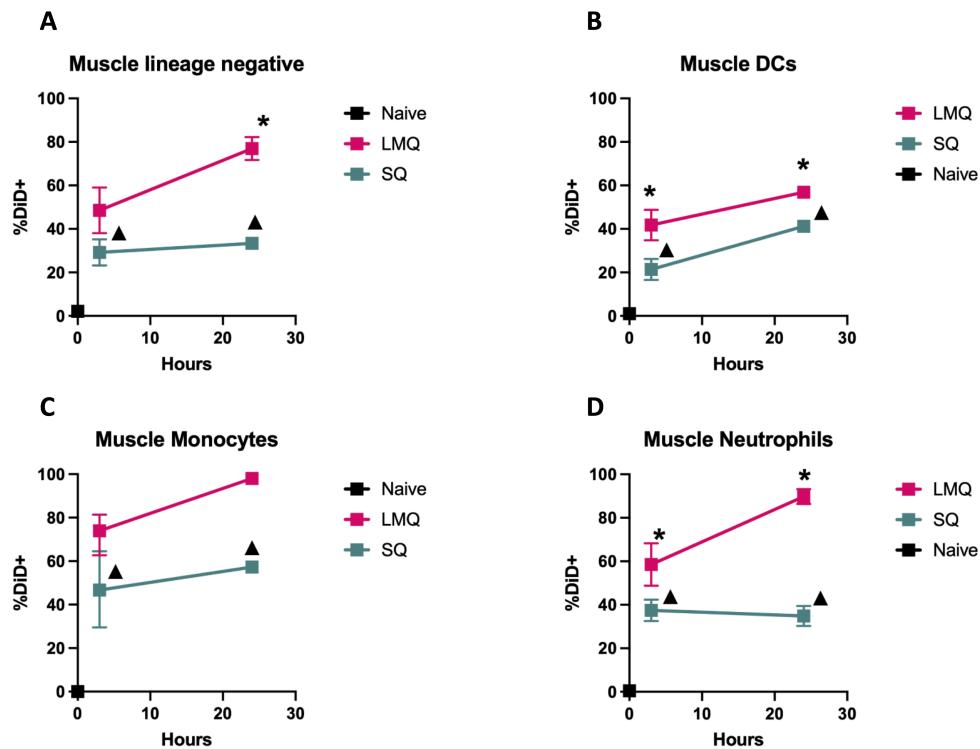


Figure 3.4: Both LMQ and SQ display uptake in myeloid cells in the muscle after immunisation. Mice were immunised i.m. with LMQ or SQ (n=6/group) and the muscle was analysed by flow cytometry. The percentage of cells in the muscle that stain positive for each adjuvant at 3 hours and 24 hours post immunisation in the cell populations: (A) Lin-cells, (B) DCs, (C) monocytes and (D) neutrophils. Mean $\pm$ SEM is shown. n=6/group from two independent experiments. *p<0.05 for the test of LMQ is significantly different to SQ at the same time point. Δ p<0.05 for the test of significant difference of SQ to PBS, LMQ is higher than PBS for all values so markers are not shown. Ordinary two-way ANOVA with main effects only, multiple comparisons are corrected for by the Holm-Šidák method.

3: Distribution of liposomal and emulsion adjuvants

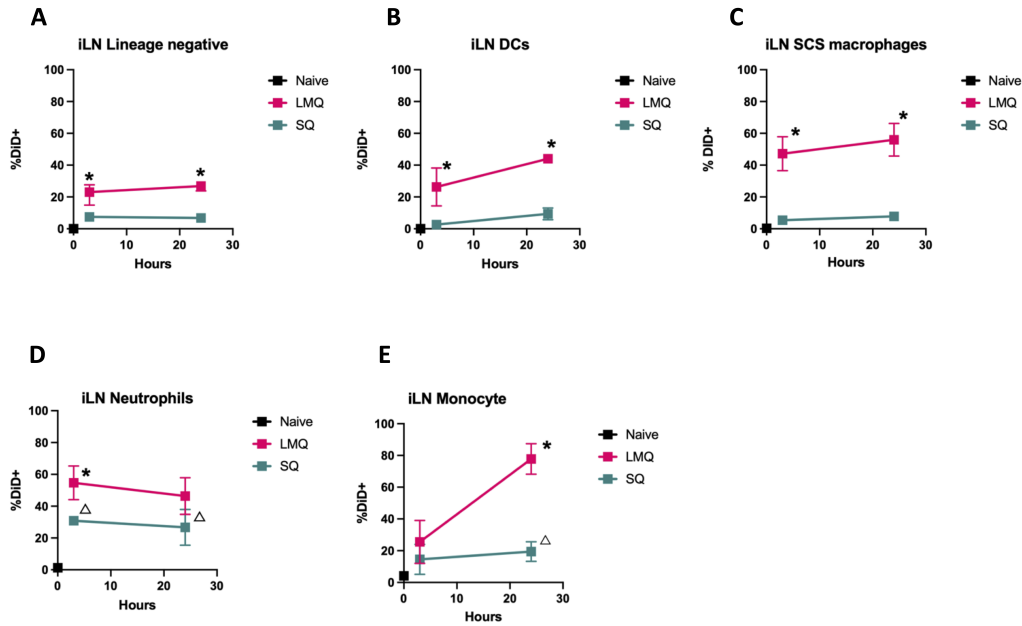


Figure 3.5: Uptake of LMQ after immunisation in myeloid cells of the lymph node is broader and more pronounced than found after SQ immunisation. Mice were immunised i.m. with LMQ or SQ (n=6/group) and the iLN was analysed by flow cytometry. The percentage of cells in the inguinal lymph node that stain positive for each adjuvant at 3 hours and 24 hours post immunisation in the cell populations: (A) Lin⁻ cells, (B) DCs, (C) SCS macrophages, (D) monocytes and (E) neutrophils. Mean $\pm$ SD is shown. n=6/group from two independent experiments. *p<0.05 for the test of LMQ is significantly different to SQ at the same timepoint. Δ p<0.05 for the test of significant difference of SQ to PBS, LMQ is higher than PBS for all values so markers not shown. Ordinary two-way ANOVA with main effects only, multiple comparisons are corrected for by the Holm-Šidák method.

To investigate if the stromal uptake of LMQ could be an artefact of the digestion process, inguinal lymph nodes from naïve mice were incubated in a digestion mixture containing fluorescent LMQ, to simulate resident cells being exposed to residual free adjuvant during the digestion process (methods). Immunised lymph node FRCs, LECs and Lin⁻ cells still had a significantly higher uptake of LMQ than cells from lymph nodes that had been incubated in LMQ in a digestion mix (Fig3.8). However, this analysis did suggest that uptake in BECs is not significantly higher than the digestion control. While uptake in LECs was statistically higher *in vivo* than in the control, the digestion control showed a high level of uptake showing a degree of artefactual LMQ uptake.

in vitro fibroblast cell cultures were also used to assess the capability of fibroblast to take up LMQ and SQ in a manner independent of other cells. Fibroblast cell cultures were formed of murine primary lymph node fibroblasts isolated by lymph node digestion, and incubated with adjuvants for 24 hours [Methods] (90). It was found that the same pattern of increased uptake of LMQ in fibroblasts compared to SQ was replicated *in vitro*. SQ showed no apparent uptake into fibroblast cultures, even at the highest concentration of adjuvants tested (Fig3.7A). Additionally, fibroblasts cultured with LMQ showed an upregulation of the inflammatory cytokine Tnf compared to SQ, which showed almost no upregulation compared to PBS (Fig3.7B) (Methods).

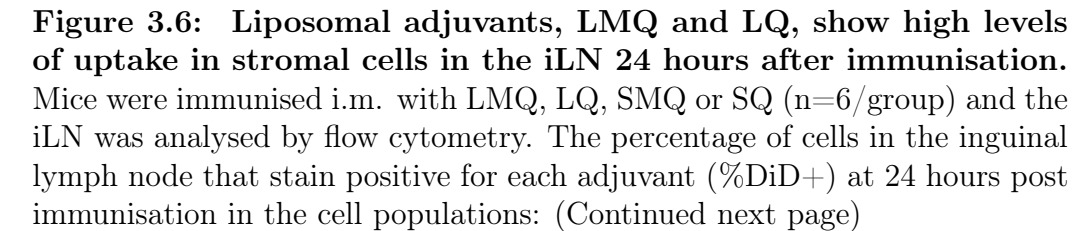


Figure 3.6: Continued from previous page: (A) FRCs, (B) LECs, (C) BECs, (D) Lin- cells, (E) B-cells and (F) T-cells. Mean $\pm$ SD is shown. $n=5/\text{group}$ from two independent experiments. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$. Ordinary two-way ANOVA, multiple comparisons are corrected for by the Holm-Sídák method.

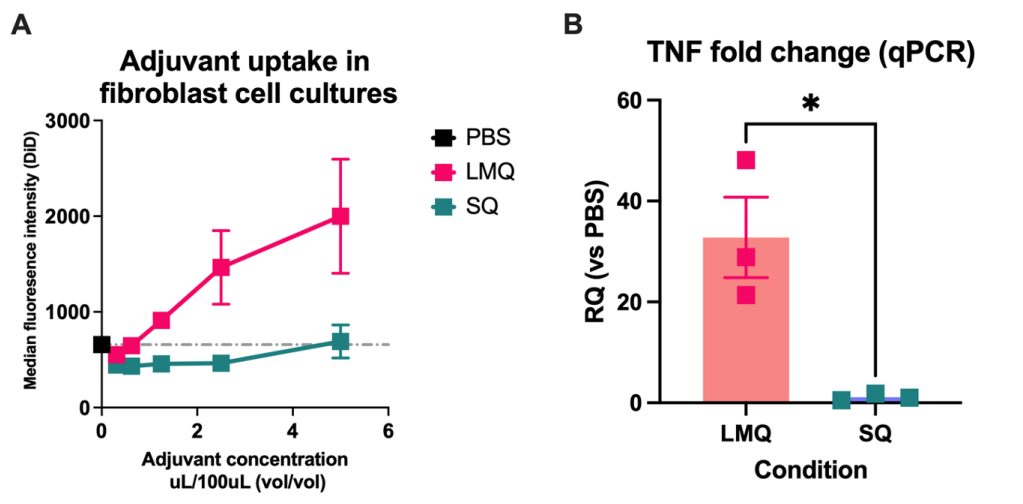


Figure 3.7: Lymph node cultured fibroblasts uptake LMQ and show changes in inflammatory gene expression after LMQ, but not SQ, treatment. (A) The median fluorescence intensity of cultured murine fibroblasts incubated for 3 hours with varying concentrations of adjuvants. Mean $\pm$ SEM is shown. $N=3/\text{group}$. (B) The Relative Quantification (RQ) showing the fold change in TNF expression of fibroblasts cultured with LMQ or SQ compared with PBS calculated by the $\Delta\Delta\text{Ct}$ method, Mean $\pm$ SD is shown, $n=3/\text{group}$. . * $p<0.05$. An unpaired two-tailed t-test is used.

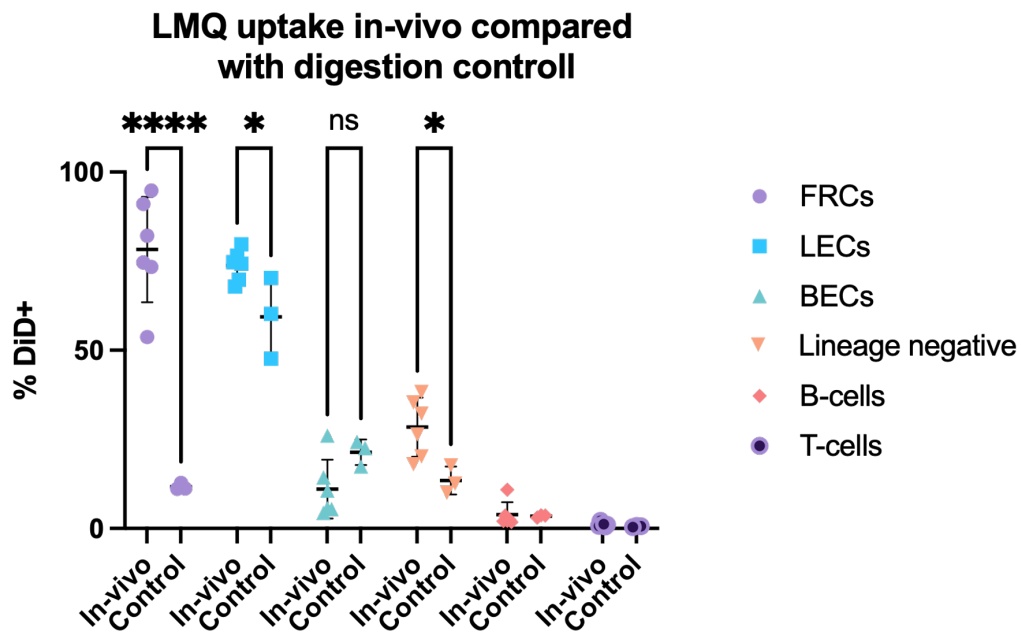


Figure 3.8: Uptake of LMQ in FRCs is not an artifact of tissue digestion. Uptake of LMQ in specific cell types is compared to uptake in a digestion control where adjuvant is added to the tissue digestion mixture. Mean $\pm$ SD is shown. $n=3-5$ /group from two independent experiments. * $p<0.05$, ** $p<0.005$, *** $p<0.0005$, **** $p<0.0001$. Ordinary two-way Anova, multiple comparisons are corrected for by the Holm-Šídák method.

3.2.5 LMQ localises in the subcapsular sinus region of draining lymph nodes

Next, to understand the spatial distribution of both adjuvants within the draining lymph nodes to investigate if specific spatial niches were being targeted by either adjuvant.

To understand the spatial distribution of adjuvants in the draining lymph nodes, immunofluorescence microscopy was employed to identify the localisation of fluorescently labelled LMQ and SQ. Immunofluorescence microscopy revealed that there was significantly higher fluorescence due to LMQ than SQ in the iLN, and that SQ was not significantly different compared to the naïve lymph node at 3 hours and 24 hours (Fig 3.9). Further analysis by staining for CD169, F4/80 and B220 showed that LMQ localises to the sinus regions of the lymph node due to the overlap of the LMQ signal with CD169 and F4/80 (Fig3.10). Curiously however, there was little fluorescent signal detected in the lymph node parenchyma, despite significant uptake of LMQ detected by flow cytometry in FRCs a cell type located in the central regions of the lymph node and not in the sinuses.

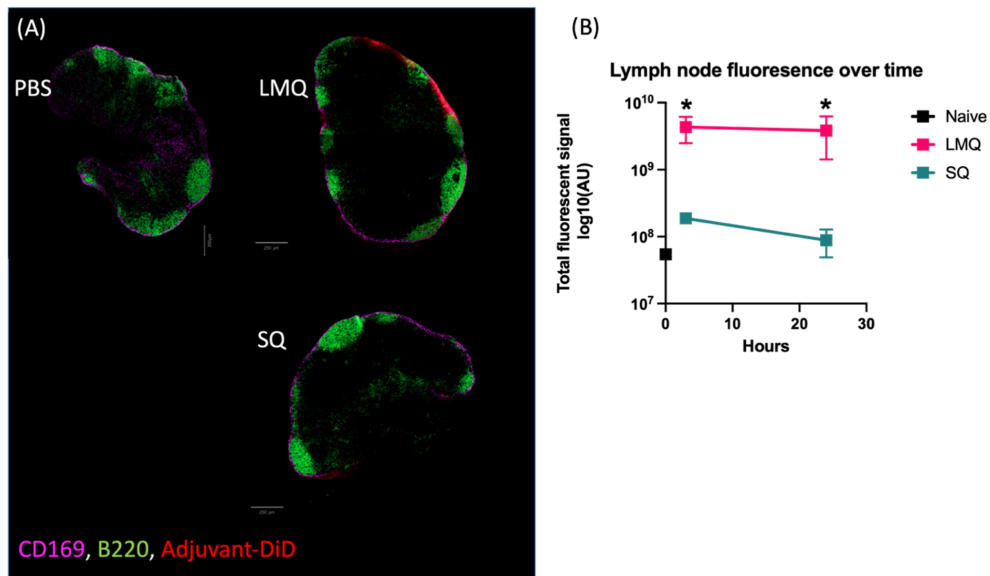


Figure 3.9: Fluorescence microscopy shows significant drainage of LMQ to the inguinal lymph nodes. (A) representative images of lymph nodes at 24 hours post immunisation, stained with CD169 (magenta) and B220 (green) showing adjuvant fluorescent signal (red). (B) Fluorescent signal from the average of 3 randomly selected sections throughout the inguinal lymph nodes at 3 and 24 hours, the fluorescent signal is displayed on a logarithmic scale with base 10. Mean $\pm$ SEM is shown. $n=3-4$ /group averaged from 3 technical replicates from each organ. * $p<0.05$ for the test of LMQ is significantly different to SQ at the same time point. Ordinary two-way Anova with main effects only, multiple comparisons are corrected for by the Holm-Šidák method.

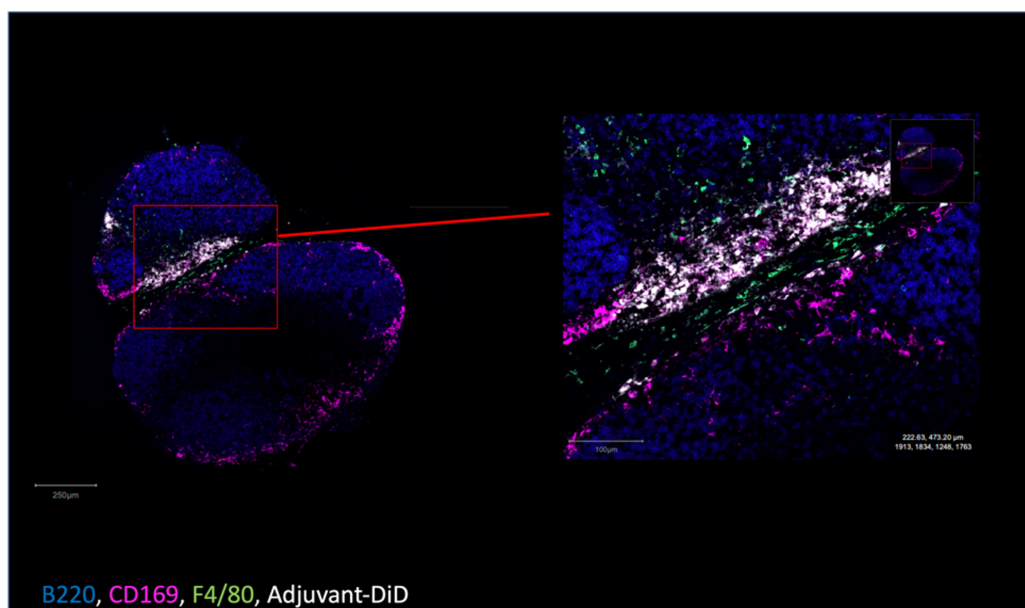


Figure 3.10: LMQ localises to the subcapsular sinus regions of the lymph node. A representative image from 3 samples of the localisation of LMQ-DiD (white), CD169 (magenta), F4/80 (green) and B220 (blue) in the inguinal lymph node 24 hours after immunisation. The left image shows the full image and the right image shows a magnification of the subcapsular sinus region showing LMQ colocalisation with CD169.

3.3 Discussion

These findings revealed that both LMQ and SQ adjuvants displayed substantial uptake in Lin⁻ immune cells at the site of injection, and in iLNs. However, the spleen exhibited minimal adjuvant presence, indicating that the systemic distribution of these adjuvants is limited within the time frame studied. This corroborates previous reports of the biodistribution of liposomal and squalene emulsion adjuvants that show presence in the injection site and draining lymph nodes (107,108). Liposomal adjuvants have variable drainage patterns with some forming a depot and some draining rapidly to the lymph node (109). LMQ appears to behave more similarly to the rapidly draining form of liposome suggesting it does not create a depot.

Both adjuvants showed increased percent-wise uptake in Lin⁻ cells compared to Lin⁺ cells, showing an apparent lack of capacity for B and T cells to directly take up adjuvant. The Lin⁻ cells are formed up of a variety of myeloid cells including dendritic cells, SCS macrophages, monocytes and neutrophils. All of these named Lin⁻ cells have the capacity to act as antigen presenting cells under certain conditions (81,89,88,115-117). Activation of APCs has been suggested as a mechanism of action for a number of other adjuvant systems and, given the uptake of LMQ and SQ in these cell types, may represent a mechanism of action for these adjuvants as well.

In the lymph node, LMQ showed a broad pattern of adjuvant uptake with significant adjuvant uptake in all examined Lin⁻ cells. SQ, however, only showed significant uptake in the neutrophil population and the monocyte population. Targeting of additional myeloid cells by LMQ compared to SQ may impact the differences in the Th polarisation of each adjuvant. For the

LMQ analogue AS01, the Ifn-gamma response and resultant Th1 polarisation is dependent on SCS macrophages (87). After immunisation only LMQ is detected at significant levels in SCS macrophages, and not SQ.

In addition to a broader distribution profile in myeloid cells, LMQ also had a capacity to be taken up by resident lymph node cells that was absent in SQ. These resident cells included both myeloid cells such as SCS macrophages, but also stromal cells such as FRCs and LECs. SQ appeared to not be taken up by any stromal cell 24 hours after immunisation, while all other adjuvants tested did. However, there is no clear correlation between targeting resident cells and vaccine protection. LQ and SMQ, which were poorly protective in a murine malaria challenge model, both showed high levels of uptake in resident cells and LQ exceeded the level of uptake seen in LMQ (18). Direct targeting of resident cells by LMQ may impact onto its mechanism of action as LECs are known to express TLR4, the receptor for the TLR4 agonist component of LMQ (25). In addition, it has been shown that TLR4 agonism impacts on LEC function in a TLR4 ligand-containing nanoparticle vaccine (113). Activation of FRCs in response to a lymphocytic choriomeningitis viral vector prevents exhaustive CD8⁺ T-cell activation, showing FRCs also play a role in productive immune responses (119).

As with previous reports of the spatial distribution of LMQ within draining lymph nodes, LMQ is localised to the subcapsular sinus region (87,88). SQ however, was not detected at a level significantly higher than the naïve lymph node suggesting minimal amounts reach the lymph node. Interestingly, a discrepancy between the flow cytometry data and the immunofluorescence data arose in what they imply about the spatial localisation of LMQ. In the FACS data, LMQ was taken up in high amounts by FRCs, a cell type located throughout the central regions of the lymph node. However,

in the immunofluorescence data, LMQ is located only in the sinus regions of the lymph node which are located around the exterior regions of the lymph node. Follow-up experiments incubating cells with adjuvant during digestion show uptake in FRCs is not an artefact of our digestion protocol, and therefore differences in processing do not explain this discrepancy. One factor of note is that during immunofluorescence microscopy, LMQ was detectable at extremely low laser power settings on the microscope. It could be that microscopy lacks the dynamic range to detect the small amounts of LMQ inside cells due to a high level of fluorescence from free adjuvant that directly drains to the lymph node. This discrepancy underscores the value of using complementary methods to study vaccine adjuvant distribution.

