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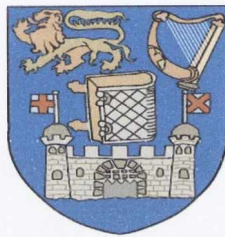
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**The Mechanism of Action
of
Particulate Vaccine Adjuvants**

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B.A (Mod)



A Thesis Submitted to
TRINITY COLLEGE DUBLIN

For the Degree
of
Doctor of Philosophy

Supervisor: Dr Ed C Lavelle

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2010



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Declaration of Authorship

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Acknowledgements

Honestly, I am not sure where to begin or how to fully express my thanks to everyone that has helped me throughout my PhD, especially over the last few months putting together this thesis.

Firstly, I would like to thank Ed. You have been a brilliant supervisor and your support and guidance throughout has been invaluable. Thank you for giving me the opportunity to be a part of this lab from the beginning. I definitely would not have made it this far without your help, thank you.

To everyone in the Lavelle lab; Edel, Anne, Eimear, Jim, Graham, Andres and Ewa. I couldn't have spent the last four years with a nicer group of people, you guys rock! I also want to thank everyone for all of the help during the numerous isolation days that we have had, I never could have done those kind of experiments without all of our help! Thank you all for your support throughout and some great nights out. I also want to thank you all for helping me through some very busy and stressful times and providing lots of laughs throughout. Darren, thanks for all of your hard work and help with the paper. I am sure that you will do great in your exams and hope that you have an amazing time in New York. You really are an amazing group of people and I will miss you guys alot!

I would especially like to thank Jim and Edel for being my proof readers. I really appreciate it and am sure that it wasn't the most exciting task in the world!

Anne, all I can say is YEAH! Cannot believe we've made it this far together, madness. You have become a close friend over the last few years, thanks for everything! Your next! I hope that you have a great time in the WHO.

Barry M, the FACs man. Thanks for all of your help and patience with me while I attempted to master the art of flow cytometry. I'll get it someday and I promise I'll make you proud! I will miss our coffee breaks and quality time with the B man.

Cheryl, Karen and Anne, thanks for some great nights out and lots and lots of tea. You guys provided lots of laughs when they were badly needed. I am lucky to have met you guys. Aine, it was great living it up with you in Ranelagh, thanks for all of your good advice and lots of fun times in our swanky apartment!

Barrington, well buddy thanks for everything and listening to my rants too! I had an awesome time living with you and Cheryl in the STRAND. We said we'd stay buddies after college and you are definitely stuck with me now.

To Laura, Chloe and my OLS girlies (Aoife, Denise, Lora, Luisa and Sarah), thanks for all of your support and for putting up with me trying to explain exactly what it is I do. I am no longer a student!

To everyone on the bottom floor of the Biotech, past and present (Sarah, Fionnula, Helen, Stephen, Jean, Padraig, Kevin, Corinna, Padraic, Caroline, Keith, Aine, Ania, Karen, Neill M, Neill F, Cheryl, Michelle, Aileen, Conor, Anna). You were great neighbours, thanks for all of your help and lots of fun nights out.

I also want to thank the ladies in the departmental office, Mary Pat and Miriam, you are both always so helpful and never hesitate to help whenever there was a crisis! Thanks for everything!

Well Cheryl, what can I say. We've been friends for nine years during which we finished our degrees together (with the same marks, heehee!) and now we are about to finished our PhDs together too. I cannot express fully how amazing it has been to have gone through this with you. You have always been so supportive and have definitely gotten me through some challenging times over the last few years, thanks! I am so lucky to have you as my friend and I know that we will remain friends for a long time, thanks for everything! Congratulations to you too, I knew we could do it!

Finally, I would like to thank my family, Mary, David, Jennifer, David and Andrew. I am happy to say that my rants about my thesis are finally over! Thank you for listening, I am sure they were the highlight of your day! You have all always supported me in everything I have set my mind to and without that support I would not be where I am today. These words are definitely enough to express my gratitude. Thanks.

Abstract

Particulate vaccine adjuvants (PVA) have been used in vaccines both experimentally and clinically for almost a century. However, the mechanisms by which adjuvants, such as alum and poly lactide-co-glycolide (PLG) microparticles promote immune responses are not well understood. This project set out to investigate the mechanisms by which PVA promote immune responses and the relationship between specific PVA characteristics and the induction of innate and adaptive immunity. Firstly, the ability of PVA to activate immune cells *in vitro* was investigated. These studies revealed that neither alum, PLG nor polystyrene (PS) microparticles promoted the maturation of dendritic cells (DC). Furthermore, the particulates did not directly promote cytokine production by DC. In contrast, PVA synergised with TLR agonists to dramatically enhance the secretion of IL-1 β , IL-18 and IL-1 α . The enhancement in IL-1 β secretion was dependent on particle uptake and potassium efflux. Furthermore, the increase in IL-1 β secretion was dependent on caspase-1 activation via the NLRP3 inflammasome. PVA promoted inflammasome activation via a process dependent on lysosomal disruption and the lysosomal protease, cathepsin B. In addition, following injection the ability of PVA to promote the recruitment and activation of innate immune cells was dependent on NLRP3. Furthermore, PVA-mediated enhancement of antigen-specific IL-6 secretion occurred in a NLRP3-dependent manner. In contrast, the ability of PVA to enhance antigen-specific humoral responses was NLRP3-independent.

While *in vitro* data suggested that PVA require the addition of a TLR agonist to enhance IL-1 β secretion, injection of PVA in the absence of a TLR agonist was sufficient to promote the local production of IL-1 β . This suggests that following injection, PVA can synergise with endogenous factors to promote IL-1 secretion. In agreement with this, PS microparticles synergised with HMGB1, a known endogenous danger signal, to enhance IL-1 β and IL-1 α secretion by DC. Furthermore, all the PVA tested were toxic to DC and splenocytes *in vitro* and induced cell death at the injection site *in vivo*. Thus, PVA may promote the release of immunostimulatory endogenous danger signals from cells around the injection site, which could synergise with the particulates to promote IL-1 secretion. In support of this, the injection of supernatants from alum-treated splenocytes together with Ova enhanced antigen-specific cell-mediated immunity.

Having established the *in vitro* effects of PVA, studies were carried out to investigate the relationship between PVA composition and adjuvant properties. Injection of alum, PLG or PS microparticles principally enhanced antigen-specific Th2 responses. The most striking finding was that injection of PVA alone exerted modulatory effects on cytokine production within 3 h of injection. In particular, injection of alum or PLG primed splenocytes to secrete IL-10, IL-6 and IFN- γ . Moreover, PLG enhanced expression of IL-21 mRNA in splenocytes 1 day after injection. These results indicate that there are shared responses to injection of particulates in terms of cytokine modulation. However, it was important to dissect the relationship between particle size and the type of adaptive immune response induced. The injection of PS nanoparticles promoted strong local inflammatory reactions and specifically enhanced the secretion of IL-17 and IFN- γ . In contrast, injection of larger PS particles induced a small discrete lesion with a low level of cellular infiltration and promoted Th2-associated cytokines. However, all PS particles, regardless of their size, enhanced the production of antigen-specific antibodies, suggesting that particle size is a key determinant of the type of cell-mediated immunity induced by particles but less important in dictating humoral immunity. Overall, these studies have revealed remarkable immunomodulatory effects of PVA and add to our understanding of how these materials induce and direct immune responses. These findings will be useful in the design and application of novel vaccine adjuvants.

Publications

James Harris, **Fiona A. Sharp**, and Ed C Lavelle. (2010) The role of inflammasomes in the immunostimulatory effects of particulate vaccine adjuvants. *European Journal of Immunology (EJI)*, Mar 40 (3); 634-638 (See Appendix I)

Fiona A. Sharp, Darren Ruane, Benjamin Claass, Emma Creagh, James Harris, Padma Malyala, Manomohan Singh, Derek T. O'Hagan, Virginie Pétrilli, Jurg Tschopp, Luke A. J. O'Neill & Ed C. Lavelle. (2009) Uptake of particulate vaccine adjuvants by dendritic cells activates the NALP-3 inflammasome. *PNAS*, Jan; 106 (3) : 870-875 (See Appendix I)

Sharp F.A and Lavelle E.C, Development of Therapeutic Agents: Discovery of Vaccine Adjuvants, John Wiley and Sons 2008 (In Press)

Abbreviations

Ag: Antigen
AGP: aminoalkyl glucosamide phosphates
AID: Activation-induced cytidine deaminase
ANCA: Anti-neutrophil circulating antibodies
APAF-1: Apoptosis protease activating factor 1
APC: Antigen presenting cell
ASC: Apoptosis associated speck-like protein
BAL: Bronchoalveolar lavage
BCL6: B cell lymphoma 6
BCR: B cell receptor
BIR: Baculovirus inhibitor of apoptosis repeat
BLIMP: B-lymphocyte induced maturation protein 1
BMDC: Bone marrow derived dendritic cell
CAPS: Cryopyrin associated periodic syndrome
CARD: Caspase activating recruitment domain
cDC: Conventional dendritic cells
CDP: Common dendritic cell precursor
CFA: Complete freunds adjuvant
CIITA: Class II transactivator
CpG: Cytosine-phosphate-guanine
CT: Cholera toxin
CTB: Cholera toxin B subunit alone
CTL: Cytotoxic T lymphocyte
CTLA-4: CTL-associated protein 4
DAI: DNA-dependent activator of interferon regulatory factor
DAMP: Danger-associated molecular pattern
DC: Dendritic Cell
DD: Death domain
DLL: Delta-like Ligand
DNA: Deoxyribonucleic acid
EAE: Experimental allergic encephalomyelitis
ELISA: Enzyme-linked Immunoabsorbant Assay
ER: Endoplasmic reticulum
ETEC: Enterotoxigenic *E.coli*
FACS: Fluorescence-activated cell sorting
FCAS: Familial cold autoinflammatory syndrome
FCS: Fetal calf serum
FOXP: Forkhead box containing protein
GALT: Gut-associated lymphoid tissue
GATA: GATA-binding protein
Gfi1: Growth factor independent 1
GLA: Glucopyranosyl lipid A
GM-CSF: Granulocyte monocyte colony stimulating factor
ICAM-1: Inter-Cellular Adhesion Molecule 1
ICOS: Inducible Co-stimulator
iDC: Inflammatory dendritic cell

IFA: Incomplete Freund's adjuvant
 IFN: Interferon
 Ig: Immunoglobulin
 IL: Interleukin
 iNKT: Invariant natural killer T cell
 IPAF: ICE-protease activating factor
 IPEX: immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
 IPS-1: Interferon- β promoter stimulator 1
 IRAK: IL-1 receptor associated kinase
 IRF: Interferon Regulatory Factor
 IS: Immunological synapse
 ITAM: Immunoreceptor tyrosine based activation motif
 iTreg: Inducible regulatory T cell
 KLH: Keyhole Limpet Hemocyanin
 LBP: LPS-binding protein
 LC: Langerhans' cells
 LFA-1: Lymphocyte Function Associated Antigen-1
 LGP2: Laboratory of genetics and physiology 2
 LPS: Lipopolysaccharide
 LRR: Leucine Rich Repeat
 LT: Heat labile enterotoxin from ETEC
 MAL: MyD88-adaptor-like protein
 mAb: Monoclonal antibody
 MAML: Mastermind like 1
 MAMP: Microbe-associated molecular pattern
 MAPK: MAP Kinase
 MDA-5: Melanoma differentiation associated gene 5
 MDP: Monocyte/macrophage/dendritic precursor
 MDP: Muramyl dipeptide
 MHC: Major histocompatibility complex
 MP: Myeloid precursor
 MPL: Monophosphoryl lipid A
 MWS: Muckle wells syndrome
 MyD88: Myeloid differentiation factor 88
 NAC: N-acetyl-cysteine
 NACHT: Nuclear oligomerisation domain (NOD), MHC II transactivator (MHC II), HET-E and TP1
 NALP: NACHT-LRR-PYD containing proteins
 NFAT: Nuclear factor of activated T cells
 NF κ B: Nuclear factor κ B
 NKT: Natural killer T cells
 NLR: Nod-like receptor
 NOD: Nucleotide oligomerisation domain
 NOMID: Neonatal onset multisystem inflammatory disease
 PCC: Pigeon cytochrome C
 PD-1: Programmed Cell Death 1
 pDC: Plasmacytoid dendritic cells
 PDI: Protein disulfide isomerase
 PGN: Peptidoglycan
 PI: Propidium iodide

PLG: Poly-lactide-coglycolide Microparticles
 PMA: Phorbol myristate acetate
 Poly (I:C): polyinosine-deoxycytidylic acid
 PRR: Pattern recognition receptor
 PS: Polystyrene microparticles
 PVA: Particulate vaccine adjuvant
 PYD: Pyrin domain
 RA: Retinoic acid
 RBPJ: recombination-signal-binding protein for immunoglobulin J region
 RICK: RIP-like interacting CLARP kinase
 RIG: Retinoic acid inducible gene
 RLR: Rig-like receptor
 RNA: Ribonucleic acid
 ROR γ t: Retinoid-related orphan receptor γ t
 ROS: Reactive oxygen species
 RT-PCR: Real time polymerase chain reaction
 SARM: Sterile alpha and HEAT/armadillo motifs
 SDS: Sodium dodecyl sulphate
 SDS-PAGE: SDS-polyacrylamide gel electrophoresis
 SEA: *Schistosoma mansoni* egg antigen
 SHM: Somatic hypermutation
 SLE: Systemic lupus erythematosus
 STAT: Signal transducer and activator of transcription
 TAP: Transporters associated with antigen presentation
 T-bet: T-box expressed in T cells
 TBK-1: TRAF family member-associated NF κ B activator (TANK)-binding kinase 1
 TCR: T cell receptor
 TDM: Trehalose dimycolate
 Tfh: Follicular helper T cell
 TGF- β : Transforming growth factor β
 Th: Helper T cell
 TipDC: TNF/inducible nitric oxide synthase producing dendritic cells
 TIR: Toll/IL-1 receptor
 TLR: Toll-like receptor
 nTreg: Natural T regulatory cells
 TNF: Tumour necrosis factor
 Treg: Regulatory T cell
 TRAM: TRIF-related adaptor molecule
 TRIF: TIR domain-containing adaptor protein inducing interferon β
 TSLP: Thymic stromal lymphopoeitin
 Xbp1: X-box binding protein-1

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Appendix I

Chapter 1

Introduction

1.1 Vaccines

The concept of vaccination dates back to the late 18th century, when Edward Jenner made the observation that milkmaids that had contracted cowpox were protected against smallpox. Subsequently he inoculated a small boy, James Phipps, with cowpox to test his theory. The procedure was termed 'vaccination' and this was the beginning of immunology as a science [1]. Jenner's approach was successful and would eventually lead to the eradication of smallpox. The next major advance in vaccination was made by Louis Pasteur, who demonstrated that attenuated micro-organisms could induce long-lived protection against infection caused by the virulent or infectious form of the organism. Pasteur developed vaccines against chicken cholera in 1879 and human rabies in 1885 [2]. Vaccination was widely introduced during the 19th century and has greatly reduced the numbers of deaths due to infectious diseases. The traditional method of vaccine development was the use of live attenuated and inactivated viruses or bacteria. These types of vaccines have been effective against a number of diseases, including smallpox, rabies and measles. Safety issues and the advances made in molecular biology and immunology has led to a push towards subunit vaccines, using just isolated components from the microorganisms. The subunits used may be peptide or protein antigens, polysaccharides or DNA. Additionally, advances made in genomics and bioinformatics have greatly changed the way in which vaccines are developed. For example, a process known as 'reverse vaccinology' involves scanning of the genome sequences of infectious micro-organisms in order to identify potential vaccine targets [3]. This technique has led to the identification of vaccine candidates for *Neisseria meningitidis* serogroup B and is currently being applied to other infectious agents, such as *Yersinia pestis* [3-4]. The increased safety profile associated with subunit vaccines comes with reduced immunogenicity, decreasing the efficacy of these vaccines. The lower immunogenicity is due the absence of microbe-associated molecular patterns (MAMP), such as LPS or flagellin, which are highly immunostimulatory. It is also thought that the particulate nature of the attenuated microorganism may present the MAMP to the immune system in a way which is more immunogenic [5].

Cells of the innate immune system have the ability to recognise these MAMP and trigger the induction of tailored pathogen-specific immune responses. There are currently two main theories on how our immune system distinguishes self from non-self; (1) Janeway's self versus non-self model and (2) Matzinger's danger theory [6-7].

1.2 Innate Immune recognition

The original model of how the immune system distinguishes self from non-self and the presence of invading microorganisms was proposed by Burnet. He hypothesised that lymphocytes possess a single receptor specific for a pathogen and that multiple copies of this receptor are expressed on their cell surface [8]. This idea was then extended to introduce the idea of a 'stimulator cell', now known as an antigen presenting cell (APC). The APC was thought to be required to provide a signal for T cell activation and that this signal was species-specific [9]. Almost 30 years later, Charles Janeway put forward the idea that APC could recognise MAMP through a set of germline encoded receptors, known as pattern recognition receptors (PRR) [6]. According to this model, APC that have not encountered pathogens are in an inactive state and once they come into contact with a pathogen they become activated and upregulate co-stimulatory molecules, which allows them to interact with naive T cells. These interactions between APC and T cells results in the generation of an antigen-specific immune response through the adaptive immune system.

The PRR proposed by Janeway equip the APC with the ability to distinguish "infectious non-self" from "non-infectious self" [6]. Although Janeway's theory is widely accepted and experimentally supported, there are examples of immune activation in disease states which do not fit into this model, such as autoimmune diseases or graft rejection. In addition, this theory cannot explain how non pathogen-derived adjuvants, such as aluminium hydroxide (alum), stimulate the immune system.

In an attempt to explain these discrepancies, Polly Matzinger proposed that APC are activated by "danger" or "alarm" signals released from dying or damaged cells [7]. This 'danger' theory suggests that the immune system recognises non-self in the context of 'danger' or damage. The identification of endogenous molecules, such as HMGB1,

uric acid, hyaluronic acid and heat shock proteins that can stimulate immune responses adds substance to this novel theory [10-12].

The common idea in both Janeway's and Matzinger's theories is the existence of a set of evolutionarily conserved receptors on cells of the innate immune system which allows them to quickly and efficiently sense the presence of invading pathogens and also damaged, injured or stressed cells.

1.3 Pathogen Recognition Receptors

PRR can be expressed on the cell surface, intracellularly or secreted. Recognition of a MAMP or danger signal triggers intracellular signalling cascades, which ultimately results in the expression of inflammatory effector molecules, such as cytokines, reactive oxygen species (ROS) and co-stimulatory molecules. During the acute phase of infection, the liver can secrete PRR, such as C-reactive protein and mannose-binding lectin, which recognise phosphorylcholine and terminal mannose residues, respectively [13-14]. Binding of these secreted PRR helps to 'label' antigens as foreign and allows their subsequent interaction with other molecules of the innate immune system. The surface expressed and intracellular PRR currently comprise of four main families: Toll-like receptors (TLR), RIG-1-like receptors (RLR), C-type lectin receptors (CLR) and the Nod-like receptors (NLR). CLR include Dectin-1, mannose receptor, DC-SIGN and Mincle [15]. It is thought that the existence of multiple PRR with the ability to sense microorganisms or danger signals, or even both, guarantees the detection of danger, even when the signalling competency of a single PRR is compromised.

1.3.1 RLR

RLR can detect viral nucleic acids and activate members of the interferon response factors (IRF) family. Retinoic acid inducible gene 1 (RIG-1) and melanoma differentiation-associated gene 5 (MDA 5) can detect the presence of dsRNA, which is present during the replication stages of many viruses (Table 1.1) [16-17]. Both PRR contain a helicase domain, which is responsible for RNA binding. In addition, both molecules contain a caspase-activating recruitment domain (CARD) which allows them

to signal downstream via homotypic interactions with other CARD-containing proteins, such as IFN- β promoter stimulator 1 (IPS-1), resulting in the activation of IRF-3 and nuclear factor kappa B (NF κ B) [18]. RIG-1 and MDA-5 are found as inactive monomers in resting cells and ligand binding induces a conformational change that exposes their CARD regions, allowing for oligomerisation and subsequent signalling [18]. More recent studies have demonstrated that, activation of MDA-5 and RIG-1 occurs in a length-dependent manner, with MDA-5 being specifically activated in response to longer poly(I:C) polymers [19]. This suggests that these dsRNA sensors may be able to distinguish between different types of RNA viruses. Database searches have also identified another helicase-containing protein, known as laboratory of genetics and physiology 2 (LGP2). Although LGP2 contains a helicase domain, it is lacking a CARD region and acts as a negative regulator of both MDA-5 and RIG-1 [20].

Additional PRR necessary for the sensing of non-self DNA have also been identified. DNA-dependent activator of interferon regulatory factor (DAI) has been shown to bind dsDNA directly and induce type I IFN secretion via activation of TRAF family member-associated NF κ B activator (TANK)-binding kinase 1 (TBK-1) and IRF3 [21]. The sensing of non-self DNA within the cytoplasm can also trigger activation of caspase-1 via inflammasome formation, induced by a DNA-sensing protein known as absent in melanoma 2 (AIM2). AIM2 has been shown to induce inflammasome activation in response to transfected DNA and vaccinia virus [22-23].

1.3.2 TLR

TLR were first discovered based on their homology with the *Drosophila* protein Toll. The *Drosophila toll* gene was initially discovered to be required for dorso-ventral patterning during embryonic development. However, it was later found that Toll induces the production of the antimicrobial protein drosomycin, suggesting a role in immune responses. Homologues of Toll have been identified in several species, ranging from plants to mammals. The first human toll-like receptor was reported in 1994 [24]. However the physiological relevance of these proteins in mammals was not identified until 1998 when Poltorak *et al* showed that the mice susceptible to infection with Gram-negative bacteria possessed a mutation in their *Tlr4* gene, suggesting that its

role is to sense the presence of pathogens through LPS [25]. Since their initial discovery, 13 mammalian TLR have been identified.

TLR are type I transmembrane proteins, which means that they have an extracellular N-terminal. The defining features of all TLR, however, is the presence of an intracellular Toll/IL-1 receptor (TIR) domain and an extracellular leucine-rich repeat (LRR) associated with pathogen sensing [18]. The leucine-rich repeat region consists of β -strands and α -helices, which run parallel to the same axis, producing a non-globular horseshoe-shaped molecule. More importantly, the LRR is associated with interactions between the receptor and its ligands [26]. The TIR domain is highly conserved and forms complexes with the TIR-containing adaptor molecules Myeloid differentiation factor 88 (MyD88), MyD88 adaptor-like protein (Mal/TIRAP), TIR domain-containing adaptor protein inducing interferon β (TRIF) and TRIF-related adaptor molecule (TRAM) [27]. In addition to these stimulatory adaptors, sterile- α and HEAT-Armadillo motifs (SARM) was identified as the first TIR-containing adaptor to have an inhibitory role [28]. MyD88 is utilised by all TLR except for TLR3, which signals through TRIF, while TLR4 has the ability to recruit either MyD88 or TRIF [29].

Multiple pathways can potentially be activated by TLR signalling, including NF κ B, MAP-kinase (MAPK) and type I interferon (IFN) regulatory factors, ultimately inducing the translocation of transcription factors into the nucleus. These transcription factors regulate the expression of pro-inflammatory genes, including cytokines, chemokines, co-stimulatory molecules and anti-microbial peptides [18]. TLR can be broadly classified based on their cellular localisation. They can be found on the cell surface (TLRs 1,2,4,5,6) or intracellular on the surface of endosomes (TLR 3,7,8,9) (Figure 1.1).

TLR3 recognises viral dsRNA and polyinosine-deoxycytidylic acid (poly I:C), which is a synthetic analogue of dsRNA [30]. TLR7 and 8 are highly conserved and both recognise the synthetic imidazoquinoline-like molecules imiquimod (R-837) and resiquimod (R848), which have anti-viral properties. In addition, TLR7 and TLR8 agonists also include guanosine- or uridine-rich ssRNA from viruses, such as HIV [31-33]. Vertebrate DNA contains CpG motifs which occur infrequently and are highly methylated. In contrast to this, unmethylated CpG motifs can be found in bacteria,

protozoa and viruses and these motifs have the ability to specifically activate TLR9 [34]. However, recently it has been shown that TLR9-dependent recognition of non-self DNA occurs in a manner that is independent of the base sequence and that signalling can be induced by sensing of the sugar backbone of DNA [35]. Furthermore, malarial DNA, which is AT-rich, induces pro-inflammatory responses in a TLR9-dependent manner [36].

Gram negative bacteria have an outer membrane, which is primarily composed of lipopolysaccharide (LPS). It is the lipid-A region of LPS, which is recognised by cell surface-expressed TLR4. The sensing of LPS occurs through a complex formed between CD14, MD2 and TLR4 on plasma membrane (Figure 1.1)[18]. There is no direct binding of LPS to TLR4, instead the LPS is delivered to the membrane bound TLR4-MD2 complex by CD14 recognition of LPS following LPS binding to LPS-binding protein (LBP) [10]. TLR2 is also expressed on the cell surface and has the ability to recognize MAMP from viruses, gram-negative and gram-positive bacteria and fungi [37]. This broad range of specificity seen with TLR2 is due to its ability to form a heterodimer with TLR6, TLR1 or non-TLR proteins, including CD36, CD14 and dectin-1. For example, the TLR2-TLR6 heterodimer recognises zymosan and diacyl-lipoproteins in conjunction with CD36, while TLR2-TLR1 binds to bacterial triacylated lipopeptides [18, 38-39]. TLR5 is highly expressed on the basolateral surface of intestinal epithelial cells, where it binds to a highly conserved region of flagellin, a protein component of bacterial flagella which is important for bacterial motility [40]. It has also been shown that TLR5 is not found on splenic DC, but is highly expressed on a subset of specialised lamina propria DC, suggesting a role for TLR5 in the regulation of innate and adaptive immune responses in the intestine [41].

In addition to their ability to sense pathogens, TLR have also been shown to have the ability to detect endogenous danger signals that can be released by necrotic, damaged or stressed cells. Both TLR2 and TLR4 have been implicated in the detection of danger signals, such as HMGB1, hyaluronic acid (HA), heat shock proteins and fatty acids [10, 42-44].

The expression of TLR can be restricted to specific cell types and this can have a major impact on the effector mechanisms of those cells. A prime example of this, are plasmacytoid DC (pDC), which play a key role in the innate response to viruses. These

cells specifically express TLR9 and TLR7, which allows them to detect viral nucleic acids and produce high levels of type I interferons (IFN) [45].

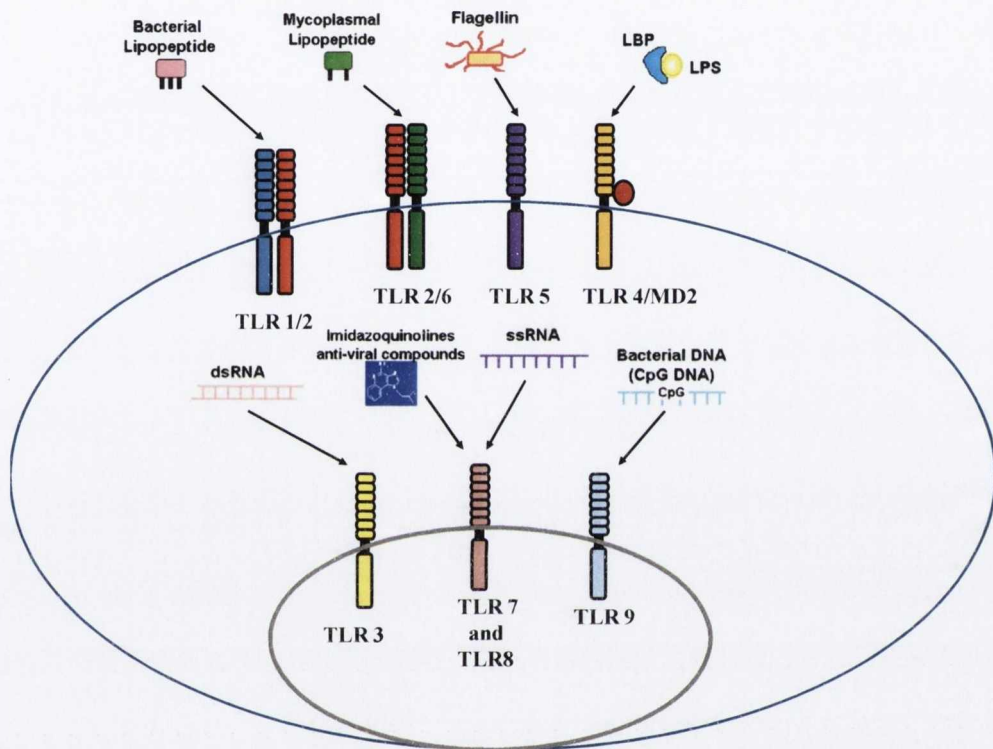


Figure 1.1: Cellular distribution of TLR and their respective ligands. TLR are pathogen recognition receptors of the innate immune system. They are expressed on the cell surface (TLR 1,2,4,5,6) and intracellularly on endosomal compartments (TLR 3,7,8,9). Their localisation allows them to detect the presence of either extracellular or intracellular pathogens. For example, TLR 3,9 have the ability to detect viral nucleic acids while TLR5 specifically interacts with bacterial flagellin.

TLR	Agonist
TLR1-TLR2	Pam ₃ CSK4, Diacyl-lipopeptides (bacteria)
TLR2	Lipoteichoic acid, peptidoglycan, porins, zymosan, HCV core and NS3 proteins, measles virus, human CMV and HSV-1
TLR3	dsRNA, mRNA, poly (I:C) polymer
TLR4	LPS(bacteria), Mannan(fungi), Glycoinositolophospholipids(protozoa), RSV fusion protein (virus)
TLR5	Flagellin
TLR6-TLR2	Triacyl-lipopeptides (bacteria)
TLR7	ssRNA, Imidazo-Quinolone-like molecules
TLR8	ssRNA, Imidazo-Quinolone-like molecules
TLR9	CpG DNA(bacteria, protozoa, virus),CpG oligos, AT rich DNA (malarial)
TLR10	Unknown
TLR11	Uropathogenic bacteria
RLR	Agonist
RIG-1	dsRNA, short poly (I:C) polymers, 5' triphosphate RNA
MDA-5	dsRNA, longer poly (I:C) polymers, RNA mesh

Table 1.1: Natural and synthetic ligands of TLR and RLR.

1.3.3 NLR

Several new families of LRR-containing proteins have been identified since the completion of the human genome project and evidence is increasing for their role in pathogen sensing and host defence. An example of one such family is the CATERPILLER (CARD, transcription enhancer, R (purine) binding, pyrin, lots of LRRs) family, distinct from TLR due to the absence of a TIR domain. These proteins are made up of three distinct regions (Figure 1.2); the C-terminal leucine-rich repeats (LRR), the central NACHT domain (domain present in neuronal apoptosis protein (NAIP) also referred to as the nuclear oligomerisation domain (NOD), MHC class II transactivator (MHC II), HET-E and TP1) and the N-terminal domain, which can consist of a pyrin domain (PYD), a caspase recruitment domain (CARD) or a baculovirus inhibitor of apoptosis repeat (BIR) domain. The leucine-rich repeat is thought to play a similar role in ligand binding as it does in TLR [46].

The NACHT or NOD region of the proteins contains seven distinct motifs, including an ATP/GTPase-specific P-loop and a Mg^{2+} binding site. It also contains a motif homologous to the NB-ARC domain in apoptosis protease activating factor 1 (APAF-1) which is involved in the binding of ATP and cytochrome-c-dependent oligomerisation, resulting in the activation of caspase-9 [47]. Based on this, the NACHT/NOD domain is thought to be required for oligomerisation and activation of NLR. The N-terminal domain is responsible for linking the receptor to downstream effector and signalling molecules. The NLR proteins can be subdivided into several families based on the class of their N-terminal effector domain; NLRA proteins containing an acidic N-terminal, NLRB proteins contain a BIR domain, NLRC proteins containing an N-terminal CARD domain and NLRP proteins containing a pyrin domain [48]. In addition to the families with known N-terminal effector domains, a protein localised to the mitochondrial membrane has recently been identified as the newest NLR. This protein has been designated NLRX1 due to the presence of an N-terminal domain. The N-terminal domain of NLRX1 has no known homology with currently identified protein domains [49-50].

The NLRA family consists of the MHC class II transactivator protein, known as CIITA and there are three different forms of this protein found, with the only one containing a CARD specifically expressed in DC [51]. The CARD region in the protein is required

for regulation of transcriptional activity within DC, through homotypic interactions with other CARD containing proteins. Furthermore, CIITA has been shown to specifically promote the transcription of MHC class II-associated genes [51]. Currently, CIITA is the only member of the NLRA family. However, further studies may identify similar proteins.

The NLRB subfamily includes NLR proteins with a BIR N-terminal domain, such as NAIP. The BIR regions are known to inhibit caspases, as shown by the ability of NAIP to abrogate the progression of apoptosis [52]. In contrast to its role as an inhibitor of caspases, NAIP5 has been shown to recognise intracellular flagellin from *Legionella pneumophila* and subsequently activates caspase-1 [53]. NAIP5 is thought to co-operate with NLRC4 in *L. pneumophila* induced caspase-1 activation, through its recognition of a specific portion of the C-terminal region of flagellin [54].

This NLRC subfamily is characterised by the presence of an N-terminal CARD domain which allows them to interact with other CARD-containing proteins via homotypic interactions. The NLRC subfamily includes the NOD proteins and ICE-protease activating factor or IPAF, now known as NLRC4 [48].

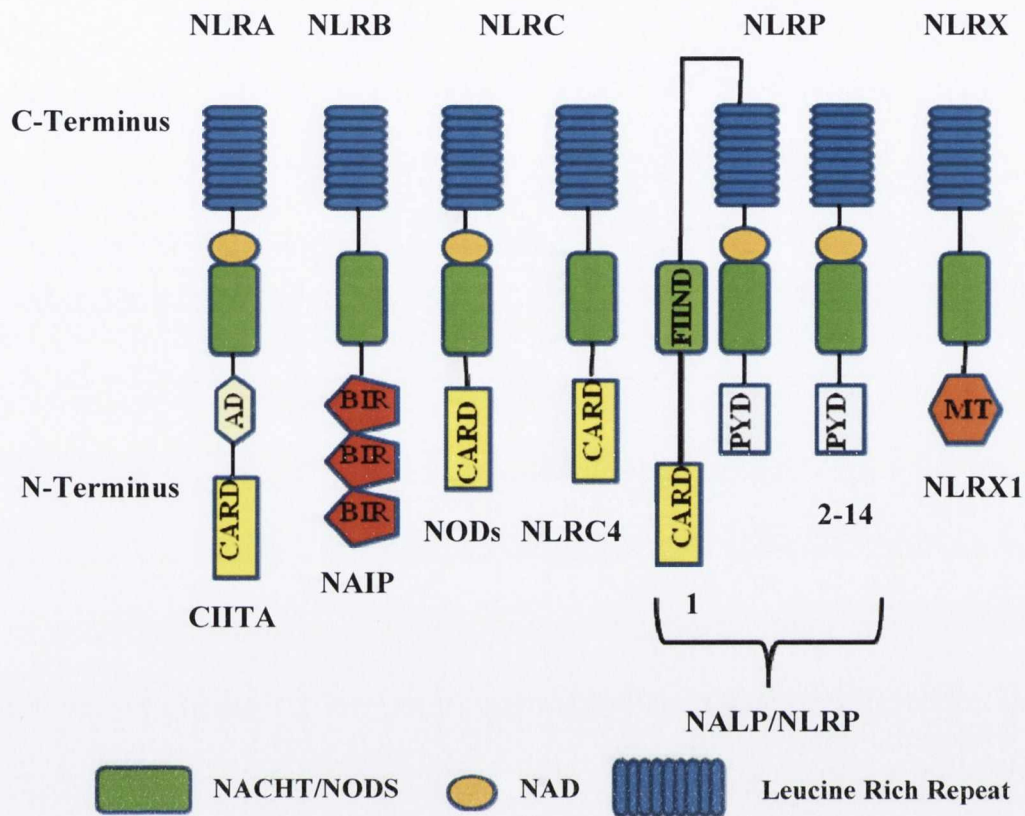


Figure 1.2: Structure of the main NLR sub-families. NLR proteins are made up of three distinct regions; the C-terminal leucine rich repeats (LRR), the central NACHT. The N-terminal domain determines the effector function of the protein. The subfamilies of NLR are classified based on their N-terminal domain, which can consist of a pyrin domain (PYD), acidic domain, a caspase recruitment domain (CARD), a baculovirus inhibitor of apoptosis repeat (BIR) domain or a mitochondrial translocation signal (MT) (Adapted from [48]).

1.3.3.1 Nucleotide oligomerisation domain (NOD) proteins

NOD1 and NOD2 were discovered based on their homology with APAF-1, a regulator of apoptosis [55-56]. They both also contain a NACHT-associated domain (NAD), the function of which is currently unknown. The only structural difference between NOD 1 and 2 is that, NOD2 contains two caspase recruitment domains in its N-terminus. Both NOD1 and NOD2 have been shown to induce activation of NF κ B when over-expressed (Table 1.2) [57]. One of the major constituents of the cell wall of gram-negative and gram-positive bacteria is peptidoglycan (PGN). It consists of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) units, cross-linked by short peptides. In gram-negative bacteria it forms part of a thin layer within the periplasmic space, while in gram-positive bacteria it makes up a large proportion of the cell wall. Bacterial remodelling of PGN results in the generation of shorter fragments, which when present in the cytosol can be detected by NOD1 and NOD2. iE-DAP (γ -glutamyl-meso-DAP), is a dipeptide fragment that can be generated by the degradation of PGN or secreted by bacteria during replication and this was identified as the core region of PGN, which can be detected by NOD1 [58-59]. NOD2 also senses PGN, although it does this by recognition of muramyl dipeptide which is the minimal common motif found in PGN from gram-negative and gram-positive bacteria [59-60]. MDP-induced activation of NOD2 has also been shown to induce activation of caspase-1 and this has been reported to involve co-operation between other members of the NLR family, including NLRP3 and NLRP1 [61-63].

It has been proposed that the NOD proteins are present in the cytoplasm in an inactive state and that conformational changes and oligomerisation are induced by ligand recognition. Once activated, the serine threonine kinase RIP-like interacting CLARP kinase (RICK also known as RIP2) is recruited, which subsequently targets the NF κ B essential modulator (NEMO/IKK γ) for degradation by the proteasome and ultimately leads to the translocation of NF κ B into the nucleus [64-65]. In addition to NF κ B, NOD1 and NOD2 activate the MAPK pathway, which also requires recruitment of RICK [66]. Triggering of both MAPK and NF κ B pathways allows activation of the NODs to induce the secretion of pro-inflammatory cytokines, chemokines and anti-microbial peptides, such as α and β defensins [67].

NOD2 has been associated with several autoimmune disorders. The uncontrolled signalling of NOD2 is linked to two disorders characterised by granulomatous inflammation in multiple organs and tissues, including the skin and eyes, known as Blau Syndrome and early onset sarcoidosis [68-69]. This over-activation of NOD2 is due to a missense mutation in the NOD domain of the protein, which is normally meant to control oligomerisation during the activation of NOD molecules. Mutations within or near the LRR region of NOD2 have been associated with an increased susceptibility to Crohn's Disease, which is characterised by trans-mural inflammation of the intestine. The exact mechanisms by which NOD2 mutations contribute to the immunopathology observed in Crohn's patients are still not clearly understood [70-71]. In addition to these abnormal responses, NOD1 and NOD2 have also been implicated in the adjuvant activities of complete Freund's adjuvant (CFA) and MDP, respectively [72-73].

1.3.3.2 NLRC4

NLRC4, otherwise known as ICE-protease activating factor (IPAF), CARD12 or CLAN, contains a C-terminal LRR, a central NACHT domain and an N-terminal CARD. However, it is distinct from other NLR due to the absence of a NAD (NACHT-associated domain) [74-75].

NLR are present in the cytosol in an inactive state until ligand binding via the LRR region. It has been shown that the removal of the LRR from NLRC4 results in a constitutively active protein. Overexpression of NLRC4 can activate caspase-1 and NLRC4 can associate with other CARD-containing proteins such as procaspase-1, NLRP1, NOD2, BCL-10 and ASC/PYCARD [74-75]. NLRC4 induces processing of caspase-1 via the formation of a multiprotein complex known as the inflammasome [76]. Activation of caspase-1 in an NLRC4-dependent manner by several bacteria has been reported, including *Salmonella typhimurium* and *Legionella pneumophila* [77-79]. In addition, studies have identified a single microbial molecule, flagellin, which is required in order to induce activation of NLRC4 [77-79]. Furthermore, activation of NLRC4 by the bacteria required an intact type III secretion system, normally used for the delivery of host cell modulating proteins into the cytosol, and NLRC4 activation could also be induced by cytosolic delivery of purified flagellin [77-78, 80-82]. NLRC4 has also been shown to be the key activator of caspase-1 in response to *Shigella*

flexneri, which is a non-motile bacteria that lack flagellin, suggesting that NLRC4 can detect an as yet unidentified bacterial molecule (Table 1.2) [83].

NLR	MAMP	Microbial Toxins	Bacteria and Fungi	Viruses	DAMP Non-Microbial
NLRP1	MDP	Lethal Factor (<i>B.anthraxis</i>)			
NLRP2	?	?	?	?	?
NLRP3	LPS Bacterial RNA MDP R838/837	Nigericin Maitotoxin Listeriolysin O Aerolysin	<i>S.aureus</i> <i>L.monocytogenes</i> <i>C. albicans</i>	Influenza Sendai Adenovirus	ATP ROS MSU Alum Silica Asbestos PLG-microparticles β -amyloid
NLRP4-14	?	?	?	?	?
NLRC4	Cytosolic flagellin		<i>S.typhimurium</i> <i>S.flexneri</i>		
NAIP	Cytosolic flagellin		<i>L.pneumophila</i>		
NOD1	ieDAP				
NOD2	MDP				

Table 1.2 : Activators of the NLR family. LPS: lipopolysaccharide, ATP: adenine triphosphate, ROS: reactive oxygen species, MSU: monosodium urate crystals, MDP: muramly dipeptide, ieDAP: γ -glutamyl-meso-DAP (Adapted from [84])

1.3.3.3 NLRP

The NLRP subfamily is characterised by the presence of an N-terminal pyrin domain (PYD). It consists of 14 members and is one of the largest subfamilies of CATERPILLER proteins [85]. NLRP have been located to two main regions on the human chromosome (Chr 11 and 19), with the exception of NLRP 1 (Chr 17) and NLRP3 (Chr 1) [46]. Due to the recent discovery of these proteins there is a limited amount of information about their functions, with the majority of our current knowledge coming from studies involving NLRP1 and NLRP3 (Table 1.2).

NLRP1

The first NLRP protein was identified based on its homology with apoptosis protease activating factor 1 (APAF-1). Initially, it was reported that NLRP1 could induce apoptosis through direct interaction with caspase-2 or -9 or indirectly through APAF-1 [86-87]. In humans, NLRP1 is the only one of the NLRP to contain a C-terminal extension, consisting of a CARD and a FIIND (function to find) region. In contrast, murine NLRP1 does not contain a FIIND region or PYD region [67]. NLRP1 has been shown to bind APAF-1 and activate caspase-1 when overexpressed [86]. Martinon *et al* first proposed the idea that IL-1 β processing occurs on a molecular platform in the cytosol and suggested that NLRP1 formed an integral part of this molecular complex through its ability to activate caspase-1 and caspase-5 [76]. By using purified recombinant forms of NLRP1 and caspase-1, Faustin *et al* showed for the first time that muramyl dipeptide (MDP) could interact with an NLR and activate NLRP1 [61]. However, this is in contrast with other studies showing a requirement for both NLRP3 and NOD2 in MD- induced IL-1 β secretion. As a result, the role of NLRP1 in the sensing of MDP remains controversial [63, 88]. Macrophages isolated from inbred mice have been found to be either resistant or susceptible to *Bacillus anthracis* lethal toxin, which causes rapid caspase-1-dependent apoptosis in these cells [89]. It has recently been reported that this difference can be associated with *Nlrp1b*, one of the three paralogues found in the *Nlrp1* locus in mice [89]. Furthermore, it has been shown that the activation of caspase-1 by *Bacillus anthracis* may involve a complex of NLRP1 and NOD2 [62].

NLRP1 is associated with a skin disorder known as familial Vitiligo, which causes depigmentation of the skin due to the absence of melanocytes [90]. It has been suggested that aberrant activation of NLRP1 enhances secretion of IL-1 β and that this could potentially prime T cells to target melanocytes. An evolutionary link has previously been suggested between apoptosomes and inflammasomes, connecting the apoptotic and inflammatory pathways. This theory is further supported by the fact that macrophages deficient in the anti-apoptotic Bcl-2 exhibit increased caspase-1 activity and IL-1 β secretion in response to stimulation with MDP, whereas the overexpression of Bcl-2 significantly reduced MDP-induced secretion of IL-1 β [91]. Furthermore, these suppressive activities have been shown to be due to direct interaction between NLRP1 and anti-apoptotic proteins Bcl-2 and Bcl-xl, suggesting the existence of cross talk between these two pathways [91].

NLRP3

NLRP3 is one of the most studied NLRP proteins. It was first identified as cryopyrin, the protein product of a gene segregated in family members suffering from Muckle-Wells (MWS) syndrome and familial cold autoinflammatory syndrome (FCAS). Several groups identified this gene around the same time, resulting in multiple names for both the gene and its protein product: CIAS-1, PYPAF1 and NALP3 [85, 92-93]. The most commonly used name is NALP3 and this has more recently become NLRP3 [48]. Dysregulation of NLRP3 has also been associated with neonatal onset multisystem inflammatory disease (NOMID) or chronic infantile neurological cutaneous and articular syndrome (CINCA) and taken together, NOMID/CINCA, MWS and FCAS are collectively known as cryopyrin-associated periodic syndromes (CAPS) [94].

The mutations (Chr 1q44) in NLRP3 result in a constitutively active form of the protein and they are clustered around the NACHT/NOD domain [95]. There are three splice variants of this protein in mice, one of which has no LRR domain [92]. NLRP3 has been reported to associate with apoptosis-associated speck-like protein containing a CARD (ASC), resulting in formation of the NLRP3 inflammasome and subsequent activation of caspase-1 [85, 96].

A wide variety of NLRP3 activators have been identified, ranging from whole microorganisms and vaccine adjuvants to environmental irritants. Several pathogens have been demonstrated to activate the NLRP3 inflammasome, including the fungi *Candida albicans* and *Saccharomyces cerevisiae* and bacteria that produce pore-forming toxins, such as *Listeria monocytogenes* and *Staphylococcus aureus* [97-99]. In addition, a range of viruses, including Sendai virus, adenovirus and influenza virus can trigger activation of the NLRP3 inflammasome [99-100]. However, it is not only exogenous stimuli that can trigger activation of the NLRP3 inflammasome, as several endogenous molecules released by dying or stressed cells have been shown to promote IL-1 β secretion via the NLRP3 inflammasome. These include uric acid, hyaluronon and extracellular ATP [99, 101-102]. A number of particulates have also been shown to activate the NLRP3 inflammasome, including silica, asbestos, cholesterol crystals and the vaccine adjuvants, alum and PLG microparticles [103-110]. It is worth noting, however, that these activators of the NLRP3 inflammasome required a 'priming' step, which involved pre-treatment of cells with a TLR agonist or DAMP. This is thought to be necessary to increase the intracellular levels of pro-IL-1 β , as most cells do not constitutively express pro-IL-1 β . However, it has also been suggested that this 'priming' step enhances the expression of NLRP3 itself via NF κ B [111]. However, it has recently been shown that the requirement may be dependent on the cell type and species, as monocytes isolated from PBMC can release active IL-1 β in response to TLR ligation alone while human alveolar macrophages require a second stimulus, such as the addition of exogenous ATP [112].

In vivo, NLRP3 has been shown to be involved in protection against invading pathogens, progression of autoinflammation and the activity of particulate vaccine adjuvants. As previously discussed, constitutive activation of NLRP3 can result in the development of autoinflammatory disorders such as muckle wells and familial cold autoinflammatory syndrome [85, 92-93]. It has also been suggested that, NLRP3 may play a role in the immunopathology associated with type 2 diabetes. Zhou *et al* have demonstrated that high extracellular glucose triggers the secretion of IL-1 β via the NLRP3 inflammasome [113]. Furthermore, the NLRP3-interacting protein TXNIP has been implicated as a mediator of pancreatic β -cell death [114-115]. It has recently been demonstrated that cholesterol crystals can activate the NLRP3 inflammasome and that their deposition and activation of NLRP3 contributes to the pathogenesis observed with

atherosclerosis [110]. NLRP3 activation has also been linked to the pathology associated with environmental irritants such as asbestos, silica, UVB irradiation and skin irritants, including trinitrophenylchloride, trinitrochlorobenzene and dinitrofluorobenzene [103-104, 116-117]. Furthermore, a role for NLRP3 in the adjuvant activities of the most commonly used particulate vaccine adjuvant alum has also been suggested. Studies have shown that NLRP3 is necessary for the enhancement of both cell-mediated and humoral responses by alum [105, 107-108, 118]. In contrast, however it has also been reported that the adjuvant properties of alum are not compromised in any way in NLRP3-deficient mice [118-119]. Therefore, the role of NLRP3 in the activity of particulate vaccine adjuvants currently remains controversial. The broad range of pathogens, vaccine adjuvants and endogenous danger signals which activate the NLRP3 inflammasome highlight its importance in innate immunity.

1.4 The Inflammasome

Interleukin-1 β (IL-1 β) is a potent endogenous pyrogen which induces the expression of inflammatory genes encoding adhesion molecules, inducible nitric oxide synthase (iNOS) and other pro-inflammatory cytokines such as IL-6. It has the potential to affect almost every cell type in the body and does so by binding to its high affinity receptor, IL-1R1, which associates with the IL-1R1 accessory protein (IL-1RAcP) once IL- β has bound, resulting in the activation of both the NF κ B and MAPK pathways [120]. Once it is released into the circulation, IL-1 β is a strong inducer of inflammation and, as a result, its secretion must be tightly regulated. The effects of IL-1 β dysregulation can be seen in the form of several autoimmune disorders, such as systemic juvenile arthritis and pyogenic arthritis where excess IL-1 β plays a major role in the uncontrolled immune responses [121]. IL-1 β is transcribed as a biologically inert protein that is 31kDa in size and lacks a signal peptide, so it is not automatically secreted; a small portion of it moves into lysosomes and the majority remains in the cytosol [122]. In order to be secreted it must be converted into its active form (17kDa). The conversion of IL-1 β into its active form is carried out by IL-1 converting enzyme (ICE) or caspase-1 which cleaves IL-1 β after an aspartic acid residue at position 116 [123-124].

Caspase-1 is part of a family of cysteine proteases which contain an N-terminal pro-domain and a CARD region and have roles in apoptosis and inflammation. The activation of caspase-1 must be tightly regulated in order to control the processing of IL-1 β . Therefore, caspase-1 is translated as a zymogen (45kDa) which must be converted into an enzymatically active heterodimer (p10 and p20 chain) by cleavage at an aspartic acid residue. In mice it has been shown that another inflammatory caspase, caspase-11, is required for IL-1 β processing, as mice deficient in caspase-11 or caspase-1 are unable to process IL-1 β [125-126]. The exact mechanism of activation of the caspases remains unknown.

In 2002 Martinon *et al* proposed the existence of the 'inflammasome', a multiprotein complex which helped to induce activation of caspases, resulting in the processing of IL-1 β . Using a cell-free system they showed that a complex comprising of caspase-1, caspase-5, NLRP1 and Pycard/ASC (apoptosis-associated speck-like protein containing a CARD) could activate both caspases and subsequently cleave IL-1 β [76]. ASC was first identified in cells undergoing retinoic acid-induced apoptosis as a detergent-insoluble protein and has since been shown to be expressed in several cell types, including epithelial cells, leukocytes and peripheral blood lymphocytes. The bipartite structure of ASC, consisting of a CARD and a PYD, allows it to interact with NLRPs and caspases [127-128]. NLRP1, 2 and 3 have all been shown to interact with ASC [76, 85, 129]. Since the discovery of the inflammasome, several have been identified including NLRP1, NLRP3 and IPAF [61, 130]. In addition, a non-NLR protein known as AIM2 has recently been shown to form an inflammasome and activate caspase-1 [22-23].

Our knowledge of how the inflammasomes are activated and assembled has expanded greatly since their discovery. The current theories suggest that NLR, such as NLRP3, are present in an autorepressed state in the cytosol. In this state the NLR is in a conformation within which the NACHT/NOD and effector domains are concealed by the LRR domain, preventing any interaction with other proteins. In agreement with this, previous studies have shown that a truncated version of IPAF/NLRC4 lacking its LRR domain was found to be constitutively active [75]. The LRR are thought to be responsible for pathogen or danger signal sensing. It is proposed that once a ligand has bound to the LRR region, the protein undergoes a conformational change, making its protein interaction domains accessible. This allows the protein to oligomerise with

other NLR as well as associate with downstream molecules containing either CARD or PYD domains such as caspases or ASC (Figure 1.3).

As an alternative to the idea that repressed NLRP proteins are activated by direct ligand binding, it has also been proposed that they are activated in response to endogenous danger signals, changes in the cytosolic ionic milieu or redox state. Several models have been proposed that may not be exclusive. Many of the studies into activation of inflammasomes have focused on the NLRP3 inflammasome.

The first model of NLRP3 activation involves extracellular ATP which activates the P2X7 ATP-gated ion channel triggering K⁺ efflux and opening of the pannexin-1-mediated pore in the cell membrane [81]. The authors proposed that opening of this pore allows direct cytosolic access for agonists of NLRP3 [81]. In agreement with this, Petrelli *et al* have demonstrated that low intracellular potassium levels, trigger formation of the NLRP3 inflammasome [131]. The second model suggests that all activators of the NLRP3 inflammasome promote the enhancement of intracellular reactive oxygen species (ROS) [103-104, 132]. In support of this model it has been shown that scavenging of reactive oxygen species (ROS) by N-acetyl-cysteine (NAC) impairs ATP, MSU, silica, malarial hemazoin and *Candida albicans*-induced inflammasome activation [97, 103-104, 133]. Furthermore, an ROS-sensitive molecule found in the cytosol, thioredoxin-interacting-protein (TXNIP), has been implicated in the activation of NLRP3 [113]. Thirdly, several studies involving activation of the NLRP3 inflammasome by particulates have demonstrated that lysosomal disruption and release of lysosomal contents into the cytosol can be detected by NLRP3 [106]. Hornung *et al* have shown a requirement for the lysosomal protease cathepsin-B, and demonstrated that acidification of lysosomes is necessary for activation of the NLRP3 inflammasome in response to silica and alum [106]. Furthermore, inhibition of cathepsin B has been shown to abrogate the ability of PLG microparticles, amyloid- β and cholesterol crystals to activate the NLRP3 inflammasome [109-110, 134].

The mechanism of activation of other inflammasomes, such as NLRP1, NLRC4/IPAF and AIM2 are less well understood. The components of the NLRP1 inflammasome differ from that of the NLRP3 inflammasome. The NLRP1 protein consists of an extra domain which contains a CARD region, allowing it to interact directly with pro-caspase-1 and bypass the need for ASC. It has been demonstrated that activation of the

NLRP1b inflammasome in macrophages does not require ASC [62]. However, in a similar manner to NLRP3, it has been reported that the NLRP1 inflammasome requires K^+ efflux for activation [135-136]. In contrast, activation of the NLRC4/IPAF inflammasome does not require K^+ efflux [131]. The AIM2 protein consists of a C-terminal HIN domain and an N-terminal PYD region, and in a similar manner to NLRP3 it requires ASC in order to recruit pro-caspase-1 [22-23]. However, formation of this inflammasome is not triggered by a central oligomerisation domain but by clustering of multiple AIM2 proteins, via its HIN domain, around the binding sites of its ligand, dsDNA [22-23].

Activation of the inflammasome is also regulated by other intracellular processes. Autophagy is a constitutively active process by which the cell sequesters, degrades and recycles the constituents of damaged proteins, organelles or pathogens within a double membrane compartment, known as an autophagosome [137]. In addition, autophagy can also be induced by various immunostimulatory stimuli, including IFN- γ , TNF- α and TLR agonists [137]. Recently, it has been shown that blockade of proteins required for autophagy, Atg16L1 and Atg7, enabled LPS-induced inflammasome activation, thus suggesting that autophagy negatively regulates activation of the inflammasome [138]. The exact mechanism of this regulation of the inflammasome by autophagy is currently unknown. However, it has been proposed that autophagosomes may target inflammasomes for degradation [139]. Furthermore, studies within our lab have shown that pro-IL-1 β is sequestered in autophagosomes in LPS-treated macrophages (Harris J and Lavelle EC *et al* unpublished findings).

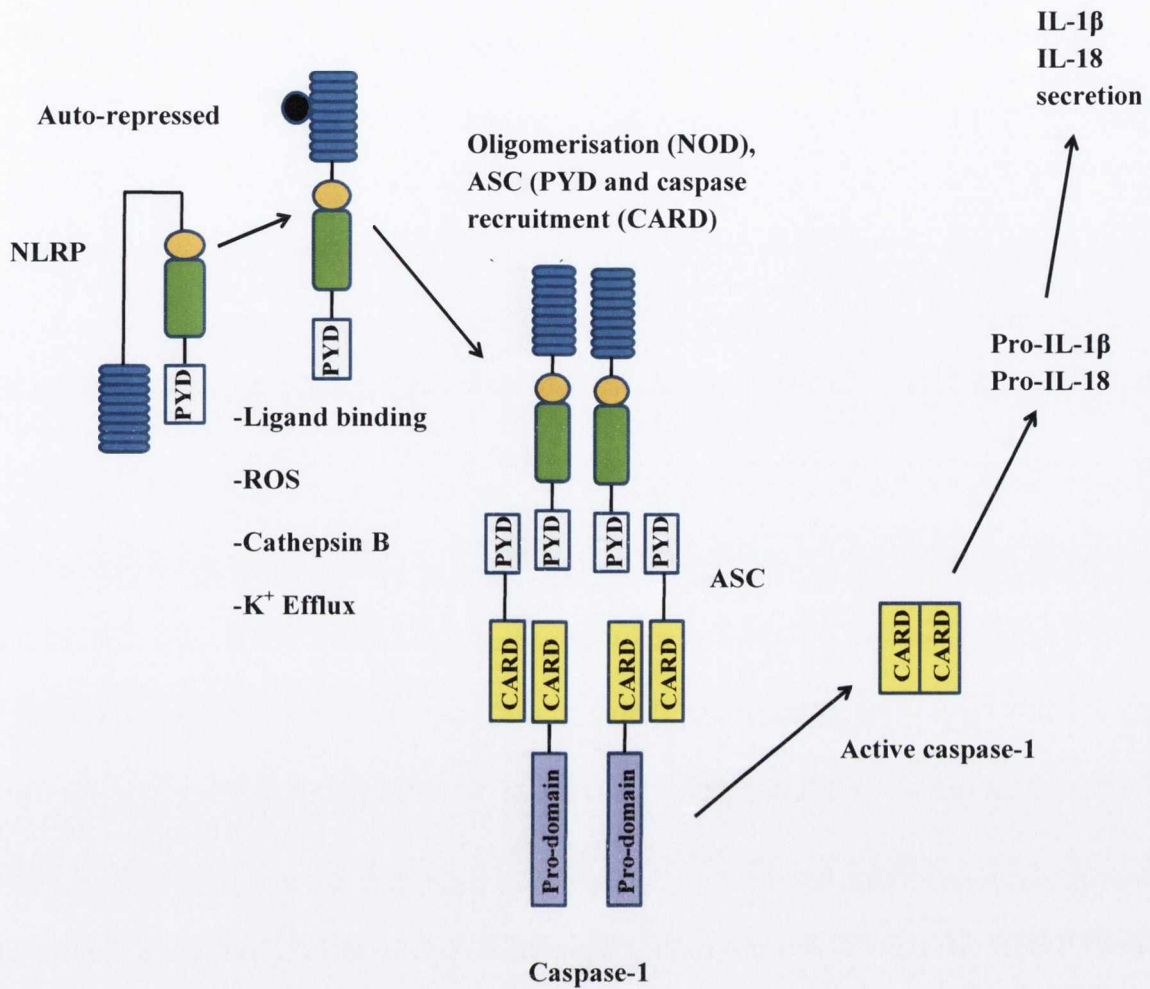


Figure 1.3: Proposed mechanism of activation of the inflammasome. Current theories suggest that NLR proteins are present in an autorepressed state in the cytoplasm, in a conformation which conceals the NACHT/NOD and effector domains, preventing any interaction with other proteins. Once the NLRP protein is activated a conformational change is induced, exposing the PYD/CARD domains. This leads to recruitment of ASC and, subsequently, caspase-1 activation which then goes on to convert pro-IL-1 β into active IL-1 β and pro-IL-18 into active IL-18 [140].

1.4.1 Regulation of the Inflammasome

Several proteins involved in the regulation of inflammasome activation have been identified. For example, pseudo-ICE and ICEBERG are proteins which contain a single CARD domain allowing them to interact with caspase-1. It is thought that they can bind to caspase-1 and prevent its recruitment into the inflammasome [141]. Another protein, protease inhibitor-9 (PI-9), can impede caspase-1 activation by binding to its active site and may also prevent the recruitment of caspase-1, thus, negatively regulating formation of the inflammasome [142]. ASC2 or POP, which is homologous to ASC but lacks a CARD, has been proposed to inhibit ASC activity, presumably by preventing caspase-1 recruitment [143]. Orthologues of NLR proteins, known as R proteins, can be found in plants and these proteins are important for host protection against invading pathogens. These R proteins have been shown to associate with the ubiquitin ligase-associated protein SGT1 and the chaperone protein heat shock protein 90 (HSP90) [144]. In a similar manner, mammalian NLR have been shown to associate with SGT1 and Hsp90 [145]. Furthermore, the activation of NLRP3, NOD1 and NOD2 requires active heat-shock protein 90 (Hsp-90) and SGT1. These proteins are proposed to associate with the autorepressed form of NLR proteins and dissociate once a pathogen or danger signal is sensed, resulting in NLRP oligomerisation and subsequent inflammasome formation [145].

The ability to recognise and react to a wide variety of pathogen and danger-associated stimuli is one of the key attributes of the innate immune system. Not only do PRR alert the immune system to the presence of danger or invading pathogens, but their activation results in the induction and development of adaptive immune responses, which are central to generating immunological memory. A key link between the innate PRR and the adaptive response is provided by dendritic cells (DC).

1.5 Dendritic Cells

The first observation of DC was made in the 1800s by Paul Langerhans, who identified what are now known as Langerhans cells in skin sections [146]. However, it was not until 1973 that Steinman and Cohn discovered DC in the secondary lymphoid organs of mice, describing them as “cells that did not look like other white blood cells”. These cells were star-like and had the ability to continually extend and retract processes. They were subsequently named dendritic cells from the greek word Dendron, meaning ‘tree’, based on their unusual morphology [147]. Following on from their initial studies, Steinman and others later demonstrated the unique ability of DC to initiate immune responses. This was reflected in their superior ability to stimulate a primary mixed leukocyte reaction in comparison to other known APC, such as B-cells and macrophages [148-149]. Further studies demonstrated a difference between the requirements for priming naive T cells and previously activated T cells, as it was found that all APC could stimulate activated T cells, while only DC had the ability to activate naive T cells [150]. These potent immunostimulators are a heterogeneous population of cells that can be further subdivided based on their localisation. For example, there are tissue-specific DC which reside in the gut and lungs, Langerhans cells are found in the epidermis of the skin, while other DC are constantly circulating. DC subtypes also differ in their migratory pathways, immunological function and the types of stimuli required for their activation or generation.

DC can be broadly classified into three main groups; pre-DC, conventional DCs and inflammatory DC [151]. Pre-DC are cells which do not exhibit the classical morphology of DC or any DC-like functions but can develop into DCs with little or no division. Plasmacytoid DC are round, long-lived circulating pre-DC that can convert into DC which characteristically produce large amounts of type-1 interferons (IFNs) and are important for innate responses to viruses [45, 152].

Conventional DC (cDC) are cells which already have both DC form and function and can be further subdivided into two categories; migratory DC and lymphoid-tissue-resident DC. These cells are generally short-lived and are replaced by blood-borne precursors [153-154]. The migratory DC are found in peripheral tissues, where they sample antigens in the surrounding area, and travel to the T and B cell zones of secondary lymphoid organs. There, they present internalised antigens to lymph node T

cells. A prime example of these cells are Langerhans cells [155]. These cells, express a high level of langerin (CD207), a type II lectin with mannose specificity, and can be found in the epidermis and epithelia of the intestines, digestive and reproductive tracts [156]. However, others have challenged the ability of LC to prime T cell responses, bringing into question the long-held idea of LC as the classical DC (refs in [146]. Lymphoid tissue-resident DC can be found in both the thymus and the spleen, where they sample self-antigen for presentation to resident T cells. Splenic cDC can be further subdivided based on their expression of CD8 α . The CD8 α^+ and CD8 α^- cells differ in their immune functions and cytokine profiles [157-158]. For example, CD8 α^+ splenic cDC have the ability to present exogenous peptides in complex with MHC class I molecules, a phenomenon known as cross-priming [157]. CD8 α^- lymphoid resident cDC can be even further subdivided based on their expression of CD4, adding another layer of complexity to the classification of these cells [159]. In addition, further studies have identified the existence of specialised subsets of cDC within the lung, gut, lamina propria, and the draining lymph nodes of the skin which express the integrin, CD103 [160-163]. This expression of CD103 by these specialised cDC has been suggested to be important for the recruitment of T cells in a tissue-specific manner during inflammation [161]. A more recent review has suggested that cDC should be subdivided based on their expression of CD11b and takes into account the differential expression of CD4, CD8 and CD103 on cDC [146]. Taken together, all of these studies suggest that cDC can be subdivided based on their expression of CD4, CD8, CD11b and CD103, which is related to the effector functions of these cells, thus highlighting the heterogeneity of cDC subsets.

The state of the immune system in a healthy mammal in the absence of any infections or inflammatory stimuli is known as 'steady state'. In contrast to pDC and cDC, inflammatory DC (iDC) are not found in the 'steady state' and are normally induced during inflammation or in response to infection. One such example is a subset of DC known as Tip DC, which appear in mice after *Listeria monocytogenes* infections and produce TNF- α and inducible nitric oxide synthase. These specialised DC were not required for adaptive antigen-specific responses, but were important for innate responses against the intracellular bacteria and the absence of these cells resulted in an inability to clear the infection [164]. Inflammatory DC have also been reported to be

recruited to the site of injection after immunisation with alum-adjuvanted Ova in a uric acid-dependent manner [165].

Both myeloid and lymphoid progenitors can develop into DC [166-167]. The development of DC occurs in the bone marrow and starts from a single myeloid precursor (MP), which gives rise to a common monocyte, macrophage and DC precursor (MDP). The MDP can subsequently become either a monocyte, some populations of macrophage or a common dendritic cell precursor (CDP) [168]. In addition, CDP can also arise from lymphoid-committed precursors. CDP have the potential to develop into both pDC and cDC, but no longer retain the ability to give rise to monocytes [153, 167, 169]. During cell differentiation, transcription factors play a determining role in deciding the fate of developing cells. Currently, the identity of all of the transcription factors necessary for DC development remain unknown, however several key players have been identified. For example, PU.1, a transcription factor which is required for the early stages of cell development within the myeloid lineage, also plays a key role in deciding the fate of MDP [170]. The presence of high levels of PU.1 will drive an MDP towards the DC pathway and prevent it from becoming a monocyte or macrophage, by inhibiting the activities of the macrophage-inducing transcription factors c-Maf and MafB [171-172]. Once a cell has committed to the DC pathway, it has the potential to become either a pDC or a cDC. The development of pDC is fully completed in the bone marrow and depends on the presence of a basic helix-loop-helix transcription factor known as E2-2 [173]. In contrast, cDC development is completed outside the bone marrow within secondary lymphoid organs during which further cell divisions are partially instructed by signals from regulatory T cells (Tregs), the fms-related tyrosine kinase receptor, Flt3 and the lymphotoxin- β receptor [153-154, 174].

1.5.1 Dendritic cell maturation

The idea that DC can exist in two different functional states was first proposed after studies showing that freshly isolated LC were more efficient at capturing and processing antigen, while cultured LC were potent immune stimulators [175-176]. Furthermore, it was shown by Austyn *et al* that DC could migrate from the blood to the spleen [177]. These studies led to the idea that DC existed in an 'immature' state in the periphery and

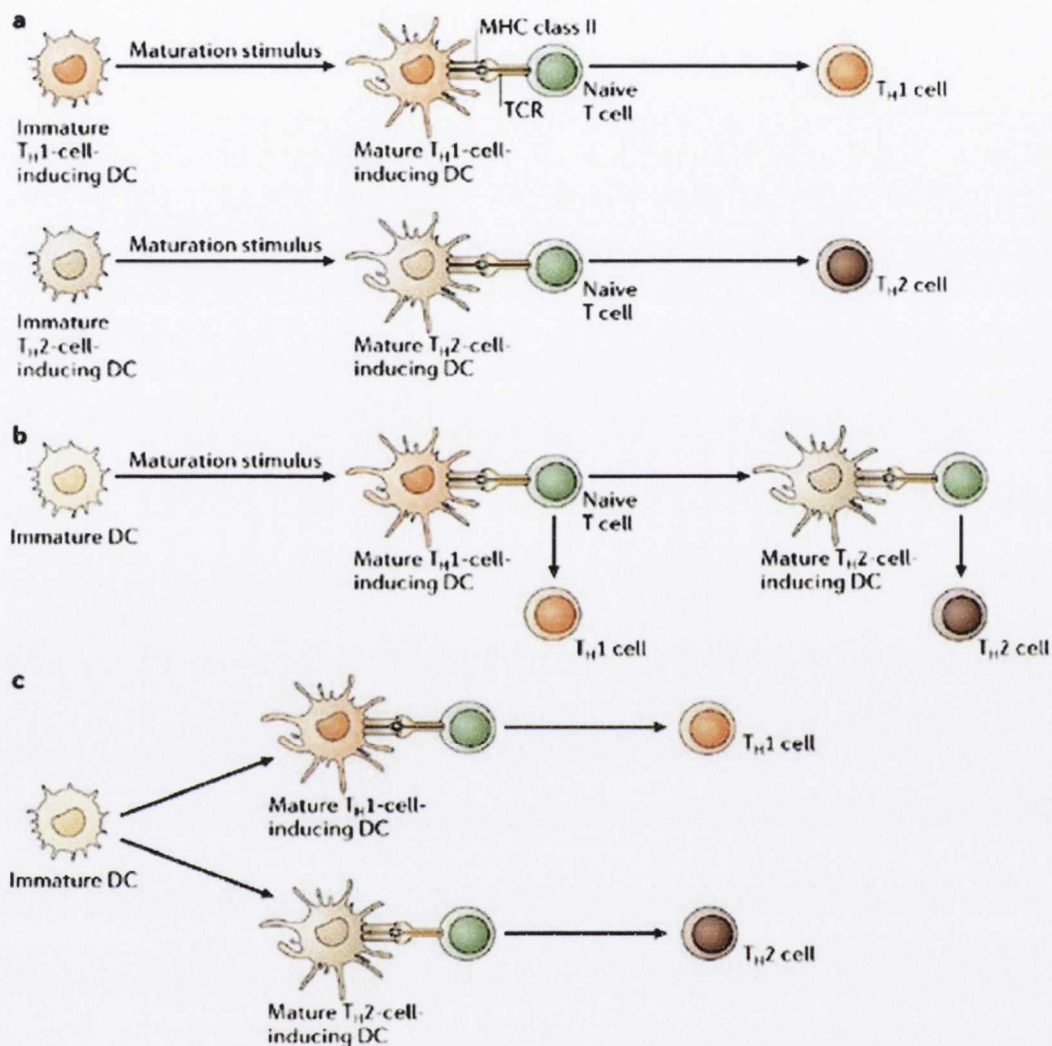
once they have captured and processed antigen they become 'mature' DC, a process that is thought to occur while the DC migrates to secondary lymph organs. Immature dendritic cells are highly efficient at antigen uptake and processing but are unable to activate T cells. Immature DC can internalise antigen in several ways, including micropinocytosis, receptor-mediated endocytosis via C-type lectins, such as DEC-205 or Fcγ receptors (CD64 and CD32), and phagocytosis [178]. Once antigen uptake has occurred, several changes are triggered within the DC which converts it into a potent APC.

The events leading to maturation involve several steps, such as the loss of endocytic receptors, upregulation of co-stimulatory molecules, including CD40, CD80 and CD86 as well as expression of peptide-containing MHC class II molecules. As a result of this process, mature DC express high levels of MHC molecules, CD80, CD86, CD40 and CD83 [179]. These molecules are necessary for T cell interaction, conferring on DC the ability to prime and subsequently activate T cells. DC maturation can be triggered by the sensing of MAMP, endogenous danger signals, local cytokines and also T-cell derived signals.

Several models have been proposed to describe DC maturation and also to explain how DC have the ability to induce several different effector responses specific to the activating signal received (Figure 1.4). The linear maturation model suggests that DC are either in an immature or mature state and that specific subsets of DC exist with the ability to prime each type of immune response required [180]. Others suggest that immature DC give rise to mature DC that initially produce high levels of IL-12-inducing Th1 cells, and that further maturation converts the DC into a cell that no longer secretes IL-12 and subsequently generates Th2 cells [181].

An alternative to both of these models presents a more flexible scenario, where the same type of DC can mature with the ability to prime both Th1 and Th2 cells [182-183]. All of the models to date are based on the idea that only mature DC are immunogenic and that tolerogenic DC, which can induce anergy, deletion or the development of regulatory T-cells, are in an immature state. However, in contrast to this, it has been shown that mature DC can be non-immunogenic and that tolerogenic DC can express high levels of co-stimulatory molecules and other markers of maturation [184-185]. Based on the confusion regarding whether maturity equals immunogenicity, it has been

proposed that DC should be described based on their phenotype, ontogeny and their effector functions, rather than simply the expression of particular surface markers [179].



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Figure 1.4: Models of DC maturation. Schematic representation of the models proposed to explain the maturation of DC. (a) Proposes the existence of specific DC subsets with the ability to prime Th1 or Th2 responses. (b) Matured DC initially secrete large amounts of IL-12 with the ability to induce Th1 type response, following further maturation the DC no longer produces IL-12 and as a result induces Th2 type responses. (c) DC exist in a more flexible state with the ability to induce both Th1 and Th2 type responses, depending on the type of activation signals received (Figure from [179]).

1.5.2 Antigen Presentation by Dendritic Cells

Antigens engulfed by DCs are processed and presented in complex with either MHC class I molecules, MHC class II molecules or CD1 molecules, depending on the nature of the antigen and the route of uptake.

1.5.2.1 MHC class I Pathway: The activation of CD8⁺ cytotoxic T cells by DC requires the presentation of peptides in the context of MHC class I molecules. The MHC class I antigen presentation pathway can be found in almost all cell types, allowing the display of endogenous peptides on the cell surface. This is central to the development of immune responses against intracellular pathogens, such as viruses.

MHC class I molecules are made up of an α chain associated with a β 2-microglobulin subunit. Folding of these molecules occurs in the endoplasmic reticulum (ER) and initially the α chain is joined to the chaperone protein calnexin, which is required for stable folding and assembly with the β 2 microglobulin subunit [186]. Once the β 2-microglobulin chain associates with the α chain, it forms a partly folded α : β 2 microglobulin complex, which then dissociates from calnexin. Subsequently, the partly folded molecules associate with a complex of proteins, containing calreticulin, Erp 57 and tapasin, known as the MHC class I loading complex. MHC class I molecules acquire peptides during their assembly within the ER. This endogenous pathway involves degradation of cytosolic proteins, initiated by labelling proteins via ubiquitin conjugation. This targets proteins to the proteasome, which consists of a catalytic subunit, 20S core particle, and two regulatory subunits, 19S regulatory particles. The proteasome recognises ubiquitinated proteins and degrades them in a process that is regulated in an ATP-dependent manner [187-189].

The degraded proteins are then translocated to the ER via transporters associated with antigen processing (TAP) 1 and 2 [190-191]. Binding of peptides to TAP1-TAP-2 induces a conformational change that stimulates ATP hydrolysis and provides the energy necessary for peptide transport [188, 192]. Studies have shown that peptide binding to the MHC class I molecules involves a process of initial binding, followed by peptide exchange and editing, in order to match the right peptides to the binding cleft [193]. It has been proposed that tapasin, a type I transmembrane glycoprotein, is responsible for peptide editing [194]. Erp57, a thiol oxidoreductase that forms a

disulfide bond with tapasin, is thought to maintain the sulphide bonds within MHC molecules during peptide loading [195].

The peptides which have access to the MHC class I presentation pathway are mostly newly translated polypeptides, including defective ribosomal products that are rapidly degraded by the proteasome into peptides of 2-25 amino acids in length [196]. In contrast to this, it has been proposed that newly synthesised polypeptides are protected from degradation immediately after translation and that the real source of the majority of peptides found on MHC class I molecules is actually pre-existing proteins [197].

As well as presenting peptides derived from endogenous sources, DC also have the ability to present exogenous peptides from the endocytic pathway to CD8⁺ T cells in the context of MHC class I molecules [198-199]. This phenomenon is known as 'cross-priming' or 'cross-presentation'. Two of the main pathways involved in cross presentation are the TAP-dependent and the TAP-independent pathways. The TAP-independent pathway is thought to involve peptide exchange in recycling endosomes or on the cell surface and a specific role for cathepsin S has been demonstrated [200]. However, the TAP-dependent pathway is thought to dominate and involves the transfer of antigens from endosomes to the cytoplasm. Cell or particle-associated antigens are more efficient at inducing cross-presentation and a subset of CD8⁺ DC found in the spleen are specialised for this function [157, 201]. Furthermore, it has been shown that the mechanism of uptake of soluble antigens may determine the method of presentation [202]. Improved understanding of this pathway may allow for vaccine targeting of antigen directly to the MHC class I pathway, allowing for the generation of CD8⁺ T cells.

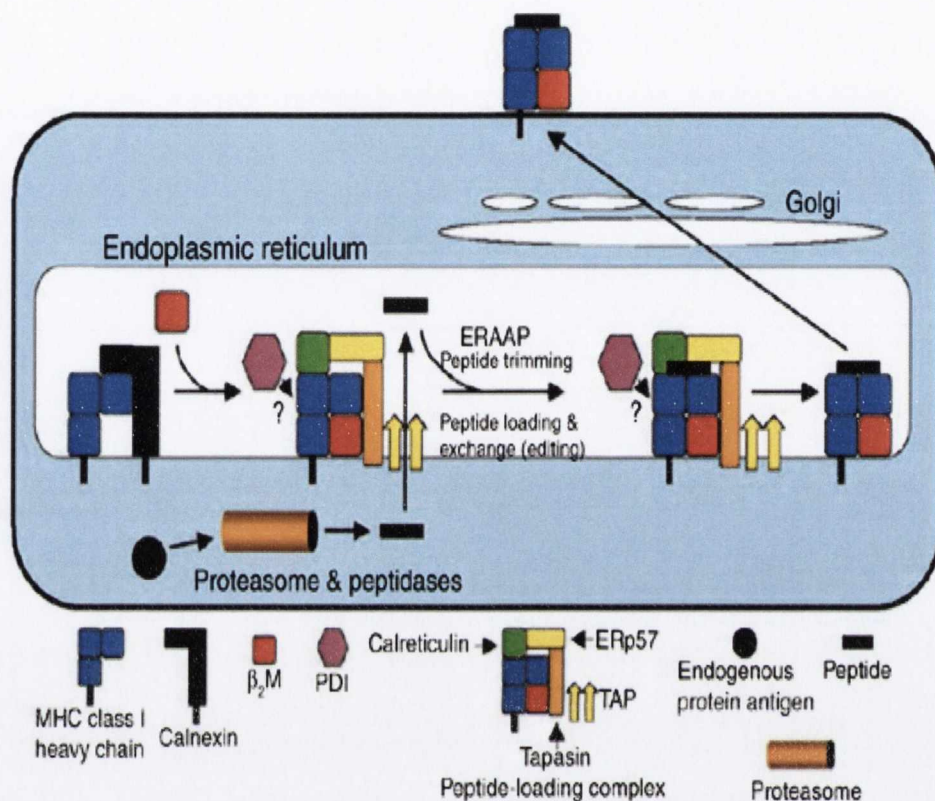


Figure 1.6: MHC class I presentation pathway. The MHC class I antigen presentation pathway can be found in almost all cell types, allowing the display of endogenous peptides on the cell surface which is important during viral infections. This endogenous pathway involves degradation of cytosolic proteins, initiated by labelling proteins via ubiquitin conjugation. The targeted proteins are then transported to the proteasome where they are degraded and translocated to the ER via TAP1 and 2 and subsequently loaded onto MHC class I molecules before transport to the cell surface for presentation (Figure from [186]).

1.5.2.2 The MHC class II presentation pathway: This pathway is only found in professional APC such as DC, B cells and macrophages. It allows exogenous antigens, phagocytosed by APC, to be presented to CD4⁺ T cells, resulting in the generation of T helper cell responses. Exogenous antigens captured by DC are directed towards lysosome-related intracellular compartments containing MHC class II molecules, known as MIIC or MHC class II-rich compartments [203-204]. While being transported to the MIICs, the phagocytosed proteins are degraded to peptides. This degradation is dependent on the fusion of the phagosome with lysosomes, which contain lytic enzymes, to form phagolysosomes, resulting in acidification and subsequent activation of enzymes necessary for antigen degradation, such as cathepsins [205]. The MHC class II molecules are heterodimers consisting of α and β chains that are assembled in the endoplasmic reticulum. During the assembly of these molecules, a type II transmembrane chaperone protein, known as the invariant chain (Ii), associates with the binding groove to prevent misfolding and promote stable assembly [206-207]. A series of proteolytic cleavage events eventually leaves a short peptide fragment, CLIP, which remains in the antigen-binding groove. Cathepsin S, a cysteine endoprotease, is responsible for cleaving the cytoplasmic domain of the Ii, which releases the MHC class II molecules from the endoplasmic retention signal [186]. The proteolytic degradation of Ii is controlled by the ratio between cathepsin S and its regulator cystatin C. During DC maturation, the levels of cathepsin S are increased, promoting the loading of antigenic peptides for subsequent presentation [208]. A protein known as H-2M (HLA-DM in humans) is also found in the MIICs, it helps to stabilise the empty MHC class II molecules and also catalyzes the removal of CLIP, while enhancing the binding of antigenic peptides.