3.3.1 Caveats and open questions

The flow cytometry and immunofluorescent techniques used in this chapter cannot confirm that fluorescent adjuvant is internalised into cells, and not associated with the surface of the cells. Adjuvant uptake into cells could be confirmed through utilisation of a pH sensitive dye, such as a lipid conjugated pHrhodo dye, formulated with the adjuvant (120). The use of a pH sensitive dye could be used to determine if adjuvants are internalised into endosomes or other acidic organelles. In addition, in this chapter it was not confirmed that DiD was stably maintained within the adjuvants after immunisation, and free fluorophore did not exit the adjuvants and cause unspecific labeling of cells without adjuvant entry. However, fixed cells have been shown to not stain positive for fluorescent adjuvant, suggesting that active uptake is required for a positive signal and minimal passive entry of free DiD occurs [data not shown]. To further confirm that free DiD is not a cause of positive DiD signal, and to confirm that adjuvant uptake is an active process, fluorescently labeled

adjuvant could be incubated in vivo with cells in the presence and absence of latrunculin or other disruptors of endocytosis.

Adjuvant trafficking by migratory cells from the site of injection to the draining lymph nodes could be explored in more detail using a photoconvertible Kaede mouse model (121). By photoconverting cells at the muscle site of injection, cell mediated adjuvant drainage to the lymph node relative could be analysed and compared with uptake in non-photoconverted cells that are resident to the lymph node or recruited from the blood.

In addition, the possibility of SQ forming a depot effect in the muscle was not explored in this chapter. Immunofluorescent imaging of muscle tissue sections taken at various timepoints to analyse the presence of an adjuvant depot would be the most simple method of analysing this effect. In vivo fluorescent imaging could also be used to test for a depot effect, however, the collection of multiple time-points would be constrained by the ethical limits of repeated anesthesia.

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4 | Adjuvant induced inflammation in the muscle and lymph nodes

4.1 Introduction

In the previous chapter, it was shown that LMQ and SQ display differential distribution patterns in the muscle and lymph nodes. Both adjuvants were found at high levels in the muscle site of injection, but SQ showed a reduced capacity to drain to the lymph node and target resident lymph node cells in comparison to LMQ. Different tissues show large differences in both the quantity of immune cells and what cell types are present, one example being the presence of SCS macrophages in the LN but not the muscle (114). Additionally, immune cells localised to different tissues are phenotypically different (115). Given the differential drainage of LMQ and SQ to the muscle and lymph node, the role of tissue-specific immune responses in LMQ and SQ was explored.

It is known that LMQ and SQ can both induce the secretion of pro-inflammatory cytokines in the muscle after immunisation (18). It was found that LMQ induced a rapid release of IL-18 in the muscle and blood serum that was not found in SQ. NF- κ B activation is predicted to occur after immunisation in both adjuvants due to the secretion of TNF- α , IL-6, G-CSF (CSF3), CXCL1 and CCL17 (18). Notably, LMQ produced a more rapid and higher level of secretion of these cytokines in comparison to SQ. Additionally, inhibition of the NLRP3 inflammasome reduced downstream cytokine secretion *in vivo* for both adjuvants, suggesting a role for the inflammasome in both adjuvants (18). LMQ contains both QS-21 and a TLR4 agonist providing the

capacity for it to generate both signals required for inflammasome activation (46). However, SQ lacks a TLR4 agonist and therefore is suspected to activate the inflammasome *in vivo* through the production of DAMPS from adjuvant-induced cell death (18).

Adjuvant-induced cell death has been shown to play an important role in the mechanism of action of a variety of different vaccine platforms including AS01, GLA-SE and alum (10,43,53,121-123). Previous work on LMQ and SQ has identified signals of cell death *in vitro* through LDH release in bone marrow-derived macrophages (18). Additionally, the QS-21 component of both LMQ and SQ has been shown to induce the loss of resident macrophages in the draining lymph node and induce the release of HMGB1. QS-21-induced cell death *in vitro* is not driven by pyroptosis, as it is independent of caspase-1/11 and NLRP3, however *in vivo* induction of pyroptosis cannot be ruled out (75). The squalene emulsion adjuvant MF-59 induces necroptotic death in lymph node macrophages in a RIPK3-dependent manner (62). Given SQ is a squalene emulsion-based adjuvant, necroptosis presents another potential form of cell death that could be induced by this adjuvant. A variety of adjuvant-induced cytokines, such as TNF, are also capable of inducing apoptosis in responding cells (124). Given adjuvants containing QS-21, or squalene emulsions, have been shown to induce apoptotic, necrotic and pyroptotic cell death *in vivo*, cell death may play a role in the mechanisms of LMQ and SQ.

4.1.1 Aims

- Identify a suitable timepoint for transcriptomic analysis of adjuvant responses *in vivo*.

- Compare the transcriptomic response to the LMQ and SQ adjuvants across the muscle and lymph node.
- Given signals of apoptotic and pyroptosis from the RNA-seq results, explore the presence of increased cell death *in vivo* in response to adjuvants.

4.2 Results

4.2.1 *in vivo* transcriptomic signatures of inflammation peak rapidly in response to LMQ and SQ

First qPCR was used to identify a timepoint where transcriptional signals of inflammation could be detected. Initially using LMQ immunisation, the transcription of selected inflammatory mediators at 1, 3, 6 and 16 hours as well as a PBS control was assessed [Fig 4.1]. The cytokines Il6, Tnf, Il1B, Il12a, Il12b and Il23a were all tested. Il6 and Tnf were analysed as early markers of inflammation, as Il6 and Tnf expression can occur directly downstream of PRR signalling (125,126). Il1B was chosen as a marker for licensing of inflammasome activation, due to increases in Il1B expression by signal 1 of the inflammasome (126,127). Il-12 is known to play a key role in the mechanism of action of AS01, and the p35 and p40 components were analysed as a result (20). IL-23 is part of the IL-12 family, and shares the IL-12b (p40) subunit with functional IL-12 (128). As Il-12a expression was not detected by qPCR, but Il-12b was, we also analysed the expression of IL-23a.. For all genes where a curve of expression was detected, the peak of expression was at 3 or 6 hours. Next, the transcriptional kinetics were confirmed for SQ by testing the expression of cytokines at 3, 24 and 48 hours [Fig 4.2]. For

the cytokines Il6 and Il1B the peak of transcription was detected at 3 hours as with LMQ.

4.2.2 Principal component analysis of bulk RNA-seq confirms the validity of the RNA sequencing experiment

Bulk RNA sequencing was performed on muscle and lymph node samples collected 3 hours after immunisation with LMQ, SQ or a PBS control [methods]. Principal Component Analysis (PCA) provides a way to summarise the large number of variables in gene expression data. PCA combines information from the expression of multiple different genes into a reduced number of variables that explain the variation in the data, known as principal components. Both the anatomical location and immunisation status are reflected in the RNA expression differences as seen in the first two principal components [Fig 4.3]. This indicates that RNA-seq can detect significant gene expression differences between immunisation statuses. Additionally, it serves as a validation check for the experiment by demonstrating a clear differentiation between muscle and lymph node tissue

Additionally, by analysing the contribution of genes to each principal component a first look at the differences between tissues and immunisations can be seen. The first principal component (PC1) which separates the lymph node samples from the muscle samples has contributions from key genes that are expressed in each tissue. Genes involved in muscle contraction such as Acta1 and Myh4, a skeletal muscle actin and myosin, contribute positively to PC1. Whereas Cxcl13 and Ighd contribute negatively. Cxcl13 is known to be produced by FDCs in the lymph node, and Ighd is expressed by B cells in lymph

4: Adjuvant induced inflammation in the muscle and lymph nodes

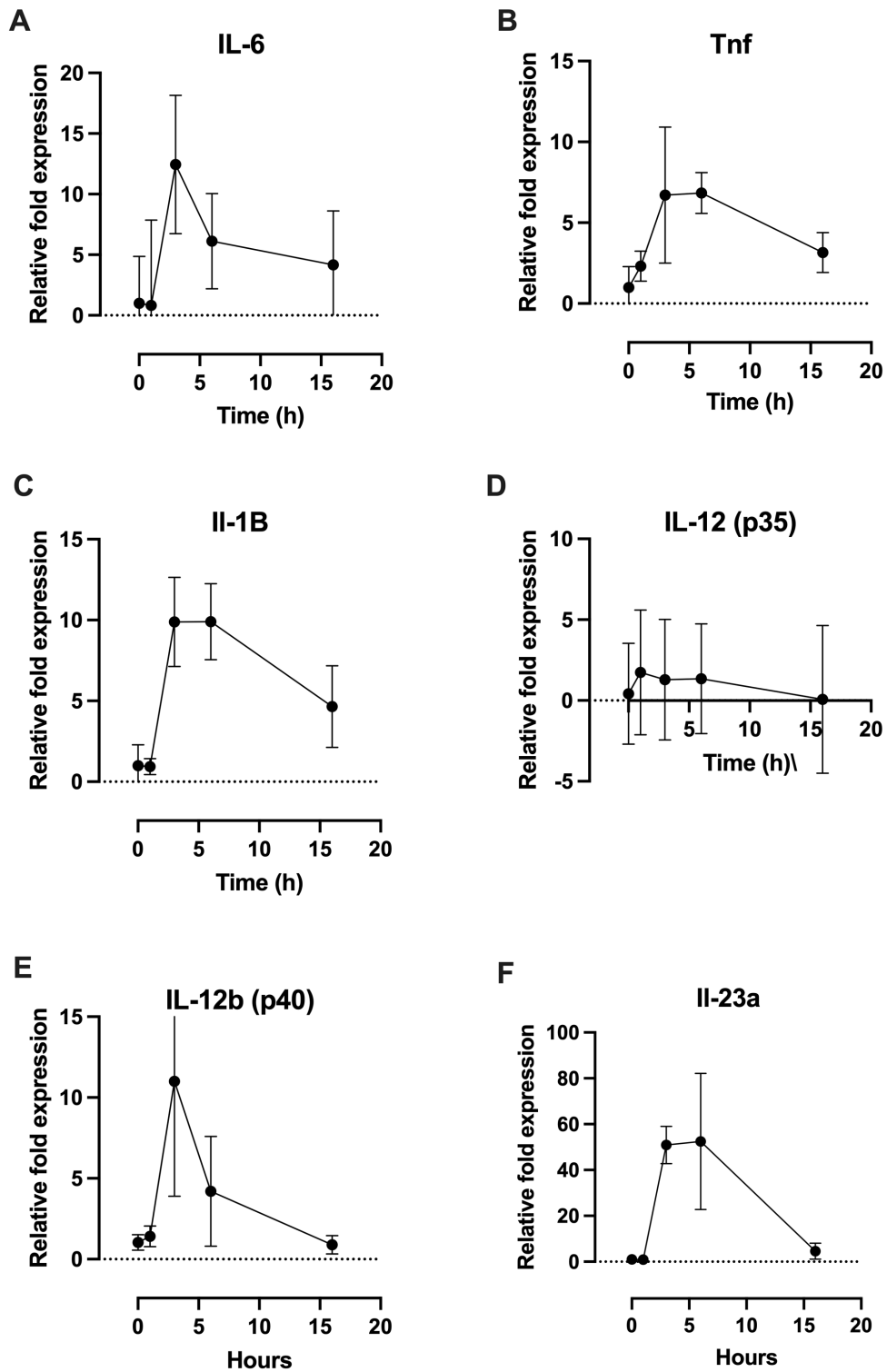


Figure 4.1: (Continued next page)

Figure 4.1: Transcriptional inflammatory markers peak rapidly after LMQ immunisation in draining LNs Time courses of the relative fold expression of (A) Il-6, (B) Tnf, (C) Il1B, (D) Il12 (p35), (E) Il-12b (p40) and (F) Il-23a induced by LMQ immunisation compared to PBS. Expression was measured at 1,3,6 and 16 hours after LMQ immunisation in the iLN. PBS is shown as the 0-hour timepoint on each graph. Mean $\pm$ SD shown (n = 4-5 mice per group)

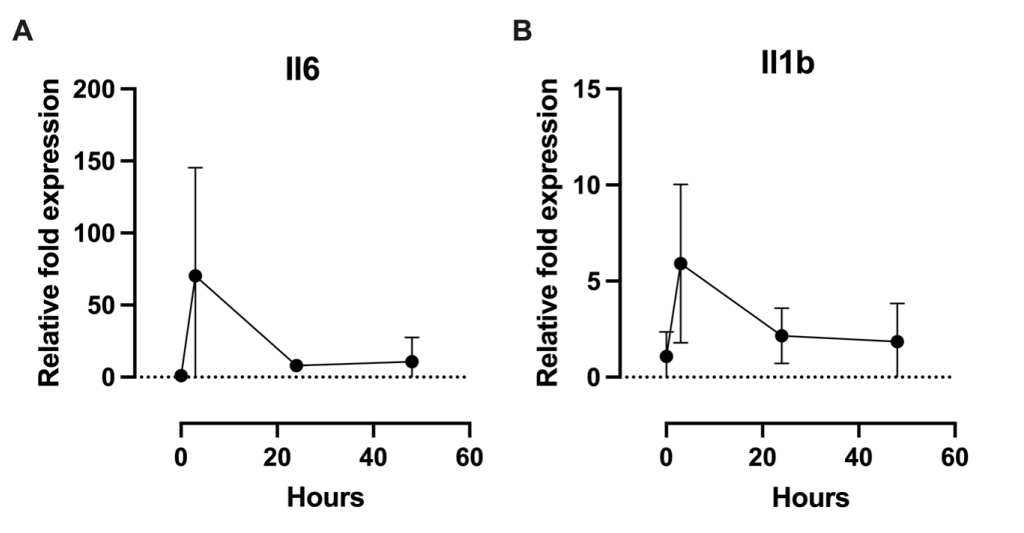


Figure 4.2: qPCR confirms an early peak in transcriptional inflammation for SQ in draining lymph nodes Time courses of the relative fold expression of (A) Il-6 and (B) Il1B induced by SQ immunisation compared to PBS. Expression was measured at 3 24 and 48 hours after SQ immunisation in the iLN. PBS is shown as the 0-hour timepoint on each graph. Mean $\pm$ SD shown (n = 4-5 mice per group)

node follicles and likely represents the increased number of B-cells found in the lymph node compared to the muscle [Fig 3.3]. The genes contributing to PC1 support the validity of the dissection and RNA extraction methodologies used, by recapitulating known gene expression differences between the muscle and lymph node.

The second principal component (PC2) separates the samples based on immunisation status, with immunised samples scoring lower than the PBS control [Fig 4.3]. Key genes that contribute negatively to PC2, and are associated with immunisation status, include the cytokines Cxcl1, Cxcl10, Csf3 (G-CSF), and Il1B. The contribution of cytokine gene expression to PC2 shows that inflammatory cytokines can differentiate the immunised samples from the control, and confirm the presence of transcriptional signals of inflammation in both tissues.

4.2.3 LMQ and SQ increase the expression of innate cytokines *in vivo*, which correspond with published cytokine secretion data.

To analyse the gene expression changes in more detail, Deseq2 was used to identify differentially expressed genes (DEGs) in murine lymph nodes and muscles after immunisation with LMQ or SQ [Methods] (93). Genes were identified as differently expressed if their Log base 2 Fold Change (Log2FC) was greater than 1.5 and the p-value of the test was lower than 0.05 after multiple comparisons correction [methods]. As discussed in the introduction, previously published data has analysed the secretion of 9 cytokines in the muscle and lymph node following LMQ or SQ immunisation at the protein level using ELISA (18). To validate the relevance of the bulk-RNA-seq, the

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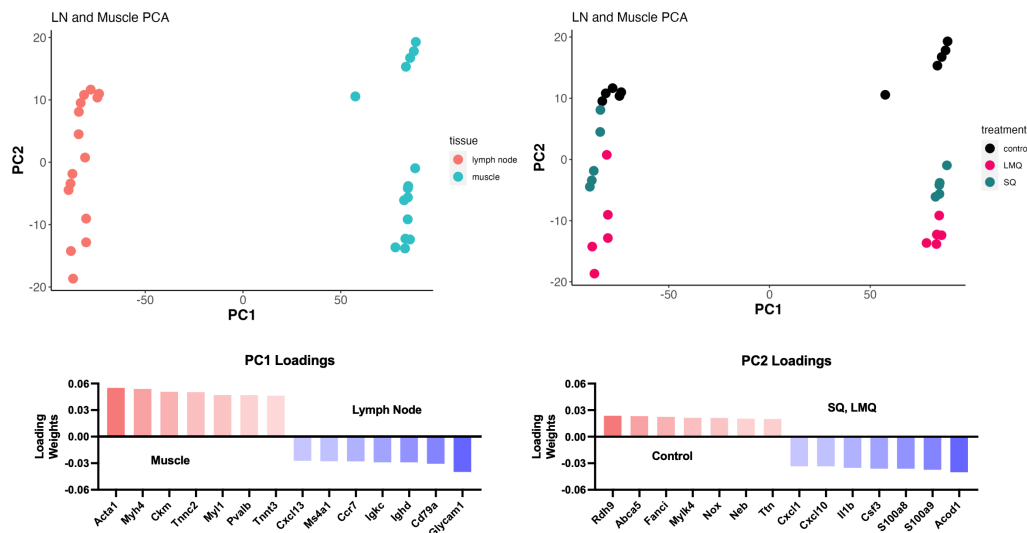


Figure 4.3: Both tissues of origin and immunisation status separate well on the first two principal components, confirming the RNAseq validity (A) All samples are shown coloured by tissue on PC1 and PC2, (B) All samples are shown coloured by their immunisation status on PC1 and PC2, (C,D) the top 7 genes that contribute positively and negatively to PC1 or PC2 and their individual weight of contribution. Samples with higher expression of genes with positive weights will score more positively on the PC, and samples with higher expression of genes with negative weights will score more negatively.

same targets analysed on the protein level were examined on the RNA level.

At the 3-hour timepoint in the muscle, LMQ and SQ induce the expression of the Tlr4 and Nfkb-induced cytokines Tnf, Il6, Csf3, Cxcl1 and Ccl17 [Fig 4.4]. In contrast to the cytokine secretion data, there is no detectable change in Il18, potentially due to Il18 release being driven by post-translational modifications that are not captured by RNAseq (128).

Cytokine secretion in the lymph node was not analysed in the previous study, so there is no direct comparator on the protein level. In the lymph node 3 hours post immunisation there was upregulation of the expression of Il-18 and Il-12b (Il12 p40) expression in response to LMQ but not SQ [Fig 4.5]. Additionally, Tnf, Il6, Csf3 (G-CSF), and Cxcl1 were upregulated in response to both adjuvants. Ccl17 was significantly upregulated by SQ but not LMQ. In summary, in both the lymph node and muscle there is evidence of TLR4 or NF- κ B-induced gene expression changes that correlate with those found on the protein level.

4.2.4 A core set of differentially expressed genes is shared after immunisation with either adjuvant across both tissues.

A total of 521 and 120 genes were differentially upregulated in the lymph node after immunisation with LMQ or SQ respectively [Fig4.6A]. However, in the muscle, there were a larger number of upregulated genes with 835 being upregulated in mice immunised with LMQ and 448 after SQ immunisation. This indicates that more genes were upregulated after LMQ immunisation than SQ, regardless of the tissue site. Moreover, in both adjuvants, a greater number of genes were upregulated in the muscle compared to the lymph node.

4: Adjuvant induced inflammation in the muscle and lymph nodes

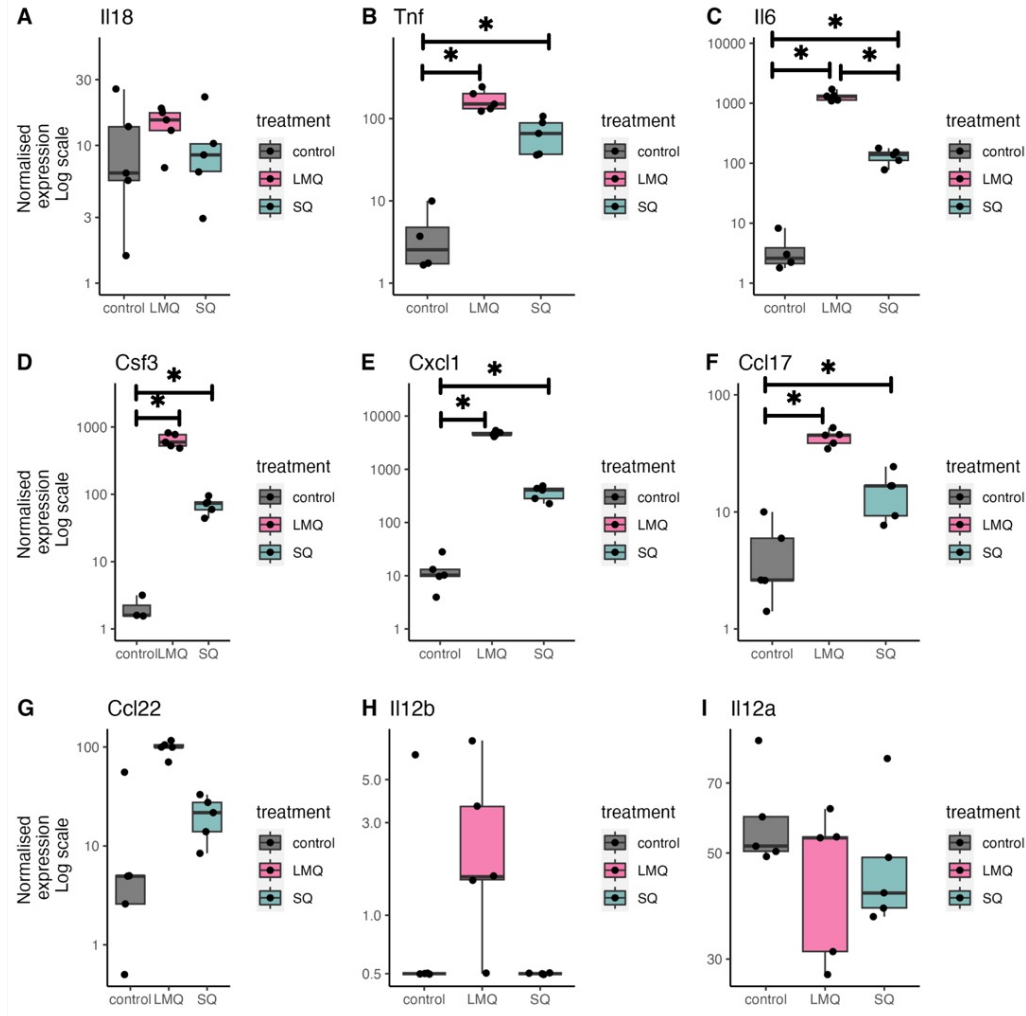


Figure 4.4: RNA-seq captures changes in the expression of genes in the muscle that correlate with changes shown in published protein level data The normalised expression of (A) Il18, (B) Tnf, (C) Il6, (D) Csf3, (E) Cxcl, (F) Ccl17, (G) Ccl2, (H) Il12b and (I) Il12a across different immunisation statuses in the muscle site of injection is shown on a log scale. Significance is determined by the results of DeSeq2 on the whole transcriptome adjusted for multiple testing with the Benjamini and Hochberg method. (* $p < 0.05$ and $\text{Log}_2\text{FC} > 1.5$).