In order for the MHC complex to be stable, it must be associated with a peptide, as empty MHC class II molecules are rapidly degraded. The available peptide pool comes from any protein that can gain access to the endosomal pathway and peptides are generated by the actions of endosomal proteases [186]. Once a stable complex has been formed between the peptide and the MHC molecules, the peptide:MHC II complex is transported to and expressed on the surface of the cell. Turnover rates and surface expression of MHC class II molecules on DC are regulated by ubiquitination of the cytoplasmic tails of the peptide-MHC complexes, therefore targeting them for degradation [209-210]. Furthermore, it was also found that ubiquitination of MHC II

molecules ceased in mature DC, allowing for the accumulation of MHC II:peptide complexes on the cell surface [210]. It has also been shown that TLR play an important role in the antigen presentation process, as signalling through TLR triggers efficient peptide loading of MHC II molecules and TLR-loaded antigens results in more potent activation of CD4⁺ T cells [211].

Autophagy, which is important to maintain cell homeostasis, is now also considered to be important for the delivery of antigens to MHC class II presentation pathways. Autophagy is considered to be a constitutively active process during which autophagosomes fuse with MIICs [212]. Also, targeting of proteins to autophagosomes is an efficient method of gaining access to the MHC II presentation pathway [212]. Therefore, through autophagy, endogenous or cytosolic antigens can be presented on MHC II molecules. The levels of autophagy within a cell can be upregulated by TLR signalling and certain cytokines suggesting interplay between TLR signalling, autophagy and antigen presentation [205, 213-214].

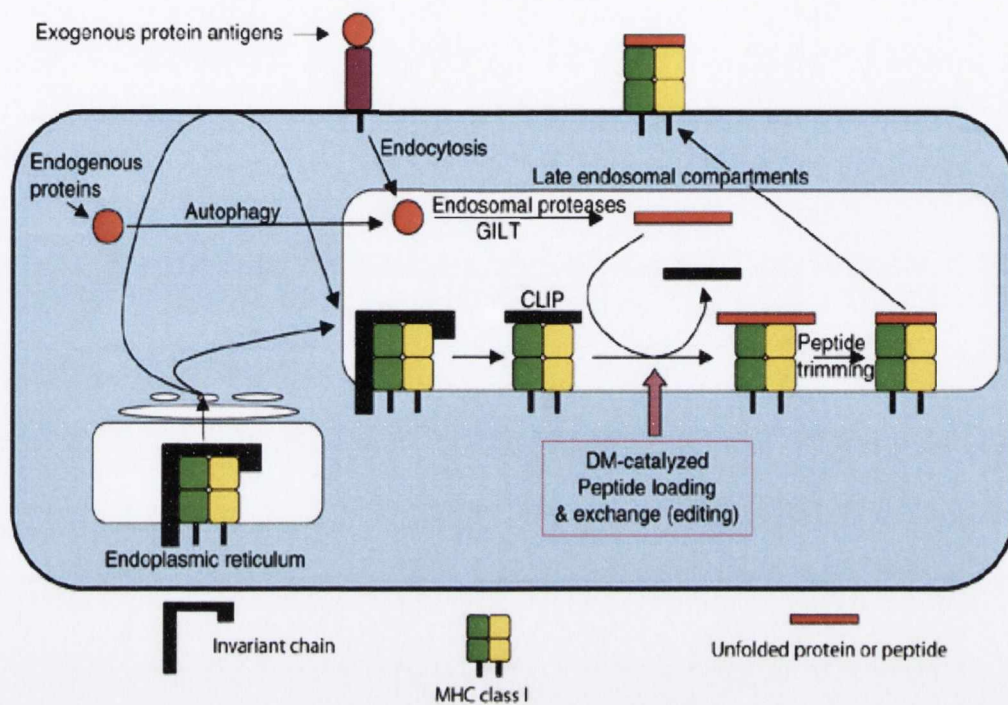


Figure 1.5: MHC class II presentation pathway. This antigen processing pathway allows exogenous antigens, phagocytosed by APC, to be presented to $CD4^+$ T cells. Exogenous antigens, captured by DC, are directed towards lysosome-related intracellular compartments containing MHC class II molecules, known as MIIC or MHC class II-rich compartments. CLIP is a short peptide which remains in the antigen-binding groove, in order to maintain stability of the MHC II complex, until HLA-DM catalyzes peptide loading onto the MHC class II molecules (Figure from [186]).

1.5.2.3 CD1 presentation pathway: CD1 proteins are a family of MHC-class-I-like glycoproteins that present lipids to T cells. They are made up of five isoforms, CD1 a-e. The structure of the CD1 molecules is similar to MHC class I molecules, consisting of one heavy chain with three globular domains that are non-covalently associated with a β 2-microglobulin subunit [215]. CD1a, b and c all present lipids to CD1-restricted T cells found within the $CD4^+$ and $CD8^+$ $\alpha\beta$ -T cell pool [216]. In contrast, only CD1d molecules present lipids to natural killer T-cells (NKT) which express an invariant TCR α chain and are innate like in their activities. Activation of NKT cells by DC via CD1 molecules results in upregulation of CD40L and IFN- γ secretion by the iNKT cells, which in turn further activates the DC [217-218]. α -galactosylceramide (α -GalCer) is a known adjuvant and also the most commonly used activator of NKT cells [219]. It is a glycolipid which is presented by CD1d-expressing APC and activates NKT cells to secrete IL-1 and IFN- γ [220]. Furthermore, it also has the potential to activate $CD8^+$ T cells via its CD1d presentation to NKT cells [221-222].

1.5.3 Dendritic cells link innate and adaptive immune systems: T cell priming

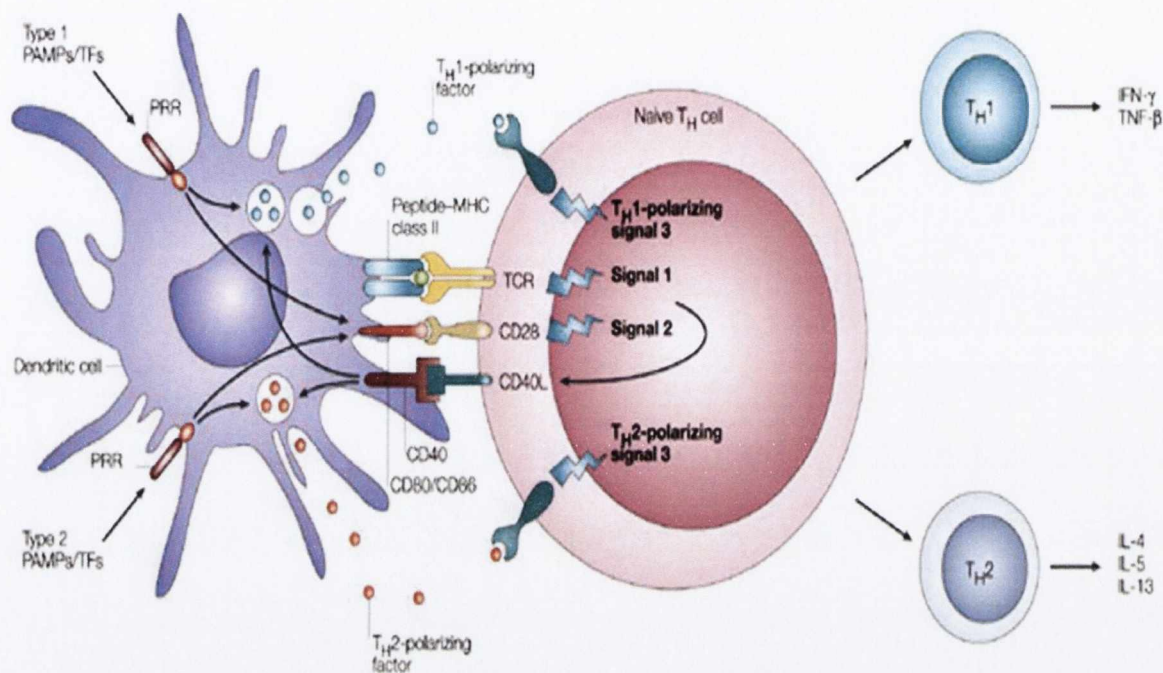
Mature DC are highly efficient at antigen presentation and express high levels of molecules required for interacting with T cells. DC have the ability to interact with and activate both $CD4^+$ and $CD8^+$ T cells through their expression of MHC class II and class I molecules, respectively. During antigen presentation, stable cell-cell junctions, known as immunological synapses (IS), are formed between DC and T cells [223]. These synapses involve interactions between, the MHC molecules expressed on the DCs and the T cell receptor (TCR), along with the CD4 or CD8 receptors on the T cell. In addition, co-stimulatory molecules, such as CD28 and LFA-1 can be found on the T cell surface and these interact with their cognate receptors expressed on activated DC. In order to fully activate naive T-cells, three signals must be provided (Figure 1.7), two of which can be found within the IS.

The pathogen-derived peptide:MHC complex, expressed on the surface of the activated DC, is recognised by the T-cell receptor (TCR). This interaction determines the antigen specificity of the response and constitutes for “signal one” [224]. In order to survive and proliferate, T cells require a co-stimulatory signal, the absence of which results in anergy and the induction of tolerance [224]. Co-stimulatory molecules, such as B7.1

and B7.2 (also known as CD80 and CD86) are upregulated on mature DC and these molecules interact with CD28 on the surface of the T cell providing “signal 2”. Other cell adhesion molecules, such as LFA-1 and ICAM-1/3, also play important roles during the interaction of the MHC-TCR. The $\alpha\beta$ TCR is associated with the CD3 complex, due to the fact that on its own the $\alpha\beta$ heterodimer is signalling incompetent. The CD3 complex consists of CD3 γ , δ and ϵ , proteins that span the plasma membrane. They contain specialised motifs for signalling, known as immunoreceptor tyrosine based activation motifs (ITAM), on their intracellular domains. These cell surface molecules are also associated with a zeta chain or ζ , which contains three ITAM on its intracellular domain and plays an important role in intracellular signalling after activation of the TCR. Ligation of the TCR results in activation of a complex intracellular signalling cascade, which begins with phosphorylation of these ITAM. This cascade ultimately results in the activation of transcription factors, such as NFAT, AP-1 and NF κ B increasing the transcription of interleukin-2 (IL-2).

Cytokines can be secreted by the activated DC, but also by other innate cells, such as granulocytes or cells in the surrounding tissue, and these can determine the fate of the naive T cell. The cytokines provide “signal 3” and the balance of these can have a strong impact on the type of immune response generated [182]. The cytokines secreted are determined by the type of pathogen-derived molecules or endogenous danger signals sensed by the DC. For example, IL-12 is a well known Th1 polarising cytokine. Other Th1-polarising factors include membrane bound factors, such as ICAM-1 and delta-like Notch ligands [225-228]. In contrast, stimulation of DC *in vitro* and *in vivo* with the egg stage of *Schistosoma mansoni* results in the development of Th2 T cells [229-231]. It is not just the presence of one key cytokine that decides the fate of a naive T cell, it is the balance of all cytokines present in the local micro-environment at the time of activation.

The T cell must receive all three signals in order to become fully activated. For example, failure to provide signal 2 and 3 in the presence of signal 1 can inactivate T cells by inducing anergy, cell death, or alternatively, the induction of tolerance by diversion into a regulatory fate [232]. In addition to the requirement for all three signals in order to fully activate naive T cells, it has been proposed that in order to induce optimal responses, all three signals must be received from the same DC that has been directly activated by the MAMP or danger signal [232].



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Figure 1.7: Activation and polarisation of T cells requires the integration of three signals. Signal one is derived from ligation of the TCR by the MHC-peptide complex and generates the antigen specificity of the immune response. Co-stimulatory molecules, such as CD28 and its ligands CD80/CD86, provide the second signal which is necessary for survival and proliferation of the T cell. The third signal is received from cytokines, the balance of which is central to the type of response that is developed. The type of cytokines will depend on the activation stimulus received by the DC, such as MAMP and endogenous danger signals (Figure from [224]).

1.6 The Adaptive Immune Response

The innate immune system is the first line of defence and has the ability to sense the presence of invading pathogens or dying or stressed cells via an array of PRR. DC act as the link between the innate and adaptive immune responses. The main cells of the adaptive immune response are the T cells and B cells. Studies carried out by Cooper and Good suggested that there were two subsets of lymphocytes in the bursa of Fabricius of chickens, one of which was necessary for antibody production (B cells) and another which required an intact thymus for their development (T cells) [233-234]. The adaptive immune response executes two major types of responses, cell-mediated immunity and humoral immunity. Activation of T cells, generates cell-mediated responses, while B cells develop into antibody-producing cells, resulting in humoral immune responses

1.6.1 T cells

T cells are a subset of lymphocytes and one of the main effector cells of the adaptive immune system. They are defined based on their development in the thymus and the presence of a heterodimeric receptor, known as the T cell receptor (TCR), expressed on their cell surface in association with CD3 molecules. T cells have several effector functions, including the provision of help to B cells to produce antibodies, activation of macrophages and killing of cells infected with intracellular pathogens, such as viruses. T cells can be subdivided based on their co-receptor expression and their effector functions. Helper T cells (Th) express CD4 on their cell surface, which acts as a co-receptor for MHC class II molecules. These cells provide help for B cells and activate macrophages to kill intracellular bacteria. Cytotoxic T cells (CTL) express CD8 on their surface, which is the co-receptor for MHC class I molecules and these cells kill virally-infected cells. Each T cell expresses a TCR that is generated by somatic gene segment re-arrangement. Most of the T cells generated possess a TCR made up of α and β chains, so-called $\alpha\beta$ T cells. However, there is also a small subset of T cells which express a $\gamma\delta$ TCR and are CD4⁻/CD8⁻; these cells are thought to play a role in immunoregulation. Once the gene re-arrangement is complete, the $\alpha\beta$ T cells undergo selection based on their recognition of self-MHC molecules and their non-

responsiveness to self antigens. Mature T cells then enter the periphery via the bloodstream and are carried to the spleen and lymph nodes, where they can encounter APC [235]. Naive $CD4^+$ T cells have the potential to develop into one of several identified effector lineages, including Th1, Th2, Th17, regulatory T-cells and follicular T-helper cells (Tfh), during their initial interaction with antigen presented on MHC class II molecules on the surface of DC (Figure 1.8).

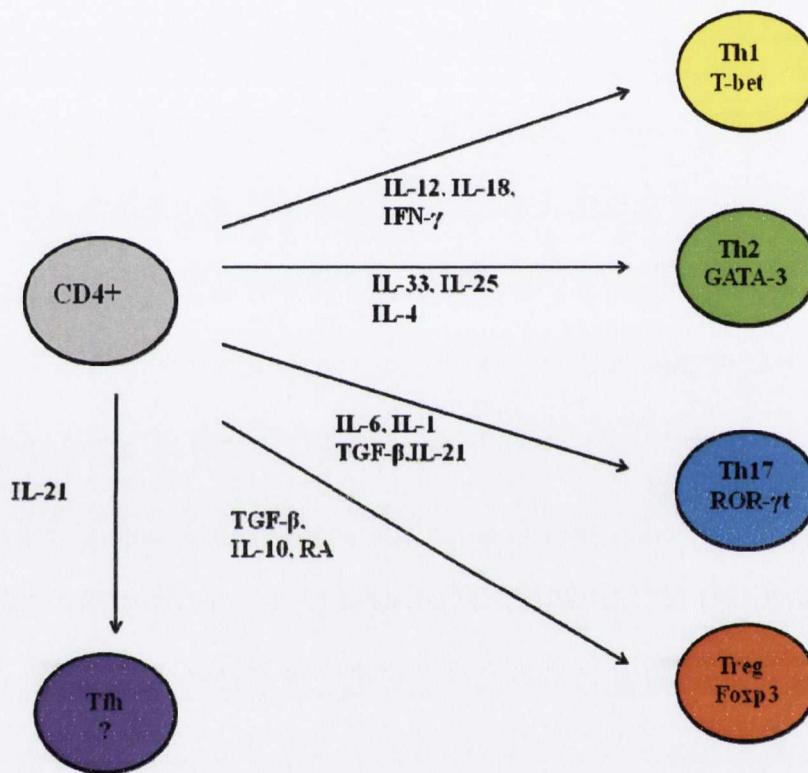


Figure 1.8: The fates of $CD4^+$ T cells. $CD4^+$ T cells have the potential to differentiate into different effector subsets. Th1 cells are induced via IL-12 and IL-18 and uniquely express the transcription factor T-bet. Th2 cells develop in the presence of cytokines, including IL-33, IL-25 and IL-4, which upregulates transcription factor GATA-3. Th17 cells are generated with a combination of IL-6, TGF- β and IL-21 and are associated with ROR- γ t expression. Exposure to IL-10 and TGF- β results in a tolerogenic phenotype and the generation of regulatory T-cells associated with the transcription factor, Foxp3. IL-21 induces development of follicular helper T cells (Tfh).

1.6.1.1 Th1

In 1986 it was formally shown that murine CD4⁺ T cells could be subdivided into Th1 and Th2 subsets based on their cytokine profile [236]. However, the existence of these sub-populations was not shown in humans until the 1990s, using samples from patients with allergic or infectious diseases [237]. T cells were isolated from immunised mice that secreted, IFN- γ , IL-2, IL-3 and GM-CSF and promoted the production of IgG2a antibodies by B cells in response to antigen or Con A. These were designated Th helper 1 (Th1) cells [236]. Th1 cells are generally associated with intracellular pathogen infections, such as *Mycobacterium tuberculosis*, autoimmune disorders and the development of Th1 cells is promoted by IL-12, the main source of which are DC and macrophages. The induction of IL-12 secretion by DC is induced by TLR stimulation. NK cells have been shown to act as an early source of IFN- γ , which can further enhance the level of DC-produced IL-12, promoting the development of Th1 cells [238]. IL-12R ligation on the surface of T cells results in the activation of STAT-4 and T-bet (T-box expressed in T cells), subsequently activating the transcription factors NF κ B and nuclear factor of activated T cells (NFAT), resulting in an increase in the expression levels of IFN- γ and a decrease in IL-4 production [239]. More recently, it has been shown that T-bet and STAT4 also co-operate in the transcription of Th1-specific genes [240]. Runx 3 is a member of the runt domain transcription factors which function mainly in cell-specific lineage decisions. One of the transcriptional targets of T-bet is the Runx3 gene. Together Runx3 and T-bet bind to the silencer region within the *Il4* gene and thereby repress transcription. In addition, this complex of T-bet and Runx3 also bind to the *Ifn γ* promoter, which enhances a positive feedback loop through the secretion of IFN- γ [241-242]. A role for IRF-1 (interferon response factor-1) in Th1 cell differentiation has been shown through its ability to regulate the expression of the IL-12R β 1 subunit and the p40 and p35 subunits of IL-12, thereby enhancing both the secretion of IL-12 and responsiveness of T cells to IL-12 [243].

While many of the earlier studies into Th1 differentiation highlighted the key role played by IL-12, it is also important to note that Th1 responses *in vivo* to certain antigens can occur in an IL-12-independent manner. For example, mice with a targeted disruption of the IL-12 p40 subunit generated a strong Th1 type response, characterised by high levels of IFN- γ and the absence of IL-4, when infected with mouse hepatitis virus (MHV) [244]. Another study demonstrated that when mice defective in IL-12

were immunised with *Toxoplasma gondii* tachyzoites, a significant number of Th1 cells were detected [245]. Based on these and other studies, it has been suggested that the role of IL-12 is not as the primary inducer of Th1 responses, but as an enhancer of effector cells already committed to Th1 differentiation [246-247].

The Notch proteins are a family of four (Notch 1-4) heterodimeric cell surface receptors, all of which have been shown to be expressed on CD4⁺ T cells [248]. The ligands for these receptors can be divided into two families, the Delta-like ligands (DLL) and the Jagged proteins [246]. Binding of ligands to the Notch receptors results in cleavage of the intracellular domain of the Notch protein and their subsequent translocation into the nucleus, where they associate with the DNA-binding factor recombination-signal-binding protein for immunoglobulin κ J region (RBPJ) and the co-activator Mastermind-like 1 (MAML-1) [249]. Stimulation of APC with known Th1-driving stimuli, such as CpG and LPS, has been correlated with enhanced expression of DLL proteins [227-228]. In addition, Notch signalling may directly activate *Tbx21*, which is the gene that encodes T-bet [250-251]. A regulatory role for ICAM-1 and LFA-1 in the Th1/Th2 fate decision has also been proposed [226]. Once Th1 cells are fully differentiated they secrete their signature cytokine IFN- γ , as well as IL-2 and TNF- α . The effector functions of IFN- γ include activation of macrophages and induction of IgG2a antibodies by B cells, which are characteristic of a type 1 response.

1.6.1.2 Th2

The second subset of cells identified by Mossman *et al* secreted IL-3, IL-4, IL-5 and enhanced B cell production of IgE and IgG1 [236]. These cells were given the name T helper 2 cells (Th2). Th2 cells are generally associated with protection against extracellular pathogens, such as *Schistosoma mansoni* and allergic reactions. IL-4 is the signature cytokine of Th2 cells and it is essential for their development from naive precursors. However, the source of IL-4 is still debated [252]. Furthermore, Th2 cells have been shown to develop in the absence of IL-4 [253]. In support of this, it has been shown *in vivo* that the absolute numbers of Th2 cells generated after helminth infection in STAT6-deficient mice (STAT6 is an essential part of the IL-4R signalling pathway) were comparable to those in wild type mice [254]. In a similar manner to IL-12, it has been proposed that IL-4 is necessary for the enhancement of Th2 responses but is not

the primary inducer of Th2 differentiation [246]. A role for IL-25 (also known as IL-17E) in Th2 cell development has also been proposed, as it can enhance IL-4, IL-5 and IL-13 secretion by signalling through IL-17RB [255-258]. Similar to IFN- γ , IL-4 acts in an autocrine manner to promote Th2 cell development and has an inhibitory effect on the development of Th1 cells.

Another cytokine associated with type 2 immune responses is IL-33 (IL-1F11), which is a member of the IL-1 family of cytokines. IL-33 has been shown to associate with the orphan receptor ST2 and has the ability to promote IL-5 and IL-13 secretion by Th2 polarised cells *in vitro*. Also, immunisation with purified IL-33 induces secretion of Th2-associated cytokines and eosinophilia [259]. Thymic Stromal Lymphopoeitin (TSLP), which is secreted by stromal cells, was originally shown to act as a B cell growth factor. It is expressed at high levels in the lungs of mice sensitised and challenged with Ova. These mice develop a Th2-type response and lung eosinophilia, both of which have been found to be reduced in TSLP-R knock-out mice, suggesting a role for TSLP in the development of Th2 responses [260].

The binding of IL-4 to its receptor results in the activation of the transcription factor STAT-6, which subsequently induces the expression of GATA-3 and c-Maf, ultimately resulting in the upregulation of IL-4 and IL-5 and downregulation of IFN- γ [261-262]. GATA-3 is a zinc finger protein which was originally found to bind to the TCR α gene enhancer, however, it is now known as the master regulator of Th2 cell differentiation. It is thought to mainly influence Th2 cytokine expression by reorganising the Th2 locus, rather than promoting proliferation of the cells [263-264]. Furthermore, GATA-3 inhibits Th1 cell differentiation by preventing upregulation of the IL-12R β chain, rendering the cells unresponsive to IL-12 and sensitive to the presence of IL-4 [263]. Other transcription factors proposed to play a role in Th2 differentiation include NFAT-1, JunB and IRF-4, all of which have been shown to enhance *Il4* gene transcription [265-266]. More recently, Dec2, a helix-loop-helix transcription factor, has been implicated in Th2 cell differentiation, as Dec2-deficient mice show reduced Th2 responses following intraperitoneal injection with *Schistosoma mansoni* eggs [267]. APC which have been stimulated by Th2 driving factors, such as *Schistosoma mansoni* egg antigen (SEA), upregulate their expression of Jagged proteins [231]. Furthermore, Notch signalling has been shown to be sufficient to drive the differentiation of Th2 cells in the absence of STAT6, in a GATA3-dependent manner. Signalling through Notch

has been implicated as a requirement of Th2 differentiation, upstream of GATA-3, further enhancing IL-4 transcription [268-269].

1.6.1.3 Th17

The recent discovery of an IL-17-producing subset of CD4⁺ T cells has provoked a rethink of the traditional Th1/Th2 paradigm. Langrish *et al* first reported the existence of a set of IL-17-secreting CD4⁺ T cells driven by IL-23. The cells secreted IL-17, IL-6 and TNF- α and were shown to be responsible for pathogenicity in experimental allergic encephalomyelitis (EAE), an inflammatory disease of the central nervous system often used as the animal model for multiple sclerosis in humans [270]. These cells have since been termed T-helper IL-17 cells or Th17 cells. IL-17, also known as IL-17A, is the founding member of the IL-17 family of cytokines, which include IL-17A-F, and has been shown to be involved in enhancing the production of chemokines and matrix proteins to recruit neutrophils [271].

After the initial discovery of Th17 cells, little was known about the signalling pathways involved in their development. As a result the idea that these cells were a separate T cell lineage was controversial and it was suggested that they had diverged from the Th1 lineage at some point during their development. The divergence theory was supported by the fact that T-bet-deficient mice were resistant to EAE, an autoinflammatory disease associated with IL-17 [272]. In addition, it was also found that the IL-23R was not expressed on naive T cells, despite previous reports that IL-23 was the initial driving factor for the differentiation of IL-17-producing T cells [273-274]. In contrast, a series of experiments by Park *et al* provided new evidence, which supported the idea that IL-17 producing T cells were, in fact, a distinct lineage of T helper cells. They demonstrated that a higher number of IL-17-producing T cells could be isolated from T-bet deficient mice [275]. Work by Harrington *et al* added further support to the Th17 lineage theory by showing that IFN- γ inhibited the secretion of IL-17 from naive T-cells but that fully differentiated IL-17-producing cells were unaffected by the presence of IL-4 or IFN- γ [276]. Moreover, further studies have identified ROR- γ t as the master transcription factor required for the development of Th17 cells [277]. A role for STAT3 in Th17 development has also been demonstrated [278-279]. Key cytokines involved in the development of Th17 cells, including IL-6 and IL-23, induce STAT3 activation

which subsequently binds to the *Il17* locus [280]. In addition to ROR γ t, a role for IRF4 has also been proposed in the differentiation of Th17 cells based on the reduced levels of ROR γ t expression in the absence of IRF4 [281].

The identity of the key cytokines involved in the differentiation of Th17 cells is somewhat controversial, but knowledge of this area is expanding rapidly. TGF- β , IL-6, IL-1 β and IL-23 are all thought to be essential for the development of Th17 cells in humans [282-283]. In mice, TGF- β , IL-21 and IL-6 are thought to be involved in the initiation of Th17 cell differentiation [284-286]. It was reported that IL-6 and TGF- β induced the production of IL-17 from naïve T cells activated with anti-CD3 and anti-CD28, and Th17 cells were absent or diminished in all tissues of TGF- β -deficient mice [285-286]. Furthermore, it was demonstrated that mice expressing a dominant negative form of the TGF- β receptor were resistant to EAE and were deficient in Th17 cells [284]. Several studies have indicated a role for IL-6 in inflammation, IL-6-deficient mice are resistant to actively induced EAE and Th17 cells are absent in the lamina propria of mice lacking in IL-6 or ROR γ t [277, 287]. Thus, it was suggested that TGF- β and IL-6 acted collectively to induce the differentiation of murine Th17 cells. Recently, it has been shown that, TGF- β promotes the differentiation of Th17 cells by inhibiting the development of Th1 and Th2 cells in both mice and humans [288-289]. TNF α and IL-1 have been implicated in increasing the differentiation of Th17 cells in the presence of IL-6 and TGF- β [286]. The role for IL-1 was further supported by the fact that, induction of Th17 cells is abrogated in IL-1R1 knock-out mice and complemented by the fact that these mice have a lower incidence of EAE than wild type mice [290]. Furthermore, it has also been shown that IL-1 and IL-23 activated $\gamma\delta$ T cells promote the secretion of IL-17 by CD4⁺ T cells [291]. In addition, a more recent study has shown that IL-1 signalling in T cells regulates the expression of ROR γ t and IRF4 [292]. Models of autoimmune disorders in mice deficient in ICOS and IL-23 have shown that these two factors are necessary for IL-17 production [293-294]. It is now thought that both IL-1 and IL-23 play important roles in the maintenance of Th17 cells and the development of memory Th17 cells.

IL-17 has been implicated in barrier functions by inducing growth, differentiation and integrity of epithelial surfaces and Th17 cells exist constitutively in the intestinal lamina propria [295]. A protective role for Th17 cells has been reported during infection with the extracellular bacteria *Klebsiella pneumoniae* [296-297]. Furthermore, IL-17 reduces Th1-mediated parasite-induced myocarditis during infection with *Trypanosoma cruzi* [298]. It has also been demonstrated that, effective protection against *Staphylococcus aureus* and *Candida albicans* requires IFN- γ and IL-17 [299]. In contrast, activation of Th17 cells has been shown to prevent the generation of protective Th1 responses against *Candida albicans* and *Aspergillus fumigatus* [300]. Therefore, the role of Th17 cells in certain infections, such as *Candida albicans* infections, remains controversial.

1.6.1.4 Regulatory T cells

The idea that thymic-derived cells had the ability to both activate and suppress the development of immune responses was first proposed in the 1970s [301]. However, it was not until 25 years later that thymic-derived cells with the ability to induce tolerance were identified. Sakaguchi *et al* were the first to describe a set of naturally occurring CD4⁺ T cells that could prevent the development of autoimmunity by inhibiting the expansion of self-reactive lymphocytes. They showed that the depletion of a population of CD4⁺ CD25⁺ cells, which constitute 5-10% of CD4⁺ T cells in mice, resulted in the spontaneous development of auto-inflammation. Furthermore, this auto-inflammation could be reversed by reconstitution of the CD4⁺ CD25⁺ cells [302]. In agreement, others have also reported aberrant T cell responses, characterised by excessive Th1 and Th2 cytokines resulting in autoimmunity, in mice deficient in these 'regulatory' T cells [303-304]. The existence of a set of T cells, now known as regulatory T cells (Tregs), with suppressive activity is currently widely accepted. Regulatory T cells can be broadly subdivided into two subsets, natural Tregs (nTregs) and inducible Tregs (iTregs) [305-306].

The CD4⁺ CD25⁺ T cells described by Sakaguchi *et al* are representative of natural T regulatory cells. These cells develop in the thymus and play a role in controlling self-reactive T cells in the periphery. It has recently been shown that TGF- β is required for their development in the thymus, as mice lacking TGF- β R1 have lower levels of naturally occurring CD4⁺CD25⁺Foxp3⁺ thymocytes [307]. Natural Tregs can be

defined by their constitutive expression of Foxp3 and CD25 (IL-2R α chain). However, both Foxp3 (intracellular) and CD25 (surface) are expressed by activated T cells, making it difficult to isolate regulatory T cells from other effector lineages [308].

Inducible Tregs (iTregs) develop in the periphery following TCR stimulation in the presence of high levels of TGF- β and the absence of any other pro-inflammatory cytokines, resulting in subsequent induction of Foxp3 expression [309-311]. These converted Foxp3⁺CD25⁺CD4⁺ cells were found to be unresponsive to TCR stimulation and displayed suppressive functions when co-cultured with other T cells [309]. The induction of Foxp3 by TGF- β requires the involvement of additional transcription factors, including STAT3 and NFAT [306, 312]. In addition to Foxp3⁺ converted Tregs, the inducible Tregs, also include Tr1 cells which secrete IL-10 and T-helper 3 cells induced during oral tolerance which secrete TGF- β , both of which are Foxp3⁻ [313]. Tr1 cells can develop in several ways; naive T-cells can convert to a regulatory phenotype following an encounter with an immature DC, they can be induced by specific pathogens as a method of evasion or alternatively can be generated by repeated exposure to antigen in the presence of IL-10, conditions which are associated with chronic infections [314].

Foxp3, a member of the forkhead/winged helix family of transcriptional regulators, is currently the main transcription factor associated with the regulatory phenotype. It was discovered as the gene mutated in scurfy mice, which spontaneously develop autoimmunity and severe inflammation [315]. Subsequently, it was shown that the immune dysregulation observed in scurfy mice was due to a deficiency in Tregs and not due to an intrinsic fault in CD4⁺CD25⁻ T cells [316-317]. Furthermore, ectopic expression or retroviral gene transfer of Foxp3 was sufficient to convert CD4⁺CD25⁻ T cells to a regulatory phenotype [316-317].

In addition to Foxp3, several other transcription factors have been implicated in the development and maintenance of both natural and inducible Treg cells. One of the members of the runt domain transcription factors, Runx1, has been suggested to be necessary for the maintenance of Foxp3 expression in both natural and inducible Treg cells (ref as unpublished data in [318]). An unlikely co-factor necessary for the differentiation of Treg cells has recently been identified. Several groups have found that the presence of retinoic acid (RA), a metabolite of vitamin A, was necessary for the

differentiation of Tregs induced by a specialised subset of DC isolated from the gut-associated lymphoid tissue (GALT) [319-321].

The exact methods of suppression employed by Tregs to prevent activation of effector lineages or control self-reactive CD4⁺ T cells are unclear. However, certain central molecules involved in the effector function of Tregs have been identified, including CTL-associated protein 4 (CTLA-4), found to be constitutively expressed at high levels by CD4⁺CD25⁺ T cells in normal mice. In addition to CTLA-4, the cytokines, IL-10 and TGF- β have been suggested to play a role in the suppressive functions of Tregs [322-323]. Recently, a multi-step process of suppression was proposed by Sakaguchi *et al* [324]. The first step requires cell-cell contact between Tregs and APC during which Tregs form aggregates around DC more efficiently than effector T cells, via an LFA-1-dependent and CTLA-4-independent mechanism. Therefore, preventing access of responder T cells to MHC class II presented antigen on the surface of APC. The second step involves the down-regulation of co-stimulatory molecules on the surface of DC, including CD80 and CD86, which occurs in a CTLA-4-dependent manner [324]. Sakaguchi *et al*, propose that this multi-step method may represent the core method of suppression and that it may function in unison with additional mechanisms of suppression that have been identified, such as the ability of Tregs to lyse cells through their expression of perforin or granzyme [233, 324].

Currently, Foxp3 is the most definitive marker of nTregs. However, it is still difficult to distinguish between converted Foxp3⁺ iTregs and nTregs. Foxp3 is also upregulated in activated T cells [308]. In addition, T cells with regulatory functions, such as Tr1 and Th3 cells do not express Foxp3. The lack of a definitive marker for regulatory T cells prevents them from being fully identified as a distinct T cell lineage rather than just a transient set of cells that have diverged from other T cell lineages.

1.6.1.5 Tfh

The idea that B cells required help from T cells in order to secrete significant levels of high affinity antigen-specific antibodies was first shown in the 1960s [325]. It was initially thought that this important role was played by Th2 cells, due to their ability to secrete large amounts of IL-4, which is known for its ability to drive class switch recombination and B cell proliferation [326]. However, it was not until the beginning of this century that a subset of CD4⁺ T helper cells that specialises in B cell help was identified. These cells were isolated from human blood and tonsillar tissue and were characterised by their high level of CXCR5 (the receptor for the chemokine CXCL13) expression. CXCL13 is specifically secreted by follicular stromal cells, thus attracting these cells to follicular regions of lymph nodes [327-328]. Furthermore, co-culture of these CXCR5⁺ CD4⁺ T cells with B cells induced activation and subsequent production of antibodies by B cells [327-328]. Based on their ability to provide B cell help and their anatomical localisation these CXCR5⁺ CD4⁺ T cells were termed follicular helper T cells (Tfh) [327-328]. Recent studies have characterised Tfh cells in more detail and have shown that they express high levels of BCL6, programmed cell death-1 (PD-1), Inducible co-stimulator (ICOS) and IL-21 [329]. While no unique transcription factor has been identified for this novel subset of Th cells, studies have shown that the high level of BCL6 expressed in these cells is a unique feature which cannot be seen in any of the other Th cell subsets [330-331]. In addition to this, it has been shown that forced overexpression of BCL6 in CD4⁺ T cells represses the differentiation of Th1 and Th17 cells. This effect is due to the binding of BCL6 to the promoters of Th1 and Th17 transcription factors, Tbet and ROR γ t respectively, thus suppressing their transcription [330-331]. Taken together, these studies suggest that BCL6 may act as the master regulator in the Tfh differentiation pathway.

It was initially thought that Tfh cells were unable to secrete significant levels of cytokines. However, recent studies have shown that Tfh cells have the ability to secrete high levels of IL-21, as well as IL-4 and IL-17 [329, 332-334]. IL-21 is thought to play a key role in the development of Tfh cells, as immunisation of IL-21^{-/-} mice with a T cell-dependent antigen results in a reduced frequency of CXCR5⁺ CD4⁺ T cells, in comparison to wild type mice [335]. Adding further complexity to the differentiation of Th subsets, a study by Tsuji *et al* showed that Foxp-3⁺ CD4⁺ cells were preferentially

converted into Tfh cells associated with a down-regulation of Foxp-3 and increased expression of IL-21, PD-1 and CXCR5 [336]. Furthermore, the increased expression of PD-1, IL-21 and CXCR5 in Tfh cells observed occurred in a B cell-dependent manner via CD40 interaction, suggesting a role for B cells in the differentiation of Tfh cells [336]. It has also been proposed that Tfh cells are preferentially generated from CD4⁺ T cells that express a TCR with high affinity for the antigen [337-338].

The main functions of Tfh cells is providing 'help' to B cells within follicles of secondary lymphoid organs, which is necessary for the progression and development of the humoral immune response. Their ability to produce large amounts of IL-21 is key to this activity, as IL-21 is a potent driver of B cell proliferation, isotype switching and differentiation into plasmablasts through its enhancement of BLIMP-1 [339]. However, if these cells are not tightly regulated they can contribute to the development of autoimmune diseases such as Systemic lupus erythematosus (SLE) where high levels of CXCR5⁺ CD4⁺ T cells expressing increased levels of PD-1 and ICOS can be found in the blood of patients [340]. A better understanding of the functions and differentiation of Tfh cells would potentially lead to the development of therapies for autoimmune disorders involving autoantibody formation and also aid the design of new vaccine adjuvants with the potential to promote Tfh development.

1.6.1.6 Plasticity among T helper subsets

The original experiments by Mosmann *et al* identified the existence of a division of labour among CD4⁺ T cells. They isolated stable mouse clones from immunised mice that produced a mutually exclusive set of cytokines which they deemed Th1 and Th2 cells. In addition, these cells were grown *in vitro* during which they were able to maintain expression of a unique cytokine profile [236]. Later studies confirmed the stability of these founding lineages by demonstrating that naive cells polarised towards a Th1 or Th2 phenotype could not be forced to change their cytokine profile even in the presence of new polarising cytokines [341]. The identification of master transcriptional regulators unique to specific subsets of Th cells helped to solidify the idea that each Th subset represents a distinct terminally differentiated lineage characterised by the expression of a unique master transcriptional regulator coupled with a distinct cytokine profile.

More recently however, the lineage model has been challenged. Several groups have reported the existence of $\text{Foxp3}^+\text{IL-17}^+\text{CD4}^+$ T cells which have been shown to have suppressive activities [342-343]. In addition, a set of $\text{Foxp3}^+\text{ROR}\gamma\text{t}^+\text{CD4}^+$ T cells have been isolated from the lamina propria of the small intestine and this dual expression of transcription factors has been mimicked *in vitro* using TGF- β [343]. Based on these studies it has been proposed that these $\text{Foxp3}^+\text{ROR}\gamma\text{t}^+\text{CD4}^+$ T cells have the ability to differentiate into either Tregs or Th17 cells and that this decision is determined by the presence of pro-inflammatory cytokines such as IL-6, IL-21 and IL-23. Furthermore, others have shown that $\text{Foxp3}^+\text{CD4}^+$ T cells can be converted into IL-17-producing $\text{ROR}\gamma\text{t}^+$ T cells upon co-culture with DC in an IL-23 dependent manner [344].

The differentiation of helper T cells into specific effector cells is intricately coupled to epigenetic changes in chromatin structure, histone and DNA modifications and the expression of small non-coding RNA [318, 345]. A recent study by Wei *et al* used genome-wide analysis to track methylation states of histones within T helper subsets in order to identify areas of DNA which were in either in a permissive or inactive state [345]. Interestingly, they found that in some T cell subsets certain genes are in what they called a bivalent state, meaning that they could be either transcribed or left in an inactive state depending on signals received from the local microenvironment. In agreement with previous findings by Krawczyk *et al* they have shown that the genes encoding Gata3 and Tbet were shown to have bivalent marks in Th2 and Th1 cells respectively, further highlighting the idea of interconversion between subsets in certain inflammatory conditions [345-346]. Treg cells were found to contain bivalent marks in several genes including *Tbx21*, *Gata3* and *Roryt*, consistent with studies demonstrating the ability of Tregs to be converted to other effector Th subsets [344-345]. Taken together, all of these studies suggest the existence of plasticity among CD4^+ T helper cells which has previously been undemonstrated and unappreciated.

1.6.2 B cells

The existence of a set of antibody-producing cells had been predicted since the discovery of serum gammaglobulins [347]. B cells can be defined as, a subset of cells that express clonally diverse cell surface immunoglobulin (Ig) receptors recognizing specific antigenic epitopes [348].

There are five main classes of antibodies, IgM, IgD, IgG, IgA and IgE, all of which have specific roles in the humoral response. Antibodies are made up of a light chain associated with a heavy chain, each of which consists of constant and variable regions. It is the constant region of the heavy chain which determines the isotype or class of the antibody and subsequently their effector functions, by interacting with other molecules and cells of the immune system via Fc receptors. The variable region of the heavy and light chains form the antigen-binding region or Fab of the antibody and it is the variability among these regions that add to the diversity of antibody specificity. The type of cytokines secreted by T cells can direct the type of antibodies produced by triggering class switch recombination within B cells in the germinal centre. For example IL-4 has been shown to induce increased expression of activation-induced cytidine deaminase (AID), an enzyme necessary for the heavy chain switch [349].

Antibodies have several functions within the immune system. They can bind to the surface of a microorganism, preventing cell invasion as well as binding to substances secreted by microorganisms such as toxins in a process known as neutralisation. Secondly, antibodies can coat the surface of an organism in a process known as opsonisation which promotes uptake by Fc receptors on phagocytic cells, which recognise the constant portion of the antibodies. Thirdly, antibodies can activate the complement system, resulting in the lysis of micro-organisms through pore-forming complement proteins. Antibodies are the main effector proteins of the humoral immune response and are important in defence against extracellular micro-organisms.

The surface receptor of the B cell is known as the B cell receptor (BCR) and consists of a membrane bound immunoglobulin M (IgM) associated with invariant Ig α and Ig β chains. The invariant Ig α and Ig β chains contain a single ITAM on their intracellular domains, which is necessary for signalling. Similar to T cells, B cells express a co-receptor composed of CD19, CD21 and CD81 [350]. Furthermore, the CD21/CD19 components of the co-receptor have been shown to be important for B cell survival

[350]. Recognition of the specific antigen epitope by the BCR triggers a complex intracellular signalling cascade, which begins with phosphorylation of the ITAM and ultimately leads to the transcription of specific genes necessary for antibody production.

B cells develop from haematopoietic stem cells within the foetal liver and adult bone marrow [351]. Immature B cells can be characterised by their short half lives, their expression of membrane bound IgM and their tendency to undergo apoptosis following BCR ligation [352-353]. Maturation occurs in the periphery and is accompanied by upregulation of membrane-bound IgD and the ability to proliferate in response to antigen stimulation [354-355]. Once immature B cells leave the bone marrow and enter the periphery their maturation involves several stages during which they 'home' or migrate to specific anatomical sites and develop into one of three subsets (Figure 1.9); B1 cells, marginal zone B cells or follicular B cells [354-355].

Bone Marrow

Periphery

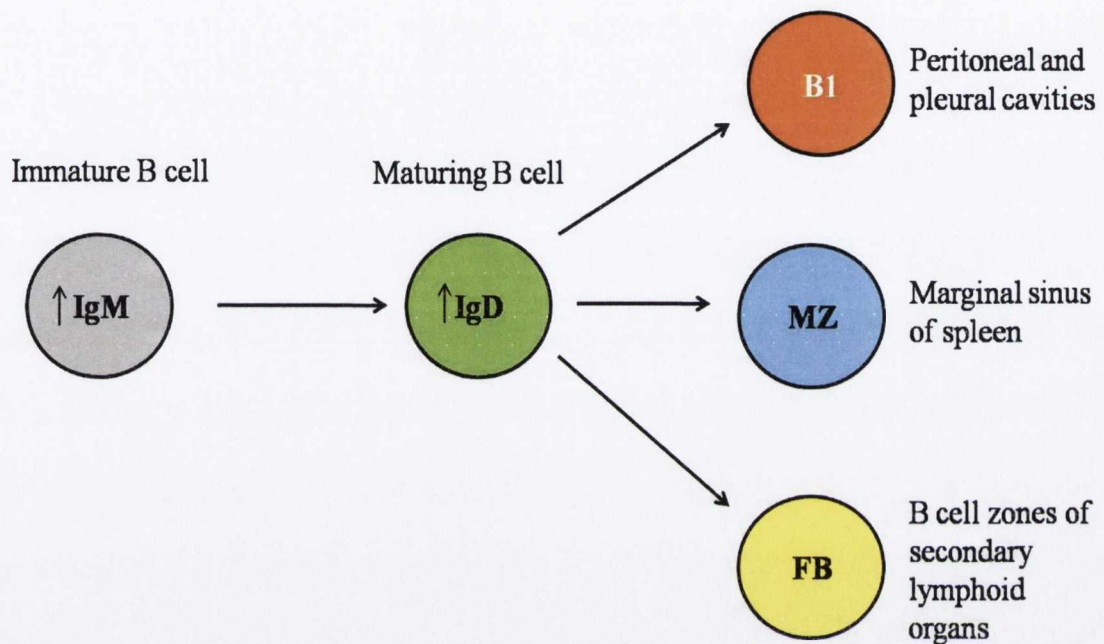


Figure 1.9: B cell subsets Immature B cells exit the bone marrow and migrate to specific areas within the periphery. These immature cells then undergo several changes within the periphery to become one of three mature B cell subsets: 1. B1 B cells (B1) found in the peritoneal and pleural cavities. 2. Marginal zone B cells (MZ) found in the marginal sinus of the spleen. 3. Follicular B cells (FB) which are located within the B cell zones of secondary lymphoid organs. (Adapted from [355])

B1 cells seed the peritoneal and pleural cavities [355]. These cells were first discovered in 1983 and have the ability to produce natural antibodies in the absence of T cell help [356-357]. T cell-independent activation of B cells occurs in response to antigens which display multiple identical epitopes in a polyvalent array on their surface, such as polysaccharides, glycolipids or a common microbial motif. As a result, these antigens have the ability to cross-link the BCR and induce antibody production in the absence of co-stimulatory signals, such as CD40L [358]. B1 cells can be further subdivided based on their responsiveness to antigens; B1a cells provide innate protection against bacterial infections and B1b cells mediate long term adaptive antibody responses against T cell-independent antigens, such as polysaccharides [359].

Marginal zone B cells are innate-like cells that reside in the marginal sinus of the spleen [355]. These cells have the ability to mediate T cell-independent responses to blood-borne pathogens, during which they can potentially differentiate into short-lived plasma cells [360]. They are also thought to play a role in T cell-dependent responses to protein antigens by delivering antigen to follicular B cells, which reside within the B cell zones of the spleen, and subsequently inducing adaptive antibody responses [355, 361-362]. Alternatively, it has been suggested that marginal zone B cells could potentially be directly activated by T cells, based on their high expression levels of co-stimulatory molecules, such as CD80 and CD86 [361-362].

Mature B cells that circulate through the B cell zones of secondary lymphoid organs, including Peyer's Patches, lymph nodes and the spleen form a subset of B cells known as follicular B cells [355]. Follicular B cells have also been reported to occupy distinct regions of the bone marrow [363]. When activated by antigen stimulation of the BCR, follicular B cells migrate towards the T cell zones within secondary lymphoid organs, where they interact with complementary antigen-activated T cells [364-365]. Once activated, follicular B cells proliferate and subsequently branch down one of two paths. The first results in the differentiation of follicular B cells into short lived plasma cells, which are non-dividing, terminally differentiated B cells that produce large amounts of antigen-specific antibodies [353, 366]. Alternatively, certain activated B cells return to the B cell follicle, where they form the germinal center [366].

Germinal centre formation is a key characteristic of T cell-dependent antibody responses. The germinal centre consist of T cells, B cells, macrophages, specialised germinal centre DC and organizational cells, known as follicular DC [367-368]. Within the germinal centre activated follicular B cells undergo several key processes, which are central to the development of the adaptive humoral response. Firstly, B cells undergo a process known as somatic hypermutation (SHM), which involves the addition of nucleotides into the variable region of the Ig heavy chain, after which they are tested for their ability to bind antigen by resident follicular DC [369]. It is this testing of BCR affinity for antigen through interaction with follicular DC which plays a pivotal role in driving affinity maturation, which is defined as an increase in the average affinity of the BCR for the antigen increases over time [351, 367, 369]. In addition, B cells undergo class switch recombination, which results in a change in the constant region of the heavy chain. The result of this is a change in the antibody isotype secreted by the B cells. In order to trigger germinal centre formation, T cell help is necessary and evidence suggests that CD40 ligation on follicular B cells is a key signal involved in the generation and maintenance of germinal centres [370-371].

Plasma cells are non-responsive to T cell signals and, therefore, are unable to switch antibody classes and do not undergo affinity maturation. Their development is associated with the presence of IL-21 and subsequent induction of the transcription factors Xbp1 (X-box binding protein-1) and B-lymphocyte induced maturation protein 1 (Blimp-1) [339, 353, 372]. T follicular helper (Tfh) cells are thought to be the source of this IL-21 and these have been shown to induce plasma cell differentiation *in vitro* [339, 373]. After antigen exposure, plasma cells are retained in the bone marrow, along with memory T and B cells. These long-lived plasma cells act as a source of antibody production, which are central to immunity, as these antibodies can provide immediate protection [374-375]. Plasma cell differentiation is a key step in the development of the humoral response, as it generates cells that can produce high levels of antigen-specific antibodies and also a source of continuous protection, even after the primary response has subsided.

Understanding the activation and effector functions of specific cells of the adaptive immune response is key to the development of vaccines. The innate immune system is the link to the adaptive immune response and the addition of adjuvants to vaccine

preparations can help to boost the innate system, ultimately resulting in more efficient activation of the adaptive immune system and protection.

1.7 Adjuvants

The development of vaccines has progressed significantly since the days of Jenner and Pasteur. Due to increased safety concerns and advances in molecular biology and immunology, we have moved away from the use of whole organism based vaccines and towards recombinant subunit vaccines. These subunit vaccines generally have an improved safety profile but the price paid for this is reduced immunogenicity. In order to enhance the efficacy of subunit vaccines, substances known as adjuvants are added to the vaccine preparations. Adjuvants were originally defined as "substances used in combination with a specific antigen that produce more immunity than the antigen alone" [376]. The adjuvant property of several compounds was first demonstrated by Gaston Ramon in the 1920s in the Pasteur Institute. Gaston Ramon tested a diverse range of substances, including tapioca, inorganic salts, oil and pyogenic bacteria with diphtheria toxoid. He demonstrated that they all had the ability to enhance antibody responses towards the toxin [376]. Following on from Ramon's observations, Alexander Glenny later showed that the combination of diphtheria toxoid and aluminium potassium sulphate or 'potash alum' resulted in enhanced antibody responses compared with the toxoid alone [377]. Today aluminium compounds remain the most widely used adjuvants.

The use of adjuvants can have several positive effects on the efficacy of vaccines, including increased stimulation of cell-mediated immunity, which is important for viral infections and the promotion of mucosal immunity, which may help the development of oral vaccines. In addition, adjuvants can reduce the amount of antigen necessary to induce protective responses and enable the use of combination vaccines by preventing antigen competition. Furthermore, they can increase the efficacy of vaccines in newborns with under-developed immune systems or in elderly and immune-compromised individuals. In all cases, safety is a prime concern with regards the use of adjuvants. Local reactions such as pain, inflammation and necrosis have been reported but these symptoms are often short-lived and self-limiting. The ideal adjuvant should

satisfy a number of criteria; it should be safe and well tolerated, biodegradable, promote long-lasting protective immune responses and remain stable before administration [378]. In addition, adjuvants should be well defined both chemically and physically, allowing for reproducibility from a manufacturing point of view as well as quality control. Current adjuvants may be broadly classified into immunostimulatory adjuvants, which are generally non-particulate in nature, and particulate adjuvants, based on the identification of a specific receptor, their ability to interact directly with the immune system and the physico-chemical nature of the substance. However, particulate vaccine adjuvants have also been shown to have immunostimulatory properties, therefore there may be some overlap between the two groups.

1.7.1 Immunostimulatory adjuvants (non-particulate)

1.7.1.1 Pathogen-derived adjuvants

Pathogen derived adjuvants stimulate the innate immune system via PRR, including TLR and NLR. Activation of these innate pathogen recognition receptors leads to the initiation of intracellular signalling pathways, which upregulate pro-inflammatory genes, such as cytokines and co-stimulatory molecules. Several species of bacteria have been used as sources of adjuvants, including *Mycobacterium* spp., *Bordetella pertussis* and *Neisseria meningitidis*. Asselineau and Lederer extracted four fractions from *Mycobacteria* with adjuvant activity and from one of these fractions they isolated trehalose dimycolate (TDM) [379]. TDM has been shown to stimulate both humoral and cellular immune responses [380]. More recently, it has been shown that TDM is recognised by the FcγR-associated C-type lectin receptor, Mincle. Furthermore, mice deficient in Mincle were unresponsive to a TDM-adjuvanted tuberculosis subunit vaccine [381]. TDM in combination with monophosphoryl lipid A (MPL), a derivative of LPS, in a metabolizable oil, referred to as the Ribi adjuvant system, is a highly potent immunostimulator but only used experimentally to date [379]. The minimal pathogen-derived component of complete Freund's adjuvant (CFA), which is a combination of an oil emulsion and heat-inactivated *Mycobacterium*, required for its adjuvanticity was identified as N-acetyl muramyl-L-alanyl-D-isoglutamine or MDP. NOD2 recognises MDP, resulting in the activation of NFκB and innate immune responses [60]. MDP, in combination with *Leishmania* antigens, can induce both cellular and humoral immune

responses and has recently been licensed for use in a veterinary vaccine against Leishmaniasis [382].

Mycobacterial DNA has previously been shown to have adjuvant activity and this was attributed to the presence of a high percentage of unmethylated CpG motifs, which are not present in vertebrates [383]. Unmethylated CpG oligonucleotides exert their adjuvant activities through stimulation of TLR9, which is expressed in B cells and plasmacytoid DC. CpG are strong inducers of Th1 immunity and CTL responses and induce protection against infectious disease, allergy and cancer in mice and primate models [384-385]. CpG has been tested clinically in a range of vaccines, including malaria, Hepatitis C and various cancers [386]. The CpG 7909 adjuvant, 24mer B-class CpG oligodeoxynucleotide, has been shown to enhance and sustain immune responses against HBV infection and boost immunity to influenza in pre-exposed subjects [387]. Additional synthetic TLR9 agonists are currently being tested in clinical trials [386]. However, CpG has been shown to promote the production of anti-neutrophil circulating antibodies (ANCA) by PBMC *in vitro* [388]. Furthermore, these ANCA have been associated with Wegner's Granulomatosis, a life threatening disease associated with vasculitis of unknown aetiology. This raises concerns for use of CpG as an adjuvant. However, studies in human subjects to date have shown that CpG are relatively safe and well tolerated.

1.7.1.2 LPS-derived adjuvants

LPS is a component of the cell wall of gram-negative bacteria and signals via TLR4 [389]. The adjuvant activity of LPS was first discovered in the 1950s and it can potentiate both humoral and cell-mediated immunity [390]. LPS is highly pyrogenic and its toxicity makes it unsafe for human use. The lipid A region of LPS is responsible for its adjuvant activity and toxicity. Derivatives of lipid A have been isolated in an attempt to identify a subunit which retains its adjuvant activity, but shows reduced toxicity. It has been shown that these lipid derivatives are only biologically active in aggregate form; therefore, manipulation of the structure of these derivatives may influence their biological activity [391].

The most promising derivative is monophosphoryl lipid A (MPL), obtained from the hydrolysis of lipid A from the LPS of *Salmonella Minnesota*, under low-acidic conditions [386]. Due to its adjuvant activities and attractive safety profile, MPL has been successfully combined with alum in a Hepatitis B vaccine (Fendrix^R), which has been approved for use in Europe [392]. It was the first TLR ligand approved for use in humans as a vaccine adjuvant.

There are a number of different formulations of MPL presently being tested in clinical trials for vaccines against *Leishmania* and allergy [386]. Alternative formulations of MPL have been produced in order to enhance its solubility and efficacy, including MPL-AF, where MPL is mixed with an aqueous solution and MPL-SE, which includes, MPL mixed with squalene oil, excipients (inactive substances used as carriers for the active ingredients) and water to produce a stable oil-in-water emulsion. MPL-SE is an excellent inducer of Th1 responses and is currently being used in clinical trials to treat and prevent *Leishmania* [386]. Further studies have led to the development of additional TLR4 agonists including aminoalkyl glucosamide phosphates (AGP) and glucopyranosyl lipid A (GLA) [386, 393]. AGP were developed as synthetic TLR4 agonists based on the structure of MPL and have been shown to induce cytokine production from human peripheral blood mononuclear cells (PBMC) and activate B cells and NK cells *in vitro*. Furthermore, they have shown strong potential as candidate adjuvants for the development of mucosal vaccines [393].

In addition to CpG and MPL, other TLR agonists currently being tested as adjuvants include the small molecule nucleoside analogues imiquimod and resiquimod, which activate TLR7 and TLR7/8 respectively [394]. These are thought to enhance immune responses via stimulation of TLR7, resulting in secretion of pro-inflammatory cytokines and activation of APC. Both imiquimod and resiquimod are currently being tested in clinical trials for melanoma, HIV and HCV vaccines [386].

1.7.1.3 QS21

Saponins are a heterogeneous group of sterol glycosides and triperenoid glycosides distributed throughout the bark, stem, roots and flowers of many plant species. Humans have used saponins for many applications for hundreds of years. For example, saponins were used in Africa to kill snails infected with Schistosomiasis [395]. The adjuvant property of saponins was first demonstrated in the early 1920s by Gaston Ramon and in the 1950s Espinet, who included saponins in a veterinary vaccine against foot and mouth disease [376, 396]. The adjuvant-active fraction of saponins from the bark of a tree (*Quilaja saponaria* Molina) found in South America, was identified by Daalsgard and named 'Quil A' [397]. Further analysis of Quil A showed that it was a heterogeneous mixture of saponins, making it unsuitable for human use. In 1991, Kensil *et al* isolated 22 fractions from Quil A using reverse phase-HPLC. One of the isolated fractions, known as QS21, has proven to be the most promising as a vaccine adjuvant, due to its strong immunostimulatory activity and reduced toxicity [398].

Studies involving QS21 suggest that, it acts as an immunostimulatory adjuvant, although no specific receptor for it has been identified [399]. Murine studies using QS21 as an adjuvant, have shown that it has the ability to induce the Th1-associated antibody isotype, IgG2a, and potent CTL activity. This gives QS21 an advantage over a number of other adjuvants, which lack the ability to induce strong cell-mediated immunity [400]. QS21 also has the ability to induce mucosal immunity following intranasal immunisation [401]. The mechanism of action of Quil A is currently not well understood. However, it has recently been shown that Quil A can activate the NLRP3 inflammasome, suggesting that this NLR protein may act as a sensor for Quil A [108]. With its strong adjuvant activity, low toxicity and ability to induce cell-mediated responses, QS21 has significant potential for use in human vaccines. There are currently several clinical trial in progress. These are using either QS21 alone or in combination with carriers (MF59) or other immunostimulatory molecules for vaccines against influenza, HBV, HIV and malaria. In addition, QS21 is also being tested in therapeutic vaccines for several cancers, including colon, melanoma and B cell lymphomas [386]. Thousands of subjects have received QS21 and only minor side effects have been registered, such as pain around the injection site.

1.7.1.4 Other Immunostimulatory adjuvants

Cholera toxin (CT) from *Vibrio cholerae* and heat-labile enterotoxin (LT) from enterotoxigenic strains of *E.coli* have both been shown to be powerful adjuvants. LT and CT belong to the AB class of bacterial toxins, the B subunit of which binds to the GM-1 receptor. The GM-1 receptor is found on the surface of a wide range of cells including, intestinal epithelial cells, DC, macrophages, T and B cells. Both LT and CT have been shown to be potent inducers of CTL responses [402]. Moreover, LT and CT have the ability to enhance immune responses via several routes of administration including, intra-peritoneal, subcutaneous, intravenous and intradermal [403-405]. In addition, they also promote strong mucosal responses when delivered orally [406-409]. However, the toxicity of these enterotoxins has caused some issues for their use in humans. CT can cause diarrhoea when delivered orally, even in small doses [410]. Furthermore, an LT-adjuvanted influenza vaccine caused Bell's Palsy in several vaccine candidates when delivered intranasally [411]. It was then demonstrated that intranasal delivery of CT resulted in binding of the toxin to the olfactory nerve [412]. The toxin was shown to travel to the olfactory bulb via retrograde transport, a mechanism which was later demonstrated to be GM-1-dependent [412-413].

In order to overcome these toxic effects several mutants and recombinant forms of these enterotoxins have been developed which retain their adjuvant effects but show reduced reactogenicity. These include LTK63, LTR72 and CTA-1DD. LTK63 and LTR72 contain mutations in the enzymatic A subunit and have been shown to retain their adjuvanticity with reduced toxicity [414-416]. Furthermore, LTK63 has been used in a clinical trial for an intranasal influenza vaccine, with no serious adverse events reported [417]. In addition, a recombinant version of CT has been developed known as CTA-1DD. It is composed of the A subunit of the CT toxin and protein A from *S.aureus*. Studies have demonstrated that CTA-1DD does not enter the olfactory nerve suggesting that CTA-1DD could potentially be used intranasally [413]. Clinical trials using CTB and killed enterotoxigenic *E.coli* (ETEC) or inactivated *Vibrio cholerae* as an oral vaccine against diarrhoeal disease have showed promise [418-422].

1.7.2 Particulate adjuvants

1.7.2.1 Emulsions

The use of emulsions in vaccines to boost immune responses can be traced back to work by LeMoignic Pinoy in 1916, who used the first oil emulsion, water and vaseline, with inactivated *Salmonella typhimurium* [423]. Less than ten years later, Ramon demonstrated that starch oil increased antibody responses to diphtheria toxoid [424]. In the 1930s, Freund developed paraffin-oil-based emulsions with or without heat-killed *Mycobacteria*, producing Freund's complete and incomplete adjuvants respectively [425]. Freund's adjuvant is an example of a water-in-oil emulsion (W/O) which is made up of antigen-containing water droplets in a continuous oil phase. Oil-in-water emulsions (O/W) are composed of antigen in a water phase with oil droplets in the solution and double emulsions can be produced with water droplets trapped within larger oil droplets dispersed in a water phase (W/O/W) [426].

Herbert showed that oil-in-water emulsions have the ability to retain an antigen at the site of injection, allowing prolonged exposure to the immune system [427]. O/W emulsions have shown the best safety profile, while W/O emulsions have proven too toxic for human use, even though they are more efficient at enhancing immune responses [426]. Syntax adjuvant formulation or SAF, is an O/W adjuvant developed in the 1980s as a possible replacement for Freund's adjuvant. SAF contains the biodegradable oil squalene, but was too toxic for clinical use due to the presence of muramyl dipeptide and induced severe local reactogenicity in a human HIV trial [386, 428]. The toxic nature of SAF led to the development of a safer emulsion adjuvant known as MF59, a micro fluidised O/W emulsion comprising squalene oil with Tween 80 and Span 85 as surfactants but with no additional immunostimulators [429]. MF59 is thought to exert its adjuvant effects by acting as a depot and subsequently directing the production of chemokines by monocytes, macrophages and granulocytes [430]. In addition, studies have demonstrated that in mice subcutaneous immunisation of MF59 promoted the differentiation of monocytes into inflammatory DC, enhanced antigen uptake and recruitment of cells to the injection site [430]. MF59 has been shown to be a potent adjuvant with an acceptable safety profile. A large number of subjects have been immunised with MF59 and the adjuvant was well tolerated and was safe in newborns and the elderly. FLUADTM is an MF59-adjuvanted influenza vaccine currently licensed

for use in the elderly by 12 European health authorities and in some non-EU countries with at least 25 million doses administered to date [431].

The Montanides are a series of W/O emulsions which are currently being developed for use as vaccine adjuvants in response to safety concerns with IFA [432-433]. Montanide ISA 51 and ISA 720 are both biodegradable emulsions which use mannide-mono-oleate as an emulsifier. They are currently being tested in clinical trials for vaccines against malaria and cancer [378]. However, during initial clinical trials, these adjuvants have induced unacceptable local reactions and have been unexpectedly reactogenic, thus preventing their progression [434]. In contrast, others have reported that Montanide ISA 51 caused no adverse effects when administered in combination with KLH and melanoma antigens. Furthermore, all of the patients which received Montanide-adjuvanted KLH produced antigen-specific antibodies and KLH-reactive CD4⁺ T cells that produced IFN- γ , TNF- α and IL-2 [435]. Therefore, the use of Montanides as adjuvants and their safety profiles requires further investigation.

1.7.2.2 ISCOMs

In 1971 Immune-stimulating complexes or ISCOMs were first visualised by electron microscopy in a preparation of virus particles treated with saponin [436]. However, it was not until 1984 that Morein *et al* first described the formation of these saponin-containing complexes with viral membrane proteins [437]. Negative stain transmission electron microscopy shows these well defined particles as spherical, cage-like structures of approximately 40 nm in diameter [438]. Their formation occurs spontaneously upon mixing cholesterol, *Quillaja saponaria* saponins and phospholipids. It is the interaction of the cholesterol and saponins that form the matrix of these particles and phospholipids are added to produce a less tightly compressed particle allowing for the insertion of amphiphatic molecules [439-440].

ISCOMs are manufactured by and registered to Isotec AB who has also developed ISCOMATRIXTM, which consists of cholesterol, phospholipids and saponins but lacks the viral membrane proteins, allowing for the insertion of antigens into the particles [441]. ISCOM-based vaccines have been shown to induce both antibody and Th1-polarised cell-mediated responses and are particularly potent in the induction of

cytotoxic T cells [438]. Watson *et al* demonstrated that the trafficking of ISCOMs from the site of injection to the spleen and the local draining lymph nodes was faster than protein alone [442].

However, the exact mode of action of ISCOMs remains largely unknown. Several theories have been suggested to explain their adjuvant activities: a) Delivery of the antigen directly to APC. b) Delivery of the antigen into the cytosol of the cell, allowing access to the MHC class I pathway, which is required for the induction of CD8⁺ T cells. c) Induction of cytokine production by APC; for example ISCOMs have been shown to induce IL-6 and IL-1 production in macrophages [395]. It has also been found that IL-12 and IFN- γ are key to the adjuvant activity of ISCOMs [443]. In addition, one of the components of ISCOMs (Quil A) has been shown to activate the NLRP3 inflammasome, suggesting that NLRP3 may be important for the adjuvant activities of ISCOMs *in vivo* [108].

However, to date, no vaccine containing ISCOMs or ISCOMATRIX has been licensed for human use. Despite this, in clinical trials, over 1000 people have been injected intramuscularly with ISCOM-based vaccines and it was reported that they were safe and generally well-tolerated and side effects were found to be self-limiting [438]. Several ISCOM-based vaccine formulations are currently in development, including viral proteins from influenza and purified recombinant proteins, such as human papilloma virus 16 (HPV) E6E7 fusion protein and NY-ESO-1, an antigen expressed in a wide variety of cancers [444-445].

1.7.2.3 Mineral Salts

Aluminium-containing adjuvants have been contributing to the efficacy of vaccines for almost a century. The adjuvant properties of these compounds were first discovered by Glenny in the 1920s. A precipitate of diphtheria toxoid and aluminium potassium sulphate (potash alum) was produced and the precipitated vaccine induced greater protective immunity than the toxoid alone [377]. Several other aluminium salts have been shown to possess adjuvant properties, including; aluminium potassium phosphate (Adju-phos), aluminium hydroxide (Alhydrogel or alum) and a combination of aluminium hydroxide and magnesium hydroxide (Imject Alum). The most commonly

used aluminium salt adjuvants are Adju-phos and Alum due to the ease of manufacturing and reproducibility. Although it is not completely correct, Adju-phos and Aldydrogel are often commonly referred to as alum. Aluminium-containing adjuvants are the most successful adjuvants based on the fact that, they are licensed for use in multiple vaccines, including Diphtheria-Pertussis-Tetanus, Diphtheria-Tetanus, Diphtheria-Tetanus combined with Hepatitis B Vaccine, *Haemophilus influenzae* B or inactivated polio virus and HPV [446].

Despite decades of use, the exact mechanism of action of these compounds has remained elusive. However, recent discoveries have shed light on this area. During Glenney's original experiments it was noticed that none of the toxoid remained in the supernatant and this led to the idea that the antigen must be completely adsorbed by the adjuvant, in order to increase the immune responses [377]. Based on these observations it was suggested that particulate vaccine adjuvants worked by forming an antigen 'depot' at the site of injection, thereby prolonging the amount of time that cells of the immune system have to 'sample' and interact with the antigen. This 'depot' effect theory is so widely accepted that the regulatory requirements by the WHO state that the antigen must be adsorbed by the aluminium-containing adjuvant [447]. In contrast to the depot theory, several studies have shown that the degree of antigen adsorption onto the adjuvant has no effect on its immunopotentiating properties [448]. In addition, antigen-aluminium salt nodules can be removed from the injection site with no subsequent effect on antibody titres [449]. Furthermore, biodistribution studies have shown that the majority of the antigen is rapidly cleared from the injection site within hours of immunisation [450].

Several studies have now demonstrated that alum mediates immunostimulatory effects. For example, injection of alum alone promotes the accumulation of IL-4-secreting Gr1⁺ cells in the spleen [451-453]. Subsequent studies identified these Gr1⁺ cells as eosinophils [451-453]. These cells were shown to induce MHC class II-mediated signalling in splenic B cells, as measured by mobilisation of intracellular Ca²⁺. Furthermore, this activation of B cells by these IL-4⁺ eosinophils was shown to be important for early (d5 or d6) alum-induced IgM responses to co-injected antigens [451, 453]. The enhancement of antigen-specific IgG1 by alum was found to be independent of these cells while antigen-specific IgG2c titres were found to be increased when Gr1⁺ cells were depleted [451-453]. Thereby, suggesting that these Gr1⁺ IL-4-secreting cells

play some role in the inhibition of Th1 responses following immunisation with alum [451-453]. Others have reported that, injection of alum is sufficient to increase the local production of pro-inflammatory mediators, including IL-1 β , CC-chemokine ligand 2 (CCL-2), CCL-11 or eotaxin and IL-5, as well as the recruitment of neutrophils and inflammatory monocytes [165, 430, 452]. The bias observed with alum-adjuvanted vaccines towards Th2 type responses also supports the idea that these adjuvants exert specific effects on the immune system. The mechanism of this Th2 bias is still unknown. However, previous studies have shown that the ability of aluminium-containing adjuvants to drive potent Th2 responses is independent of IL-4 and IL-6 [454-456].

The mechanism by which alum exerts its adjuvant activities is currently unknown. However, roles for uric acid and the NLR protein NLRP3 have recently been proposed. It was demonstrated that alum injection results in the local release of uric acid, subsequently promoting the recruitment of inflammatory monocytes to the injection site [165]. These inflammatory DC were found to play a key role in the activation of CD4⁺ T cells, suggesting that alum-mediated uric acid release is central to the promotion of cell-mediated responses by alum [165]. Several studies have demonstrated the ability of alum to activate the NLRP3 inflammasome resulting in enhanced secretion of IL-1 β [105, 107-108, 457-458]. *In vivo* studies have shown that NLRP3-deficient mice show a reduction in recruitment of neutrophils, eosinophils and monocytes into the injection site and a decrease in the secretion of antigen-specific IL-5 in local lymph nodes following immunisation with alum-adjuvanted Ova [105, 107-108, 457-458]. These studies suggest that in addition to uric acid, NLRP3 activation by alum may be a key step in the promotion of cell-mediated responses.

One of the main attributes associated with alum as an adjuvant is its ability to strongly enhance humoral immunity. Previous findings have demonstrated that alum-enhanced antibody responses occur independently of TLR [459-460]. To date the only molecule implicated in the enhancement of antibody responses by alum has been NLRP3. However, this is surrounded with some controversy. Two groups have reported that NLRP3-deficient mice produce significantly less antigen-specific IgG1 and IgE with no effects on levels of IgG2 [105, 107]. In contrast to these findings, others have demonstrated that NLRP3- and caspase-1-deficient mice produce levels of antigen-specific IgG, IgG1, IgG2 and IgE that are comparable to wild type mice [118-119].

Overall, the requirement of NLRP3 for alum-mediated humoral immunity remains controversial. Unfortunately, the role of uric acid in alum-mediated humoral immunity has not been directly investigated. Although these recent findings have greatly increased our understanding of the mechanism of action of aluminium based adjuvants, they have also raised more questions, which currently remain unanswered. For example, it is not completely clear how aluminium salts activate the inflammasome or whether its activation is necessary for the enhanced antibody production by aluminium salts and why aluminium salt adjuvants bias the immune response towards the development of Th2 type responses.

Despite their acceptable safety profile, aluminium-containing adjuvants are poor inducers of cell-mediated immunity making them unsuitable for use in vaccines against diseases such as tuberculosis, malaria and HIV, which require long lasting antibody responses along with potent cell-mediated immunity, based on CD4⁺ and CD8⁺ T cell responses [386].

1.7.2.4 Biodegradable microparticles

Biodegradable, particularly poly (lactide-co-glycolide) (PLG) microparticles, have been used for decades in sutures, for drug delivery and controlled release vaccine delivery systems [461]. The adjuvant activity of PLG microparticles was first demonstrated in the early 1990s [462-463].

Microparticles can be formulated from a range of different polymers. However, PLG microparticles are the prime candidates for development as vaccine adjuvants [464]. PLG are biodegradable *in vivo*, undergoing non-enzymatic hydrolysis of the ester bonds to produce lactic and glycolic acids. The half life of PLG is dependent on the molecular weight of the polymer and the lactic to glycolic acid ratio and it can range from days to weeks and possibly years. The optimal size range for vaccination is likely to depend on the application but it has been proposed that microparticles of 1-3 μm are optimal in some settings [461]. Antigens can be encapsulated within PLG using a technique known as microencapsulation. During this process, antigens can be exposed to various potentially damaging conditions such as low pH, high shear and organic solvents [465-466]. This and the susceptibility of encapsulated antigen to acid-mediated hydrolysis,

encouraged scientists to develop alternative means of associating antigens with microparticles [467-468]. O'Hagan *et al* described the preparation of charged microparticles, using SDS to produce anionic PLG, which were easier to adsorb proteins onto [469]. Factors such as, protein charge and hydrophobicity, affect antigen adsorption and the optimal adsorption conditions are protein-specific [470]. Cationic particles were developed by the same group and these have been optimised for the delivery of DNA vaccines [471].

The exact mode of action of PLG is unclear; although, uptake by macrophages and DC seems to play an important role in their adjuvanticity [463, 472]. Microparticles facilitate multimeric presentation of antigens on their surface which is required for optimal B cell activation [473]. In addition to promoting strong antibody responses, studies in rodents indicate that PLG also effectively promote cell-mediated immunity [474-475]. Others have also shown that while PLG do not activate DC, they do enhance antigen presentation [476]. More recent studies have shown that, PLG can activate the NLRP3 inflammasome and enhance IL-1 secretion by DC *in vitro* [109]. Cationic PLG have been tested in nonhuman primates as a delivery system for a Hepatitis C vaccine and induced seroconversion in all animals tested [470]. In addition, cationic PLG have been evaluated in a phase I clinical trial with a candidate HIV vaccine which was recently completed. However, the results have not yet been published [477].

1.7.2.5 Other particulate vaccine adjuvants

Virus like particles (VLP) and virosomes consist of self-assembling viral envelope proteins. They form particles ranging from 20-100 nm in size [478]. Both act, essentially, as non-infective viruses, retaining the ability to penetrate cells in a similar manner to infective viral particles. However, they do not contain any viral nucleic acids and therefore lack the ability to replicate and infect [479].

VLP can be generated using yeast, baculovirus, mammalian or even plant expression systems. More importantly, VLP and virosomes have the ability to stimulate both cell-mediated and humoral immunity [480-482]. Currently, there are several vaccines on the market which have taken advantage of the VLP/virosome technology. Virosome-based vaccines include EpaxalTM, which is a vaccine for hepatitis A that has been registered

for use in several European countries [483]. The first VLP vaccine to be marketed expressed Hepatitis B surface antigens PreS1 and PreS2. More recently, a vaccine against Human Papilloma Virus (HPV), marketed as Gardasil^R, is a VLP-based vaccine, composed of LI (major capsid protein) from several HPV serotype, including HPV types 6, 11, 16 and 18 [484-486]. Gardasil has been shown to reduce HPV infection by 90% and HPV types 16 and 18 have been implicated in almost 70% of cervical cancers [484, 487]. In addition to these marketed vaccines, VLP are currently being evaluated in pre-clinical studies for vaccines against influenza, hepatitis C and Ebola virus [481].

Liposomes were developed in 1965 by Alec Bangham, who extracted lipids from egg yolk and found that when they were submerged into an aqueous solution they spontaneously re-arranged as double layered bubbles approximately the same size as a cell [488]. These 'bubbles', were defined as liposomes, which are particulate structures composed of either natural or synthetic phospholipids. The biomedical applications of liposomes include drug delivery and vaccines and they are also used as red blood cell substitutes for hemorrhagic injuries when blood is not available. Allison and Gregoriadis were the first to show that encapsulation of an antigen into a liposome led to increased antibody responses [489]. 'Conventional liposomes' consist of a mixture of phospholipids and have been shown to be rapidly cleared by phagocytic cells of the reticulo-endothelial system and by macrophages [490]. This discovery prompted the development of 'stealth-like' liposomes, which contain a small proportion of sterically stabilising phospholipids, and sometimes cholesterol, to further boost membrane stability and rigidity [491]. The increased stability of these 'stealth-like' liposomes, allows them to persist as long as 2 days in the blood stream after injection [491]. Physicochemical properties, such as charge and fluidity of liposomes have been reported to have an effect on their immunogenicity. Targeting of antigens to specific cells of the immune system by liposomes has been shown for, example, anionic liposomes comprised of phosphatidylcholine, cholesterol and phosphatidylglycerol or phosphatidylserine have been reported to interact specifically with human and murine DC [492]. 'Immunoliposomes' allow for more specific targeting to cells, as they contain a cell-specific receptor-targeting monoclonal antibody incorporated into their membrane [492].

In 1992 Alving *et al* immunised a group of thirty healthy males with a liposome-encapsulated recombinant protein (R32NS1₈₁) containing epitopes from *Plasmodium falciparum* [493]. The liposomes used also contained the MPL and were adsorbed to alum. Overall, the vaccine was found to increase R32-specific antibody production and no serious adverse events were reported [493]. Despite these positive early results, there are currently no marketed liposome-based vaccines. However several are currently under investigation. A liposome-based vaccine containing MPL known as Stimuvax^R has been developed for the treatment of non-small cell lung cancer [494]. This vaccine has moved on to Phase III trials and Stimuvax^R has also been tested for the treatment of breast cancers and multiple myelomas. However, one of the patients involved in the multiple myeloma trial developed encephalitis resulting in the FDA putting all trials involving Stimuvax^R on hold for the foreseeable future [495-496].

In addition to these lipid-based systems, particulate delivery systems may also be generated from proteins and these are known as proteosomes. Proteosomes are nanoparticles composed of the outer membrane proteins from bacteria. The most commonly used proteins are, the outer membrane proteins (OMP) from *Neisseria meningitides* [483]. These particles are hydrophobic in nature and both apolar and amphiphilic antigens may be incorporated into this delivery system [497]. Proteosomes composed of OMP from *Neisseria meningitides* are 80-100 nm in size and have TLR2 adjuvant activity, due to the presence of high levels of the protein Porin B [498-500]. In addition, proteosomes can be complexed with LPS, which converts the particle into a TLR4 agonist, to produce an adjuvant known as ProtollinTM [501-503]. It has been reported that proteosomes and ProtollinTM promote the secretion of chemokines from TLR-transfected HEK cells and that enhanced uptake of proteosomes by M cells within Peyer's Patches is TLR2-dependent [498]. These delivery systems have proven to be safe for intra-nasal delivery and have been shown to promote the development of high titres of serum antibodies and in nasal secretions when proteosomes were formulated with influenza A-H1/N1 proteins [497, 504].

1.7.2.6 Combination Vaccine Adjuvants

Increased understanding of the activation of the innate immune system and the relationship between innate and adaptive immunity has allowed for engineering of the next generation of adjuvants. These encompass a combination of immunostimulatory and particulate vaccine adjuvants. This new found knowledge will allow for the development of more sophisticated and tailored adjuvants. The next generation of adjuvants will need to have attributes that are lacking in currently used adjuvants. This is necessary in order to aid the development of vaccines to target diseases for which there are currently no vaccines available, including HIV and malaria. These attributes will include: the ability to promote the development of more heterologous antibodies, thus allowing for increased cross reactivity among different strains of the same microorganism, the induction of more effective T cell responses for killing of pathogens and the induction of more potent functional antibody responses to allow for pathogen killing or neutralisation [378]. In addition the future adjuvants will be expected to allow for more practical applications, such as a reduction in antigen dose.

A prime example of the next generation of adjuvants, are the adjuvant systems (AS) developed by GSK. The AS, include AS01, AS02, AS03 and AS04. AS01 is a liposomal formulation containing MPL. It has been shown to produce strong cell-mediated responses and is currently being tested in GSKs malaria vaccine candidate [505]. Another combination vaccine adjuvant, known as AS02, is currently being investigated. It is a combination of MPL and QS21 in an O/W emulsion and has been shown to induce both strong humoral and cell-mediated responses [386]. In addition, the GSK RTS/S malaria vaccine combined with AS02 has been shown to provide 65% protection against first time infections in infants. However, earlier trials in children (1-4 yrs) were only effective in 35 % of candidates [506]. GSKs H1N1 vaccine, PandemrixTM, is adjuvanted with AS03, which is composed of α -tocopherol, a synthetic form of vitamin E, in an O/W based emulsion [507]. AS04 is composed of the TLR4 agonist MPL and the particulate vaccine adjuvant, alum. A recent study has shown that this combination of MPL and alum induces the local secretion of cytokines, which in turn enhances the uptake of antigen by and activation of DC [508]. AS04 is one of the first new generation vaccines to be approved for use in a vaccine against HPV (Cervarix) by the FDA [509]. Cervarix contains two types of VLP, HPV 16 and 18 [510]. Adding further complexity to the design of adjuvants, GSK have also developed

AS15 which consists of, AS01 (Liposomal based MPL) and CpG. AS15 is currently being tested as an adjuvant for therapeutic cancer vaccines [511]. These new adjuvant systems have been proven to have a high safety profile with over 68,000 people receiving AS04 and an estimated 29 million people in Europe receiving the AS03 adjuvanted GSK flu vaccine [512].