4: Adjuvant induced inflammation in the muscle and lymph nodes

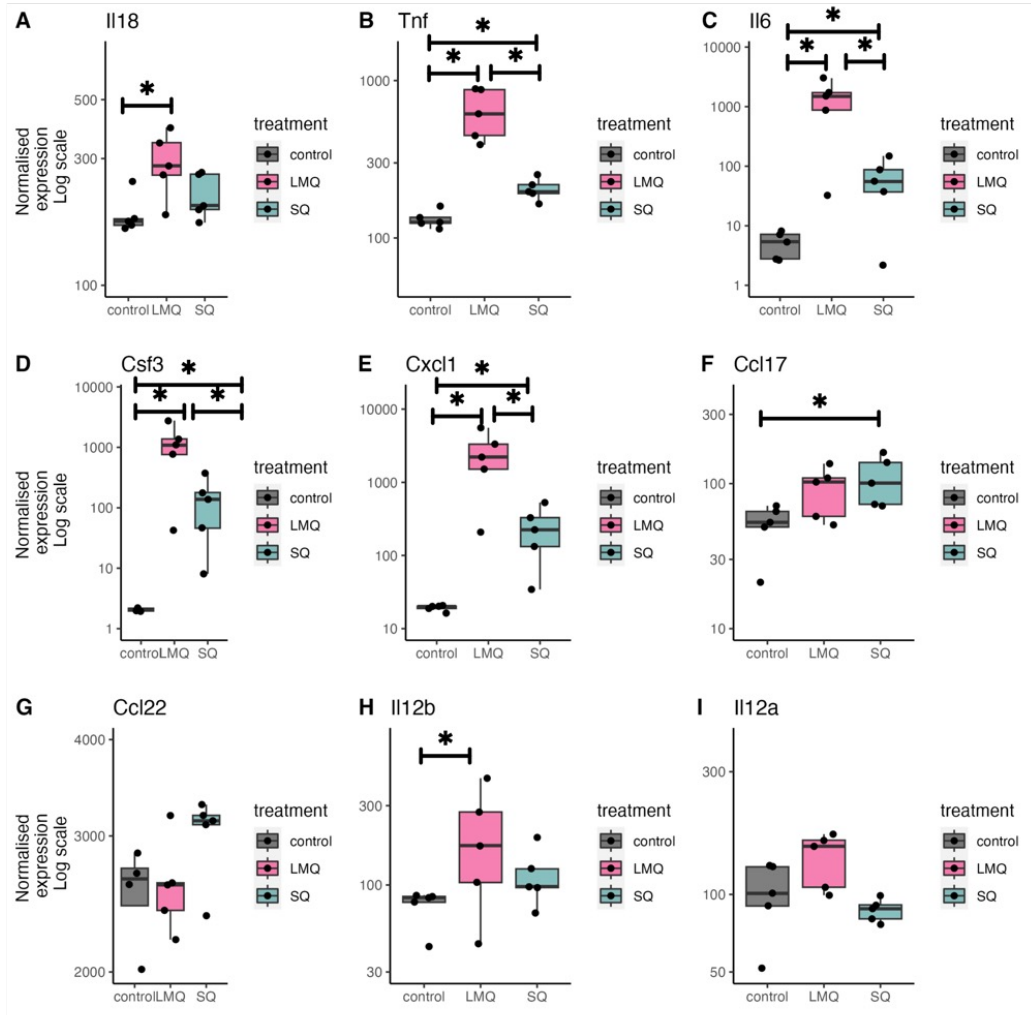


Figure 4.5: RNA-seq captures changes in the expression of selected cytokines and chemokines in the iLN after immunisation with LMQ or SQ. The normalised expression of (A) Il18, (B) Tnf, (C) Il6, (D) Csf3, (E) Cxcl1, (F) Ccl17, (G) Ccl2, (H) Il12b and (I) Il12a across different immunisation statuses in the iLN is shown on a log scale. Significance is determined by the results of DeSeq2 on the whole transcriptome adjusted for multiple testing with the Benjamini and Hochberg method. (*p<0.05 and Log2FC>1.5).

This trend is consistent with the adjuvant uptake data, which demonstrated that higher levels of uptake were found for LMQ compared to SQ, and higher levels of uptake were found in the muscle compared to the lymph node.

Next, the overlap of differentially expressed genes across tissues and adjuvants was analysed. From this analysis, a set of 70 genes that were shared in both tissues for both immunisations was identified [Fig 4.6B]. Selected genes in this shared group were categorised by the class of protein [Fig4.7]. Cytokines and chemokines such as Il6, Il33, Ccl7 and Csf3 were upregulated, as well as pattern recognition receptors including Clec4e (Mincle) and Clec7a (dectin-1). Interestingly, there was upregulation of histidine decarboxylase (Hdc) in both tissues in response to both adjuvants. Hdc is the only enzyme required for histamine synthesis in a single-step reaction (129). Performing overrepresentation analysis on the shared set of genes showed enrichment for cytokine signalling and cellular migration functional terms [Fig 4.8].

4.2.5 Both adjuvants show enrichment of functional terms for cytokine signalling, cell migration and cell death in the muscle and lymph node.

Enrichment analysis can be used to test sets of genes from differential expression analysis for the enrichment of functional biological terms. Although the exact genes may not be shared between tissues, different genes with similar functions may be upregulated in each tissue and immunisation status. For each tissue and immunisation status, enrichment analysis was performed to test for enrichment of Gene Ontology (GO) terms [Fig 4.9].

A clear signal for activation of the innate immune response was seen across tissues and immunisations with the terms Innate immune response (GO:0045087),

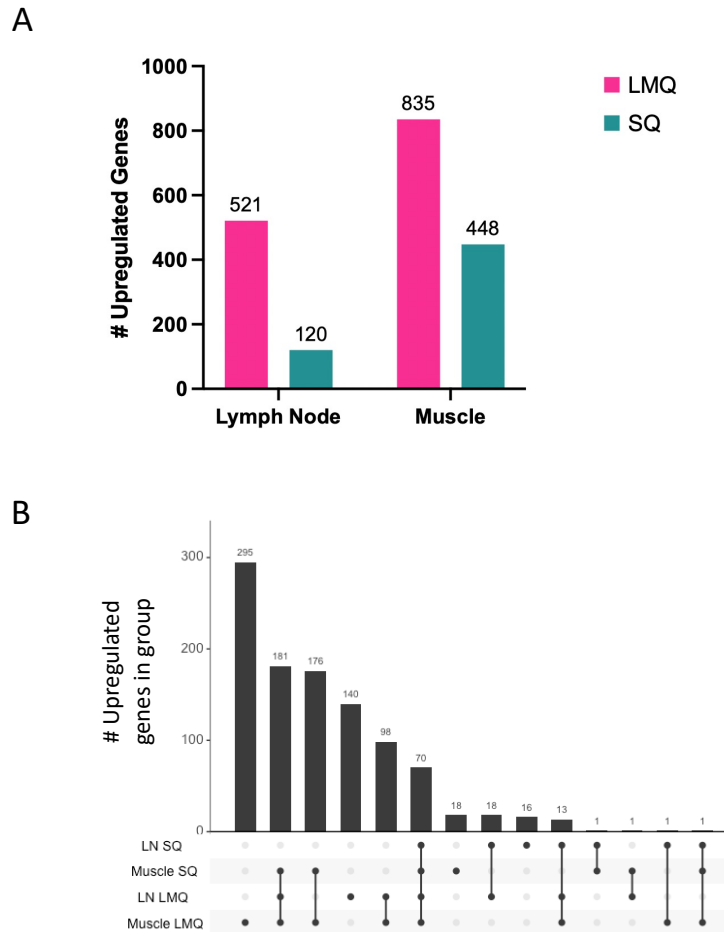


Figure 4.6: Gene expression changes are distinct between both immunisation and tissue. (A) A plot showing the number of differentially expressed genes in the muscle and lymph node after immunisation with LMQ or SQ compared to a PBS control. Genes are defined as upregulated by DeSeq2 by the threshold $p < 0.05$ and $\text{Log}_2\text{FC} > 1$. (B) A plot showing the overlap of differentially upregulated genes across tissues and immunisation status. Dots along the bottom legend show the conditions that are being shown, and where multiple are coloured they indicate the overlap of differentially upregulated genes between the different conditions. The muscle site shows a higher number of DE genes than the lymph node, while LMQ-treated mice have a higher number of DE genes than SQ-treated mice. Interestingly, there is a core set of 70 genes that are upregulated in all tissues by both adjuvants.

4: Adjuvant induced inflammation in the muscle and lymph nodes

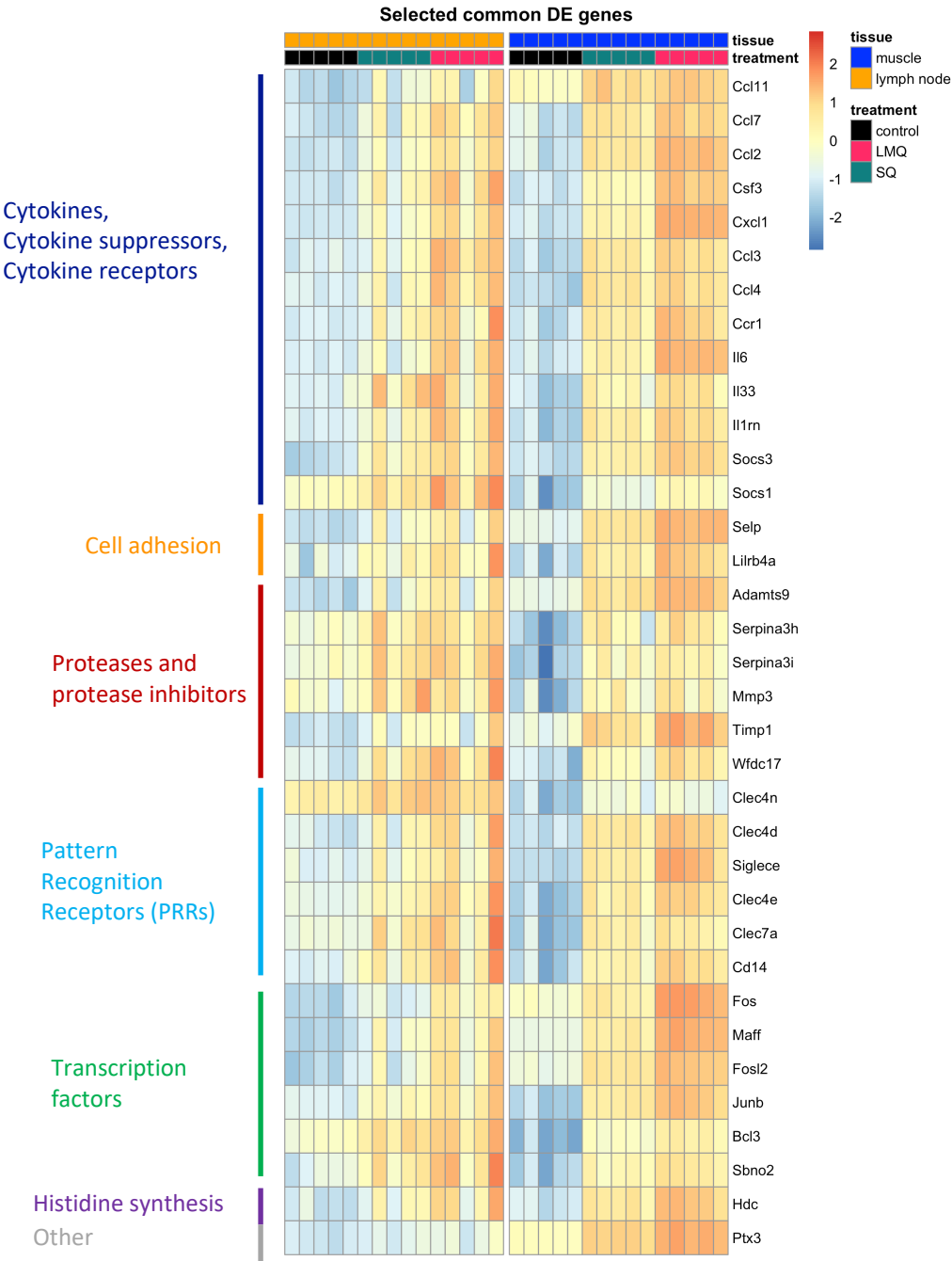


Figure 4.7: A core set of differentially upregulated genes are shared across all tissues and adjuvant status. The Z-Score of gene expression for each gene is shown for genes that are upregulated in both tissues by both immunisations. Genes are defined as upregulated by DeSeq2 by the threshold $p < 0.05$ and $\text{Log}_2\text{FC} > 1$. (continued next page)

Figure 4.7: Continued from previous page: The top two rows of the heatmap indicate the tissue and immunisation status indicated by colour. Genes are grouped by categories shown on the left-hand side.

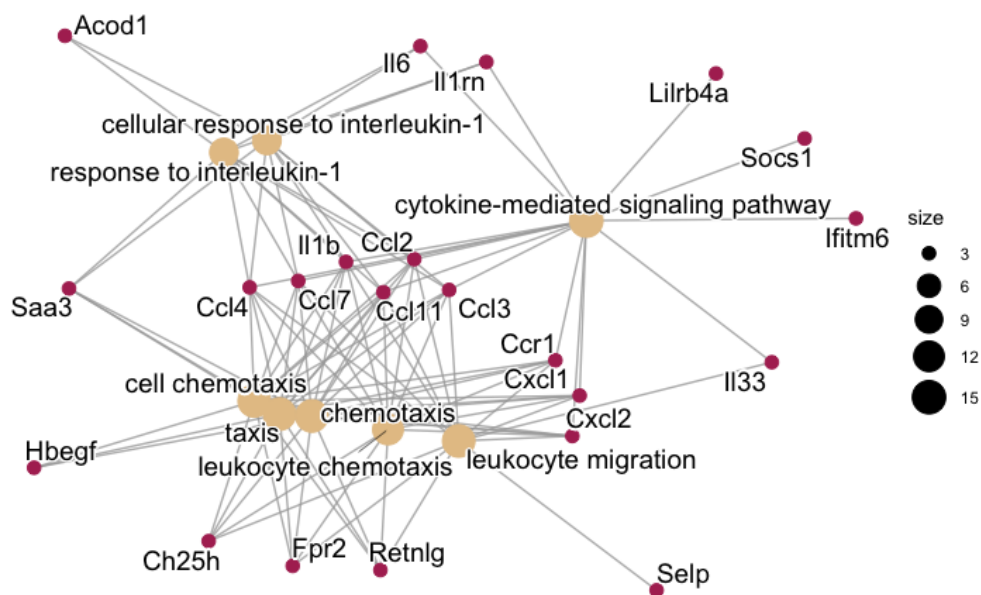


Figure 4.8: The common set of DEGs found in all conditions are overrepresented in cell migration and cytokine signalling functions. A network plot of the connections between enriched functional terms and genes. Enriched GO terms are shown in orange, and genes that take part in these terms are shown in red. Lines between go terms and genes indicate that a gene is associated with the relevant function.

Neutrophil Migration (GO:1990266), Monocyte chemotaxis (GO:0002548) and Canonical NF kappa B signal transduction (GO:0007249) upregulated. Signals of mast cell activation were seen in the muscle and lymph nodes of mice immunised with LMQ, but not SQ. Along with the upregulation of Hdc this signal of mast cell activation is consistent with other adjuvants containing both a TLR4 agonist and a saponin, which have been shown to activate mast cells and enhance lymphatic drainage to proximal lymph nodes (118).

Interestingly, the extrinsic apoptotic signalling pathway (GO:0097191) was enriched after immunisation in the muscle and lymph node after immunisation with both adjuvants. Additionally, Pyroptosis (GO:0070269) was also enriched after immunisation in the muscle for both adjuvants and the lymph node after LMQ immunisation. Previously, it has been shown that both LMQ and SQ can induce *in vitro* cell death in bone marrow-derived macrophages. The gene expression changes seen *in vivo* also point to a role for cell death in the mechanism of LMQ and SQ.

4.2.6 Immunisation with LMQ produces an enhanced antiviral and type II interferon response in comparison to immunisation with SQ

After immunisation with LMQ numerous genes were upregulated that were not detected after immunisation with SQ [Fig 4.7B]. Interestingly, there were minimal genes that were unique to SQ immunisation. In the LMQ immunised lymph node and muscle, numerous cytokines and chemokines were upregulated including Il22, Il11, Il23a, Ccl24 and Cxcl16 [Fig 4.10A]. Overrepresentation analysis performed when directly comparing the expression of LMQ to SQ revealed enhancement of Cytokine Production, Type II interferon

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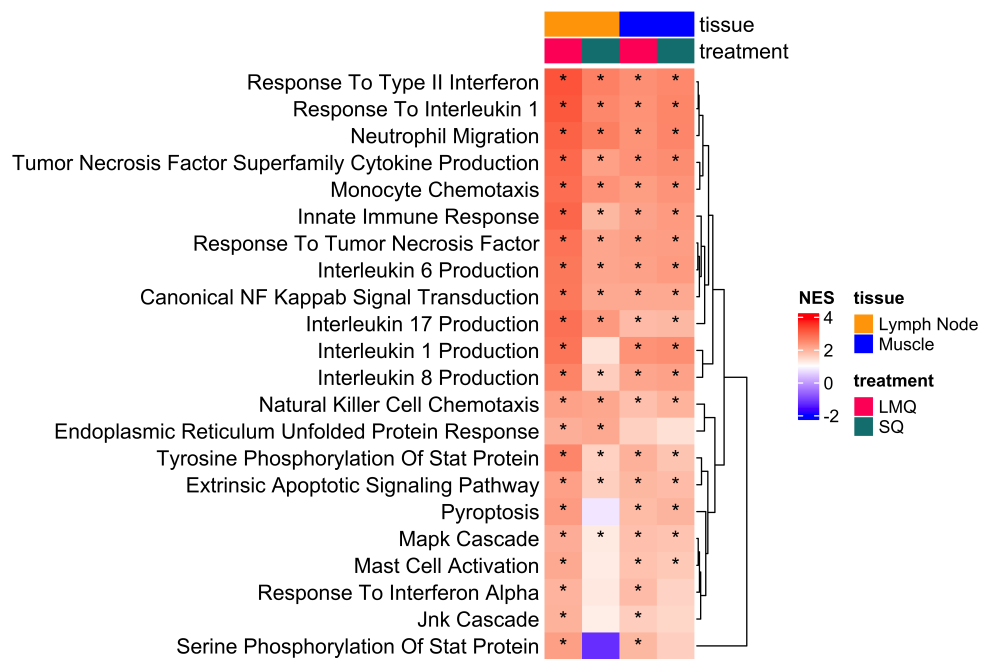


Figure 4.9: GO-Terms across tissues and treatments A heatmap of GO terms that are enriched across the lymph node and muscle for both adjuvant immunisations. The normalised enrichment score (NES), and significantly enriched GO terms are indicated (*p<0.01).

production and leukocyte migration above that found in SQ [4.10B]. LMQ also induced higher upregulation of the effector gene *Gzmb*, characteristic of LMQs induction of an anti-viral gene expression pattern [Fig 4.11A].

While immunisation SQ showed enhancement of the Type II interferon production (GO:0032609) GO term when compared to PBS, immunisation with LMQ was superior in its induction of this gene expression pattern over SQ [Fig 4.10B]. Additionally, only LMQ showed a significant upregulation of *Ifng*, the only member of the type II interferon family, within the LMQ immunised lymph node [Fig 4.10C]. This suggests that the upregulation of the Type II interferon GO term after immunisation with SQ may be driven by an overlap of the genes in this pathway with other pathways, given the lack of *Ifng* expression after SQ immunisation.

4.2.7 The TUNEL assay shows minimal detectable cell death in adjuvant immunised muscle.

Given the overrepresentation of the extrinsic apoptotic signalling pathway (GO:0097191) and pyroptosis (GO:0070269) in muscle after immunisation with LMQ or SQ, the role of cell death in the mechanism of these adjuvants was explored further. The TUNEL assay detects double-stranded DNA breaks, which form during apoptotic, necrotic and pyroptotic cell death (44,130,131). Therefore the TUNEL assay was chosen as a non-selective marker for a variety of forms of cell death that could be induced by adjuvants. TUNEL assay was used on PFA fixed paraffin embedded tissue sections collected from muscles immunised with LMQ, SQ or a PBS control 6 hours after immunisation to provide time for cell death to occur. A positive control was created by using DNase-I to generate double-stranded DNA

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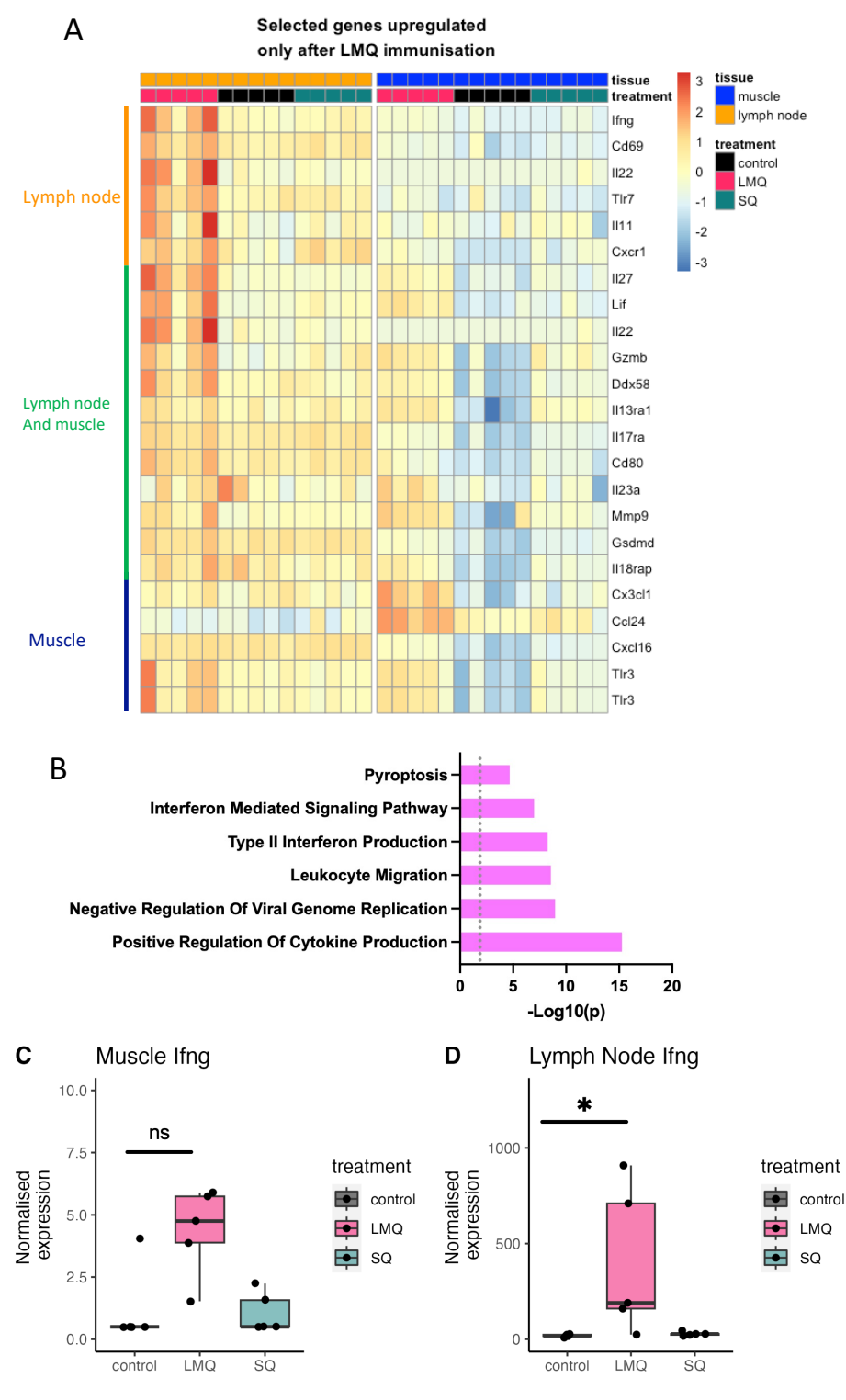


Figure 4.10:

Figure 4.10: A heatmap showing the Z scores of exemplar genes that are upregulated in LMQ immunised muscles or lymph nodes, but are not found after immunisation with SQ. (B) Enrichment analysis comparing LMQ directly to SQ showing the p values of enrichment for specific functional GO terms. (C) The expression of *Ifng* in the muscle and lymph nodes of mice immunised with LMQ, SQ or control. Significance is determined by the results of DeSeq2 on the whole transcriptome adjusted for multiple testing with the Benjamini and Hochberg method. (* $p < 0.05$ and $\text{Log}_2\text{FC} > 1.5$).

breaks and confirm that the TUNEL reaction was successful.

Fluorescent microscopy of sections of muscle tissue revealed low levels of TUNEL staining for both PBS and adjuvant immunised tissues, while the positive control showed a high level of TUNEL staining confirming a successful reaction [Fig 4.11]. Quantification of the microscopy images confirmed the minimal TUNEL staining in the muscle in response to either adjuvant. The absence of TUNEL staining suggests at this time point, there is minimal *in vivo* cell death in the muscle. This contrasts with previous publications showing cell death in response to LMQ and SQ in bone marrow-derived macrophage cell cultures (18).

4.3 Discussion

In this chapter, the transcriptomic mechanisms of LMQ and SQ across both the injection site and the draining lymph node were analysed. This was of particular interest due to the differential drainage of LMQ and SQ to the lymph node, with LMQ showing a higher level of uptake in resident cells including fibroblasts and SCS macrophages.

Initial analysis found that the 3-hour timepoint is suitable for capturing robust transcriptional signals of inflammation in response to LMQ and SQ.

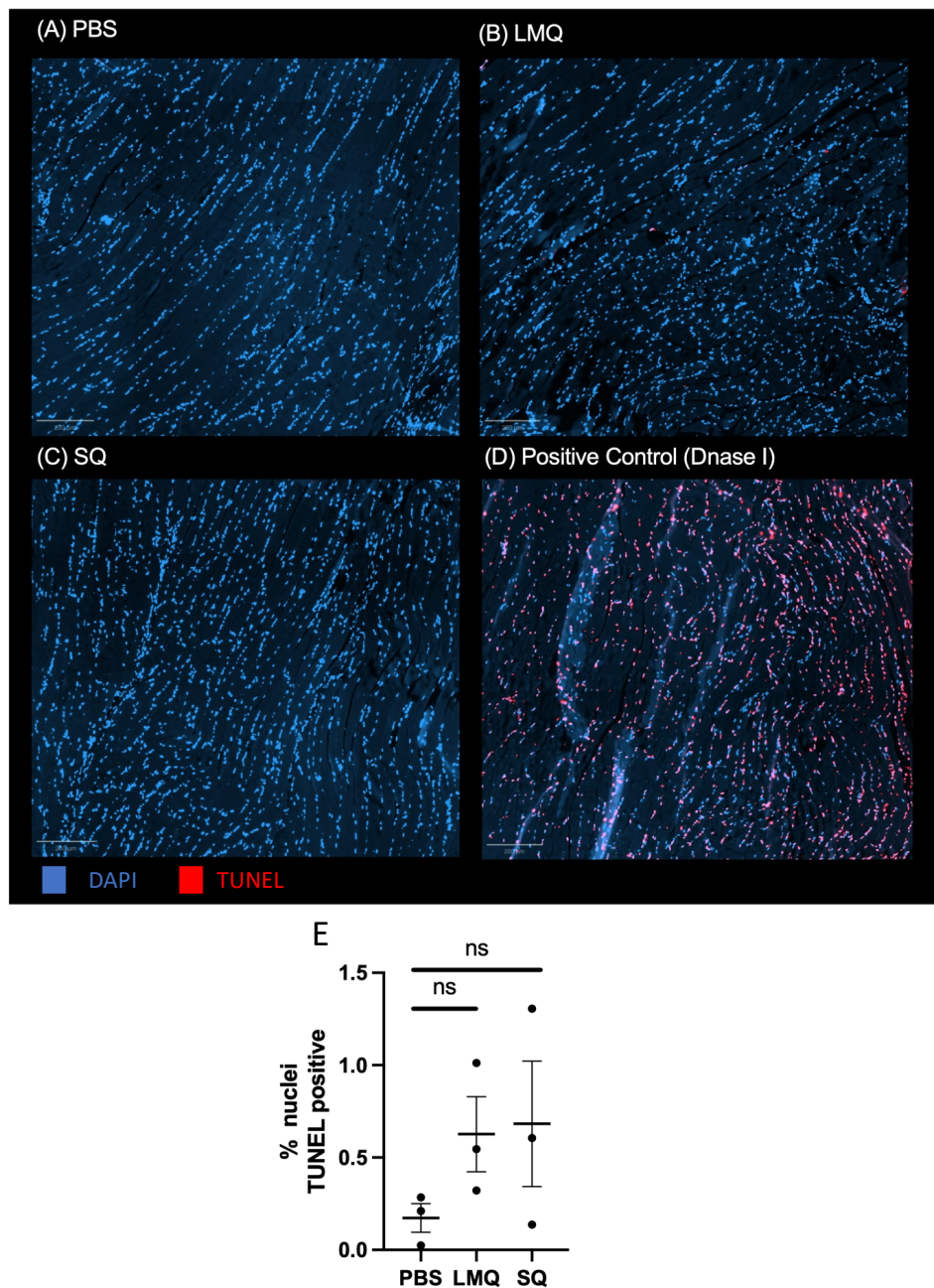


Figure 4.11: A TUNEL assay shows no evidence of significant adjuvant-induced cell death *in vivo* at the 6h timepoint (A-D) Representative fluorescent microscopy images of murine muscle 6 hours after immunisation with PBS, LMQ, SQ or a positive control. Blue indicates DAPI staining, while Red Indicates TUNEL positive cells (cells with double-stranded DNA breaks, indicative of multiple types of cell death). Continued next page:

Figure 4.11: Continued from previous page: All images were taken with the Zeiss Axioscan 7 microscope slide scanner. (E) Quantification of TUNEL results passed on the percentage of nuclei in the muscle that stain positive on the TUNEL assay, statistical analysis is performed using an ordinary one-way ANOVA [Methods]. Mean $\pm$ SEM is shown.

Transcriptional signals of inflammation peak rapidly after immunisation, and return to near baseline levels by 24 hours. This rapid, but transient, inflammation is consistent with the kinetics observed for other vaccine adjuvants such as AS01 and MF59 (20,132). The full impact of the transient property of adjuvant-induced inflammation is unknown. However, strategies to prolong adjuvant-induced inflammation are being explored as a method to enhance the efficacy of adjuvants (133) Prolonging adjuvant-induced inflammation may enhance antibody and T-cell responses (130). However, the safety and tolerability of longer-term inflammation from adjuvants have not been explored.

From the RNA-seq analysis, a core set of differentially expressed genes across tissues and for both adjuvants was found. Many of the genes in this set were chemokines including Ccl11, Ccl7, Ccl2, Cxcl1, Ccl3 and Ccl4. Cxcl1 and Ccl2 are known neutrophil chemoattractants (126). Csf3 which was also up-regulated across tissues and immunisations promotes survival of neutrophils and increases the number of neutrophils at the site of inflammation (132). Ccl2, Ccl3 are Ccl4 are known to attract or recruit dendritic cells (131). Monocytes have also been previously shown to be recruited by Ccl7 and Ccl2 (132). While Ccl11 is a known eosinophil and basophil chemoattractant through its interaction with CCR3, however, the presence of these cells in the muscle or lymph node has not been explored in this thesis (129).

Also among the upregulated genes after immunisation with either adju-

vant in both tissues are multiple PRRs of the C-type lectin class, including Clec4e (Mincle), Clec7a (Dectin-1) and Clec4d (MCP). These PRRs recognise a variety of endogenous DAMPS including galectin-9, Annexins and β -glucosylceramide (136-138). Therefore, their upregulation after immunisation may contribute to the mechanism of action of both adjuvants through the sensing of DAMPS that are suspected to be released by both adjuvants due to tissue damage or cell death.

When viewing the transcriptional changes purely in terms of the number of upregulated genes, immunisation with LMQ produced numerous gene expression changes that were not seen in SQ. Interestingly, immunisation with SQ produced a much smaller number of unique gene expression changes. The majority of genes that were upregulated by SQ were also upregulated by LMQ. Therefore, SQ's gene expression profile was characterised by a lack of expression of LMQ-induced genes rather than having its own unique profile.

LMQ, however, induced the upregulation of numerous unique genes including *Ifng* in the lymph node. LMQ induced an enhanced type II interferon signalling and antiviral gene expression programme in comparison to SQ after immunisation. This is strongly suggestive that LMQ can induce the same synergistic interaction of TLR4 and QS-21 inflammasome activation as AS01, as this is mediated through *Ifng* release in the lymph node (49).

Enrichment of genes involved in apoptosis and pyroptosis after immunisation with both adjuvants in the muscle suggested that cell death may contribute to the mechanism of action of both adjuvants. This was supported by previous evidence of *in vitro* cell death in a bone marrow-derived macrophage model when treated with LMQ or SQ. The TUNEL assay was used to assess cell death in the muscle through the detection of double-stranded DNA breaks

that are formed during apoptosis, pyroptosis and necroptosis (146,131). However, the results showed no evidence of significant cell death, contrasting with published *in vitro* experiments and the transcriptional profiling. The discrepancy between the *in vivo* and *in vitro* results could be due to a variety of factors such as compensatory mechanisms protecting against cell death *in vivo* or by clearance of dead cells by phagocytic cells that are recruited to the muscle.

To summarise, in this chapter, it was found that both LMQ and SQ induce a rapid but transient upregulation of transcriptional signatures of inflammation and activation of the innate immune response. While LMQ showed a unique interferon response gene expression programme, SQ did not show any significant differences to LMQ except for the lack of LMQ unique genes. While there were transcriptional signatures of pyroptosis and apoptosis detected after immunisation, minimal cell death was observed in the muscle in contrast to *in vitro* stimulation data.

4.3.1 Caveats and open questions

The question of if LMQ and SQ cause cell death *in vivo* is still open, and has not been ruled out by a lack of TUNEL positive staining. Cell death *in vivo* could be explored through a variety of techniques including LDH assays on muscle tissue following immunisation. In addition further microscopy work could be done to examine through cell death, such as through H&E staining to look for gross structural signals of tissue damage or through immunofluorescent staining for markers of programmed cell death such as caspases. In addition, functional consequences of cell death for immunisation with LMQ and SQ have not been verified. Previous studies have used ATPase enzymes to block the DAMP signalling effect of ATP after immunisation with MF59,

4: Adjuvant induced inflammation in the muscle and lymph nodes

similar strategies could be used with LMQ and SQ if appropriate DAMPs could be identified (61).

5 | Single cell mechanisms of liposomal and squalene emulsion adjuvants

5.1 Introduction.

Early innate signalling from specific cell types in response to vaccine adjuvants is known to significantly affect downstream adaptive immune responses (10). While the results from the previous chapter and other publications have informed our understanding of inflammatory mediators induced by LMQ and SQ adjuvants, the cellular sources of inflammation are poorly understood *in vivo* (18). Identifying these cellular sources, and understanding the transcriptional changes induced in them, could aid in the understanding of vaccine adjuvant mechanisms and inform rational vaccine design. To target this knowledge gap a comprehensive cellular and transcriptional map of murine early immune responses to LMQ and SQ in draining iLNs was generated.

Single-cell RNA sequencing (scRNA-seq) has emerged as a powerful tool for understanding the transcriptomic immune responses on a single-cell level. Previous sequencing studies on murine and human lymph nodes have transcriptionally categorised the sub-populations of cells in this tissue (140-143). From the adjuvant uptake experiments in Chapter 3, a variety of cells are shown to interact with both LMQ and SQ. However, it is unclear which cell types are responding and in what way within the iLN.

High levels of cellular influx and trafficking of adjuvants by neutrophils to the iLN suggest a potential role for neutrophils in the mechanisms of action of both LMQ and SQ [Fig 3.5]. The most widely used scRNA-seq platform, 10X Chromium, has previously performed poorly in capturing the transcriptomes

of granulocytes in comparison to the BD Rhapsody whole transcriptome sequencing platform (144). Transcriptomes of granulocytes captured by 10X Chromium have previously been shown to have significantly lower levels of total RNA and unique genes detected relative to those captured by BD Rhapsody (144). Low levels of RNA capture have even led to granulocytes being excluded from downstream analysis by falling below the level needed to be defined as a cell by the Cellranger pre-processing software. However, new developments in the software used for processing scRNA-seq raw data by 10X Chromium have reopened the question as to its performance in capturing granulocyte transcriptomes. The newer cellranger v7.0 software includes intronic RNA when counting RNA reads, which may improve the biological information that can be extracted from these cells due to their higher proportion of intronic RNA (145).

5.1.1 Aims

- Identify the optimal scRNA-seq platform for characterising immune responses to adjuvants in iLNs.
- Transcriptionally characterise the cells found in the iLN after LMQ or SQ immunisation.
- Characterise the profiles of inflammation driven by LMQ and SQ in the iLN on a cellular level.

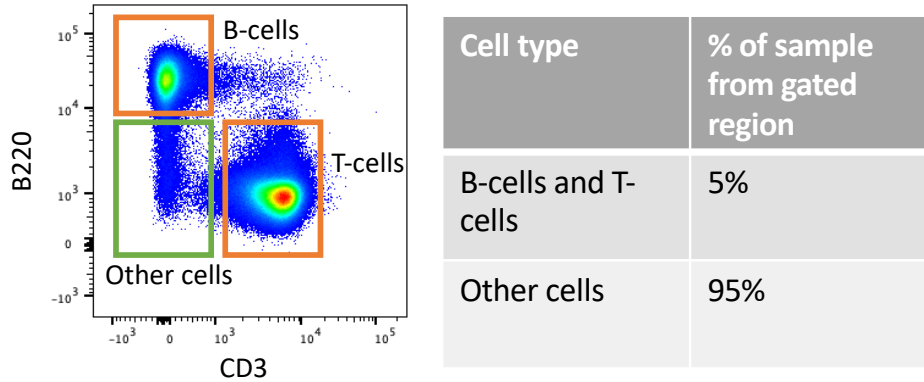


Figure 5.1: B and T cells are depleted prior to sequencing Exemplar gating strategy used for the enrichment of non-B non-T cells, and a chart showing the cytometer settings for the percentage of the total sample taken from each gated region.

5.2 Results.

5.2.1 BD Rhapsody marginally outperforms 10X Chromium in capturing granulocyte transcriptomes.

The study was began by performing a head-to-head comparison of two alternative scRNA-seq technologies, 10X Chromium and BD Rhapsody, to assess their relative performance in capturing the transcriptomes of granulocytes, hypothesised to be an important cellular mediator of adjuvant mechanisms of action. Given the lymph node is comprised of $\sim 95\%$ B and T cells, rarer cell types must be enriched [Fig 3.3]. To enrich rarer cell types in the LN, B220+ and CD3E+ cells were depleted by FACS [Figure 5.1]. Each sample of cells was divided in half and processed by both the BD Rhapsody and 10X Chromium workflows to perform a head-to-head comparison. Each sample was sequenced to the same depth ($\sim 50,000$ reads per cell).

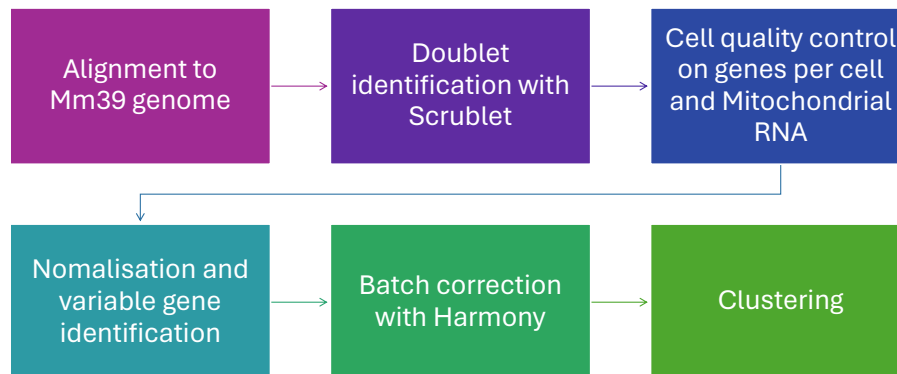


Figure 5.2: An overview of the scRNA-seq processing pipeline.

Unique Molecular Identifiers (UMIs) are short barcoding sequences used to tag each unique RNA molecule in the single-cell sequencing library. UMIs for each gene are totalled to give the quantity of RNA per cell. After sequencing, both technologies were run through their respective preprocessing pipelines to produce UMI data on a per-cell basis. The UMI data from both platforms were later processed using the same QC pipeline to filter out doublets and low-quality cells and normalise the data (Fig 5.2, 5.3) [Methods]. Cell types from both platforms were integrated with the Harmony package and then clustered using the Louvain algorithm and manually annotated by marker genes [Fig 5.3D-F, 5.4A, B]. The cell types of interest were identified, including T-cells, B-cells, monocytes, stromal cells, and granulocytes, such as neutrophils (Cd14+, Cxcr2+) and a mixed eosinophil and basophil population (Itgam+, Ccr3+). Both technologies were able to capture the same cell types, but there was a slight bias for eosinophil and basophil capture when using BD Rhapsody [Fig. 5.4C]. Neutrophil capture, however, was roughly equivalent for both technologies [Fig5.4C].

The transcriptome quality of each cell type was assessed by evaluating the number of unique genes detected, the number of UMIs detected, riboso-

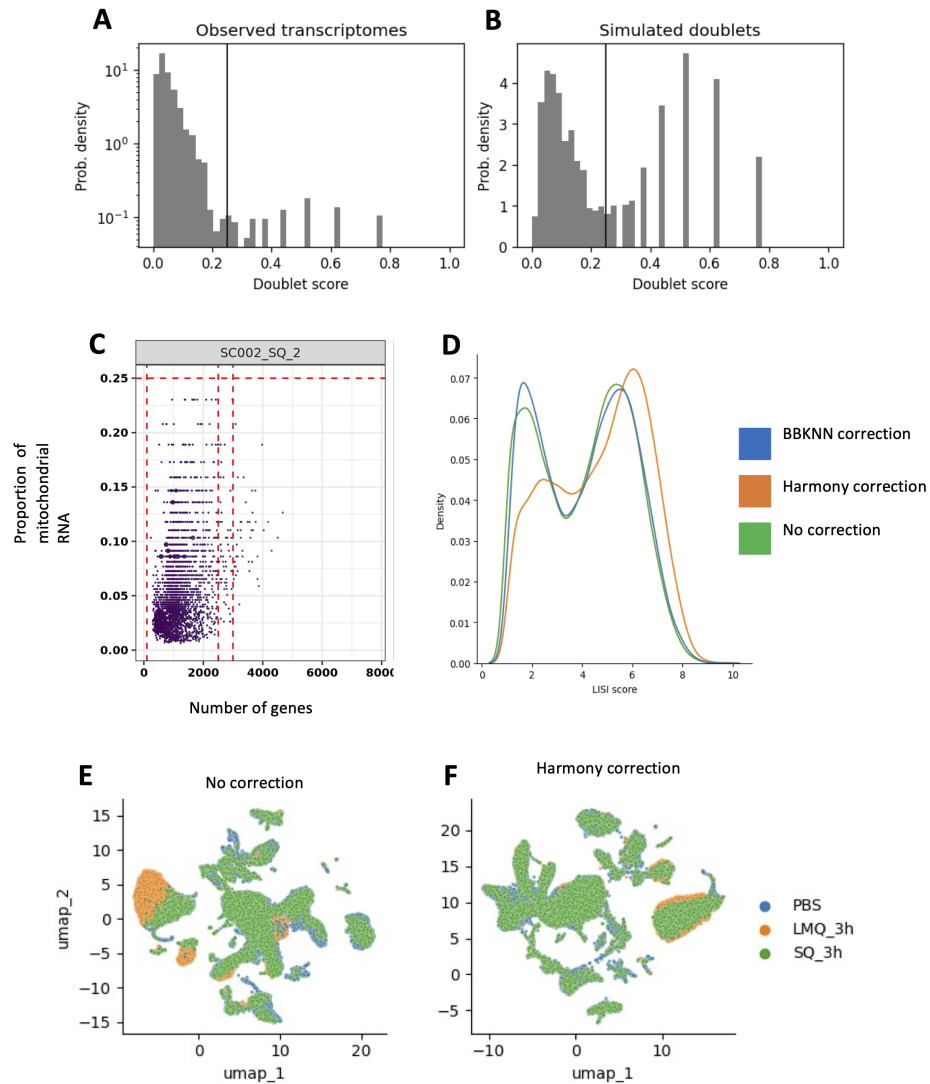


Figure 5.3: scRNA-seq quality control exemplar figures. (A, B) Results from Scrublet showing the observed and simulated doublets within a sample. The vertical line indicated the cut-off value for removing doublets, and is set by the local minimum of the simulated doublets. (C) The number of genes plotted by the proportion of mitochondrial RNA, this is used to filter low quality cells on high mitochondrial RNA and low numbers of genes. (D) the LISI score of the scRNA-seq dataset before and after correction with BBKNN or Harmony. Harmony led to the largest increase in LISI score indicating it performed best for batch correction. (E, F) UMAP plots before and after integration with harmony coloured by the treatment. Harmony integration reduces the separation of samples based on batch on the UMAP visualisation.