In addition to GSK, others have also reported the successful use of combination adjuvant systems. For example, PLG-encapsulated CpG has been shown to promote a four-fold enhancement in antibody titres in comparison to soluble CpG [513]. Another study reported enhanced mucosal antigen-specific IgA responses when the Group B streptococcus antigen (GBS) was co-encapsulated with CpG in PLG [514]. Furthermore, the adjuvant was shown to enhance immune responses via several routes of immunisation, including oral, vaginal, nasal, intra-muscular and intraperitoneally [514].

IC31^R is a two component adjuvant system developed by Intercell. It consists of KLK, an antimicrobial peptide, and a TLR9-activating oligodeoxynucleotide, ODN1a. Studies have demonstrated that IC31 promotes the formation of a depot at the injection site via KLK. In addition, IC31 has been shown to activate APC and strongly enhance both humoral and cell-mediated responses, with a bias towards Th1 type responses [515-517]. Furthermore, IC31 has been shown to enhance immune responses to several influenza antigens in aged mice, suggesting that it may be a prime candidate for use in flu vaccines for the elderly [517]. This novel adjuvant is currently being tested in vaccines against tuberculosis and influenza. Reports so far suggest that IC31 is well tolerated in subjects, with no serious adverse events observed [518].

Vaccine development has progressed from the use of whole microorganisms to specific proteins or the subunits of the pathogen. This has provided increased safety profiles but the cost for this was immunogenicity. In the case of vaccine adjuvants, the challenge is to increase their immunogenicity as well as maintaining a high safety profile. Advances in molecular and immunological techniques give us the ability to fully understand how particulate vaccine adjuvants work. Given the high safety profile that particulate adjuvants, such as alum and PLG display, gaining a better understanding of how these adjuvants activate the immune system could be the key to development of future adjuvants and subsequently better vaccines.

Hypothesis: Previous studies have demonstrated that particulates, such as uric acid, have the ability to enhance the secretion of IL-1 by immune cells. Therefore, particulate vaccine adjuvants (PVA) may also promote the secretion of IL-1 subsequently promoting innate and adaptive responses. In addition, it has been reported that PVA, such as alum, cause local necrosis when injected subcutaneously. Therefore, PVA may also promote the release of endogenous danger signals with pro-inflammatory activities.

Aims:

- Determine the ability of particulate vaccine adjuvants to activate immune cells, specifically analyse the ability of PVA to promote IL-1 secretion
- Investigate the relationship between particle characteristics and the adjuvant properties of particulates
- Examine the toxicity of particulate vaccine adjuvants and establish the consequences of particle-induced toxicity on the immune response

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Tissue culture reagents

Ammonium Chloride Red Blood Cell Lysis Solution

0.77 g Ammonium Chloride (NH_4Cl) dissolved in 100 ml dH_2O and filter sterilized.

Cell Culture Medium

Roswell Park Memorial Institute (RPMI)-1640 medium (Biosera) was supplemented with 8% (v/v) heat inactivated (56°C for 30 min) foetal calf serum (FCS, Biosera), 100 mM L-Glutamine (Gibco) and 100 $\mu\text{g/ml}$ penicillin streptomycin (Gibco). Complete RPMI (cRPMI) was used to culture all cells *in vitro*.

X-vivo (Lonza) medium was used for *in vivo* studies. It is formulated with L-glutamine and gentamicin and is serum free.

2.1.2 Buffers and Solutions for ELISA

PBS 10X (5L)

400 g NaCl

58 g Na_2HPO_4

10 g KH_2PO_4

10 g KCl

Made up in 5 L of dH_2O and brought up to pH 7.2

Wash Buffer

PBS 0.05% (v/v) Tween 20

1 L 10x PBS pH 7.0

5 ml Tween 20

Made up to 10 L with dH₂O

Reagent Diluent

BD Pharmingen ELISA kits (IL-6, IL-5, IL-4, IFN- γ): PBS

R & D Systems Duoset ELISA kits (IL-1 β , IL-1 α , IL-17, IL-10, TNF- α):

1% (w/v) Bovine serum albumin (BSA) in 1X PBS

MBL (IL-18): Assay diluents provided with kit

Block Solution

BD Pharmingen kits: 10% (w/v) Marvel in PBS (except IL-6: 3% (w/v) BSA)

R & D Duosets: 1% (w/v) Bovine serum albumin (BSA) in 1X PBS

Phosphate Citrate Buffer

10.19 g anhydrous citric acid

36.9 g Na₂HPO₄·12H₂O

Made up to 1L with dH₂O and brought up to pH 5.5

Stop Solution (1M H₂SO₄)

26.74 ml 18 M H₂SO₄

473.26 ml dH₂O

Carbonate Buffer

4.2 g NaHCO₃

1.78 g Na₂CO₃

Made up to 500 ml with water and brought up to pH 9.5

Table 2.1: Antibodies used in cytokine ELISAs

Antibody	Supplier	Sample dilution
IL-1 α	R&D Systems	1/2 in 1% (w/v) BSA/PBS
IL-1 β	R&D Systems	1/2 in 1% (w/v) BSA/PBS
IL-4	Pharmingen	1/2 in PBS
IL-5	Pharmingen	1/2 in PBS
IL-6	Pharmingen	1/2 in PBS
IL-10	R&D Systems	1/2 in 1% (w/v) BSA/PBS
IL-12p40	Pharmingen	1/2 in PBS
IL-12p70	R&D Systems	1/2 in 1% (w/v) BSA/PBS
IL-17	R&D Systems	1/2 in 1% (w/v) BSA/PBS
IL-18	MBL Ltd	1/5 in kit diluent
TNF- α	R&D Systems	1/2 in 1% (w/v) BSA/PBS
IFN- γ	Pharmingen	1/200 in PBS

All supernatants were diluted as outlined above for cytokine analysis.

Note:

- (1) All supernatants from re-stimulated were diluted 1/2 for *in vivo* studies.
- (2) All antibodies come in a purified (coating) and biotinylated (detection) form (see 2.2.4 for use)

Table 2.2: Antibodies used in antibody ELISAs

Antibody	Supplier	Starting dilution	sample	Blocking buffer
Total IgG	Sigma	1/100		10% (w/v) Marvel/PBS
IgG ₁	BD Pharmingen	1/100		10% (w/v) Marvel/PBS
IgG _{2a}	BD Pharmingen	1/100		10% (w/v) Marvel/PBS
IgG _{2b}	BD Pharmingen	1/100		10%(w/v) Marvel/PBS
IgG ₃	BD Pharmingen	1/100		10%(w/v) Marvel/PBS
IgE	BD Pharmingen	1/10		10% (v/v) FCS/PBS

All serum samples were diluted in PBS (2.1.5.1)

2.1.3 Buffers and Solutions for Flow Cytometry

FACS Buffer

2% (v/v) Fetal calf serum (FCS; Biosera)

0.1% (w/v) Sodium azide

Made up in 1X PBS.

Table 2.3: Fluorochrome labelled antibodies for flow cytometry

Antibody specificity	Fluorescent label	Supplier	Dilutions
CD80	FITC	BD-Pharmingen	1/400
CD86	PE	BD-Pharmingen	1/1000
CD40	FITC	BD-Pharmingen	1/400
MHC II	PE	BD-Pharmingen	1/1333
CD11c	APC	BD-Pharmingen	1/2666
Ly6C	FITC	BD-Pharmingen	1/2000
Ly6G	PE	BD-Pharmingen	1/2000
CD3	FITC	BD-Pharmingen	1/2000
CD19	PerCP-Cy5.5	BD-Pharmingen	1/2000
F4/80	PerCP-Cy5.5	BD-Pharmingen	1/2000

2.1.4 Buffers and solutions used in Western Blotting

1.5 M Tris-HCl pH 8.8

18.15 g Tris base

75 ml dH₂O

Made up to 100ml with water and brought to pH 8.8.

0.5 M Tris-HCl pH 6.8

6 g Tris base

60 ml dH₂O

Made up to 100 ml with water and brought to pH 6.8.

5X Running Buffer

15 g Tris Base

72 g Glycine

5 g SDS

Made up to 1L with dH₂O and brought to pH 8.3

Transfer Buffer

0.19 g Tris Base

4.32 g Glycine

240 ml dH₂O

60 ml MeOH

0.15 g SDS

Brought to pH 8.3

SDS-PAGE Sample Buffer

- 62.5 mM Tris pH 6.8
- 2% (w/v) sodium dodecyl sulphate (SDS)
- 10% (v/v) Glycerol
- 0.1% (w/v) Bromophenol blue
- 50 mM DL-Dithiothreitol (DTT)

Table 2.4: Components of stacking and resolving gels

For 4 gels:

	Stacking Gel 5% (v/v)	Resolving Gel 15% (v/v)
dH ₂ O	2.7 ml	6.6 ml
30% Bis-acrylamide Mix	0.67 ml	8.0 ml
1M Tris pH 6.8	0.5 ml	
1.5M Tris pH 8.8		5 ml
10% (w/v) Amonium persulphate (APS)	40 µl	200 µl
10% (w/v) SDS	40 µl	200 µl
TEMED	4 µl	8 µl

Table 2.5: Primary antibodies for western blot analysis

Antibody	Supplier	Dilution
IL-1β	National Institute for Biological Standards and Control (NIBSC)	1/200 3% (w/v) Marvel/PBST
Caspase-1 (p10)	Santa Cruz	1/400 3% (w/v) Marvel/PBST
β-Actin	Sigma	1/5000 5% (w/v) Marvel/PBST

Table 2.6: Secondary antibodies for western blot analysis

Antibody	Supplier	Dilution
Anti-mouse IgG, peroxidase conjugate	Sigma	1/1000 5% (w/v) Marvel/PBST
Anti-sheep IgG, peroxidase conjugate	Sigma	1/1000 3% (w/v) Marvel/PBST
Anti-rabbit IgG, peroxidase conjugate	Sigma	1/1500 3% (w/v) Marvel/PBST

Table 2.7: Pathogen recognition receptor (PRR) agonists used *in vitro*

Ligand	Receptor	Supplier	Concentration Range
LPS	TLR-4	Alexis Biochemicals	50 pg/ml-100 ng/ml
CpG	TLR-9	Sigma Genesis	4-50 µg/ml
Poly I:C	TLR-3	Sigma	50 µg/ml
Pam3CSK4	TLR-2	Invivogen	50 ng/ml

Table 2.8: List of inhibitors used *in vitro*

Inhibitor	Target	Supplier	Concentration Range
YVAD-FMK	Caspase-1	Bachem	40 μ M
Uricase	Degrades uric acid	BioChemika, Sigma	0.1 – 10 U/ml
N-acetyl cysteine (NAC)	Scavenges free radicals	Sigma	5 - 50 mM
Diphenyliodonium (DPI)	NADPH oxidase iNOS	Sigma	5 – 10 μ M
Cytochalasin B	Actin polymerisation	Sigma	2.5 – 10 μ M
Bafilomycin	H ⁺ ATPase system	Sigma	250 nM
CA-074-Me	Cathepsin B	Sigma	10 μ M
ZVAD-FMK	Caspases	Bachem	40 μ M

Table 2.9: Particulate vaccine adjuvants

Adjuvant	Source
Alum (Alhydrogel)	Brenntag Biosector, Frederikssund, Denmark
Polystyrene Microparticles	Microspheres-Nanospheres, Cold Spring, NY, USA
Poly(lactide-co-glycolide) (PLG) Microparticles	Novartis Vaccines, Emeryville, USA

2.2 Methods

2.2.1 Animals

Female C57BL/6 mice were obtained from Harlan Olac (Bicester, U.K.) and were used at 8–16 wk of age. NLRP3^{-/-} breeding pairs were provided by Prof Jurg Tschopp (Dept of Biochemistry, University of Lausanne, Switzerland) and bred in the TCD Bioresources Unit. IL-1R1^{-/-} mice were bred in the Bioresources unit in TCD. HeN/HeJ mice were obtained from Harlan and bred in the TCD Bioresources Unit. Animals were maintained according to the regulations of the European Union and the Irish Department of Health.

2.2.2 Cell culture

Cells were cultured at 37°C with an atmosphere maintained at 95% humidity and 5% CO₂.

2.2.2.1 Cell Counts

Cell viability was determined by trypan blue exclusion. A 10 µl volume of the cell suspension was then loaded into a Hycor Glasstic slide with grids (Hycor) and the number of viable cells was counted using a light microscope. The number of cells per ml was determined using the following formula: number of cells/ml = cell number x 10⁴ x dilution factor.

2.2.2.2 Murine Bone Marrow-Derived Dendritic Cell Culture

Bone marrow-derived dendritic cells (BMDC) were generated from mice using a method similar to that described by Lutz *et al* [519]. Mice were euthanized and their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with complete RPMI medium using a 27G needle. The cell aggregates were broken up using a 19G needle and the cell suspension was pelleted by centrifugation (1200 rpm/225 g for 5 min at 4°C). The pellet was resuspended in 1 ml of ammonium chloride (0.88%) lysis solution (heated to 37°C) for 2 min, to lyse red blood cells.

The cells were then washed in complete RPMI, pelleted by centrifugation (225 g for 5 min at 4°C) and resuspended in 10 ml of complete RPMI. Cell viability was assessed by the trypan blue exclusion method (2.2.2.1).

Immature BMDC were seeded at 4.0×10^5 cells/ml in 30 ml of complete RPMI supplemented with 40 ng/ml of GM-CSF in the form of supernatant from the GM-CSF expressing J558 cell line (Generously provided by Nathalie Winter, Institut Pasteur, Paris). After 3 days of incubation, 30 ml of fresh medium containing 40 ng/ml GM-CSF was added to the flasks. On day 6, the flasks were removed from the incubator and cell culture supernatant removed to eliminate non-adherent cells (e.g. granulocytes) from the culture. 30 ml of sterile pre-heated (37°C) PBS was added to each flask and cells were removed by continuous pipetting before the PBS was added to 50 ml tubes containing 10 ml of complete RPMI. 30 ml of sterile pre-heated (37°C) EDTA (0.02%; Sigma) was added to each flask and then flasks were placed in the incubator for 10 min. Meanwhile, the cells removed by the PBS step were pelleted by centrifugation (1200 rpm/225 g for 5 min at 4°C) and resuspended in complete RPMI. After 10 min, the EDTA flasks were removed from the incubator, cells were removed by continuous pipetting, added to 10 ml of complete RPMI and pelleted by centrifugation (225 g for 5 min at 4°C) followed by resuspension in complete RPMI. Cells removed using PBS and EDTA were pooled and cell viability was assessed by the trypan blue method (2.2.2.1). Cells were then seeded at 4.2×10^5 cells/ml in complete RPMI supplemented with 40 ng/ml of GM-CSF. After a further 3 days of incubation, 30 ml of complete RPMI containing 40 ng/ml of GM-CSF was added to each flask. Two days later, the loosely

adherent cells were harvested by gentle pipetting. Cell viability was assessed and cells were plated in complete RPMI containing 20 ng/ml of GM-CSF.

2.2.2.3 Culture of J558 Cell line

The mouse gene for granulocyte-monocyte colony stimulating factor (GM-CSF) was cloned into mammalian expression vector and transfected into the plasmacytoma line X63-AgS. Subsequently, these cells secrete large amounts of GMCSF into their growth medium, the levels of which can be quantified and used to grow BMDC.

Cells were retrieved from frozen stock (liquid nitrogen) and grown in complete RPMI medium containing G418 (Geneticin 1mg/ml, Gibco) for two passages at 37°C in a 5% CO₂ incubator. After the second passage, cells were washed in complete RPMI medium and re-seeded (1×10^6 cells/ml) without G418. For the first culture without G418 the cells were grown up to 'medium' density and then re-seeded at lower concentrations (2.5×10^5 cells/ml) for future passages. The supernatant was collected at each passage until passage number 9. The concentration of GM-CSF was quantified by ELISA (R&D systems, 2.2.5).

2.2.3 Flow cytometry

2.2.3.1 Determination of surface marker expression on cells

BMDC and spleen cell surface marker expression was analysed by flow cytometry using a FACS CaliberTM flow cytometer (Cyan ADP, Dako, Colorado). The flow cytometry was calibrated using Autocomp software in conjunction with commercially available fluorescent beads (Calibrate Beads, Dako, Colorado). BMDCs or spleen cells (0.5×10^6 cells/ml) were harvested in 200 µl of FACS buffer (2% (v/v) FCS, 0.1% (v/v) NaN₃, in PBS) and then incubated with Fc-receptor block (1 µl per 10^6 cells, BD Pharmingen) for 10 min. Cells were then incubated for 30 min on ice in the dark with fluorochrome labelled antibodies (Table 2.1.3, BD Pharmingen). After 30 min, cells were washed in 2 ml of FACS buffer (2% FCS (v/v), 0.1% (v/v) NaN₃, in PBS), re-suspended in 200 µl of FACS buffer and analyzed for immunofluorescence. Samples

were acquired using summit software (Dako, Colorado) and data was analysed using Flowjo™ software (Treestar, Oregon). 10,000 cells were analysed per sample, unless otherwise stated.

2.2.3.2 Analysis of adjuvant-mediated cell death

Adjuvant-mediated cell death was assessed by the addition of propidium iodide directly to FACS tubes (final concentration 1 µg/ml), immediately prior to the acquisition of samples on the flow cytometer (Cyan ADP, Dako, Colorado). Non viable cells take up the propidium iodide and its fluorescence is detected the FL3 channel.

2.2.3.3 Acridine orange staining

Incorporation of acridine orange was measured using flow cytometry. Cells were incubated with acridine orange (1 µg/ml; Sigma) for 15 min and washed. After removing all excess stain, cells were treated with particulate adjuvants. After 3 h, cells were harvested and re-suspended in 200 µl of FACS buffer. Acridine orange fluoresces green in its monomeric state and binds to cytosolic and nucleic DNA and RNA. It also becomes concentrated in acidic vesicles, due to its cationic nature, where it forms dimers and emits red fluorescence (650-680 nm, FL3). The amount of red fluorescence directly correlates with the amount of acidic vesicles within the cells.

2.2.4 TLR Agonist and Inhibitor Assays

Murine BMDC (generated as described in section 2.2.2.2) were plated at 6.25×10^5 cells/ml in 96 well round bottomed plates (Greiner BioOne). In general cells were stimulated with particles alone for 1 h before the addition of TLR agonists or TLR agonists alone and then left for 24 h before the supernatants were collected and analysed for cytokines by ELISA (2.2.5).

In more specific experiments either the particles or the TLR agonist was added to the cells for 6 or 24 hr prior to addition of the second stimulus or alternatively both stimuli (particle and TLR agonist) were added to the cells simultaneously.

For inhibitor assays, the cells were first incubated with inhibitors for 30 min or 1 h before addition of the particles. The exception to this was uricase which was incubated with particles overnight at 37°C before being added to the dendritic cells prior to particle and TLR agonist stimulation.

2.2.5 Measurement of Cytokine Production

Determination of IL-12p70, IL-1 α , IL-1 β , TNF- α , IL-17, IL-10 and IL-18

Concentrations of the cytokines IL-10, IL-12p70, IL-1 α , IL-1 β , TNF- α and IL-17 were measured using commercially available ELISA kits (R&D Systems; IL-18 from MBL). High binding 96 well plates (Greiner Bio-one) were coated overnight with 40 μ l/well of rat anti-mouse IL-10 (2 μ g/ml), IL-12p70 (4 μ g/ml), TNF- α (0.8 μ g/ml), IL-1 α (2 μ g/ml), IL-1 β (4 μ g/ml) or IL-17 (2 μ g/ml). The IL-18 ELISA kit included capture antibody pre-coated plates. After washing, non-specific binding sites were blocked with 100 μ l/well of blocking solution (1% (w/v) BSA in PBS) for 2 h at room temperature.

Plates were washed and then 40 μ l of test supernatant (diluted as per table 2.1) or serially diluted IL-10 (0-2000 pg/ml), IL-12p70 (0-1500 pg/ml), TNF- α (0-2000 pg/ml), IL-1 α (0-1000 pg/ml), IL-1 β (0-1000 pg/ml), IL-17 (0-1000 pg/ml) or IL-18 (0-1000 pg/ml) standard in 1% BSA/PBS were added to plates and incubated overnight at 4°C.

Plates were then washed and 40 μ l/well of biotinylated goat anti-mouse IL-10 (500 ng/ml), IL-12p70 (400 ng/ml), TNF- α (150 ng/ml), IL-1 α (50 ng/ml), IL-1 β (400 ng/ml), IL-17 (200 ng/ml) or IL-18 (100 ng/ml) in 1% (w/v) BSA/PBS was added for 2 h at room temperature. After this incubation, plates were washed and IL-10, IL-12p70, IL-1 α , IL-1 β , TNF- α , IL-17 and IL-18 were detected by incubating plates in the dark for 30 min with 40 μ l/well of horseradish-peroxidase (HRP)-conjugated streptavidin (1:200 in 1% (w/v) BSA/PBS). Finally plates were washed and 40 μ l of Ortho-

Phenylenediamine (OPD) substrate in phosphate citrate buffer (0.4 mg/ml) was added per well. The enzyme reaction was quenched by the addition of 25 µl per well of 1M H₂SO₄. The OD values were determined by measuring absorbance at 492 nm using a microtitre plate reader (Versamax, Molecular Devices). Cytokine concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse IL-10, IL-12p70, IL-1α, IL-1β, TNF-α, IL-17 and IL-18 of known concentrations.

Determination of IL-6, IL-12p40, IL-4, IL-5 and IFN-γ

Concentrations of the cytokines IL-4, IL-5, IL-12p40, IFN-γ and IL-6 were measured using assays developed using commercially available antibodies (BD Pharmingen). High binding 96 well plates (Greiner Bio-one) were coated overnight with 40 µl/well of rat anti-mouse IL-4 (1 µg/ml), IL-5 (1 µg/ml), IL-12p40 (2 µg/ml), IFN-γ (1 µg/ml) or IL-6 (1 µg/ml) capture antibody in PBS. After washing, non-specific binding sites were blocked with 100 µl/well of blocking solution, [5% (w/v) dried milk (marvel)/PBS except IL-6 (3% (w/v) BSA/PBS)], for 2 h at room temperature. Plates were washed and then 40 µl/well of test supernatant (diluted as per table 2.1) or serially diluted IL-4 (0-2000 pg/ml), IL-5 (0-2500 pg/ml), IL-12p40 (0-2000 pg/ml), IFN-γ (10 ng/ml) or IL-6 standard (0-5000 pg/ml) diluted in PBS were added to plates and incubated overnight at 4°C.

Plates were then washed and 40 µl/well of biotinylated goat anti-mouse IL-4 (1 µg/ml), IL-5 (1 µg/ml), IL-12p40 (2 µg/ml), IFN-γ (1 µg/ml) or IL-6 (2 µg/ml) in PBS was added for 1 h at room temperature. After this incubation, plates were washed and IL-4, IL-5, IL-12p40, IFN-γ or IL-6 was detected by incubating plates in the dark for 30 min with 40 µl/well of horseradish-peroxidase (HRP)-conjugated streptavidin (Sigma, 1:750). Finally, plates were washed and 40 µl per well of Ortho-Phenylenediamine (OPD) substrate in phosphate citrate buffer (0.4 mg/ml) was added. The enzyme reaction was quenched by the addition of 25 µl per well of 1M H₂SO₄. The OD values were determined by measuring absorbance at 492 nm using a microtitre plate reader (Versamax, Molecular Devices). Cytokine concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse IL-4, IL-5, IL-12p40, IFN-γ or IL-6 of known concentrations.

Determination of granulocyte-monocyte colony stimulating factor (GM-CSF)

The concentration of GM-CSF in the J558 cell line supernatant was measured using a commercially available ELISA kit (R&D systems) (Table 2.1.1). High-binding ELISA plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/ well of rat anti-mouse GM-CSF capture antibody (2 µg/ml) in PBS. After washing, non-specific binding sites were blocked by adding 100 µl/well of blocking solution (1% (w/v) BSA in PBS) and incubating the plates for 2 h at room temperature. Plates were washed and 50 µl of test supernatant (neat or at various concentrations in 1% (w/v) BSA/PBS) or serially diluted GM-CSF standard (0-2000 pg/ml) in 1% (w/v) BSA/PBS solution were added and plates were incubated overnight at 4°C. After washing, plates were incubated with 50 µl/well of biotinylated goat anti-mouse GM-CSF (50 ng/ml), in 1% BSA/PBS for 2 h at room temperature.

Plates were washed and GM-CSF was detected by incubating plates in the dark for 30 min with 50 µl/well of horse-radish peroxidase (HRP) conjugated streptavidin (1 in 200 in 1% (w/v) BSA/PBS) followed by washing and the addition of 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml). The colour reaction was allowed to develop for approximately 10 min after which time 25 µl/well of 1 M H₂SO₄ was added to stop the enzymatic reaction. The OD values were determined by measuring absorbance at 492 nm using a microtitre plate reader and cytokine concentrations of test samples were evaluated using a standard curve prepared using recombinant mouse GM-CSF of known concentrations.

2.2.6 Western Blot Analysis

2.2.6.1 Preparation of whole cell lysates

BMDC were plated in 24 well plates (0.5×10^6 cells/ml), in a volume of 500 µl per well. After appropriate stimulation, supernatants were removed and cells were lysed with 100 µl of 1X sample buffer (2.1.5). Samples were then heated to 95°C for 5 min.

2.2.6.2 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Gels were poured as described in materials section (table 2.4). Once set, 10 µl of each sample was loaded per well in addition, 5 µl of a protein molecular weight marker (Biorad Dual Colour 5-75kDa). After samples were loaded, gels were run at 120 V for 90 min.

2.2.6.3 Transfer of proteins to nitrocellulose membrane

The transfer of proteins to the nitrocellulose membrane was carried out using a semi-dry transfer system. Gels were removed from the glass plates, kept moist with transfer buffer (2.1.4) and placed onto the nitrocellulose membrane between sheets of moist (transfer buffer) filter paper. Care was taken to ensure there were no air bubbles in the system and the proteins were transferred for 1 h at 300 mA.

2.2.6.4 Immunodetection of proteins

All steps during the detection stage of western blotting were carried out on a rocker. Membranes were blocked using blocking buffer (3-5% (w/v) dried milk, marvel, in PBS) optimised for the specific antibody in question for 1 h at room temperature. Blots were then washed in PBS-tween, six times for 5 min. After washing, the membranes were incubated with the primary antibody overnight at 4°C. Membranes were washed in PBS-tween as previously described and then incubated with the secondary antibody for 1 h at room temperature. Blots were then washed and developed by chemiluminescence as per manufacturer's instructions (Amerhsam ECL).

2.2.7 Preparation of nanoparticles and microparticles

PLG nanoparticles were prepared by the solvent displacement method (This was carried out by Padma Malyala, Manomohan Singh and Derek T O'Hagan in Novartis, Cambridge, MA, USA) [470] [469]. An organic phase with PLG dissolved in acetone was added drop wise to pure, distilled water under magnetic stirring and the acetone was allowed to evaporate overnight. There was no surfactant present in the aqueous

phase. Particles were sterile filtered through a Pall (East Hills, NY) Acrodisc 0.2 μm Supor Membrane syringe filter.

Microparticles with 0.05% Dioctyl Sodium Sulfosuccinate (DSS), of size $\sim 1\ \mu\text{m}$ were prepared as previously described using a double emulsion solvent evaporation method [470] [469]. Anionic microparticles were prepared by homogenizing 10ml of 6% w/v polymer solution in Methylene Chloride with 2.5 ml of phosphate-buffered saline (PBS) using a 10-mm probe (Ultra-Turrax T25 IKA-Labortechnik, Germany) with a water : oil ratio of 1:4. After homogenizing for two minutes, the water-in-oil emulsion formed was then added to 50ml of distilled water containing Dioctylsulfosuccinate surfactant, and the mixture was homogenized at very high speed with a 20-mm probe (ES-15 Omni International, Jarrenton, VA) for 5 minutes in an ice bath. This procedure resulted in the formation of water-in-oil-in-water emulsion that was then stirred at 1000 rpm for 12 hours at room temperature; the Methylene Chloride was allowed to evaporate.

Microparticles with 0.05% Dioctyl Sodium Sulfosuccinate (DSS), of size $\sim 30\ \mu\text{m}$ were prepared as described above using a double emulsion solvent evaporation method. Anionic microparticles were prepared by homogenizing 4 ml of 15% (w/v) polymer solution in Methylene Chloride with 1 ml of phosphate-buffered saline (PBS) using a 10-mm probe (Ultra-Turrax T25 IKA-Labortechnik, Germany) with a water : oil ratio of 1:4. After homogenizing for one minute, the water-in-oil emulsion formed was then added to 30 ml of distilled water containing Dioctylsulfosuccinate surfactant and Poly Vinyl Alcohol (10 % (w/ v)), and the mixture was homogenized at high speed with a 20-mm probe (ES-15 Omni International, Jarrenton, VA) for 5 min. This procedure resulted in the formation of awater-in-oil-in-water emulsion that was then stirred at 1000 rpm for 12 h at room temperature; the Methylene Chloride was allowed to evaporate. The resulting suspension was washed thrice to remove PVA and the PLG microparticles reconstituted to the original volume.

2.2.7.1 Lyophilisation

Excipients, comprising of Mannitol and Sucrose, were added to the particle suspensions immediately prior to Lyophilisation. The concentrations of Mannitol and Sucrose were 4.5 % and 1.5 % of the reconstitution volume respectively. The suspensions were placed in glass vials and frozen at -80°C for two hours. Lyophilization was carried out in a Labconco Freeze Dry System, Freezone 4.5 (Kansas City, MO) operating at -49°C and vacuum less than 133×10^{-3} mBar for approximately 24 h. All samples were reconstituted with distilled water. The osmolarity for all formulations was in the range 270-330 mOsm/mL.

2.2.7.2 Particle Characterisation

The size distribution was determined with a Zetasizer 3000HSA (Malvern Instruments, UK) for the nanoparticles. Microparticles were sized with a Horiba LA-930 (Irvine, CA). This instrument is based on static light scattering to determine particle size, while the Zetasizer used dynamic light scattering to detect the smaller particles.

2.2.8 Confocal Microscopy

The confocal microscopy section of this project was carried out with help from Dr. James Harris (Adjuvant Research Group, TCD).

BMDC (0.5×10^6 cells/ml) were added plated into 24 well tissue culture plates onto 19 mm coverslips (Marenfeld, Germany) and left overnight at 37°C. The cells were then treated with fluorescent polystyrene microparticles (0.6 mg/ml; Corpuscular) of 420 nm, 1 µm, 10 µm and 32 µm in size. Each sample was set up in duplicate. After 1 hr the supernatants were removed and cells were fixed in 2% (v/v) paraformaldehyde (in PBS) for 20 min at room temperature. Samples were then washed 3 times in PBS. One set of samples was permeabilised by incubation in 0.1% (v/v) Triton-X100 (Sigma) in PBS for 10 min at room temperature. Both sets of samples were then blocked in 1% (w/v) BSA and 1% (v/v) goat serum in PBS for 20 min at room temperature. After blocking, samples were stained with Alexa-Fluor 594-conjugated wheat germ agglutinin (10 µg/ml) for 30 min and then washed 5 times in PBS.

Once stained coverslips were removed from wells and dipped in dH₂O and excess dH₂O was removed before mounting onto a glass slide with one drop of mounting medium (Dako, Colorado).

2.2.9 Removal of LPS from Ova

Commercially available Ova is contaminated with LPS. Therefore, LPS from the Ova (Sigma) used for *in vivo* studies was removed to ensure that the Ova was endotoxin free. The Detoxi-GelTM endotoxin removing kit (Thermo scientific) was used according to manufacturer's protocol as outlined below. The Detoxi-GelTM resin column was regenerated by washing with resin-bed volumes of 1% sodium deoxycholate five times followed by 5 washes with pyrogen free H₂O (Baxters) to remove the detergent. Post-regeneration, the Ova solution was added to the columns. The first 900 µl of fluid to run through the columns was discarded as this represents the column bed volume which does not contain any Ova. The sample was run through three columns sequentially three times.

Once the sample has been collected the column was washed through with pyrogen free H₂O (Baxter) and the first 900 µl of this is also collected as this is the column bed volume left from the sample run through. Once the entire sample was collected it was tested for protein concentration (BCA assay) in order to quantify the amount of Ova that passed through the column. In addition, the level of LPS following the detoxification process was analysed using an endotoxin detection kit (Lonza).

2.2.10 *In vivo* studies

2.2.10.1 Role of NLRP3 in the adjuvant properties of particles

(i) Female C57BL/6 (6-8 weeks old, Harlan, UK) and NLRP3^{-/-} mice (bred in the Bioresources unit TCD) were injected subcutaneously in the left flank or intraperitoneally with, PBS, LPS-free Ova (50 µg/mouse) or PLG-microparticles (4 mg; Novartis) with Ova. Mice were immunised on day 0 and subsequently boosted on day 14 of the experiment in an identical manner. Blood samples were collected prior to the boost immunisation.

After 21 days mice were sacrificed, spleens, inguinal lymph nodes, mesenteric lymph nodes and mediastinal lymph nodes were removed and blood samples were collected. Isolated single cell suspensions (spleens 2×10^6 cells/ml, peritoneal cells 1×10^6 cells/ml, lymph node cells 1×10^6 cells/ml) were re-stimulated *in vitro* with either medium alone, LPS-free Ova (0.02-1 mg/ml). After 3 days, supernatants were collected and subsequently analysed for a range of cytokines by ELISA (2.2.5).

Blood samples were centrifuged (30,680 g for 5 min at 4°C) and serum was collected. The serum collected was subsequently analysed for antigen-specific antibodies by ELISA (2.2.11).

The skin surrounding the subcutaneous injection site was removed and preserved (10% Formalin; Sigma) for histological analysis (2.2.11).

(ii) C57BL/6 mice and NLRP3^{-/-} mice (bred in Bioresources unit TCD) were injected intra-peritoneally with PBS or PLG microparticles (4 mg/ mouse). Mice were sacrificed 18 h after immunisation. Cells were collected from the peritoneal cavity by injecting 10 ml of ice cold PBS (Biosera) into the peritoneum using a 23G needle. The peritoneal fluid was agitated using a vortex and removed using a 23G needle. Cell subtypes and activation states were analysed by flow cytometry (2.2.3, table 2.3).

2.2.10.2 Comparison of the Efficacy of Particulate Vaccine Adjuvants

C57BL/6 mice were injected subcutaneously with alum (2.5 mg), PLG (2.5 mg) or PS (2.5 mg) in the right and left flank. Each particulate adjuvant was co-administered with keyhole limpet hemocyanin, KLH (5 $\mu\text{g}/\text{mouse}$; Calbiochem). Control groups were immunised with either PBS alone or KLH alone. Mice were immunised on day 0 and sacrificed after 7 days. Spleens and inguinal lymph nodes were removed and blood samples were collected. Isolated single cell suspensions (spleens 2×10^6 cells/ml, lymph node cells 1×10^6 cells/ml) were stimulated with either medium alone, KLH (2 $\mu\text{g}/\text{ml}$, 10 $\mu\text{g}/\text{ml}$ and 50 $\mu\text{g}/\text{ml}$) or a combination of proliferative stimuli as follows; anti-CD3 (1 $\mu\text{g}/\text{ml}$) or anti-CD3 (1 $\mu\text{g}/\text{ml}$) with PMA (250 ng/ml).

The right and left injection sites were both removed. The left injection site was homogenised in 500 mM NaCl/50 mM Hepes, pH 7.4, containing 0.1% (v/v) Triton X-100, protease inhibitor cocktail (contains serine/cysteine/acid protease inhibitors and aminopeptidases, Sigma) and 0.02% (v/v) NaN₃. The homogenates were then sonicated (pulsed three times for 10 sec) and centrifuged at 6000 g for 20 min at 4°C. Supernatants were collected and analysed for cytokines by ELISA (2.2.5). The right injection site was preserved (10% v/v formalin; Sigma) and analysed by histology (2.2.12).

Blood samples were centrifuged (30,680 g for 5 min at 4°C) and serum was collected. Subsequently, serum antigen-specific antibody concentrations were analysed by ELISA (2.2.11).

2.2.10.3 Kinetics of immune responses to injection with alum

Wild type and IL-1R1^{-/-} C57BL/6 mice (bred in Bioresources unit TCD) were injected with alum (3mg/mouse) subcutaneously in their right flank. Mice were sacrificed at 3 h, 1, 3, 5 and 7 days post immunisation. Injection sites were removed and preserved (10% v/v Formalin;Sigma) for histological analysis (2.2.12). Spleens were removed, cells were isolated and stimulated (2x10⁶ cells/ml) with either anti-CD3 (1 µg/ml) alone or in combination with PMA (0.2 ng/ml, 2 ng/ml, 20 ng/ml) or LPS (10 ng/ml, 100 ng/ml, 1000 ng/ml). After 3 days, supernatants were removed and analysed for cytokines by ELISA (2.2.5).

2.2.10.4 Kinetic study of PLG microparticles

C57BL/6 mice were injected subcutaneously with PLG (4 mg; Novartis) in the left flank. Mice were sacrificed at 3 h, 1, 3, 5, 7 and 14 days post immunisation. Spleens, inguinal lymph nodes, mesenteric lymph nodes and mediastinal lymph nodes were removed.

Isolated single cell suspensions (spleens 2x10⁶ cells/ml, peritoneal cells 1x10⁶ cells/ml, lymph node cells 1x10⁶ cells/ml) were stimulated *in vitro* with either medium alone or a

combination of proliferative stimuli as follows; anti-CD3 (0.05 µg/ml), anti-CD3 (0.5 µg/ml) or anti-CD3 (1 µg/ml) with PMA (250 ng/ml). A second set of single spleen cell suspensions were stimulated with either medium alone or ultrapure LPS (10, 100, 1000 pg/ml; Alexis Biochemicals). After 3 days, supernatants were removed and analysed for cytokines by ELISA (2.2.5).

2.2.10.5 Effect of particle size on the efficacy of PVA

C57BL/6 mice were injected, either subcutaneously in the left flank or intraperitoneally, with 4 mg of PS particles of varying size; 50 nm, 200 nm, 430 nm, 1 µm, 10 µm and 30 µm. Each particulate was co-administered with LPS-free Ova (50 µg/mouse). Control groups was immunised with either PBS or ova alone. Mice were immunised on day 0 and subsequently boosted on day 14 (right flank for subcutaneous study) in a similar manner as described above. Blood samples were collected prior to the boost immunisation.

After 21 days mice were sacrificed, the peritoneal cavity cells and blood samples were collected and spleens, medistinal, mesenteric and inguinal lymph nodes were removed. Cells were collected from the peritoneal cavity by injecting the peritoneum with 10 ml of ice cold PBS (Biosera) using a 23 G needle. Isolated single cell suspensions (spleens 2×10^6 cells/ml, peritoneal cells/lymph nodes 1×10^6 cells/ml) were stimulated *in vitro* with either medium alone, LPS-free Ova (20, 100 or 500 µg/ml), anti-CD3 (1 µg/ml) alone or anti-CD3 (1 µg/ml) with PMA (250 ng/ml). After 3 days, supernatants were removed and analysed for cytokines by ELISA (2.2.5).

Blood samples were centrifuged (30,680 g for 5 min at 4°C) and serum was collected and subsequently analysed for antigen-specific antibodies by ELISA (2.2.12).

The skin surrounding the subcutaneous injection sites (day 0 and day 14) were removed and preserved (10% Formalin; Sigma) for histological analysis (2.2.11).

2.2.11: Histology

The histology and immunohistochemistry work in this project was carried out in collaboration with; Prof Brian Sheehan and Alexandra Whelan in University College Dublin (School of Agriculture, Food Science and Veterinary Medicine UCD, Belfield, Dublin 4, Ireland; alum kinetics study), Dr. Sean Coyle (School of Agriculture, Food Science and Veterinary Medicine UCD, Belfield, Dublin 4, Ireland Particle size study). The experimental set up and section isolation was carried out in Trinity while the embedding, sectioning and staining was carried out in UCD under supervision.

For both *in vivo* studies the injection site was removed and preserved in 10% formalin (Sigma) for further histological examination. Preserved injection sites were then trimmed into the appropriate size and shape and placed in embedding cassettes. The cassettes hold the sample in place, they contain holes to allow for the penetration of chemicals used during the embedding process.

The cassettes were then paraffin embedded using the following protocol:

1. 70% v/v Ethanol, two changes, 1 hr each
2. 80% v/v Ethanol, two changes, 1 hr each
3. 95% v/v Ethanol, two changes, 1 hr each
4. 100% v/v Ethanol, two changes, 1hr each
5. Xylene, three changes, 1 hr each
6. Paraffin wax (56-58⁰C), two changes, 1 hr 30 min each
7. Embedding tissues into paraffin blocks (Histocentre 3 Thermoshandon)

Paraffin blocks were then trimmed and cut into 3µm sections (Leica RM2135 Microtome). The paraffin ribbon obtained was placed in a water bath (40⁰C) and mounted onto a glass slide. Slides were left to dry. The paraffin was then removed from the sections by reversing the schedule outlined above with the addition of a rinse in distilled water to finish. Sections were then ready for staining and immunohistochemistry. Slides were then stained with haemotoxylin and eosin (Thermo Shandon Varistain Gemini, autostainer).

2.2.12: Antibody ELISA

IgG, IgG1, IgG2a, IgG2b, IgG2c

Serum antigen-specific antibody concentrations were analysed using commercially available antibodies (BD-Pharmingen or Sigma). Medium binding plates (Greiner Bio) were coated (50 µl/well) with KLH (5 µg/ml, Calbiochem) or Ova (50 µg/ml, Sigma) in carbonate buffer (2.1.2) overnight at 4°C. Excess antigen was removed by washing plates with wash buffer (PBS/0.05% v/v Tween 20) and non-specific binding sites were blocked by incubating plates with 10% (w/v) of dried milk (Sigma) for 2 h at room temperature. Plates were washed and then serum samples were added to the plate (50 µl/well) at a starting dilution of 1/100 or 1/50 and subsequently serially diluted 1/2 across the plate in PBS and left overnight at 4°C. After sample incubation, plates were washed and biotinylated antibodies (Pharmingen) specific for IgG, IgG1, IgG2a, IgG2b or IgG2c, (2.1.2) were added to the plates (50 µl/well) for 1 hr. Plates were washed and IgG, IgG1, IgG2a, IgG2b and IgG2c were detected by incubating plates in the dark for 30 min with 50 µl/well of horseradish-peroxidase (HRP)-conjugated streptavidin (1:750; Sigma). Finally, plates were washed and 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml) was added. The enzyme reaction was quenched by the addition of 25 µl/well of 1 M H₂SO₄ following a 30 min incubation in the dark at 37°C.

The OD values were determined by measuring absorbance at 492 nm using a microtitre plate reader. Antibody concentrations are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut off point of 2 standard deviations.

IgE

IgE concentrations were analysed using a commercially available kit (BD-Pharmingen). Medium binding plates (Greiner Bio) were coated (50 µl/well) with IgE capture antibody (1/250) in carbonate buffer (2.1.4.1). Excess antibody was removed by washing plates with wash buffer (PBS/0.05% v/v Tween 20) and non-specific binding sites were blocked by incubating plates with 10% (v/v) FCS in PBS (Sigma) for 2 h at room temperature.

Plates were washed and then serum samples were added to the plate (50 µl/well) at a starting dilution of 1/10 or an IgE standard (100 ng/ml) and subsequently serially diluted 1/2 across the plate in PBS and left overnight at 4°C. After sample incubation, plates were washed and a mixture of anti-IgE detection antibody (1/1000) and horseradish-peroxidase (HRP)-conjugated streptavidin (1:250) in PBS was added to the plates (50 µl/well) for 1 hr. Finally, plates were washed and 50 µl/well of OPD substrate in phosphate citrate buffer (0.4 mg/ml) was added. The enzyme reaction was quenched by the addition of 25 µl/well of 1 M H₂SO₄ following a 30 min incubation in the dark at 37°C. IgE concentrations for test samples were evaluated using a standard curve prepared using recombinant mouse IgE of known concentration.

Statistical Analysis

Statistical analyses were performed using the computer based mathematical package Instat 3. Statistical differences between experimental and control groups were determined by one-way ANOVA. Where significant differences were found, the Tukey-Kramer multiple comparisons test was used to identify differences between individual groups. P-values of 0.05 or less were considered to be significant.

Chapter 3

The Role of NLRP3 in the Adjuvant Activity of Particulate Vaccine Adjuvants

3.1 Introduction

Vaccination has had a profound effect on mortality and morbidity rates due to infectious disease since its widespread introduction in the 19th century. The development of vaccines has progressed from the use of whole live or attenuated organisms to the use of specific antigenic components of microorganisms. This more refined process can be attributed to significant developments in molecular biology and immunology, coupled with advances in genomics which now allows for whole genome sequencing and subsequent identification of putative vaccine antigens. Although modern subunit vaccines display improved safety profiles compared to their predecessors, they are less immunogenic, which significantly decreases their efficacy. In order to compensate for this, adjuvants can be added to the vaccines during the formulation process.

The adjuvant properties of several compounds were first demonstrated by Gaston Ramon in the 1920s with subsequent compounds identified by Alexander Glennie [376-377]. Adjuvants are now a common feature of modern vaccine formulations. Vaccine adjuvants can be broadly subdivided into two groups; immunostimulatory adjuvants which can directly activate the immune system through interaction with known receptors and particulate vaccine adjuvants (PVA) or delivery systems which, in most cases, have no known receptors.

PVA, particularly alum, have been in use since the 1920s and are the most commonly used vaccine adjuvants to date. However, the exact mechanism of action of these “delivery systems” remains unknown. Several theories have been proposed in order to explain the immunostimulatory activities of particulates. During Glennie’s original experiments, it was noticed that none of the tetanus toxoid that had been mixed with the adjuvant remained in the supernatant and this led to the idea that the antigen must be completely adsorbed by the adjuvant in order to increase the immune responses [377]. Based on these observations it was suggested that aluminium-containing adjuvants worked by forming an antigen ‘depot’ at the site of injection, thereby prolonging the amount of time that cells of the immune system have to ‘sample’ and interact with the antigen. The efficacy of PVA has also been attributed to the conversion of soluble antigens into particulate form, which enhances phagocytosis by antigen-presenting cells (APC). Contrary to the depot theory, it has been shown that excision of the antigen and alum complex from the injection site after injection does not affect the efficacy of this

PVA [449]. Furthermore, it has been demonstrated that aluminium-containing adjuvants can enhance the immune response to rapidly desorbed antigens as well as non-adsorbed antigens [520-521]. More recently, the attention of adjuvant research has been re-focused towards PVA and further studies have added to the concept that these adjuvants act as more than elaborate delivery systems.

DC have been described as nature's adjuvant. These professional APC are unique in their ability to activate naive T cells, acting as the central link between the innate and adaptive arms of the immune system. These sentinel cells have the ability to phagocytose, process and present antigen to naive T cells thus activating and directing the adaptive responses. This unique characteristic of DC has prompted investigations into the role of DC in the adjuvant properties of particulates. Previous studies have demonstrated that treatment of human PBMC or murine BMDC with alum enhances expression of co-stimulatory molecules [458, 522]. In contrast, others have shown that incubation of BMDC *in vitro* with PVA alone was not sufficient to enhance the surface expression of MHC class II or co-stimulatory molecules, suggesting that PVA cannot act directly on DC [165, 476]. However, DC isolated from the peritoneum after injection of alum displayed increased expression of CD86 and the authors postulated that this enhancement was due to the release of endogenous signals which induced activation of DC [165]. In addition, Kool *et al* found that intraperitoneal injection of alum enhanced the recruitment of inflammatory monocytes, which are known precursors of DC, into the peritoneum [165]. Furthermore, depletion of these inflammatory monocytes significantly decreased the proliferation of antigen-specific T cells in the draining lymph nodes [165]. Taken together these studies suggest that DC play a central role in the adjuvanticity of particulates.

In view of the crucial role played by DC in the activation of adaptive immune responses, the main aim of this study was to investigate the effects of particulates on DC. These studies focused on the PVA aluminium hydroxide (Alhydrogel) which will be referred to as alum throughout, poly lactide-co-glycolide (PLG) microparticles and polystyrene (PS) microparticles. This range of particulate vaccine adjuvants was used to determine if common mechanisms of action could be identified for particulates.

3.2 Results

3.2.1. PVA alone do not promote maturation of DC

DC that have not come into contact with any microorganism-derived or endogenous danger signals are generally said to be in an immature state and are extremely efficient at phagocytosis. Once a DC has encountered a pathogen or host-derived danger signal, it undergoes several changes to become a mature dendritic cell, making it more effective at antigen presentation. During this maturation process, DC upregulate several cell surface markers important for interaction with T cells, including the co-stimulatory molecules CD86, CD80, CD40 and MHC class II molecules [179]. Whether or not PVA, such as alum, have the ability to induce maturation of DC currently remains controversial. Some groups have shown that Ova-conjugated alum can enhance the expression of both CD80 and CD86 by DC [458]. Conversely, others have reported that alum has no effect on the expression of co-stimulatory markers by DC *in vitro* [165, 476]. In order to determine whether PVA have the ability to induce DC maturation *in vitro*, BMDC were incubated with alum (5 mM), PLG (PLG) microparticles (1µm; 0.6 mg/ml) or polystyrene (PS) microparticles (430nm; 0.6 mg/ml) alone. As a positive control LPS (5 ng/ml) was used to treat BMDC. LPS stimulation of DC resulted in enhanced expression of MHC class II, CD40 and CD86, as measured by flow cytometry (Figure 3.1). In contrast to this, treatment of BMDC with alum (5 mM) had no effect on the surface expression of MHC II, CD86 and CD40 on BMDC (Figure 3.1). Similarly, treatment of BMDC with PLG (0.6 mg/ml) or PS (0.6 mg/ml) had no effect on the surface the expression of CD40, MHC class II or CD86 (Figure 3.1). Overall, these results show that PVA do not promote maturation of BMDC.

3.2.2 PVA promote the secretion of IL-1 β and IL-1 α by BMDC

The next question addressed was whether PVA could directly induce cytokine production alone or modulate cytokine production by dendritic cells in response to TLR agonists. The concentrations used were identified initially by titration of stock PVA. These amounts are slightly higher than those given to humans (0.5mg/ml of saline) but are however, comparable to those used in peer reviewed publications. The particulates alone did not induce the secretion of IL-6, TNF- α , IL-12, IL-10, IL-1 α or IL-1 β . When added before a TLR agonist, particulates did not enhance the secretion of IL-6, TNF- α , IL-12 or IL-10 (Data not shown). However synergistic effects were observed on the secretion of IL-1 β and IL-1 α . Stimulation of BMDC with LPS alone was inefficient at inducing IL-1 β and IL-1 α production. However, if the cells were pre-incubated with either alum (Figure 3.2), PLG (1 μ m; Figure 3.3), or PS (430nm; Figure 3.4) for 1 h prior to the addition of the TLR agonist, significantly higher concentrations of IL-1 β and IL-1 α were secreted than with LPS alone. Alum enhanced IL-1 β and IL-1 α secretion by BMDC in the presence of LPS with a concentration of 1 mM having the most marked effect (Figure 3.3). PLG and PS synergised with LPS to enhance IL-1 β and IL-1 α secretion by BMDC with 3 mg/ml being the most potent concentration tested (Figure 3.2). Furthermore, alum, PLG and PS, enhanced the secretion of IL-1 α in the absence of LPS (Figure 3.2-3.4). This enhancement of IL-1 α was observed at several concentrations with alum (25mM, 5mM, 1mM), with 5 mM having the most marked effect (Figure 3.2). In the case of PLG, a significant enhancement of IL-1 α was only observed at the top concentration tested (3mg/ml) (Figure 3.3). BMDC treated with PS at 3 mg/ml and 0.6 mg/ml secreted significantly higher levels of IL-1 α than unstimulated BMDC, with 3 mg/ml having the most marked effect (Figure 3.4).

3.2.3 PVA can synergise with a range of TLR agonists to induce IL-1 β production by BMDC

In order to determine if the synergistic effect between PVA and LPS on IL-1 β secretion was restricted to agonists of TLR4, which is expressed on the cell surface, BMDC were pre-incubated with particles for 1 h and then stimulated with a range of TLR agonists. The following TLR agonists were used; the TLR4 agonist LPS, the TLR2 agonist PAM3CSK4, the TLR3 agonist poly I:C and the TLR9 agonist CpG. TLR2 and TLR4 are expressed on the cell surface, while TLR3 and TLR9 are expressed on the surface of endosomes within the cytosol. Synergistic effects on IL-1 β production were observed between all four TLR agonists and alum, PLG (1 μ m) and PS (430nm) (Figure 3.5). However, when alum and CpG were combined, no significant increase in IL-1 β was observed (Figure 3.5).

3.2.4 The enhancement of IL-1 β secretion by PVA does not require simultaneous presentation of the TLR agonist and the PVA to the DC

Having shown that alum, PLG and PS could synergise with a range of TLR agonists to enhance the secretion of IL-1 β by DC, the next key question to answer was whether the particle and the TLR agonist must be presented to the dendritic cell simultaneously. In order to investigate this, BMDC were stimulated with alum, PLG (1 μ m) or PS (430nm) either simultaneously or 6 h or 24 h prior to or after LPS addition. For both PS and alum, addition of the particles 6 h after LPS stimulation resulted in the greatest enhancement of IL-1 β production (Figure 3.6). In contrast, the addition of PLG 6 h before LPS stimulation induced optimal secretion of IL-1 β (Figure 3.6). It is also worth noting that even when addition of the TLR agonist and the particulate is separated by 24 h, enhancement of IL-1 β secretion can still be observed (Figure 3.6).

3.2.5 PVA promote processing of pro-IL-1 β (31kDa) into its active (17kDa) form by activation of caspase-1

Having established that PVA enhanced the secretion of IL-1 β , the underlying mechanism was addressed. The secretion of IL-1 β is tightly regulated; it is controlled at the level of pro-IL-1 β transcription and the conversion of the inactive protein (31 kDa) into its biologically active form (17 kDa) before secretion. Pro-IL-1 β is cleaved between Asp¹¹⁶ and Ala¹¹⁷ by caspase-1 [124]. Given the complex nature of IL-1 β secretion, it is possible that PVA could enhance the secretion of IL-1 β by either induction or enhancement of IL-1 β transcription within the nucleus or by increasing the processing of pro-IL-1 β into its active form. In order to determine whether PVA were enhancing transcription or increasing the processing or secretion of IL-1 β , western blots were run on the cell lysates and supernatants of BMDC treated with PLG (1 μ m), PS (430nm), alum or LPS alone or LPS in combination with particulates for 24 h. LPS at 5 ng/ml induced pro-IL-1 β (31kDa) and pre-incubation with PLG, PS or alum had no effect on the level of pro-IL-1 β found in cell lysates as detected by western blot (Figure 3.7). Stimulation with particles alone had no effect on the expression of pro-IL-1 β . Western blot analysis of the supernatants showed that LPS at 5 ng/ml was not sufficient to induce processing of IL-1 β into its biologically active form, 17 kDa (Figure 3.7). However, pre-incubation of cells with PLG, PS or alum prior to LPS treatment resulted in the secretion of processed IL-1 β (17 kDa) into the supernatant. This effect was most marked at the highest concentrations of particulates (Figure 3.7).

The activation of caspase-1 must be tightly regulated in order to control the processing of IL-1 β , therefore caspase-1 is translated as a zymogen (45 kDa) which must be converted into an enzymatically active heterodimer (p10 and p20 chain) by cleavage at an aspartic acid residue. In order to determine if the particulates were enhancing IL-1 β processing by activating caspase-1, western blots were run on the cell lysates and supernatants of BMDC treated with LPS and particulates. LPS at 5 ng/ml did not activate caspase-1 (Figure 3.8). Similarly, stimulation of BMDC with PLG (1 μ m), PS (430nm) or alum alone did not activate caspase-1 (Figure 3.8).

However, pre-incubation of cells with PLG, PS or alum prior to the addition of LPS resulted in activation of caspase-1 as evidenced by detection of the p10 fragment in cell lysates (Figure 3.8). This enhancement of caspase-1 activation was strongest at the highest concentration of all three particulates. Furthermore, the caspase-1 p10 fragment was detected in concentrated supernatants from PLG pre-treated cells (Figure 3.8).

3.2.6 The ability of PVA to enhance IL-1 β secretion by BMDC is caspase-1 dependent

Having established that PVA could synergise with TLR agonists to activate caspase-1, the role of caspase-1 in the ability of PVA-mediated IL-1 β secretion was investigated. To determine if particle-mediated enhancement of IL-1 β was dependent on caspase-1 activation, BMDC were pre-incubated with a caspase-1 inhibitor (YVAD-fmk). Pre-treatment with the caspase-1 inhibitor prevented the enhancement of IL-1 β secretion by alum, PLG (1 μ m) and PS (430nm) demonstrating that the increase in processing mediated by the particles is caspase-1 dependent (Figure 3.9).

Treatment with the caspase-1 inhibitor also significantly reduced the secretion of IL-1 α in response to particles and LPS (Figure 3.9). However the effect of YVAD-fmk on particle-mediated enhancement of IL-1 α was not comparable to that of IL-1 β . While IL-1 α secretion was significantly reduced by YVAD-fmk it was not completely abrogated (Figure 3.9).

3.2.7 The ability of PVA to enhance IL-1 β secretion by BMDC is caspase-1- and ASC-dependent

In 2002 Martinon *et al* proposed the existence of the 'inflammasome', a multiprotein complex which helped to induce activation of caspases resulting in the processing of IL-1 β . They suggested that this multiprotein complex was composed of apoptosis-associated speck-like protein containing a CARD (ASC), a member of the NLR protein family and caspase (1 or 5) [76].

Given that the PVA can synergise with TLR agonists to activate caspase-1, it is possible that they are inducing activation of caspase-1 by triggering formation of an

inflammasome. This experiment examined whether or not ASC, a known component of the inflammasome, was necessary for particle-mediated enhancement of IL-1 β and IL-1 α . BMDC were generated from ASC-deficient and wild type (WT) mice. Treatment of BMDC with PLG (1 μ m) or PS (430nm) enhanced the secretion of IL-1 β in WT mice, however this effect was compromised in the ASC^{-/-} mice (Figure 3.10). In agreement with previous studies using a caspase-1 inhibitor, caspase-1^{-/-} mice also showed a significant defect in their ability to secrete IL-1 β when treated with PVA and LPS (Figure 3.10).

In contrast, the ability of PVA to enhance the secretion of IL-1 α was not abrogated in the ASC^{-/-} mice (Figure 3.11). The particulates tested exhibited a differential requirement for ASC. While the ability of PS to enhance IL-1 α was not abrogated it was, however, decreased in the ASC^{-/-} mice (Figure 3.11). In contrast to this, PLG retained the ability to enhance the secretion of IL-1 α in the absence of ASC (Figure 3.11). The ability of both PS and PLG to enhance the secretion of IL-1 α was reduced, but not completely abrogated, in the caspase-1^{-/-} mice (Figure 3.11).

3.2.8 Particle-mediated enhancement of IL-1 β and IL-18 is NLRP3-dependent

As demonstrated in previous experiments PVA enhanced the secretion of IL-1 β in a caspase-1 and ASC-dependent manner, which suggested a role for an inflammasome (Figure 3.10, 3.11). Several members of the NLR family, including NLRP1, NLRP2 and NLRP3, have been shown to interact with ASC and it has been predicted that they have the potential to form inflammasomes [76, 85, 129]. Uric acid, the causative agent of gout, was identified as an activator of the NLRP3 inflammasome [101]. Based on the shared particulate nature of uric acid and alum, PLG and PS, it was predicted that PVA were enhancing the secretion of IL-1 β via the NLRP3 inflammasome. In order to investigate whether particulate-mediated enhancement of IL-1 β secretion required the NLRP3 inflammasome, BMDC were generated from wild type (WT) or NLRP3^{-/-} mice. Pre-treatment of DC with PLG (1 μ m), PS (430nm) or alum before LPS stimulation resulted in enhanced secretion of IL-1 β in WT cells (Figure 3.12).

In the case of PLG and PS, a concentration of 3 mg/ml induced the most marked enhancement of IL-1 β secretion, while alum induced a significant increase of IL-1 β

secretion at concentrations of 25 mM, 5mM and 1 mM (Figure 3.12). In contrast to the WT BMDC, pre-incubation of NLRP3^{-/-} BMDC with PLG, PS or alum followed by LPS stimulation did not promote IL-1 β secretion. There was no detectable IL-1 β found in the supernatants of NLRP3^{-/-} cells, in comparison to the high concentrations of the cytokine secreted by the WT cells (Figure 3.12). IL-18 also requires processing by caspase-1 in order to be converted into its active form and secreted from the cell. Therefore, the ability of PVA to enhance the secretion of IL-18 by BMDC was also investigated. In a similar manner to IL-1 β , pre-treatment of BMDC with particulate adjuvants prior to LPS stimulation significantly enhanced the secretion of IL-18 by BMDC (Figure 3.13). This enhancing effect on IL-18 was completely abrogated in the NLRP3^{-/-} cells (Figure 3.13).

NLRP3 deficiency also significantly reduced IL-1 α secretion in response to PVA with LPS (Figure 3.14). In a similar manner to the requirement of caspase-1, PVA showed a differential requirement for NLRP3 in their ability to enhance the secretion of IL-1 α in comparison to IL-1 β (Figure 3.14). NLRP3 was not essential for the enhancement of IL-1 α by PVA as the effect was not abrogated in NLRP3^{-/-} cells, although it was reduced (Figure 3.14).

Although IL-1 β and IL-18 secretion was abrogated in NLRP3^{-/-} BMDC, TNF- α secretion was unaffected (Figure 3.15), demonstrating that NLRP3 is not required for the induction of all pro-inflammatory cytokines and that the NLRP3^{-/-} cells were not inherently defective in their ability to produce and secrete cytokines (Figure 3.15).

Having established that particulate mediated enhancement of IL-1 β secretion was NLRP3-dependent, it was necessary to investigate whether or not this result would be reflected in the ability of PVA to enhance the activation of caspase-1 and subsequent processing of IL-1 β . In order to investigate this, western blot analysis was carried out on lysates and supernatants from WT and NLRP3^{-/-} mice treated with LPS alone or LPS with alum or PLG. There was no difference in the levels of pro-caspase-1 found in cell lysates from WT and NLRP3^{-/-} BMDC (Figure 3.16). However, while combining PVA and LPS induced processing of IL-1 β in WT BMDC this processing was completely absent in the NLRP3^{-/-} BMDC (Figure 3.16). Similarly, while the activation of caspase-1 by the PVA plus LPS was detected in WT BMDC, this activation was abrogated in the NLRP3^{-/-} BMDC (Figure 3.16). The processing of caspase-1 was most marked with the

highest concentrations of alum (5 mM and 1 mM) and the p10 band was absent in the NLRP3^{-/-} cells (Figure 3.16).

3.2.9 Particle-mediated enhancement of IL-1 β but not IL-1 α is dependent on K⁺ efflux

Findings from this study have demonstrated that PVA have the ability to synergise with TLR agonists to activate the NLRP3 inflammasome (Figure 3.16). Following on from this, the underlying mechanism was investigated. It has been shown that NLRP3 inflammasome activation by monosodium urate crystals (MSU), the TLR 7/8 agonist R837 and Nigericin, a pore forming toxin, requires K⁺ efflux from the cell [131]. In order to investigate the requirement for K⁺ efflux in the case of particulate-mediated inflammasome activation, the normal growth medium was replaced with KCl-supplemented medium therefore preventing the efflux of K⁺ ions from the cells. BMDC were subsequently pre-treated with alum, PLG (1 μ m) or PS (430nm) prior to the addition of LPS. The prevention of K⁺ efflux abrogated particle-mediated enhancement of IL-1 β (Figure 3.17). Particle-mediated enhancement of IL-1 α was also significantly reduced when K⁺ efflux was blocked. However, this was not comparable to the complete abrogation observed with IL-1 β (Figure 3.17).

3.2.10 Particle uptake is necessary for optimal activation of the inflammasome and subsequent secretion of IL-1 β

Given that the ability of PVA to synergise with TLR agonists and enhance the secretion of IL-1 β does not require presentation of the TLR and particle simultaneously, the role of microparticle uptake in the induction of IL-1 β secretion was investigated. To determine the relationship between microparticle size and the enhancement of IL-1 β , cells were treated with polystyrene (PS) particles of 420 nm, 1 μ m, 10 μ m and 32 μ m in size and analysed by confocal microscopy. PS particles of 420 nm and 1 μ m in size were efficiently taken up by DC (Figure 3.18). DC were also able to phagocytose PS particles of 10 μ m in size (Figure 3.18). In contrast, PS particles of 32 μ m were not efficiently taken up by the DC however the cells were seen to attach themselves to the surface of the particles (Figure 3.18).

Having identified the limits of phagocytosis in terms of particle size, the relationship between uptake of particles and their ability to enhance secretion of IL-1 β was investigated. Of all the particles tested, the 1 μ M PS particles were most effective at enhancing IL-1 β secretion, followed by the 420 nm particles with the 10 μ m and 32 μ m proving to be the least potent (Figure 3.19).

The relationship between particle uptake and the enhancement of IL-1 α secretion was also determined. The augmentation of IL-1 α secretion by PS was found to be dependent on particle uptake and, as with IL-1 β , the 1 μ m particles were the most potent at driving secretion of IL-1 α (Figure 3.20). Furthermore, PS alone could significantly enhance the secretion of IL-1 α and this occurred in a size-dependent manner (Figure 3.20). The 1 μ m and 10 μ m PS particles were the most efficient at driving IL-1 α secretion with the highest concentrations of both particles having the most marked effect (Figure 3.20). It is also worth noting that the larger PS particles (10 μ m and 32 μ m) were more efficient at promoting the secretion of IL-1 α than IL-1 β thus suggesting the existence of different mechanisms of enhancement for each cytokine by the larger particles.

To further confirm the requirement for particle uptake, experiments were performed using cytochalasin B to prevent phagocytosis. Cytochalasin B acts by inhibiting actin polymerisation, which is necessary for remodelling of the cytoskeleton. BMDC were pre-treated with cytochalasin B (5 μ M or 10 μ M) prior to the addition of alum, PLG (1 μ m) or PS (430nm) either alone or in combination with LPS. The presence of cytochalasin B significantly reduced and almost abrogated, at higher concentrations of the inhibitor, the enhancement of IL-1 β by all three PVA (Figure 3.21). In a similar manner to the previous particle size experiments, the secretion of IL-1 α was significantly reduced by the prevention of phagocytosis but not abrogated, demonstrating a partial dependency on particle uptake rather than an absolute requirement (Figure 3.21). Taken together, these results show that particle uptake is necessary for optimal enhancement of IL-1 β secretion by PVA.

3.2.11 Degradation of uric acid does not inhibit the enhancement of IL-1 β secretion by PVA

Several endogenous danger signals have been identified to date, such as HMGB1 and uric acid or monosodium urate crystals (MSU) [11-12]. Furthermore, it has been proposed that the adjuvant effect of alum *in vivo* is partly dependent on uric acid [165]. Thus, it was possible that the effect of PVA on IL-1 β secretion was due to an increase of uric acid in the supernatant during cell culture. To assess a potential role for uric acid, BMDC were stimulated with either MSU, alum, PLG (1 μ m) or PS (430nm) with LPS in the presence or absence of uricase, an enzyme with the ability to degrade uric acid. In the case of MSU, the uricase blocked its ability to induce the secretion of IL-1 β (Figure 3.22). In contrast to MSU, the uricase had no effect on the ability of alum and PS to enhance the secretion of IL-1 β (Figure 3.22). However, the uricase did significantly reduce the level of enhancement of IL-1 β secretion by PLG, although it did not completely abrogate this effect (Figure 3.22).

3.2.12 ROS play no role in activation of the NLRP3 inflammasome by PVA

NLR proteins are thought to be present in the cytosol in an auto-repressed state which prevents formation of the inflammasome. In this auto-repressed form, NLR proteins are in a conformation which hides their NACHT domains, which are required for oligomerisation with other NLR proteins, triggering formation of an inflammasome. It has been suggested that binding of a ligand to the LRR region of the NLR will induce the conformational change necessary to expose the regions of the protein required for formation of the inflammasome. However, to date a direct interaction between the NLR and their respective ligands has not been documented. In response to this discrepancy, an alternative activation theory has been put forward which suggests that the NLR conformational change is induced indirectly by endogenous danger signals, changes in the cytosolic ionic milieu or the redox state of the cell. In support of this theory, it has been shown that scavenging of reactive oxygen species (ROS) by N-acetyl-cysteine impairs ATP-, MSU-, TLR7/8 agonist R837- and silica-induced inflammasome activation [104]. To assess the role that reactive oxygen species play in PVA-mediated inflammasome activation, the ROS inhibitor diphenyliodonium (DPI) was used to pre-treat BMDC prior to stimulation with particles and a TLR agonist. DPI inhibits the

activation of NADPH-oxidase, one of the enzymes responsible for the production of ROS. BMDC pre-treated with DPI showed no significant reduction in their secretion of IL-1 β or IL-1 α in response to stimulation with PVA and LPS in comparison to BMDC that were not pre-treated with DPI (Figure 3.23). Furthermore, the presence of DPI had no effect on the secretion of IL-6 by the BMDC (Figure 3.23). In addition, it is also worth noting that BMDC pre-treated with DPI and subsequently stimulated with LPS secreted higher levels of IL-1 β and IL-1 α in comparison to LPS alone (Figure 3.23).

3.2.13 PVA activate the NLRP3 inflammasome through lysosomal disruption and subsequent release of Cathepsin B

It has been proposed that silica crystals and alum activate the inflammasome by causing lysosomal disruption and subsequent release of the lysosomal enzyme cathepsin B, a process also dependent on lysosomal acidification [106]. Having established that PVA-mediated inflammasome activation occurs independently of ROS, the ability of PVA to cause lysosomal disruption was investigated.

Lysosomes contain many enzymes which require the acidic environment to become activated. Based on this, the role of lysosomal acidification in particulate-mediated NLRP3 activation was assessed. Bafilomycin A1 is a macrolide antibiotic derived from *Streptomyces griseus* that acts as a specific inhibitor of the H⁺ ATPase vacuolar acidification system. Blocking of lysosomal acidification with bafilomycin A1 significantly reduced particulate-mediated secretion of IL-1 β and IL-1 α by BMDC, while no significant effect was observed on the ability of the cells to secrete IL-6 (Figure 3.24).

BMDC were pre-incubated with the dye acridine orange, which becomes concentrated in acidic vesicles. Due to the cationic nature of acridine orange, it forms dimers in acidic vesicles and the fluorescence spectra of this compound can be detected by flow cytometry (650-680 nm, FL3). This dye can be used to quantify lysosomal content as the amount of red fluorescence directly correlates with the amount of acidic vesicles within the cells [106]. After incubation with acridine orange, BMDC were stimulated with alum, PS (430nm) or PLG (1 μ m) and the level of red fluorescence was analysed by flow cytometry. A reduction in the level of fluorescence is indicative of a loss of

fluorescence, therefore a loss of acidic vesicles or lysosomes in the cells. Treatment of DC with PVA resulted in a reduction in fluorescence, suggesting increased lysosomal disruption in comparison with control cells (Figure 3.25).

The data in figures 3.24 and 3.25 suggested the possibility that one of the enzymes activated within the acidic environment of the lysosome and subsequently released due to lysosomal disruption could be required for particle-mediated activation of the NLRP3 inflammasome. Based on the role demonstrated for cathepsin B in both silica- and alum-mediated inflammasome activation, the cathepsin B inhibitor CA-074-Me was tested [106]. Pre-treatment of BMDC with CA-074-Me prior to stimulation with, alum, PLG or PS in combination with LPS resulted in a significant decrease in particle-mediated enhancement of IL-1 β (Figure 3.25). The ability of both alum and PLG to enhance the secretion of IL-1 α was significantly compromised when cathepsin B activity was inhibited (Figure 3.25). In contrast, the inhibitor did not reduce PS-mediated IL-1 α secretion (Figure 3.25).

Taken together these experiments suggest that PVA activate the NLRP3 inflammasome by causing lysosomal disruption and subsequent release of cathepsin B. Furthermore, acidification of lysosomes is necessary for optimal activation of the inflammasome suggesting that cathepsin B requires activation prior to its release.

3.2.14 PVA enhance the secretion of IL-1 α by BMDC in a TLR4-independent manner

Results from previous experiments have demonstrated that treatment of BMDC with PVA alone is sufficient to promote the secretion of IL-1 α (Figure 3.2-3.4). However, it was a possibility that this was due to contaminating microbial products, such as LPS. In order to investigate this, C3H/HeJ mice, which contain a mutation in the signalling domain of TLR4, making them hypo-responsive to LPS, were treated with PLG (1 μ m: 3 and 0.6 mg/ml) and IL-1 α secretion was analysed by ELISA. Treatment of BMDC with PLG and LPS resulted in an enhancement in the amount of IL-1 α secreted in comparison to LPS alone (Figure 3.26). However, this enhancement was abrogated in the C3H/HeJ mice (Figure 3.26). PLG alone were sufficient to enhance the secretion of IL-1 α by BMDC in comparison to untreated cells (Figure 3.26). This enhancing effect

was still observed in the C3H/HeJ mice, suggesting, that it occurs in a TLR4-independent manner (Figure 3.26). Therefore, it is not due to the presence of contaminating LPS.

3.2.15 NLRP3 is necessary for the recruitment and activation of innate immune cells in response to PVA

Having established that PVA activate the NLRP3 inflammasome *in vitro* it was then important to investigate the role of NLRP3 in the adjuvant activities of particulates *in vivo*. The role of the NLRP3 inflammasome in adaptive immune responses induced by aluminium-containing adjuvants has been investigated by several groups [105, 107-108, 118-119]. However, results from these studies have not been conclusive and the role of NLRP3 in the adjuvant properties of aluminium-containing adjuvants currently remains controversial. This study aimed to investigate the role of NLRP3 in the adjuvant properties of PLG. Wild type (WT) and NLRP3^{-/-} mice were immunized intra-peritoneally or subcutaneously with PBS, Ova alone (50 µg/mouse) or Ova (50 µg/mouse) in combination with PLG (4 mg/mouse).

The mice were immunized intra-peritoneally in order to investigate the role of NLRP3 in cell recruitment to the injection site, as previous studies have shown a requirement for NLRP3 in the recruitment of innate cells such as neutrophils, eosinophils and monocytes into the injection site [105, 107-108, 118-119]. To assess the role of NLRP3 in particle-mediated innate cell recruitment, cells were isolated from the peritoneum of mice injected with PLG alone and analysed by flow cytometry for their expression of specific cell type associated markers including CD11c, CD11b, Gr1 and F4/80. Specific cell types can be identified based on their expression of these markers as follows according to Kool *et al*: neutrophils (CD11b⁺Gr1⁺F4/80⁻), eosinophils (CD11b⁺Gr1^{int}F4/80^{int}) and monocytes (CD11b⁺Gr1⁻F4/80^{int}) (int: intermediate) [107]. The recruitment of CD11b⁺Gr1⁻ cells into the peritoneum was significantly reduced in the absence of NLRP3 suggesting a role for NLRP3 in particulate-mediated cell recruitment (Figure 3.27). The activation status of these cells was also investigated. The expression of CD86 on the surface of these CD11b⁺Gr1⁻ cells was significantly reduced in NLRP3^{-/-} mice in comparison to WT mice (Figure 3.27). Based on their

surface expression of Gr1, these cells are not granulocytic cells. Their expression of Gr1 and CD11b suggests that they are monocytic cells.

In addition to the role of NLRP3 in innate cell recruitment, it has been reported that NLRP3^{-/-} mice display a significant reduction in their secretion of antigen-specific cytokines when cells isolated from draining lymph nodes are stimulated *ex vivo* [105, 107]. To assess whether NLRP3-deficient mice displayed defective cell-mediated responses, the levels of antigen-specific cytokines (IL-4, IL-5, IL-6, IFN- γ , IL-17, IL-10) secreted by stimulated cells isolated from inguinal lymph nodes, mediastinal lymph nodes and the spleen were analysed by ELISA. The absence of NLRP3 had no effect on the secretion of IL-4, IL-5, IL-6, IFN- γ , IL-17 or IL-10 by stimulated cells isolated from inguinal lymph nodes or mediastinal lymph nodes (Data not shown). Similarly, no effect on the secretion of IL-4, IL-5, IFN- γ , IL-17, or IL-10 by stimulated splenocytes was observed (Data not shown). In contrast, splenocytes isolated from NLRP3^{-/-} mice showed a significant deficit in their ability to secrete antigen-specific IL-6 when re-stimulated *ex vivo* in comparison to WT cells (Figure 3.28).

These studies suggest a role for NLRP3 in the recruitment and activation of innate cells and in adaptive cell-mediated immunity induced by PVA.

3.2.16 NLRP3 is not required for PLG microparticles to promote antigen-specific antibody responses

Having established that the promotion of cell-mediated responses by PLG requires NLRP3, the role of NLRP3 in particulate-mediated humoral responses was investigated. Previous studies involving aluminium-containing adjuvants have reported mixed results on the role of NLRP3 in particulate adjuvant-driven antibody responses [105, 107-108, 118-119].

Some groups have reported a decrease in titres of antigen-specific IgG1 in NLRP3^{-/-} mice [105, 107-108], while others propose that NLRP3 is dispensable for particulate-mediated enhancement of humoral responses [118-119]. To assess the role of NLRP3 in particulate-driven humoral responses, WT and NLRP3^{-/-} mice were immunized intraperitoneally with PBS, Ova alone (50 μ g/mouse) or Ova (50 μ g/mouse) in combination with PLG (4 mg/mouse). Mice were immunised on day 0 and boosted on day 14 and

serum samples were collected 21 days post immunization, in order to assess the level of antibodies. Consistent with other studies, IgG1 and IgG2b titres were comparable in WT and NLRP3^{-/-} mice (Figure 3.29) [118-119]. Furthermore, NLRP3^{-/-} mice immunised subcutaneously with PLG also showed no defect in particulate-mediated humoral responses (Data not shown).

In contrast to the role for NLRP3 in particle-driven cell-mediated immunity, NLRP3 is dispensable for the enhancement of particulate adjuvant-driven humoral immune responses.

3.3 Discussion

Dendritic cells are the principal APC of the innate immune system, unique in their ability to present antigen to and activate naive T cells [150]. They are thought to exist in two distinct functional states; immature DC which are more efficient at antigen capture and processing or mature DC which are potent antigen-presenting cells, primed for interactions with naive T cells [175-176]. DC maturation can be triggered by the sensing of a PAMP, an endogenous danger signal, cytokines or by T cell derived factors. Mature DC are characterised by their increased surface expression of MHC molecules, CD40, CD80, CD86 and CD83, all of which are important for interacting with T cells [179]. Mature DC are thought to be immunogenic with the ability to activate and direct adaptive immune responses. Based on the central role played by DC within the immune system, acting as the link between the innate and adaptive arms of the response, the effect of PVA on DC was investigated. It is possible that the interaction between the DC and particulates induces upregulation of co-stimulatory markers, including CD80 and CD86 and MHC class II molecules, resulting in maturation of the DC and subsequent enhancement of adaptive responses.

Previous studies have been inconclusive regarding whether or not PVA can directly induce the maturation of dendritic cells. Two groups have reported enhanced expression of maturation-associated molecules by BMDC and human PBMC treated with alum, including CD86 and MHC class II molecules, respectively [458, 522]. These studies are in agreement with earlier studies, which showed that alum could directly activate human PBMC [523]. In contrast, others have shown that incubation of BMDC with alum is insufficient to enhance surface expression of MHC class II or co-stimulatory molecules [165, 476]. However, DC isolated from the peritoneum of mice injected with alum showed enhanced expression of CD86 [165, 476]. This suggested that while particles could not activate DC directly *in vitro*, that they could induce maturation of DC indirectly *in vivo*, possibly through an endogenous molecule. In agreement with this, the results from this study, suggests that PVA are inefficient at promoting the upregulation of co-stimulatory molecules on the surface of BMDC. Taken together, these current findings, and those of others, suggest that particulates do not have the ability to directly activate BMDC *in vitro*. Further studies are necessary to investigate whether a similar effect is observed *in vivo*.

Following on from these experiments, studies were conducted into whether the presence of PVA could affect the way in which DC respond to stimulation with MAMP. BMDC were stimulated with a range of TLR agonists in the presence of PS, PLG or alum. PVA alone were inefficient at promoting cytokine secretion by BMDC. However, stimulation of DC with alum, PLG or PS in the presence of a TLR agonist resulted in enhanced secretion of IL-1 β and IL-18. Others have confirmed the ability of alum to enhance the secretion of IL-1 β and IL-18 [457-458]. These current findings demonstrate that PLG and PS can also enhance the secretion of IL-1 β and IL-18, suggesting that this is a general property of all particulate adjuvants. Furthermore, pre-treatment of DC with PLG, PS or alum induces enhanced secretion of IL-1 α . This enhancement of cytokines by particles *in vitro* is dependent on the presence of a TLR agonist. However, it is not specific to LPS as a range of TLR agonists tested, including CpG and poly I:C, synergised with the particles to enhance the secretion of IL-1 β and IL-1 α by dendritic cells. In addition to MAMP, further studies have also demonstrated that particulates can synergise with HMGB1, a known danger associated molecular patterns (DAMP) (Figure 5.9). These data demonstrate that the presence of a PVA modulates the response of a dendritic cell to both MAMP and DAMP.

Interleukin-1 β is a potent endogenous pyrogen which induces the expression of inflammatory genes, including those that regulate the translation of cytokines, chemokines and inducible nitric oxide synthase (iNOS) [524]. IL-1 β is translated in the cytosol into a biologically inert protein, 31kDa in size, that lacks an N-terminal hydrophobic domain and is therefore excluded from the classical secretory pathway via the endoplasmic reticulum (ER) and the Golgi apparatus [525]. IL-1 β is not automatically secreted and as a result a small portion of it moves into lysosomes while the majority remains in the cytosol [122]. In order to be secreted, IL-1 β must be converted into its active form (17kDa), which is catalysed by IL-1 converting enzyme (ICE) or caspase-1 which cleaves IL-1 β after an aspartic acid residue at position 116 [123-124]. As a result, IL-1 β secretion requires two signals, one to induce transcription of pro-IL-1 β and the other to activate caspase-1. These current results show that PVA enhance the activation of caspase-1, and subsequently increase the processing of IL-1 β into its active form. Other studies have confirmed this enhancement of caspase-1 activation and subsequent secretion of IL-1 β by alum [457]. However, the ability of PLG and PS to induce caspase-1 activation in the presence of a TLR agonist was not

previously appreciated. Others have suggested that caspase-1 processing can occur in response to stimulation with aluminium-containing adjuvants alone [457]. However, these current studies have been unable to reproduce these results and show that caspase-1 processing can only occur in the presence of a TLR agonist. The requirement for caspase-1 activity in particle-mediated enhancement of IL-1 β was confirmed using YVAD-FMK, a cell permeable caspase-1 inhibitor, which abrogated particle-mediated secretion of IL-1 β . In addition, this requirement for caspase-1 was further confirmed by the abrogation of particle-mediated enhancement of IL-1 β in caspase-1-deficient mice.