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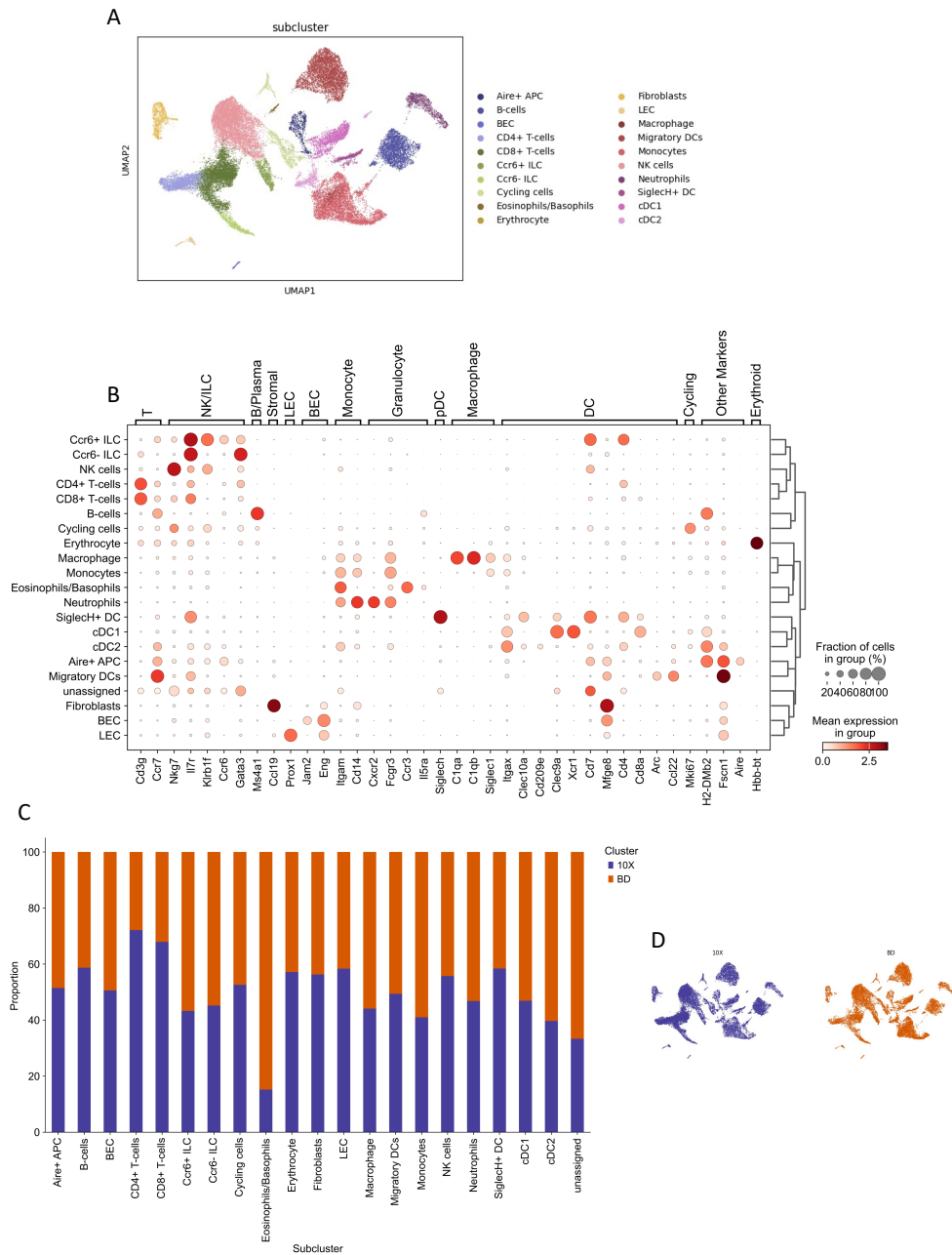


Figure 5.4: Cell types of interest are captured by both sequencing platforms. (A) A UMAP projection of cells from the combined BD rhapsody and 10X chromium dataset (B) A dot plot depicting the expression of selected marker genes for each cell cluster in the combined dataset. Continued next page:

Figure 5.4: Continued from previous page: (C) Stacked bar plots depicting the proportion of cells that each sequencing technique contributes to a cell cluster, with both technologies detecting the same cells but to differing degrees (D) UMAP projections split by sequencing technology.

mal protein RNA content and mitochondrial RNA content for both 10X Chromium and BD Rhapsody [Fig5.5]. Notably, Neutrophils, Eosinophils, and Basophils displayed a decreased total RNA and unique gene count when assessed with 10X Chromium relative to BD Rhapsody, aligning with prior findings [Fig5.5A, B](143). However, the observed disparity was less pronounced compared to earlier reports, where a 12-fold elevation in total RNA and an 8-fold increase in the number of detected genes was reported for human blood neutrophils (144). A more modest 2-fold increase in total RNA and a 1.6-fold rise in unique genes within neutrophils were detected in this experiment [Fig 5.5A, B]. In summary, while BD Rhapsody exhibited superior RNA capture and gene detection in murine lymph node cells, the discrepancies between the two methodologies were notably reduced compared to findings in human blood or bone marrow samples

BD Rhapsody showed lower levels of ribosomal protein RNA compared to 10X Chromium [Fig 5.5C]. Ribosomal proteins can form a large part of the transcriptome but are often not biologically relevant to a particular research question. Reduced ribosomal protein RNA capture can therefore represent large cost savings on sequencing experiments. High mitochondrial RNA can be indicative of dying cells, as apoptotic cells upregulate mitochondrial RNA expression. BD Rhapsody showed higher levels of mitochondrial RNA expression compared to 10X [Fig 5.5D]. Given that the same pooled samples were used for BD and 10X, this suggests that the 10X sequencing process was less damaging to cells. However, the level of mitochondrial mRNA found

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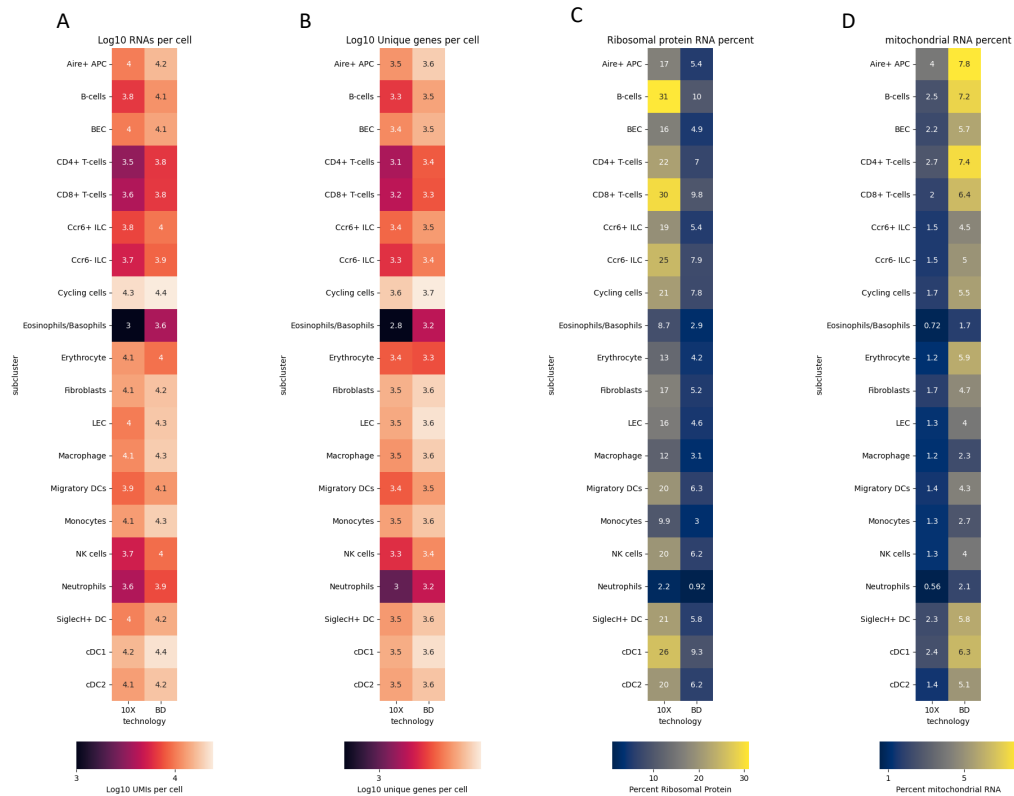


Figure 5.5: BD Rhapsody captures marginally more useful RNA compared to 10X Chromium Heatmaps of cell clusters from BD Rhapsody and 10X Chromium showing (A) mean log10 UMIs per cell, (B) mean Log10 unique genes per cell, (C) mean percentage of total RNA that encodes ribosomal proteins and (D) mean percentage of total RNA that encodes mitochondrial proteins.

after BD Rhapsody was still relatively low, and mitochondrial RNA at this level is generally not considered a concern.

10X Chromium is currently the most widely used platform for scRNA-seq, and multiple tools exist that enable cross-comparison of scRNA-seq data even from across institutions. Given the marginal difference between the two techniques and 10X's potential for integration with a variety of additional data, 10X Chromium was used as the sequencing technology for further experiments.

5.2.2 Cell type identification of the CD3-ve and B220-ve compartment in murine lymph nodes upon adjuvant treatment.

Sequencing was performed on iLNs from mice immunised with LMQ, SQ or PBS control at 3 hours post immunisation (n=3 mice per condition) (Fig 5.6) [Methods]. After quality control, a total set of 34,731 cells were captured in our dataset and 21 cell clusters were identified by Louvain clustering and annotation with marker genes (Fig 5.7A, B) [Methods].

Upon grouping cell clusters into broader cell groups of T and B cells, myeloid, stromal, ILCs and granulocytes it was found that 20% of the dataset was composed of B and T cells compared to the expected value of 5% from the designed sorting strategy [Fig5.8C]. The largest cell group on average across LMQ,SQ and PBS were ILCs (44% of cells) followed by granulocytes (18%), monocytes (14%) and stromal cells (2%).

Details of the different cell clusters and their markers can be found in table 5.1. In contrast to the observations in the head-to-head comparison of

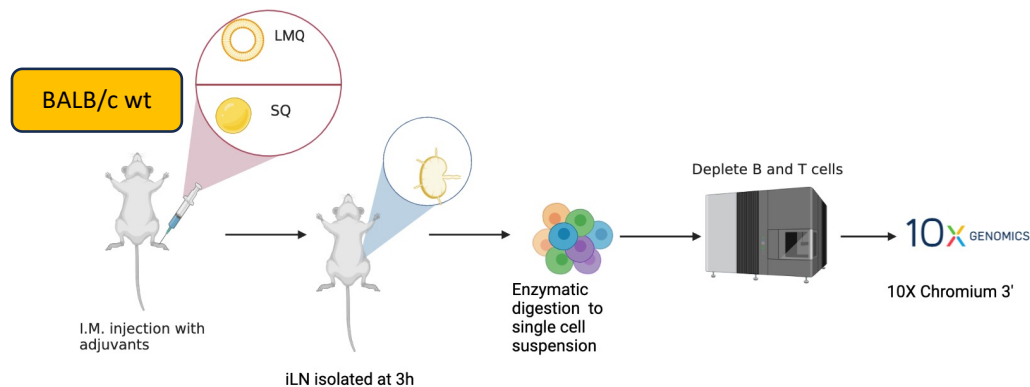


Figure 5.6: A schematic of the sequencing methodology used to compare LMQ, SQ and PBS.

sequencing technologies, no eosinophil or basophil population was found. Additionally, two neutrophil populations were found that appear to represent different levels of neutrophil activation, given enrichment for matrix metalloproteinase genes in the Neutrophil-2 population. Additionally only 56 pDCs were detected in our dataset, a minimal number compared to other lymph node datasets. This is likely due to differences in the sorting strategy. B220 was used as a marker to deplete B cells while other lymph node datasets used CD19, however B220 is also displayed on the surface of pDCs (146).

No cell cluster was fully unique to any immunisation status [Fig 5.8 A, B]. However, differential abundance testing revealed that there were significantly higher numbers of neutrophil-1 (Cd14+, Cxcr2+), neutrophil-2 (CD14+, Cxcr2+, Mmp8+, Mmp9+) and monocytes (Cd14+, Fcgr3a+, Itgam+) in the LNs from mice immunised with LMQ [Fig 5.8D] [Methods]. In the mice that received SQ only neutrophil-1 population was differentially abundant across all cell types [Fig 5.8D]. This is consistent with our FACS findings of a rapid neutrophil and monocyte influx into the draining lymph nodes after adjuvant administration [Chapter 3 Fig3.5].

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

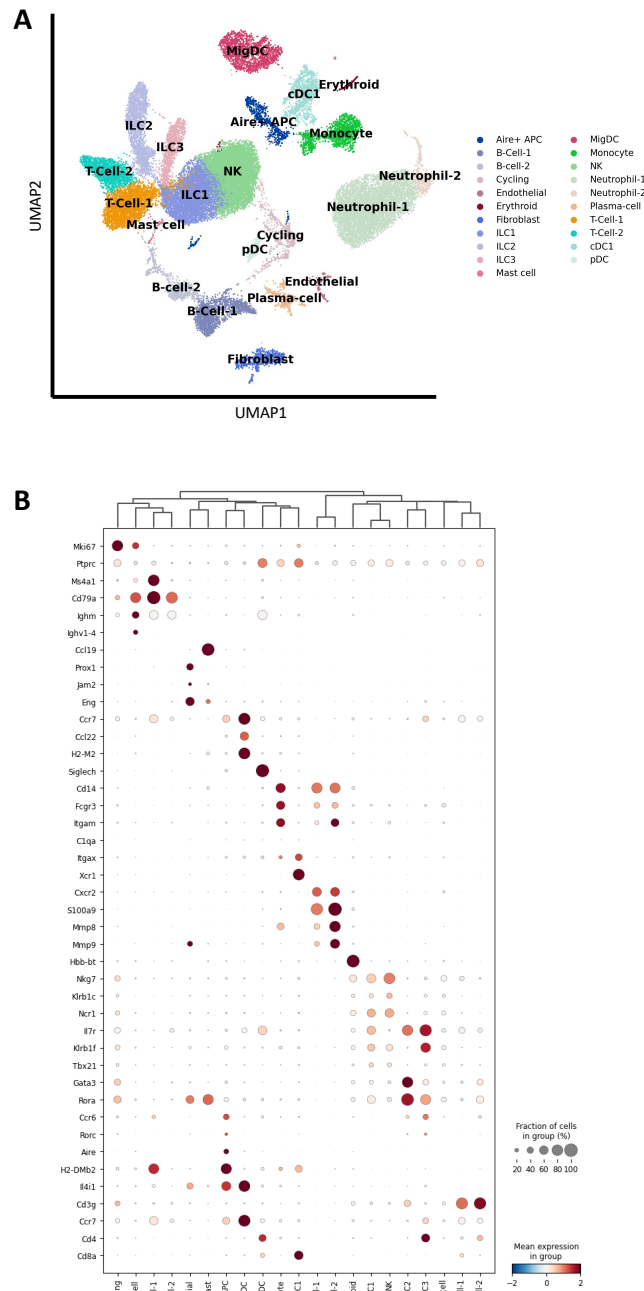


Figure 5.7: scRNA-seq identifies cell populations in the draining lymph node after immunisation. (A) UMAP projection of cells from mice immunized with LMQ or SQ displaying cell clusters (n=3-4 mice per condition) (B) Dot plots of Z scaled expression of selected marker genes in each cell cluster.

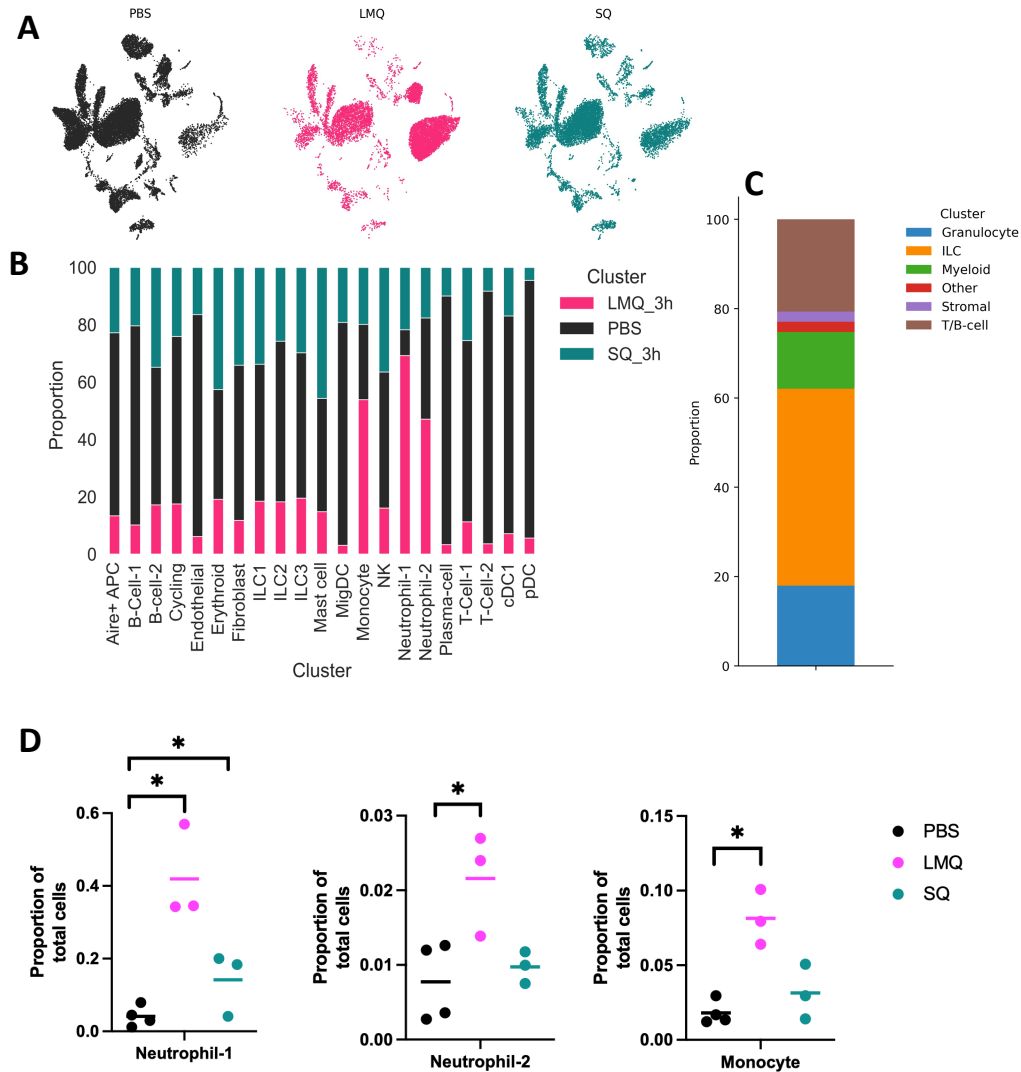


Figure 5.8: scRNA-seq detects changes in abundance of neutrophils and monocytes after immunisation. (A) UMAP projection of cells showing each treatment condition individually and the overlap of cells from each condition in the UMAP projection, (B) stacked barplots showing the proportion that each treatment condition contributes to each cell cluster. Continued next page:

Figure 5.8: Continued from previous page: (C) a stacked bar plot showing the proportion of the total sample that is classified as granulocytes, ILCs, Myeloid, Stromal, T/B cells or other cell types. (D) Compositional analysis of the dataset showing cell clusters that are differentially abundant between conditions, non-significant changes in cell abundance are not shown.* = statistically credible effect as defined under SCCODAs Bayesian framework [Methods].

5.2.3 Differential expression testing identifies global transcriptomic changes after LMQ immunisation compared to more restricted changes with SQ.

To analyse changes in gene expression among all cell clusters, each adjuvant immunisation status was compared to PBS using the MAST model [Methods] (147). Genes were identified as differentially upregulated within a cell type if $\text{Log}_2\text{FC} > 1.5$ and Bonferroni adjusted $P < 0.05$. Upregulated genes were totalled to produce an overview of where the greatest changes in gene expression were occurring across cells for each adjuvant [Fig5.9A]. In LMQ-immunised samples, there is a broad distribution of gene upregulation across multiple cell types. In contrast, in SQ-immunised samples, most of the gene expression changes are primarily restricted to monocytes and neutrophils. Similar to the distribution of SQ uptake in cells, SQ gene expression changes are largely restricted to cells that are known to be highly migratory in response to inflammation.

5.2.4 Gene ontology overrepresentation.

Neutrophils and monocytes were the only cell populations that showed a large change in gene expression after either LMQ or SQ immunisation, therefore these cell types were chosen as the starting point for the analysis. Gene

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

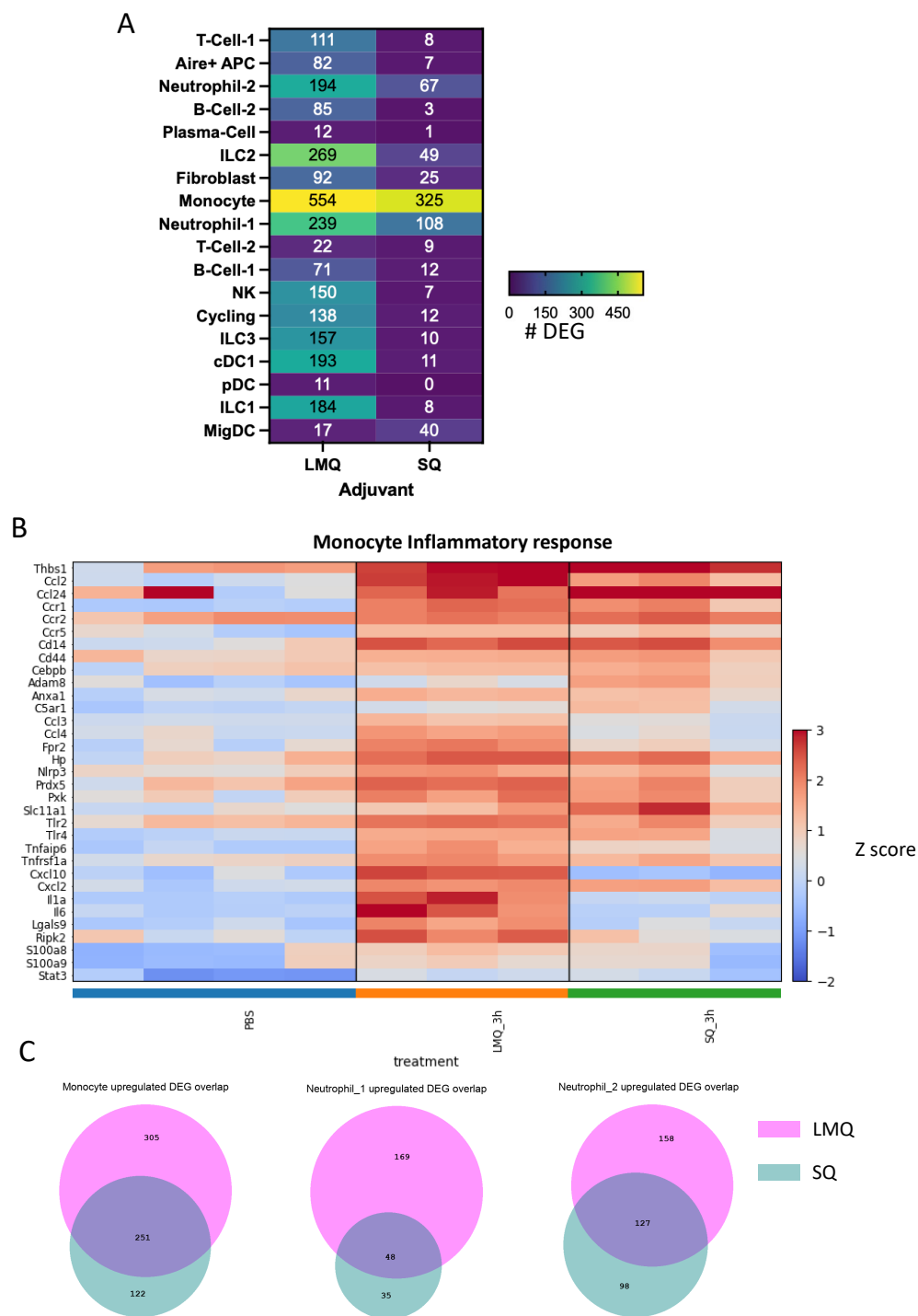


Figure 5.9: LMQ shows broader and different patterns of gene upregulation in comparison to SQ. Continued next page:

Figure 5.9: Continued from previous page: (A) A heatmap showing the number of DEGs (Log2FC >1.5 and P<0.05) across cell clusters for LMQ and SQ when compared to PBS as a baseline, (B) Heatmap of the Z-scored expression of selected from the GO term Inflammatory response (GO:0006954) in monocytes.(C) VENN diagrams of the overlap of upregulated DEGs between LMQ and SQ for monocytes, neutrophils-1 and neutrophils-2 showing overlap of gene upregulation between the two adjuvants.

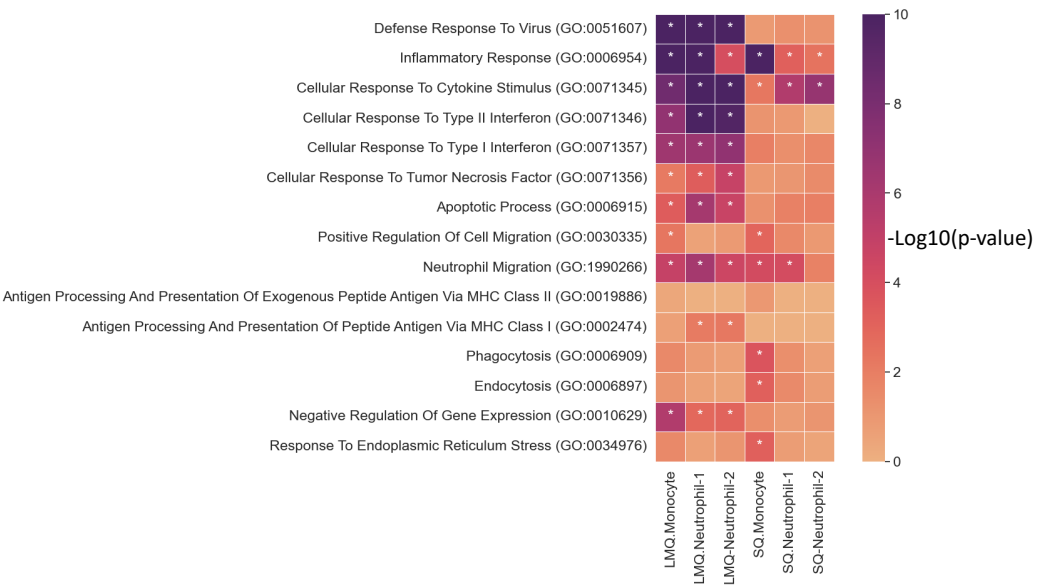


Figure 5.10: Monocytes and neutrophils from mice immunised with LMQ display a unique antiviral and type II interferon response programme, while SQ immunised mice show increased endocytosis and endoplasmic reticulum stress Heatmap of overrepresented GO terms in monocytes, neutrophils-1 and neutrophils-2 (*p<0.01) showing common and unique GO terms overrepresented for each adjuvant.

Table 5.1: Cell marker genes

Cell Group	Cell Clusters	Marker genes
B and T cells	T-Cell-1	Cd3e+, Cd4+
	T-cell-2	Cd3e+, Cd8a+
	B-cells-1	Ms4a1+, Cd79a+
	B-Cell-2	Ms4a1-, Cd79a+
	Plasma Cells	Mki67+, Ighv+
Stromal Cells	Fibroblasts	Ccl19+, Ptpre-
	Endothelial Cells	Prox1+, Eng+, Jam2+, Ptpre-
ILCs	ILC1s	Il7r+, Nkg7+
	ILC2s	Il7r+, Gata3+
	ILC3s	Il7r+, Rorc+
	NK cells	Nkg7+, Ncr1+
	Aire+ve APCs	Aire+, Rorc+, H2-DMb2+
Myeloid Cells	cDC1s	Itgax+, Xcr1+
	Monocytes	Itgam+, CD14+
	Migratory DCs (MigDCs)	Ccr7+, Ccl22+
	Plasmacytoid DCs	Siglech+
Granulocytes	Neutrophil_1	Cd14+, Cxcr2+
	Neutrophil_2	Cd14+, Cxcr2+, Mmp9+, Mmp8+
	Mast Cells	Cpa3+
Other Populations	Cycling Cells	Mki67+, Top2a+
	Erythroid Cells	Hbb-bt+

ontology (GO) terms were tested for over-representation in those genes that were defined as differentially upregulated ($\text{Log2FC} > 1.5$, $P < 0.05$) in each cell type. General GO terms were identified that were enriched across both adjuvants, along with GO terms associated with only one adjuvant [Fig 5.8].

In both LMQ- and SQ-immunised mice, and across neutrophils and monocytes, the transcriptional signatures of inflammatory response (GO:0006954) and cellular response to cytokine stimulus (GO:0071345) were significantly overrepresented [Fig 5.10]. There was also an overlap in the specific genes that underly the inflammatory response GO term in monocytes that are differentially expressed for both LMQ and SQ. Genes such as Ccr1, Ccl2,

Tlr4, Cxcl2 and Ccl24 were upregulated under both conditions [Fig 5.9B]. However, there are certain immunisation-specific genes that were not shared, including Il6, Il1a and Cxcl10 which were upregulated only by LMQ.

Upregulation of cellular migration was detected through the overrepresentation of positive regulation of cell migration genes (GO:0030335) and neutrophil migration genes (GO:1990266) for both LMQ- and SQ-immunised mice. This corresponds with the increased cell count of Lin⁻ cells, neutrophils and monocytes detected in the iLN after immunisation by flow cytometry and cellular abundance in scRNA-seq [Figs 3.3, 3.5 and 5.8D].

Monocytes and neutrophils showed a pattern of anti-viral and interferon response GO term enrichment in response to LMQ, exemplified by enrichment in the defence response to virus (GO:0051607), cellular response to type II interferon (GO:0071346) and cellular response to type I interferon (GO:0071357) terms [Fig 5.10]. Additionally, there was an overrepresentation in the transcription of genes involved in the cellular response to tumour necrosis factor (GO:0071356) and the apoptotic process (GO:006915). Monocytes in SQ-immunised mice were overrepresented with genes associated with Phagocytosis (GO:0006909), Endocytosis (GO:0006897) and response to endoplasmic reticulum stress (GO:0034976) that were not seen after LMQ immunisation.

5.2.5 Interferon Signalling underlies transcriptional programmes in the lymph node in response to LMQ immunisation.

There was a significant overrepresentation of GO terms associated with the interferon response (GO: 0071346 and GO:0071357) in neutrophils and mono-

cytes from LMQ-treated lymph nodes, which were not observed in SQ treated populations [Fig 5.10]. Therefore, the interferon signature in LN cells from LMQ-immunised mice were explored in more detail. Overrepresentation analysis showed that the cellular response to type II interferon (GO:0071346) and cellular response to type I interferon (GO:0071357) terms were found across a variety of cell types [Fig 5.11A]. Interestingly, while many cell types showed an overrepresentation of both terms, some cell types were enriched for either Type I or Type II interferon signatures. The Aire⁺ APC (Aire⁺, H2-DMb2⁺, Rorc⁺) and B-Cell-1 (Ms4a1⁺, Cd79a⁺) clusters were significantly enriched for the type I interferon response only, while fibroblasts (Ccl19⁺, Coll1a1⁺) showed only a significant Type II response.

A variety of type II interferon-stimulated genes are expressed across multiple cell types [Fig 5.11B-D]. These include genes from the interferon-stimulated genes (ISG) group such as *Isg15* and *Isg20*, and Interferon regulatory factors (IRFs) including *Irf1*, *Irf2*, *Irf7*. Interferon-induced transcription factors such as *Stat1* and *Stat2* were also found to be differentially expressed. To identify the potential sources of type II interferon, upregulated *Ifng* expression across cell types was examined. NK cells and ILC1 cells were found to be the cell types that differentially upregulated their expression of *Ifng*, and are therefore suspected to be the sources of the Type II interferon signalling [Fig 5.12A, B].

Cell-cell communication analysis was performed with NicheNet to examine signalling from ILC1s and NK cells to all other cells in the dataset. NicheNet predicts signalling ligands and their downstream effects from single cell data using prior knowledge of receptor ligand interactions and intracellular signalling pathways, and can account for different treatment conditions (102). NicheNet analysis confirmed the presence of *Ifng* signalling in the dataset,

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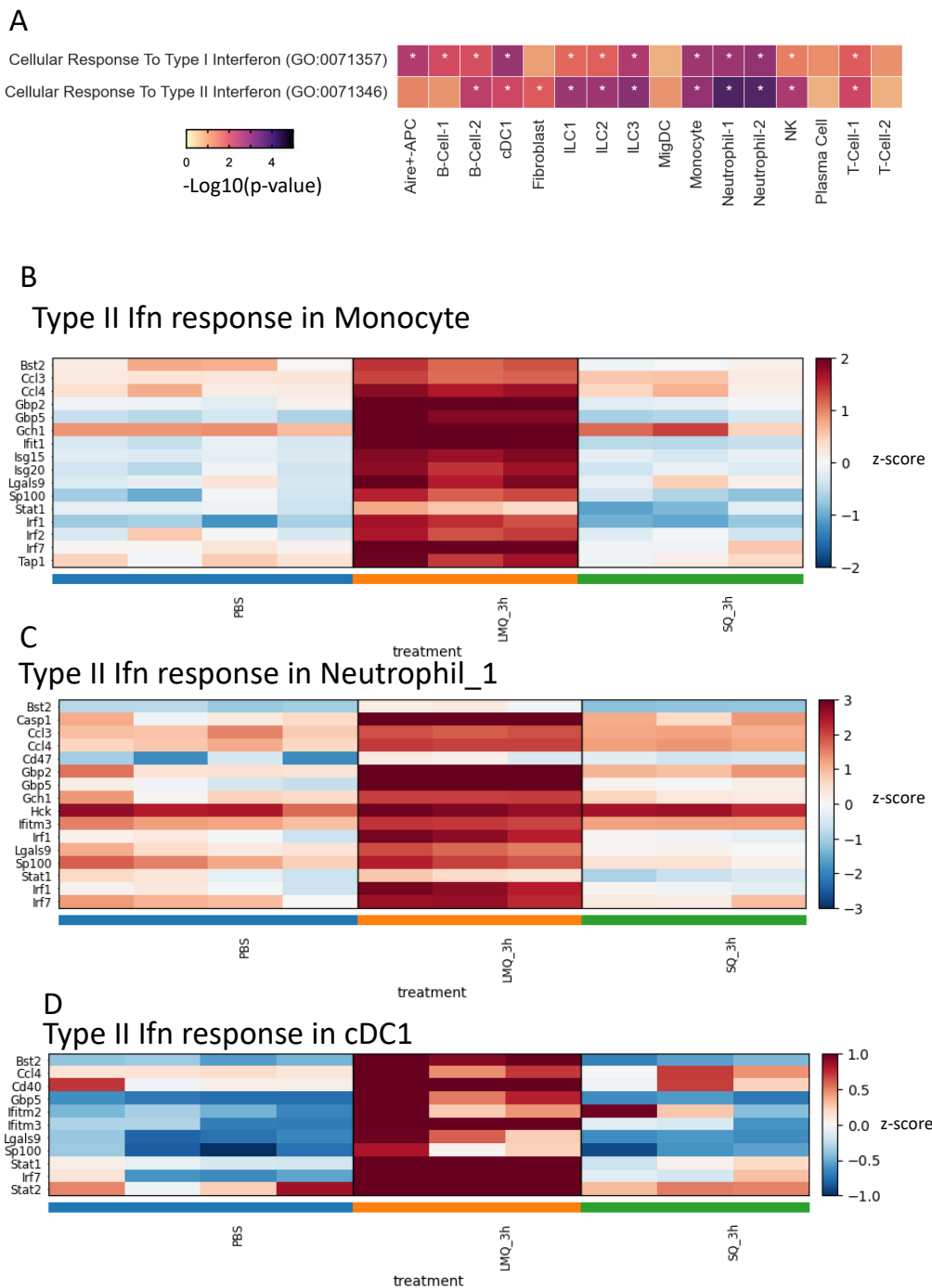


Figure 5.11: LMQ immunised mice display transcriptional signatures of interferon signaling across multiple cell types. Continued next page:

Figure 5.11: Continued from previous page: a) A heatmap showing over-representation of the cellular response to Type I Interferon (GO:0071357) and Cellular response to Type II interferon (GO:0071346) GO terms across all cell subclusters (* $p < 0.01$) comparing LMQ treated samples to PBS, (b-d) Heatmaps showing the sample level z-scored gene expression for selected DEGs from the cellular response to Type II interferon (GO:0071346) term across selected myeloid cells: monocytes, neutrophils and cDC1s.

along with other key signalling ligands such as Il1B, Tnf and Il6 [Fig5.12C].

5.2.6 Neutrophils are identified as a putative source of inflammasome signalling in the iLN, and cellular sources of known cytokines are identified.

From a previous publication it is known that cytokines are secreted following immunisation with LMQ and SQ, and transcription of these cytokines using bulk RNA-seq was confirmed [Fig 4.5, 4.6] (18). In addition, the inflammasome appeared to be involved in the mechanism of action of both adjuvants, as inhibition of the inflammasome with the NLRP3 inhibitor MCC950 resulted in a reduction of cytokine secretion in the muscle and blood sera. In the iLN, LMQ and SQ both resulted in increased Il6 expression in Fibroblasts [Fig 5.13A]. Il6 expression was also increased in monocytes and the neutrophil-1 population after immunisation with LMQ [Fig 5.13A, B]. Csf3 was also upregulated in fibroblasts after immunisation with LMQ, suggesting stromal cells as the source of G-CSF after immunisation [Fig 5.13C].

Inflammasome activation cannot be definitively confirmed using transcriptomic methods, as much of the processes involved are post-translational. However, it is known that part of inflammasome activation, signal 1, involves an increased expression of Il1B and Nlrp3 that licences NLRP3 in-

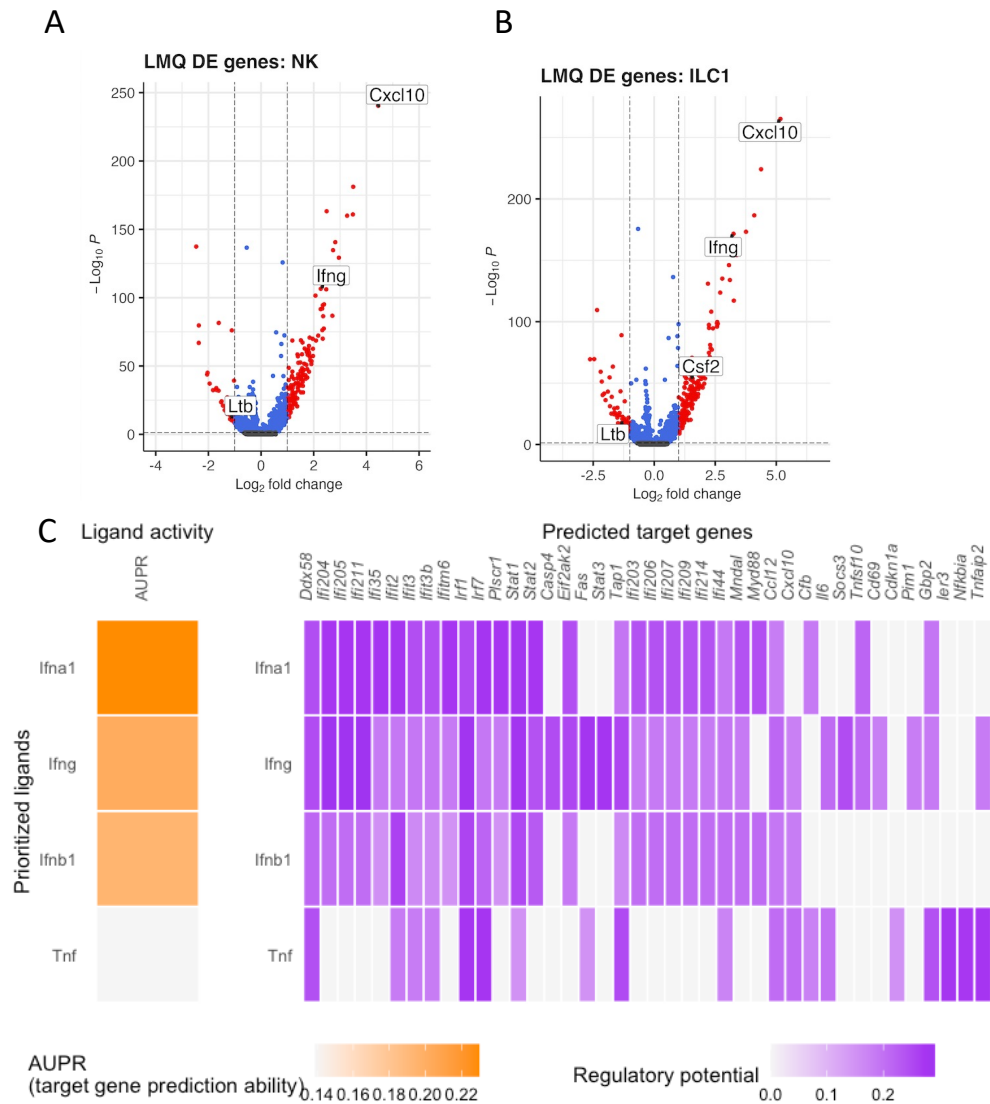


Figure 5.12: Ifng expression by NK cells and ILC1s underlies the interferon signalling programmes observed after LMQ immunisation. (A, B) Volcano plots showing the expression of genes in NK cells and ILC1s displaying significant upregulation of Ifng in LMQ-treated samples. (C) Results of NicheNet cell-cell communication analysis, a heatmap showing the predicted LMQ-induced signaling ligand activity from the dataset and a heatmap showing the regulatory effect these ligands have on downstream genes in cells.

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

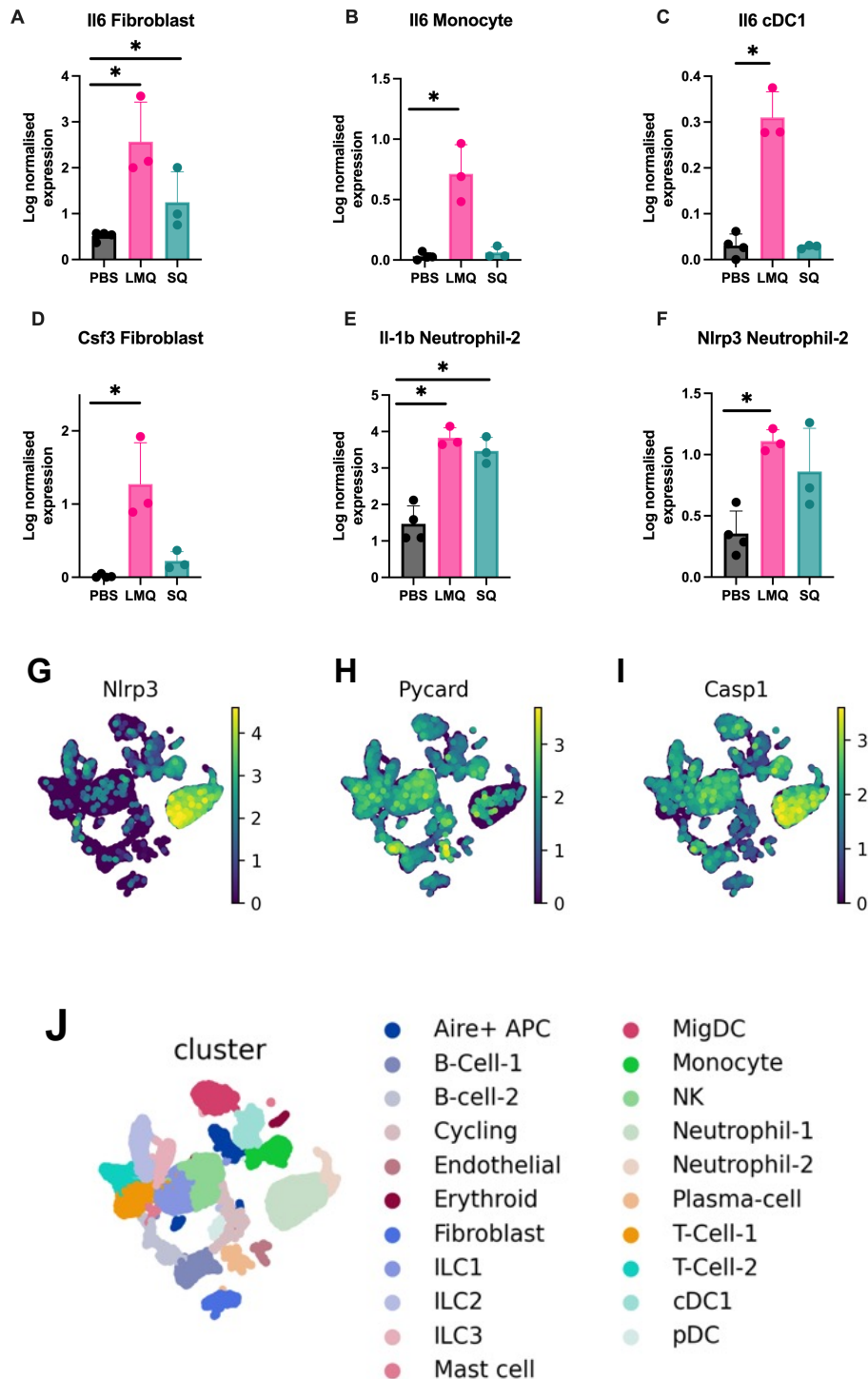


Figure 5.13: scRNA seq identifies cellular sources of inflammation and Signal 1 of inflammasome activation. Continued next page:

Figure 5.13: Continued from previous page: The log normalised expression for each sample is shown for (A) Il6 expression in fibroblasts, (B) Il6 expression in fibroblasts, (C) Il6 expression in cDC1s, (D) Csf3 expression in fibroblasts, (E) Il1B and (F) Nlrp3 expression in the activated Neutrophil-2 population. Plots are shown cell types only where a significant gene expression is shown for Il6, Csf3 or Il1B. Significance is determined by transcriptome-wide testing with MAST (* $p < 0.05$ and $\text{Log2FC} > 1.5$). Log normalised expression of (G) Nlrp3, (H) Pycard (also known as ASC), (I) and Casp1 displayed on a UMAP projection. (J) indicates the cell cluster identity also displayed on a UMAP projection. This displays NLRP3 inflammasome components expressed in Neutrophils consistent with the upregulation of Il1B.

flammasome activation (123,124). Il1B was upregulated in the neutrophil-2 population after LMQ or SQ immunisation, but not other myeloid cells such as monocytes, cDC1s or the neutrophil-1 population [Fig 5.13E]. Nlrp3 was also upregulated in this population, but this was only statistically significant after immunisation with LMQ [Fig 5.13F]. In addition, the neutrophil-2 population expressed multiple components of the NLRP3 inflammasome including Nlrp3, Pycard (ASC) and Casp 1 [Fig 5.13G-I]. This provides further evidence that these neutrophils express the correct components for NLRP3 inflammasome activation.

5.2.7 An integrated human single-cell atlas reveals differences in the expression of key adjuvant sensing receptors.

Given the transcriptional inflammation induced by immunisation with LMQ or SQ in mice, the overlap of mouse and human PRR expression was explored. By exploring the expression of key receptors for LMQ and SQ, such as Tlr4 and Nlrp3 inflammasome components, the potential applicability of the murine results to humans can be assessed. At the time the analysis was

performed, there was no one human lymph node scRNA-seq atlas that captured all of the cell types in the lymph node, due to the overrepresentation of B and T cells that must be removed by depletion. Therefore, an integrated single-cell atlas from 5 different publications was generated (140, 142, 150-152). The cell types were then reclassified from this integrated atlas using canonical cell markers [Figs 5.14-5.17]. After integration and cell quality control, this human lymph node atlas contained a total of 102,199 cells with 29 identified cell types. A murine dataset which was depleted of B and T cells was used as the murine comparator to the human data [Covid statement] (141).