IL-1 α was discovered during cloning studies when two forms of IL-1 were isolated, differing in their amino acid sequence and their isoelectric point [526-528]. It was originally thought that IL-1 α was translated to a biologically inert form that required processing in order to become active [529]. However, it is currently thought that IL-1 α does not require any processing in order to become active and it has been shown that the full length form of IL-1 α can associate with its receptor [530]. The current data suggest that particulate-mediated enhancement of IL-1 α is partially dependent on caspase-1, as the enhancement of IL-1 α was significantly reduced, but not completely abrogated, in the presence of a caspase-1 inhibitor and in caspase-1-deficient BMDC. Processing of IL-1 α was not investigated and it is possible that this reduction was due to impairment of export rather than biological activity. In agreement with this, Kuida *et al* have previously reported that mice deficient in caspase-1 show altered export of IL-1 β and IL-1 α [125]. More recently it has been shown that caspase-1 is required for the export of proteins lacking signal peptides, including pro-angiogenic growth factor FGF-2 and Bid, and therefore not released via the conventional golgi-ER network [531]. Furthermore, these studies demonstrated that full length IL-1 α can associate with the p20 subunit of caspase-1 [531]. Adding further evidence to the role of caspase-1 in the secretion of leaderless proteins, the small GTPase Rab39a has been shown to associate with caspase-1. It has been predicted that it is required to traffic IL-1 β in vesicles out of the cell, as blocking of its activity prevents secretion of IL-1 β but has no effect on IL-1 β mRNA levels [532]. Both IL-1 α and IL-1 β lack a signal peptide suggesting that caspase-1 plays a role in the secretion of these cytokines in addition to its role in processing IL-1 β .

Caspase-1 is part of a family of cysteine proteases, produced as zymogens, which contain an N-terminal pro-domain and a CARD region. The exact mechanism of activation of the caspases remains unknown. However, in 2002 Martinon *et al* proposed the existence of the 'inflammasome', a multiprotein complex which helped to induce activation of caspases. Using a cell-free system, they showed that a complex comprising caspase-1, caspase-5, NLRP-1 and Pycard/ASC (apoptosis-associated speck-like protein containing a CARD) could activate caspases and subsequently cleave IL-1 β [76]. ASC was first identified in cells undergoing retinoic acid-induced apoptosis as a detergent-insoluble protein and has since been shown to be expressed in several cell types, including epithelial cells, leukocytes and peripheral blood lymphocytes. The bipartite structure of ASC, consisting of a CARD and a PYD, allows it to interact with NLRPs and caspases [127-128].

Based on the ability of PVA to activate caspase-1 and given that caspases are now known to be activated within inflammasomes, it was predicted that particulates were activating caspase-1 by inducing formation of the inflammasome. In order to confirm this hypothesis, ASC-deficient BMDC were treated with particles and LPS. As postulated, the ability of PVA to enhance the secretion of IL-1 β was completely abrogated in ASC^{-/-} mice, suggesting that particulates can somehow induce formation of the inflammasome. In contrast, the ability of particles to enhance IL-1 α secretion was reduced in the ASC^{-/-} BMDC but not completely abrogated. In addition, the enhancement of IL-1 α by PLG was unaffected by the absence of ASC, implying that the regulation of IL-1 α is not only different to that of IL-1 β but that the mechanism of its enhancement is also dependent on the nature of the particulate.

The most studied inflammasomes are the NLRP1, NLRP3 and NLRC4 inflammasome. The NLRP3 inflammasome is activated by monosodium urate crystals (MSU) and calcium pyrophosphate dihydrate crystals (CPPD), the causative agents of gout and pseudogout, respectively [101]. Based on the particulate nature of these stimuli, it was predicted that PVA were also activating the NLRP3 inflammasome. Using NLRP3^{-/-} BMDC it was shown that particulates enhance the secretion of IL-1 β and IL-18 in a NLRP3-dependent manner, suggesting the existence of a general pathway activated in response to phagocytosis of particulates. The ability of the NLRP3^{-/-} BMDC to produce TNF- α in response to LPS was not compromised, confirming that these knock-out cells are responsive to stimuli and that their inability to produce IL-1 β is not due to some

intrinsic defect. In agreement with these observations, several other studies have recently shown that other particulate substances, including silica crystals, asbestos, and amyloid- β , activate the NLRP3 inflammasome [104, 106, 134]. Several other groups have also demonstrated the ability of alum to activate the NLRP3 inflammasome *in vitro* [105, 107-108]. The ability of particles to activate the NLRP3 inflammasome, thereby activating caspase-1, was further confirmed by western blot showing that the p10 band of processed caspase-1 could not be detected in NLRP3^{-/-} BMDC treated with particles and LPS. The level of enhancement of IL-1 α by particulates was reduced in NLRP3 deficient BMDC but was not completely abrogated. Overall this study shows that PVA can activate the NLRP3 inflammasome, thereby enhancing the processing and subsequent secretion of IL-1 β .

Having identified that the NLRP3 inflammasome is specifically activated by particulates, the mechanism of activation was investigated. Firstly, the role of particle uptake was determined using confocal microscopy. Results from these studies, suggest that uptake of particles is necessary for optimal activation of the NLRP3 inflammasome. Using confocal microscopy, the ability of BMDC to phagocytose particles up to 10 μ m in size was demonstrated, but smaller particles are more readily phagocytosed and are more efficient at driving the secretion of IL-1 β . The requirement for particle uptake was further confirmed using an inhibitor of actin polymerisation, which prevents phagocytosis. This data is consistent with previous studies which have shown that particles, such as MSU and silica also require uptake in order to activate the inflammasome [104, 106, 134].

Our knowledge of how inflammasomes are activated and assembled has expanded greatly since their discovery. The current theories suggest that NLR, such as NLRP3, are present in an autorepressed state in the cytoplasm, in a conformation which conceals the NACHT/NOD and effector domains, preventing any interaction with other proteins. The LRR are thought to be responsible for pathogen or danger signal sensing. It has been proposed that once a ligand has bound to the LRR region, the protein undergoes a conformational change, making its protein interaction domains accessible and allowing it to oligomerise with other NLR. Thus, allowing it to interact with downstream molecules containing either CARD or PYD domains, such as caspases or ASC.

Given the wide range of stimuli that have been shown to activate the inflammasome, in particular the NLRP3 inflammasome, it has also been suggested that activation of the inflammasome occurs in an indirect manner through endogenous danger signals, changes in the cytosolic ionic milieu or redox state within the cytosol of the cell. Based on recent studies, three models of inflammasome activation have emerged. The first suggests pore formation in the membrane of the cell induced by either ligation of the P2X7 receptor by ATP which subsequently induces the opening of a pore mediated by pannexin-1, or by pore forming toxins [533-534]. The formation of these pores allows entry of MAMP into the cytosol and facilitates the efflux of ions from the cell.

Secondly, Dostert *et al* have proposed a role for reactive oxygen species (ROS), which are free radicals generated by the enzymatic activity of NADPH oxidases and cyclooxygenases [104, 535]. They suggest that increased levels of ROS are the trigger for the induction of inflammasome activation. In addition, it has recently been shown that thioredoxin-interacting protein (TXNIP) which negatively regulates thioredoxin (TRX), a neutralizer of ROS, directly interacts with NLRP3 [113]. It was proposed that during times of oxidative stress, which can be induced by prolonged increases in ROS levels, TXNIP is released as TRX becomes activated and that TXNIP can then interact with NLRP3 and induce formation of the inflammasome [113]. Thirdly, it has also been demonstrated that lysosomal damage induced by the phagocytosis of particulates results in the release of cathepsin B into the cytosol. Following its release, cathepsin B can potentially modify host proteins, which subsequently activate the inflammasome [106, 134].

It has been known for some time that ATP and Nigericin, a pore forming toxin, both activate caspase-1 and this is dependent on potassium ion (K^+) efflux from cells [536-537]. More recently, Petrelli *et al* have shown that low intracellular potassium levels triggers formation of the NLRP3 inflammasome [131]. In agreement with this, most of the known activators of the NLRP3 inflammasome require K^+ efflux, resulting in low intracellular levels of K^+ [80, 104, 106, 131, 134]. In order to investigate whether K^+ efflux was a prerequisite for NLRP3 inflammasome activation in response to PVA, BMDC were incubated in potassium-supplemented medium, to prevent K^+ efflux. Consistent with other NLRP3 activators, the ability of particulates to enhance the

secretion of IL-1 β by BMDC was abrogated when K⁺ efflux was blocked. In addition, levels of IL-1 α were significantly reduced when K⁺ efflux was blocked but they were not abrogated. Although K⁺ efflux is necessary for activation of the NLRP3 inflammasome, it is not a prerequisite for the formation of the NLRC4 inflammasome, suggesting that there are differences in the activation of inflammasomes depending on the cytosolic NLR involved [131].

Two of the current theories of inflammasome activation (ROS and lysosomal disruption models) share a commonality, both of them have been proposed based on particulate activators of the NLRP3 inflammasome such as MSU, silica crystals, alum and amyloid- β [104, 106, 134]. Based on this, the pathways involved in particle-mediated NLRP3 inflammasome activation were investigated. NADPH oxidases (NOX) are a family of enzymes that generate ROS by carrying electrons across biological membranes. Blocking of NOX activity had no effect on IL-1 β secretion induced by particulates, which implies that

ROS is not necessary for NLRP3 inflammasome activation by PVA. Furthermore, BMDC in which NOX activity was blocked and were treated with LPS produced higher levels of IL-1 β than cells stimulated with LPS alone, suggesting that ROS may actually negatively regulate activation of the NLRP3 inflammasome. Consistent with the idea that ROS levels do not control activation of the inflammasome, treatment of BMDC with DPI, an inhibitor of NOX activity, in combination with LPS enhanced secretion of IL-1 β and IL-1 α by DC. Others have shown that, caspase-1 activity is suppressed in murine cells deficient in superoxide dismutase, an anti-oxidant which neutralises the effects of ROS [538]. Chronic granulomatous is a disease that is associated with defective NADPH activity preventing the production of ROS. Interestingly, PBMC from patients with chronic granulomatous disease produce significantly higher levels of IL-1 β when stimulated with MSU in comparison to PBMC from unaffected subjects [539].

Having established that ROS levels play no role in the activation of the NLRP3 inflammasome by PVA the effect of particles on lysosomes was determined. Results from these studies have shown that treatment of BMDC with alum, PLG or PS induces lysosomal damage, as characterised by an increased loss of acidic vesicles in comparison to untreated cells. These findings are in agreement with other studies that

have demonstrated that other particulate substances, including silica, alum and amyloid- β , promote lysosomal disruption [106, 134]. Lysosomes contain a plethora of enzymes that require the low pH in order to become activated. Blocking of the H^+ ATPase acidification system, which is necessary to maintain the low pH levels within lysosomes, significantly reduced the levels of IL-1 β secretion induced by particulates. Furthermore, inhibition of the lysosomal protease cathepsin B impaired the ability of particulate vaccine adjuvants to enhance the secretion of IL-1 β . These studies suggest that uptake of PVA induces lysosomal damage which allows the translocation of cathepsin B into the cytosol where it either directly or indirectly activates the NLRP3 inflammasome.

While these studies demonstrate that lysosomal disruption and cathepsin B both play key roles in the activation of NLRP3 by particulates, blocking of either did not completely abrogate the enhancement of IL-1 β , suggesting a requirement for alternative pathways in particle-mediated inflammasome activation. A recent paper demonstrated that malarial hemazoin, a by-product of haemoglobin metabolism, activates the NLRP3 inflammasome in a mechanism that required cathepsin B, ROS and was independent of lysosomal rupture [133].

Adding another layer of complexity is the recent identification of a requirement for the tyrosine kinase Syk in the activation of the inflammasome in response to malarial hemazoin and *Candida albicans* [97, 133]. Syk is normally activated via ligation of receptors, including antigen binding receptors, such as the TCR or BCR, and FcR. These receptors contain immunoreceptor tyrosine based activation motifs (ITAM) that are phosphorylated by Src family kinases, which leads to the recruitment and activation of Syk [540]. Both studies demonstrated that, blockade of Syk activity inhibited caspase-1 activation in response to hemazoin or *C.albicans* [97, 133]. The role of Syk in inflammasome activation was found to be signal-specific, as inhibition of Syk had no effect on the ability of ATP or nigericin to activate the inflammasome, while it strongly inhibited caspase-1 activation in response to *C.albicans* [97]. Hemazoin and *C.albicans* promote Syk phosphorylation and activation of Syk is necessary for the enhancement of IL-1 β secretion by hemazoin or *C.albicans* [97, 133]. Furthermore, inhibition of Syk prevented the activation of cathepsin B induced by hemazoin [133].

Therefore, it is possible that Syk or other tyrosine kinases may also play a role in particulate mediated activation of Cathepsin B. More importantly, hemazoin requires both cathepsin B and ROS, which suggests that cross talk between the two pathways can occur. However, in the case of PVA ROS do not appear to be required. Therefore one could postulate that phagocytosis of particulates may increase the levels of, some as of yet unidentified, endogenous molecules that act in concert with cathepsin B.

Findings from these studies have shown that PVA enhance the secretion of IL-1 α by BMDC to a similar extent as IL-1 β . Although the secretion of both of these pro-inflammatory cytokines is greatly enhanced, the regulation of their secretion in response to particulates is very different. IL-1 α induces similar responses to IL-1 β *in vivo*, including fever, release of acute phase proteins and activation of lymphocytes. Both IL-1 α and IL-1 β lack a signal peptide and so are not secreted via the conventional golgi-ER pathway [528, 541]. In contrast to IL-1 β , IL-1 α does not require processing in order to become biologically active and the full length form of IL-1 α is fully active when it engages the IL-1R. IL-1 α is found constitutively within most cells of healthy individuals, including epithelial cells and keratinocytes [542]. In addition to its extracellular activities, it has been shown that IL-1 α can translocate into the nucleus where it is involved in the transcription of genes [543].

The current data demonstrate that the enhancement of IL-1 α is partially dependent on caspase-1 and activation of the inflammasome, since the absence of NLRP3, inhibition of cathepsin B and blocking of lysosomal acidification reduced the secretion of IL-1 α but did not abrogate it. Although IL-1 α does not require processing, the requirement for caspase-1 may be explained by a recent report which demonstrated a role for caspase-1 in the secretion of proteins lacking a signal peptide [531]. Consistent with this unconventional role for caspase-1, it has recently been demonstrated that the small GTPase, Rab39a associates with caspase-1 and that it is required for the secretion of IL-1 β , which also lacks a signal peptide [532]. Findings from these current studies have shown that inhibition of caspase-1 using a chemical inhibitor (YVAD-fmk) had a more marked effect on the level of particle-mediated enhancement of IL-1 α than caspase-1-deficient BMDC. These differences could be due to non-specific activities of the inhibitor, which resulted in a greater effect on the secretion of IL-1 α . This dependency on components of the NLRP3 inflammasome for particulate-mediated IL-1 α secretion

may also be due to possibility that NLRP3-dependent factors, such as IL-1 β or IL-18, are promoting the enhancement of IL-1 α in an autocrine manner.

In addition to a possible role for caspase-1 in the secretion of IL-1 α , it has also been suggested that IL-1 α is rarely found in the extracellular compartment and is in general sequestered in the cytosol or associated with the cell in a membrane-associated form [544]. Furthermore, others have proposed that IL-1 α is only made available when the cell dies by necrosis, therefore releasing its cellular and nucleic contents [542, 545]. Taking all of these studies together, they strongly suggest the existence of two unconventional pathways for the secretion of IL-1 α . Consistent with this, current studies found that while a proportion of the IL-1 α secretion that is induced by particulates is dependent on caspase-1 and other components of the NLRP3 inflammasome there is always a certain amount of IL-1 α which is released independently of caspase-1 and inflammasome components. In agreement with this, particles alone are sufficient to induce the secretion of IL-1 α by DC which is more than likely caspase-1-independent as particles alone cannot activate caspase-1. Furthermore, the ability of particulates to drive IL-1 α secretion occurs in a TLR4-independent manner which rules out the possibility that the enhanced IL-1 α is due to contaminating microbial products such as LPS. Therefore, it is possible that at high concentrations particulate vaccines adjuvants may be toxic to cells, inducing a necrotic type of cell death and that this may account for the caspase-1-independent secretion of IL-1 α and also the differential regulation observed between IL-1 α and IL-1 β .

Having established the ability of PVA to activate the NLRP3 inflammasome thereby increasing the secretion of IL-1 β , the role of NLRP3 in the adjuvant activities of these particulates was determined. NLRP3-deficient mice showed impaired recruitment and activation of innate cells into the injection site when immunised intraperitoneally with PLG. In addition, splenocytes from NLRP3-deficient mice showed a significant reduction in their ability to produce antigen-specific IL-6 when restimulated *ex vivo*. These findings are consistent with other studies, which have demonstrated a requirement for NLRP3 in innate cell activation, recruitment and cellular immunity mediated by alum [105, 107].

In contrast to the requirement for NLRP3 in innate and cell mediated responses, it was found that NLRP3 activation was dispensable for the enhancement of humoral immune responses by PLG. NLRP3-deficient mice produced comparable levels of IgG1 and IgG2b to wild type mice when immunised with PLG and Ova. These findings suggest that NLRP3 is dispensable for particulate-mediated enhancement of humoral responses. Consistent with these results, others have also found that NLRP3 is not necessary for the enhancement of antigen-specific IgG1, IgG2b or IgG2a by alum [105, 107-108, 118-119]. Overall, these results suggest that NLRP3 is not required for the enhancement of humoral immune responses by PVA. However, the role of NLRP3 in the induction of alum enhanced IgG1 and IgE is controversial, with some groups showing an absolute requirement for NLRP3, while others report that it is not essential [105, 107-108, 118-119].

Adding further complexity to the issue, it has been reported that NLRP3-deficient mice produce significantly higher levels of antigen-specific IgG2c when immunised with alum and Ova [107]. The authors propose that this enhanced IgG2c observed in the NLRP3-deficient mice may reflect a switch in the type of response and that NLRP3 activation may be responsible for the skew in T helper cell responses observed with alum [107]. However, this switch in antibody isotypes in NLRP3-deficient mice has not been reported by any other groups and findings from these current studies did not show any increase in particle-promoted antigen-specific antibodies. In agreement with the idea that NLRP3 is not required for particulate enhancement of humoral responses, it has previously been shown that MyD88, a component of the IL-1R1 signalling pathway, is not necessary for alum-induced antibody responses [459-460]. Furthermore, it has also been shown that IL-6-deficient mice produce comparable levels of antigen-specific antibodies to WT mice when immunised with alum [454]. This is in agreement with results from this study which demonstrate that although antigen-specific IL-6 is significantly reduced in the NLRP3-deficient mice, no changes in antibody levels were observed. Results from these studies have demonstrated that NLRP3 is not required for the enhancement of humoral responses by PLG. However, whether this is a general requirement of all PVA remains controversial and requires further studies.

Thus, it appears that NLRP3 is important for particle-induced innate responses, such as the recruitment and activation of innate cells and adaptive cellular immunity. However, the role of NLRP3 in the development of humoral responses remains unclear. It has

been proposed that the different physico-chemical properties of PVA may account for some of the differences. This could explain, in part, the differences observed between current findings with PLG and data published by others on alum. It is also possible that the route of immunisation, experimental set-up and the choice of antigen may also have an impact on the results. In order to determine the role of NLRP3 in the adjuvant properties of particulates in general a standard assay would need to be carried out in order to limit the variables which may be affecting the outcome. Therefore, it is currently impossible to say whether or not NLRP3 is central to the ability of all particulates to enhance humoral immune responses. Based on these current findings, it can be concluded that NLRP3 is dispensable for humoral responses induced by PLG but is necessary for cellular immunity.

Overall, this study has shown that PVA activate a general pathway *in vitro* in BMDC. The phagocytosis of particulates by BMDC results in lysosomal disruption, which triggers NLRP3 inflammasome activation and the subsequent secretion of IL-1 β in a cathepsin B-dependent manner. The finer mechanistic details of inflammasome activation remain elusive and several questions remain, including what are the downstream targets of cathepsin B, or does cathepsin B activate a currently unidentified pathway?

Having evaluated the role of NLRP3 *in vivo*, there remains one major question; how do particulate vaccine adjuvants enhance humoral immunity? In order to investigate this, the *in vivo* effects of PVA need to be studied in more detail.

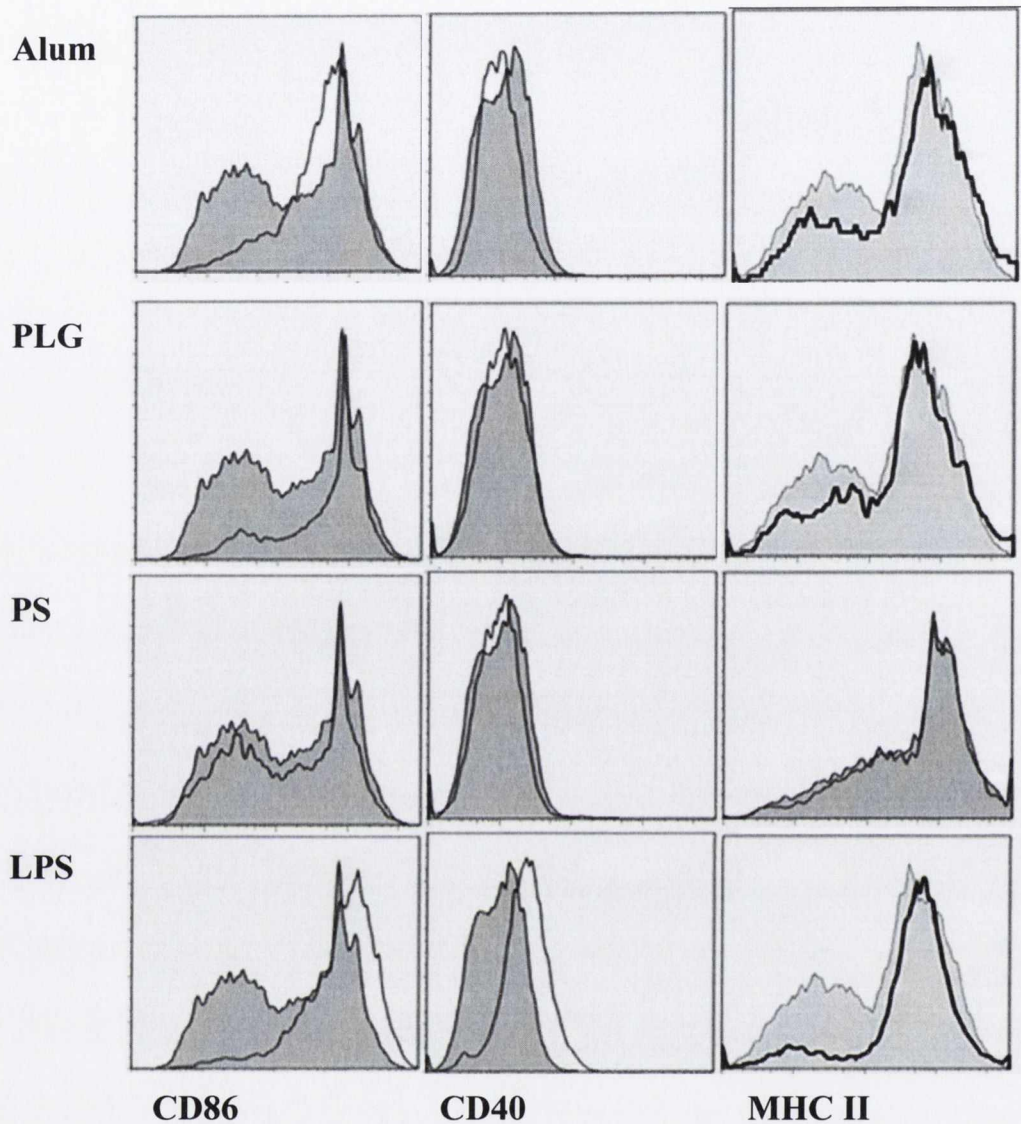


Figure 3.1: The effect of PVA on the expression of MHC class II molecules, CD40 and CD86 by dendritic cells. BMDC (1×10^6 cells/ml) were stimulated with alum (5mM), PLG ($1 \mu\text{m}$: 0.6 mg/ml), PS (430nm: 0.6 mg/ml) or LPS at 5 ng/ml. Stimulated BMDC were left for 24 hr. Cells were harvested and analysed for expression of maturation markers by flow cytometry (2.2.3). Solid colour represents control samples with treated samples overlayed as dark line.

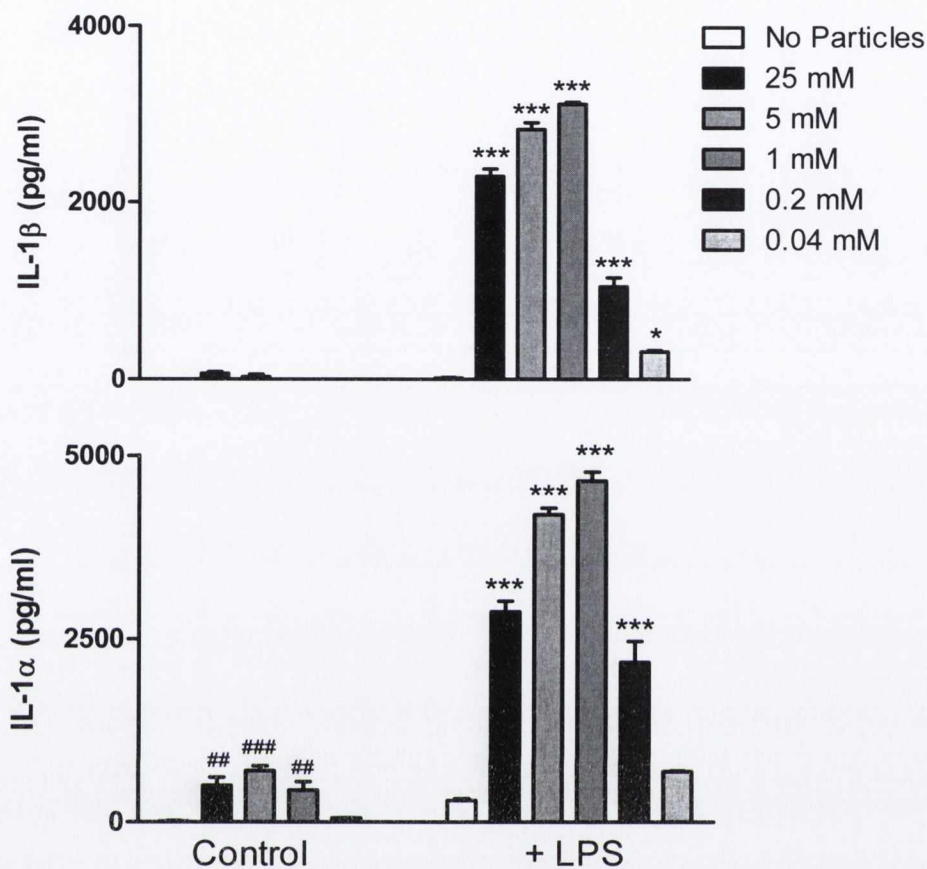


Figure 3.2: Alum synergises with LPS to induce IL-1 β and IL-1 α secretion by BMDC. BMDC (6.25×10^5 cells/ml) were pre-incubated with medium or alum (0.04-25 mM) for 1 h before the addition of medium or LPS (5 ng/ml). After 24 h, supernatants were collected and analysed for IL-1 β and IL-1 α by ELISA (2.2.5). Control v LPS only or Particles only, ### $p < 0.001$, Particles + LPS v LPS only, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

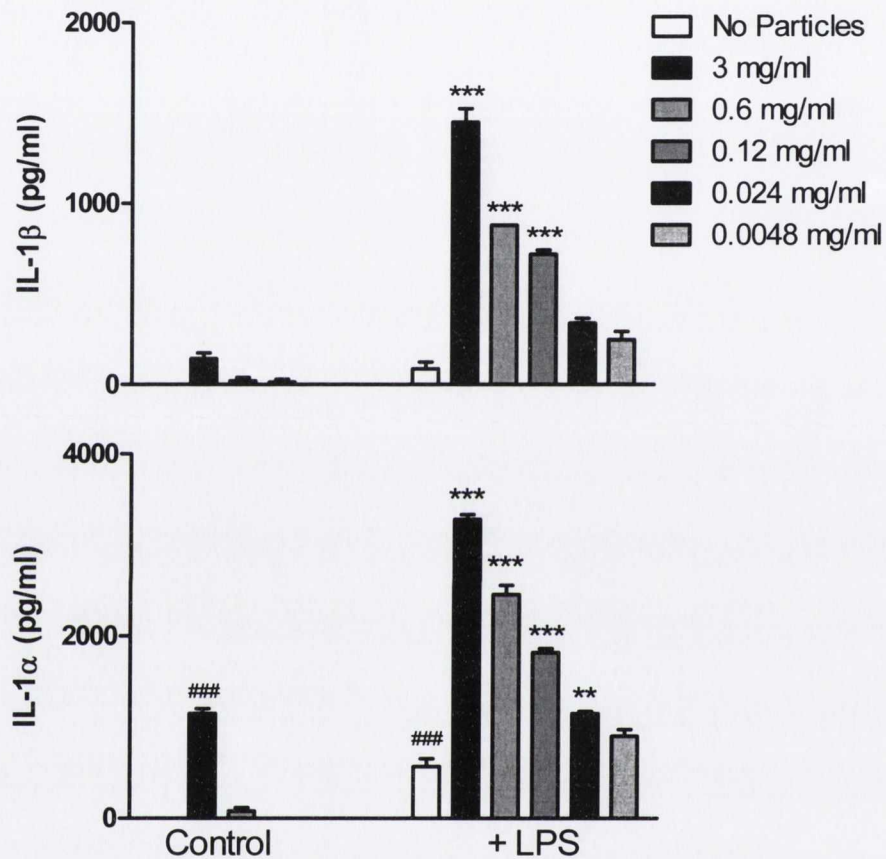


Figure 3.3: PLG-microparticles synergise with LPS to induce IL-1 β and IL-1 α secretion by BMDC. BMDC (6.25×10^5 cells/ml) were pre-incubated with medium or PLG ($1 \mu\text{m}$; 3-0.0048 mg/ml) for 1 h before the addition of medium or LPS (5 ng/ml). After 24 h, supernatants were collected and analysed for IL-1 β and IL-1 α by ELISA (2.2.5). Control v LPS only or Particles only, ### $p < .001$, Particles + LPS v LPS only, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

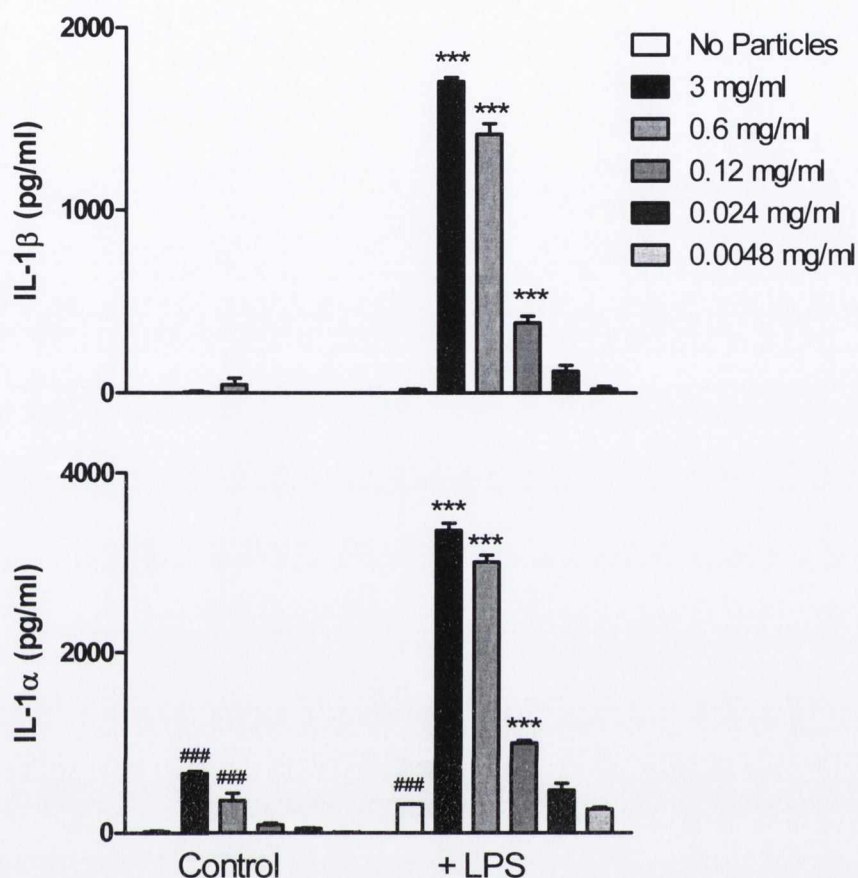


Figure 3.4: Polystyrene microparticles synergise with LPS to induce IL-1 β and IL-1 α secretion by BMDC. BMDC (6.25×10^5 cells/ml) were pre-incubated with medium or PS (430nm; 3-0.0048 mg/ml) for 1 h before the addition of medium or LPS (5 ng/ml). After 24 h, supernatants were collected and analysed for IL-1 β and IL-1 α by ELISA (2.2.5). Control v LPS only or Particles only, ### $p < .001$, Particles + LPS v LPS only, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

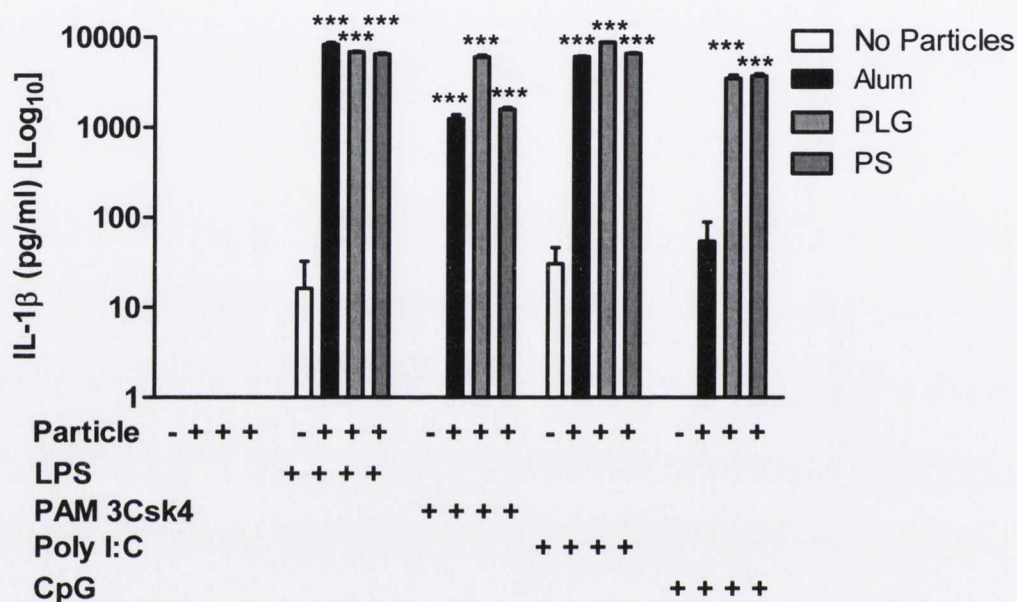


Figure 3.5: PVA can synergise with a range of TLR agonists to induce IL-1 β production by BMDC. BMDC (6.25×10^5 cells/ml) were pre-incubated with medium or particles (alum 25 mM, PLG $1 \mu\text{m}$; 3 mg/ml, PS 430nm; 3 mg/ml) alone for 1 h before the addition of medium or TLR agonists (LPS 5 ng/ml, Pam3Csk4 10 ng/ml, poly I:C 50 $\mu\text{g/ml}$ or CpG 50 $\mu\text{g/ml}$). After 24 h, supernatants were collected and analysed for IL-1 β by ELISA (2.2.5). Particles + TLR agonist v TLR agonist only, *** $p < 0.001$.

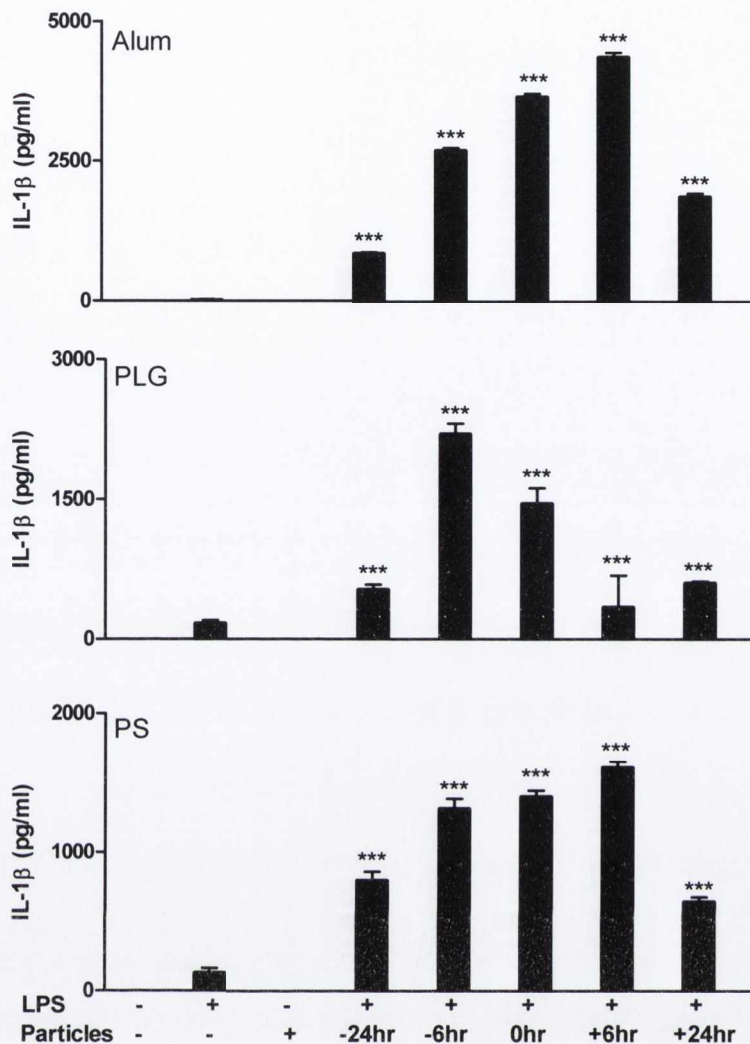
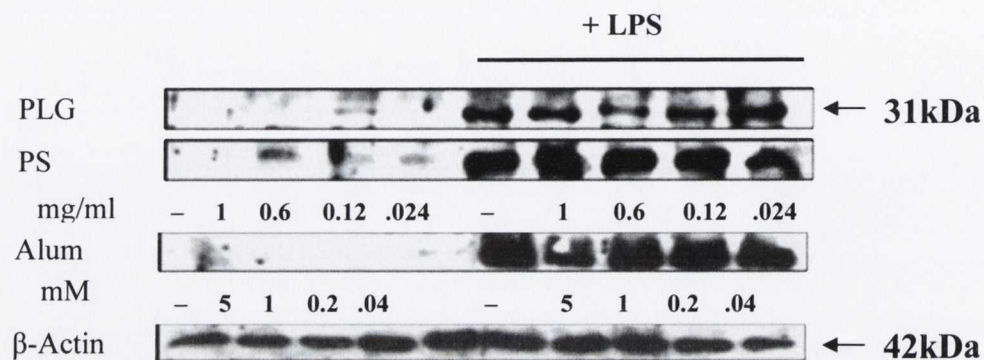


Figure 3.6: The enhancement of IL-1 β secretion by PVA does not require simultaneous presentation of the TLR agonist and the PVA to the DC. BMDC (6.25×10^5 cells/ml) were stimulated with medium or alum (5 mM), PLG (1 μ m; 0.6 mg/ml) or PS (430nm; 0.6 mg/ml) either simultaneously or 6 h or 24 h prior to or after the addition of medium or LPS (5 ng/ml). Once the second stimulus was added the cells were left for 24 h before supernatants were collected and analysed for IL-1 β by ELISA (2.2.5). Particles + LPS v LPS only, *** $p < 0.001$.

(a)



(b)

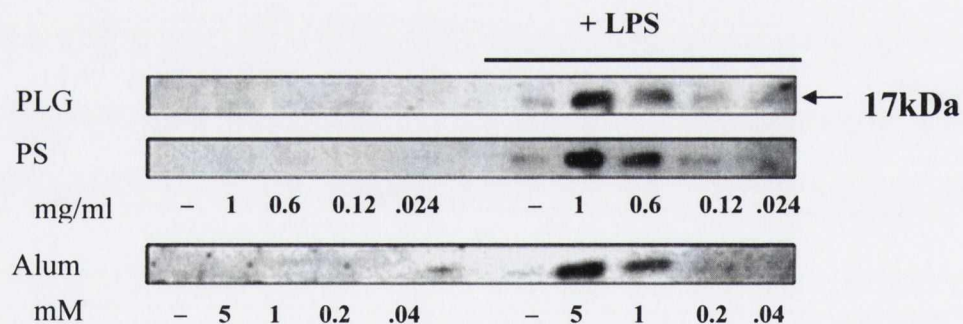


Figure 3.7: PVA promote processing of pro-IL-1 β (31kDa) into its active form (17kDa). (a) Pro-IL-1 β in cell lysates. (b) Processed IL-1 β in supernatants. β -Actin loading control, 42kDa. BMDC (0.5×10^6 cells/ml) were stimulated with medium, LPS (5 ng/ml) alone, PLG (1 μ m), PS (430nm) or alum alone; or for 1 h with particles before the addition of medium or LPS. After 24 h, supernatants were removed and cells were lysed. Cell lysates and supernatants were analysed for IL-1 β by western blot (2.2.6).

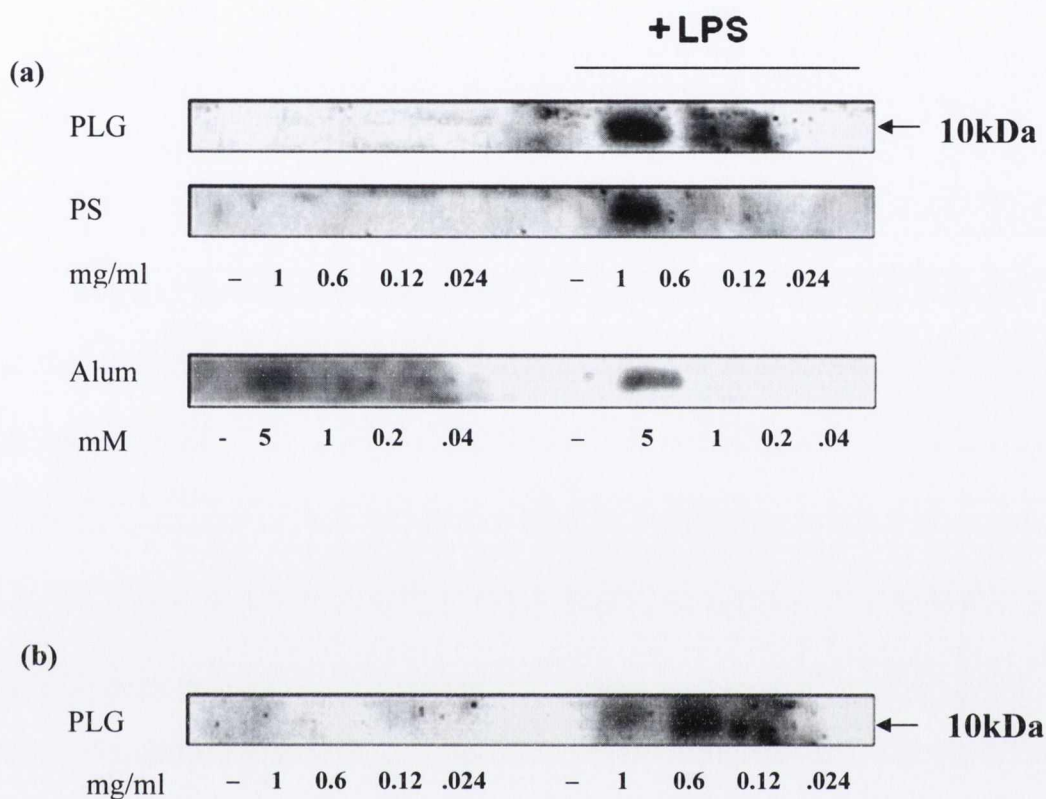


Figure 3.8: PVA synergise with LPS to promote caspase-1 activation. **(a)** Caspase-1 p10 in cell lysates. **(b)** Caspase-1 p10 in concentrated supernatant. BMDC (0.5×10^6 cells/ml) were stimulated with medium or LPS (5 ng/ml) alone or alum, PLG (1 μ m) or PS (430nm) alone; or for 1 h with particles before the addition of medium or LPS. After 24 h, supernatants were removed and cells were lysed. Cell lysates and supernatants were analysed for processed caspase-1 (p10) by western blot (2.2.6).

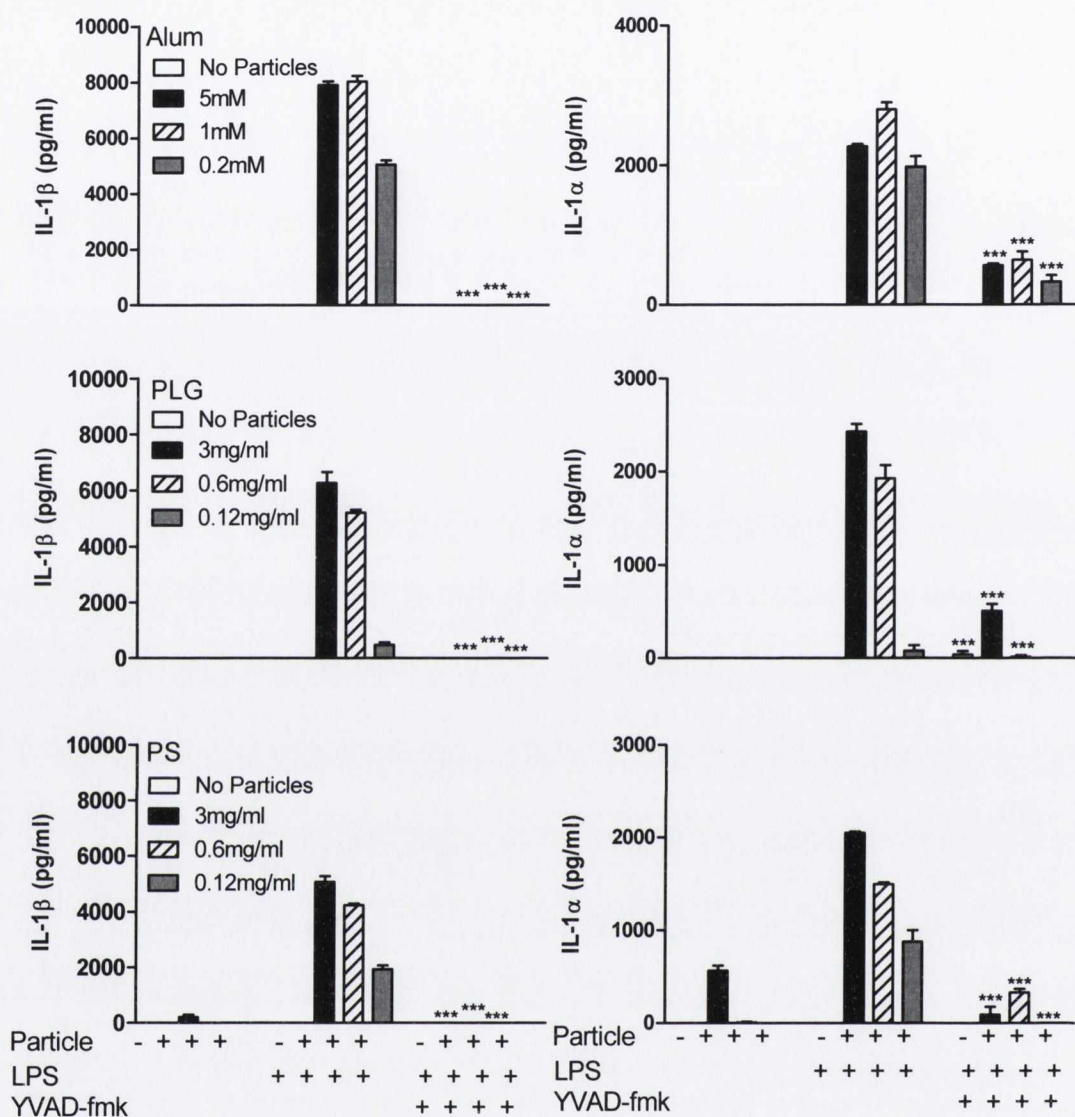


Figure 3.9: The ability of PVA to enhance IL-1β secretion by BMDC is abrogated by pre-treatment with YVAD-fmk while IL-1α secretion is significantly reduced. BMDC (6.25×10^5 cells/ml) were incubated with medium or the caspase-1 inhibitor (YVAD-FMK, 40 μM) for 30 min followed by 1 h incubation with medium, alum, PLG (1μm) or PS (430nm). 1 h later medium or LPS (5 ng/ml) was added to the cells. 24 h post-stimulation, supernatants were collected and IL-1β and IL-1α were determined by ELISA (2.2.5). Particles + LPS v Particles + LPS + caspase-1 inhibitor, *** p<0.001.

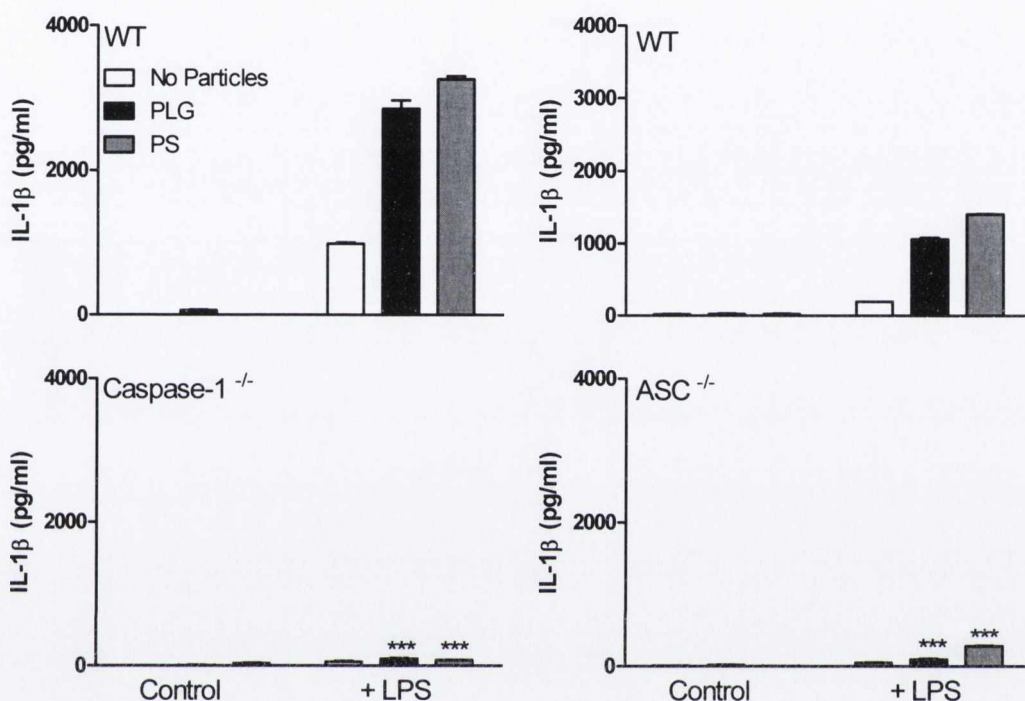


Figure 3.10: The ability of PVA to enhance IL-1 β secretion by BMDC is caspase-1 and ASC dependent. BMDC (6.25×10^5 cells/ml) from WT C57BL/6 and caspase-1^{-/-} or ASC^{-/-} mice were pre-incubated with medium, PLG (1 μ m;3mg/ml) or PS (430nm;3 mg/ml) alone for 1 h before the addition of medium or LPS (5 ng/ml). 24 h post-stimulation supernatants were collected and analysed for IL-1 β by ELISA (2.2.5). Particle + LPS WT mice v Particle + LPS caspase-1^{-/-} or ASC^{-/-} mice, *** p<0.001.

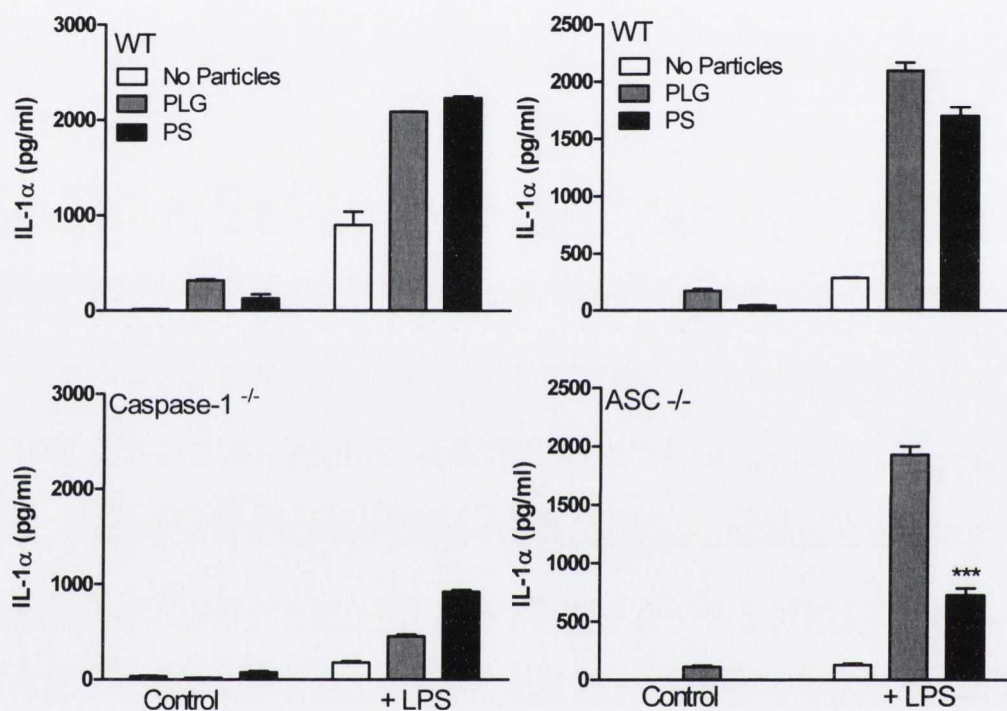


Figure 3.11: The ability of PVA to enhance IL-1 α secretion by BMDC is not caspase-1- or ASC-dependent. BMDC (6.25×10^5 cells/ml) from WT C57BL/6 and caspase-1^{-/-} or ASC^{-/-} mice were pre-incubated with medium, PS (430nm;0.6mg/ml) or PLG (1 μ m;0.6 mg/ml) alone for 1 h before the addition of medium or LPS (5 ng/ml). 24 h post-stimulation supernatants were collected and analysed for IL-1 α by ELISA (2.2.5). Particle + LPS WT mice v Particle + LPS caspase-1^{-/-} or ASC^{-/-} mice, *** $p < 0.001$.

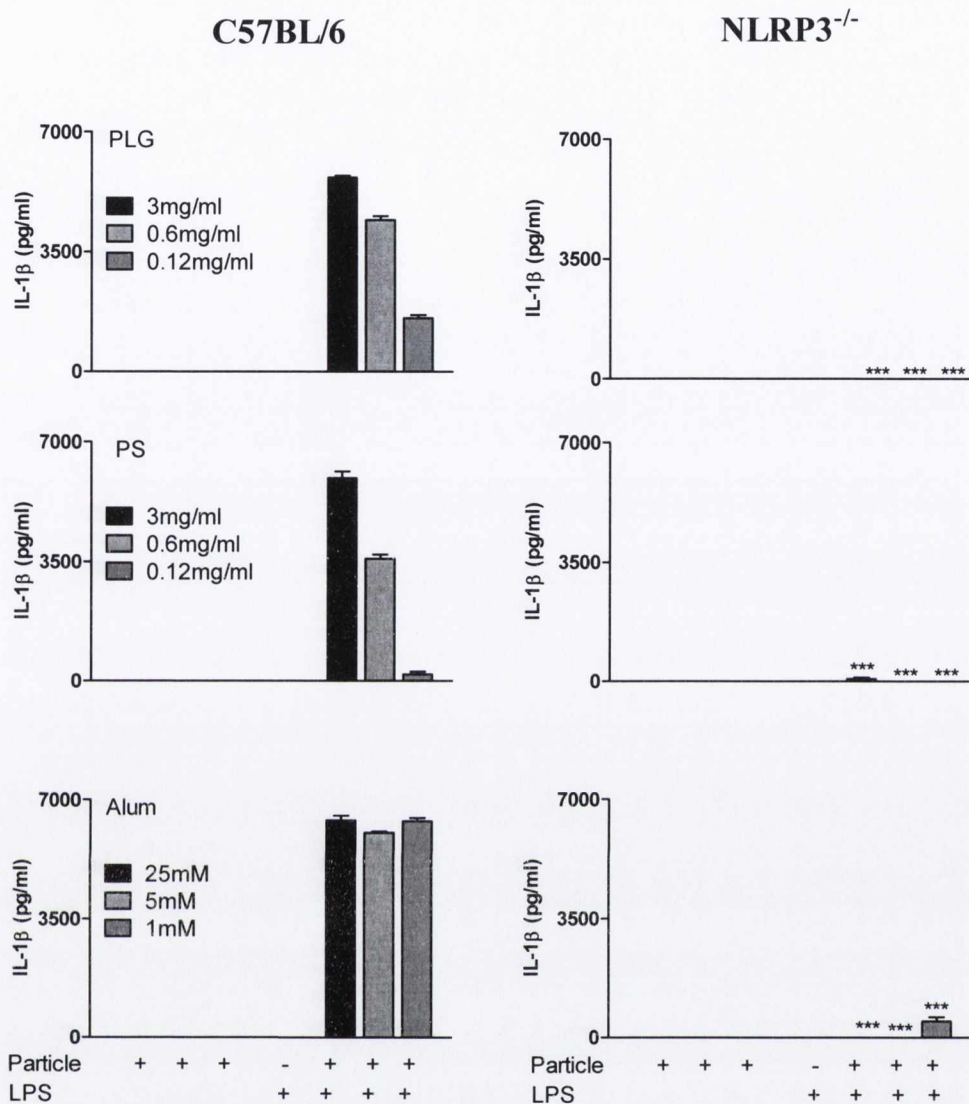


Figure 3.12: Particle-mediated enhancement of IL-1 β is NLRP3-dependent. BMDC (6.25×10^5 cells/ml) from WT C57BL/6 mice and NLRP3^{-/-} mice were pre-incubated with medium or particle alone for 1 h before medium or LPS (5 ng/ml) was added to the cells. After 24 h, supernatants were removed from the cells and analysed for IL-1 β by ELISA (2.2.5). Particles + LPS WT C57BL/6 v Particles + LPS NLRP3^{-/-} mice, *** $p < 0.001$.

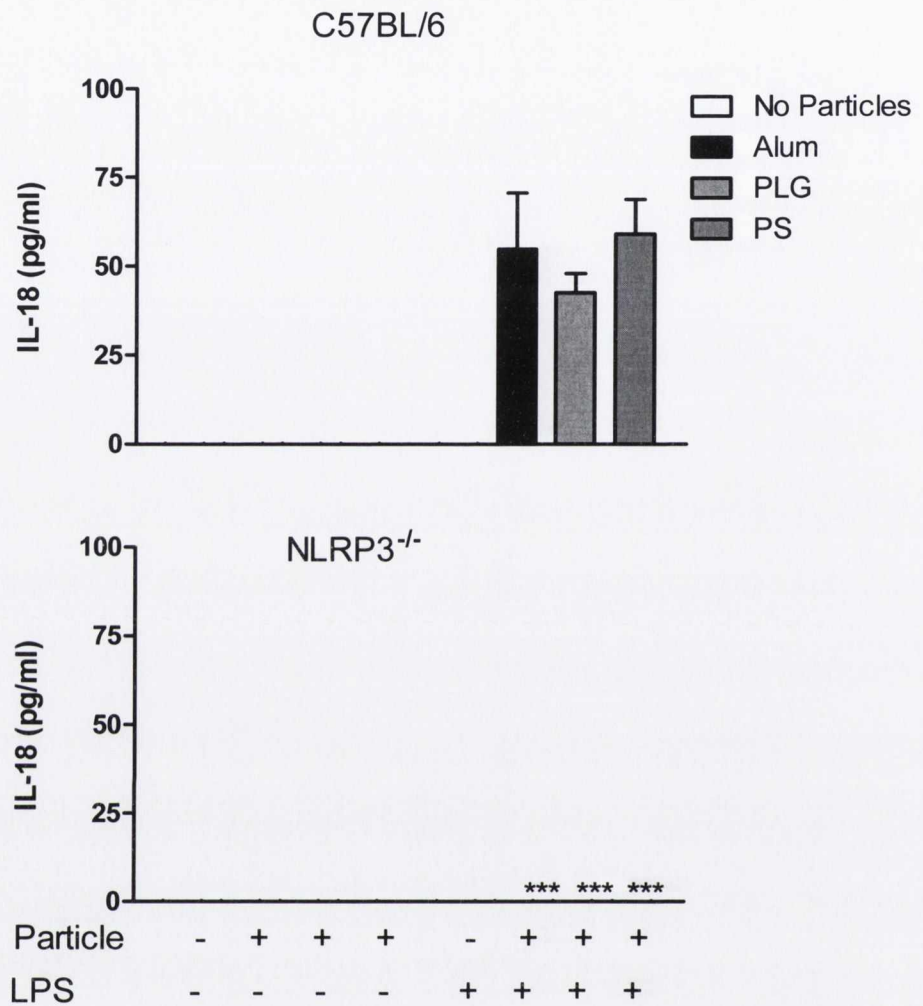


Figure 3.13: PVA synergise with LPS to promote IL-18 secretion in a NLRP3-dependent manner. BMDC (6.25×10^5 cells/ml) from NLRP3^{-/-} and WT C57BL/6 mice were pre-incubated with medium or particles (Alum 25 mM, PLG 1 μ m;3 mg/ml, PS 430nm;3 mg/ml) alone for 1 h before LPS (5 ng/ml) was added to the cells. After 24 h, supernatants were removed from the cells and analysed for IL-18 by ELISA (2.2.5). Particles + LPS WT C57BL/6 mice v Particles + LPS NLRP3^{-/-} mice ,*** p<0.001.

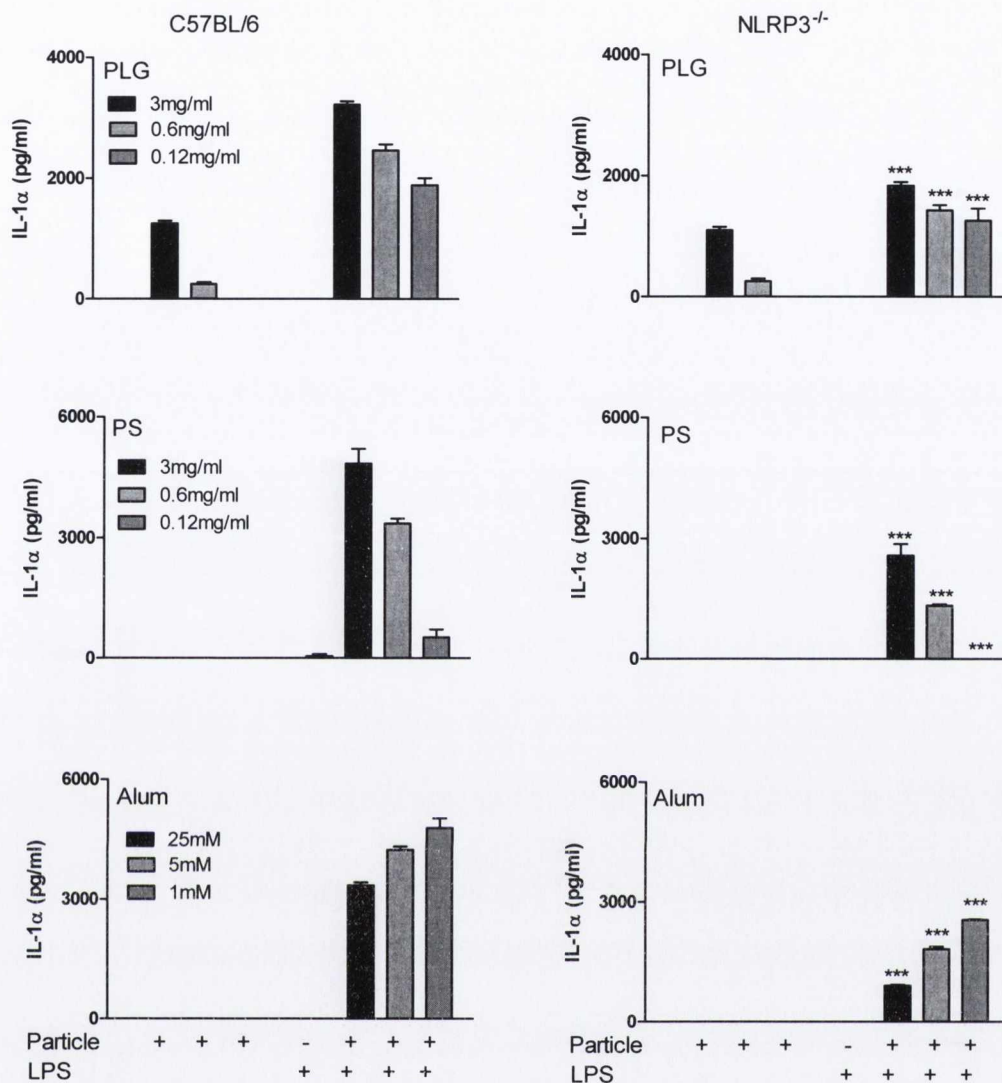


Figure 3.14: Particle-mediated enhancement of IL-1α secretion is partly dependent on NLRP3. BMDC (6.25×10^5 cells/ml) from NLRP3^{-/-} and WT C57BL/6 mice were pre-incubated with medium or particles alone for 1 h before medium or LPS (5 ng/ml) was added to the cells. After 24 h, supernatants were removed from the cells and analysed for IL-1α by ELISA (2.2.5). Particles + LPS WT C57BL/6 v Particles + LPS NLRP3^{-/-} mice, *** p<0.001.

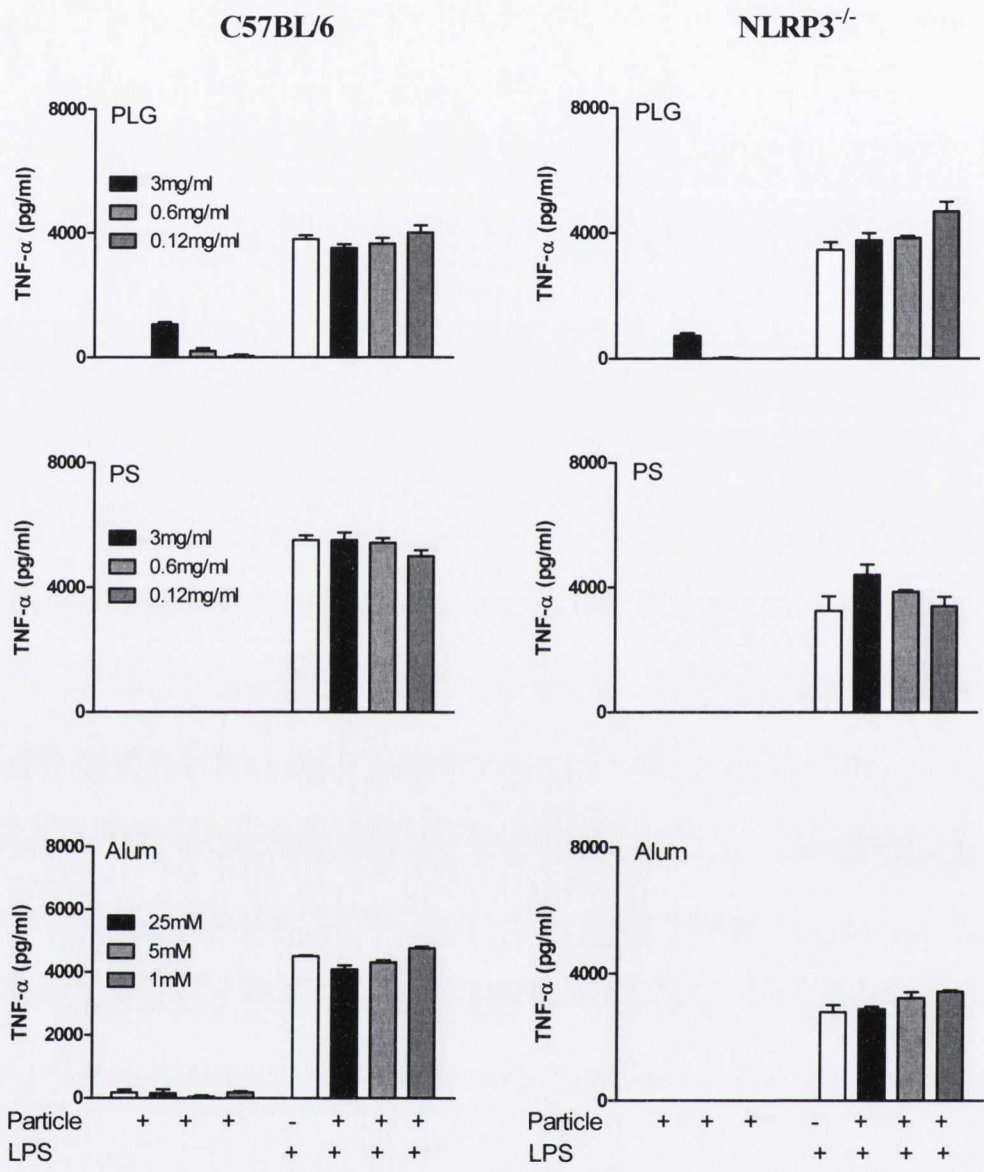


Figure 3.15: TNF- α secretion by BMDC in response to LPS and particle stimulation does not require NLRP3. BMDC (6.25×10^5 cells/ml) from NLRP3^{-/-} and WT C57BL/6 mice were pre-incubated with medium or particles alone for 1 h before medium or LPS (5 ng/ml) was added to the cells. After 24 h, supernatants were removed from the cells and analysed for TNF- α by ELISA (2.2.5). Particles + LPS WT C57BL/6 v Particles + LPS NLRP3^{-/-} mice, *** $p < 0.001$.

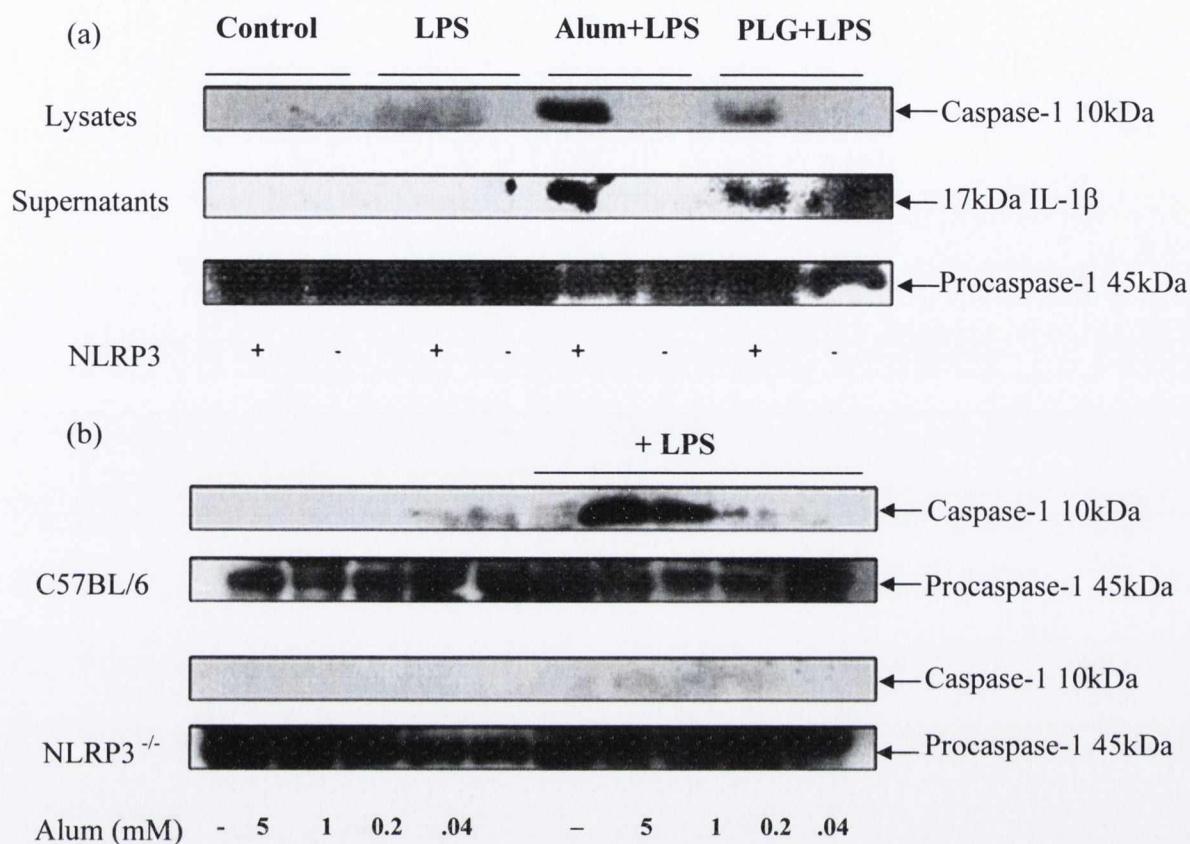


Figure 3.16: Particle-mediated caspase-1 activation is NLRP3-dependent. (a) Caspase-1 activation and IL-1 β processing is absent in NLRP3-deficient BMDC. (b) Caspase-1 activation is most marked with the highest concentrations of alum and is absent in the NLRP3-deficient BMDC. BMDC (6.25×10^5 cells/ml) from NLRP3^{-/-} and WT C57BL/6 mice were pre-incubated with medium or particles alone for 1 h before medium or LPS (5 ng/ml) was added to the cells. After 24 h, supernatants were removed and cells were lysed. Cell lysates were analysed for activated caspase-1 (p10) and supernatants were analysed for IL-1 β by western blot (2.2.6).

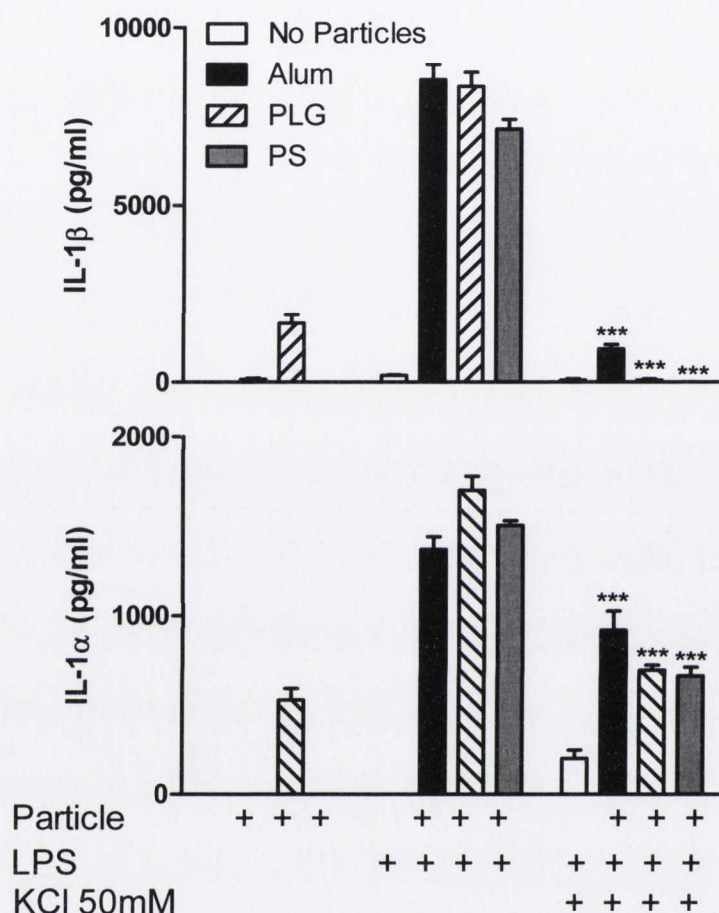


Figure 3.17: Particle mediated enhancement of IL-1 β but not IL-1 α is dependent on K⁺ efflux. BMDC (6.25×10^5 cells/ml) were incubated in medium with or without KCl (50 mM) for 30 min. Cells were stimulated with medium or particles alone (Alum 25 mM, PLG 1 μ m; 3 mg/ml, PS 430nm; 3 mg/ml). After 1 h, medium or LPS (5 ng/ml) was added. 24 h post-stimulation, supernatants were collected and analysed for IL-1 β and IL-1 α by ELISA (2.2.5). Particle + LPS v Particle + LPS + KCL 50mM, *** $p < .001$.

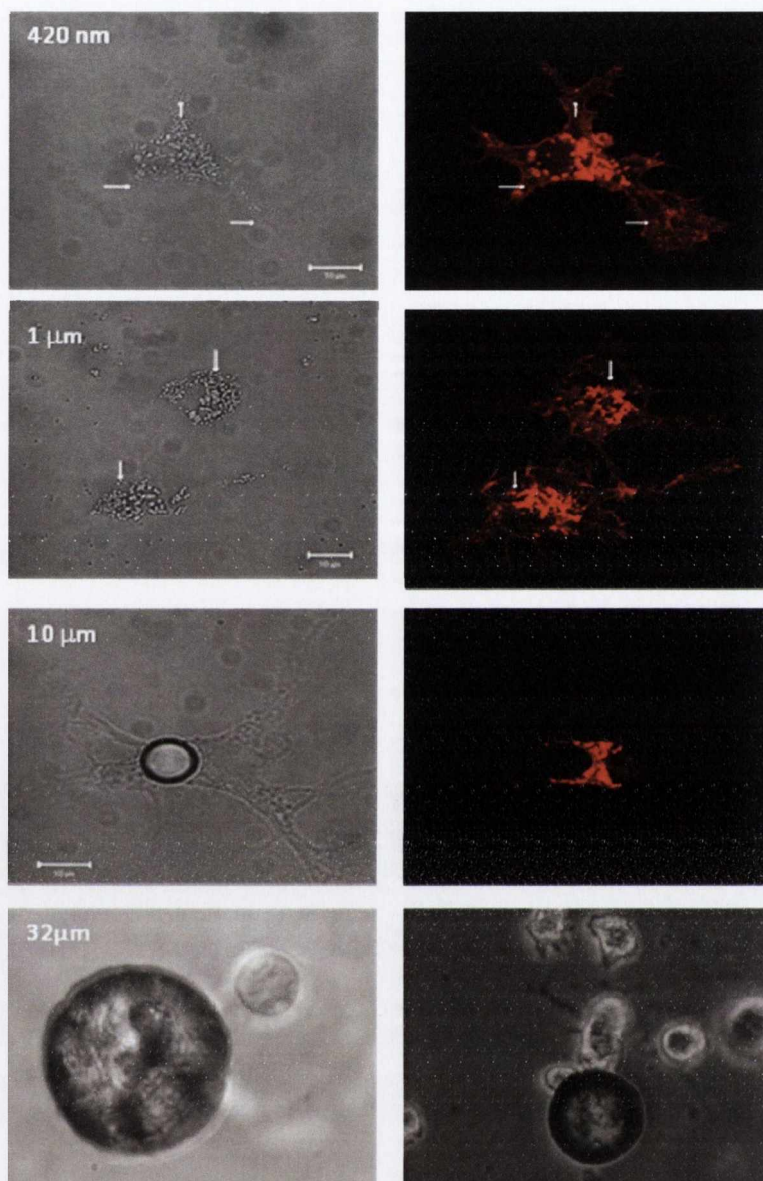


Figure 3.18: Dendritic cell uptake of PS particles is optimal at particle sizes $\leq 10 \mu\text{m}$. (Arrows point to internalised particles) BMDC were incubated with PS ranging from in size from 420 nm up to 32 μm (0.6 mg/ml) for 18 h on 19 mm coverslips. Samples were then fixed in 2% paraformaldehyde (in PBS) for 20 min and stained with Alexa-Fluor 594 conjugated wheat germ agglutinin (10 $\mu\text{g/ml}$) for 30 min (2.2.8). Cells were mounted on glass slides and analyzed on a Zeiss LSM 510CF with LSM 5 software (version 4) and Adobe Photoshop.

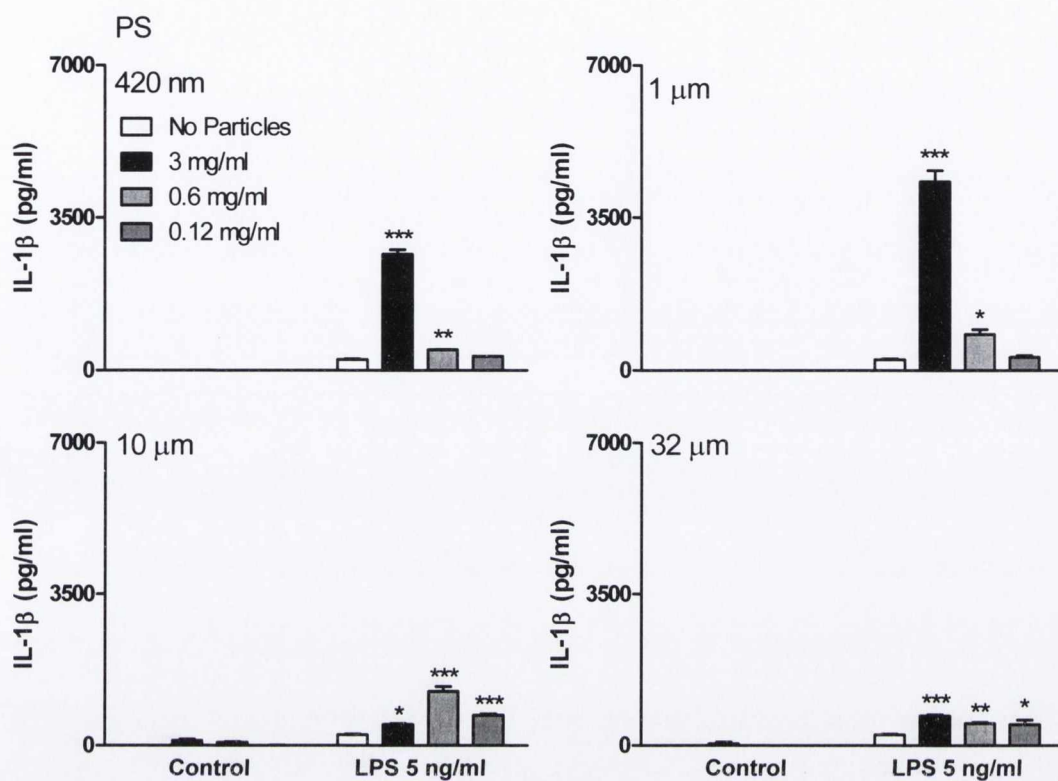


Figure 3.19: Optimal promotion of IL-1 β secretion by PS particles requires particle uptake. BMDC (6.25×10^5 cells/ml) were stimulated with medium or PS (420 nm, 1 μ m, 10 μ m, or 32 μ m) alone or for 1 h before the addition of medium or LPS (5 ng/ml). After 24 h, the supernatants were collected and analysed for IL-1 β by ELISA (2.2.5). LPS only v Particle + LPS, * p<0.05, ** p<0.01, *** p<0.001.