The integration of human single-cell data across various studies was carried out using batch removal tools such as BBKNN and Harmony. To measure the quality of integration, the Local Inverse Simpsons Index (LISI) was used as a metric [Fig 5.18]. Based on the median LISI scores, the data could be ranked in order of the mixing of samples from lowest to highest: without integration (median LISI = 2.18), BBKNN (median LISI = 2.91) and Harmony (median LISI = 4.78). Due to increased LISI scores the Harmony tool was used for integration. Tlr4 and its co-receptors CD14 and Ly6g (MD2) are critical for recognising TLR4 ligand adjuvants such as 3D-6-acyl-PHAD, the ligand found in LMQ. In human lymph nodes, Tlr4 expression was primarily localised to the innate immune compartment with expression found in monocytes macrophages and neutrophils [Fig 5.19]. While in the murine lymph node, Tlr4 expression was observed in both stromal cells such as LECs and Fibroblasts as well as in neutrophils. In both humans and mice, components of the Nlrp3 inflammasome were only found expressed in innate immune cells [Fig 5.20]. Humans showed Nlrp3 expression in DCs and monocytes but had a relatively lower level of expression in neutrophils. While in mice Nlrp3 was

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

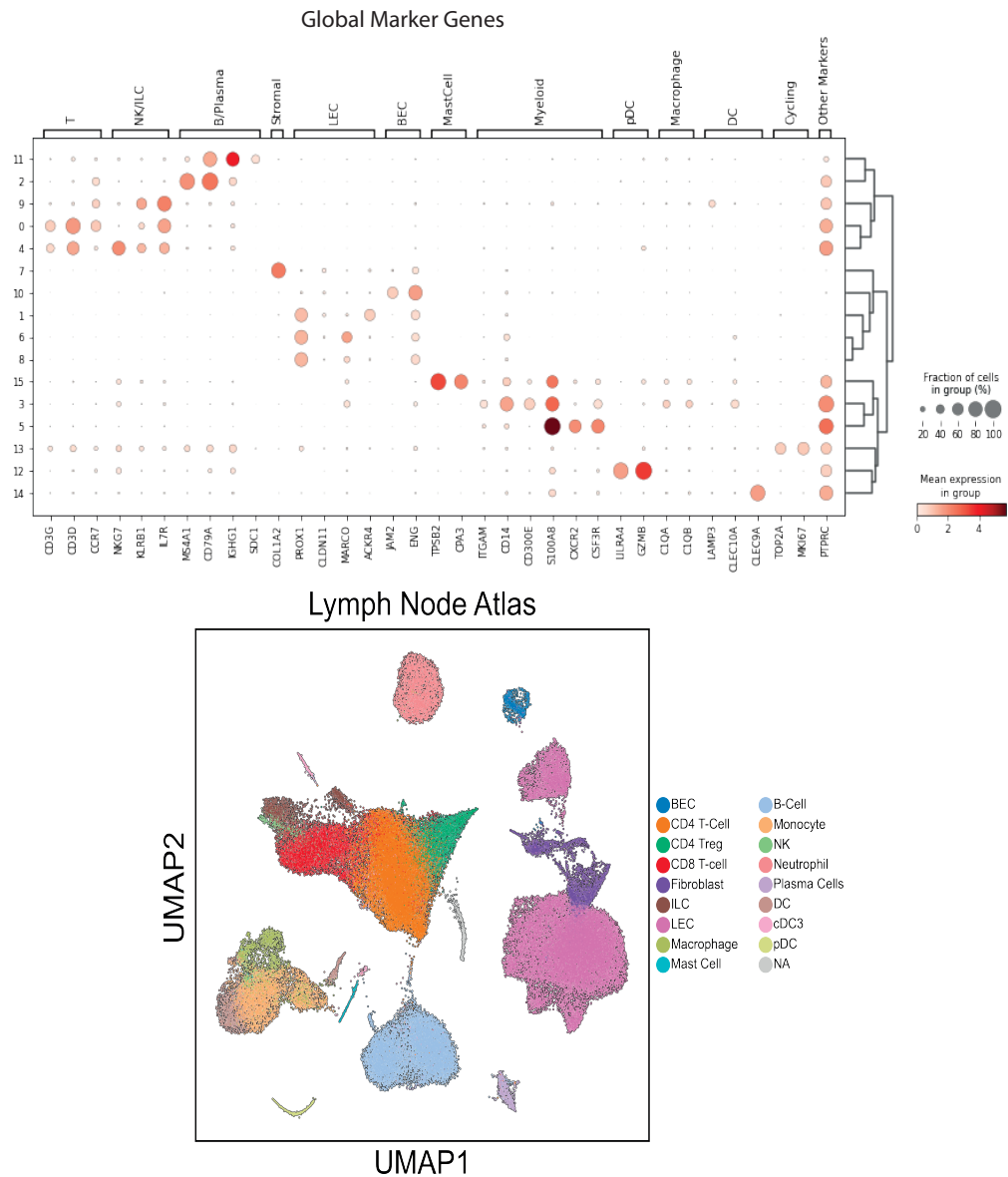


Figure 5.14: Marker gene expression for cells in the human lymph node atlas. A dotplot of the normalised log scaled gene expression of marker genes for cell clusters. And a graph the cell clusters shown on a UMAP projection.

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

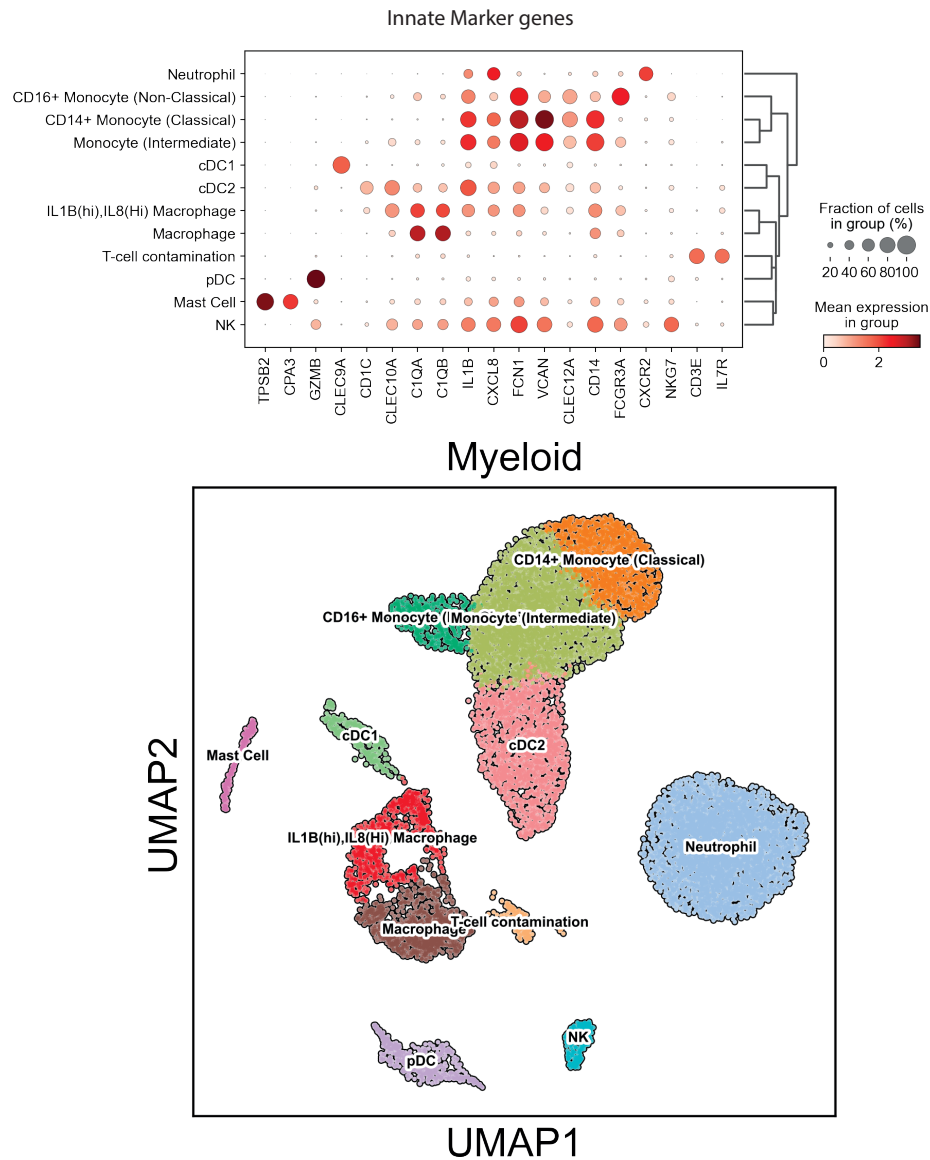


Figure 5.15: Marker gene expression for cells in the myeloid compartment of the human lymph node atlas. A dotplot of the normalised log scaled gene expression of marker genes for cell clusters. And a graph the cell clusters shown on a UMAP projection.

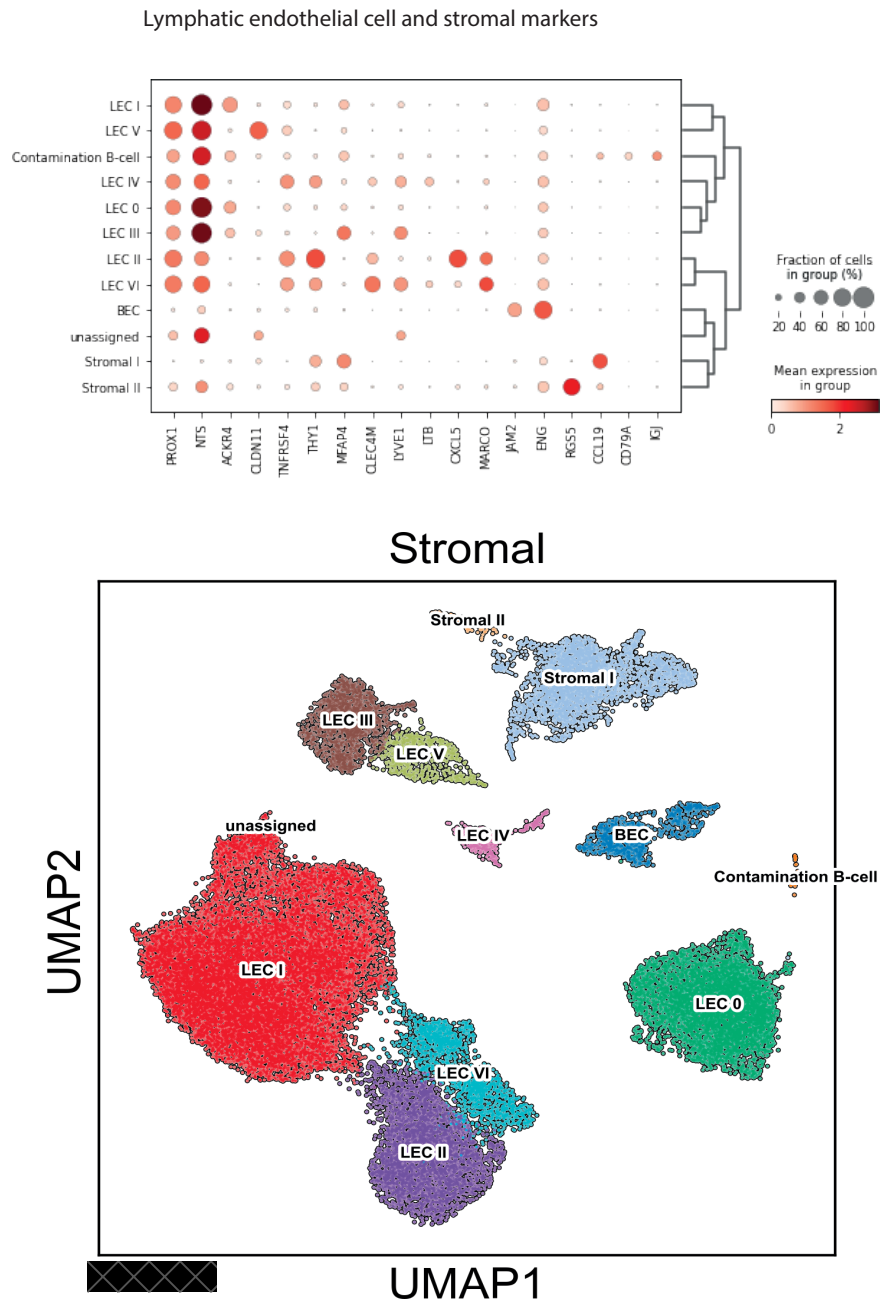


Figure 5.16: Marker gene expression for cells in the stromal compartment of the human lymph node atlas. A dotplot of the normalised log scaled gene expression of marker genes for cell clusters. And a graph the cell clusters shown on a UMAP projection.

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

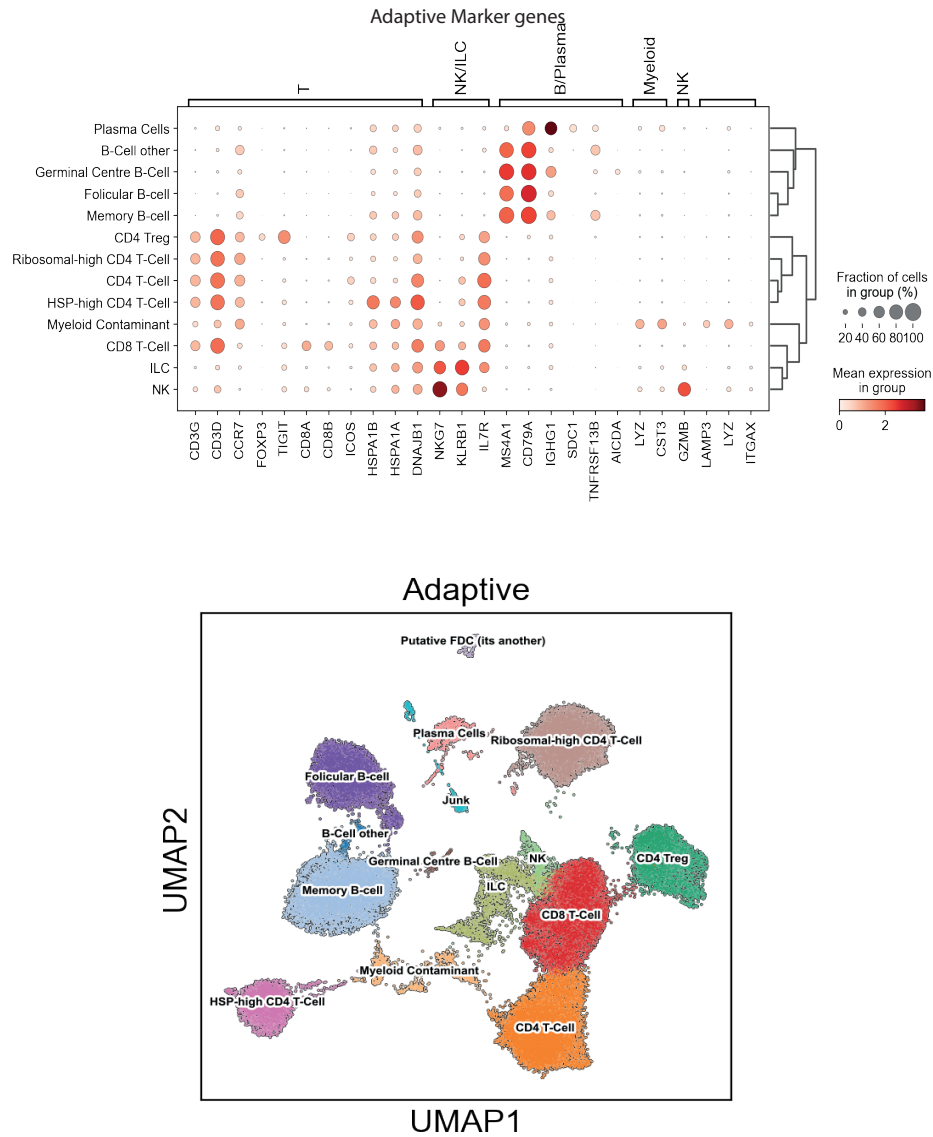


Figure 5.17: Marker gene expression for cells in the adaptive immune compartment of the human lymph node atlas. A dotplot of the normalised log scaled gene expression of marker genes for cell clusters. And a graph the cell clusters shown on a UMAP projection.

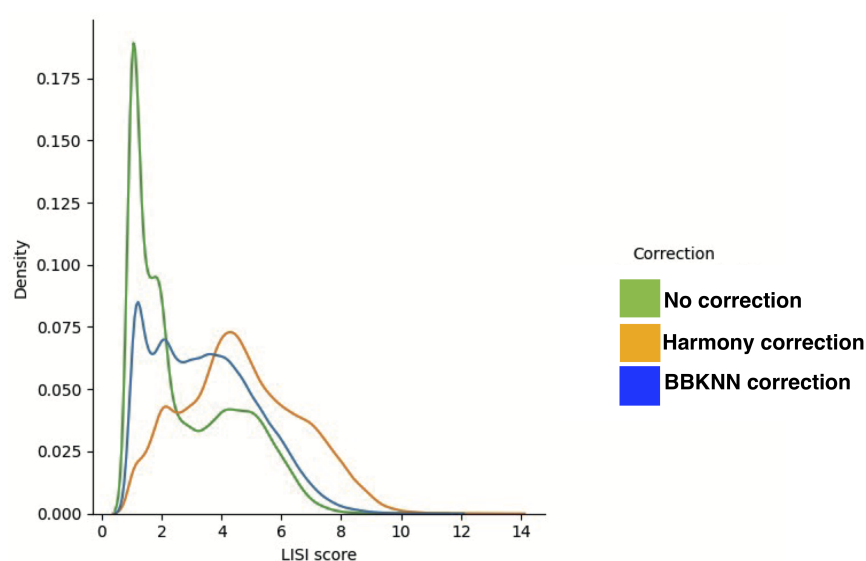


Figure 5.18: Harmony correction removes batch effects from different literature sources of human lymph node datasets. LSI scores for the human lymph node atlas showing the degree of mixing of data sets from different publication after no correction, BBKNN and Harmony batch correction methods.

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

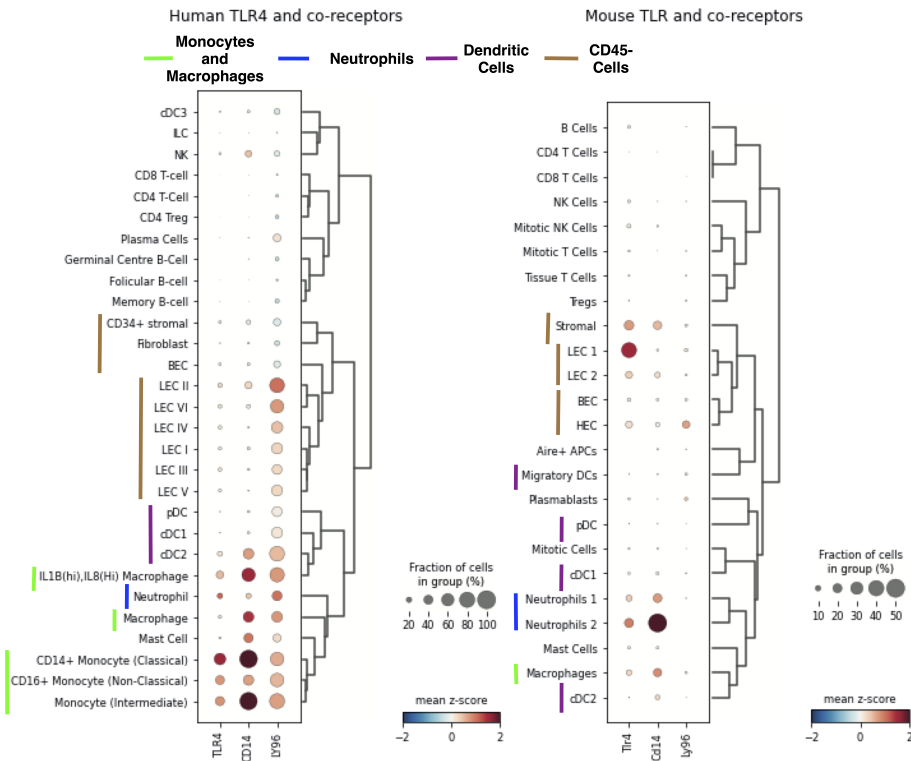


Figure 5.19: Murine, but not human, CD45- cells express Tlr4. Dot plots of z-score scaled expression of selected Tlr4 receptor complex genes in each cell cluster. Related cell types are indicated with colors as shown in the legend.

only significantly expressed in Neutrophils, consistent with the Il1B expression found after LMQ immunisation [Fig 5.13].

Myeloid cells both take up high levels of LMQ and SQ, and respond transcriptionally to both adjuvants and express pro-inflammatory cytokines. This suggests they play a key role in the response to vaccine adjuvants, therefore the similarity between these cells on a more global scale than simply individual receptor expression was compared. To compare cells between mice and humans, only one-to-one orthologues were retained and the Pearson correlation between the expression of the top 100 marker genes for each cell type

5: Single cell mechanisms of liposomal and squalene emulsion adjuvants

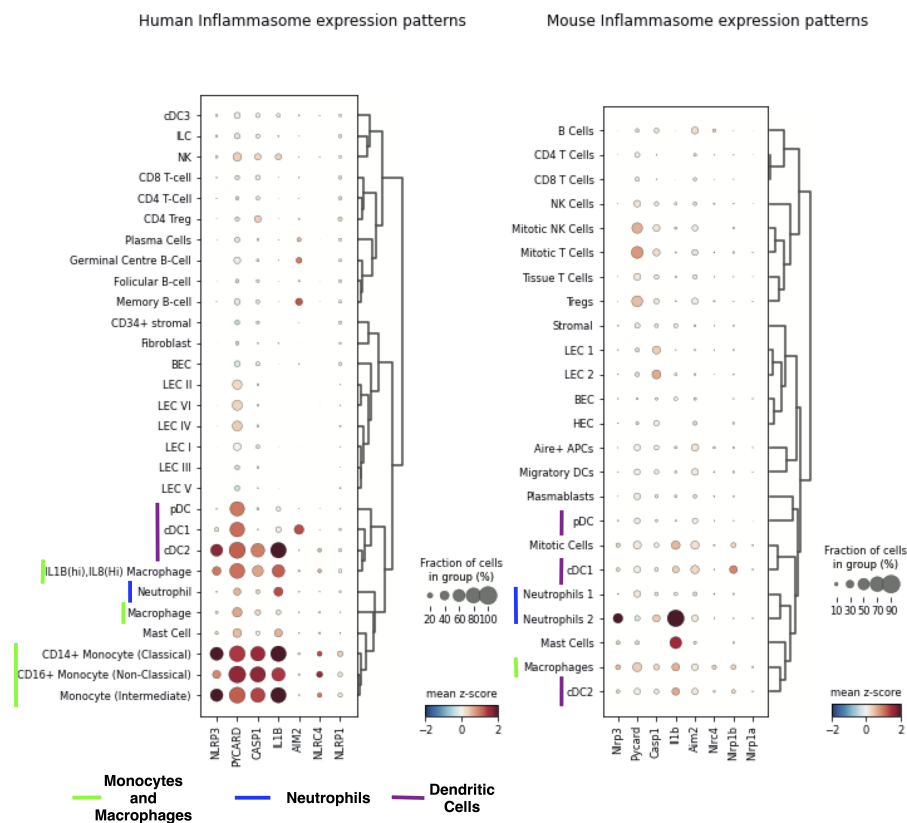


Figure 5.20: Nlrp3 inflammasome expression is restricted to murine and human myeloid cells. Dot plots of z-score scaled expression of selected Nlrp3 inflammasome genes in each cell cluster. Related cell types are indicated with colors as shown in the legend.

was calculated [Methods]. This revealed a generally high level of correlation of gene expression between the murine and human myeloid cells [Fig 5.21]. However, murine cDC2s did not correlate well with any human cell type including human cDC2s [Fig 5.21]. This lack of correlation suggests that there could be phenotypic differences between murine and human cDC2s. Overall, the correlation analysis showed a high degree of transcriptomic similarity between murine and human myeloid cells and supports the validity of the adjuvant receptor comparison.