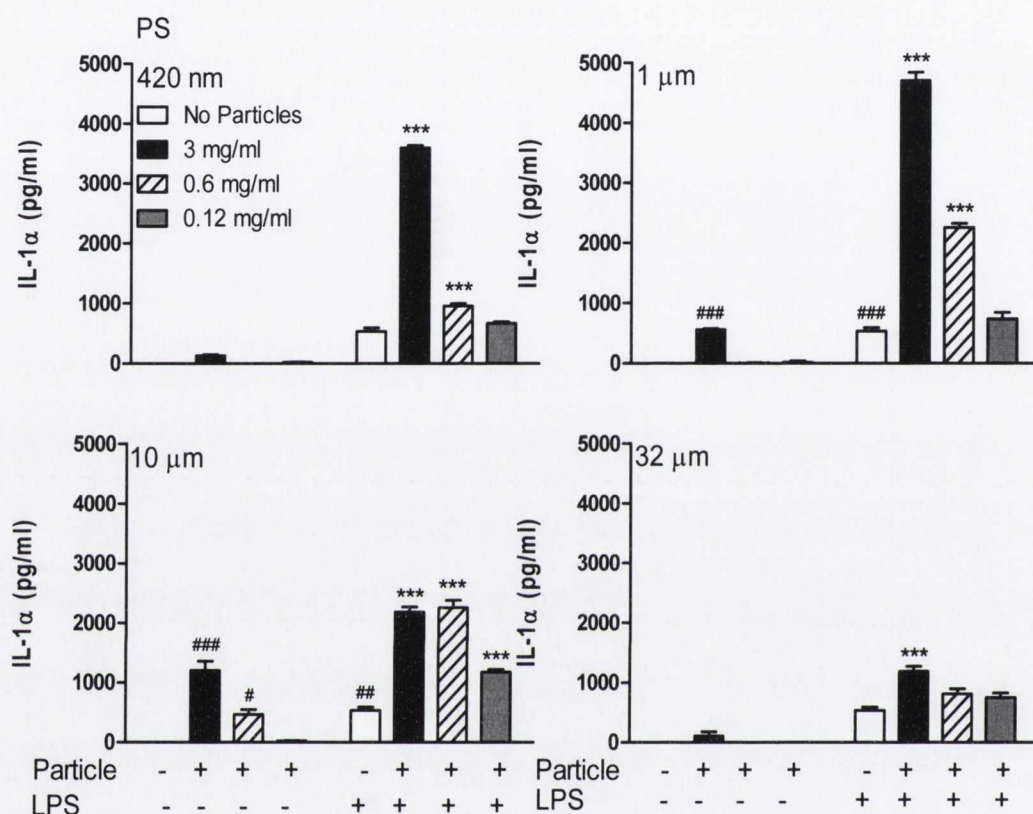


Figure 3.20: Optimal promotion of IL-1α secretion by PS particles requires particle uptake. BMDC (6.25×10^5 cells/ml) were stimulated with medium or PS (420 nm, 1 μm, 10 μm or 32 μm) alone or for 1 h before the addition of medium or LPS (5 ng/ml). After 24 h, the supernatants were collected and analysed for IL-1α by ELISA (2.2.5). LPS only v Particle + LPS, *** $p < 0.001$; Control v LPS alone or particle alone, ### $p < 0.001$, ## $p < 0.01$, # $p < 0.05$.

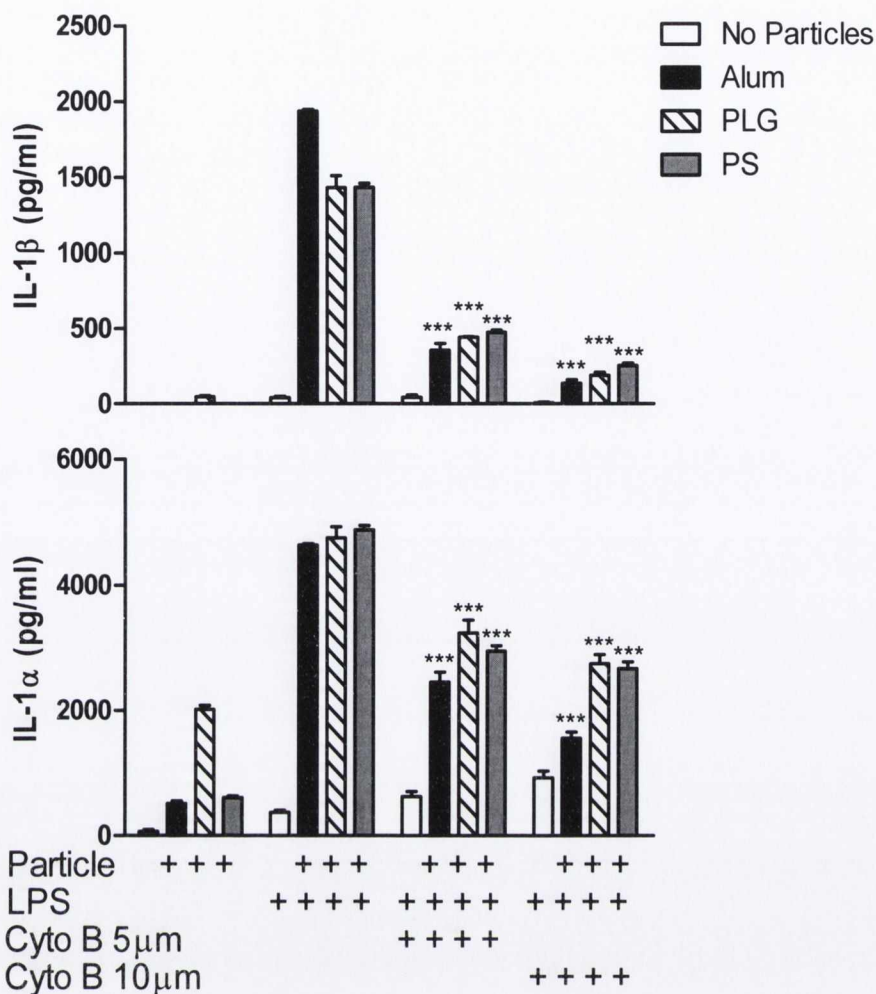


Figure 3.21: Inhibition of actin polymerisation significantly reduces the ability of PVA to enhance secretion of IL-1 β by BMDC. BMDC (6.25×10^5 cells/ml) were incubated with medium or cytochalasin B (10 μ M, 5 μ M) for 30 min before the addition of medium or alum (5 mM), PLG (1 μ m;3mg/ml) or PS (430nm;3 mg/ml) alone. After 1 h, medium or LPS (5 ng/ml) was added and cells were left for 24 h. Supernatants were removed and analysed for IL-1 β and IL-1 α by ELISA (2.2.5). Particles + LPS v Particle + LPS + Cytochalasin B, *** $p < 0.001$.

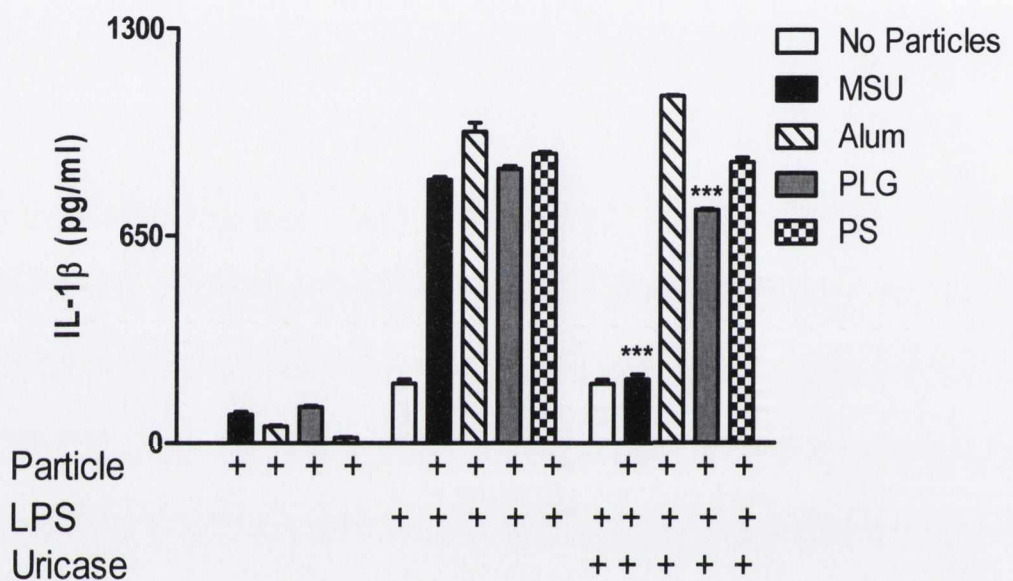


Figure 3.22: Degradation of uric acid does not inhibit the enhancement of IL-1 β secretion by PVA. MSU (200 μ g/ml), alum (25 mM), PLG (1 μ m;3 mg/ml) and PS (430nm;3 mg/ml) alone were incubated with uricase (0.1 Units/ml) overnight at 37 $^{\circ}$ C. Medium, control particles or uricase-treated particles alone were then added to BMDC (6.25 $\times$ 10 5 cells/ml). After 1 h, medium or LPS (5 ng/ml) was added and cells were left to incubate overnight. Post-stimulation, supernatants were removed and analysed for IL-1 β by ELISA (2.2.5). Particle + LPS v Particle + LPS + uricase, *** p<.001.

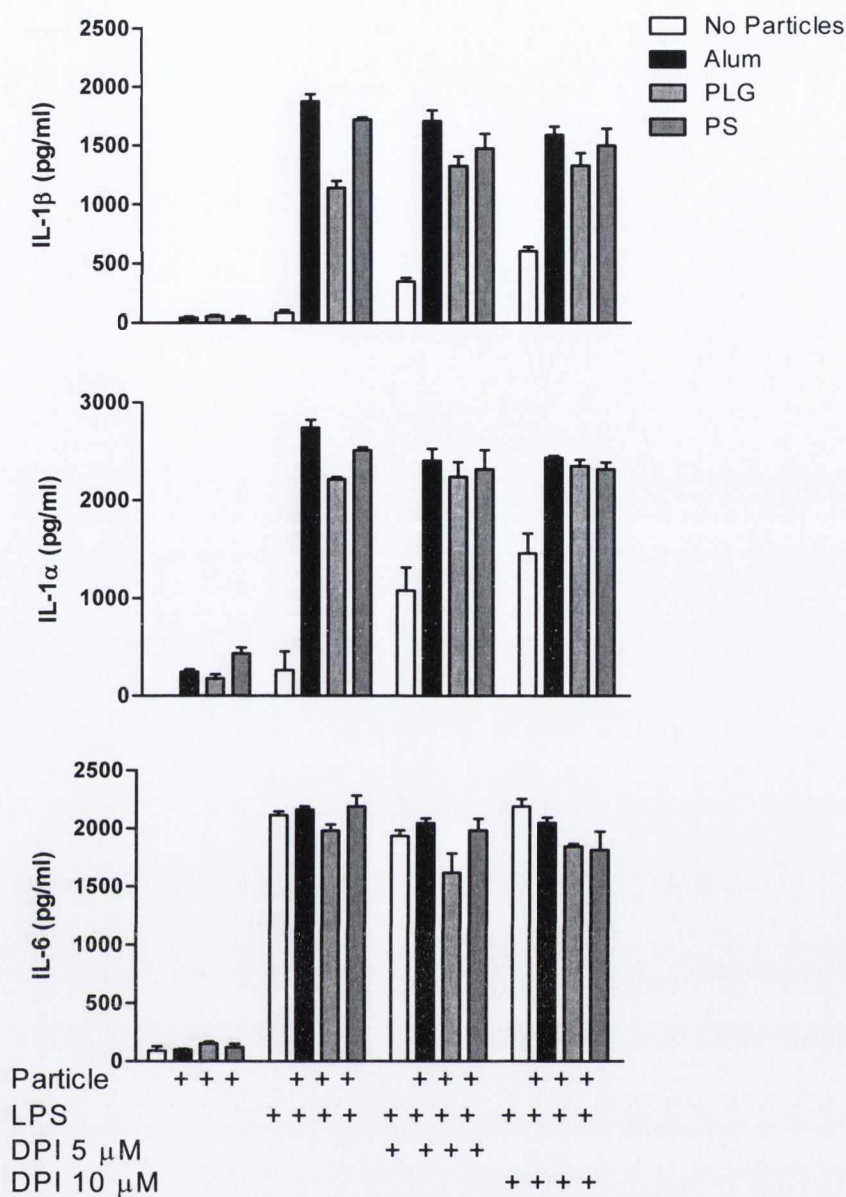


Figure 3.23: ROS are not required for activation of the NLRP3 inflammasome by PVA. BMDC (6.25×10^5 cells/ml) were incubated with medium or DPI (5 μ M, 10 μ M) for 1 h before the addition of medium, alum (5 mM), PLG (1 μ m;3mg/ml) or PS (430nm;3 mg/ml) alone. After 1 h, medium or LPS (5 ng/ml) was added and cells were left for 24 hr. Supernatants were removed and analysed for IL-1 β , IL-1 α and IL-6 by ELISA (2.2.5).

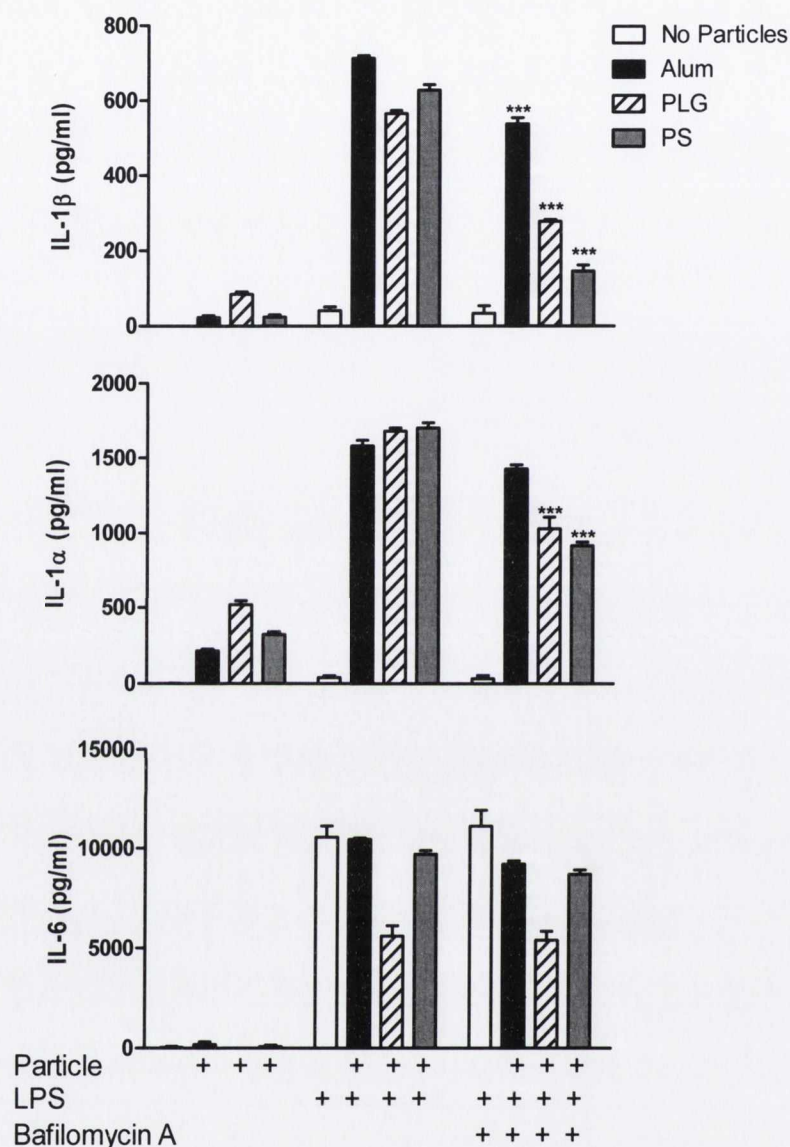
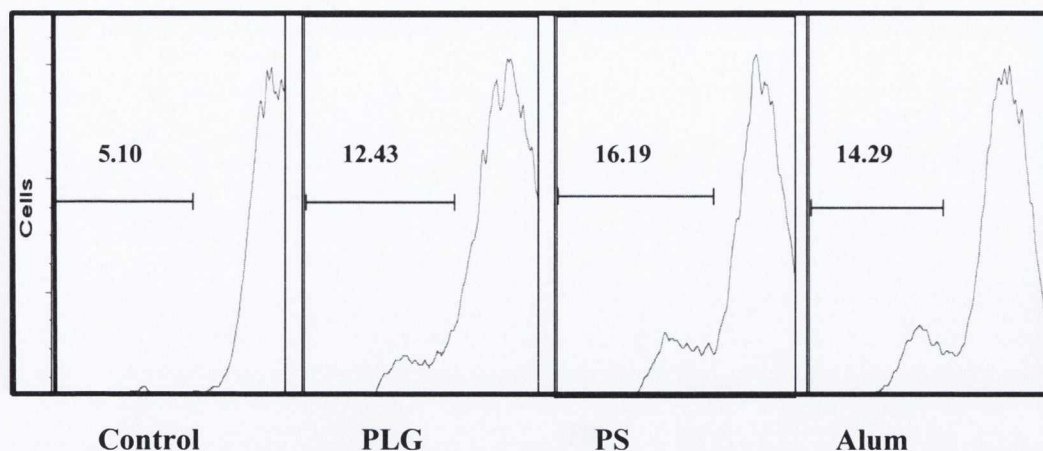


Figure 3.24: Blocking of the H^+ ATPase system reduces the ability of PVA to enhance IL-1 β and IL-1 α secretion by DC. BMDC (6.25×10^5 cells/ml) were incubated with medium or bafilomycin A1 (250 nM) for 1 h before the addition of medium, alum (5 mM), PLG ($1 \mu\text{m}$; 3 mg/ml) or PS (430 nm; 3 mg/ml) alone. After 1 h, medium or LPS (5 ng/ml) was added and cells were left for 24 h. Supernatants were removed and analysed for IL-1 β , IL-1 α and IL-6 by ELISA (2.2.5). Particle + LPS v Particles + LPS + Bafilomycin A, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

(a)



(b)

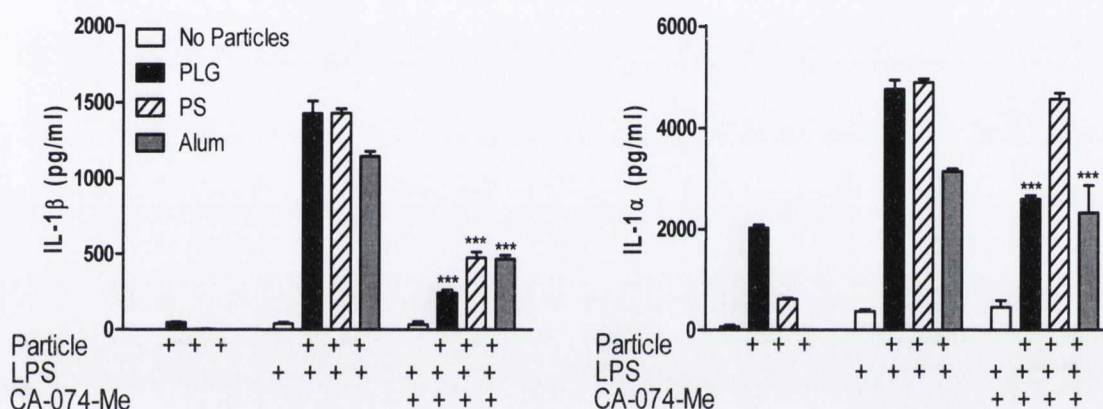


Figure 3.25: Particulates cause lysosomal disruption and require Cathepsin B activation for their enhancement of IL-1. (a) BMDC (6.25×10^5 cells/ml) were incubated with medium or particles alone (alum 5 mM, PS or PLG 0.6 mg/ml). After 3 h cells were harvested, resuspended in FACS buffer (2.1.5.2) and stained with acridine orange. Incorporation of acridine orange was analysed by flow cytometry (2.2.2.3). (b) BMDC (6.25×10^5 cells/ml) were incubated with medium or CA-074-Me (10 μ M) for 1 hr before the addition of medium, PLG (1 μ M; mg/ml), PS (430nm; 3 mg/ml) or alum (5 mM) alone. After 1 h, medium or LPS (5 ng/ml) was added and cells were incubated for 24 h. Supernatants were removed and analysed for IL-1 β , and IL-1 α by ELISA (2.2.5). Particle + LPS v Particle + LPS + CA-074-Me, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

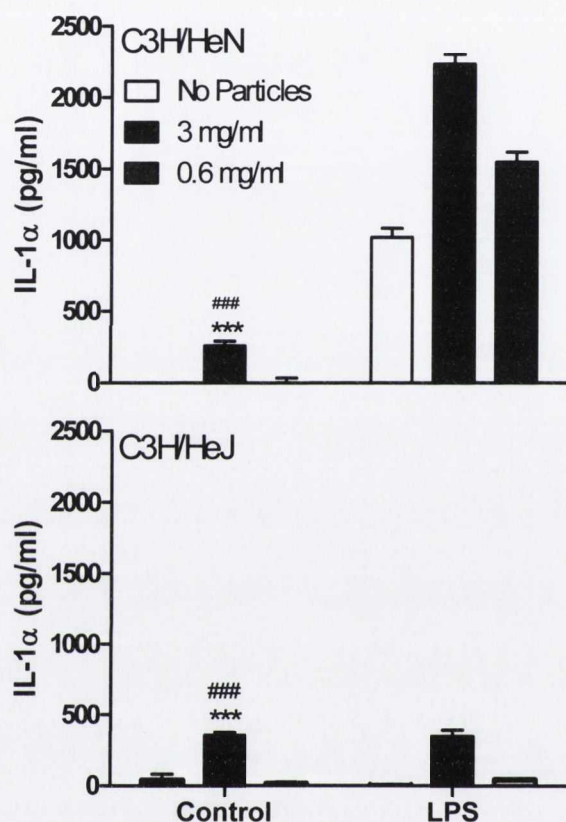


Figure 3.26: PVA enhance the secretion of IL-1 α by BMDC in a TLR4-independent manner. BMDC (6.25×10^5 cells/ml) from C3H/HeN (LPS responsive) or C3H/HeJ (LPS hypo-responsive; TLR4 mutation) mice were pre-incubated with medium or PLG (1 μ m) alone. After 1 h, medium or LPS (5 ng/ml) was added and cells were incubated for 24 h. Supernatants were removed and analysed for IL-1 α by ELISA (2.2.5). Control v Particle treated BMDC, *** $p < 0.001$; PLG treated HeN BMDC v PLG treated HeJ BMDC, ### $p < 0.001$.

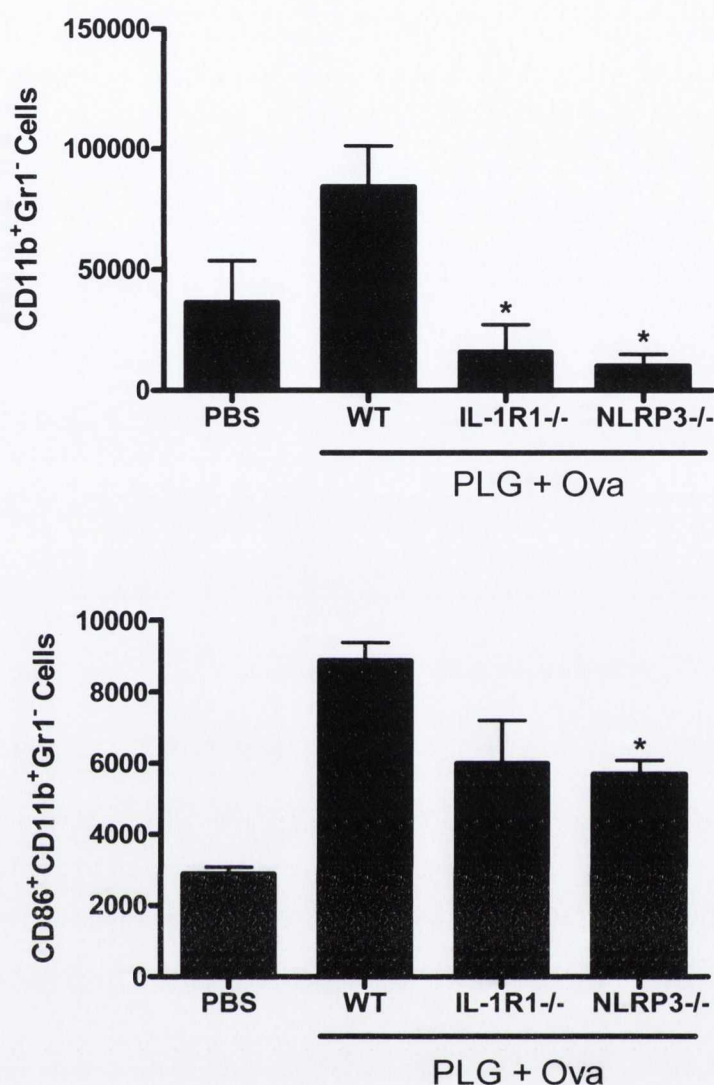


Figure 3.27: PLG-mediated recruitment of innate cells to the injection site is NLRP3-dependent. WT, IL-1R1^{-/-} and NLRP3^{-/-} C57BL/6 mice were immunised intra-peritoneally with PBS, Ova alone or PLG with Ova. After 18 h, the peritoneal cavity was washed with PBS. Isolated cells were stained with cell subtype specific markers and the cell numbers was determined using flow cytometry (2.2.3.1). Total cell counts WT v Total cell counts in NLRP3^{-/-} mice or Total cell counts in IL-1R1^{-/-} mice, * p<0.05.

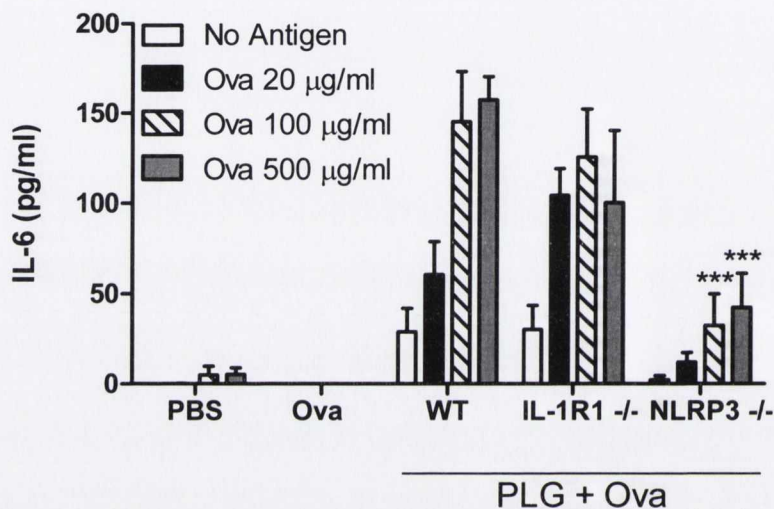


Figure 3.28: PVA-mediated enhancement of antigen-specific IL-6 is NLRP3-dependent. WT, IL-1R1^{-/-} and NLRP3^{-/-} C57BL/6 were immunised with PBS, Ova (50 µg/mouse) alone or Ova (50 µg/mouse) with PLG (4 mg/mouse) subcutaneously on day 0 and day 14. After 21 days the mice were sacrificed and spleens were removed. Cells isolated from spleens were re-stimulated with Ova (20, 100, 500 µg/ml). After 3 days, supernatants were removed and analysed for IL-6 by ELISA (2.2.5). Ova (20, 100 or 500 µg/ml) WT mice v Ova (20, 100 or 500 µg/ml) IL-1R1^{-/-} mice or Ova (20, 100 or 500 µg/ml) NLRP3^{-/-} mice, *** p<0.001.

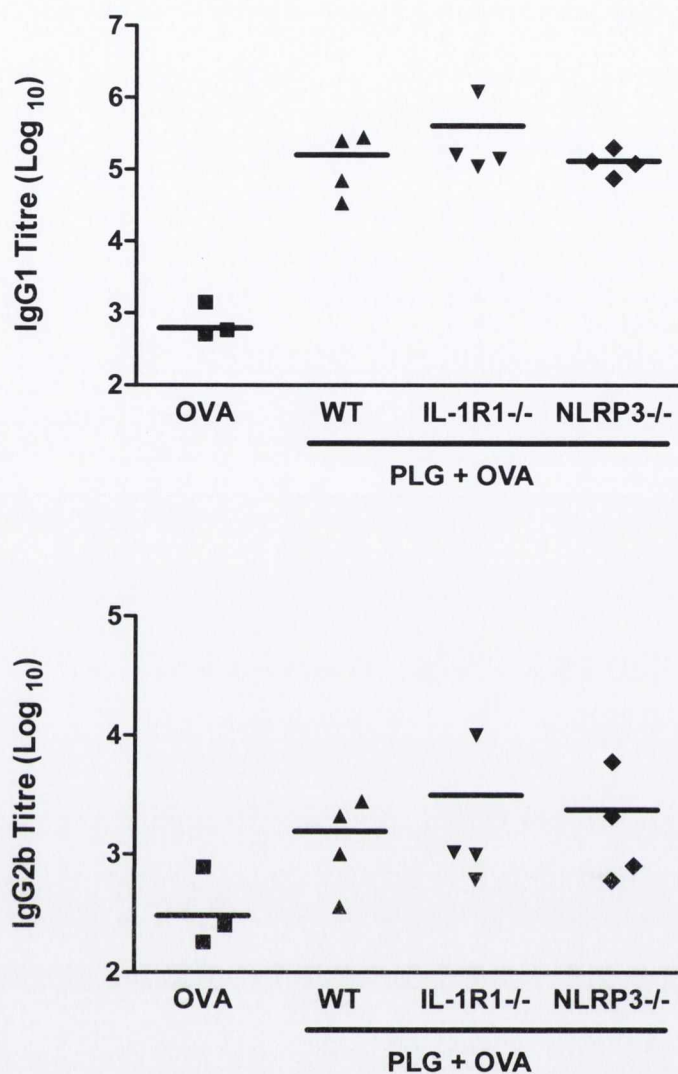


Figure 3.29: NLRP3 is not required for PVA to enhance antigen-specific antibodies. WT, IL-1R1^{-/-} and NLRP3^{-/-} C57BL/6 mice were immunised intraperitoneally with PBS, Ova alone or PLG with Ova. Serum samples were collected 21 days post-immunisation and analysed for antigen-specific antibodies by ELISA (2.2.12).

Chapter 4

An Investigation of the Relationship between Particle Characteristics and the Adjuvant Properties of Particulates

4.1 Introduction

Aluminium-containing adjuvants are clinically the most widely used vaccine adjuvants. Despite their widespread use, our knowledge of the mechanisms underlying how these particulate substances induce their adjuvant activities *in vivo* remains limited. It was proposed that particulates acted principally as a depot, facilitating sustained antigen presentation and thereby enhancing the immune response. However, this idea has been contested and it is now widely accepted that particulate vaccine adjuvants (PVA) are acting as more than antigen depots.

Although alum and related adjuvants are not known to activate any currently identified receptors, studies suggest that these adjuvants can exert antigen-independent immunostimulatory effects on the immune system *in vivo*. For example, injection of mice with alum was found to enhance the level of intracellular calcium in B cells within the spleen. This exerted a priming effect, preparing B cells for activation by MHC class II aggregation [451-453]. The injection of alum also induced the accumulation of IL-4⁺ Gr1⁺ cells in the spleen, which were later identified as eosinophils. These cells were also shown to be responsible for alum-mediated B cell priming [451-453]. The ability of alum and other PVA, such as the O/W emulsion MF59, to promote the recruitment and conversion of monocytes into DC has been reported [165, 430]. Both alum and MF59 have been shown to induce the release of chemoattractants, including CCL2, CCL3, CCL4 and IL-8, all of which are involved in the recruitment of white blood cells into the injection site [430]. In addition, microarray studies have shown that alum and MF59 promote the upregulation of hundreds of genes in muscle tissue within several hours following intramuscular injection [546]. This study also demonstrated that alum and MF59 enhanced the expression of 168 common genes which they termed ‘adjuvant core response genes’. This cluster of genes included genes encoding cytokines, chemokines and adhesion molecules [546]. Overall, there is a strong body of evidence to suggest that PVA exert immunomodulatory effects in addition to enhancing antigen uptake and antigen presentation *in vivo*.

PVA share their ability to enhance immune responses, however the reality is that this class of adjuvants actually represents a diverse mixture of compounds which differ greatly in their physico-chemical properties. Despite this, results presented in chapter 3 demonstrate that a range of PVA can activate the NLRP3 inflammasome and enhance

the secretion of IL-1 α and IL-1 β by BMDC (Figures 3.3-3.6). NLRP3 was required for the development of cell-mediated responses, but was dispensable for particle-mediated humoral responses (Figures 3.27-3.29). Given the ability of all particulates to activate the NLRP3 inflammasome it was predicted that PVA may also activate similar pathways *in vivo*. The relationship between the nature of the PVA and the type of immune response induced *in vivo* was therefore investigated.

As outlined above, others have shown that alum and MF59 have the ability to induce antigen-independent immunopotentiating effects *in vivo*, which were previously unappreciated [165, 430, 451-453, 546]. In this current study, the kinetics of the immune response induced by PVA *in vivo* was investigated, in order to identify the mediators of the immunomodulatory effects of particulates.

In addition to their different compositions, PVA also differ in their size. Results from chapter 3 have shown that particle size determines the ability of particles to activate the NLRP3 inflammasome (Figures 3.19-3.21). Given the role of NLRP3 in particle driven cell-mediated responses, it is therefore possible that the size of the particle may be a determining factor in the type of immune response induced by PVA *in vivo* (Figure 3.27-3.28). Several studies have investigated the importance of size in the ability of PVA to enhance immune responses. These studies have found that particle size can have a major impact on the type of immune response induced [547-549]. However, none of these studies have provided a detailed account of the effects of particle size on local inflammatory reactions, the production of key T cell associated cytokines and on the induction of humoral responses.

The overall aims of this chapter were to:

- Investigate the relationship between the composition of the particulate and the type of immune response induced.
- Study the kinetics of the immune response promoted by particulate vaccine adjuvants.
- Determine the importance of size in the adjuvant properties of polystyrene particles.

4.2 Results

4.2.1 The induction of innate and adaptive immune responses by PVA

The aim of this study was to compare and contrast the immune responses to a bystander antigen promoted by different particulate vaccine adjuvants (PVA) *in vivo*. C57BL/6 mice were immunised with PBS or Keyhole Limpet Hemocyanin (KLH) alone or with alum, polystyrene (PS) microparticles (420 nm) or PLG (PLG) microparticles (1 μ m) subcutaneously at two injection sites on the right and left flank. All mice were sacrificed 7 days post immunisation.

4.2.1.1 Histopathological analysis of the injection site

In order to establish the local effects of each particulate adjuvant, the injection site was excised and preserved for histological examination. No visible lesions were observed at the site of injection with KLH (Figure 4.1). In contrast to this, injection of KLH and PLG resulted in considerable changes in the local anatomy. The lesion was characterised by infiltration of inflammatory cells, including neutrophils, as well as areas of necrosis (Figure 4.2). Areas of necrosis were characterised by fragmentation and loss of pyknotic nuclei, which are a specific form of irreversibly condensed nuclei normally observed in dying cells. The local response suggests an attempt to control or wall-off PLG by formation of a granuloma, which was characterised by its lipid-like appearance; however this may be due to the nature of the microparticles themselves (Figure 4.2). Similar to PLG, the injection of PS and KLH had significant effects on the local tissue and induced considerable infiltration of inflammatory cells (Figure 4.3). However, in contrast to PLG, the reaction seemed to be less controlled as the granuloma formed was less demarcated and characterised by large numbers of neutrophils, representing a pyogranuloma (Figure 4.3). The area contained fewer remnants of the microparticles themselves and more inflammatory cells which suggested that the PS were more efficiently cleared from the injection site (Figure 4.3). PS injection also caused a considerable amount of necrosis in the local area (Figure 4.3).

Co-administration of alum and KLH induced the formation of a large lesion at the injection site (Figure 4.4). The type of lesion formed by alum injection resembled that of the PLG injection site, as it was characterised by a large well-demarcated granuloma (Figure 4.4). While there was a significant inflammatory cell infiltration, it was less

than that observed at the PS injection site (Figure 4.4). There was a significant amount of necrotic cell death observed within the alum injection site (Figure 4.4).

4.2.1.2 Local IL-1 β production

Having established the ability of PVA to activate the inflammasome and induce IL-1 β secretion *in vitro* (Figures 3.3-3.6), the ability of particulates to promote IL-1 β *in vivo* was investigated. In order to investigate whether IL-1 β enhancement could be seen locally *in vivo* the injection site was excised, homogenised and analysed for IL-1 β . When KLH was combined with alum, PLG or PS, local IL-1 β could be detected within the excised tissue (Figure 4.5). In contrast, injection with PBS or KLH alone did not induce local production of IL-1 β , suggesting that the enhanced IL-1 β was due to specific effects induced locally by the particles (Figure 4.5). This demonstrates that PVA can enhance IL-1 β production *in vivo* in the absence of an exogenous second signal, suggesting that particulates may have the ability to induce the release or secretion of an endogenous molecule which can prime cells to produce IL-1 β , thereby enhancing local inflammation.

4.2.1.3 The effect of PVA on adaptive cell-mediated responses

Following on from investigations into the local effects *in vivo* of PVA, the effects of particulates on antigen-specific adaptive responses were analysed. In order to achieve this, inguinal lymph nodes (draining lymph node) and spleens were removed from the immunised mice and cells were isolated and stimulated *ex vivo* with KLH at a range of concentrations (2, 10, 50 $\mu\text{g/ml}$). Following *ex vivo* stimulation, supernatants were collected and analysed for cytokines.

IL-4, IL-6, IL-17 and IFN- γ were all undetectable in the supernatants from re-stimulated inguinal lymph node cells (Figure 4.6). However, inguinal lymph node cells isolated from mice immunised with KLH and PLG spontaneously secreted IL-5 (Figure 4.6). In addition to IL-5, small amounts of antigen-specific IL-10 were secreted by inguinal lymph node cells taken from mice immunised with either PLG and KLH or PS and KLH re-stimulated with KLH (10 or 50 $\mu\text{g/ml}$) (Figure 4.6). However, the

concentrations of IL-10 and IL-5 produced were not significantly greater than in cells from mice immunised with antigen alone. Overall, these data suggest that PVA do not induce strong cellular responses in the inguinal lymph nodes after subcutaneous injection.

A similar trend was observed in the spleen with no detectable secretion of IL-4, IL-6 or IL-17 (Figure 4.7). However, injection of PLG and KLH induced the secretion of IL-5 and IL-10 by splenocytes (Figure 4.7). In contrast to the inguinal lymph nodes, trace amounts of antigen-specific IFN- γ (not significant) were secreted by splenocytes from mice immunised with PS and KLH (Figure 4.7). Overall, these data suggest that injection of PVA with KLH does not induce strong antigen-specific cellular immune responses in the spleen.

To determine the responsiveness of cells to polyclonal stimuli, spleen and lymph node cells from immunised mice were also stimulated with anti-CD3 alone or anti-CD3 and PMA.

In response to anti-CD3 and PMA stimulation, isolated inguinal lymph node cells from mice immunised with PS and KLH secreted significantly higher concentrations of IL-5 than inguinal lymph node cells isolated from mice injected with KLH alone (Figure 4.8). Similarly, injection of alum or PLG with KLH enhanced IL-5 secretion by inguinal lymph node cells in response to anti-CD3 and PMA, however these differences were not statistically significant (Figure 4.8). Stimulation of inguinal lymph node cells with anti-CD3 produced a similar trend, where injection of PVA with KLH enhancing the secretion of IL-5, however these effects were not statistically significant (Figure 4.8). Inguinal lymph node cells isolated from mice immunised with PS and KLH produced significantly higher amounts of IL-4 than cells isolated from mice immunised with KLH alone when re-stimulated with anti-CD3 and PMA *ex vivo* (Figure 4.8). No effect on the secretion of IL-4 by inguinal lymph node cells was observed when cells were re-stimulated with anti-CD3 only (Figure 4.8). In comparison to KLH alone, subcutaneous injection of KLH combined with alum, PS or PLG induced enhanced IL-6 secretion by inguinal lymph node cells in response to re-stimulation with anti-CD3 alone or anti-CD3 with PMA (Figure 4.8). These effects on IL-6 were most marked when inguinal lymph node cells were re-stimulated with anti-CD3 and PMA (Figure 4.8).

In contrast to their enhancing effects on Th2 cytokines, the injection of PVA with KLH did not enhance the secretion of IL-17, IL-10 or IFN- γ by inguinal lymph node cells re-stimulated with either anti-CD3 or anti-CD3 and PMA (Figure 4.8). In the case of IL-17, re-stimulation with PMA exerted a strong negative effect, as cells re-stimulated with anti-CD3 and PMA secreted less IL-17 than cells stimulated with anti-CD3 alone (Figure 4.8). Although KLH is thought to be weakly immunogenic and act as a bystander antigen, it was found that injection with KLH alone has modulatory effects on the secretion of IL-10 and IFN- γ by inguinal lymph node cells re-stimulated with anti-CD3 alone or anti-CD3 and PMA (Figure 4.8).

Splenocytes from mice injected with PS or PLG and KLH produced higher concentrations of IL-4 than cells from mice immunised with KLH alone, when re-stimulated with anti-CD3 (Figure 4.9). Furthermore, splenocytes from mice immunised with PLG and KLH secreted higher concentrations of IL-4 when re-stimulated with anti-CD3 and PMA (Figure 4.9). In comparison to KLH alone, injection of KLH with alum, PS or PLG, enhanced IL-5 secretion by splenocytes re-stimulated with anti-CD3 or anti-CD3 and PMA (Figure 4.9). In a similar manner to inguinal lymph node cells, splenocytes isolated from mice immunised with PLG and KLH spontaneously secreted IL-5 when cultured *ex vivo* (Figure 4.9). Injecting PLG and KLH enhanced IL-10 secretion by splenocytes, but not significantly (Figure 4.9).

In contrast to its supposed role as a by-stander antigen, immunisation with KLH alone resulted in a significant enhancement in IFN- γ secretion when splenocytes were re-stimulated *ex vivo* with either anti-CD3 or anti-CD3 with PMA in comparison to cells isolated from mice immunised with PBS or PVA and KLH (Figure 4.9). Furthermore, the presence of the particulates reduced this enhancing effect on KLH-induced IFN- γ secretion, which suggests that particles may suppress Th1 responses (Figure 4.9). These results suggest that, KLH is not an appropriate bystander antigen, as it clearly displays immunomodulatory effects. Overall, these results suggest that injection with PVA primarily promotes the secretion of Th2 cytokines by inguinal lymph node cells and splenocytes.

4.2.1.4 The effect of PVA on humoral responses

Having established the effects of PVA on the cellular response, the effect of these particulates on antigen-specific humoral responses was investigated. To achieve this, serum from immunised mice was collected 7 days post injection with KLH alone or KLH combined with alum, PLG or PS. The titres of antigen-specific antibodies were measured by ELISA. Injection of PLG or alum with KLH induced a significant enhancement in the level of circulating antigen-specific IgG in comparison to immunisation with KLH alone (Figure 4.10). In contrast to this, immunisation with PS and KLH did not significantly enhance antigen-specific IgG titres compared to injection of KLH alone (Figure 4.10). The titres of the Th2-associated antibody subclass IgG1, were also analysed. Immunisation with alum and KLH was the most efficient combination for promoting the production of IgG1, in comparison to injection with KLH alone, followed by PLG and KLH (Figure 4.10). PS was the least efficient adjuvant at driving antigen-specific humoral responses (Figure 4.10).

4.2.2 Injection of alum strongly enhances production of IL-6 and IL-10

In order to determine the kinetics of cellular infiltration and immune cell activation after PVA injection, a kinetic study was carried out using alum. Mice were immunised with alum (3mg/mouse) alone subcutaneously in the flank and sacrificed at 3 h, 1, 3, 5 and 7 days post injection. Based on the enhancing effect that alum has on IL-1 secretion *in vitro*, wild-type (WT) and IL-1R1^{-/-} mice were immunised, to determine whether the immunomodulatory effects of alum are mediated by IL-1.

In order to determine the type of immune responses induced by alum injection, the spleens of injected mice were removed, splenocytes were plated and re-stimulated *in vitro* with PMA and anti-CD3 or LPS and supernatants were analysed for cytokines.

No significant effects were observed on the secretion of IL-4, IL-5 or IL-17 by splenocytes isolated from mice injected with alum when re-stimulated with anti-CD3 and PMA or LPS (Data not shown). Splenocytes isolated from mice injected with alum and re-stimulated with anti-CD3 and PMA (2 or 20 ng/ml) did not secrete higher amounts of IL-6 in comparison to stimulated splenocytes isolated from mice injected with PBS (Figure 4.11). In contrast to this, splenocytes isolated from mice 5 days post-

injection with alum secreted significantly higher concentrations of IL-6 when re-stimulated with LPS (1 μ g/ml) compared to mice injected with PBS (Figure 4.11). This enhancement of IL-6 by alum was observed in both WT and IL-1R1^{-/-} mice. The kinetics of the enhancement was similar in both strains of mice, suggesting that this effect is independent of IL-1 (Figure 4.11).

In addition to IL-6, injection of alum primed splenocytes to secrete significant concentrations of IL-10 when stimulated with anti-CD3 alone or anti-CD3 and PMA (Figure 4.12). In comparison to mice injected with PBS, enhanced IL-10 secretion was measured by splenocytes isolated from WT mice 1 day and 3 days following injection of alum when stimulated with anti-CD3 and PMA (2 or 20 ng/ml) (Figure 4.12). Furthermore, cells taken from mice 1, 3 and 5 days post injection of alum spontaneously secreted IL-10 when cultured *ex vivo* (Figure 4.12). This spontaneous secretion of IL-10 was most marked by splenocytes isolated from mice 3 days after administration of alum (Figure 4.12).

In a similar manner to WT mice, splenocytes from IL-1R1^{-/-} mice injected with alum produced greater concentrations of IL-10 than those that received PBS (Figure 4.12). However, the kinetics of these effects was different to that observed in WT mice (Figure 4.12). Detectable IL-10 was measured as early as 3 h after injection of alum when cells were re-stimulated with anti-CD3 and PMA (2 or 20 ng/ml) (Figure 4.12). Furthermore, this priming effect on splenocyte IL-10 production was detected in spleen cells isolated from mice from 3 h up to 7 days following injection of alum (Figure 4.12).

The enhancing effect of alum injection on IL-10 production by splenocytes was sustained for a longer period of time in the IL-1R1^{-/-} mice than WT mice (Figure 4.12). This suggests that IL-1 may play a regulatory role in the induction of IL-10 in response to subcutaneous injection with alum.

Although alum is known as a strong inducer of Th2 type responses, injection of alum enhanced the production of IFN- γ by splenocytes stimulated with anti-CD3 or anti-CD3 and PMA (2 or 20 ng/ml) (Figure 4.13). Splenocytes isolated from WT mice 1 day following administration of alum secreted enhanced concentrations of IFN- γ in comparison to mice injected with PBS (Figure 4.13). However, this effect was not significant. In contrast, splenocytes isolated from IL-1R1^{-/-} mice as early as 3 h following injection of alum secreted significantly higher amounts of IFN- γ than controls

when re-stimulated with anti-CD3 and PMA (Figure 4.13). This enhancing effect on IFN- γ was maintained for up to 5 days after injection of alum (Figure 4.13). Consistent with observations on the production of IL-10 and IL-6, the differences in the kinetics and scale of IFN- γ secretion in response to injection with alum between the WT and IL-1R1^{-/-} mice suggests that IL-1 may suppress the early production of IFN- γ . In contrast to these effects, when cells were re-stimulated with anti-CD3 and PMA, no significant effects on IFN- γ secretion were observed when splenocytes were re-stimulated with LPS (Data not shown).

Data presented in chapter 3 (Figures 3.3-3.5) and chapter 4 (Figure 4.5) highlight the ability of PVA to enhance secretion of IL-1 β , both *in vitro* and locally *in vivo*. In light of these results, the ability of alum to promote the secretion of IL-1 β by splenocytes after injection of alum was determined. There was negligible secretion of IL-1 β by splenocytes isolated from WT mice injected with alum following stimulation with PMA and anti-CD3 (Figure 4.14). In contrast, splenocytes isolated from IL-1R1^{-/-} mice 7 days post injection of alum produced significantly higher concentrations of IL-1 β in comparison to cells isolated from mice injected with PBS (Figure 4.14). Furthermore, little difference was observed in IL-1 β secretion by the splenocytes when stimulated with anti-CD3 alone or PMA (2 or 20 ng/ml) with anti-CD3 suggesting that, the cells were strongly primed to produce IL-1 β . Interestingly, no significant enhancement of IL-1 β was observed when the spleen cells were stimulated with LPS (Figure 4.14).

Taken together, results from this study demonstrate previously unappreciated immunostimulatory effects of PVA *in vivo*. They show that, subcutaneous injection of alum is sufficient to prime splenocytes to produce IL-6, IL-10, IL-1 β and IFN- γ in a time-dependent manner. The kinetics of the responses demonstrates that subcutaneous injection of alum can have effects on cells in secondary lymphoid organs, such as the spleen, as early as 3 h post-injection suggesting that immunisation with particulates induces a very rapid response. Furthermore, these results also suggest that the production of all of these cytokines in response to alum-injection is in part regulated by IL-1, as the responses differed in the WT and IL-1R1^{-/-} mice.

4.2.3 Injection of PLG microparticles strongly enhances IL-6, IL-10 and IL-21 production

To determine if the immunomodulatory effects observed with alum were specific to this material or reflected a common responses to PVA, mice were injected with PLG (PLG) microparticles (4 mg) subcutaneously and sacrificed at 3 h, 1, 3, 5 and 7 days post injection.

Cells from the spleen, mediastinal lymph nodes, mesenteric lymph nodes and inguinal lymph nodes of injected mice were removed, single cell suspensions were isolated from each organ, plated and stimulated with PMA and anti-CD3 or LPS and supernatants were analysed for cytokines.

No significant modulatory effects of PLG injection were observed in lymph nodes (mediastinal lymph nodes, mesenteric lymph nodes or inguinal lymph nodes) on the secretion of IL-4, IL-5, IL-17, IL-6, IL-10 or IFN- γ following cell stimulation with anti-CD3 and PMA (Data not shown).

Additionally, no significant modulatory effects were observed on the secretion of IL-4, IL-5, IL-17, or IFN- γ by splenocytes following cell re-stimulation. However, in comparison to PBS, injection with PLG resulted in the priming of splenocytes to secrete enhanced concentrations of IL-10 and IL-6 (Figure 4.15). Splenocytes isolated from mice 1 or 3 day after injection of PLG secreted significantly higher concentrations of IL-10 than controls when re-stimulated with LPS (100 ng/ml and 1 μ g/ml) (Figure 4.15). The priming effect of PLG on IL-10 in spleen cells was most marked 1 day after injection (Figure 4.15).

In a similar manner to IL-10, splenocytes isolated from mice injected with PLG were also primed to secrete IL-6 when stimulated with LPS (100 ng/ml and 1 μ g/ml) (Figure 4.15). In comparison to PBS, splenocytes isolated from mice 3h, 1 or 7 days following injection of PLG secreted significantly higher concentrations of IL-6 when stimulated with LPS (100 ng/ml and 1 μ g/ml) (Figure 4.15). The priming effect of PLG on IL-6 secretion by spleen cells was most marked 1 day after injection of PLG (Figure 4.15).

IL-21 is the key cytokine produced by a subset of T helper cells known as T follicular helper cells (Tfh), whose primary role is to provide help for the production of antibodies by B cells [550]. The secretion of IL-21 by re-stimulated spleens was analysed,

however IL-21 was not detected in any of the treatment groups (Data not shown). Therefore, the expression of IL-21 mRNA was analysed by real time polymerase chain reaction (RT-PCR) (This analysis was carried out by a Health Research Board PhD programme rotation student in the lab, Ewa Oleszycka). Significant levels of IL-21 mRNA were detected in spleens from mice injected with PLG using RT-PCR (Figure 4.16). This increase in the levels of IL-21 mRNA was first evident in splenocytes isolated from mice 3 h following injection of PLG (Figure 4.16). In addition, this enhancement of IL-21 mRNA was also observed in splenocytes isolated from mice 1 day after injection of PLG in comparison to mice injected with PBS (Figure 4.16). The enhancement of IL-21 mRNA is rapid and transient effect as it occurred within 3 h, was most marked after 1 day and IL-21 mRNA levels return to basal levels within 3 days after the subcutaneous injection of PLG (Figure 4.16).

In summary, as in the case of alum, subcutaneous injection of PLG enhanced the production of IL-6 and IL-10. Furthermore, injection of PLG significantly enhanced the transcription of the Tfh-associated cytokine IL-21 in the spleen, within 1 day following injection.

4.2.4 The effect of particle size on the adjuvant properties of particulates following subcutaneous immunisation

Having investigated the relationship between the composition of particulates and their adjuvant properties and demonstrated the immunomodulatory effects of PVA when injected alone, subsequent studies aimed to investigate the relationship between particle size and adjuvanticity.

In order to study the effects of particle size on the type of immune responses induced, mice were immunised subcutaneously with PBS, Ova alone or Ova with polystyrene (PS) particles of 200 nm, 430 nm, 1 μ m, 10 μ m or 30 μ m in diameter. In the study mice were immunised subcutaneously so that the local effects of particulates could be analysed by histopathology. The injection site was excised 21 days post immunisation and embedded in paraffin wax for histopathological examination.

4.2.4.1 Histopathological analysis of the injection site

Previous findings in this chapter (Figure 4.2-4.4) have shown the dramatic effects of injecting PVA on the local injection site which, included granuloma formation, increased cellular infiltration and local necrosis. Based on these results, this study aimed to investigate the importance of particle size in driving these responses. Injection of mice subcutaneously with either PBS or Ova alone resulted in no significant local changes; the injection sites from these mice were overall unremarkable (Figure 4.17). In contrast, injection of PS particles of 200 nm in size with Ova resulted in the development of a large well-demarcated non-encapsulated inflammatory focus (Figure 4.17). Inflammatory cells, which included lymphocytes, neutrophils and macrophages, were noted in the periphery of the mass and also individual cells were found among the particles in the core of the mass (Figure 4.17). Similarly, the injection of PS particles of 430 nm in size in conjunction with Ova induced the formation of multiple large well demarcated inflammatory foci (Figure 4.17). However, the areas of inflammation were well defined with more structure and were encapsulated around the outer area of the particles by a large infiltration of inflammatory cells (Figure 4.17). A higher number of particles remained at the injection site in comparison to the amount of particles left in the 200 nm injection site, which suggests that the 200 nm particles were more efficiently cleared but that this was associated with a less controlled responses within the injection site (Figure 4.17).

The local injection site in mice injected with PS particles of 1 μm in size with Ova was reminiscent of that observed in the mice injected with 430 nm PS particles (Figure 4.17). The injection of 1 μm polystyrene particles induced the formation of a large inflammatory focus in the local area, which was well-demarcated and well defined due to its encapsulation by inflammatory cells surrounding the outer edges of the mass (Figure 4.17). However, the amount of cellular infiltration appeared to be less than that observed in mice injected with 430 nm or 200 nm PS particles (Figure 4.17). Furthermore, the remaining injected particles appeared brown in colour, a phenomenon that was not observed following injection of any of the other particles (Figure 4.17).

The injection of PS particles of 10 μm in size with Ova resulted in the development of a small discrete lesion with low cellular infiltration but in contrast to other particles the cellular infiltrate was mainly composed of neutrophils (Figure 4.17). The injection site

contains mainly the remaining particles, which are clearly visible as small almost translucent spheres (Figure 4.17). In a similar manner to previous injection sites, the lesion is well demarcated but is, however, not encapsulated by a large number of infiltrating cells (Figure 4.17). Similar to 10 μm PS particles, injection of particles 30 μm in size with Ova resulted in the development of discrete lesions with low, almost non-existent cellular infiltration (Figure 4.17). The lesion is mainly characterised by the presence of the remaining particles, which are visible as medium sized translucent spheres (Figure 4.17).

Overall, this overview of the injection sites post immunisation with various sized PS particles demonstrates that smaller particles (200 nm, 430 nm, 1 μm), induce prominent local inflammatory reactions characterised by high cellular infiltration and in all cases the formation of granulomas. In contrast, the larger particles (10 μm , 30 μm) did not induce strong local inflammatory responses. The injection was analysed both 21 days (prime injection) and 7 days (boost injection) following immunisation. The results presented here represent the injection 21 days following injection of mice, however, no significant differences were observed within the between the prime and boost injection sites (Data not shown). Therefore, suggesting, that the injection is quite a stable environment with a constant maintenance of cellular infiltration.

On closer inspection of the injection sites the type of inflammatory cells recruited into the lesions were identified. As previously outlined, injection with PS particles of 200 nm in size and Ova induced a strong mixed cellular influx into the local area (Figure 4.18). The types of cells recruited, included lymphocytes, neutrophils and macrophages (Figure 4.18). In addition, these macrophages were quite swollen (foamy) as their cytoplasm was filled with phagocytosed particles (Figure 4.18). Subcutaneous injection of PS particles 430 nm in size with Ova also induced a strong local inflammatory response (Figure 4.19). The 430 nm particles promoted the recruitment of macrophages and neutrophils into the injection site, in as similar manner to the 200 nm particles (Figure 4.19). Furthermore, “foamy” macrophages swollen with phagocytosed microparticles can also be identified within the granuloma (Figure 4.19).

In a similar manner to the 430 nm particle injection site, injection of 1 μm PS particles with Ova induced a strong local inflammatory response and granuloma formation (Figure 4.20). Neutrophils and macrophages were the main types of inflammatory cells recruited (Figure 4.20). Injection of PS particles of 10 μm in size and Ova induced a local inflammatory reaction; however the level of this response was much lower than those observed in the injection sites of smaller polystyrene microparticles (Figure 4.21). Although the inflammatory response induced was on a smaller scale, it was primarily neutrophilic in nature and no significant numbers of macrophages were identified (Figure 4.21). In particular, no foamy macrophages were observed (Fig 4.21). This strongly suggests that, the increase in particle size from 1 μm to 10 μm dramatically changed the nature of the local inflammatory response. Injection of 30 μm PS particle with Ova did induce formation of a lesion however; no specific inflammatory cells were present within the injection site, which was mainly characterised by the remaining particles (Figure 4.22).

Overall, the results from the histological assessment demonstrate that smaller particles (200nm, 430nm, 1 μm) recruited a mix of macrophages, lymphocytes and neutrophils into the local area while particles of 10 μm in size induced a more specific response mainly recruiting neutrophils, a specificity that is gone when the particle size is increased up to 30 μm .

4.2.4.2 The effect of particle size on adaptive cell-mediated responses induced by PS particles

Following on from investigations into the local inflammatory responses to particles, the effects of particle size on antigen-specific adaptive immunity was analysed. For these experiments, mesenteric lymph nodes, inguinal lymph nodes, mediastinal lymph nodes and spleens were removed from the immunised mice. Cells were isolated and stimulated with Ova at 500 $\mu\text{g/ml}$. Supernatants were collected after 3 days and analysed for cytokines.

No significant antigen-specific responses were detected in the inguinal lymph nodes (Data not shown) but immunomodulatory effects were observed in both the spleen and the mesenteric lymph nodes (Figure 4.24).

The injection of PS particles with Ova had no significant immunomodulatory effects on the secretion of antigen-specific IL-17, IL-10 or IFN- γ by splenocytes (Figure 4.23). In contrast, the injection of PS particles of various sizes with Ova enhanced the levels of IL-4, IL-5 and IL-6 secreted by stimulated splenocytes; these effects were not observed in all mice and were dependent on particle size (Figure 4.23). No enhancing effects on antigen-specific cytokines were observed in mice immunised with Ova and 200nm PS particles (Figure 4.23). In contrast, injection of Ova with 430nm PS particles significantly enhanced the secretion of Ova-specific IL-5 and IL-6 by splenocytes in comparison to controls (Figure 4.23). Enhanced levels of Ova-specific IL-10 were also observed, although these effects were not statistically significant (Figure 4.23). In comparison to controls, a significant increase in the levels of Ova-specific IL-5 secreted by re-stimulated splenocytes was observed when mice were immunised with 1 μ m PS particles and Ova (Figure 4.23). In addition, injection of 1 μ m PS particles primed splenocytes to spontaneously secrete significant amounts of IL-5 and to a lesser extent, IL-6 (Figure 4.23). Splenocytes isolated from mice immunised with Ova and 10 μ m PS particles secreted higher amounts of antigen-specific IL-4 and IL-5 than controls (Figure 4.23). These cells also secreted enhanced levels of IL-5 in an antigen-independent manner (Figure 4.23). Co-administration of 30 μ m PS particles with Ova enhanced Ova-specific IL-6 and IFN- γ , however these effects were not statistically significant (Figure 4.23).

In addition to the spleen, the subcutaneous injection of PS particles with Ova had significant effects on cytokine production by cells isolated from the mesenteric lymph nodes (Figure 4.24). Injection of PS particles of 200 nm, 430 nm or 1 μ m in size with Ova had no significant effects on cytokine production by mesenteric lymph node cells (Figure 4.24). Additionally, no significant effects of PS particles were observed on the secretion of IL-10, IL-17, IL-5 or IFN- γ by antigen-stimulated mesenteric lymph nodes cells (Figure 4.24). In contrast, injection of 10 μ m or 30 μ m PS particles with Ova significantly enhanced the secretion of IL-4 by mesenteric lymph node cells (Figure 4.24). This increased secretion of IL-4 occurred without antigen re-stimulation and there was no further enhancement observed when the cells were re-stimulated with Ova (Figure 4.24). In addition, injection of mice with 30 μ m PS particles also resulted in enhanced secretion of IL-6 by mesenteric lymph node cells (Figure 4.24).

Taken together, these results demonstrate that there is a strong relationship between the size of the particulate and the type of cellular immune response induced. Subcutaneous injection with 30 μm PS particles specifically enhanced the production of IL-4 and IL-6, both Th2 associated cytokines, in the mesenteric lymph nodes. PS particles of 10 μm in size promoted the production of IL-4 by spleen and mesenteric lymph node cells, as well as IL-5 in the spleen. Immunisation with 1 μm PS particles and Ova primed splenocytes to secrete IL-5 and IL-6 in an antigen-independent manner, while no effects on cytokines in the mesenteric lymph nodes were observed. Overall, this suggests that immunisation with particles above 1 μm in size will preferentially promote Th2 type responses. In this study, no significant effects were observed with PS particles of 200 nm in size on Th1 associated cytokines such as IFN- γ or IL-17. Thus, it was of interest to investigate the effect of even smaller particles on antigen-specific cellular immunity, in order to determine if these could bias the response in an opposite direction to the larger particles by promoting the production of IFN- γ and/or IL-17.

4.2.4.3 The effect of particle size on the ability of PS particles to promote antigen-specific humoral immunity

Having established that the injection of different sized particles can have significantly different effects on cell-mediated immune responses, the relationship between particle size and the induction of humoral responses was investigated. Blood samples were collected 21 days post immunisation and the levels of serum antigen-specific IgG, IgG1, IgG2b and IgG2c were determined by ELISA.

Injection of all PS particles with Ova, regardless of their size, increased levels of Ova-specific IgG in the serum in comparison to injection of Ova alone (Figure 4.25). The IgG antibody isotypes, IgG2b, IgG2c and IgG1, were also measured. In a similar manner to IgG, all particles enhanced the production of antigen-specific IgG1, IgG2b and IgG2c when compared to Ova alone (Figure 4.25). Remarkably, there were no significant differences in the titres of antigen-specific antibody isotypes between the different sized particles (Figure 4.25). However, injection of 200 nm PS particles with Ova resulted in the highest titres of antigen-specific IgG2c in comparison to the other PS particles (Figure 4.25). In contrast, there were no differences in the ability of different sized PS to promote the production of antigen-specific IgG1 and IgG (Figure

4.25). In the case of IgG2b, however, injection of 430 nm PS did not enhance antigen-specific titres in comparison to Ova alone (Figure 4.25). Overall, these results suggest that particle size is not a decisive factor in the ability of PS to enhance humoral responses following subcutaneous administration.

4.2.5 The effects of particle size on the adjuvant properties of PS particles following intraperitoneal immunisation

Following on from the results of the initial particle size study, it was predicted that the induction of more robust cell-mediated responses would require very small particles. In agreement with this, studies have shown that nanoparticles of 40 nm in size have the ability to promote the development of CD8⁺ T cell responses [547, 549]. Mice were immunised intraperitoneally with PBS, Ova alone or Ova with PS particles of various sizes (50 nm, 200 nm, 430 nm, 1 µm, 10 µm or 30 µm). The peritoneal cells were isolated post-immunisation in order to investigate the local cytokine response. The spleen, mesenteric lymph nodes, mediastinal lymph nodes and inguinal lymph nodes was also removed so that systemic effects on cytokine production could be analysed. Cells were isolated and re-stimulated with medium or Ova (500 µg/ml). Supernatants were collected after 3 days and analysed for cytokines.

4.2.5.1 Effect of particle size on adaptive cell-mediated responses induced by PS particles

No significant effects on cytokine secretion by re-stimulated mesenteric, inguinal or mediastinal lymph node cells were observed (Data not shown). Additionally, injection of PS particles with Ova did not have any immunomodulatory effects on the secretion of IL-4, IL-5 or IL-6 by peritoneal cells. In contrast, significant effects of particle injection were observed on the local secretion of antigen-specific IFN-γ and IL-17 (Figure 4.26). Peritoneal cells isolated from mice injected with Ova and 50 nm PS particles secreted significantly higher concentrations of Ova-specific IL-17 than cells isolated from mice injected with Ova alone (Figure 4.26). In addition, peritoneal cells from mice injected with Ova and 200 nm PS particles also secreted increased amounts of Ova-specific IL-17 (Figure 4.26). However, this effect was not statistically

significant. Interestingly, injection of the 50 nm particles did not induce the secretion of significant concentrations of any other cytokine (IL-4, IL-5, IL-10, IFN- γ , IL-6), either spontaneously or antigen-stimulated (Figure 4.26).

Peritoneal cells isolated from mice injected with Ova and 1 μ m PS particles produced significant amounts of IL-10 spontaneously compared to mice injected with PBS or Ova alone (Figure 4.26). However, no antigen-specific IL-10 was detected by peritoneal cells isolated from any of the immunised mice (Figure 4.26).

Injection of 50 nm, 10 μ m or 30 μ m PS particles with Ova led to an increase in secretion of antigen-specific IFN- γ in comparison to cells taken from mice injected with Ova alone (Figure 4.26). However, the increase was only significant with the 10 μ m particles (Figure 4.26).

In addition to local cytokine secretion, the effect of particle size on cytokine secretion by spleen cells was examined. In agreement with the data obtained following subcutaneous injection, intraperitoneal injection of 10 μ m PS particles with Ova enhanced antigen-specific IL-4 production by splenocytes (Figure 4.27). This effect on antigen-specific IL-4 was not observed with the other particles (Figure 4.27). In addition to IL-4, intraperitoneal injection of 10 μ m PS particles with Ova also significantly enhanced the production of IL-10 by splenocytes in both an antigen-dependent and antigen-independent manner (Figure 4.27).

In contrast to earlier findings, no significant effects were observed on the secretion of IL-5 by splenocytes isolated from mice injected with PS particles (all sizes) and Ova (Figure 4.27). While the effects were not statistically significant, injection of PS particles also enhanced the production of antigen-specific IL-6, IFN- γ and IL-17 in the spleen (Figure 4.27). In particular, splenocytes isolated from mice injected with 50 nm PS particles secreted higher concentrations of Ova-specific IL-17 than cells taken from mice injected with Ova alone (Figure 4.27). This is consistent with the enhanced secretion of local IL-17 observed by Ova-stimulated peritoneal cells from mice injected with 50nm PS particles and Ova (Figure 4.26). Injection of 50 nm, 200 nm or 10 μ m PS particles with Ova enhanced the secretion of antigen-specific IFN- γ (Figure 4.27).

In the case of IL-6, injection of Ova with PS particles of 50 nm, 10 μ m and 30 μ m in size resulted in enhanced Ova-specific IL-6 in comparison to cells taken from mice injected with Ova alone (Figure 4.27). However, these effects were not significant (Figure 4.27).

Overall, this study shows that IP injection of smaller PS particles (≤ 200 nm) is an efficient means of driving the Th1 and Th17 associated cytokines, IFN- γ and IL-17. In contrast, larger PS particles ($\geq 10\mu$ m) are more effective at promoting the Th2 associated cytokines, IL-10 and IL-4 (Figures 4.26-4.27).

4.2.5.2 Particle size is not a key determinant of the efficiency of PS particles to enhance humoral immune responses following intraperitoneal immunisation

Having established that different sized particles can have significantly different effects on cell-mediated immune responses after intraperitoneal injection, the relationship between particle size and the induction of humoral responses was investigated.

In a similar manner to the subcutaneous studies, intra-peritoneal injection of PS particles with Ova, regardless of their size, enhanced the production of antigen-specific antibodies (Figure 4.28). As well as enhancing total IgG titres, all particles enhanced the production of antigen-specific IgG1, IgG2b and IgG2c when compared to Ova alone (Figure 4.28). Remarkably, there were no significant differences in the titres of antigen-specific antibody isotypes between the different sized particles (Figure 4.28). Furthermore, no particular sized particle showed any bias towards the production of any specific antibody isotype (Figure 4.28).

These findings are similar to those observed when mice were immunised subcutaneously with various sized PS particles (Figure 4.25) and taken together indicate that in the range of 50nm to 30 μ m, PS particle size is not the key factor in determining the magnitude or nature of antigen specific antibody responses induced.

4.3 Discussion

The demonstration that PVA can activate the NLRP3 inflammasome was an important discovery, but it is not required for the induction of humoral immunity. Therefore, the mediators of adjuvanticity promoted by particulates remain elusive. Here, a number of *in vivo* experiments were carried out to investigate the innate and adaptive responses driven by PVA. Firstly, a study was designed to investigate the relationship between the composition of the PVA and the type of immune response induced.

All three particulates, alum, PLG (PLG) microparticles and polystyrene (PS) microparticles enhanced local cellular infiltration and induced the formation of a granuloma at the injection site. These local reactions to PVA have previously been reported for alum [551-552]. Furthermore, necrotic cells were observed within the injection site following administration of all three particulates, suggesting that PVA are toxic to cells *in vivo*.

In addition to the local inflammatory response, injection with PVA promoted the local production of IL-1 β , no effects on IL-1 α , IL-10, IFN- γ , IL-4, IL-5 or IL-6 locally were observed. This enhancement of IL-1 β is in agreement with other studies that have shown that intraperitoneal injection with alum enhances the local secretion of IL-1 β as rapidly as 4 h post-immunisation [119]. What is most interesting is that this enhancement of IL-1 β occurs in the absence of an exogenously administered second stimulus. This is in contrast to *in vitro* studies, where a second signal in the form of a TLR agonist is required to promote particle-mediated enhancement of IL-1 β . Histological analysis demonstrated that subcutaneous administration of PVA induces necrosis. Therefore, it is possible that these dying cells are releasing endogenous danger signals, which provide the priming signal necessary to enhance the transcription of pro-IL-1 β . Several endogenous danger signals with the ability to enhance immune responses have been identified, including uric acid and HMGB1 [11-12]. Data presented in chapter 3, demonstrated that uric acid was not required for the enhancement of IL-1 β secretion by PVA *in vitro* (Figure 3.23). In contrast, exogenous HMGB1 can replace TLR agonists to provide signal 1 for IL-1 β secretion by DC treated with PVA (Figure 5.9). It is therefore possible that, HMGB1 released by necrotic cells within the injection site, could act as the priming signal for PVA to activate the inflammasome, thus promoting the local secretion of IL-1 β . It would be of interest to

investigate local HMGB1 release by immunohistochemistry and to carry out further studies with blocking antibodies to HMGB1 to fully investigate the role of HMGB1 in promoting local inflammation in response to PVA.

Following on from the analysis of local cytokine production, the effect of PVA administered with KLH on cytokine production by re-stimulated spleen and inguinal lymph node cells was analysed. Overall, the particulates (alum, PLG, PS) were poor inducers of antigen-specific cell-mediated responses. However, when re-stimulated with polyclonal stimuli (anti-CD3 or anti-CD3 and PMA), cells isolated from mice injected with KLH and PVA secreted higher levels of Th2-associated cytokines particularly IL-4 and IL-5, in comparison to cells isolated from mice injected with KLH alone. Despite the poor antigen-specific cell-mediated responses induced by the particulates, these adjuvants strongly enhanced the production of antigen-specific antibodies. Overall, this suggested that the three PVA analysed (alum, PLG, PS) promote similar types of immune responses when injected *in vivo*.

Since the study comparing alum, PLG and PS clearly demonstrated that these adjuvants can modulate spleen cell responses to anti-CD3 or anti-CD3 and PMA, a more detailed kinetic analysis was carried out to investigate alum- and PLG-mediated modulation of spleen and lymph node cytokine production. These studies have identified several cytokines, including IL-6, whose secretion was enhanced by injection of PVA.

Injection with alum alone resulted in the priming of splenocytes to produce significant amounts of IL-6. This enhancement in IL-6 production was also observed in spleen cells from mice injected with PLG or alum. The kinetics of this priming for IL-6 production differed between the adjuvants; in the case of alum, the enhancement of IL-6 production was found in spleen cells isolated from mice 5 d following injection, whereas, IL-6 was detected as early as 3 h post-injection with PLG. Overall, these studies suggest that the enhancement of IL-6 production is a common feature of particulates. In agreement with this, others have shown that injection of alum induces the production of local IL-6 as rapidly as 4 h after injection [119]. The differences in kinetics between this and current findings with alum may be explained by, the site of the IL-6 production and the route of injection, as the study by McKee *et al* demonstrated local production of IL-6 in the peritoneum following intraperitoneal injection. Furthermore, in their study, IL-6 was detected in the peritoneal lavage and

the cells did not require any subsequent stimulation. Therefore, it is also possible that the source of the IL-6 secreted in the spleen and the peritoneum may also differ.

IL-6 is a pro-inflammatory cytokine with pleiotropic effects on the immune system. It has effects on the innate response, such as promoting the production of acute phase proteins within the liver and stimulating the generation of neutrophils in the bone marrow. In addition, IL-6 is required for the development of Th17 cells. IL-6-deficient mice lack Th17 cells in the lamina propria, which are normally constitutively present [277]. Of most interest, however, is the fact that IL-6 has the ability to promote the proliferation of activated antibody-producing B cells. It is also one of the key cytokines required for the development of Tfh cells, CD4⁺ T cells which are the main providers of B cell help, through the induction of BCL6 [330]. The key role played by IL-6 in B cell activities is highlighted by the fact that, IL-6-deficient mice have a reduced number of germinal centre B cells, reduced numbers of Tfh cells and display defects in their ability to promote germinal centre formation [330, 332, 553]. Based on these findings, it is possible to predict that this enhanced IL-6 is important for the promotion of humoral immune responses by particulates.

Contrary to this theory, it seems that these B cell-activating roles of IL-6 are redundant in the case of alum as IL-6-deficient mice show no defect in their level of IgG or IgG1 production when immunised with alum-adjuvanted Ova [454]. However, IL-6-deficient mice produced significantly higher levels of IgG2a and IFN- γ in response to immunisation with Ova and alum in comparison to their WT controls [454]. While the levels of IgG2a in the IL-6^{-/-} mice indicate a greater Th1 response, the cytokine data showed that along with increased IFN- γ levels, the absence of IL-6 also promoted the production of IL-4, IL-5 and IL-10, suggesting that IL-6 regulates the development of Th1 and Th2 cell-mediated responses [454]. Taken together results from Brewer *et al* suggest that, while the absence of IL-6 allowed for the enhancement of IFN- γ and IgG2a it also promoted the production of Th2 associated cytokines, which ultimately allowed the Th2 response to dominate [454].

It will be important to identify the source of the IL-6, as this may provide information about the type of cells that are activated either directly or indirectly *in vivo* by PVA. Given the fact that this enhancement of IL-6 was only observed when splenocytes were stimulated with a TLR agonist, LPS, one would predict that a cell from the myeloid

lineage is the primary producer of the IL-6. In agreement with this hypothesis, a recent study has demonstrated that injection of alum induced a rapid induction of local IL-6 production [119]. This enhancement of IL-6 was dependent on the presence of mast cells and macrophages, as depletion of either ablated the local production of IL-6 [119]. However, depletion of both cell types had no effect on the ability of alum to enhance antigen-specific antibody production, suggesting that IL-6 is not required for alum to promote antibody production [119].

In addition to the enhanced secretion of IL-6, splenocytes isolated from mice injected subcutaneously with alum produced significantly greater amounts of IL-10 than mice injected with PBS when stimulated with anti-CD3 or anti-CD3 and PMA. This enhancement of IL-10 occurred rapidly, with significant levels detected within 3 h post-injection of alum and was maintained for up to 7 days. Moreover, injection with PLG alone also primed splenocytes to produce IL-10 when stimulated *ex vivo*. However, the stimulus used to promote the secretion of IL-10 by the PLG-primed splenocytes was different to that used in the alum kinetics study. In the case of alum, splenocytes treated with anti-CD3 and PMA displayed enhanced IL-10 secretion, whereas PLG-primed cells secreted significant amounts of IL-10 when stimulated with LPS. In addition to the stimuli used to promote the secretion of the IL-10, there was also a difference in the kinetics of its release. Despite these differences, both studies show that the ability to prime cells to produce IL-10 is a common feature observed with a range of PVA and this could provide some insight into their mechanism of action. In contrast to our findings, a recent study investigating the effect of alum-injection on the local production of chemokines and cytokines, did not find any effects on the levels of local IL-10 [119]. However, McKee *et al* looked at local cytokine production 4 h post alum administration, whereas the effect of PVA on IL-10 secretion was observed 24 h following subcutaneous injection of alum. Furthermore, in the study by McKee *et al* the local production was analysed whereas, these current studies investigated more systemic effects, by analysing secondary lymphoid organs for changes in cytokine production. Therefore, these differences in kinetics and anatomical locations of the response may explain the discrepancies between the studies.

IL-10 is a type II cytokine, which was first identified as a product of Th2 cells. It was initially shown to be an inhibitor of Th1 cell development [554]. The biological effects of IL-10 range from, activation of NK and B cells, to regulation of APC activity through IL-10-induced downregulation of MHC class II and co-stimulatory molecules [555-556]. IL-10 plays an important role in regulating immune responses, maintaining steady state homeostasis and reducing immunopathology induced by overactive responses. For example, IL-10-deficient mice develop spontaneous colitis, succumb to sublethal doses of LPS and parasitic infections, including *T.gondii* and *T.cruzi* [557-560]. In contrast, the absence of IL-10 promotes increased resistance to pathogens, such as *Mycobacterium avium* and lymphocytic choriomeningitis (LCMV), which require strong Th1 responses for clearance [561-562]. The different effects on protective immunity observed in IL-10-deficient mice, suggests that IL-10 can have either protective or detrimental effects depending on the type of response that is required.

Of most relevance to these current studies, however, is the role played by IL-10 in the priming and determination of T helper effector responses. Since its discovery, it has been demonstrated that IL-10 has inhibitory effects on the development of Th1 type immune responses [554]. Moreover, it has been shown that the co-administration of an IL-10R blocking antibody with the protein antigen Ova promoted the development of Th1 type responses, as characterised by high levels of antigen-specific IFN- γ [563]. However, this study also demonstrated that this enhancement of Ova-specific IFN- γ in the absence of IL-10 only occurred when the Ova was contaminated with LPS, suggesting the need for a pathogen derived signal [563]. Based on these studies and the inability of PVA to promote the development of Th1 responses, one can propose that the rapid IL-10 production induced by particulates may dampen down or inhibit Th1 responses. In addition to the enhancement of IL-10 observed post-injection of particles, it was also found that PVA have the ability to prime for IFN- γ secretion at early time points, specifically in IL-1R1^{-/-} mice. This suggests that the inability of PVA to promote cell-mediated responses is not due to the absence of Th1 promoting factors. The development of Th2 type cell-mediated responses may be due to the presence of high levels of IL-10, which dominates the priming stage of the response and favours the Th2 phenotype.

In agreement with this, studies in our lab have shown that IL-10R^{-/-} mice immunised with alum-adjuvanted Ova produced significantly higher levels of antigen-specific IFN- γ when stimulated *ex vivo* in comparison to WT controls (Oleszycka E *et al* unpublished findings). This data adds to the hypothesis that the induction of IL-10 by alum is central to the Th2 bias observed *in vivo* with this adjuvant. This switch in the cell-mediated responses observed in alum-immunised IL-10R^{-/-} mice towards a more Th1 bias was not reflected in the humoral responses, as no significant differences in antibody titres were observed between the IL-10R^{-/-} and WT mice. Ongoing studies are investigating the effects of blocking this initial burst of IL-10 using a neutralising antibody to the IL-10R.

It is important to identify the source of this early IL-10 production. Although IL-10 was originally classified as Th2-associated cytokine, the spectrum of immune cells identified with the ability produce IL-10 has expanded greatly [554]. In addition to Th2 cells, IL-10 has also been shown to be produced by other CD4⁺ T cells including Th1, Th17, natural Tregs, antigen-specific Tregs and the newly identified IL-9 producing T cells [564-567]. As well as CD4⁺ T cells, CD8⁺ T cells, B cells and neutrophils have also been shown to produce IL-10 [556, 568-569]. The main sources of IL-10 are the innate APC, including DC and macrophages. Adding further complexity is the fact that splenocytes isolated from mice injected with alum or PLG produced IL-10 in response to different stimuli. In the case of alum, significant amounts of IL-10 were produced when cells were stimulated with classical T cell activating stimuli, including anti-CD3 and PMA. However, this does not rule out the possibility that the activated T cells are stimulating other cell types to produce the IL-10, thus the production of IL-10 could be due to either a direct or indirect effect of the stimuli used. Splenocytes were also stimulated with LPS. However, no significant effects on IL-10 production by splenocytes isolated from alum-injected mice were observed suggesting that the source of the IL-10 is not an APC. In contrast to alum-injected mice, splenocytes isolated from mice injected with PLG only secreted IL-10 when treated with LPS. Overall, the source of the IL-10 cannot currently be predicted based on the stimuli used, due to the wide range of both innate and adaptive cells that produce IL-10 and the range of stimuli that promote its secretion.

Given the ability of PVA to activate the inflammasome and promote the secretion of IL-1 β *in vitro* and at the site of injection, the levels of IL-1 β produced by splenocytes isolated from alum-injected mice were analysed. Interestingly, splenocytes isolated from mice injected with alum produced significant levels of IL-1 β when stimulated with anti-CD3 alone or anti-CD3 and PMA. This effect on IL-1 β was only observed 7 days following alum administration in the IL-1R1^{-/-} mice and no significant effects were observed in the WT mice. This difference may be due to rapid uptake of IL-1 β by its receptor in the WT cells, therefore preventing detection of IL-1 β by standard assays. In addition, it is also possible that there is a negative feedback promoted by IL-1 β , thereby limiting its own production. This would also explain the absence of IL-1 β secretion from the stimulated WT splenocytes isolated from alum-injected mice.

Recent studies have demonstrated that IL-1 has the ability to promote the expansion of antigen activated CD4⁺ T cells, suggesting a role for IL-1 β in the promotion of T cell responses [570]. The authors transferred antigen-specific (pigeon cytochrome C;PCC) lymph node cells into lymphocyte-deficient mice, the recipients were then immunised with PCC alone or in combination with LPS or IL-1. They found that, the mice that received the PCC in combination with IL-1 had a greater number of antigen-specific T cells following immunisation than the mice that had received PCC alone or PCC in combination with LPS [570]. In addition, the presence of IL-1 boosted T cell-mediated effects as shown by enhanced production of Ova-specific IgG1 in WT mice immunised with Ova and rIL-1 in comparison to mice immunised with Ova alone. Furthermore, the addition of IL-1 β during boost immunisations significantly enhanced the expansion of memory antigen-specific T cells [570]. Therefore, this study shows that IL-1 has the potential to enhance primary and secondary T cell-mediated responses by promoting the expansion of antigen-specific T cells, a previously unappreciated role for IL-1 β . Given this recently identified effect of IL-1 β , it is possible that the physiological consequence of the enhanced IL-1 production observed in the spleen 7 days post alum-injection may be important for the expansion and maintenance of T cell responses mediated by PVA. Furthermore, mice deficient in NLRP3, which is necessary for IL-1 β secretion, show defective cell-mediated responses, such as a reduction in antigen-specific IL-5 and IL-6 when immunised with alum or PLG and Ova, respectively, compared to WT mice, further adding to data suggesting that IL-1 β positively activates cellular immunity [105] (Figure 3.28).

One of the most interesting findings from the PVA kinetics studies was the enhancement of IL-21 mRNA detected in spleen cells taken from mice 24 h post injection with PLG. This enhancement of IL-21 mRNA occurred in an antigen-independent manner and was detected in unstimulated spleen cells. The expression of IL-21 mRNA was not measured in mice injected with alum alone therefore it is not possible to say whether this is a common feature of particles. IL-21 is a type I cytokine, which signals via the IL-21R and common γc receptor chain, shared by IL-2, IL-4, IL-7, IL-9 and IL-15. Ligation of the IL-21R and γc receptor complex triggers activation of the Jak/STAT3 signalling pathway [571-573]. The main functions of IL-21 include, promoting B cell proliferation, isotype switching and differentiation of B cells into long lived plasma cells through its enhancement of BLIMP-1 [339, 372, 574]. In addition, IL-21 has also been shown to activate NK cells, NKT cells and promote the proliferation of CD8⁺ T cells and the development of Th17 cells [572, 575-577]. While the IL-21R is expressed on a wide variety of cells, it is predominantly found on B cells [578].

The important role of IL-21 in the activation of B cells is highlighted by the fact that overexpression of IL-21 *in vivo* results in an increase in the number of immature B cells, isotype switched memory B cells and plasma cells. In addition, these mice also produce enhanced levels of serum IgG and IgM [372]. Furthermore, IL-21-deficient mice show a decrease in isotype switching in activated B cells, specifically reduced IgG1 which is the main isotype enhanced by particulates. Mice deficient in IL-21 also display impaired development of germinal centres, which are central to processes such as somatic hypermutation and affinity maturation [372]. Given the role played by IL-21 in B cell homeostasis, proliferation and activation it is possible that, the physiological consequence of this PLG-enhanced IL-21 mRNA may be central to the ability of the particles to promote humoral immunity. Previous studies into the mechanism of action of PVA have shed light on cellular responses induced by particulates, such as the requirement of NLRP3 for cell recruitment and the enhancement of chemokine and cytokine production by particulates *in vivo* [105, 107-108, 118-119]. However, the mechanism of their ability to enhance humoral responses remains controversial [105, 107-108, 118-119]. The ability of PLG to enhance the expression of IL-21 mRNA in spleen cells has previously been unappreciated and may represent a key observation to help elucidate the basis of robust humoral responses induced by PVA.

IL-21 was discovered in the supernatant of activated CD3⁺ cells and originally shown to activate NK cells [572]. More recently however, it has been shown that IL-21 exerts an autocrine role in the development of Th17 cells. It is also produced by NKT cells, both CD4⁻ and CD4⁺ and follicular T helper cells (Tfh) [575, 577-578]. The main source of IL-21 is the Tfh cell, which was originally identified in human blood and tonsillar tissue [327-328]. The original studies into Tfh cells demonstrated that, co-culture of B cells and Tfh cells induced activation and subsequent production of antibodies by the B cells [327-328]. The essential role played by Tfh cells in the development of humoral responses, makes them an attractive candidate for the source of enhanced IL-21 mRNA following PLG-injection. However, the rapid induction of the PLG-mediated IL-21 suggests that the source of the IL-21 may be an innate cell. It is also possible that an innate cell may represent the early source of IL-21 prior to Tfh cell activation. It will be of interest to investigate whether or not this is enhancement of IL-21 is a common effect induced by all particulates and also to look at antigen-specific production of IL-21 by repeating the study using an antigen-adjuvant combination.

Overall, these kinetics studies have led to the identification of several important factors that are promoted by injection of PVA alone. Injection of particles is sufficient to promote the enhancement of IL-6, IL-10 and IL-21. All of which have been shown to play key roles in B cell activity, thereby promoting the development of humoral immunity. Furthermore, the rapid induction of IL-6 and IL-10 by particulates may be responsible for the Th2 bias that is observed with particulate adjuvants. In addition, PVA also promote the production of IL-1 β following injection, which may be important for particle-induced cell-mediated responses. Taken together, the results from these studies strongly support the idea that, PVA are immunomodulatory and have identified several important cytokines which may explain the basis for their mechanism of action.

It has previously been suggested that, the size of particulate adjuvants may play a significant role in determining their ability to induce immune responses. The size of particles used as adjuvants are the same as many of the pathogens that are encountered by our immune system, such as viruses, which are normally less than 0.5 μ m and bacteria, which are generally greater than 0.5 μ m [547, 579]. Several studies have investigated the relationship between the size of the particles and the type of immune response induced [547-549, 579-580]. While these studies have given insight into the relationship between particle size and immunogenicity, none of them have provided

detailed studies of the effects on innate, and adaptive, humoral and cell-mediated responses. Microparticles can be manufactured from several different substances, with the most popular being biodegradable poly-lactide-co-glycolide microparticles, due to their biocompatibility and their high safety profile for human use [579]. However, PS particles were chosen for this current study due to the fact that their size can be sharply defined. Furthermore, the use of inert nano-beads (polystyrene) as adjuvants has been demonstrated by several studies [547, 549]. This study aimed to investigate the effect of particle size on the induction of immune responses by analysing the local effects, cytokine production both locally and systemically, as well as the production of antigen-specific antibodies. Furthermore, as others have shown that different routes of administration potentially elicit different types of immune reactions based on differential uptake by resident APC [547, 580], two routes of administration were tested.

The initial study examined the effect of particle size on the local inflammatory response by immunising subcutaneously, which allowed for histopathological analysis of the tissue surrounding the injection site. Overall, smaller particles (200-1000 nm) induced a strong local inflammatory response characterised by, increased cellular infiltration, granuloma formation and areas of local necrosis. In contrast, the larger particles (10 μm and 30 μm) resulted in a lower level of cellular infiltration into the area and no necrosis. Despite this low level of local inflammation, it is worth noting that the majority of the cells which were recruited in response to injection of 10 μm PS particles and Ova, were neutrophils. This specific recruitment observed in response to the 10 μm PS particles was in contrast to the smaller PS particles which attracted neutrophils and macrophages, the latter being identified by their “foamy” nature due to phagocytosed particles. In general the smaller particles, ranging from 200 nm- 1 μm in size, caused more local inflammation than the larger particles. This could be due in part to, the ability of different sized particles to be phagocytosed and subsequently activate local APC, which could produce cell recruiting chemokines. Furthermore, the necrosis and damage induced by the smaller particles may also contribute to the local secretion of cell recruiting chemokines, through the release of endogenous danger signals from dying cells.

The ability of larger particles (polystyrene: 4 μm) to promote neutrophil recruitment, specifically, after intratracheal immunisation has been previously reported (Samuelsen, Nygaard et al. 2009). Current findings have demonstrated that PS particles of 10 μm in size specifically recruit neutrophils. Therefore, it would be of interest to carry out further studies with particles ranging from 1-10 μm in size, in order to identify exactly at what size this switch to selective neutrophil recruitment occurs. In addition, the effect of particle size on the local production of cell recruiting chemokines and cytokines should be investigated.

Following on from the analysis of the injection site reaction, the systemic effects of particle size were also analysed to determine if there was any relationship between the degree of local inflammation and the induction of cellular immune responses. Based on the large amount of inflammation induced locally by the smaller particles (200 nm and 430 nm) it was predicted that these particles may promote the development of a Th1 type response. However, no enhancement in the production of IFN- γ , a key Th1 associated cytokine, was observed in either the spleen or the mesenteric lymph nodes following subcutaneous injection with smaller PS particles (200 nm and 430 nm) and Ova. In fact the smaller PS particles (200 nm and 430 nm), were inefficient at driving cell-mediated immune responses in general. In contrast, when the larger PS particles (1 μm , 10 μm and 30 μm) were injected subcutaneously, they specifically promoted the production of the Th2 associated cytokines, IL-4 and IL-5, in secondary lymphoid tissues. Despite the low level of local inflammation observed with the larger particles, which cannot be phagocytosed efficiently, injection of these with Ova promoted the production of IL-4 and IL-5 in the spleen and mesenteric lymph nodes. However, the mechanism by which these larger particles promote cell-mediated responses remains unknown.

As previously discussed, IL-1 plays an important role in the enhancement of effector T cell responses [570]. Furthermore, others have demonstrated that even particles that cannot be phagocytosed by APC can still activate the inflammasome, subsequently enhancing the secretion of IL-1 [104]. The authors propose that, this activation of the inflammasome by larger particles occurs by enhanced levels of intracellular reactive oxygen species triggered by the attempted phagocytosis of the particles by the APC. A process known as frustrated phagocytosis [104]. Therefore, it is possible that the attempted phagocytosis of these larger PS particles (10 μm and 30 μm) could have

activated the inflammasome and subsequent secretion of IL-1 by local APC. This potential enhancement of IL-1 via frustrated phagocytosis by local APC could subsequently play a role in the enhancement of effector T cell responses as previously reported [570]. Further studies involving the immunisation of IL-1R1^{-/-} mice may help to identify whether or not IL-1 enhancement is required for the larger particles to promote cell-mediated responses.

It was previously demonstrated that smaller particles (40-50 nm: polystyrene nanobeads) strongly promoted the development of CD8⁺ IFN- γ producing T cells [547, 549]. However, they showed that the optimal particle size range for the induction of these IFN- γ secreting T cells was, between 40-50 nm [547, 549]. This is roughly five times smaller than the particles tested in the subcutaneous vaccine study, suggesting that in order to promote stronger cell-mediated responses requires the use of particles less than 200 nm in size.

In order to test this theory, particles 50 nm in size were also tested for their ability to promote local and systemic cell-mediated responses and humoral immune responses. These particles were compared to the particles tested in the original study (200 nm-30 μ m). In contrast to the original subcutaneous study the particles were injected intraperitoneally in combination with Ova, so that the local production of cytokines could be analysed. In agreement with the idea that smaller particles would promote stronger Th1 and Th17 cell-mediated responses, intra-peritoneal injection of 50 nm PS particles specifically enhanced the production of the pro-inflammatory T cell cytokines IL-17 and IFN- γ . The enhancement of IL-17 occurred both locally and in the spleen. Furthermore, this enhancement of IL-17 was specific to the smaller particles, as the 200 nm particles also enhanced IL-17, while no effect was observed in any of the mice immunised with particles larger than 200 nm. Interestingly, the 10 μ m PS particles also promoted the production of IFN- γ locally and in the spleen, although this was not statistically significant. However, in the spleen the 10 μ m PS particles significantly enhanced the secretion of antigen-specific IL-4 and IL-10 suggesting a Th2 type response.

Overall, the findings from this study suggest that in order to promote a strong cell-mediated response that, ultrafine nanoparticles of 50 nm in size or larger particles 10 μm in size should be used. Furthermore, the smaller particles will promote a more Th1 type response, while the larger particles bias the response towards a Th2 phenotype. The enhancement of cell-mediated responses with ultrafine PS particles was previously reported [547, 549]. However, the ability of the larger particles to promote strong cell-mediated responses has previously been unappreciated.

Having established the cytokine profiles induced, both locally and systemically, in response to various sized PS particles, it was subsequently necessary to analyse their ability to promote humoral immune responses. The findings from the this current study show that there were no significant differences observed between the various sized particles (50 nm- 30 μm) in their ability to enhance the production of antigen-specific IgG. Regardless of the size of the particle, a significant increase in Ova-specific IgG was detected in the serum of particle and Ova immunised mice in comparison to Ova alone. Therefore, there is no direct relationship between the size of the particle and its ability to promote humoral immune responses. In contrast to this, others have reported differences in the level of antibody production by various sized particles [548, 580].

One such study entrapped tetanus toxoid (TT) within PLA microparticles of various sizes (<2 μm , 2-8 μm , 10-70 μm , 50-150 μm) and compared the anti-TT antibody responses induced following a single intramuscular injection [548]. In this study, Katare *et al* demonstrated that, the optimal size range for enhanced anti-TT antibody production is between 2-8 μm , while PLA particles less than 2 μm and greater than 10 μm elicited lower anti-TT antibody titres [548]. These results suggest that the size of the particle can affect the ability of the particulate to enhance humoral responses. One explanation for the differences between the study by Katare *et al* and current findings is that they used PLA microparticles which cannot be formulated so that each particle is the exact same size. As a result, particle preparations contain a range of particle sizes [548]. In contrast, PS particles were used in this current study, which can be well defined on the basis of size. An additional explanation for the differences observed between current results and what has been reported by Katare *et al* is the route of immunisation, intraperitoneal or subcutaneous versus intramuscular, as it has previously been suggested that different routes of immunisation elicit different types of responses [581].

Despite the fact that particle size does not affect the enhancement of antigen-specific IgG, the cytokine profiles suggested that different sized particles were inducing different types of responses. Therefore, this may be reflected in the subclasses of antibodies. It has also been shown that different size particles display differential uptake by APC and it was proposed that this may direct the induction of adaptive immune responses [547, 549]. Based on these observations, the serum levels of several IgG isotypes were analysed. These current studies showed no significant differences were found in the induction of IgG1, IgG2a or IgG2c induced by the various sized particles suggesting that, the size of the particle does not determine the type of humoral immune response that is induced.

Despite the fact that the particles size does not determine the strength or type of humoral response that is induced, it is surprising that the larger particles (10 μm and 30 μm) were able to enhance the production of antigen-specific antibodies at a level comparable to the smaller particles, despite their inability to be phagocytosed. However, other studies have demonstrated the ability of larger microparticles to elicit strong antibody responses. One such study reported that a single vaccination, with a mixture of small (<10 μm) and larger (10-100 μm) PLA particles encapsulated with Hepatitis B surface antigen, elicited stronger more robust antigen-specific antibody responses than three doses of an antigen-alum combination [582]. It was proposed that these enhanced responses using mixed particle sizes were the result of biphasic responses, the initial rapid enhancement of responses which was promoted by the smaller particles and a slower boost of responses much later due to the slow release of the antigen by the larger particles [582]. Since this current system did not rely on adsorbed, entrapped or conjugated antigen this concept of slow antigen release may not explain the ability of larger particles to enhance antibody responses.

Although the larger particles are not phagocytosed, previous findings from chapter 3 have demonstrated that APC still attempt to phagocytose these particles and attach themselves to the surface of the particles (Figure 3.19). It is therefore possible that these APC could potentially still be activated by the larger particles while they attempting to engulf the particle via frustrated phagocytosis [104]. Therefore, the frustrated phagocytosis of these larger particles may activate APC sufficiently to promote the enhancement of humoral immune responses. However, further studies

would be necessary in order to fully elucidate the mechanism of action which is induced by larger particles in order to promote humoral immune responses.

In general, smaller particles (50-200nm) promote both strong cell-mediated and humoral immune responses along with local inflammation. In contrast, particles of 430 nm-1 μ m in size are poor stimulators of cellular immune responses but do however promote humoral responses and induce local inflammation. At the other end of the spectrum, larger particles (10-30 μ m) do not induce strong local inflammatory immune reactions but they are efficient at promoting the production of antibodies as well as Th2 type cell-mediated immune responses. Overall, this study suggests that, while the size of the particles does not determine the strength or type of humoral immune responses it does affect local inflammation and determines the type of antigen-specific T cell response generated. These are potentially key factors that should be taken into account in the future development of particulate vaccine adjuvants and vaccine formulation.

(a)



(b)



Figure 4.1: Injection of KLH alone causes no visible lesions at the site of injection.

(a) Injection site 7 days post-immunisation (hemotoxylin and eosin;H&E,x100). **(b)** Injection site 7 days post-immunisation (H&E,x200). C57BL/6 mice were immunised with KLH (5 μ g/mouse) subcutaneously in the right flank and sacrificed 7 days post-injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histological analysis (2.2.11).

(a)



(b)

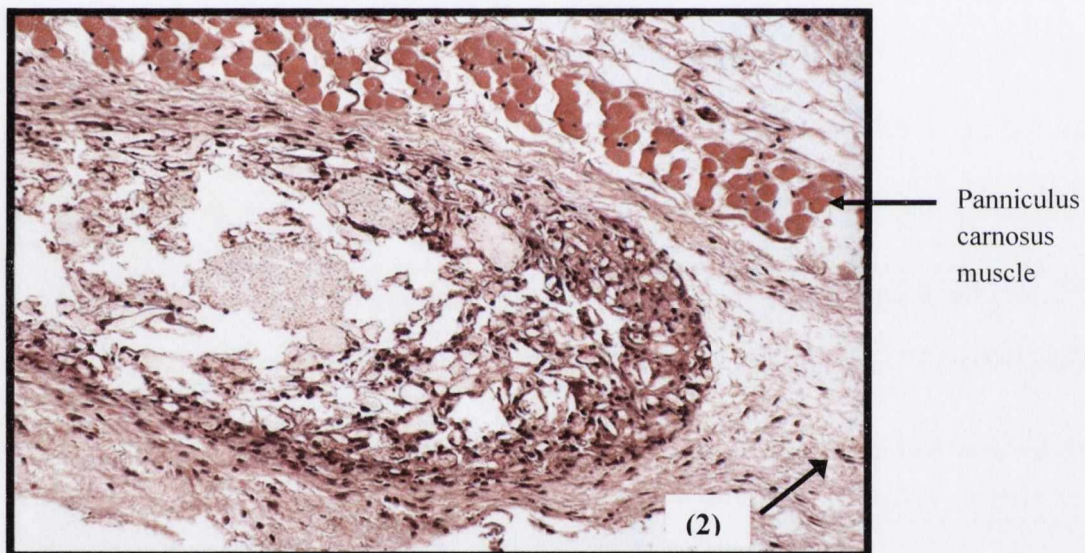


Figure 4.2: Immunisation with PLG microparticles (1 μm) and KLH induces the formation of a lipid granuloma at the injection site (1) and causes necrosis (2). (a) Injection site 7 days post-immunisation (hemotoxylin and eosin;H&E,x100). (b) Injection site 7 days post-immunisation (H&E,x200). C57BL/6 mice were immunised with KLH (5 $\mu\text{g}/\text{mouse}$) and PLG (1 μm :2.5mg) subcutaneously in the right flank and sacrificed 7 days post-injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histological analysis (2.2.11).

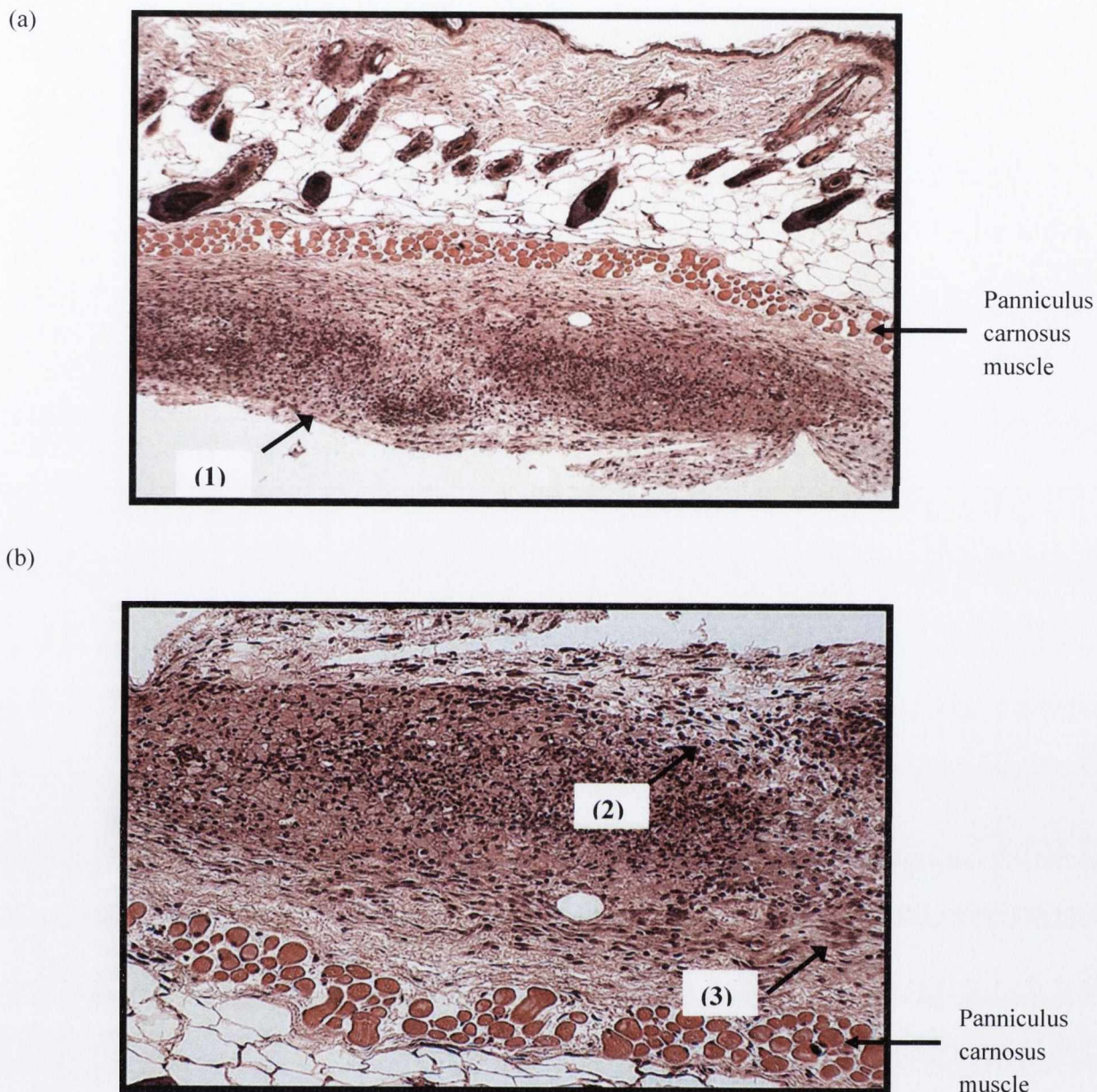
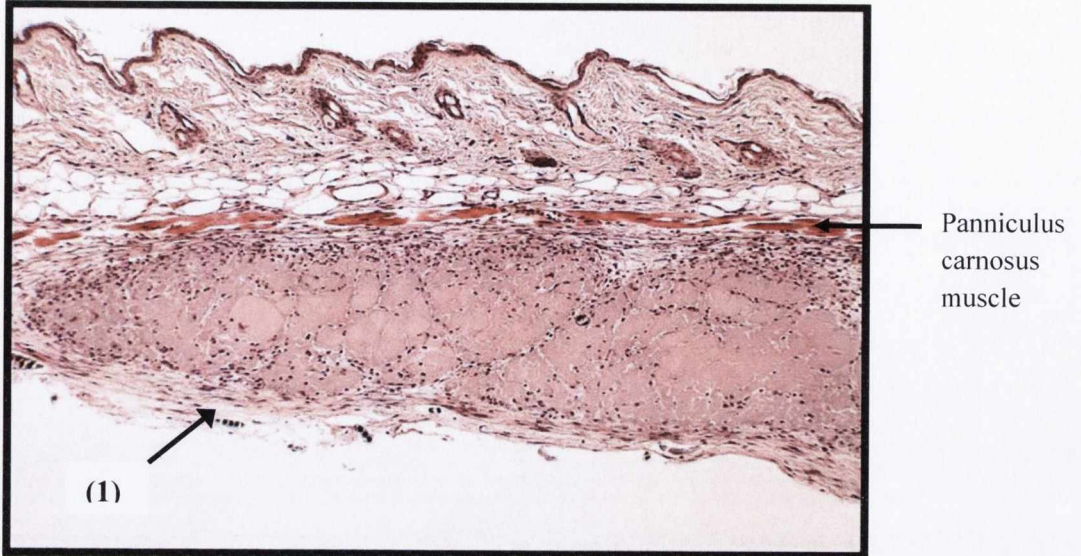


Figure 4.3: Immunisation with PS and KLH induces the formation of a pyogranuloma (1), characterised by neutrophils (2) at the injection site and causes necrosis (3). (a) Injection site 7 days post-immunisation (hemotoxylin and eosin; H&E,x100). (b) Injection site 7 days post-immunisation (H&E,x200). C57BL/6 mice were immunised with KLH (5 µg/mouse) and PS (430nm:2.5 mg) subcutaneously in the right flank and sacrificed 7 days post-injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histological analysis (2.2.11).

(a)



(b)

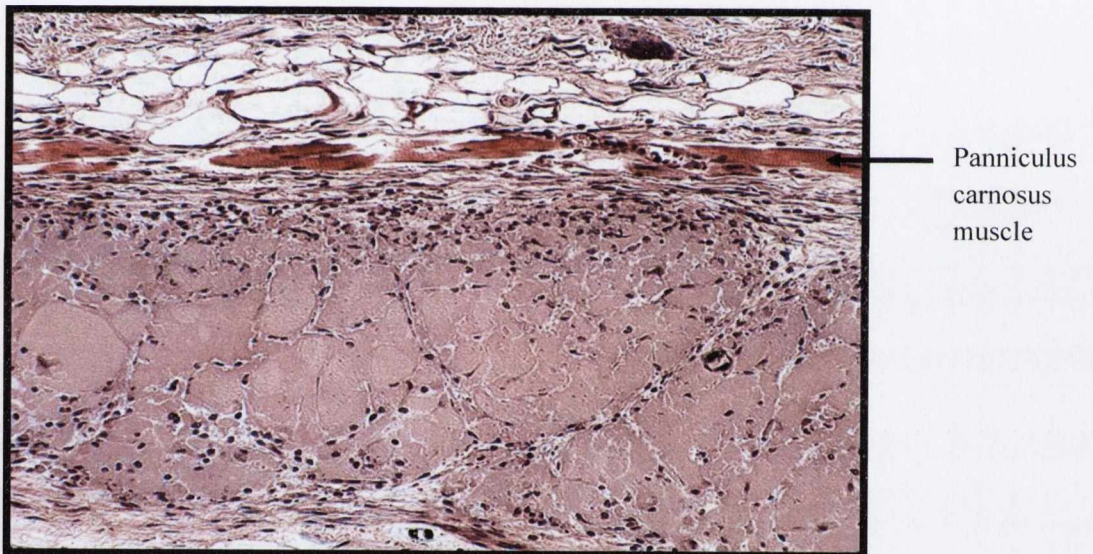


Figure 4.4: Immunisation with Alum and KLH causes necrosis and induces granuloma formation (1) at the injection site. (a) Injection site 7 days post immunisation. H&E,x100. (b) Injection site 7 days post-immunisation. H&E,x200. C57BL/6 mice were immunised with KLH (5 μ g/mouse) and alum (2.5 mg) subcutaneously in the right flank and sacrificed 7 days post-injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histological analysis (2.2.11).

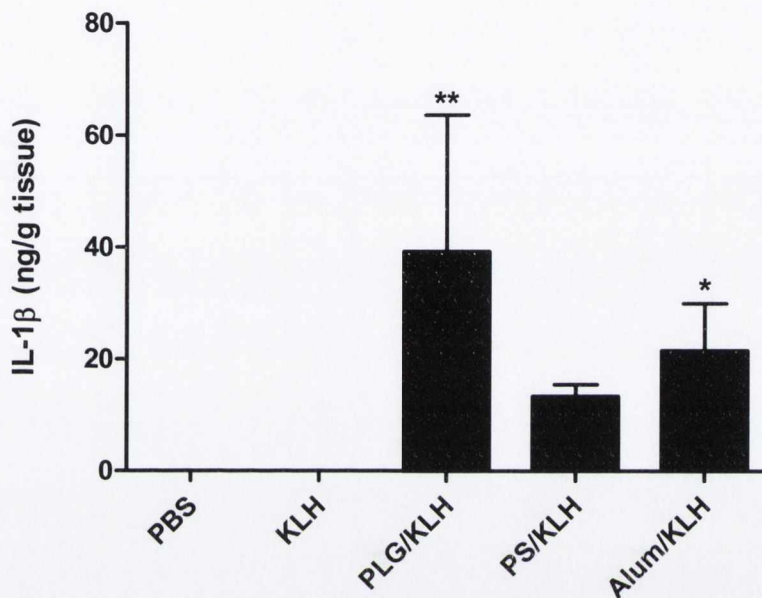


Figure 4.5: Injection of PVA induces local IL-1 β production. C57BL/6 mice were immunised with PBS, KLH (5 μ g/mouse) alone or KLH in combination with alum (2.5 mg), PLG (1 μ m:2.5 mg) or PS (430 nm:2.5 mg) subcutaneously in the right flank. After 7 days, the mice were sacrificed and the injection site was removed. The tissue was homogenised and sonicated. Following centrifugation, supernatants were removed and analysed for IL-1 β by ELISA (2.2.5). Results are expressed as nanogram of cytokine per gram of tissue. KLH alone v KLH+Particle, * p <0.05, ** p <0.01. p <0.0

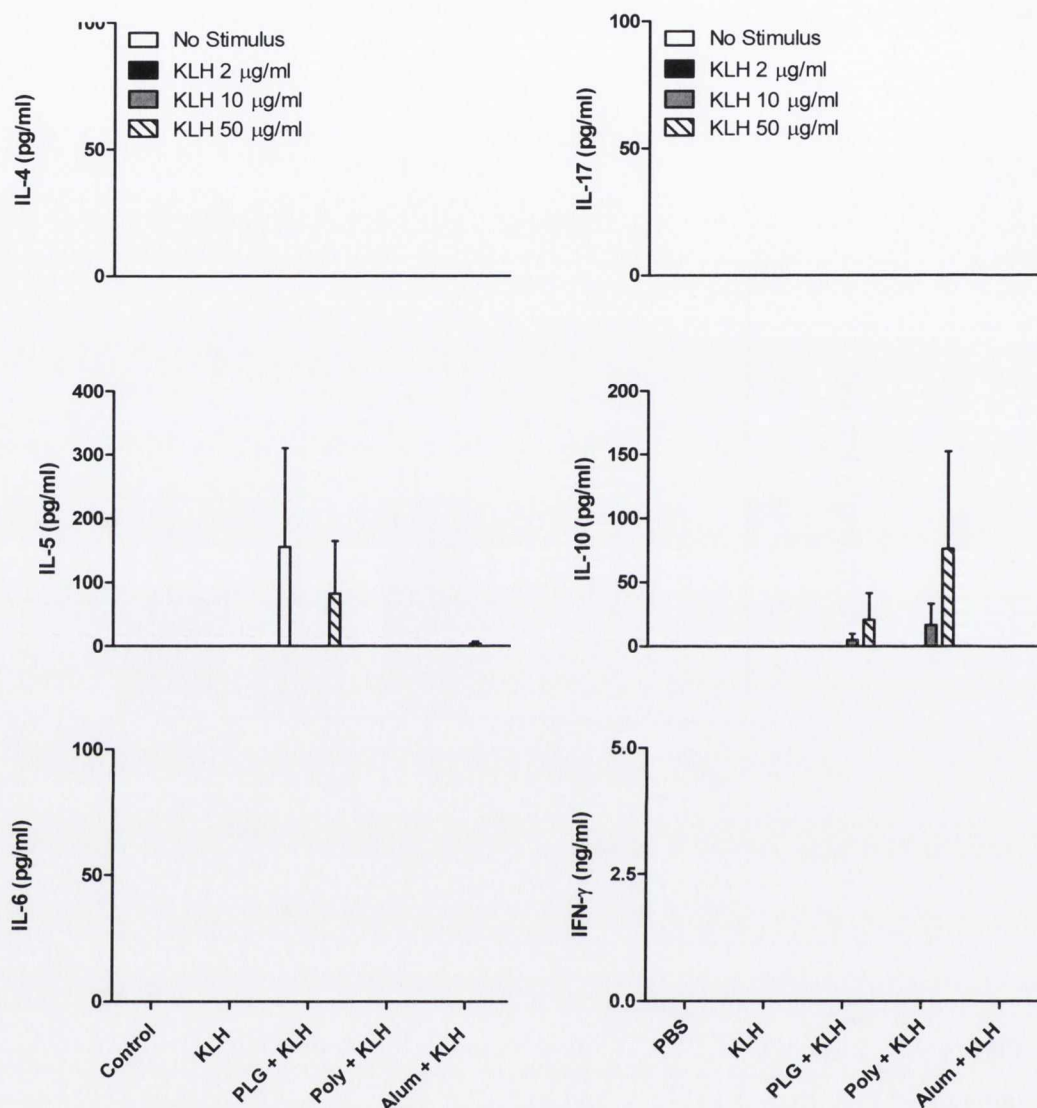


Figure 4.6: Immunisation with KLH and PVA does not induce strong antigen specific cell mediated responses in the inguinal lymph nodes. C57BL/6 mice were immunised with PBS, KLH (5 μ g/mouse) alone or KLH in combination with alum (2.5mg/200 μ l PBS), PLG (1 μ m:2.5mg) or PS (430nm:2.5mg) subcutaneously in the right flank. After 7 days, the mice were sacrificed and the inguinal nodes were removed. Cells isolated from the inguinal nodes were stimulated with medium or KLH (2, 10, 50 μ g/ml). After 3 days, supernatants were removed and analysed for IL-4, IL-5, IL-6, IL-17, IL-10 and IFN- γ by ELISA (2.2.5).

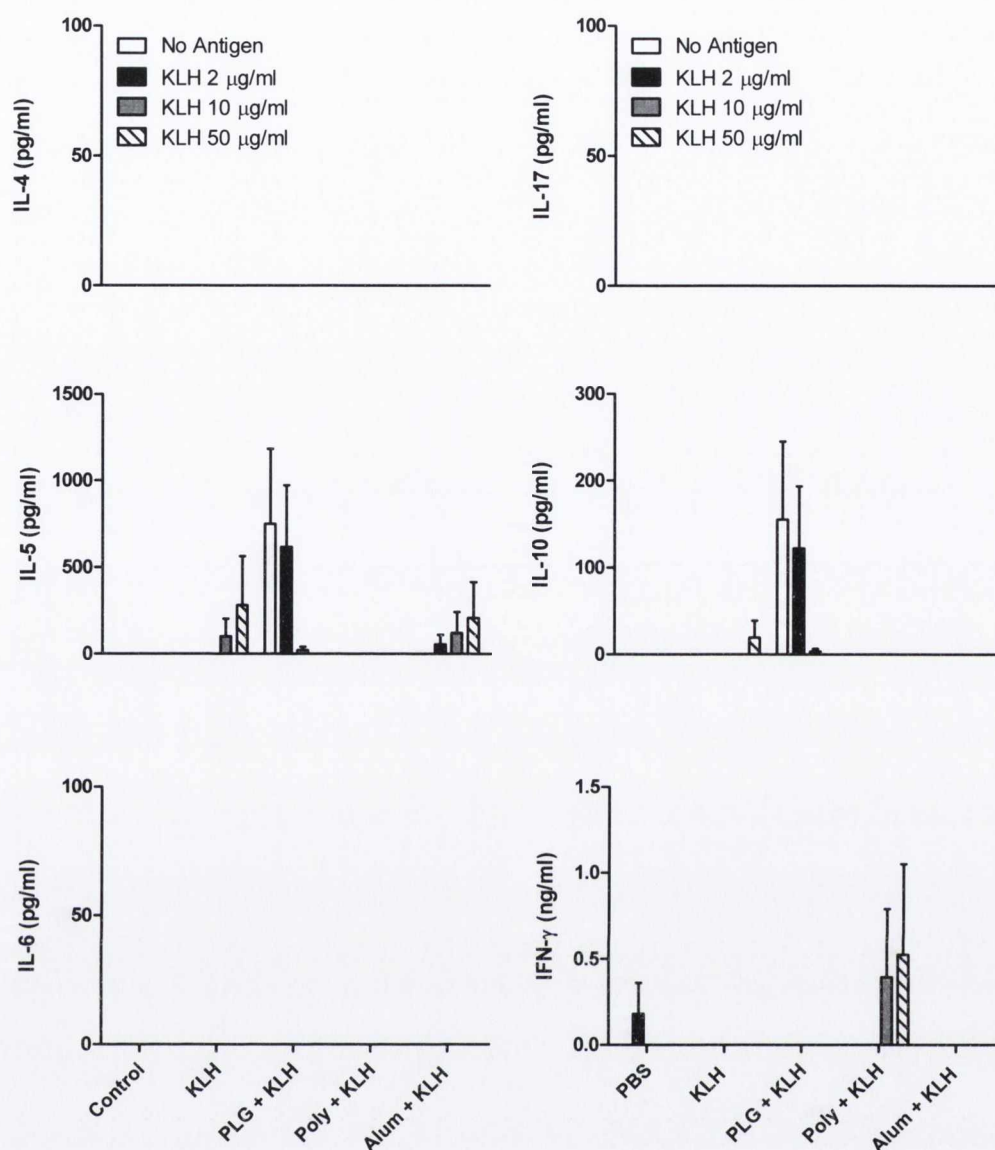


Figure 4.7: Immunisation with KLH and PVA induces spontaneous and antigen-specific IL-5 and IL-10 secretion by splenocytes. C57BL/6 mice were immunised with PBS, KLH (5 μ g/mouse) alone or KLH in combination with alum (2.5mg), PLG (1 μ m:2.5mg) or PS (430nm:2.5mg) subcutaneously in the right flank. After 7 days, the mice were sacrificed and the spleens were removed. Cells isolated from the spleens were stimulated with medium or KLH (2, 10, 50 μ g/ml). After 3 days, supernatants were removed and analysed for IL-4, IL-5, IL-6, IL-17, IL-10 and IFN- γ by ELISA (2.2.5).

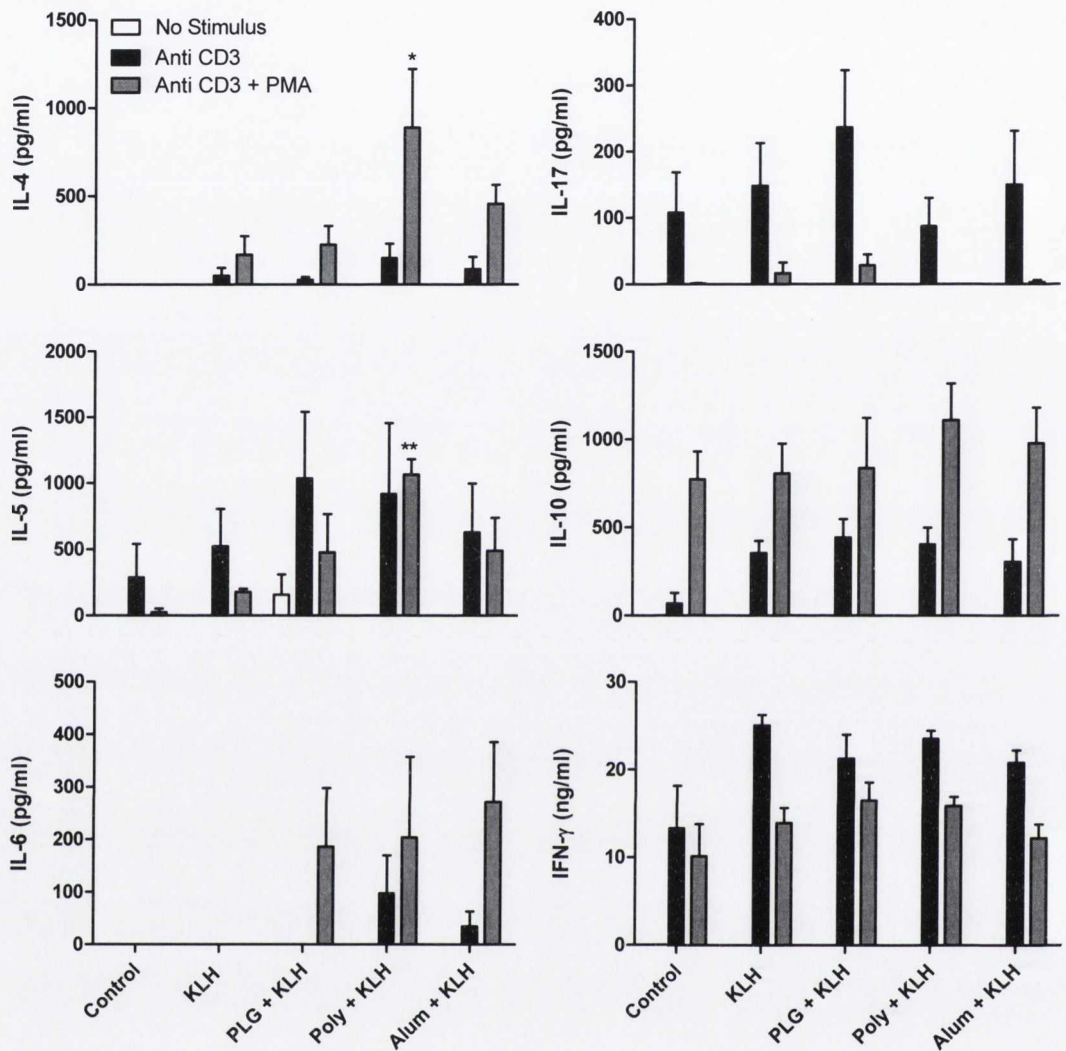


Figure 4.8: Injection of PVA enhances the secretion of IL-4 and IL-5 by inguinal lymph node cells. C57BL/6 mice were immunised with PBS, KLH (5µg/mouse) alone or KLH in combination with alum (2.5mg), PLG (1µm:2.5mg) or PS (430nm:2.5mg) subcutaneously in the right flank. After 7 days, the mice were sacrificed and the inguinal nodes were removed. Cells isolated from the inguinal nodes were stimulated with medium, anti-CD3 (1 µg/ml) or anti-CD3 (1 µg/ml) with PMA (250 ng/ml). After 3 days, supernatants were removed and analysed for IL-4, IL-5, IL-6, IL-17, IL-10 and IFN-γ by ELISA (2.2.5). KLH alone v KLH + adjuvant, * p<0.05, ** p<0.01.

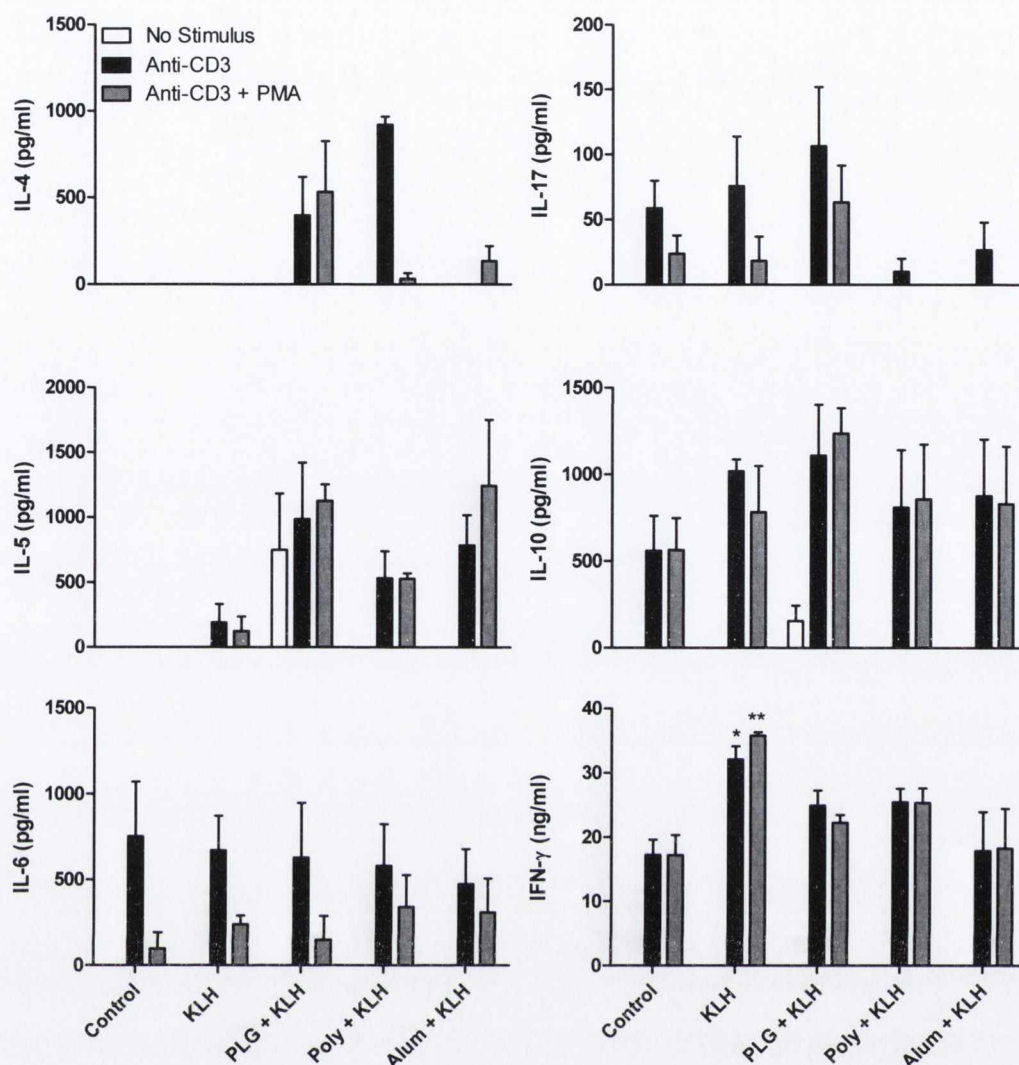


Figure 4.9: Immunisation with KLH alone enhances IFN- γ secretion by splenocytes. C57BL/6 mice were immunised with PBS, KLH (5 μ g/mouse) alone or KLH in combination with alum (2.5mg), PLG (1 μ m:2.5mg) or PS (430nm:2.5mg) subcutaneously in the right flank. After 7 days the mice were sacrificed and the spleens were removed. Cells isolated from the spleens were stimulated with anti-CD3 (1 μ g/ml) or anti-CD3 (1 μ g/ml) with PMA (250 ng/ml). After 3 days, supernatants were removed and analysed for IL-4, IL-5, IL-6, IL-17, IL-10 and IFN- γ by ELISA (2.2.5). KLH alone v KLH + adjuvant, * $p < 0.05$, ** $p < 0.01$.

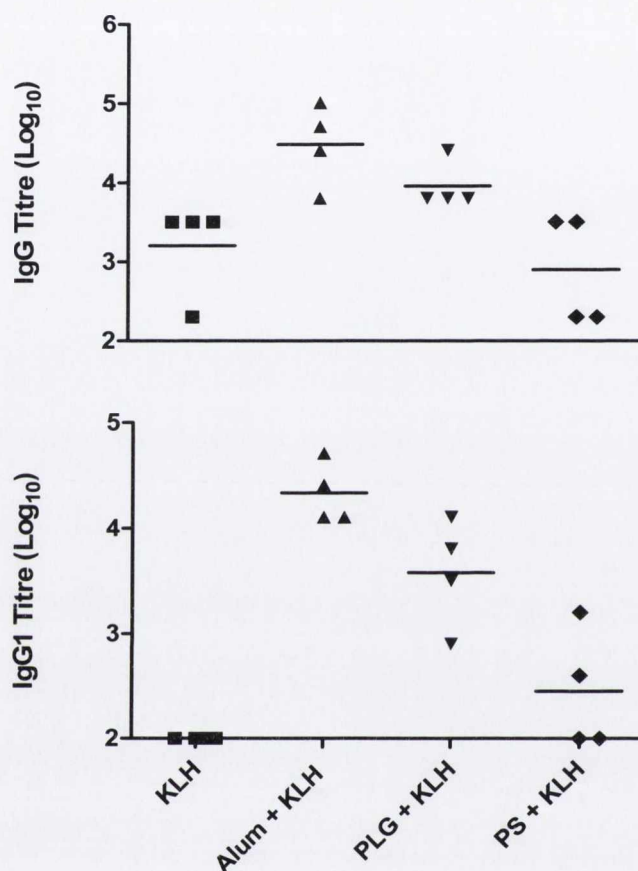


Figure 4.10: PVA enhance antigen-specific IgG1 responses to co-administered KLH. C57BL/6 mice immunised with PBS, KLH (5µg/mouse) alone or KLH in combination with alum (2.5mg), PLG (1 µm:2.5mg) or PS (430nm:2.5mg) subcutaneously in the right flank. After 7 days, the mice were sacrificed and blood was collected. The serum samples were analysed for total IgG and IgG1 titres by ELISA (2.2.12). Antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut off point of 2 standard deviations above sera for PBS injected mice.

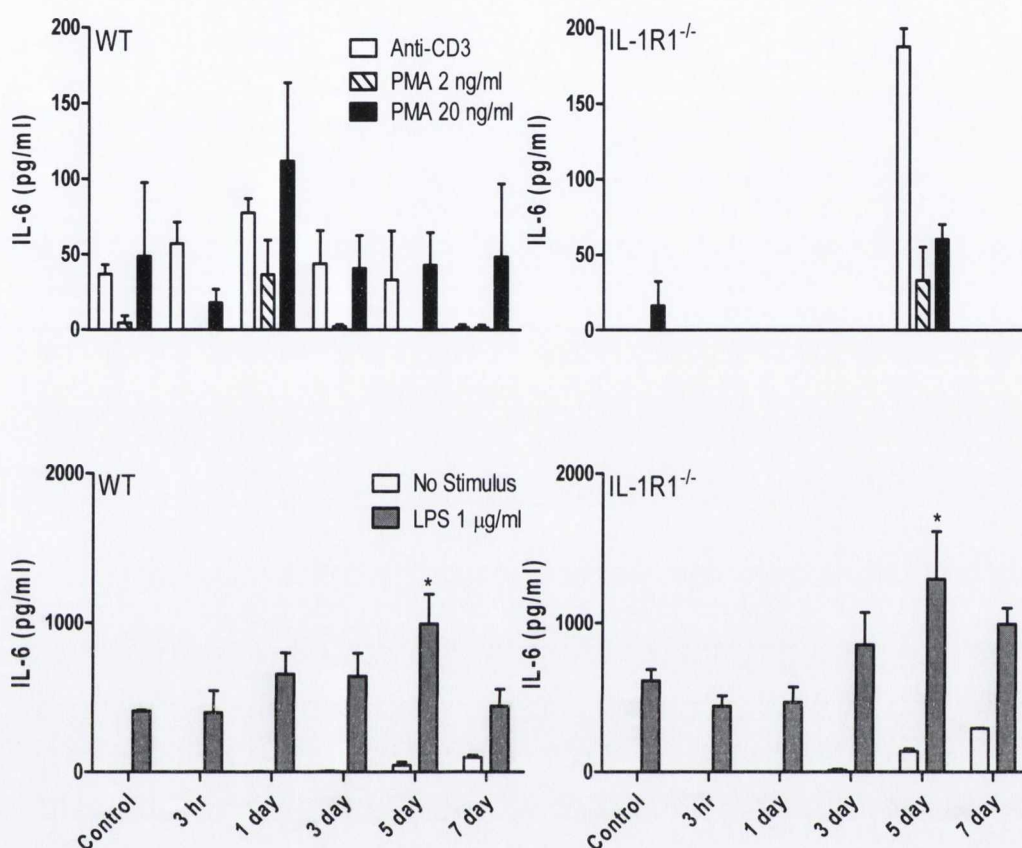


Figure 4.11: Injection of mice with alum enhances IL-6 secretion by splenocytes in an IL-1-independent manner. WT and IL-1R1^{-/-} C57BL/6 mice were injected with PBS only or alum (3mg/mouse) subcutaneously in the right flank. Mice were sacrificed at 3 h, 1, 3, 5 and 7 days following injection and spleens were removed. Splenocytes (2x10⁶ cells/ml) were stimulated with plate bound anti-CD3 (1 µg/ml) alone or with PMA (2 or 20 ng/ml) or medium or LPS (1 µg/ml). After 3 days, supernatants were collected and analysed for IL-6 by ELISA (2.2.5). PBS (3hr, 1day, 3day, 5day or 7day) v alum (3hr, 1day, 3day, 5day or 7day), * p<0.05.

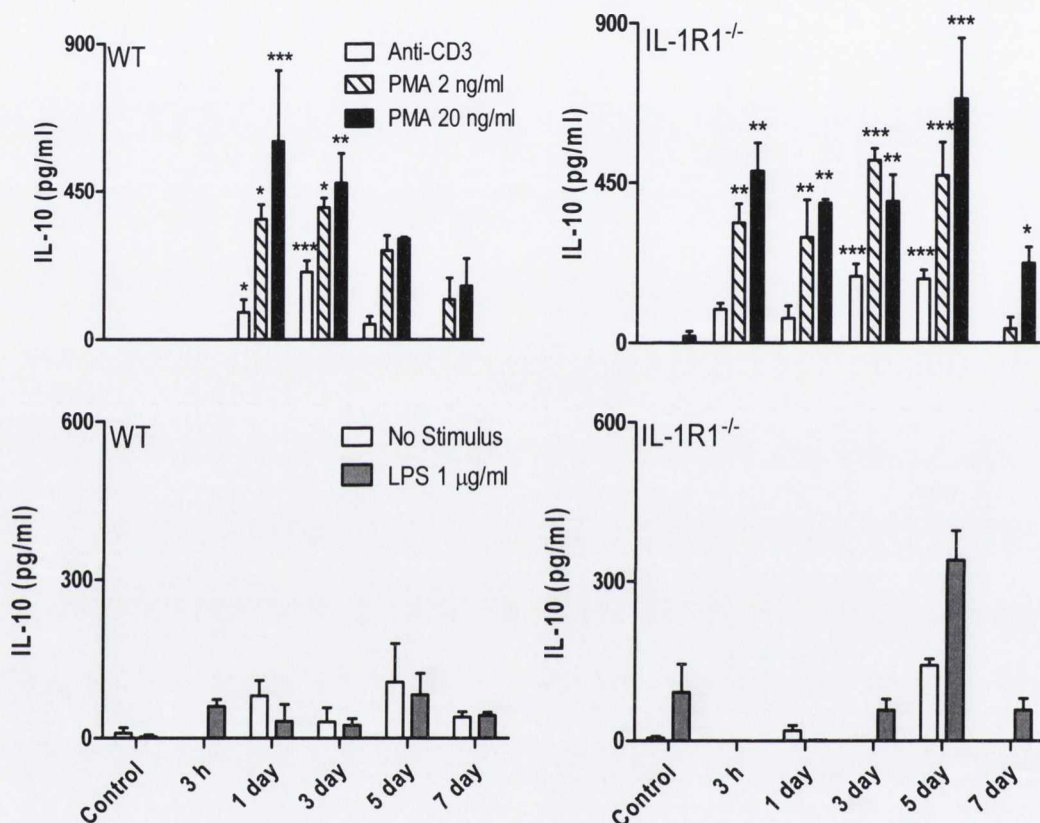


Figure 4.12: Injection of alum primes splenocytes to secrete enhanced levels of IL-10. WT and IL-1R1^{-/-} C57BL/6 mice were injected with PBS only or alum (3mg/mouse) subcutaneously in the right flank. Mice were sacrificed at 3 h, 1, 3, 5 and 7 days following injection and spleens were removed. Splenocytes (2x10⁶ cells/ml) were stimulated with plate bound anti-CD3 (1 µg/ml) alone or with PMA (2 or 20 ng/ml) or with medium or LPS (1 µg/ml). After 3 days, supernatants were collected and analysed for IL-10 by ELISA (2.2.5). PBS (3h, 1day, 3day, 5day or 7day) v alum (3h, 1day, 3day, 5day or 7day), * p<0.05, ** p<0.01, ***p<0.001.

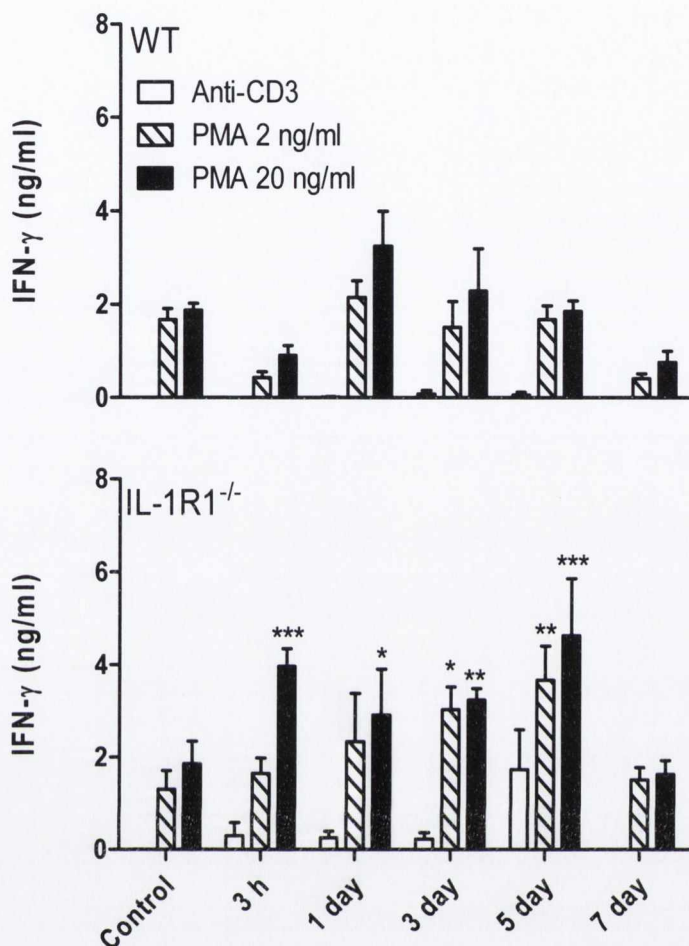


Figure 4.13: Subcutaneous injection of alum primes splenocytes to secrete IFN- γ . WT and IL-1R1^{-/-} C57BL/6 mice were injected with PBS only or alum (3mg/mouse) subcutaneously in the right flank. Mice were sacrificed at 3 h, 1, 3, 5 and 7 days post injection and spleens were removed. Splenocytes (2×10^6 cells/ml) were stimulated with plate bound anti-CD3 (1 μ g/ml) alone or with PMA (2 or 20 ng/ml). After 3 days, supernatants were collected and analysed for IFN- γ by ELISA (2.2.5). PBS (3h, 1day, 3day, 5day or 7day) v alum (3h, 1day, 3day, 5day or 7day), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

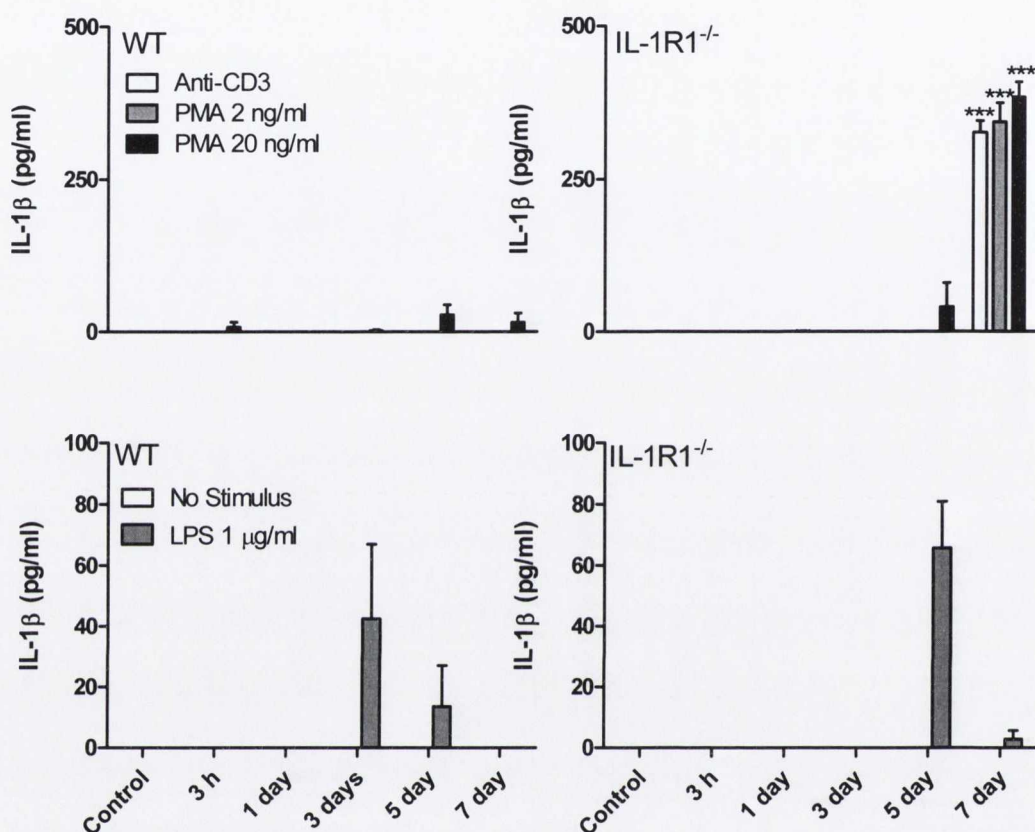


Figure 4.14: The secretion of IL-1 β by IL-1R1^{-/-} splenocytes is significantly enhanced after subcutaneous injection of alum. WT and IL-1R1^{-/-} C57BL/6 mice were injected with PBS only or alum (3mg/mouse) subcutaneously in the right flank. Mice were sacrificed at 3 h, 1, 3, 5 and 7 days following injection and spleens were removed. Splenocytes (2×10^6 cells/ml) were stimulated with plate bound anti-CD3 (1 μ g/ml) alone or with PMA (2 or 20ng/ml) or LPS (1 μ g/ml). After 3 days, supernatants were collected and analysed for IL-1 β by ELISA (2.2.5). PBS (3h, 1day, 3day, 5day or 7day) v alum (3h, 1day, 3day, 5day or 7day), *** $p < 0.001$.

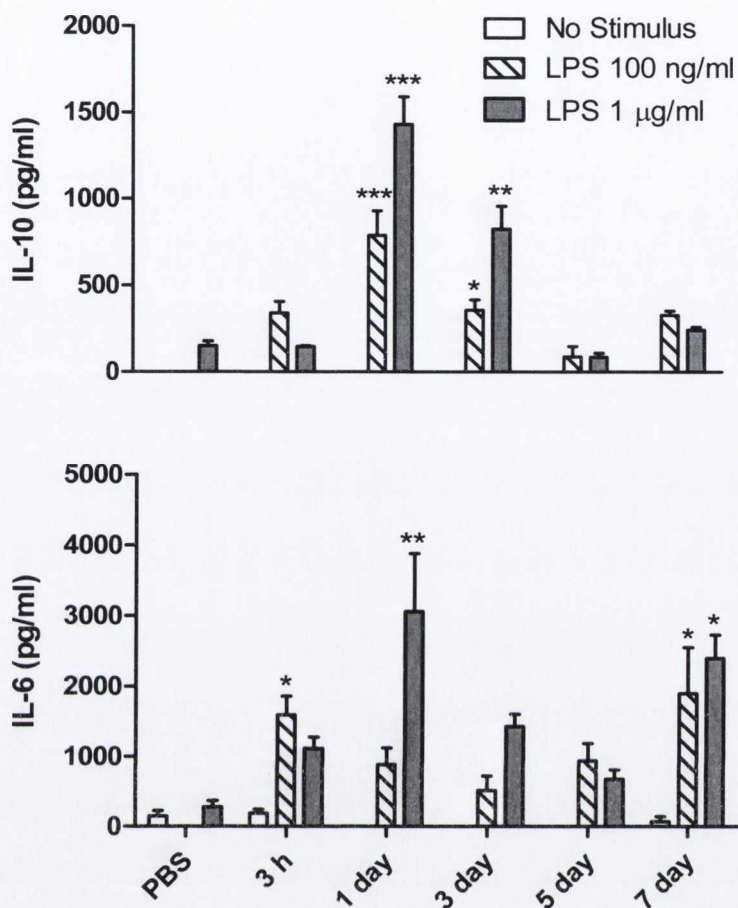


Figure 4.15: Injection of PLG primes splenocytes for enhanced secretion of IL-10 and IL-6. WT C57BL/6 mice were injected with either PBS or PLG (1µm: 4mg/mouse) subcutaneously. Mice were sacrificed at 3 h, 1, 3, 5 and 7 days following injection, spleens were removed. Splenocytes (2×10^6 cells/ml) were stimulated with medium or LPS (100 ng/ml or 1 µg/ml). After 3 days, supernatants were collected and analysed for IL-10 and IL-6 by ELISA (2.2.5). PBS (3h, 1day, 3day, 5day or 7 day) v PLG (3h, 1day, 3day, 5day or 7 day), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

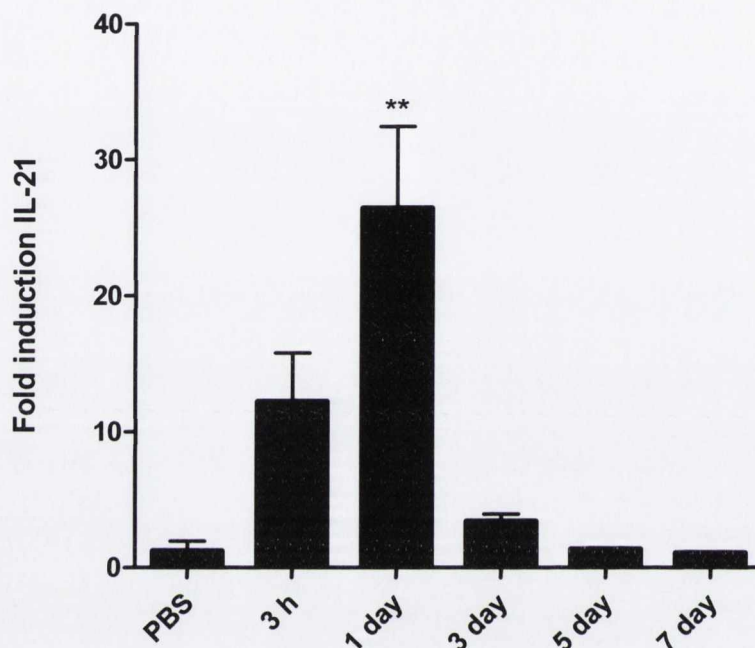


Figure 4.16: Subcutaneous injection of PLG significantly enhances the transcription of IL-21 in the spleen. WT C57BL/6 mice were immunised with either PBS or PLG (1µm: 4mg/mouse) subcutaneously. Mice were sacrificed at 3 h, 1, 3, 5 and 7 days post immunisation. RNA was isolated from spleen cells and levels of IL-21 mRNA were analysed by RT-PCR. IL-21 mRNA fold induction in PBS-injected mice v PLG-injected mice (3h, 1, 3, 5, 7days),** $p<0.0.1$. (The RTPCR analysis of these samples was carried out by Ewa Oleszycka)

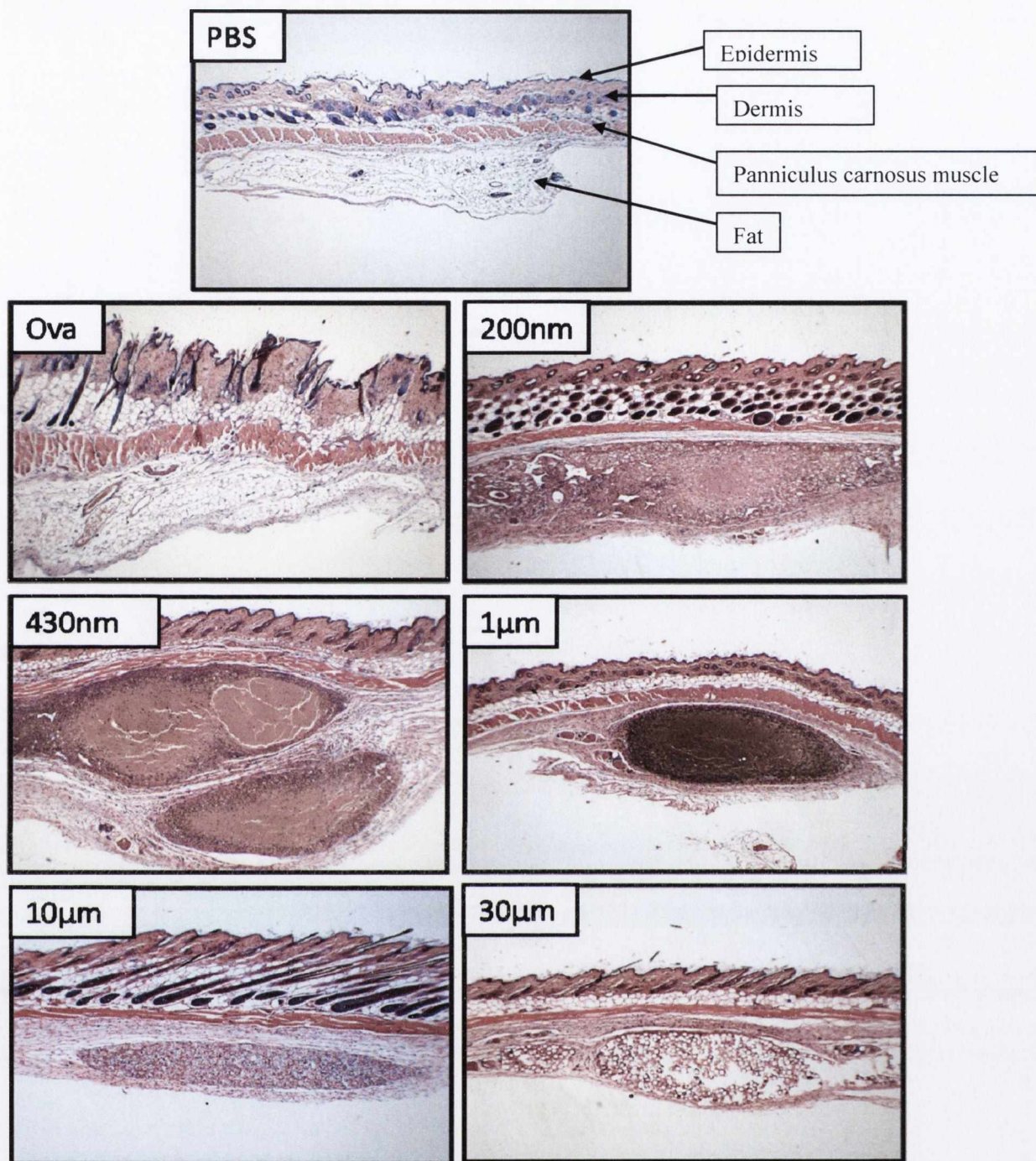


Figure 4.17: Photomicrographs of the subcutaneous granulomas induced by various sized PS particles (H&E,x40). C57BL/6 mice were immunised with either PBS or Ova (50 µg/mouse) or a combination of PS particles of varying size (200 nm, 430 nm, 1 µm, 10 µm, or 30 µm) with Ova (50 µg/mouse) subcutaneously in the right flank. The injection site was removed 21 days after immunisation and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.11).

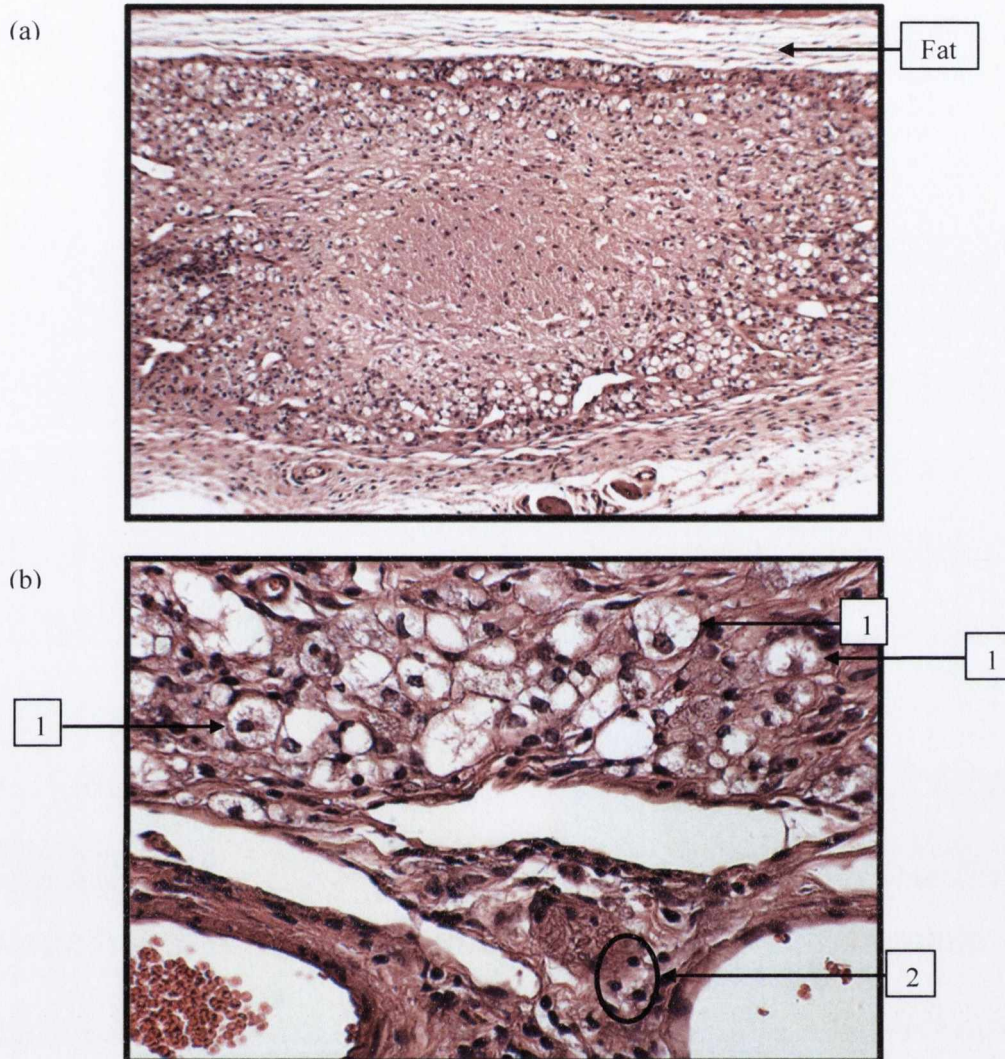


Figure 4.18: Subcutaneous injection of 200nm PS particles induces a strong local inflammatory response characterised by a core of particulate material surrounded by a mix of inflammatory cells which includes foamy macrophages (1) and lymphocytes (2). (a) 100x,H&E. (b) 400x,H&E. C57BL/6 mice were immunised with PS particles of 200 nm in size (4 mg/mouse) and Ova (50µg/mouse) subcutaneously in the right flank and sacrificed 21 days post injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.11).

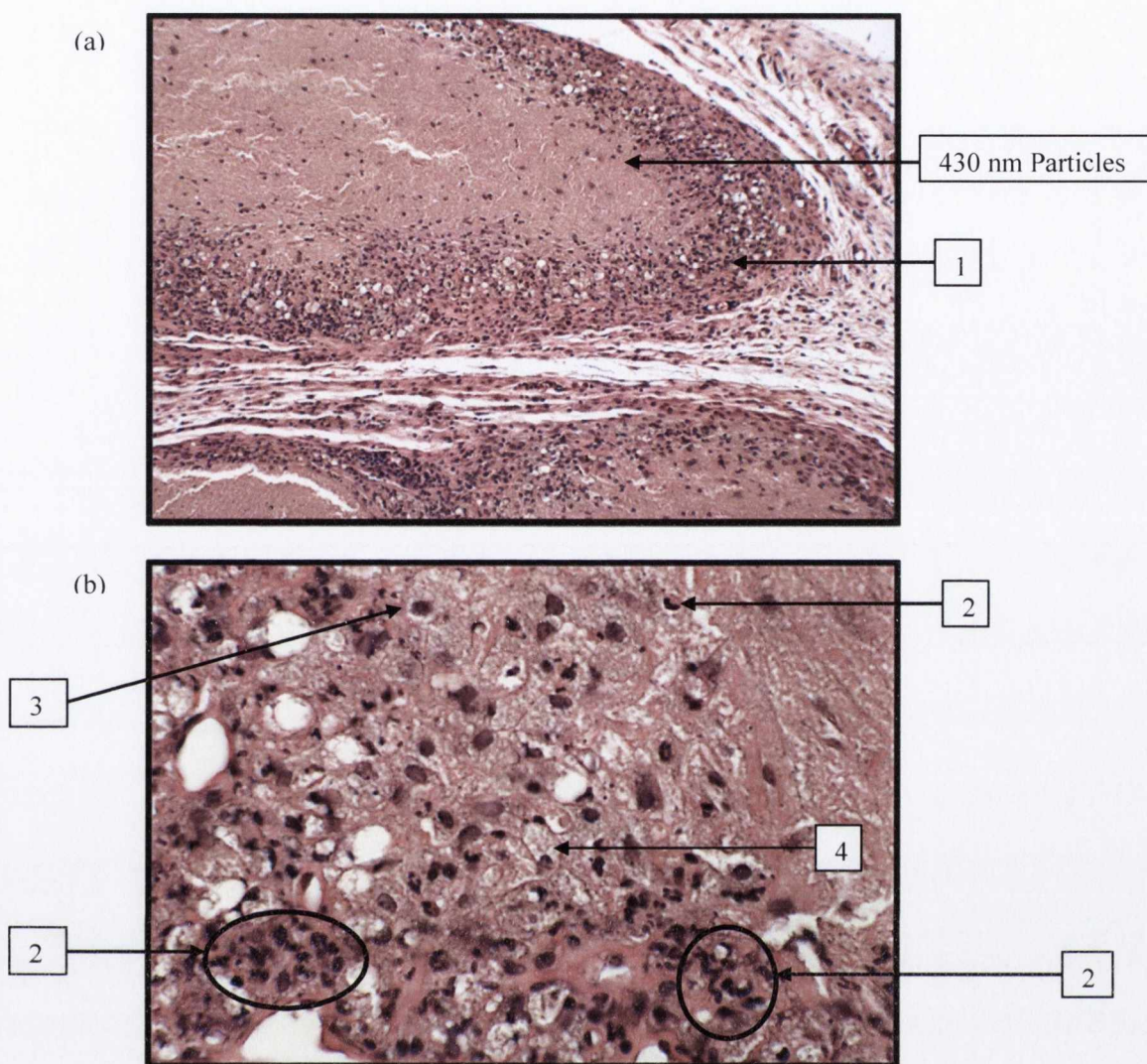


Figure 4.19: Subcutaneous injection of 430nm PS particles induces a granuloma (1) containing a core of particulate material surrounded by neutrophils (2), lymphocytes (3) and foamy macrophages (4). (a) 100x,H&E. (b) 400x,H&E. C57BL/6 mice were immunised with PS particles of 430 nm in size (4 mg/mouse) and Ova (50 μ g/mouse) subcutaneously in the right flank and sacrificed 21 days post injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.11)

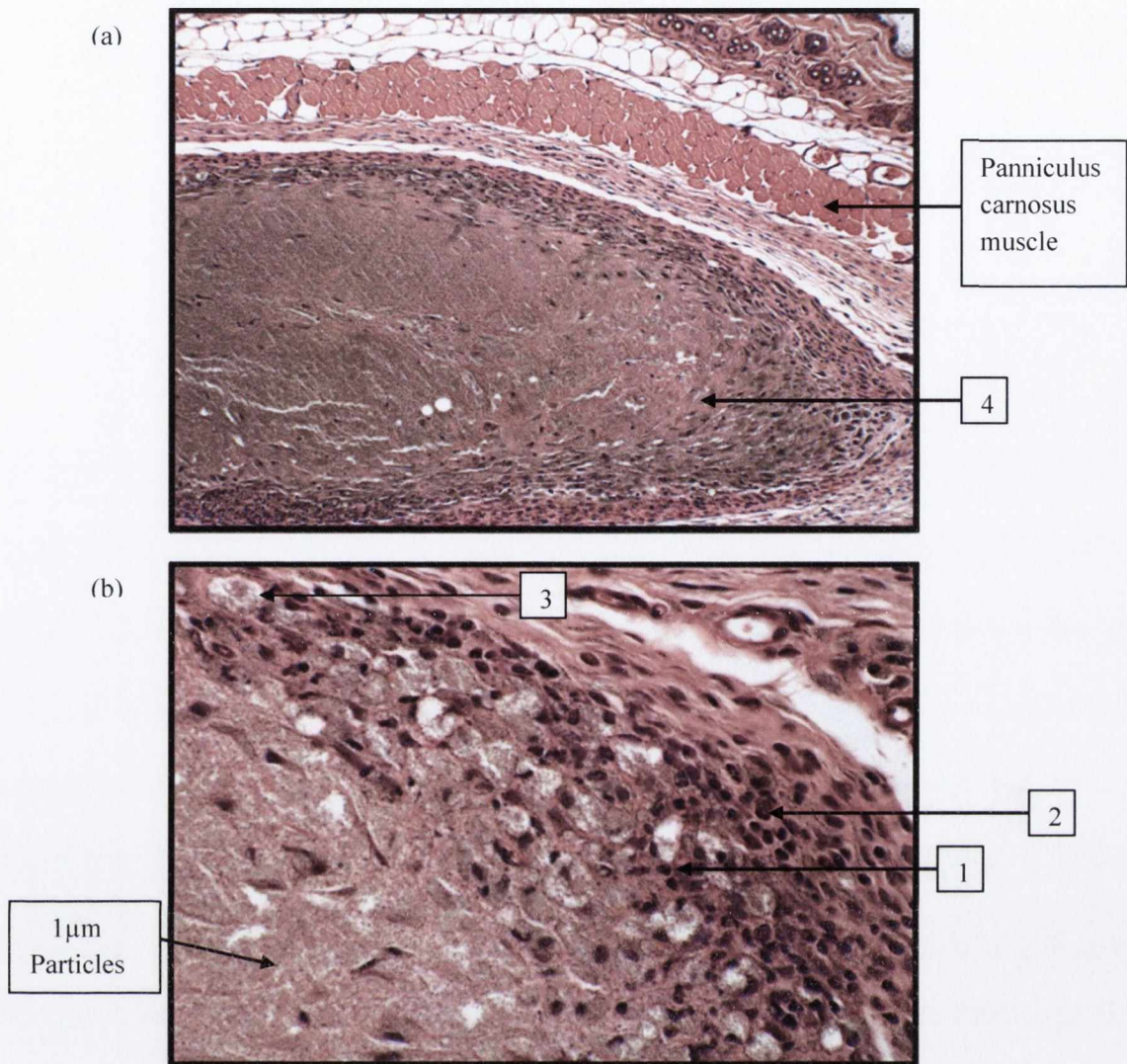


Figure 4.20: Subcutaneous injection of 1 µm PS particles promotes the recruitment of neutrophils (1), lymphocytes (2) and foamy macrophages (3) and local granuloma (4) formation. (a) 100x,H&E. (b) 400x,H&E. C57BL/6 mice were immunised with PS particles of 1 µm in size (4 mg/mouse) and Ova (50µg/mouse) subcutaneously in the right flank and sacrificed 21 days post injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.11).