5.3 Discussion.

To identify the optimal scRNA-seq platform for analysing adjuvant immune responses in murine lymph nodes, a head-to-head comparison of BD Rhapsody and 10X Chromium was performed. Contrary to expectations, based on previously reported studies, there was minimal difference in the quality of granulocyte transcriptomes captured using either 10X Chromium or BD Rhapsody (144). BD Rhapsody captures more RNA and genes and had evidence of an increased number of Eosinophils and Basophils being detected. However, the magnitude of the difference was smaller than in previous studies by an order of magnitude (144). Multiple factors could influence this difference including organism-specific effects, tissue-specific effects and changes to the Cellranger software used for 10X Chromium. Previous studies had focused on human cells, whereas ours focused on fresh mouse tissues which may impact cell capture, viability and RNA content. Additionally, while previous studies used blood and bone marrow cells, our study focused on the lymph node. Finally, Cellrangers v7.0 update has claimed to increase the quality of granulocyte transcriptomes by incorporating intron RNA information by

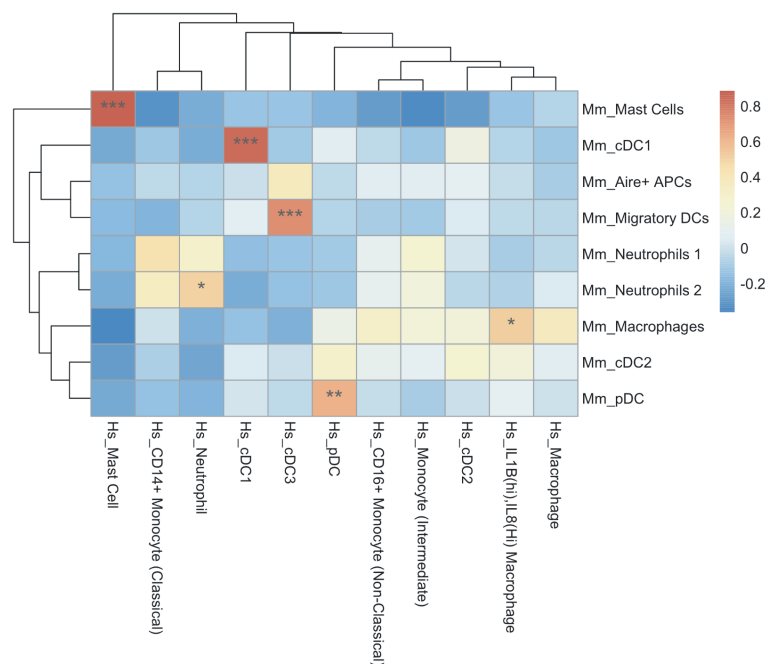


Figure 5.21: Human and mouse myeloid cells show highly correlated patterns of gene expression in the steady state. A heatmap comparing human (x-axis) and mouse (y-axis) myeloid cells based on the correlation marker genes expression. Statistically significant correlations based on the false discovery rate (FDR) are indicated by * FDR < 0.05, ** FDR < 0.005, *** FDR < 0.0005. Permutation testing with benjamini hochberg FDR correction is used to assess statistical significance of correlation.

default. This new update may have reduced the magnitude of the previously reported differences between BD Rhapsody and 10X Chromium. In conclusion, due to the fast moving developments in the field of scRNA-seq technologies, performing small in-house comparisons provides lots of utility. Relying only on previous reports, especially where there are differences in tissue or organism of interest, can lead to poor choices of sequencing platform.

This sequencing experiment characterised similar cell types as found in other lymph node single-cell atlases (142,141). Lymph node resident macrophages were a population of interest for this chapter. However, this dataset lacked a clearly defined macrophage population, which can be identified in other datasets by the expression of C1 complex genes. The differential abundance analysis confirmed the previous observations of monocyte and neutrophil influx [Fig 3.5].

Putative sources of cytokines known to be secreted *in vivo* after immunisation with LMQ or SQ were identified. Sources for Il-6, Csf3 and Il-1 were identified, however other known secreted cytokines were not differentially expressed in this dataset. Tnf, Cxcl1 and Il18 were not identified as differentially expressed in this dataset, despite being upregulated in bulk RNA sequencing of the lymph node. Bulk RNA sequencing gene expression changes can represent both upregulation of gene expression but also represent cellular migration increasing the number of cells that express a particular gene, a confounder that is removed in scRNA-seq (152). This same property applies to analysis of sera and muscle lysates, in that increased protein secretion could be driven by an increased quantity of cytokine secreting cells. Given there is significant influx into the lymph node after immunisation, increased cell migration could explain the absence of differential gene expression found on

the bulk level compared to the single cell level. In support of this, Tnf and Cxcl1 were expressed in Neutrophils and MigDCs in both the control and immunised lymph nodes, these cells are known highly migratory cell types in the lymph node and influx of these cell types may alter cytokine levels [Fig 5.8D] (154). Changes in Il-18 were not detected in any cell type for either adjuvant, the production of which by macrophages has been shown to induce Ifng responses in AS01 (20). A variety of factors could have influenced this, including that a clearly defined SCS macrophage population was not identified in the sequencing strategy. Additionally, active IL-18 is produced from pro-IL-18 by caspase cleavage of the unmodified protein (130). ScRNA-seq is limited in its ability to detect changes in genes that are largely or partially driven by post-transcriptional mechanisms such as this.

Ifng signalling is essential for the development of antigen-specific T-cells, and cell infiltration after immunisation with AS01 (20). Immunisation with AS01 results in Ifng expression by NK cells and CD8⁺ T-Cells, through an IL18 and IL12-driven mechanism (20). Significant upregulation of Ifng expression was found 3 hours after immunisation with LMQ in NK cells and ILC1s, but not in the CD8⁺ T-Cell-2 population. Type II interferon response programmes were induced across a variety of cells in the lymph node. Importantly this strong type II interferon signalling was not observed after SQ immunisation.

SQ showed a more restricted pattern of transcriptional changes mostly focused on migratory cells such as monocytes and neutrophils. While overrepresentation analysis showed upregulation of inflammation in SQ that overlaps with those found in LMQ, there was no evidence of interferon response programmes being activated. This is consistent with recently published work that shows that both LMQ and SQ are equally protective against malaria challenge in mice, but operate through different pathways and Th biases (18).

The squalene emulsion-based adjuvant AS03 induces the endoplasmic reticulum stress response through IRE1 α in myeloid cells, which contributes to cytokine production and adaptive immune responses. (66). Evidence of ER stress responses in monocytes after SQ immunisation was found, which was consistent with previous research on AS03 (65). Interestingly, SQ-stimulated monocytes also show enhanced phagocytic and endocytic activation. Increased antigen processing and presentation by APCs is a known mechanism of adjuvant function, and enhanced phagocytosis and endocytosis may contribute to this property (155).

Exploration of the cross-species patterns of adjuvant recognising receptors revealed differences between humans and mice. Tlr4 was restricted to myeloid cells in humans but was also expressed on stromal cells in mice [Fig 5.19]. Murine fibroblasts express Csf3 and Il-6 response to immunisation, both genes that are induced by NF- κ B activation potentially through Tlr4 signalling [Fig 5.13] (18). Lack of Tlr4 expression in human fibroblasts may impact onto downstream immune responses to vaccination. Given these differences were in stromal cells, care must be taken in human models of vaccination to consider the role of CD45- cells. Many human models, for example PBMCs and cultured cell models cannot take into account stromal cell contribution to inflammation.

In this chapter, a cellular and transcriptional map of the murine response to liposomal and squalene emulsion adjuvants was generated. It was identified that both adjuvants share a key underlying pattern of inflammation in monocytes and neutrophils, however, LMQ showed signals of inflammation across a wider range of cells in the lymph node. Additionally, it was found that interferon signalling on the transcriptional level is seen only after immunisation with LMQ but not SQ, despite their equal level of protection

against malaria.

5.3.1 Caveats and open questions

One major caveat of this chapter it is that the direct and indirect effects of adjuvant immunisation cannot be distinguished. Given a single time point the direct effect of adjuvants on a cell cannot be distinguished from cytokine mediated signalling between cells. This caveat could be addressed with a large scRNAseq experiment containing multiple time points and by adapting the sorting strategy to separate cells that are positive or negative for fluorescent adjuvant. By sorting cells based on adjuvant signal, they could subsequently be bar-coded with hashing antibodies and the transcriptional profiles of cells that are positive for adjuvant could be compared to those that are negative. The inclusion of multiple time points could enable the analysis to track cytokine signaling between cells over time, which may further elucidate the direct effect of adjuvants from downstream signaling events. In addition it would also provide greater detail on the kinetics of the transcriptional signals of inflammation in the lymph node.

6.1 Summary of findings.

This thesis seeks to characterise the cellular distribution and transcriptional innate pathways induced post immunisation with liposomal or squalene emulsion adjuvants. The following findings were established in the thesis:

- LMQ and SQ induce the influx of immune cells into the muscle site of injection and the draining iLN. Transcriptomic analysis of both sites revealed the upregulation of numerous chemokines that may regulate this process.
- Squalene emulsion and liposomal adjuvants show different patterns of drainage and cell uptake in the lymph node. Using fluorescent adjuvants, LMQ showed high levels of fluorescence in the lymph node sinus and uptake by resident lymph node cells that was not detected with SQ.
- LMQ and SQ do not induce double-stranded DNA breaks, a signal of cell death, in the muscle after at 6 hours post immunisation.
- SQ shows a highly restricted pattern of gene expression changes in the draining lymph node, with the majority of changes occurring in monocytes and neutrophils. LMQ, however, displays a broad upregulation of genes across multiple cell types consistent with its broader range of uptake in cells.
- Increased expression of cytokines, consistent with signal 1 of the inflammasome, is found in neutrophils but not cDC1s or monocytes in

the draining lymph node after immunisation with LMQ or SQ.

The following sections will discuss and interpret these results in more detail, and suggest future work that could be done to explore the themes further [Fig 6.1].

6.2 Differential uptake of squalene emulsion and liposomal adjuvants by cells in the muscle and draining lymph node.

This thesis has produced a detailed map of the cellular localisation of liposomal (LMQ) and squalene emulsion based (SQ) adjuvants after immunisation. Previous studies had primarily used immunofluorescence imaging to localise adjuvants, which understates the diversity of cell types that take up adjuvants (83,85). It was found that LMQ has a higher capacity to be taken up by resident lymph node cells such as FRCs, LECs and SCS macrophages after immunisation [Fig 3.6]. In addition, LMQ showed higher levels of uptake in DCs, neutrophils and monocytes in both the lymph node and muscle [Figs 3.4, 3.5]. There is no known other report of adjuvant uptake by the stromal cells in the draining lymph nodes, and this thesis provides the first evidence for this. Bone marrow chimaera models suggest that TLR4 signalling in stromal cells is dispensable for IgG titres and antigen-specific CD8⁺ T cell responses to immunisation with AS01 (32). However, this does not rule out a role for stromal cells in adaptive immune responses after immunisation through alternative signalling pathways or different effector mechanisms.

Conventionally it is considered that the molecular weight limit for passive entry into the lymph node parenchyma is approximately 70 kD, with the

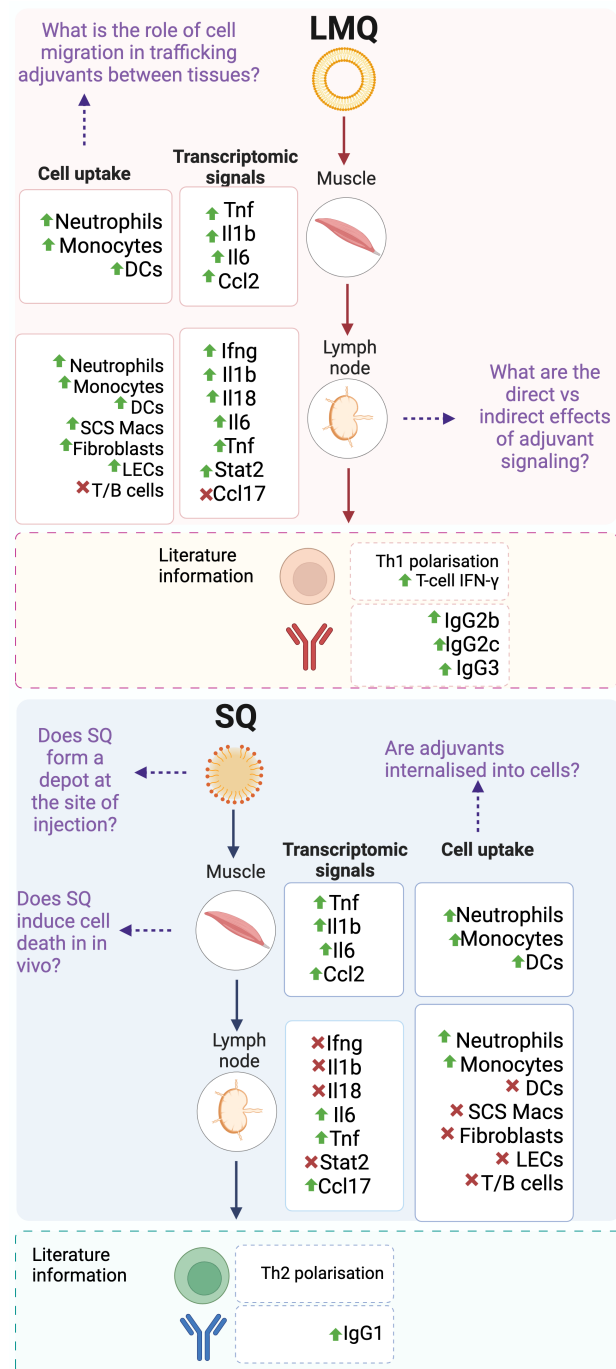


Figure 6.1: Graphical summary

median human protein size being 42kD (156). Substances above 70kD are generally restricted to the subcapsular sinus region, and cannot access stromal cells such as FRCs in the lymph node parenchyma. Adjuvants such as LMQ and SQ are significantly larger than this limit, suggesting active methods of transport of LMQ or a breakdown of the barrier to entry in the lymph node occur after immunisation. However, the path of adjuvant entry into fibroblasts located in the lymph node parenchyma remains unknown. Targeting of adjuvants to the lymph node has been previously shown to enhance immune responses in both tumour and infection models (157,156). Therefore, increased lymph node targeting by LMQ represents a potential mechanism underlying its strong level of protection.

SQ uptake was only significant within neutrophils and monocytes in the lymph node, and not stromal cells or resident SCS macrophages. This leads to the hypothesis that SQ must be trafficked to the lymph node by these cells, and is not taken up directly by resident cells in this tissue. Neutrophils and monocytes are known to have the capacity to undergo lymphatic migration from sites of inflammation to the lymph node (159,160). In the context of vaccination, they are also known to be able to traffic antigens from peripheral tissues to the lymph node, but their involvement in trafficking antigens has not been previously studied (61,161). For neutrophils lymphatic trafficking has been shown to be ICAM-1 and VCAM-1 dependant (160,162). In addition, anti CD11b antibodies have been previously used to inhibit transendothelial trafficking of CD11b+ cells such as neutrophils (163). To further analyse the role of monocytes and neutrophils in trafficking antigen to the lymph node an antibody blocking experiment could be used, targeting VCAM-1, ICAM-1 or CD11b, to assess the impact on adjuvant uptake in the lymph node. An experiment was performed using i.v. anti-ICAM-1

and anti-VCAM-1 but this approach was not well tolerated by Balb/c mice, therefore alternative delivery routes and dosing schedules should be tested [data not shown].

6.3 Cell death *in vivo*.

Cell death has previously shown an important role in the mechanisms of action of both squalene and liposomal vaccine adjuvants (10). Previous research on LMQ and SQ has shown that both adjuvants are able to induce cell death *in vitro* in BMDM (18). In addition, immunisation with the squalene emulsion adjuvant MF59 induces cell death in both the muscle and lymph node (60,61). QS-21 formulated in liposomes has also been shown to induce cell death in the lymph node macrophages (87). Transcriptional signatures of cell death were found in both the muscle and lymph nodes after immunisation with LMQ and SQ [Fig 4.10]. However, assessing cell death using the TUNEL assay in the muscle revealed no apparent cell death at 6 hours [Fig 4.12].

Programmed cell death is influenced by multiple factors *in vivo* that are not captured by *in vitro* assays. *in vitro* assays contain only a single cell type, and cell survival and proliferation can be enhanced by factors produced by hematopoietic and stromal cells (164,165). It is possible that the lack of cell death observed here is due to pro-survival factors produced *in vivo* but not *in vitro*, but this has not been established. In addition, clearance of dead and dying cells occurs *in vivo*, and is enhanced by infiltrating neutrophils as is the case after immunisation; this could suppress the detection of cell death *in vivo* (166,167).

While the TUNEL assay does not show any evidence of cell death *in vivo*, It

also does not rule out the involvement of DAMPs in the mechanism of action of LMQ and SQ as DAMPs can be released from cells without requiring cell death and DNA fragmentation (55). In addition, the negative TUNEL assay does not rule out inflammasome activation *in vivo* because myeloid cells, including DCs and neutrophils, can activate the inflammasome and release active IL-1B without pyroptotic cell death (168,169). Further research could analyse the secretion of major DAMPs such as HMGB1 and ATP in the supernatant of immunised muscle cells and lymph nodes to test for DAMP upregulation independent of cell death.

6.4 Cellular sources of inflammation in adjuvant draining lymph nodes.

This thesis has also explored the cellular sources of inflammation in the iLN (the draining LN) after immunisation with LMQ and SQ. In chapter 4, programmes of gene expression induced after immunisation with these adjuvants were identified. After immunisation with SQ gene expression changes were largely restricted to monocytes and neutrophils, which were also the only cell types in the iLN that showed significant SQ uptake [Figs 5.8A, 3.5]. LMQ, however, had a broad pattern of gene expression changes over the majority of cell types in the lymph node and this is partially driven by interferon response programmes in these cells [Figs 5.8A, 5.11A-D].

LMQ induced a stronger type II interferon response programme in comparison to SQ in both the muscle and lymph node. It was also identified using scNRA-seq that the sources of type II interferon expression after immunisation with LMQ, but not SQ, were NK cells and ILC1s. *Ifng* expression

was induced in the absence of antigen, and therefore can be driven by the action of LMQ alone. For AS01 it has been previously shown that NK cells and CD8⁺ T cells produce Ifng 6 hours after immunisation with antigen and adjuvant (20). Ifng production by ILC1s has been observed in the lymph node after immunisation with subunit vaccines and viral vectors, and in the muscle during viral infection (165-167).

It is known from protein expression data that IL-1B is secreted after immunisation by both LMQ and SQ (18). However, the cellular sources of IL-1B were not understood. Triggering of signal 1 of the inflammasome, which is required for inflammasome activation, results in the expression of IL-1B and other inflammasome components such as Nlrp3 (122,123). It was found in the lymph node that after immunisation with LMQ and SQ IL-1B and Nlrp3 were upregulated in neutrophils-2 cells, but not cDC1s or monocytes, which is strongly suggestive of inflammasome signalling in neutrophils given the known presence of active IL-1B secretion [Fig 5.13E, F] (18). It appears that neutrophils are the sources of early IL-1B after immunisation, but this remains to be confirmed on the protein level. An ASC reporter mouse in combination with imaging flow cytometry or immunohistochemistry with neutrophil markers could be used to confirm activation of the inflammasomes in neutrophils (173).

6.5 Mechanistic studies as a resource to inform rational vaccine design.

Subunit vaccines are typically studied using both their antigen and adjuvant in combination. This thesis, however, was focused on the innate immune

mechanisms of action of adjuvants alone. The inclusion of different antigens using the same adjuvant can lead to alterations of the Th bias of the immune response, with lower affinity antigens leading to stronger Th2 bias, and altered cytokine production (174). Therefore, the absence of antigens in the immunisations used in this thesis removes this confounding factor on adjuvant mechanisms. However, each antigen-adjuvant combination must likely be validated individually, to confirm that mechanisms from adjuvant-only studies apply to that specific combination. Adjuvant only studies provide a knowledge base from which validating experiments can be designed and performed.

This thesis made significant use of transcriptomic methods such as RNA-seq and scRNA-seq. Both of these methods enable an unbiased transcriptome-wide screen of gene expression changes as a result of adjuvant immunisation, and are not restricted to analysing a small number of target genes or proteins. As a result of this, these datasets provide a valuable resource for exploring immune responses to adjuvants and hypothesis generation, and will be soon made publicly available. One limitation of the use of transcriptomic studies is the absence of protein-level information, and transcription does not necessarily correlate with protein abundance. Fortunately, ELISA data on selected proteins is available for LMQ and SQ from previous publications (18).

Expanding mechanistic studies to humans remains an important next step in investigating the mechanisms of action of protective vaccine adjuvants. It was shown in this thesis, that baseline human and mouse lymph nodes show differential distribution of PRRs that can detect TLR4 and saponin adjuvants [Figs 5.19, 5.20]. In mice, Tlr4 is expressed strongly on stromal cells such as FRCs and LECs, while Tlr4 is poorly expressed on similar cell types in humans. This emphasises the need for complementary mechanistic

studies in both mouse and human models.

While blood is the most used method to explore immunological readouts in humans, the use of PBMCs presents multiple limitations. PBMCs cannot provide information on localised immune responses in tissues, as was found in the muscle and iLN after immunisation with LMQ and SQ. The lack of stromal cells in PBMCs also prevents the investigation into the role of stromal cells in adjuvant responses, which is relevant due to the known receptor expression differences between humans and mice. In addition, rare cells such as antigen-specific T Cells can be hard to detect in the blood with single-cell transcriptomic methods that only collect thousands of cells for each sample.

Despite the challenges in studying the mechanisms of action of adjuvants in humans, multiple methods have been developed to address this. Skin blister models provide a minimally invasive method to study human immune responses *in vivo* (175). By forming a blister on the skin and separating the dermis from the epidermis, immune cell infiltration into the blister fluid can be analysed. This has been used for studying cytokine responses, immune cell infiltration kinetics and antigen recall responses after vaccination *in vivo* in humans (176â178). As skin blister models study dermal immune responses, they are limited in their ability to inform on the immune responses in vaccine-draining lymph nodes. Fine needle aspiration is another minimally invasive method to study human immune responses *in vivo* and can directly access human lymph nodes (179). Fine needle aspiration has been used to study adaptive immune responses to vaccination directly in the lymph node draining the vaccination site (179). However, this method can only capture a small number of cells, mostly B and T Cells, and cannot capture stromal cells (180).

Live ex vivo slices of lymph node tissue enable the analysis of immune responses in human lymph node tissue with a large degree of control. By using slices instead of disassociated tissue, lymph node slices retain the spatial organisation of cells found in the lymph node and include stromal cells (181). While this technique is in its infancy it shows strong promise to be used for detailed mechanistic studies of the responses to vaccine adjuvants. An alternative approach to using ex vivo tissue is through creating organoids. Tonsil organoids have been developed that spontaneously form lymphoid tissue like structures, including B Cell follicles (182). These organoids have been used to study adaptive immune responses over multiple days but could be adapted for also analysing early innate immune responses by human cells.

It is hoped that investigating the mechanisms of action of protective vaccine adjuvants will lead to a better understanding of how adjuvants drive protective immunity, and in the future aid in the rational design of vaccines. Failure of investigational vaccines to lead to protection is common, even for established developers and manufacturers of vaccines. A better understanding of vaccine adjuvant mechanisms may be able to reduce the risk of failure and lead to more efficacious vaccines.

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