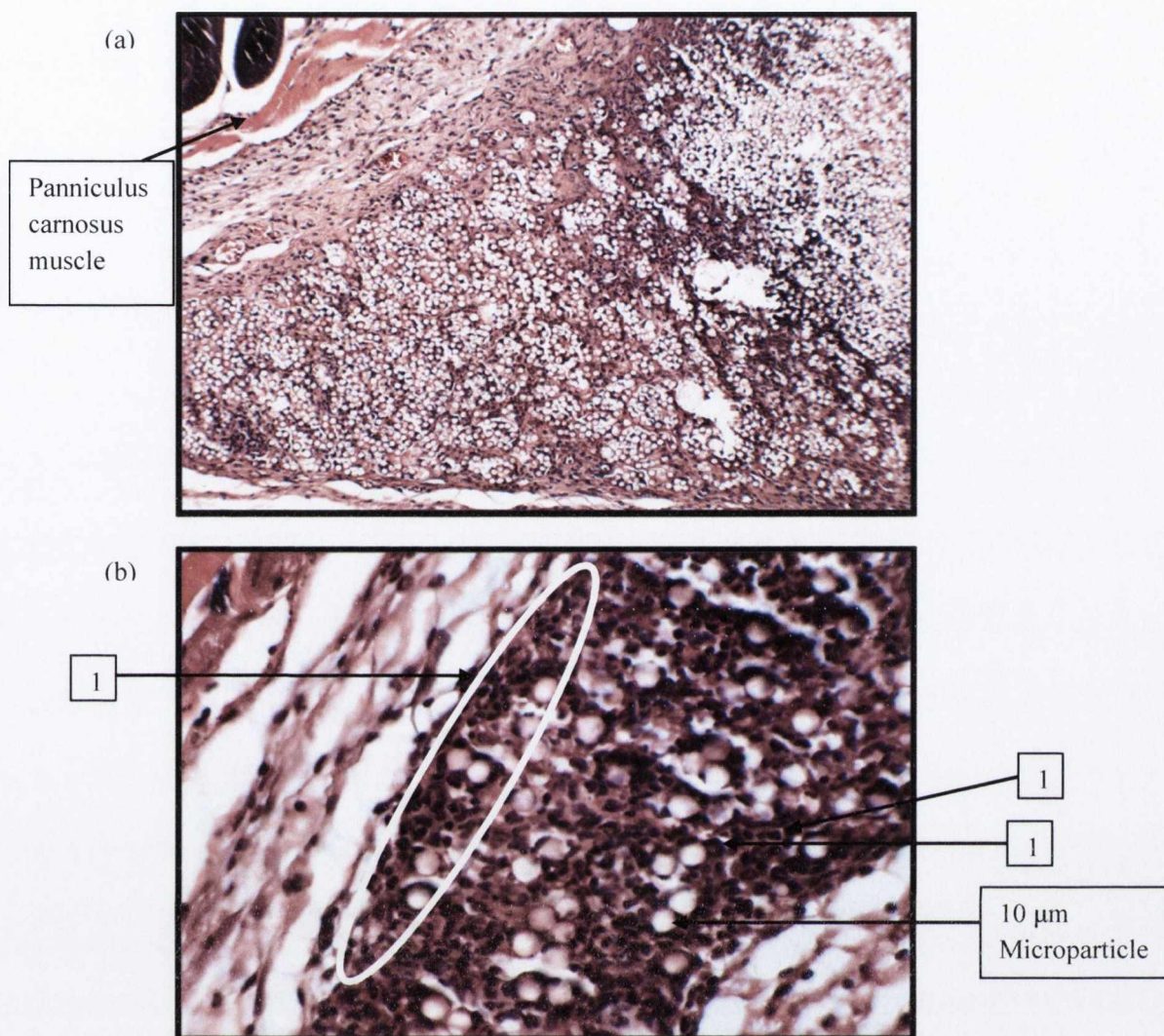


Figure 4.21: Subcutaneous injection of 10 µm PS particles induces a local inflammatory response characterised by the presence of large numbers of neutrophils (1) with small numbers of macrophages at the periphery (not shown). (a) 100x,H&E. (b) 400x,H&E. C57BL/6 mice were immunised with PS particles of 10 µm in size (4 mg/mouse) and Ova (50µg/mouse) subcutaneously in the right flank and sacrificed 21 days post injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.11).

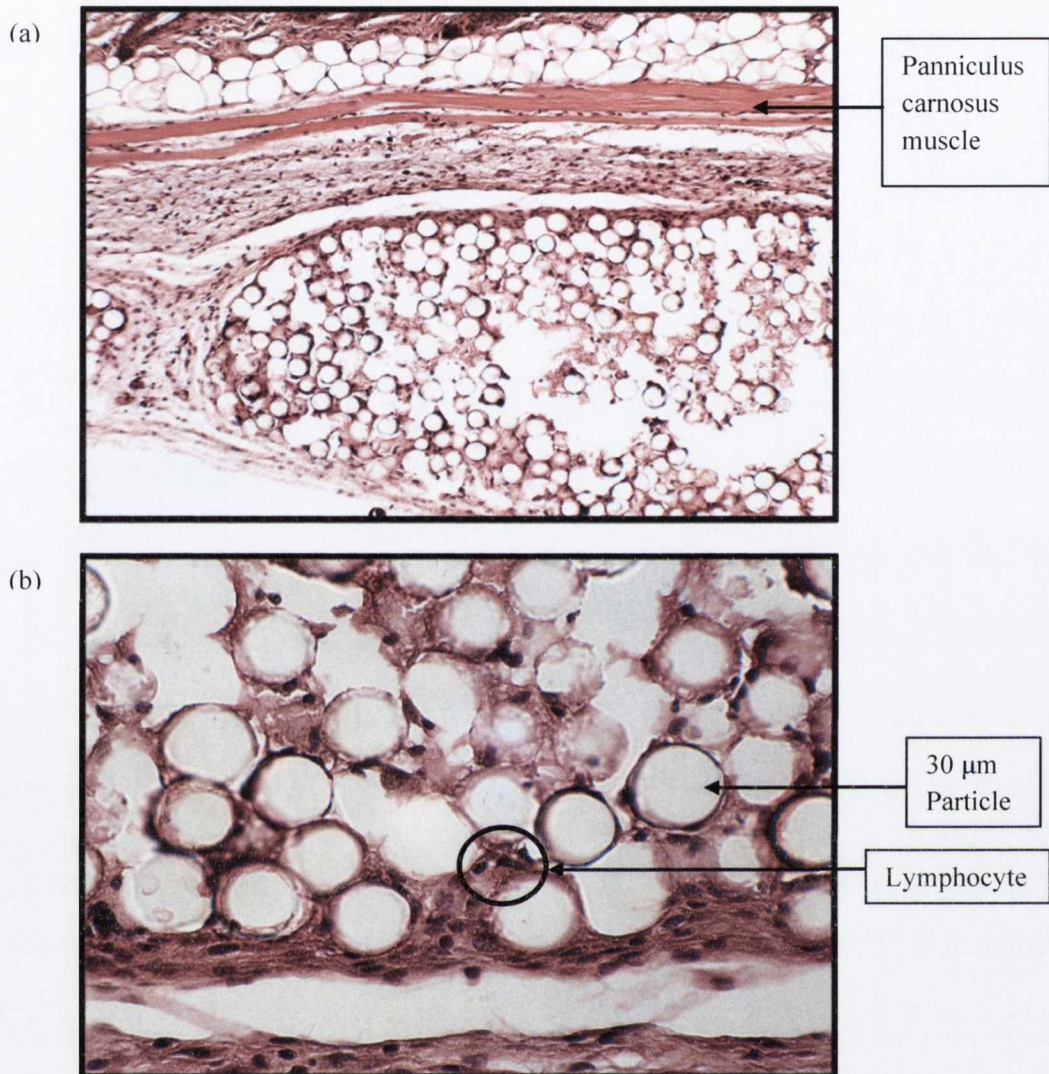


Figure 4.22: Subcutaneous injection of 30 µm PS particles induces formation of a small discrete lesion with very little evident local inflammation (a) 100x,H&E. (b) 400x,H&E. C57BL/6 were immunised with PS particles of 30 µm in size (4 mg/mouse) and Ova (50µg/mouse) subcutaneously in the right flank and sacrificed 21 days post injection. The injection site was removed and preserved in 10% formalin. The tissue was embedded in paraffin wax, sectioned and stained with hemotoxylin and eosin (H&E) for histopathological analysis (2.2.11).

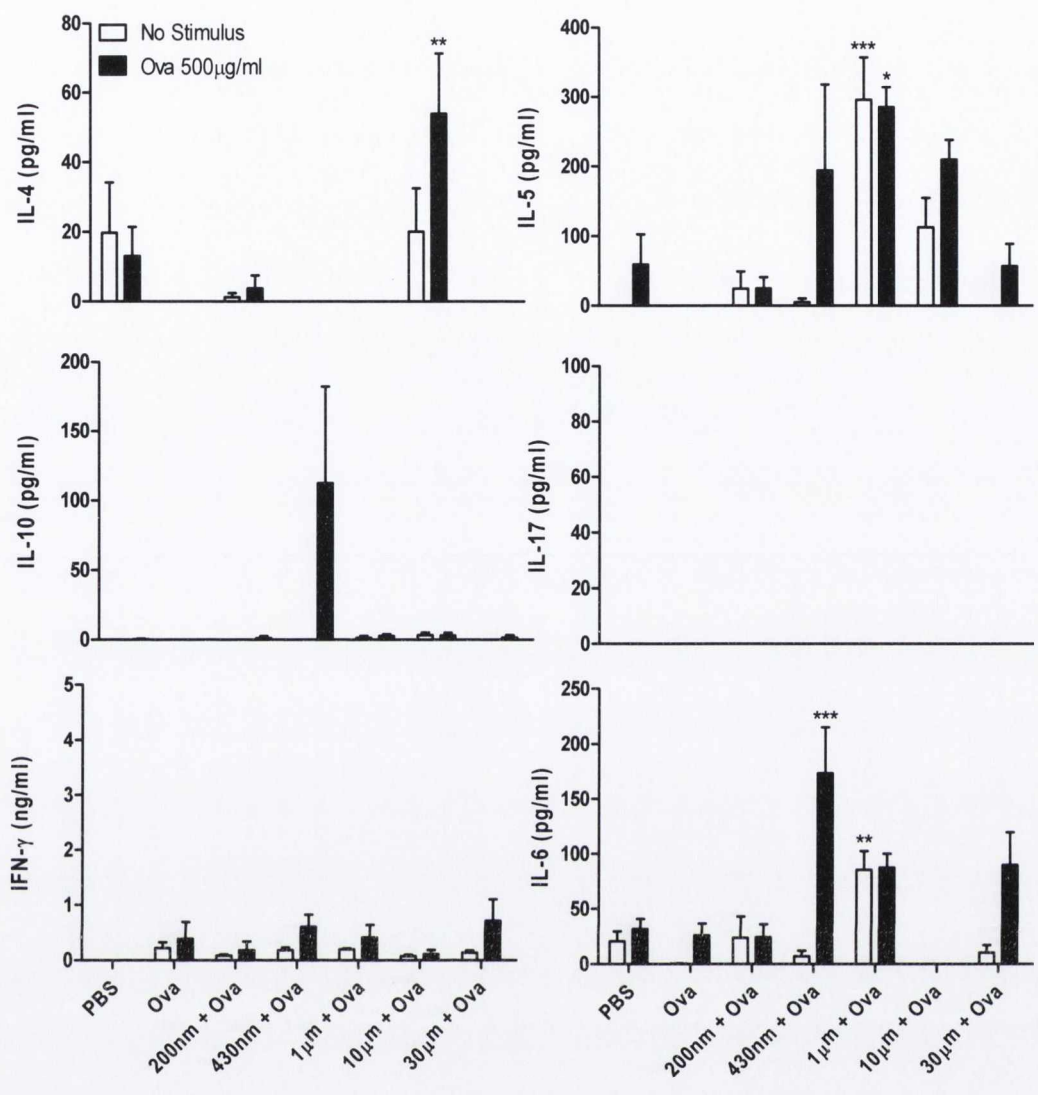


Figure 4.23: Subcutaneous injection of PS particles enhances the secretion of IL-4, IL-5 and IL-6 by splenocytes in a size-dependent manner. C57BL/6 mice were immunised subcutaneously with either PBS or Ova (50 μg/mouse) or a combination of PS particles (200 nm, 430 nm, 1 μm, 10 μm, or 30 μm; 4mg/mouse) with Ova (50 μg/mouse). After 21 days, mice were sacrificed and spleens were removed. Isolated cells were stimulated (2×10^6 cells/ml) with medium or Ova (500 μg/ml). After 3 days, supernatants were collected and analysed for IL-10, IL-17, IL-6, IL-4, IL-5 and IFN-γ by ELISA (2.2.5). Ova injected mice v Ova + PS particle injected mice, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

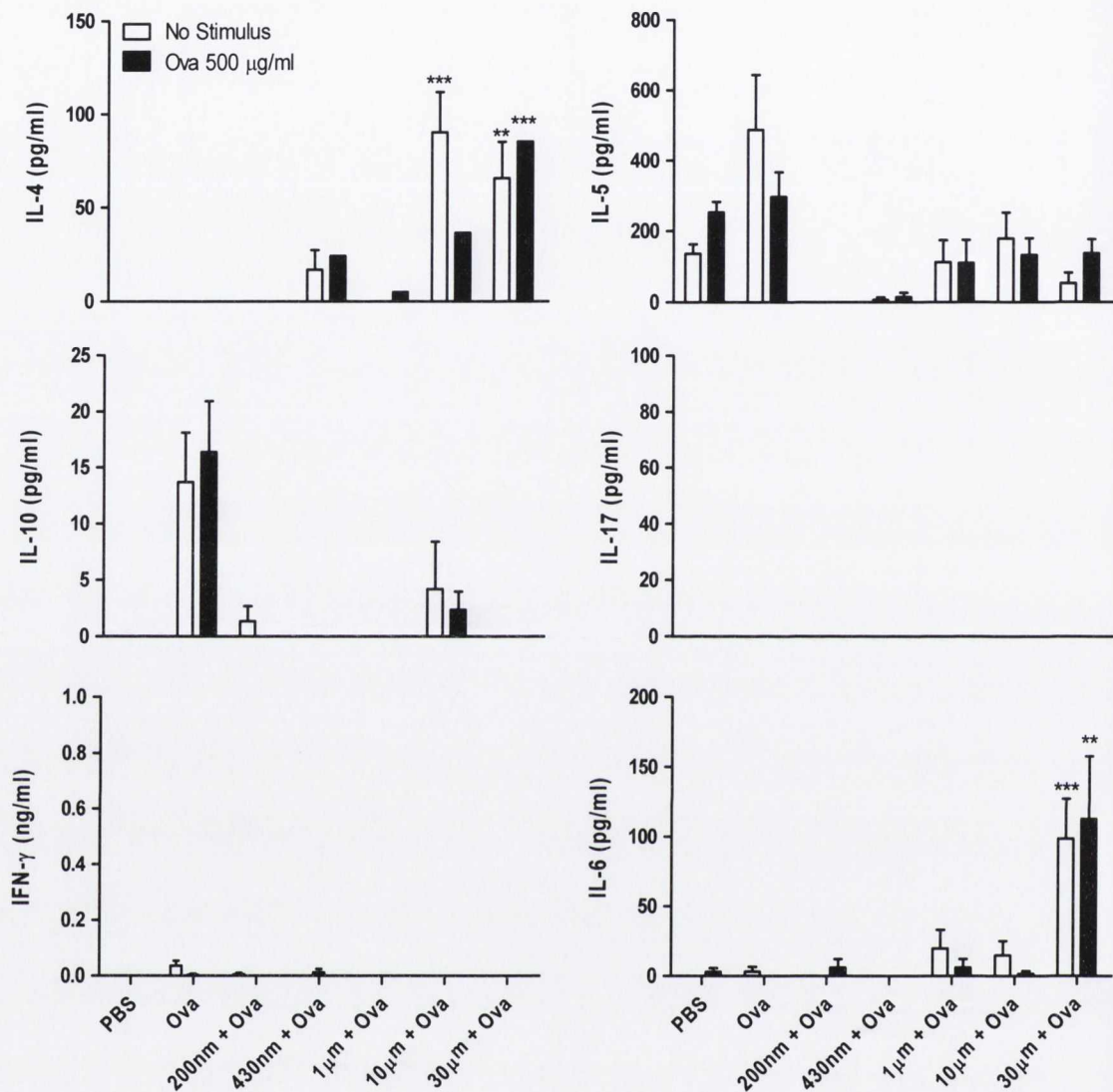


Figure 4.24: Subcutaneous injection of larger PS particles specifically drive IL-4 and IL-6 secretion by mesenteric lymph node cells. C57BL/6 mice were immunised subcutaneously with either PBS or Ova (50 µg/mouse) or a combination of PS particles (200 nm, 430 nm, 1 µm, 10 µm, or 30 µm) with Ova (50 µg/mouse). After 21 days, mice were sacrificed and mesenteric nodes were removed. Isolated cells were stimulated (2×10^6 cells/ml) with medium or Ova (500 µg/ml). After 3 days, supernatants were collected and analysed for IL-10, IL-17, IL-6, IL-4, IL-5 and IFN-γ by ELISA (2.2.5). Ova injected mice v Ova + PS particle injected mice, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

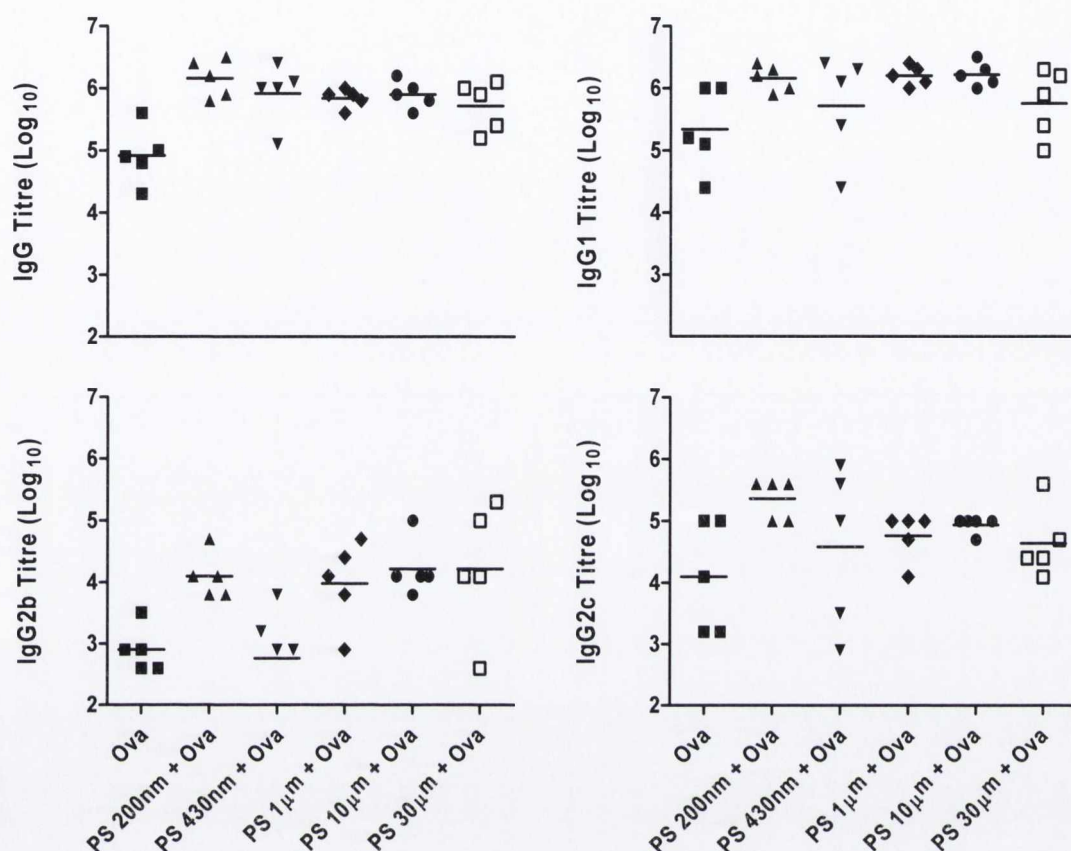


Figure 4.25: Injection of PS particles enhances antigen-specific antibody responses in a size-independent manner. C57BL/6 mice were immunised subcutaneously with either PBS or Ova (50 µg/mouse) or a combination of PS (200 nm, 430 nm, 1 µm, 10 µm, or 30 µm) with Ova (50 µg/mouse). After 21 days, mice were sacrificed and blood samples were collected. The serum samples were analysed for antigen-specific IgG, IgG1, IgG2b and IgG2c titres by ELISA (2.2.12). Antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut off point of 2 standard deviations above sera for PBS injected mice.

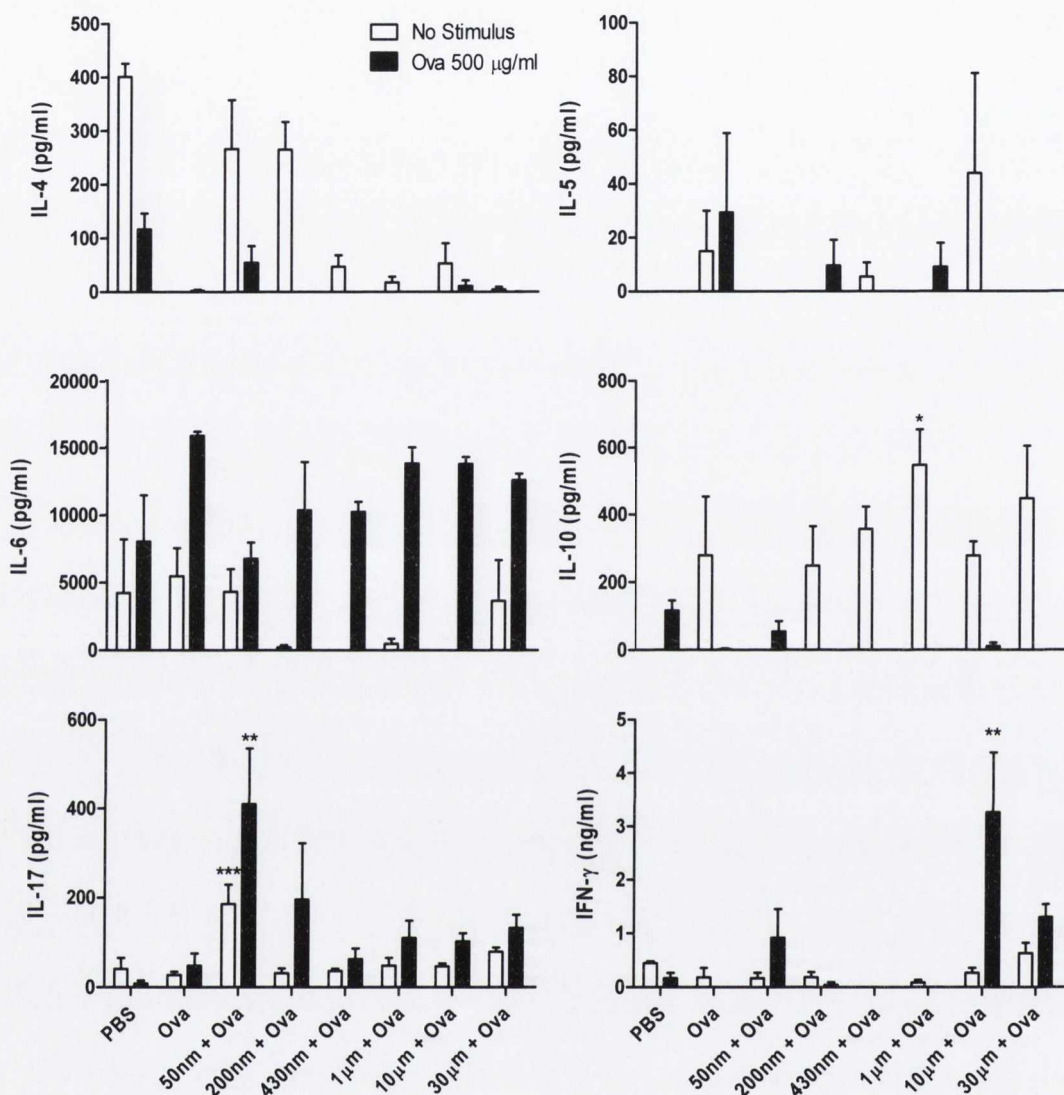


Figure 4.26: Intraperitoneal injection of PS particles enhances local IL-10, IL-17 and IFN-γ secretion in a size-dependent manner. C57BL/6 mice were immunised intra-peritoneally with either PBS or Ova (50 μg/mouse) or a combination of PS particles (50 nm, 200 nm, 430 nm, 1 μm, 10 μm, or 30 μm) with Ova (50 μg/mouse). After 21 days, mice were sacrificed and peritoneal cells were collected. Isolated cells were stimulated (1×10^6 cells/ml) with medium or Ova (500 μg/ml). After 3 days, supernatants were collected and analysed for IL-4, IL-5, IL-6, IL-10, IL-17 and IFN-γ by ELISA (2.2.5). Ova injected mice v Ova + PS particle injected mice, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

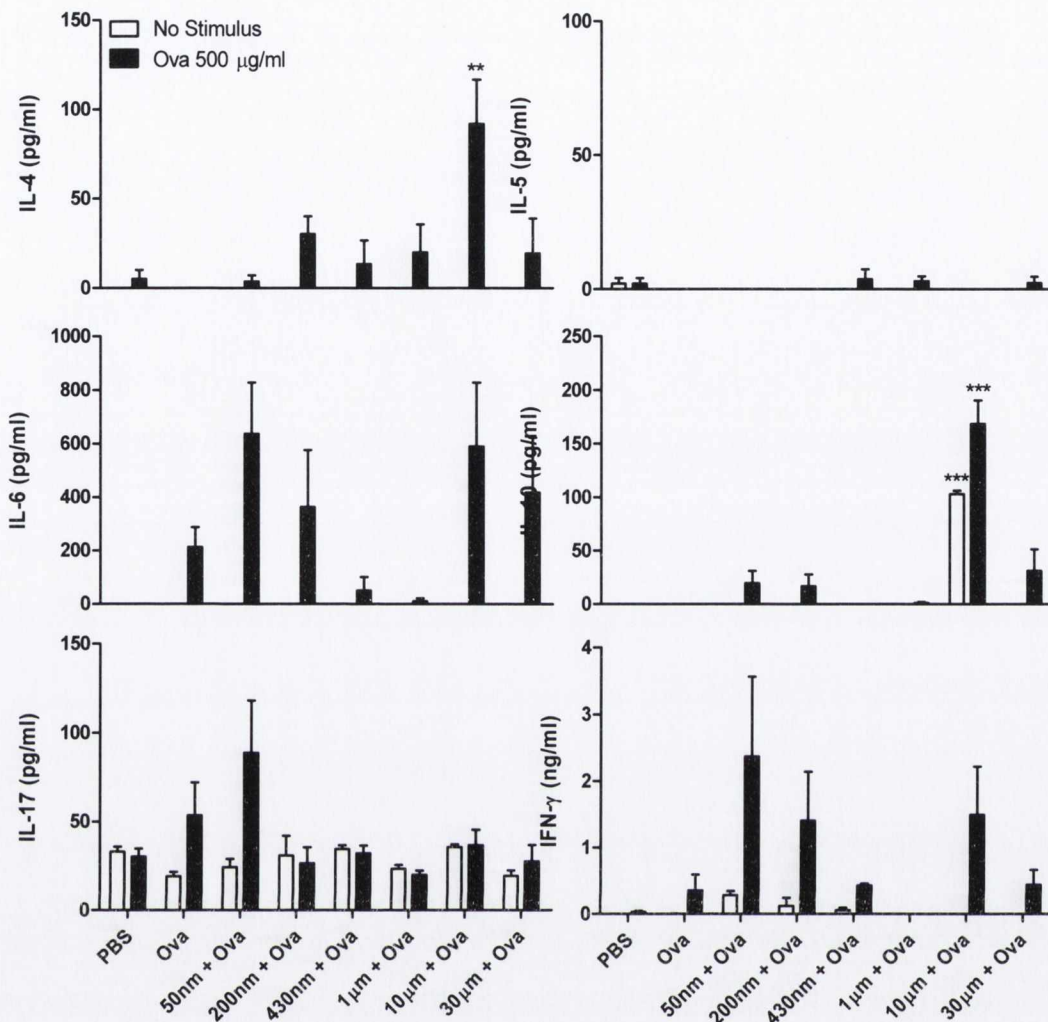


Figure 4.27: Injection of PS particles enhances IL-10 and IL-4 secretion by splenocytes in a size-dependent manner. C57BL/6 mice were immunised intra-peritoneally with either PBS or Ova (50 μg/mouse) or a combination of PS particles (50 nm, 200 nm, 430 nm, 1 μm, 10 μm, or 30 μm) with Ova (50 μg/mouse). After 21 days, mice were sacrificed and spleens were removed. Isolated cells were stimulated (2×10^6 cells/ml) with medium or Ova (500 μg/ml). After 3 days, supernatants were collected and analysed for IL-4, IL-5, IL-6, IL-10, IL-17 and IFN-γ by ELISA (2.2.5). Ova injected mice v Ova + PS particle injected mice, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

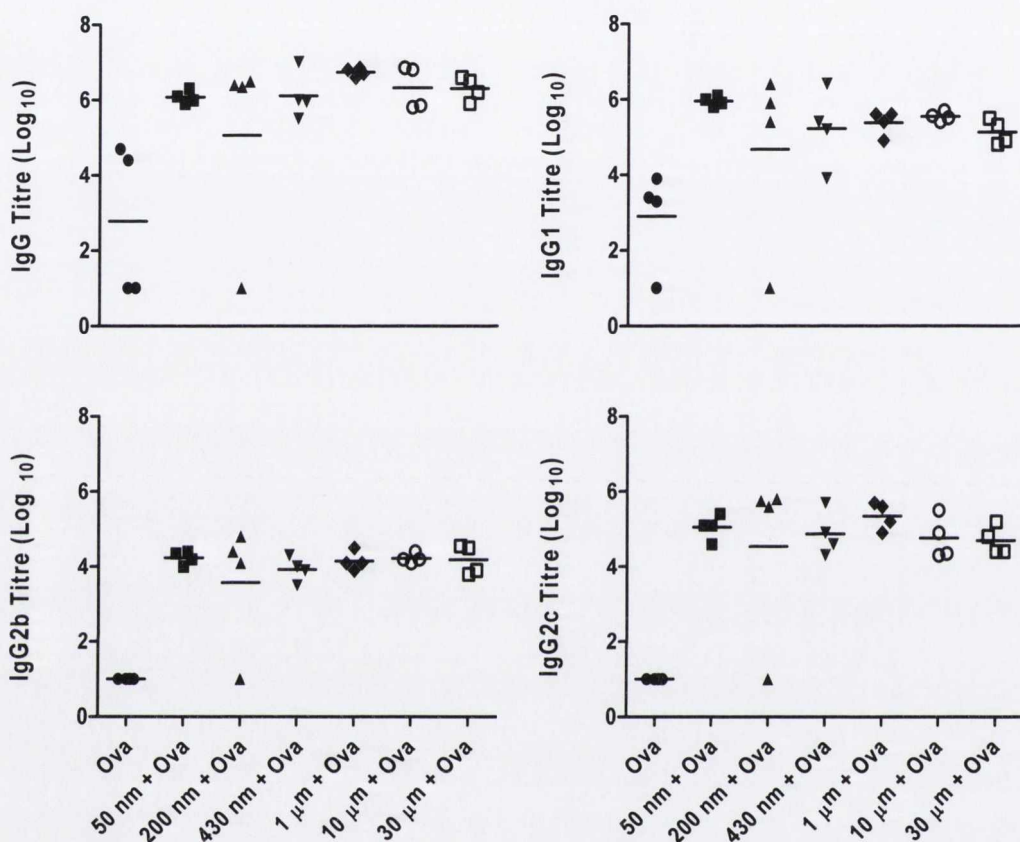


Figure 4.28: Intraperitoneal injection of PS particles enhance antigen-specific antibody responses in a size-independent manner. C57BL/6 mice were immunised intra-peritoneally with either PBS or Ova (50 µg/mouse) or a combination of PS particles (50 nm, 200 nm, 430 nm, 1 µm, 10 µm, or 30 µm) with Ova (50 µg/mouse). After 21 days, mice were sacrificed and blood samples were collected. The serum samples were analysed for total IgG, IgG1, IgG2b and IgG2c titres by ELISA (2.2.12). Antibody responses are expressed as end point titres calculated by regression of a curve of OD values versus reciprocal serum levels to a cut off point of 2 standard deviations above sera for PBS injected mice.

Chapter 5

An Investigation of the Toxic Effects of Particulate Vaccine Adjuvants

5.1 Introduction

One of the most studied questions in immunology is: how do cells of the immune system recognise the presence of pathogenic microorganisms or potential danger? Despite much progress, particularly the discovery of multiple receptors with the ability to recognise pathogens and damage, the exact mechanism of recognition and discrimination remains an area of much debate.

The original model of how the immune system distinguishes self from non-self and the presence of invading microorganisms was formulated by Burnet. He proposed that lymphocytes possess a single receptor specific for a pathogen and that multiple copies of this receptor are expressed on their cell surface [8]. This idea was then extended to introduce the idea of a ‘stimulator cell’, now known as an antigen presenting cell, required to provide a signal for T cell activation and that this signal was species-specific [9]. Almost 30 years later, Janeway put forward the idea that antigen presenting cells (APC) could recognise microbe-associated molecular patterns (MAMP) through a set of germline encoded receptors known as pattern recognition receptors (PRR) [6]. According to this model, APC that have not encountered pathogens are in an inactive state and once they come into contact with a pathogen they become activated and upregulate costimulatory molecules which allows them to interact with naive T cells and generate an antigen-specific immune response.

The PRR proposed by Janeway equip the APC with the ability to distinguish “infectious non-self” from “non-infectious self” [6]. Although Janeway’s theory is widely accepted and experimentally supported, there are examples of immune activation in disease states which do not fit into this model, such as autoimmune diseases or graft rejection. In addition, this theory cannot explain how non-pathogen derived adjuvants, such as alum, stimulate the immune system. Furthermore, no PRR that can directly identify alum or any particulate vaccine adjuvants (PVA) have been identified to date.

In an attempt to explain these discrepancies Polly Matzinger proposed that APC are activated by “danger or alarm” signals released from dying or damaged cells [7]. This ‘danger’ theory suggests that the immune system recognises non-self in the context of ‘danger’ or damage. Matzinger proposed that programmed cell death results in the formation of apoptotic bodies which are rapidly scavenged before they undergo secondary necrosis and are therefore non-immunogenic. In contrast, cells that die by

necrosis or other forms of cell death that are associated with the loss of membrane integrity will release their intracellular contents and therefore potentially release endogenous molecules that signal danger to other immune cells [583]. The identification of endogenous molecules, such as high mobility group box-1 (HMGB1), uric acid, hyaluronic acid and heat shock proteins that can stimulate immune responses adds substance to this theory [10-12]. Furthermore, several studies have since demonstrated the pro-inflammatory activities of necrotic cells, demonstrating that cells dying by necrosis possess immunostimulatory activities *in vivo* [11, 545, 584-585]. The danger theory proposes that the immune system is only activated in the presence of dangerous non-self.

Data presented in chapter 4 demonstrated that injection of PVA induced necrosis and tissue damage (Figures 4.1-4.4). This local cellular damage could therefore promote the release of endogenous danger signals. Therefore, in line with the danger theory, it is possible that PVA promote cellular necrosis leading to the release of normally cell-enclosed endogenous danger signals and subsequent induction of immune responses. Others have demonstrated that the injection of alum alone into the peritoneum enhances the local levels of uric acid [165]. They then went on to show that this promoted the recruitment of inflammatory monocytes into the injection site [165]. Furthermore, this study also demonstrated that these inflammatory monocytes were required for the enhancement of antibody production by alum, which suggests that the release of endogenous danger signals plays an important part in the adjuvant activities of alum [165]. Based on data presented in chapter 4 (Figures 4.1-4.4) demonstrating the necrotic effects of particulates *in vivo* and the role of uric acid in the *in vivo* effects of alum [165], this study aimed to further investigate the toxic effects of particulate vaccine adjuvants.

Specifically, this work set out to:

- Establish whether particulate vaccine adjuvants are toxic to dendritic cells and spleen cells
- Identify the type of cell death promoted by particulate vaccine adjuvants
- Investigate whether treatment of cells with particulate vaccine adjuvants promoted the release of endogenous danger signals with immunostimulatory properties

5.2 Results

5.2.1 PS are toxic to BMDC at high concentrations

The increase in local cell death observed around the injection site of PVA (alum, PLG (PLG) microparticles and polystyrene (PS) microparticles (4.1-4.4, 4.18-4.23) prompted further investigation into the toxicity of these adjuvants. To assess the toxicity of particulates, BMDC were incubated with PS (430 nm; 3mg/ml) alone for 24 h. Cell viability was determined by incorporation of propidium iodide (PI), analysed by flow cytometry. PI is a DNA intercalating fluorochrome, which is only incorporated into cells that have lost membrane integrity. There was an enhancement in the number PI⁺ cells in BMDC treated with PS in comparison with control DC (Figure 5.1). These results and the large amounts of necrotic cell death observed at the injection site *in vivo* suggests that PVA are clearly toxic to immune cells.

5.2.2 Optimal particulate-induced cell death occurs 6 h after stimulation with PVA

Having established that PS are toxic to DC, it was necessary to determine if this was a shared property of other PVA (alum and PLG (PLG) microparticles) and the kinetics of these toxic effects. Particulate-induced cell death was examined at 15 min, 1 h, 2 h, 4 h and 6 h after the addition of alum, PLG (1µm) or PS (430 nm) to BMDC. Cell viability was determined by incorporation of PI, analysed by flow cytometry. Stimulation of BMDC with alum resulted in an increase in the number of PI⁺ cells in comparison to controls (Figure 5.2). This enhancement in the number of PI⁺ cells was most marked after 6 h (Figure 5.2). A similar trend was observed with PLG, where the highest numbers of PI⁺ cells were detected 6 h following stimulation with PLG (Figure 5.2). Stimulation of BMDC with PS enhanced the number of PI⁺ cells (Figure 5.2). Again, the most marked enhancement in PI⁺ cells was observed 6 hr post stimulation (Figure 5.2). Particulate-induced cell death was observed as early as 1 or 2 h following the addition of particulates to DC (Figure 5.2).

Given the toxic nature of PVA at high concentrations, the effect of lower concentrations of particulates was determined. BMDC were incubated with alum, PLG or PS alone at a range of concentrations for 6 h and cell viability was determined by incorporation of PI, analysed by flow cytometry. *In vitro* treatment of BMDC with alum, PLG or PS

resulted in increased levels of PI⁺ cells in comparison with control (Figure 5.3-5.4). This toxicity was most marked at the highest concentration of particles with the lowest concentration tested only inducing levels of cell death comparable to that seen in the controls (Figure 5.3-5.4). All of the PVA were toxic to the BMDC. However, alum proved to be the most toxic, followed by PLG, while PS induced least amount of cell death (Figure 5.3-5.4).

5.2.3 Particle-induced BMDC death occurs independently of NLRP3, cathepsin B and caspases

Previous findings have demonstrated that particulates have the ability to activate the NLRP3 inflammasome (chapter 3). Therefore the relationship between NLRP3 and the toxicity of particulates was investigated. Furthermore, several recent reports have described a necrotic type of cell death that requires NLRP3 [586-588]. In order to investigate the role of NLRP3 in particle-induced cell death, NLRP3^{-/-} BMDC and wild type (WT) BMDC were stimulated with alum (25mM, 5mM), PLG (1µm; 3 mg/ml, 0.6 mg/ml) or PS (430nm; 3 mg/ml, 0.6 mg/ml). The percentage of PI⁺ cells was analysed by flow cytometry 6 h after the addition of particles.

Incubation of WT BMDC with alum resulted in an increase in the percentage of PI⁺ cells, with this effect being most marked at the highest concentration of alum tested (Figure 5.5). Similar to the WT cells, NLRP3^{-/-} BMDC also showed a significant enhancement in the percentage of PI⁺ cells when treated with alum in comparison to control cells (Figure 5.5). There was no significant difference in the number of PI⁺ cells in alum-treated WT and NLRP3^{-/-} BMDC (Figure 5.5). Similarly, treatment of WT and NLRP3^{-/-} BMDC with PLG significantly enhanced the percent of PI⁺ cells with no significant differences between the NLRP3^{-/-} and WT BMDC (Figure 5.5). In further agreement with these results, stimulation with PS significantly enhanced the percentage of PI⁺ WT and NLRP3^{-/-} BMDC to comparable levels (Figure 5.5).

The toxic effect of alum and PLG was observed at both concentrations tested, although the increase in PI⁺ cells was most marked at the highest concentrations (25 mM, 3 mg/ml) (Figure 5.5). In the case of PS, only the highest concentration (3mg/ml) was significantly toxic to BMDC (Figure 5.5). Overall, these findings demonstrate the

toxicity of PVA occurs independently of NLRP3. Pyronecrosis has been shown to be induced by *Shigella flexneri* and requires the lysosomal protease cathepsin B and NLRP3 [588]. Therefore, these results suggest that, the type of cell death induced by PVA is not pyronecrosis.

Cathepsin B has also been proposed to be required for pyronecrosis. Therefore, the role of cathepsin B in particle-induced cell death was also determined. In order to fully rule out the possibility that PVA were promoting pyronecrosis in BMDC, BMDC were pre-treated with either the cathepsin B inhibitor CA-074-Me or medium alone prior to the addition of alum. BMDC were then analysed for their uptake of PI by flow cytometry either 1 h or 2 h after stimulation with alum. There were increased numbers of PI⁺ cells in the alum-treated group, in comparison to controls, at both 1 and 2 h following stimulation (Figure 5.6). Pre-treatment with CA-074-Me had no effect on the enhancement of PI⁺ cells observed with alum (Figure 5.6). These findings show that cathepsin-B is not required for particle-induced cell death. Furthermore, they are in agreement with earlier findings (Figure 5.5), which indicated that NLRP3 was dispensable for particle-induced cell death. These results confirm that the type of cell death induced by particulates is not pyronecrosis.

Having established that particle-induced cell death is not dependent on NLRP3 or cathepsin B, it was necessary to further investigate the mechanism of this toxicity and to attempt to identify the type of cell death. Several other forms of cell death rely on the activation of caspases, including apoptosis and pyroptosis [589-592]. Given the important role of caspases in cell death, their role in particle-induced cell death was investigated. BMDC were pre-treated with a pan-caspase inhibitor (ZVAD-fmk) prior to the addition of alum. Either 1 or 2 h post-stimulation with alum, BMDC were analysed for PI incorporation by flow cytometry.

Similar to previous findings, stimulation of BMDC with alum strongly enhanced the percentage of PI⁺ cells in comparison to controls (Figure 5.6). Pre-treatment with ZVAD-fmk had little or no effect on this enhancement of PI⁺ cells by alum (Figure 5.6). Although the percentage of PI⁺ cells was slightly reduced 2 h post-stimulation in comparison to 1 hr, a similar trend was observed and the pan-caspase inhibitor (ZVAD-fmk) had no effect on toxicity of alum (Figure 5.6). These findings demonstrate that there is no role for caspases in particulate-induced cell death, which suggests that it is

not a form of apoptosis or pyroptosis. Moreover, these results also suggest that there is no direct connection between toxicity and the ability of PVA to enhance the secretion of IL-1 β , as this can be inhibited by pre-treatment with CA-074-Me (cathepsin B) and caspase-1 inhibitors.

5.2.4 Particulate-induced cell death requires particle uptake

Having established that particulate-induced cell death occurs independently of NLRP3, cathepsin B and caspases, further studies were carried out to investigate whether particle uptake was necessary.

BMDC were pre-treated with cytochalasin D prior to the addition of alum. Cytochalasin D is an inhibitor of actin polymerisation which prevents phagocytosis. 6 h following the addition of alum to the BMDC, cells were analysed for PI incorporation. Treatment of BMDC with alum strongly enhanced the numbers of PI⁺ cells in comparison to controls (Figure 5.7). However, pre-treatment with cytochalasin D reduced the number of PI⁺ cells to a level comparable to controls (Figure 5.7). These results suggest that particle uptake is necessary for particulate-induced cell death.

In order to further confirm that phagocytosis of particles is necessary them to exert their toxic effects, BMDC were stimulated with PS particles either 50 nm or 10 μ m. Particles less than 1 μ m in size are efficiently phagocytosed while larger 10 μ m PS particles are not easily taken up by DC (Figure 3.18). Therefore, the relationship between particle uptake and cell death could be investigated. After 6 h cells were analysed for PI incorporation. Stimulation of BMDC with PS particles 50 nm in size resulted in an enhancement in the percentage of PI⁺ cells in comparison to controls (Figure 5.7). In contrast, BMDC treated with PS 10 μ m in size showed no enhancement in numbers of PI⁺ cells than controls (Figure 5.7). Therefore, smaller particles (50 nm) are more toxic to BMDC than larger particles (10 μ m). These results further confirm that phagocytosis of particles is necessary for particle-mediated cell death.

5.2.5 PVA are toxic to splenocytes

The necrosis observed around the injection site post-immunisation with PVA suggests that these toxic effects of particulates are not restricted to BMDC (Figure 4.2-4.4). In order to investigate this, spleen cells were isolated from naive mice and stimulated with alum (25 mM and 5 mM). Spleen cell viability was then analysed using PI incorporation by flow cytometry, 1 h and 2 h following stimulation with alum.

Treatment of spleen cells with alum increased the numbers of PI⁺ cells at both time points tested (Figure 5.8). This enhancement was greatest with the highest concentration of alum (25 mM) (Figure 5.8). These findings indicate that the toxicity of alum is not specific to BMDC and confirms observations from *in vivo* studies that demonstrated the presence of necrotic neutrophils within the injection site (Figures 4.2-4.4).

5.2.6 PVA synergise with HMGB1 to enhance IL-1 secretion by BMDC

Previous findings indicated that PVA synergise with TLR agonists to enhance the secretion of IL-1 β by BMDC (Figure 3.3-3.6). Furthermore, when particulates are injected subcutaneously they have the ability to enhance the local secretion of IL-1 β in the absence of a TLR agonist, which suggests that they are synergising with an endogenous molecule (Figure 4.5). Cells that die by necrosis release their cellular contents, potentially including endogenous danger signals.

PVA may promote the release of endogenous danger signals which could potentially act as the priming signal for IL-1 β . In agreement with this, previous studies from within our lab have shown that PVA alone promote the release of HMGB1 from BMDC (Coleman M and Lavelle EC, Unpublished findings). In order to test whether HMGB1 could replace TLR agonists as the second signal necessary for the secretion of IL-1 β by BMDC, cells were pre-incubated with PS (430 nm) and subsequently stimulated with recombinant HMGB1. Treatment of BMDC with HMGB1 was insufficient to drive the secretion of IL-1 β (Figure 5.9). However, pre-incubation of BMDC with PS (430 nm) prior to the addition of HMGB1 was significantly enhanced the secretion of both IL-1 β and IL-1 α from BMDC (Figure 5.9). In addition, PS alone promoted the secretion of IL-1 α from BMDC, with no effect on IL-1 β (Figure 5.9).

These findings indicate that HMGB1 can replace the need for a TLR agonist and synergise with particles to enhance the secretion of IL-1 β and IL-1 α by BMDC. Furthermore, they provide evidence to support the theory that the ability of PVA to promote the local secretion of IL-1 β *in vivo* (Figure 4.5) is through particle-induced cell death and subsequent release of endogenous danger signals, such as HMGB1.

5.2.7 Supernatants from alum-treated splenocytes display adjuvant activity *in vivo*

Findings from this study so far have demonstrated that particulates are toxic to cells and that they can synergise with endogenous danger signals to promote the secretion of IL-1 by BMDC. In addition, it has previously been shown that PVA can promote the release of endogenous danger signals, such as HMGB1 and uric acid from cells [165]. Combining the fact that PVA are toxic to cells both *in vitro* and *in vivo* with their ability to promote the release of endogenous danger signals, it is tempting to predict that the release of endogenous danger signals induced by particulates plays a role in their adjuvanticity.

In order to investigate this theory, the supernatants from spleen cells treated with alum were used as potential adjuvants and co-administered with Ova intraperitoneally into mice. Some of the endogenous danger signals that have been identified to date have been shown to signal through TLR4. Therefore, C3H/HeJ mice, which have a mutation in TLR4 making them hyporesponsive to TLR4 agonists [42-44], and WT (C3H/HeN) mice were immunised.

Mice were immunised as follows:

1. PBS alone
2. Ova alone
3. Supernatant from untreated splenocytes + Ova
4. Supernatant from alum-treated splenocytes + Ova
5. Supernatant from alum-medium (no cells) + Ova

21 days post-immunisation (prime d0 and boost d14) the spleens and peritoneal cells were isolated. Isolated cells were re-stimulated with Ova (100 µg/ml) or medium, supernatants were removed and analysed for cytokines.

When spleen cells were isolated and re-stimulated with Ova *ex vivo* no significant effects were observed on the production of IL-6 or IL-10 (Data not shown). However, co-administration of supernatants from alum-treated spleen cells with Ova enhanced the production of IL-4, IL-5, IL-17 and IFN-γ.

Injection of supernatants from alum-treated spleens with Ova significantly enhanced the secretion of Ova-specific IL-5 by splenocytes, in comparison to cells taken from mice injected with Ova alone (Figure 5.10). Furthermore, this enhancement was observed in C3H/HeN mice and not in the C3H/HeJ mice, which suggests a role for TLR4 (Figure 5.10). In comparison to mice immunised with Ova alone, spleen cells isolated from C3H/HeN mice immunised with supernatant from alum-treated splenocytes with Ova produced higher concentrations of Ova-specific IL-4 (Figure 5.10). In a similar manner to IL-5, this enhancement of IL-4 was only evident in the C3H/HeN mice suggesting a role for TLR4 in the enhancement of IL-4 (Figure 5.10). In addition to IL-4, splenocytes isolated from mice immunised with supernatants from alum-treated cells and Ova produced significantly higher amounts of Ova-specific IL-17 in comparison to mice injected with Ova alone (Figure 5.10). This effect was only observed in the C3H/HeJ mice and not in the C3H/HeN immunised mice, which suggests that TLR4 may play some role in the downregulation of this enhanced IL-17 (Figure 5.9).

In contrast to splenocytes, there was no significant enhancement in antigen-specific IL-4, IL-5 or IL-17 production by peritoneal exudate cells (PEC) isolated from the peritoneum of mice (PEC) immunised with supernatant from splenocytes treated with alum and Ova (Figure 5.11). Injection of mice with supernatant from alum-treated splenocytes with Ova promoted the secretion of significant amounts of Ova-specific IFN-γ by PEC in comparison to injection of Ova alone (Figure 5.11). This enhancement in IFN-γ was absent in the C3H/HeJ mice, which suggests that TLR4 is necessary for the promotion of IFN-γ by endogenous danger signals (Figure 5.11).

Overall, these results demonstrate that supernatant from alum-treated cells have the ability to act as an adjuvant and enhance antigen-specific cytokine production both locally and systemically. Furthermore, some of these effects are TLR4-dependent as differences in cytokine profile were observed between C3H/HeN and C3H/HeJ mice.

5.3 Discussion

The danger theory was first proposed by Matzinger over 20 yrs ago [7]. Matzinger presented this idea as an alternative to Janeway's theory that the immune system was able to discriminate between "infectious non-self" and "non-infectious self" and react appropriately [6]. Although Janeway's original theory is still widely accepted and supported by a wealth of experimental evidence it does not account for certain immune reactions, such as autoimmunity, transplant rejection or why a mother's immune system does not mount an attack against a developing foetus [583]. Matzinger's proposal was that the immune system recognised endogenous danger signals released by dying, stressed or injured cells and was therefore only fully activated in response to damage [7].

Originally, this proposal was theoretical, but it is now supported by some convincing experimental evidence. At the core of the danger theory is the idea that cells that die by a type of death that is characterised by a loss of membrane integrity, facilitates the subsequent expulsion of cellular contents. More importantly, it promotes the release of endogenous danger signals that can activate other cells of the immune system. Since the idea of endogenous danger signals was first proposed several have been identified, including uric acid, HMGB1, biglycan and heat shock proteins [11-12, 44, 584, 593]. Furthermore, necrotic cells have been shown to promote the upregulation of co-stimulatory molecules on DC and several groups have demonstrated the pro-inflammatory properties of necrotic cells *in vivo* [12, 545, 584, 594-595]. The identification of endogenous danger signals and the adjuvant activities of necrotic cells have added experimental substance to Matzinger's original theory.

Findings presented in chapter 4 (Figures 4.1-4.4) demonstrate that PVA cause necrosis and increased cellular infiltration when injected subcutaneously. Others have also reported that injection of alum causes local cellular damage, with marked injury to local cells and increased recruitment of neutrophils [551-552]. These *in vivo* effects prompted further investigation into the toxicity of particulates *in vitro*. BMDC that were treated with alum, PLG (1 μ m) or PS (430 nm) alone, induced higher levels of cell death in comparison to controls, as measured by PI incorporation. Furthermore, this enhancement in cell death by PVA occurred rapidly with cell death observed as early as 1 h after treatment with particulates and maximal death 6 h after treatment. These

findings support the observation of necrosis around the injection site following injection with particulates and demonstrate that PVA are toxic to DC at high concentrations.

Up until recently cell death was generally separated into two main types; programmed or controlled (apoptosis) and uncontrolled (necrosis). Apoptosis is a type of cell death that was initially characterised by distinct morphological features, including chromatin condensation, membrane blebbing, exposure of phosphatidylserine on the outer leaflet of the cell membrane and the final stage, which involves the fragmentation of the cell into apoptotic bodies [590, 596-597]. The apoptotic bodies are generally engulfed by phagocytes, leaving no remnants of the dead cells [590, 597]. This process is heavily dependent on caspases, which can be subdivided into initiator caspases (2,8,9,10) and effector caspases (3,6,7) and are activated in response to external and internal stimuli [590-592]. In general, apoptotic cell death is considered to be non-immunogenic, due to the fact that membrane integrity is maintained throughout the process and cellular contents remain sequestered. In contrast, necrosis was considered to be a type of cell death that was uncontrolled and induced as a result of trauma and infection, which usually led to local inflammation. Necrosis is morphologically characterised by a gain in cell volume and swelling of cell organelles, while DNA remains uncondensed and membrane integrity is lost [598-599]. Although necrosis was originally thought to be an uncontrolled process, recent evidence suggests that it may in fact be tightly regulated [600-601]. More recently, the term “necroptosis” has been used to classify a type of cell death which results in loss of membrane integrity, is triggered by stimulation of death receptors (eg. TNFR, Fas) in the presence of caspase inhibitors and is regulated by RIP1 and RIP3 kinases [602-606].

In addition to necrosis, several other forms of cell death that result in loss of membrane integrity have also been identified, including pyroptosis and pyronecrosis [588, 607-609]. Pyroptosis has been shown to be induced by several bacteria, including *Salmonella typhimurium* and *Listeria monocytogenes* [135, 589, 608, 610]. It is associated with activation of the inflammatory caspase, caspase-1, and the subsequent release of IL-1 β . Furthermore, depending on the stimulus, NLRC4 and NLRP1 have been implicated in the induction of pyroptosis [135, 589]. In a similar manner to pyroptosis, pyronecrosis also employs the use of an NLR protein, NLRP3 [588]. However, pyronecrosis occurs independently of caspase-1. It requires activation of the lysosomal protease cathepsin B and the bipartite adaptor protein ASC [588]. During

pyronecrosis, membrane integrity is lost and the endogenous danger signal HMGB1 is released [588].

Having established that PVA are toxic to cells, it was important to determine the type of cell death induced by particulates. As outlined above, cell death is a complex process. It often requires the activation and synchronisation of several pathways. Furthermore, the classification of cell death is almost as complicated as the process itself, as different types of cell death can be classified based on morphological characteristics, requirement of specific enzymes, physiological consequences and whether or not it is pro-inflammatory [599]. A necrotic type of cell death that coincides with IL-1 β and IL-18 release and requires activation of NLRP3 has previously been reported [588]. PVA have the ability to enhance the secretion of IL-1 β and IL-18 in a NLRP3-dependent manner (Figures 3.13-3.14). Furthermore, concentrations that are able to optimally promote the secretion of IL-1 β are also toxic to BMDC. Therefore, it is possible that particulates promote a NLRP3-dependent cell death, resulting in the secretion of IL-1 β . However, NLRP3^{-/-} BMDC were also susceptible to particle-induced cell death. Moreover, the levels of cell death were comparable in WT and NLRP3^{-/-} mice. These findings demonstrate that particulate-induced cell death occurs independently of NLRP3. Pyronecrosis has also been shown to require the lysosomal protease cathepsin B. Therefore, in order to fully exclude pyronecrosis, the level of particle-induced cell death was measured in BMDC pre-treated with the cathepsin B inhibitor Ca-074-Me. Pre-treatment of BMDC with the cathepsin-B inhibitor were also susceptible to particle-induced cell death, suggesting that there is no role for cathepsin B in particle-mediated cell death. Overall, these findings indicate that the type of cell death induced by particulates occurs independently of NLRP3 and cathepsin B. Furthermore, this demonstrates that the toxic effect of particulates is not related to their ability to activate the NLRP3 inflammasome.

Many of the morphological characteristics associated with apoptosis and pyroptosis, are initiated by caspases [589-592]. In order to investigate whether caspases were required for particulate-induced cell death, BMDC were pre-treated with the pan-caspase inhibitor ZVAD-fmk. Pre-treatment of BMDC with ZVAD-fmk had no effect on the level of cell death induced by PVA, suggesting that particulate-induced cell death

occurs independently of caspases. However, ZVAD-fmk does not inhibit all caspases equally and also has non-specific activities on other proteases such as cathepsins and calpains [599]. Furthermore, a recent report has suggested that when this inhibitor is used and has an effect, that it should be reported that the cell death is 'ZVAD-fmk inhibitable' rather than caspase-dependent [599]. Therefore, the results from this current study demonstrate that the type of cell death induced by particulates is not 'ZVAD-fmk inhibitable'. Overall, these studies into the type of cell death induced by particulates have demonstrated that it results in loss of membrane integrity, occurs independently of NLRP3 and cathepsin B and is not blocked by ZVAD-fmk. However, chemical inhibition of phagocytosis by cytochalasin D abrogated the cytotoxic effects of alum. Furthermore, smaller particles (50 nm) which are readily phagocytosed are more toxic to DC than larger particles (10 μ m), which are not easily taken up by DC. Taken together, these results strongly suggest that particle-induced cell death requires phagocytosis of the particle. This provides some insight into the mechanism of particle-mediated cell death.

These studies into particulate-induced cell death indicate that particles do not induce apoptosis, as shown by loss of membrane integrity or pyroptosis due to the fact that the release of IL-1 β by particulate adjuvants seems to be unrelated to their toxic nature. Finally, they do not promote pyronecrosis as they can still induce cell death in the absence of NLRP3 and when cathepsin B is inhibited. However, the particles must be phagocytosed in order to exert their cytotoxic effects. Further studies will be necessary to investigate the exact morphological changes that are induced during particle-induced cell death and also to identify potential mediators of this process.

PVA can synergise with TLR agonists to enhance the production of IL-1 β by BMDC (Figures 3.3-3.6). Although this enhancement is reliant on the presence of a second signal *in vitro*, further studies have demonstrated that subcutaneous injection of particulates alone is sufficient to drive the local production of IL-1 β (Figure 4.5). These findings suggest the existence of an endogenous molecule with the potential to synergise with particles to enhance IL-1 β . Based on the fact that particulates have been shown to cause local necrosis *in vivo* (Figures 4.2-4.4), it is possible that this cell death leads to the release of an endogenous molecule with the potential to synergise with particulates.

HMGB1 is one of the most studied danger signals and has been shown to be released by necrotic cells [11]. Moreover, HMGB1 was initially identified as a mediator of endotoxin-induced septic shock and has been associated with the immunopathology observed in autoimmune disorders, such as systemic lupus erythematosus (SLE) [611-614]. In addition, previous findings have shown that treatment of BMDC with alum alone is sufficient to drive the release of the endogenous danger signal, HMGB1 (Coleman M and Lavelle EC Unpublished findings). Given the established role of HMGB1 as an endogenous danger signal, its ability to synergise with PVA was analysed. In a similar manner to earlier studies using TLR agonists (Figures 3.3-3.6), treatment of BMDC with HMGB1 alone was inefficient at driving the secretion of IL-1 β . However, when BMDC were pre-treated with PS, a significant enhancement in the secretion of IL-1 β by cells was observed. These findings demonstrate that particulates have the ability to synergise with endogenous danger signals, suggesting that the local IL-1 β observed at the site of particulate injection maybe due to the release of endogenous danger signals and that these molecules replace microbe-derived TLR agonists as the second signal *in vivo*. HMGB1 has previously been shown to promote the recruitment and activation of DC. Therefore, its release could be important for the promotion of the immune response through its actions on APC [613, 615]. Furthermore, HMGB1 has been shown to associate with other pro-inflammatory molecules including IL-1 β and this is a beneficial partnership as it enhances the activity of IL-1 β [616]. Thus, HMGB1 released as a result of particulate-induced cell death could potentially promote the local secretion of IL-1 β and subsequently enhance the activity of the released IL-1 and activate recruited APC. However, before any of these putative effects of HMGB1 can be investigated, further studies are necessary to confirm the local release of HMGB1 *in vivo* and also the possibility that other danger signals may also be involved.

Several studies have demonstrated the adjuvant properties of necrotic cells and the cytosolic contents of cells [11, 545, 584-585]. Particulate-induced cell death promotes loss of membrane integrity, thereby allowing for the release of endogenous molecules with the potential to act as adjuvants. Therefore, supernatants from splenocytes treated with alum were used as a putative adjuvant and co-administered with Ova. The original hypothesis suggested that the adjuvant activities of particulates may be due to their ability to induce the release of endogenous pro-inflammatory molecules by promoting a

necrotic-like cell death. In agreement with this, the supernatant isolated from alum treated spleen cells displayed adjuvant properties when co-injected with Ova. Supernatant from alum-treated cells injected with Ova resulted in enhanced production of antigen-specific IL-5, IL-4, IFN- γ and IL-17 in the spleen and increased local IFN- γ , in comparison to injection of Ova alone. These results are in agreement with other studies that have demonstrated the adjuvant properties of necrotic cells [11, 545, 584-585].

Several of the identified endogenous danger signals, including HMGB1 and biglycan, have been shown to signal via TLR4 [42-44]. Therefore, the role of TLR4 in the adjuvant properties of supernatants isolated from alum-treated cells was also investigated. The enhancement of antigen-specific IL-5, IL-4 and IFN- γ was dependent on the presence of TLR4. These effects were abrogated in C3H/HeJ mice, which contain a mutation in the signalling domain of TLR4 rendering them hypo-responsive to TLR4 agonists. In contrast, the enhancement of antigen-specific IL-17 was only present in the C3H/HeJ mice, suggesting that somehow TLR4 signalling prevents the secretion of IL-17 promoted by supernatant isolated from splenocytes treated with alum. This negative regulation of IL-17 production by TLR4 is in agreement with a recent study, which demonstrated a role for TLR4 in the regulation of CD4 T cell effector responses [617]. This study demonstrated that in a model of spontaneous colitis, mice defective in TLR4 and IL-10 signalling displayed signs of inflammation earlier than mice deficient in IL-10 alone [617]. Given the enhanced inflammation observed in the absence of TLR4, the authors suggested that TLR4 plays a regulatory role in the activation of TCR-dependent CD4 effector cells [617]. The findings from this current study demonstrate that the supernatant from alum-treated cells have adjuvant properties, with the ability to enhance cell-mediated responses in a manner which is dependent on TLR4.

The results from these current studies into the toxicity have demonstrated that PVA are toxic to DC and splenocytes. Moreover, particulate-induced cell death promotes loss of plasma membrane integrity. Furthermore, they have demonstrated that the toxic effect of particles occur independently of their ability to activate the NLRP3 inflammasome. However, particle-induced cell death is dependent on phagocytosis of the particles, giving some insight into the possible mechanisms of particle-mediated cell death. Although the exact mechanism of particulate-induced cell death remains elusive, these studies have ruled out several types of cell death, including pyronecrosis, apoptosis and

pyroptosis and demonstrated that particle uptake is necessary. Particulate-induced cell death does promote the release of soluble mediators and potential danger signals, which demonstrate adjuvant properties *in vivo*. Furthermore, these endogenous molecules could act as mediators of the adjuvant effects of PVA.

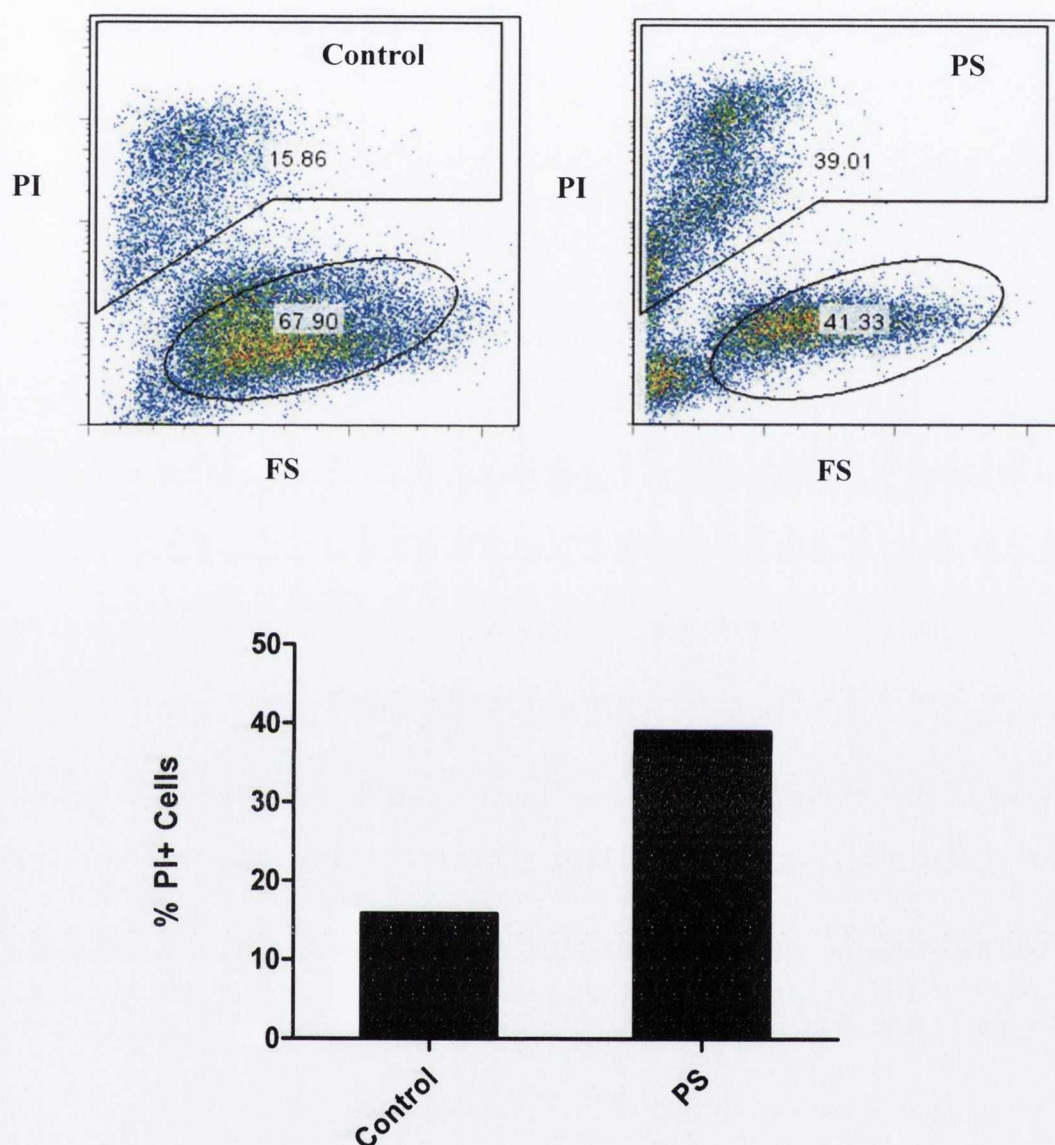


Figure 5.1: PS are toxic to BMDC. BMDC (6.25×10^5 cells/ml) were incubated with medium alone or PS (430nm; 3 mg/ml) for 24 h. After stimulation, cells were harvested, re-suspended in FACS buffer (2.1.5.2) and cell viability was determined by uptake of PI by flow cytometry (2.2.3). Toxicity is expressed as the percentage of PI-positive cells. (FS: Forward scatter, PI: Propidium iodide)

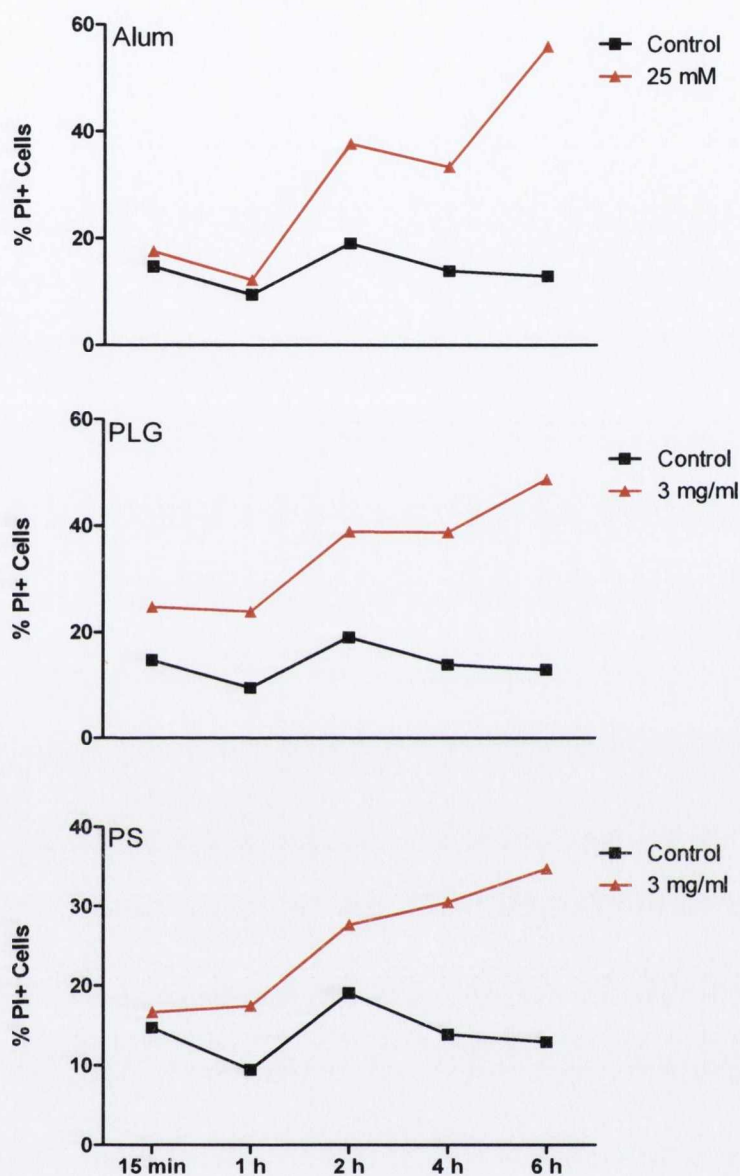


Figure 5.2: Kinetics of the toxic effect of particles on BMDC. BMDC (6.25×10^5 cells/ml) were incubated with medium alone or either alum, PLG (1 μ m) or polystyrene PS (430 nm) for 15 min, 1, 2, 4 or 6 h. After stimulation, cells were harvested and re-suspended in FACS buffer (2.1.5.2). PI (1 μ g/ml) was added to samples and cells were analysed for PI uptake by flow cytometry (2.2.3). Cell death is expressed as the percentage of PI-positive cells.

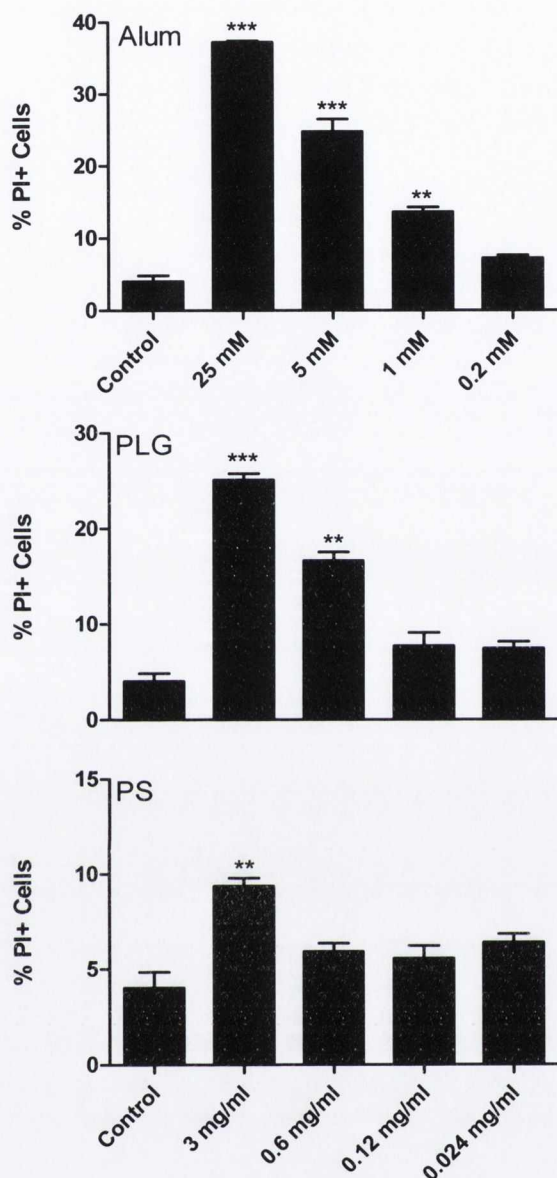


Figure 5.3: Alum, PLG and PS are toxic to BMDC at high concentrations. BMDC (6.25×10^5 cells/ml) were incubated with medium alone or either alum, PLG (1 μ m) or PS (430nm) for 6 h. After stimulation, cells were harvested and re-suspended in FACS buffer (2.1.5.2). PI (1 μ g/ml) was added to samples and cells were analysed for PI uptake by flow cytometry (2.2.3). Cell death is expressed as the percentage of PI-positive cells. Control v Particle, ** $p < 0.01$, *** $p < 0.001$

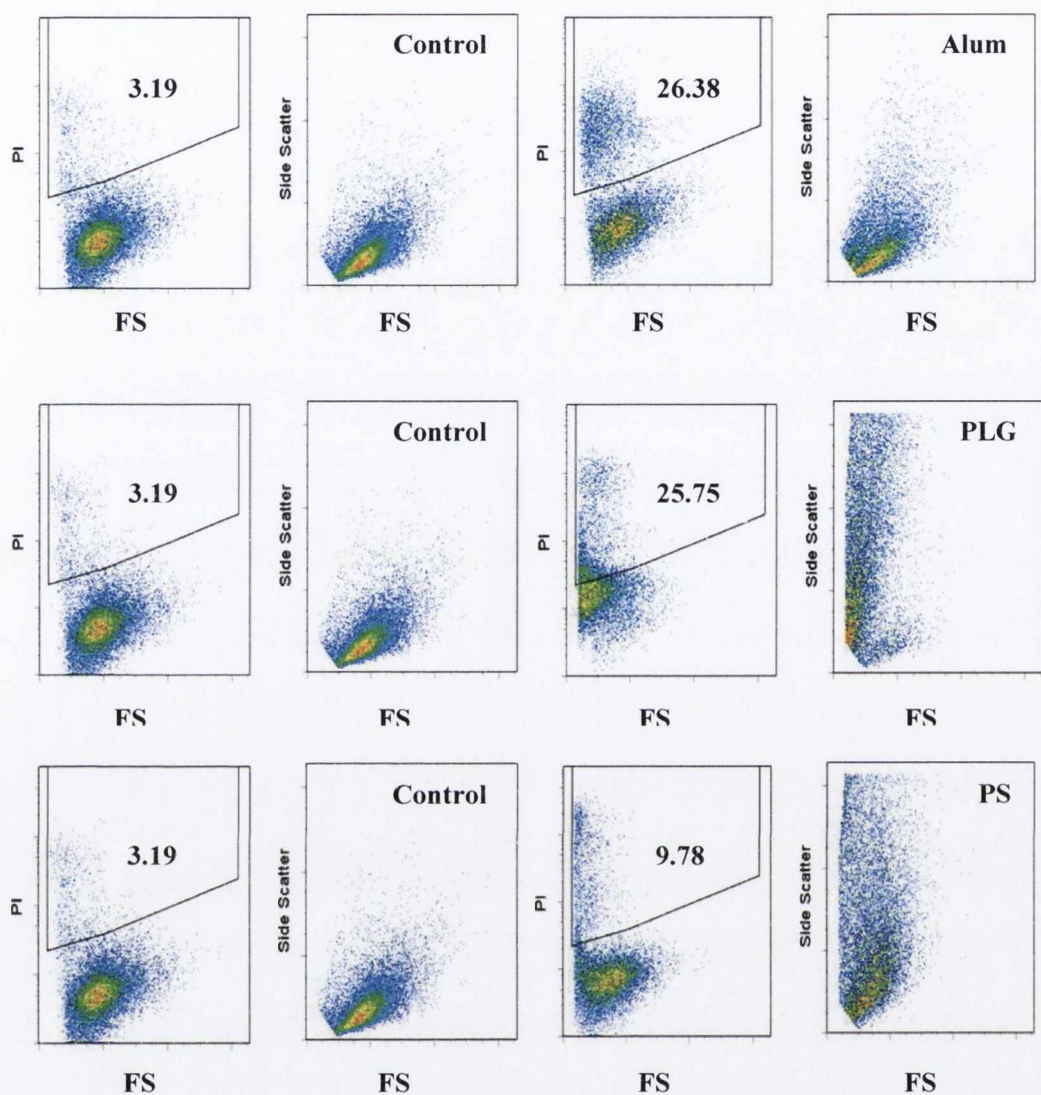


Figure 5.4: Scatter profile of BMDC treated with alum, PLG or PS. BMDC (6.25×10^5 cells/ml) were incubated with medium alone or either alum (25mM), PLG ($1 \mu\text{m}$; 3mg/ml) or PS (430nm; 3mg/ml) for 6 h. After stimulation, cells were harvested and re-suspended in FACS buffer (2.1.5.2). PI ($1 \mu\text{g/ml}$) was added to samples and cells were analysed for PI uptake by flow cytometry (2.2.3). (FS: Forward scatter)

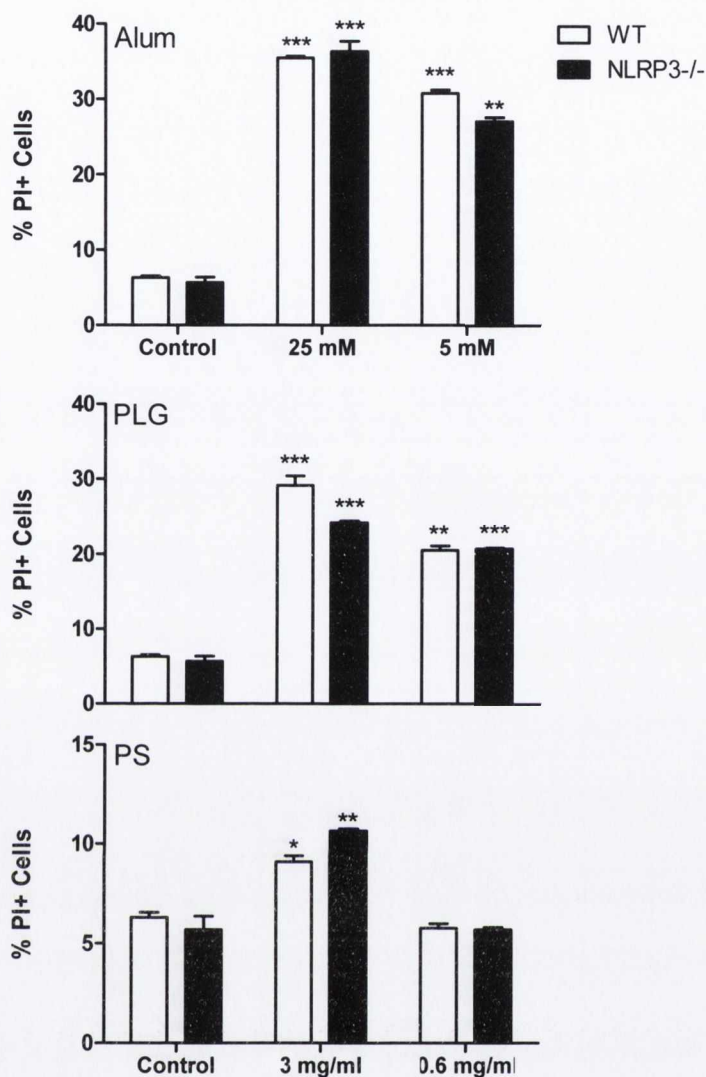


Figure 5.5: Particle-induced cell death occurs independently of NLRP3. BMDC (6.25×10^5 cells/ml: WT or NLRP3^{-/-}) were incubated with medium alone or alum (25 or 5 mM), PLG (1 μ m) or PS (430nm: 3 or 0.6 mg/ml) for 6 h. After stimulation, cells were harvested and re-suspended in FACS buffer (2.1.5.2). PI (1 μ g/ml) was added to samples and cells were analysed for PI uptake by flow cytometry (2.2.3). Cell death is expressed as the percentage of PI-positive cells. Control v Particle, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Particle WT v Particle NLRP3^{-/-} # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$.

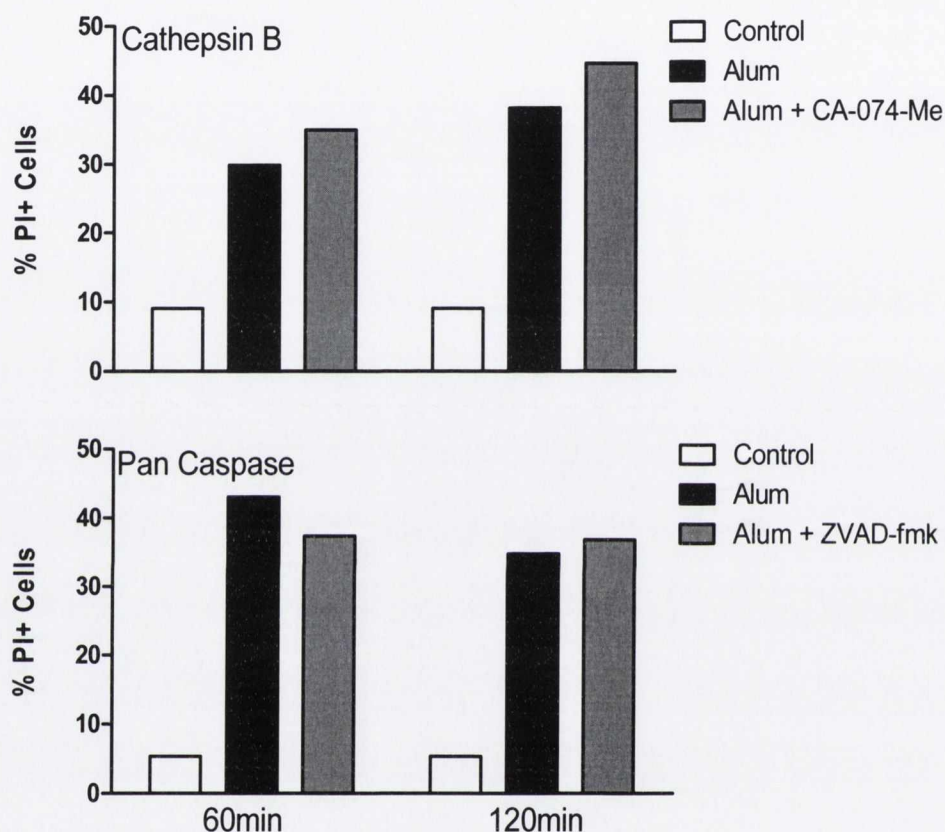


Figure 5.6: Particulate-induced cell death does not require the activation of caspases or cathepsin B. BMDC (6.25×10^5 cells/ml) were incubated with either medium, CA-074-Me (10 μ M) or ZVAD-fmk (40 μ M) for 30 min followed by alum (25 mM). After 60 or 120 min, cells were harvested and re-suspended in FACS buffer (2.1.5.2). PI (1 μ g/ml) was added to samples and cells were analysed for PI incorporation by flow cytometry (2.2.3). Cell death is expressed as the percentage of PI-positive cells.

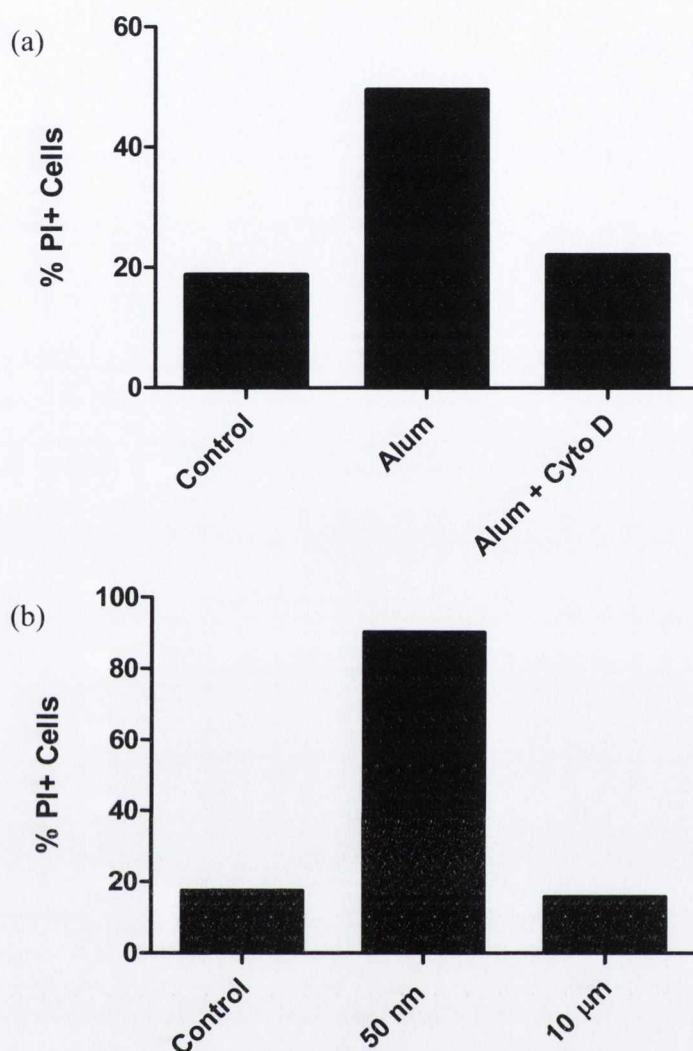


Figure 5.7: Particulate-induced cell death requires particle uptake. (a) BMDC (6.25×10^5 cells/ml) were incubated with either medium, or cytochalasin D ($5 \mu\text{M}$) for 30 min followed by alum (25 mM). (b) BMDC (6.25×10^5 cells/ml) were incubated with either medium, or PS (50 nm or 10 μm). (a,b) After 6 h, cells were harvested and re-suspended in FACS buffer (2.1.5.2). PI (1 $\mu\text{g/ml}$) was added to samples and cells were analysed for PI incorporation by flow cytometry (2.2.3). Cell death is expressed as the percentage of cells out of the total cell number that have incorporated PI.

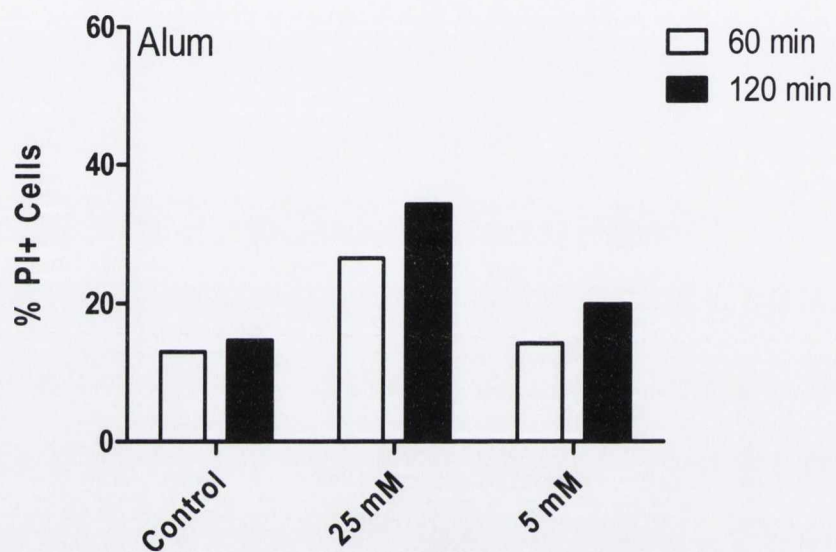


Figure 5.8: PVA are toxic to splenocytes. Isolated splenocytes (6.25×10^5 cells/ml) were incubated with either medium alone or alum (25 or 5 mM) for either 60 or 120 min. After stimulation, cell viability was determined by PI incorporation using flow cytometry (2.2.3). Cell death is expressed as the percentage of PI-positive cells.

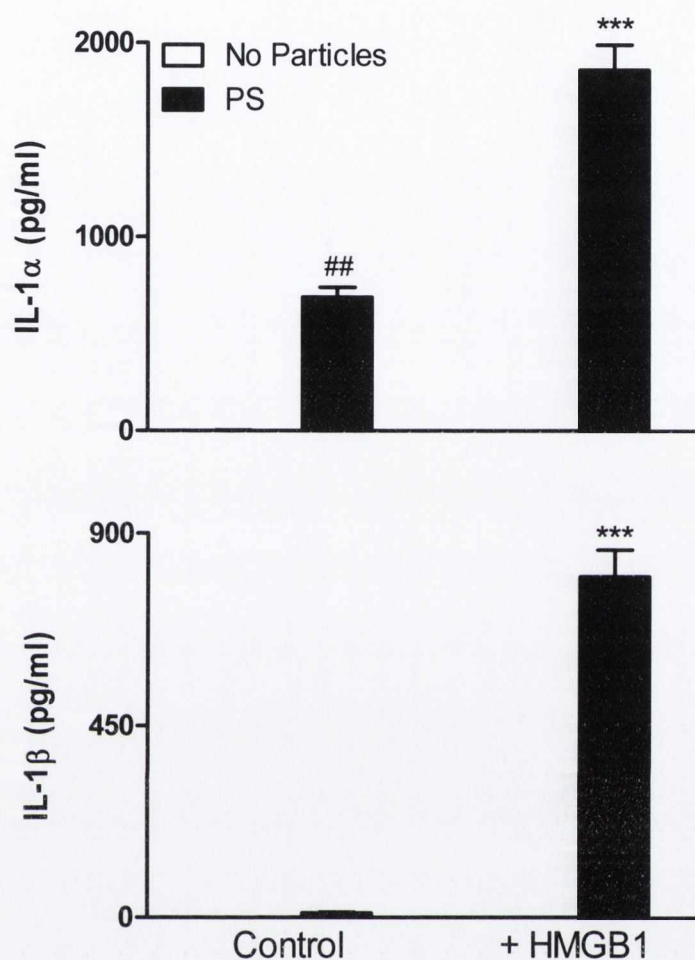


Figure 5.9: PVA synergise with the endogenous danger signal HMGB1 to enhance IL-1 secretion by BMDC. BMDC (6.25×10^5 cells/ml) were pre-incubated with medium or PS (430nm; 3 mg/ml) alone for 1 h before the addition of HMGB1 (50 μ g/ml) or medium. After 24 h, supernatants were collected and analysed for IL-1 β and IL-1 α by ELISA. Particles + HMGB1 v HMGB1 only, *** $p < 0.001$; Control v HMGB1 alone or Particle alone, ## $p > 0.01$

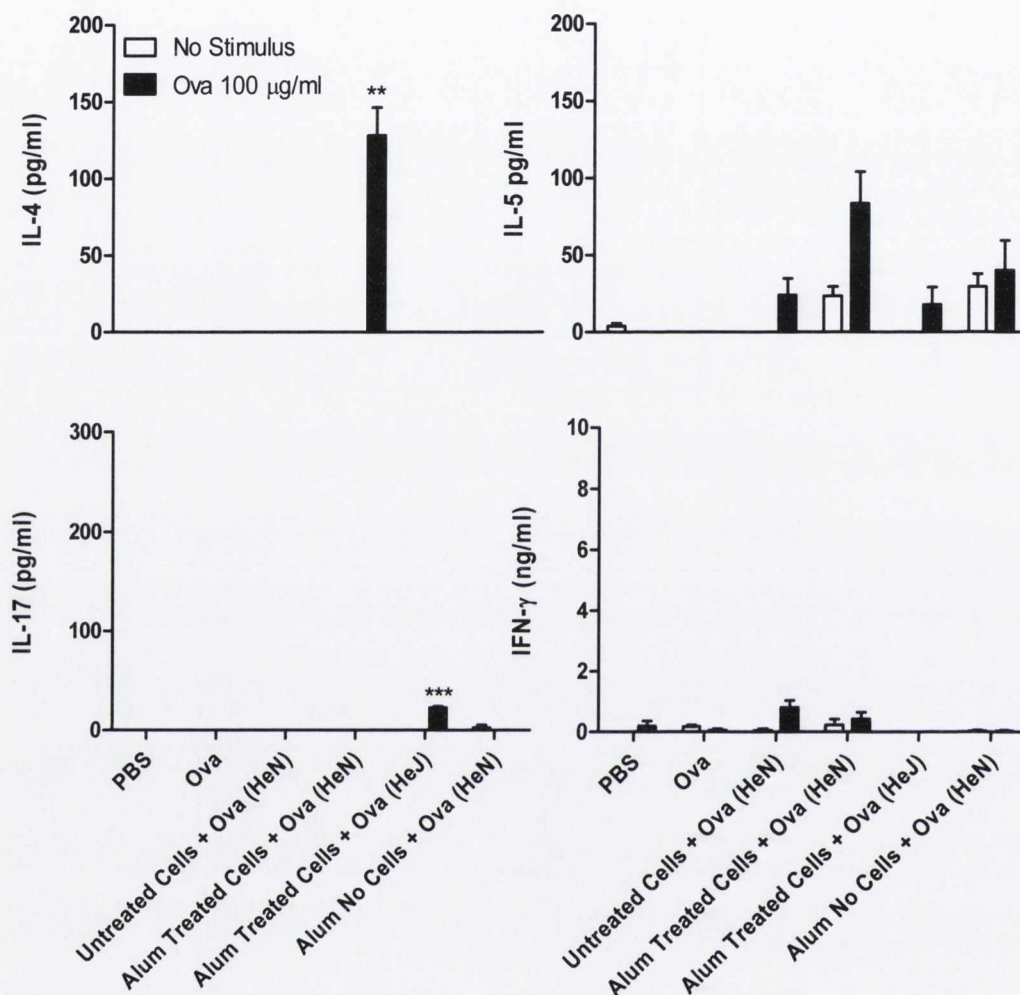


Figure 5.10: Immunisation with supernatant from alum-treated splenocytes induces enhanced secretion of antigen-specific IL-4, IL-5 and IL-17 in the spleen. Female C3H/HeN and C3H/HeJ (TLR4 mutation) mice were immunised intraperitoneally with PBS, Ova alone, or Ova with supernatant from untreated splenocytes, splenocytes treated with alum (25 mM) or medium with alum alone. Splenocytes were isolated and stimulated (2×10^6 cells/ml) with medium or Ova (100 μg/ml). After 3 days, supernatants were collected and analysed for cytokines by ELISA (2.2.5). Ova alone v Ova + adjuvant, * $p < 0.05$.

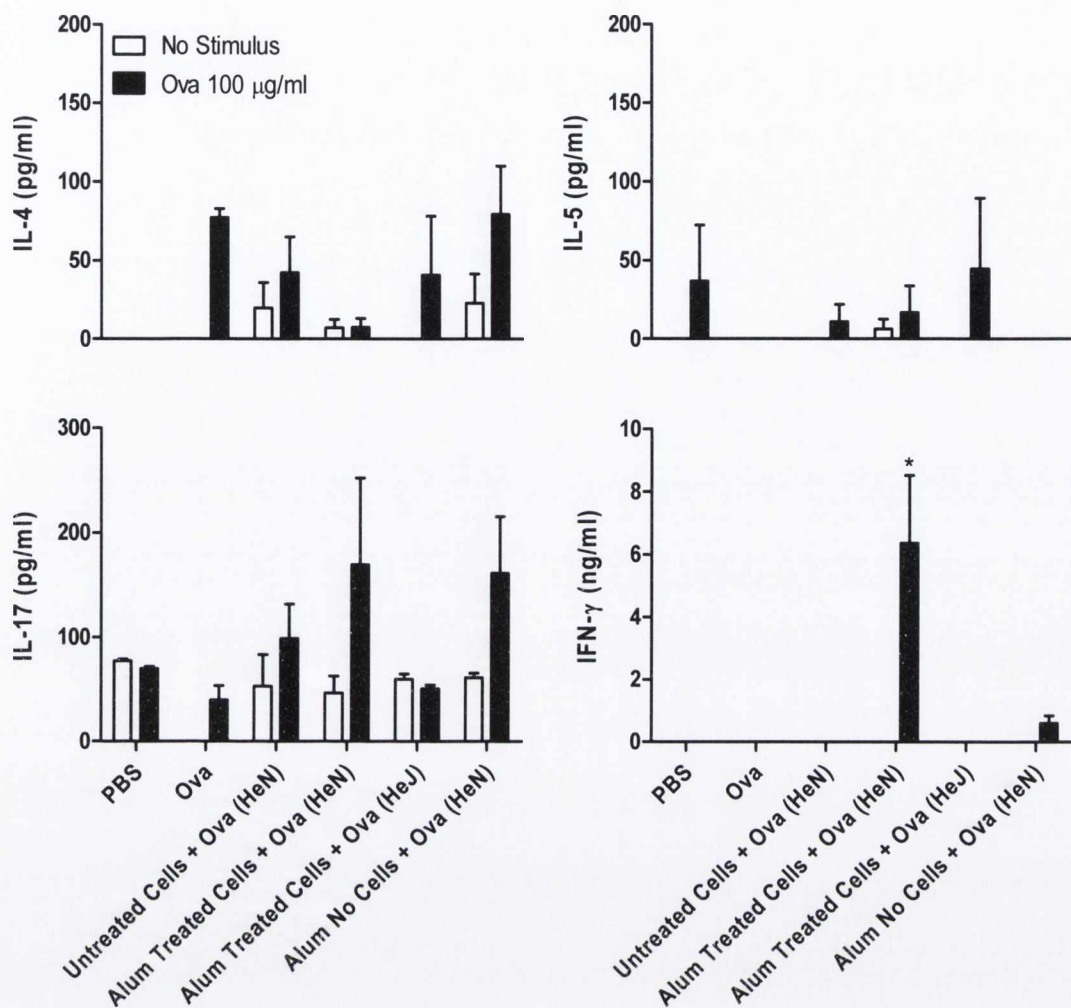


Figure 5.11: Immunisation with supernatant from alum-treated splenocytes induces enhanced secretion of antigen-specific IFN- γ by PEC. Female C3H/HeN and C3H/HeJ (TLR4 mutation) mice were immunised intra-peritoneally with PBS, Ova alone, or Ova with supernatant from untreated splenocytes, splenocytes treated with alum (25 mM) or medium with alum alone. Peritoneal exudate cells (PEC) were isolated and stimulated (1×10^6 cells/ml) with medium or Ova (100 μ g/ml). After 3 days, supernatants were collected and analysed for cytokines by ELISA (2.2.5). Ova alone v Ova + adjuvant, * $p < 0.05$.

Chapter 6

General Discussion

6. General Discussion

Some of the first vaccine adjuvants tested were particulate in nature. For example, Glenny used 'potash' alum in the early 1920s as an adjuvant for tetanus toxoid [377]. Despite the use of particulate vaccine adjuvants (PVA) for over 80 years, the exact mechanism of their action remains elusive. This present study investigated the mechanism of action of PVA using alum, PLG microparticles and polystyrene microparticles. Initial studies focused on the effect of particulates on DC *in vitro*. Following on from these studies, the effects of PVA *in vivo* both locally and systemically were investigated. The overall aim of this study was to improve our understanding of how particulates enhance both cellular and humoral immune responses.

PVA can activate the NLRP3 inflammasome

Recently, there has been a renaissance of interest in the area of PVA and research in this area has led to significant progress in understanding how they boost immune responses. One major discovery has been the ability of alum to promote the secretion of IL-1 β and IL-18 through activation of the NLRP3 inflammasome [105-107, 118, 165, 457-458]. Findings from this current study have also demonstrated that alum can activate the NLRP3 inflammasome and promote the secretion of IL-1 β and IL-18. In addition, pre-treatment of BMDC with PLG or PS prior to the addition of a TLR agonist enhanced secretion of IL-1 β and IL-18 suggesting that the ability to activate the NLRP3 inflammasome is a common feature of all particulates. Furthermore, activation of the NLRP3 inflammasome by particulates is dependent on K⁺ efflux and phagocytosis, which results in lysosomal disruption and subsequent release of cathepsin B. These results suggest the existence of a general pathway in DC which is activated in response to the phagocytosis of particulates. In agreement with this, other particulates including asbestos, silica and amyloid- β have all been shown to activate the NLRP3 inflammasome [104, 106, 134].

Given the ability of PVA to activate the NLRP3 inflammasome, several studies have investigated the role of NLRP3 in the adjuvant activities of alum *in vivo* [105, 107-108, 118-119]. Despite unanimous agreement with respect to the ability of alum to activate the NLRP3 inflammasome *in vitro*, the role of NLRP3 in the adjuvant effects of alum *in vivo* remains controversial. Several studies have reported a role for NLRP3 in the

recruitment and activation of innate immune cells, which impacts on antigen-specific adaptive responses, as indicated by a reduction in antigen-specific T cell cytokines and accumulation of antigen-reactive T cells in local lymph nodes [105, 107]. In agreement with these studies, NLRP3^{-/-} mice immunised with PLG showed a decrease in the number of activated CD11b⁺ Gr1⁺ cells recruited into the injection site. Furthermore, production of antigen-specific IL-6 was also significantly reduced in NLRP3^{-/-} mice. Taken together, these current findings suggest that PVA activate the NLRP3 inflammasome *in vivo* and that this is required for their ability to promote cell-mediated responses. In contrast to this however, McKee *et al* have reported that NLRP3^{-/-} and caspase-1^{-/-} mice show no defects in their ability to prime either CD4 or CD8 antigen-specific cells when immunised with alum and Ova [119].

In addition to cell-mediated responses, studies have reported mixed results with respect to the role of NLRP3 in particulate vaccine-mediated humoral responses [105, 107-108, 118-119]. Some studies have shown a requirement for NLRP3 in alum-enhanced antibody production [105, 107-108], while others reported that there were no defects in alum-induced humoral responses in NLRP3^{-/-} mice [118-119]. In agreement with the latter studies, these current findings have shown that PLG-enhanced antibody production was not compromised in NLRP3^{-/-} mice.

The current data suggest that, NLRP3 is required for cell-mediated responses and dispensable for humoral responses induced by particulate vaccine adjuvants, which is in agreement with some studies and in contrast to others involving alum (Figure 6.1). However, a study using a chitosan based particulate adjuvant adds further support to the results from the PLG study, demonstrating that while cell-mediated immunity was reduced in NLRP3-deficient mice the chitosan-mediated humoral responses were unaffected. Furthermore, studies involving chitosan have also demonstrated that the nature of the adjuvant system is a key determining factor as to whether or not NLRP3 is required. In this case, the combination of a TLR agonist, CpG, with chitosan induced enhanced levels of antigen-specific IL-17 and IFN- γ in a NLRP3-dependent manner (Mori A and Lavelle EC Manuscript in preparation). These results strongly suggest that, while NLRP3 is required for the promotion of cell-mediated responses it is dispensable for the enhanced production of antibodies. Moreover, they suggest that distinct combinations of adjuvants, such as chitosan and CpG, are perceived differently

by the innate immune system and that this determines whether or not NLRP3 is required by the adjuvant.

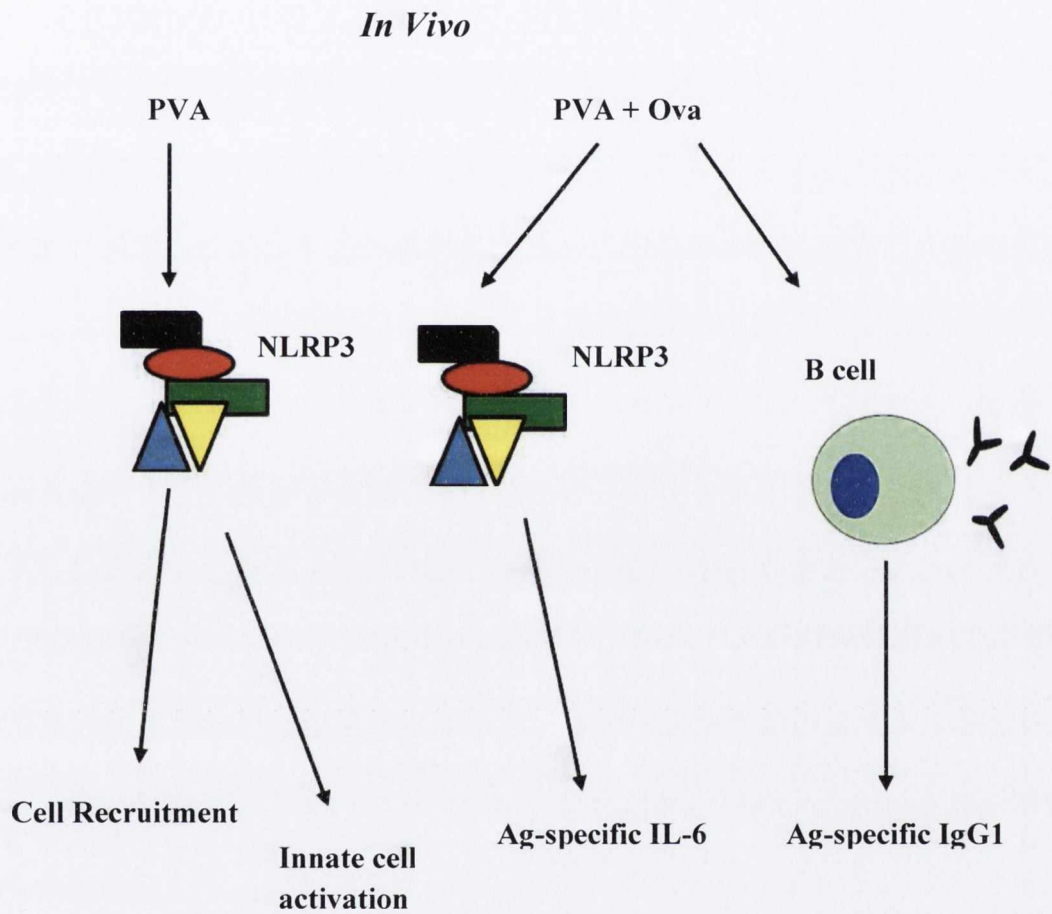


Figure 6.1: PVA activate the NLRP3 inflammasome and are dependent on NLRP3 for cell-mediated responses. However, PVA-mediated humoral responses occur independently of NLRP3.

PVA display immunostimulatory activities in vivo

Previous studies have suggested that IL-1 may have the ability to promote the development of CD4 helper T cell responses and also boost primary responses when co-injected with antigens [618-619]. Furthermore, it has recently been suggested that IL-1 has the ability to enhance the proliferation of CD4 T cells thus promoting the development of helper T cell effector functions [570]. Kinetics studies of the immunomodulatory effects of alum have demonstrated that, splenocytes isolated from IL-1R1^{-/-} mice 7 days following injection of alum are primed to secrete significantly higher amounts of IL-1, an effect which was not observed in WT mice. These differences may be due to rapid receptor binding of IL-1 in the WT mice, preventing detection of IL-1. The kinetics of this IL-1 production strongly suggests that this enhancement of IL-1 by alum may be key to its ability to promote cell-mediated immunity. This increase in IL-1 may be important for the alum-promoted expansion of CD4 T helper cells and subsequent cell-mediated responses. Moreover, spleen cells from NLRP3^{-/-} mice which are unable to produce IL-1 secrete less IL-6 than spleen cells from wild type mice when stimulated with antigen *ex vivo*. In further agreement with these results and the findings from the NLRP3^{-/-} study, IL-1R1^{-/-} mice display differential kinetics in their development of cell-mediated responses when injected with alum alone. In general, the enhancement of IFN- γ and IL-10 by alum injection occurred earlier and was maintained for longer in IL-1R1^{-/-} mice immunised with alum in comparison to wild type mice. These results suggest that IL-1 may play a regulatory role in the activation of cell-mediated responses by alum.

One of the major limitations of alum as a vaccine adjuvant is its inability to promote strong cell-mediated responses and its natural bias towards a Th2 phenotype [454, 620]. So far, there is no explanation for the Th2 bias observed with alum. It has previously been suggested that inflammasome-dependent members of the IL-1 family, including IL-1 β and IL-18 may be responsible [105]. In agreement with this, Eisenbarth *et al* have reported a reduction in the Th2-associated cytokine IL-5 in an alum-dependent asthma model in NLRP3^{-/-} mice, suggesting that activation of the NLRP3 inflammasome may be required for the cell-mediated Th2 bias observed with alum [105]. In contrast to the cellular immune responses, the antibody profile reported for these NLRP3-deficient mice did not suggest that there was a switch in the type of humoral response, but merely a reduction in the titres of antigen-specific IgG1 and IgE antibodies [105, 107]. One

study has reported that antigen-specific IgG2c titres, a Th1-associated antibody isotype, were significantly higher in the NLRP3^{-/-} mice, however, the titres of IgG1 were also enhanced in the NLRP3^{-/-} mice in comparison to wild type mice [107]. Furthermore, it has recently been shown that IL-33, a known inducer of Th2 responses, does not require processing by caspase-1 in order to become biologically active and is in fact inactivated by proteolysis by apoptotic caspases including caspase-3 and caspase-7 [621]. While this demonstrates that the NLRP3 inflammasome and caspase-1 are not required for the production of bioactive IL-33, it does not however rule out a role for IL-33 in the Th2 bias of alum. A role for IL-18 in the Th2-promoting effects of alum has been investigated and it was reported that IL-18^{-/-} mice produced less IL-4 than their wild type counterparts, while the absence of IL-18 had no effect on Th2-associated antibodies [622].

Current theories with regard to the Th2 bias observed with PVA suggest that, the Th2 phenotype is favoured by the production of Th2-promoting factors, such as IL-4, which suppress the development of Th1 cells [452, 456]. In contrast to this, the current work suggests that it is the enhancement of soluble factors with the potential to inhibit the development of Th1 responses that is the driving force for the Th2 bias. Injection of alum alone is sufficient to prime spleen cells to secrete IFN- γ . This suggests that alum has the ability to promote Th1 responses. However, in addition to this enhancement of IFN- γ , alum also primes spleen cells to produce IL-10. Previous studies have demonstrated that, IL-10 has the ability to inhibit the development of Th1 responses in chronic viral infections, such as lymphocytic choriomeningitis (LCMV) and during infection with the intracellular pathogen *Mycobacterium avium* [561-562]. The result of the increase of IL-10 in both of these cases is reduced clearance and increased persistence due to defective protective immune responses. Furthermore, it has been shown that the co-administration of an IL-10R blocking antibody with LPS-contaminated Ova promoted the development of Th1 type responses as characterised by high levels of antigen specific IFN- γ [563]. Given the ability of IL-10 to prevent the development of Th1 responses *in vivo*, it is possible that this early increase in IL-10 promoted by alum is sufficient to dampen the IFN- γ and promotes Th2 responses. In agreement with this, studies in the lab have demonstrated that, following intraperitoneal injection of Ova with alum, IL-10^{-/-} mice produced significantly higher levels of antigen-specific IFN- γ than wild type mice (Ewa Oleszycka and Lavelle EC

Unpublished Data). No effects were observed on the ability of alum to promote humoral immunity in IL-10^{-/-} mice. These preliminary findings support the theory that the early IL-10 induced by PVA plays an important role in the development of T helper cell effector functions.

Findings from these studies suggest that enhancement of IL-1 may be central to the ability of particulates to promote cell-mediated responses and that early IL-10 production promotes the development of Th2 responses and dampens down the production of Th1-associated cytokines. Neither of these results however, explains the potent humoral immune responses observed with PVA. In agreement with the findings of the NLRP3 study, others have demonstrated that alum-induced antibody responses occur independently of the TIR adaptor, MyD88, which rules out a role for IL-1 or IL-18 [459-460]. Previous studies have shown that alum has the ability to prime B cells for MHC class II stimulation via enhanced intracellular Ca₂⁺ levels and promotes the accumulation of IL-4-producing Gr1⁺ cells, later identified as eosinophils, which were shown to be required for early IgM responses [451-453]. However, studies by Brewer *et al* have suggested that antibody production by particulates occurs independently of IL-4, suggesting that these previously identified IL-4 producing eosinophils are not required for the production of antibodies [455]. Furthermore, eosinophils have been shown to be dispensable in the adjuvant effects of alum as intact antibody and T cell responses to alum-adjuvanted Ova have been reported in eosinophil-deficient mice [119]. Therefore, studies to date have been unable to identify a central mediator of particulate induced humoral immune responses.

In addition to the enhancement of IL-10 observed when PVA are injected subcutaneously, these kinetics studies have also demonstrated that PLG induce enhanced expression of IL-21 mRNA in spleen cells. A subset of CD4⁺ T cells expressing high levels of CXCR5 with the ability to activate B cells and promote antibody production were identified in human tonsillar tissue [327-328]. These cells are now known as T follicular helper cells (Tfh) and have previously been shown to produce significant levels of IL-21 [578]. Tfh cell development requires IL-21 and these cells are thought to play key roles in the formation of germinal centres which are essential to the maturation of humoral responses [332, 339]. Furthermore, IL-21 induces the expression of BLIMP-1 in B cells, promoting their differentiation into plasma cells which produce significant amounts of antibodies and a few remaining

plasma cells act as a continuous source of antibodies, which provides immediate protection during subsequent pathogen encounters [339]. More recently, studies have shown that IL-21 acts directly on B cells and that IL-21^{-/-} mice display impaired affinity maturation along with reduced germinal centre persistence and plasma cell differentiation [623-624]. Therefore the ability of PLG microparticles alone to promote the transcription of IL-21 could potentially promote the development of Tfh cells with the ability to promote the development and maturation of B cell responses.

Subcutaneous injection of PLG or alum alone also primes splenocytes to produce significant amounts of IL-6. IL-6 itself can induce the production of IL-21 and Tfh cells have been shown to require IL-6 for their development [332, 577]. Therefore, the combined enhancement of both IL-21 and IL-6 by PVA potentially provides the optimal conditions for Tfh cell development. However, NLRP3^{-/-} mice showed a significant reduction in their ability to secrete antigen-specific IL-6 and had no defect in the levels of antigen-specific antibodies produced. Furthermore, previous studies have demonstrated that IL-6^{-/-} mice produce normal levels of antibodies when immunised with alum [454]. These studies suggest that IL-6 may have a redundant role in the production of antibodies in response to particulates. However, the enhancement of IL-6 was observed very early on in the response and was produced in an antigen-independent manner. Therefore, it is possible that this early IL-6 promotes IL-21 expression and the development of Tfh cells. Further studies will be necessary in order to identify whether or not this enhancement of IL-21 is a common trait of all particles and to investigate the role of IL-21 in the adjuvant properties of particulates. However, it is tempting to speculate that IL-21 may be the key to particulate-induced antibody responses.

In addition to its immunostimulatory effects, IL-21 has also been shown to exert negative effects, such as inducing B cell apoptosis and inhibiting the function and maturation of DC [372, 625-626]. More recently it has been suggested that some of the suppressive effects exerted by IL-21 are the result of its ability to promote the secretion of IL-10 [627]. Furthermore, cells incubated in Th1 polarising conditions in the presence of IL-21 secreted less IFN- γ and showed an increase in their expression of IL-10 mRNA, in comparison to cells that were not stimulated with IL-21 [627]. The Th1 cells stimulated with IL-21 were shown to co-produce IL-10 and IFN- γ and these double producers showed reduced proliferation in response to antigen [627]. Given the ability of IL-21 to mediate suppressive effects through the enhancement of IL-10, it is

possible that a positive interaction could exist between the initial IL-21 and IL-10 induced by PVA. The early IL-21 enhancement could promote the induction of IL-10. Furthermore, both IL-10 and IL-21 have been shown to have inhibitory effects on the development and activities of Th1 cells, respectively [563, 627]. Therefore, this combination may be important in determining the type of effector T cell responses that develop in response to particulates. It has also been reported that IL-6^{-/-} mice immunised with alum show enhanced production of both Th1 and Th2 associated cytokines suggesting that IL-6 plays a regulatory role in the development of cell-mediated responses by PVA [454]. Given the ability of IL-6 to promote the expression of IL-21 and the fact that IL-21 can promote the secretion of IL-10, these cytokines have the potential to regulate each other and in some cases themselves, ultimately determining the development of T helper effector cells [332, 577, 627].

The effect of particle size on the adjuvant effects of particulate vaccine adjuvants

One of the most interesting findings from these current studies, with significant relevance to the design and development of future vaccine adjuvants, is the relationship between the size of the particle and the type of immune responses induced. Smaller particles (50 nm) promoted significant cellular recruitment into the injection site, production of antigen-specific IL-17 and IFN- γ , as well as strong humoral responses. On the other end of the spectrum, larger particles (10 μ m), which were not phagocytosed, specifically recruited neutrophils into the injection site, primed cells to produce antigen-specific IL-4, IL-5, IL-10 and interestingly IFN- γ , although to a lesser extent than the other cytokines (Figure 6.2). In addition, these large particles also promoted strong humoral responses (Figure 6.2). The bias towards different types of cell-mediated responses observed with the smaller (50 nm), and larger particles (10 μ m) may reflect the different responses that are observed during infection with microorganisms that differ greatly in their size. For example, a virus, such as herpes simplex virus 1 (HSV1) specifically promotes strong cell-mediated responses, while a parasite, such as *Schistosoma mansoni* induces a more Th2 type humoral response [628-629].

The size of the particle had a dramatic effect on the type of antigen-specific cell-mediated response generated. The superiority of nanoparticles (polystyrene) for the induction of strong pro-inflammatory cell-mediated responses has previously been reported [547, 549]. These studies demonstrated that these polystyrene nanoparticles (40nm) were efficiently taken up by and activated DC specifically, by enhancing surface expression of co-stimulatory molecules, such as CD86 [547]. Further studies have shown that smaller particles exploit different mechanisms of uptake and are therefore targeted to different intracellular compartments. This in turn determines the efficiency of antigen presentation and subsequently the type of response that is elicited [630]. It is possible that the difference in size results in different cellular targeting, differential uptake pathways and altered antigen presentation which would explain the superior ability of smaller particles to promote IL-17 and IFN- γ . However, this cannot explain the ability of larger particles (10 and 30 μ m) to promote cellular responses distinct from those observed with the smaller particles (50 nm). The larger (≥ 10 μ m) cannot be phagocytosed by cells therefore the idea of distinct intracellular pathways post uptake cannot explain the ability of these larger particles to enhance cellular responses in a specific manner. *In vitro*, larger particles of up to 30 μ m in size still have the ability to synergise with TLR agonists to enhance the secretion of IL-1 β in comparison to stimulation with TLR agonists alone which suggests that despite their inability to be phagocytosed they can still potentially activate the inflammasome. Furthermore, stimulation of BMDC with polystyrene particles alone, regardless of their size, is sufficient to enhance the secretion of IL-1 α . Therefore, it is possible that these particles are enhancing local IL-1 production sufficiently to promote the recruitment of innate cells into the injection site and the subsequent development of cell mediated responses.

The mechanism by which these larger particles activate the inflammasome is as of yet unidentified. However, it is possible that ROS may be involved. Frustrated phagocytosis occurs when an APC attempts to phagocytose a particle which is too large to be engulfed. Previous studies have shown that 'frustrated phagocytosis' can enhance the levels of intracellular ROS and that the ROS then acts as a signal for formation and activation of the inflammasome and subsequent secretion of IL-1 [104]. In agreement with the idea of frustrated phagocytosis leading to inflammasome activation, the data presented in chapter 3 demonstrated that although larger particles cannot be

phagocytosed, APC still attempt to engulf them and these cells can be seen on the surface of the particles. Others have also found that APC can be observed attempting to phagocytose larger particles by associating with the surface of the particles [359, 631]. As previously mentioned, IL-1 has been shown to play a role in the expansion of CD4 effector functions and it has also been demonstrated that co-injection of IL-1 α enhances primary immune responses via direct effects on APC [570, 618-619]. Therefore, larger particles may be promoting cell-mediated responses by their ability to activate APC and promote ROS production by frustrated phagocytosis thus enhancing IL-1. Furthermore, 10 μ m polystyrene particles preferentially recruit neutrophils, which could also be explained by their ability to promote the secretion of IL-1 α , a known neutrophil chemoattractant, via enhanced ROS production and subsequent activation of the inflammasome [545, 632]. It is also possible that prolonged enhancement of ROS could lead to metabolic stress and subsequent cell death, which could also lead to release of IL-1 α , further potentiating the pro-inflammatory effects of these particles [633]. These current findings indicate that by simply changing the size of the particle the entire immune response can be re-directed.

Injection of polystyrene particles of any size enhanced the production of antigen-specific antibodies to comparable levels, which suggests that particle size and therefore particle uptake is not a determining factor in the promotion of humoral immune responses by PVA (Figure 6.2). In addition, no differences in IgG1 or IgG2b/c were observed between groups suggesting that the size of the particle does not determine the type of humoral response that is generated (Figure 6.2). Furthermore, despite the distinct cell-mediated responses observed with different sized particles there were no differences in the humoral responses suggesting that there is no correlation between the cell-mediated and humoral responses promoted by particulates. Several questions remain; including, how do the larger particles promote antibody production and what is the common pathway that is triggered by all particulates regardless of their size or nature that promotes humoral immunity? While there is a potential role for IL-1 in the development of cell-mediated responses it is unlikely that IL-1 also plays a role in the enhanced production of antibodies. In agreement with this, it has previously been shown that MyD88^{-/-} mice show no defect in their ability to promote antibody responses when immunised with alum [459-460]. Since injection of PLG enhanced the expression of IL-21 mRNA rapidly and enhanced levels of IL6 (chapter 3), these may mediate

particulate-induced antibody responses. Both IL-21 and IL-6 are required for the development of the main cells responsible for B cell help, Tfh cells [327-328, 332]. Therefore it is possible that the particulates promote the development of Tfh cells, thus contributing to their ability to enhance humoral immunity. Based on this, it will be of interest to investigate whether or not all particles can enhance the expression of IL-21 mRNA regardless of their size.

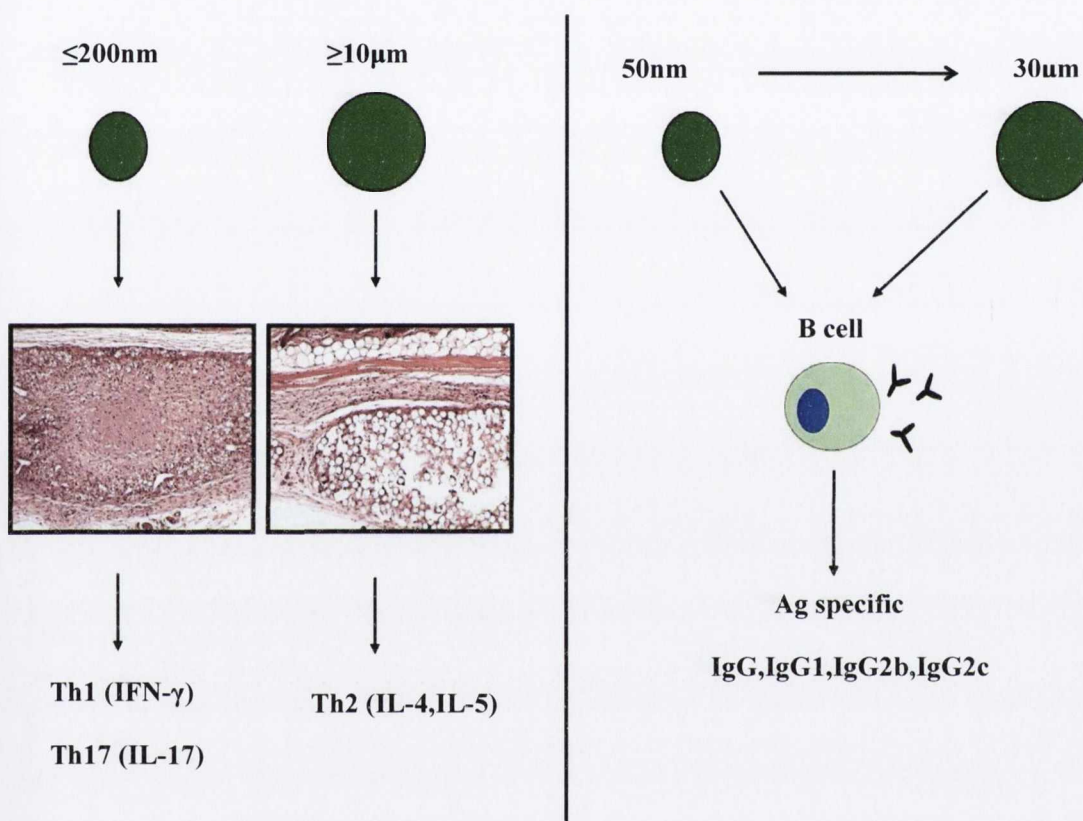


Figure 6.2: The effect of particle size on the adjuvant properties of particulates. The size of the particle determines the type of cell-mediated responses that is induced but does not effect the humoral responses.

The role of particle-induced cell death in the adjuvant properties of particulates

Another key characteristic of PVA which has been identified is their toxicity. The idea that uncontrolled cell death results in the release of pro-inflammatory endogenous danger signals is widely accepted [7, 583]. Previous reports have demonstrated that injury at the time of infection can boost protective responses [634]. Results from current findings demonstrate that at high concentrations, particulates are toxic to BMDC and spleen cells. Furthermore, this toxicity has been observed *in vivo*, where areas of necrosis can be found around the injection site when particles are injected subcutaneously. Further investigations suggest that this is a necrotic type of cell death, which results in loss of plasma membrane integrity and the release of endogenous danger signals such as HMGB1 (Coleman M and Lavelle EC unpublished findings). It has also been reported that injection of alum alone is sufficient to increase local levels of the endogenous danger signal uric acid [165]. Furthermore, the recruitment and activation of inflammatory monocytes as well as T cell proliferation in response to alum was dependent on uric acid [165]. Therefore, the ability of particulate adjuvants to promote the release of endogenous danger signals via their necrotic effects on cells is potentially important to their ability to enhance immune responses. In further support of the role of endogenous danger signals in the adjuvant effects of particulates, findings from these current studies have shown that supernatants from spleen cells treated with alum display adjuvant properties. These results demonstrated that co-administration of supernatant from alum treated spleen cells with Ova resulted in enhanced production of antigen-specific IL-4, IL-17, IL-5 and IFN- γ (Figure 6.3). Furthermore, the enhancement of some of these cytokines was dependent on TLR4, which has been shown to be required for the signalling of several endogenous danger signals, including biglycan and HMGB1 [42-44].

In addition to classical danger signals, certain cytokines can be released by necrotic cells in biologically active forms, such as IL-1 α and IL-33 [542, 621, 635]. Furthermore, IL-1 α has been shown to play a key role in the recruitment of neutrophils during sterile inflammation, which further supports the idea that IL-1 α released in response to particulates may play a key role in the recruitment of neutrophils and possibly other innate cells [545]. IL-1 α released by injured cells has also been shown to be a key mediator of adaptive responses during allograft rejection [636]. Taking into account the fact that certain cytokines are only released via necrosis, it is possible that

particle-mediated cell death may promote the release of pro-inflammatory cytokines, such as IL-1 α and IL-33, which can further potentiate the immune response promoted by PVA.

In chapter 5 it was shown that smaller particles are more toxic than larger particles and that particle uptake is a necessary step in particulate-induced cell death, suggesting that smaller particles may promote enhanced release of pro-inflammatory cytokines, such as IL-1 α from necrotic cells. In addition, smaller particles are also more efficient at driving activation of the inflammasome and subsequent release of IL-1 β . Therefore, smaller particles can promote IL-1 production more efficiently than larger particles. This potent enhancement of IL-1 and the toxicity of smaller particles may account for their ability to drive a more robust Th1-like response. Furthermore, necrotic cells are potent drivers of CTL and delayed type hypersensitivity reactions [585, 594]. In addition, current findings have demonstrated that nanoparticles (50 nm) specifically promote the secretion of IL-17 and IFN- γ when injected intraperitoneally. IL-1 is required for the development of Th17 cells, which adds further evidence to the idea that nanoparticles are more efficient drivers of IL-1 and this could explain their specific enhancement of IL-17 [290, 292].

Overall, smaller particles are more toxic and therefore have the potential to promote the release of endogenous danger signals more efficiently than larger particles, suggesting that toxicity promotes the development of a more pro-inflammatory response. In further agreement with this, larger particles which are non-toxic and did not cause any local necrosis when injected subcutaneously promoted the secretion of mainly Th2-associated cytokines, including IL-4, IL-5 and IL-10. Taken together, the toxic nature of PVA, the relationship between particle size and cell death and the fact that different sized particles induce distinct cytokine profiles *in vivo*, it is possible that increased levels of cell death are related to the enhancement of cytokines, such as IL-17 and IFN- γ . However, the toxicity of the particulates has no effect on the ability of particles to promote humoral responses, suggesting that particle size and relative toxicity are key factors that should be taken into account during the development of vaccines and adjuvant systems.

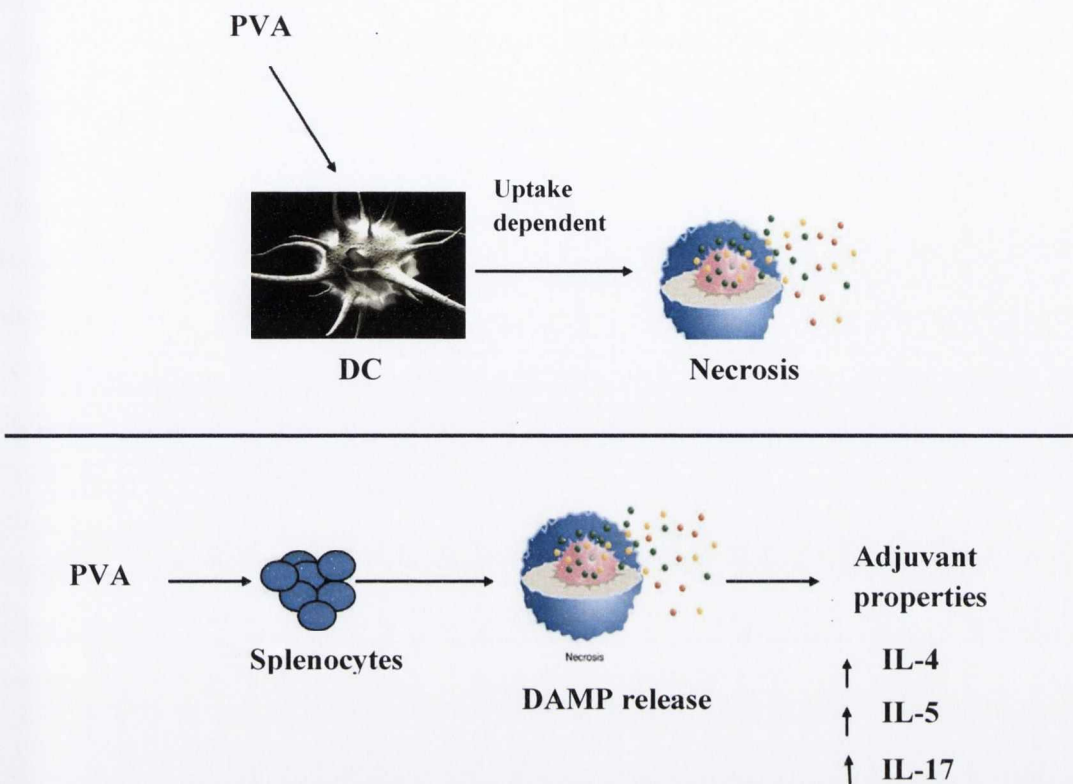


Figure 6.3: PVA are cytotoxic which promotes the release of endogenous molecules with proinflammatory effects. Supernatants from PVA-treated splenocytes promote the secretion of antigen-specific IL-4, IL-5 and IL-17 when co-administered subcutaneously with Ova.

Overall, the results from these studies have contributed significantly to our understanding of the mechanism of action of PVA (Figure 6.4). In agreement with several other studies, they have re-affirmed the idea that particulates have immunostimulatory properties and that they act as more than an antigen depot [119, 430, 451-452, 459]. PVA promote activation of the NLRP3 inflammasome *in vivo* and while this is not required for the enhancement of humoral responses, it is important for the recruitment and activation of innate cells as well as antigen-specific cytokine responses. Furthermore, IL-1 is also enhanced both locally and systemically post-

immunisation with particulates and may play a key role in both the initial development of cell-mediated responses and the expansion of later cell-mediated responses. The enhancement of IL-10 by PVA may help to explain their predominant Th2 bias and inability to promote strong cell-mediated responses such as CD8⁺ T cells. The early enhancement of IL-21 and IL-6 may promote the development of Tfh cells, which could potentially be central to the enhancement of humoral responses by PVA. The toxic effects of these adjuvants promote the release of endogenous danger signals, which also have immunostimulatory effects and have the potential to associate with and enhance the activity of other pro-inflammatory mediators, such as IL-1 [616]. Furthermore, the level of cell death could also contribute to determining the type of T helper cell response that is induced by PVA. These studies have identified particle size as a key factor in determining the type of immune response induced by particulates. By simply changing the size of the particle the type of cell mediated response is completely altered without affecting the humoral response. Therefore, the particle size should be considered when designing future particulate vaccine adjuvants and vaccines depending on what elements of the immune response are most important for protection.

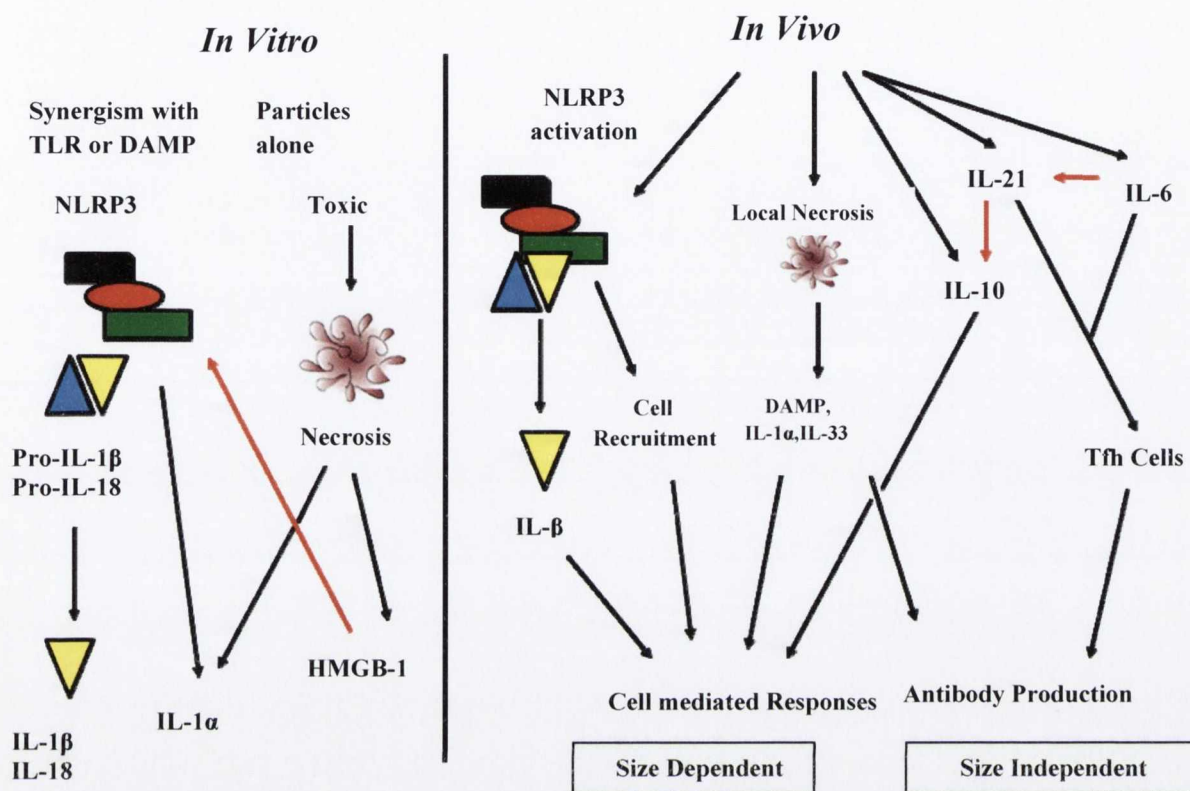


Figure 6.4: Immunostimulatory effects of particulate vaccine adjuvants.

A better understanding of how particulate adjuvants enhance immune responses will help us to overcome one of the major limitations associated with particulate vaccine adjuvants, which is their inability to produce strong cell-mediated responses and allow for the development of improved adjuvants in the future and subsequently better vaccines. Studies like this help us to unravel the specific mechanisms underlying the observed effects of particulate vaccine adjuvants on immune responses *in vitro* and *in vivo*. This, in turn, will lead to the development of more efficient and efficacious vaccines.

Chapter 7

References

1. Wilkinson, G.W. and P.J. Lehner, *Jenner's irony: cowpox taps into T cell evasion*. Cell Host Microbe, 2009. **6**(5): p. 395-7.
2. Steinman, R.M., *Dendritic cells in vivo: a key target for a new vaccine science*. Immunity, 2008. **29**(3): p. 319-24.
3. Pizza, M., et al., *Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing*. Science, 2000. **287**(5459): p. 1816-20.
4. Comanducci, M., et al., *NadA, a novel vaccine candidate of Neisseria meningitidis*. J Exp Med, 2002. **195**(11): p. 1445-54.
5. O'Hagan, D.T. and N.M. Valiante, *Recent advances in the discovery and delivery of vaccine adjuvants*. Nat Rev Drug Discov, 2003. **2**(9): p. 727-35.
6. Janeway, C.A., Jr., *Approaching the asymptote? Evolution and revolution in immunology*. Cold Spring Harb Symp Quant Biol, 1989. **54 Pt 1**: p. 1-13.
7. Matzinger, P., *Tolerance, danger, and the extended family*. Annu Rev Immunol, 1994. **12**: p. 991-1045.
8. Burnet, F.M., *The clonal selection theory of acquired immunity*. Vanderbilt University Press, Nashville, 1959.
9. Lafferty, K.J. and A.J. Cunningham, *A new analysis of allogeneic interactions*. Aust J Exp Biol Med Sci, 1975. **53**(1): p. 27-42.
10. Miyake, K., *Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors*. Semin Immunol, 2007. **19**(1): p. 3-10.
11. Scaffidi, P., T. Misteli, and M.E. Bianchi, *Release of chromatin protein HMGB1 by necrotic cells triggers inflammation*. Nature, 2002. **418**(6894): p. 191-5.
12. Shi, Y., J.E. Evans, and K.L. Rock, *Molecular identification of a danger signal that alerts the immune system to dying cells*. Nature, 2003. **425**(6957): p. 516-21.
13. Fraser, I.P., H. Koziel, and R.A. Ezekowitz, *The serum mannose-binding protein and the macrophage mannose receptor are pattern recognition molecules that link innate and adaptive immunity*. Semin Immunol, 1998. **10**(5): p. 363-72.
14. Schwalbe, R.A., et al., *Pentraxin family of proteins interact specifically with phosphorylcholine and/or phosphorylethanolamine*. Biochemistry, 1992. **31**(20): p. 4907-15.
15. Ferwerda, G., et al., *The role of Toll-like receptors and C-type lectins for vaccination against Candida albicans*. Vaccine, 2010. **28**(3): p. 614-22.
16. Yoneyama, M., et al., *Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity*. J Immunol, 2005. **175**(5): p. 2851-8.
17. Yoneyama, M., et al., *The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses*. Nat Immunol, 2004. **5**(7): p. 730-7.
18. Kawai, T. and S. Akira, *The roles of TLRs, RLRs and NLRs in pathogen recognition*. Int Immunol, 2009. **21**(4): p. 317-37.
19. Kato, H., et al., *Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5*. J Exp Med, 2008. **205**(7): p. 1601-10.
20. Rothenfusser, S., et al., *The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I*. J Immunol, 2005. **175**(8): p. 5260-8.
21. Takaoka, A., et al., *DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response*. Nature, 2007. **448**(7152): p. 501-5.

22. Fernandes-Alnemri, T., et al., *AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA*. *Nature*, 2009. **458**(7237): p. 509-13.
23. Hornung, V., et al., *AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC*. *Nature*, 2009. **458**(7237): p. 514-8.
24. Nomura, N., et al., *Prediction of the coding sequences of unidentified human genes. I. The coding sequences of 40 new genes (KIAA0001-KIAA0040) deduced by analysis of randomly sampled cDNA clones from human immature myeloid cell line KG-1*. *DNA Res*, 1994. **1**(1): p. 27-35.
25. Poltorak, A., et al., *Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene*. *Science*, 1998. **282**(5396): p. 2085-8.
26. Kobe, B. and A.V. Kajava, *The leucine-rich repeat as a protein recognition motif*. *Curr Opin Struct Biol*, 2001. **11**(6): p. 725-32.
27. Bortoluci, K.R. and R. Medzhitov, *Control of infection by pyroptosis and autophagy: role of TLR and NLR*. *Cell Mol Life Sci*, 2010.
28. Carty, M., et al., *The human adaptor SARM negatively regulates adaptor protein TRIF-dependent Toll-like receptor signaling*. *Nat Immunol*, 2006. **7**(10): p. 1074-81.
29. O'Neill, L.A. and A.G. Bowie, *Sensing and signaling in antiviral innate immunity*. *Curr Biol*, 2010. **20**(7): p. R328-33.
30. Alexopoulou, L., et al., *Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3*. *Nature*, 2001. **413**(6857): p. 732-8.
31. Diebold, S.S., et al., *Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA*. *Science*, 2004. **303**(5663): p. 1529-31.
32. Heil, F., et al., *Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8*. *Science*, 2004. **303**(5663): p. 1526-9.
33. Hemmi, H., et al., *Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway*. *Nat Immunol*, 2002. **3**(2): p. 196-200.
34. Hemmi, H., et al., *A Toll-like receptor recognizes bacterial DNA*. *Nature*, 2000. **408**(6813): p. 740-5.
35. Haas, T., et al., *The DNA sugar backbone 2' deoxyribose determines toll-like receptor 9 activation*. *Immunity*, 2008. **28**(3): p. 315-23.
36. Parroche, P., et al., *Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9*. *Proc Natl Acad Sci U S A*, 2007. **104**(6): p. 1919-24.
37. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity*. *Cell*, 2006. **124**(4): p. 783-801.
38. Gantner, B.N., et al., *Collaborative induction of inflammatory responses by dectin-1 and Toll-like receptor 2*. *J Exp Med*, 2003. **197**(9): p. 1107-17.
39. Hoebe, K., et al., *CD36 is a sensor of diacylglycerides*. *Nature*, 2005. **433**(7025): p. 523-7.
40. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5*. *Nature*, 2001. **410**(6832): p. 1099-103.
41. Uematsu, S., et al., *Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells*. *Nat Immunol*, 2006. **7**(8): p. 868-74.
42. Park, J.S., et al., *High mobility group box 1 protein interacts with multiple Toll-like receptors*. *Am J Physiol Cell Physiol*, 2006. **290**(3): p. C917-24.
43. Park, J.S., et al., *Involvement of toll-like receptors 2 and 4 in cellular activation by high mobility group box 1 protein*. *J Biol Chem*, 2004. **279**(9): p. 7370-7.

44. Schaefer, L., et al., *The matrix component biglycan is proinflammatory and signals through Toll-like receptors 4 and 2 in macrophages*. J Clin Invest, 2005. **115**(8): p. 2223-33.
45. Liu, Y.J., *IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors*. Annu Rev Immunol, 2005. **23**: p. 275-306.
46. Ting, J.P. and B.K. Davis, *CATERPILLER: a novel gene family important in immunity, cell death, and diseases*. Annu Rev Immunol, 2005. **23**: p. 387-414.
47. Koonin, E.V. and L. Aravind, *The NACHT family - a new group of predicted NTPases implicated in apoptosis and MHC transcription activation*. Trends Biochem Sci, 2000. **25**(5): p. 223-4.
48. Ting, J.P., et al., *The NLR gene family: a standard nomenclature*. Immunity, 2008. **28**(3): p. 285-7.
49. Moore, C.B., et al., *NLRX1 is a regulator of mitochondrial antiviral immunity*. Nature, 2008. **451**(7178): p. 573-7.
50. Tattoli, I., et al., *NLRX1 is a mitochondrial NOD-like receptor that amplifies NF-kappaB and JNK pathways by inducing reactive oxygen species production*. EMBO Rep, 2008. **9**(3): p. 293-300.
51. Nickerson, K., et al., *Dendritic cell-specific MHC class II transactivator contains a caspase recruitment domain that confers potent transactivation activity*. J Biol Chem, 2001. **276**(22): p. 19089-93.
52. Liston, P., et al., *Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes*. Nature, 1996. **379**(6563): p. 349-53.
53. Molofsky, A.B., et al., *Cytosolic recognition of flagellin by mouse macrophages restricts Legionella pneumophila infection*. J Exp Med, 2006. **203**(4): p. 1093-104.
54. Lightfield, K.L., et al., *Critical function for Naip5 in inflammasome activation by a conserved carboxy-terminal domain of flagellin*. Nat Immunol, 2008. **9**(10): p. 1171-8.
55. Ogura, Y., et al., *Nod2, a Nod1/Apaf-1 family member that is restricted to monocytes and activates NF-kappaB*. J Biol Chem, 2001. **276**(7): p. 4812-8.
56. Inohara, N., et al., *Nod1, an Apaf-1-like activator of caspase-9 and nuclear factor-kappaB*. J Biol Chem, 1999. **274**(21): p. 14560-7.
57. Inohara, N., et al., *Human Nod1 confers responsiveness to bacterial lipopolysaccharides*. J Biol Chem, 2001. **276**(4): p. 2551-4.
58. Chamaillard, M., et al., *An essential role for NOD1 in host recognition of bacterial peptidoglycan containing diaminopimelic acid*. Nat Immunol, 2003. **4**(7): p. 702-7.
59. Girardin, S.E., et al., *Peptidoglycan molecular requirements allowing detection by Nod1 and Nod2*. J Biol Chem, 2003. **278**(43): p. 41702-8.
60. Inohara, N., et al., *Host recognition of bacterial muramyl dipeptide mediated through NOD2. Implications for Crohn's disease*. J Biol Chem, 2003. **278**(8): p. 5509-12.
61. Faustin, B., et al., *Reconstituted NALP1 inflammasome reveals two-step mechanism of caspase-1 activation*. Mol Cell, 2007. **25**(5): p. 713-24.
62. Hsu, L.C., et al., *A NOD2-NALP1 complex mediates caspase-1-dependent IL-1beta secretion in response to Bacillus anthracis infection and muramyl dipeptide*. Proc Natl Acad Sci U S A, 2008. **105**(22): p. 7803-8.
63. Pan, Q., et al., *MDP-induced interleukin-1beta processing requires Nod2 and CIAS1/NALP3*. J Leukoc Biol, 2007. **82**(1): p. 177-83.

64. Girardin, S.E., et al., *CARD4/Nod1 mediates NF-kappaB and JNK activation by invasive Shigella flexneri*. EMBO Rep, 2001. **2**(8): p. 736-42.
65. Inohara, N., et al., *An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways*. J Biol Chem, 2000. **275**(36): p. 27823-31.
66. Park, J.H., et al., *RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs*. J Immunol, 2007. **178**(4): p. 2380-6.
67. Franchi, L., et al., *Function of Nod-like receptors in microbial recognition and host defense*. Immunol Rev, 2009. **227**(1): p. 106-28.
68. Kanazawa, N., et al., *Early-onset sarcoidosis and CARD15 mutations with constitutive nuclear factor-kappaB activation: common genetic etiology with Blau syndrome*. Blood, 2005. **105**(3): p. 1195-7.
69. Miceli-Richard, C., et al., *CARD15 mutations in Blau syndrome*. Nat Genet, 2001. **29**(1): p. 19-20.
70. Hugot, J.P., et al., *Association of NOD2 leucine-rich repeat variants with susceptibility to Crohn's disease*. Nature, 2001. **411**(6837): p. 599-603.
71. Ogura, Y., et al., *A frameshift mutation in NOD2 associated with susceptibility to Crohn's disease*. Nature, 2001. **411**(6837): p. 603-6.
72. Fritz, J.H., et al., *Nod1-mediated innate immune recognition of peptidoglycan contributes to the onset of adaptive immunity*. Immunity, 2007. **26**(4): p. 445-59.
73. Kobayashi, K.S., et al., *Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract*. Science, 2005. **307**(5710): p. 731-4.
74. Damiano, J.S., et al., *CLAN, a novel human CED-4-like gene*. Genomics, 2001. **75**(1-3): p. 77-83.
75. Poyet, J.L., et al., *Identification of Ipaf, a human caspase-1-activating protein related to Apaf-1*. J Biol Chem, 2001. **276**(30): p. 28309-13.
76. Martinon, F., K. Burns, and J. Tschopp, *The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta*. Mol Cell, 2002. **10**(2): p. 417-26.
77. Amer, A., et al., *Regulation of Legionella phagosome maturation and infection through flagellin and host Ipaf*. J Biol Chem, 2006. **281**(46): p. 35217-23.
78. Franchi, L., et al., *Cytosolic flagellin requires Ipaf for activation of caspase-1 and interleukin 1beta in salmonella-infected macrophages*. Nat Immunol, 2006. **7**(6): p. 576-82.
79. Mariathasan, S., et al., *Differential activation of the inflammasome by caspase-1 adaptors ASC and Ipaf*. Nature, 2004. **430**(6996): p. 213-8.
80. Franchi, L., et al., *Differential requirement of P2X7 receptor and intracellular K⁺ for caspase-1 activation induced by intracellular and extracellular bacteria*. J Biol Chem, 2007. **282**(26): p. 18810-8.
81. Kanneganti, T.D., et al., *Pannexin-1-mediated recognition of bacterial molecules activates the cryopyrin inflammasome independent of Toll-like receptor signaling*. Immunity, 2007. **26**(4): p. 433-43.
82. Miao, E.A., et al., *Cytoplasmic flagellin activates caspase-1 and secretion of interleukin 1beta via Ipaf*. Nat Immunol, 2006. **7**(6): p. 569-75.
83. Suzuki, T., et al., *Differential regulation of caspase-1 activation, pyroptosis, and autophagy via Ipaf and ASC in Shigella-infected macrophages*. PLoS Pathog, 2007. **3**(8): p. e111.
84. Bryant, C. and K.A. Fitzgerald, *Molecular mechanisms involved in inflammasome activation*. Trends Cell Biol, 2009. **19**(9): p. 455-64.

85. Manji, G.A., et al., *PYPAF1, a PYRIN-containing Apaf1-like protein that assembles with ASC and regulates activation of NF-kappa B*. J Biol Chem, 2002. **277**(13): p. 11570-5.
86. Chu, Z.L., et al., *A novel enhancer of the Apaf1 apoptosome involved in cytochrome c-dependent caspase activation and apoptosis*. J Biol Chem, 2001. **276**(12): p. 9239-45.
87. Hlaing, T., et al., *Molecular cloning and characterization of DEFCAP-L and -S, two isoforms of a novel member of the mammalian Ced-4 family of apoptosis proteins*. J Biol Chem, 2001. **276**(12): p. 9230-8.
88. Martinon, F., et al., *Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome*. Curr Biol, 2004. **14**(21): p. 1929-34.
89. Boyden, E.D. and W.F. Dietrich, *Nalp1b controls mouse macrophage susceptibility to anthrax lethal toxin*. Nat Genet, 2006. **38**(2): p. 240-4.
90. Jin, Y., et al., *NALP1 in vitiligo-associated multiple autoimmune disease*. N Engl J Med, 2007. **356**(12): p. 1216-25.
91. Bruey, J.M., et al., *Bcl-2 and Bcl-XL regulate proinflammatory caspase-1 activation by interaction with NALP1*. Cell, 2007. **129**(1): p. 45-56.
92. Aganna, E., et al., *Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis*. Arthritis Rheum, 2002. **46**(9): p. 2445-52.
93. Hoffman, H.M., et al., *Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome*. Nat Genet, 2001. **29**(3): p. 301-5.
94. Feldmann, J., et al., *Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes*. Am J Hum Genet, 2002. **71**(1): p. 198-203.
95. Tschoopp, J., F. Martinon, and K. Burns, *NALPs: a novel protein family involved in inflammation*. Nat Rev Mol Cell Biol, 2003. **4**(2): p. 95-104.
96. Agostini, L., et al., *NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder*. Immunity, 2004. **20**(3): p. 319-25.
97. Gross, O., et al., *Syk kinase signalling couples to the Nlrp3 inflammasome for anti-fungal host defence*. Nature, 2009. **459**(7245): p. 433-6.
98. Joly, S., et al., *Cutting edge: Candida albicans hyphae formation triggers activation of the Nlrp3 inflammasome*. J Immunol, 2009. **183**(6): p. 3578-81.
99. Mariathasan, S., et al., *Cryopyrin activates the inflammasome in response to toxins and ATP*. Nature, 2006. **440**(7081): p. 228-32.
100. Muruve, D.A., et al., *The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response*. Nature, 2008. **452**(7183): p. 103-7.
101. Martinon, F., et al., *Gout-associated uric acid crystals activate the NALP3 inflammasome*. Nature, 2006. **440**(7081): p. 237-41.
102. Yamasaki, K., et al., *NLRP3/cryopyrin is necessary for interleukin-1beta (IL-1beta) release in response to hyaluronan, an endogenous trigger of inflammation in response to injury*. J Biol Chem, 2009. **284**(19): p. 12762-71.
103. Cassel, S.L., et al., *The Nalp3 inflammasome is essential for the development of silicosis*. Proc Natl Acad Sci U S A, 2008. **105**(26): p. 9035-40.
104. Dostert, C., et al., *Innate immune activation through Nalp3 inflammasome sensing of asbestos and silica*. Science, 2008. **320**(5876): p. 674-7.

105. Eisenbarth, S.C., et al., *Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants*. *Nature*, 2008. **453**(7198): p. 1122-6.
106. Hornung, V., et al., *Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization*. *Nat Immunol*, 2008. **9**(8): p. 847-56.
107. Kool, M., et al., *Cutting edge: alum adjuvant stimulates inflammatory dendritic cells through activation of the NALP3 inflammasome*. *J Immunol*, 2008. **181**(6): p. 3755-9.
108. Li, H., et al., *Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3*. *J Immunol*, 2008. **181**(1): p. 17-21.
109. Demento, S.L., et al., *Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy*. *Vaccine*, 2009. **27**(23): p. 3013-21.
110. Duewell, P., et al., *NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals*. *Nature*, 2010. **464**(7293): p. 1357-61.
111. Bauernfeind, F.G., et al., *Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression*. *J Immunol*, 2009. **183**(2): p. 787-91.
112. Netea, M.G., et al., *Differential requirement for the activation of the inflammasome for processing and release of IL-1beta in monocytes and macrophages*. *Blood*, 2009. **113**(10): p. 2324-35.
113. Zhou, R., et al., *Thioredoxin-interacting protein links oxidative stress to inflammasome activation*. *Nat Immunol*, 2010. **11**(2): p. 136-40.
114. Parikh, H., et al., *TXNIP regulates peripheral glucose metabolism in humans*. *PLoS Med*, 2007. **4**(5): p. e158.
115. Shalev, A., *Lack of TXNIP protects beta-cells against glucotoxicity*. *Biochem Soc Trans*, 2008. **36**(Pt 5): p. 963-5.
116. Sutterwala, F.S., et al., *Critical role for NALP3/CIAS1/Cryopyrin in innate and adaptive immunity through its regulation of caspase-1*. *Immunity*, 2006. **24**(3): p. 317-27.
117. Watanabe, H., et al., *Activation of the IL-1beta-processing inflammasome is involved in contact hypersensitivity*. *J Invest Dermatol*, 2007. **127**(8): p. 1956-63.
118. Franchi, L. and G. Nunez, *The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity*. *Eur J Immunol*, 2008. **38**(8): p. 2085-9.
119. McKee, A.S., et al., *Alum induces innate immune responses through macrophage and mast cell sensors, but these sensors are not required for alum to act as an adjuvant for specific immunity*. *J Immunol*, 2009. **183**(7): p. 4403-14.
120. Sims, J.E., *IL-1 and IL-18 receptors, and their extended family*. *Curr Opin Immunol*, 2002. **14**(1): p. 117-22.
121. Dinarello, C.A., *Blocking IL-1 in systemic inflammation*. *J Exp Med*, 2005. **201**(9): p. 1355-9.
122. Andrei, C., et al., *The secretory route of the leaderless protein interleukin 1beta involves exocytosis of endolysosome-related vesicles*. *Mol Biol Cell*, 1999. **10**(5): p. 1463-75.
123. Cerretti, D.P., et al., *Molecular cloning of the interleukin-1 beta converting enzyme*. *Science*, 1992. **256**(5053): p. 97-100.

124. Thornberry, N.A., et al., *A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes*. Nature, 1992. **356**(6372): p. 768-74.
125. Kuida, K., et al., *Altered cytokine export and apoptosis in mice deficient in interleukin-1 beta converting enzyme*. Science, 1995. **267**(5206): p. 2000-3.
126. Wang, S., et al., *Murine caspase-11, an ICE-interacting protease, is essential for the activation of ICE*. Cell, 1998. **92**(4): p. 501-9.
127. Masumoto, J., et al., *ASC, a novel 22-kDa protein, aggregates during apoptosis of human promyelocytic leukemia HL-60 cells*. J Biol Chem, 1999. **274**(48): p. 33835-8.
128. Masumoto, J., et al., *Expression of apoptosis-associated speck-like protein containing a caspase recruitment domain, a pyrin N-terminal homology domain-containing protein, in normal human tissues*. J Histochem Cytochem, 2001. **49**(10): p. 1269-75.
129. Martinon, F., et al., *NALP inflammasomes: a central role in innate immunity*. Semin Immunopathol, 2007. **29**(3): p. 213-29.
130. Schroder, K. and J. Tschopp, *The inflammasomes*. Cell, 2010. **140**(6): p. 821-32.
131. Petrilli, V., et al., *Activation of the NALP3 inflammasome is triggered by low intracellular potassium concentration*. Cell Death Differ, 2007. **14**(9): p. 1583-9.
132. Cruz, C.M., et al., *ATP activates a reactive oxygen species-dependent oxidative stress response and secretion of proinflammatory cytokines in macrophages*. J Biol Chem, 2007. **282**(5): p. 2871-9.
133. Shio, M.T., et al., *Malarial hemozoin activates the NLRP3 inflammasome through Lyn and Syk kinases*. PLoS Pathog, 2009. **5**(8): p. e1000559.
134. Halle, A., et al., *The NALP3 inflammasome is involved in the innate immune response to amyloid-beta*. Nat Immunol, 2008. **9**(8): p. 857-65.
135. Fink, S.L., T. Bergsbaken, and B.T. Cookson, *Anthrax lethal toxin and Salmonella elicit the common cell death pathway of caspase-1-dependent pyroptosis via distinct mechanisms*. Proc Natl Acad Sci U S A, 2008. **105**(11): p. 4312-7.
136. Wickliffe, K.E., S.H. Leppla, and M. Moayeri, *Anthrax lethal toxin-induced inflammasome formation and caspase-1 activation are late events dependent on ion fluxes and the proteasome*. Cell Microbiol, 2008. **10**(2): p. 332-43.
137. Deretic, V. and B. Levine, *Autophagy, immunity, and microbial adaptations*. Cell Host Microbe, 2009. **5**(6): p. 527-49.
138. Saitoh, T., et al., *Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1beta production*. Nature, 2008. **456**(7219): p. 264-8.
139. Harris, J., J.C. Hope, and E.C. Lavelle, *Autophagy and the immune response to TB*. Transbound Emerg Dis, 2009. **56**(6-7): p. 248-54.
140. Petrilli, V., et al., *The inflammasome: a danger sensing complex triggering innate immunity*. Curr Opin Immunol, 2007. **19**(6): p. 615-22.
141. Druilhe, A., et al., *Regulation of IL-1beta generation by Pseudo-ICE and ICEBERG, two dominant negative caspase recruitment domain proteins*. Cell Death Differ, 2001. **8**(6): p. 649-57.
142. Annand, R.R., et al., *Caspase-1 (interleukin-1beta-converting enzyme) is inhibited by the human serpin analogue proteinase inhibitor 9*. Biochem J, 1999. **342 Pt 3**: p. 655-65.
143. Stehlik, C., et al., *The PAAD/PYRIN-only protein POP1/ASC2 is a modulator of ASC-mediated nuclear-factor-kappa B and pro-caspase-1 regulation*. Biochem J, 2003. **373**(Pt 1): p. 101-13.

144. Pedra, J.H., S.L. Cassel, and F.S. Sutterwala, *Sensing pathogens and danger signals by the inflammasome*. Curr Opin Immunol, 2009. **21**(1): p. 10-6.
145. Mayor, A., et al., *A crucial function of SGT1 and HSP90 in inflammasome activity links mammalian and plant innate immune responses*. Nat Immunol, 2007. **8**(5): p. 497-503.
146. Heath, W.R. and F.R. Carbone, *Dendritic cell subsets in primary and secondary T cell responses at body surfaces*. Nat Immunol, 2009. **10**(12): p. 1237-44.
147. Steinman, R.M. and Z.A. Cohn, *Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution*. J Exp Med, 1973. **137**(5): p. 1142-62.
148. Nussenzweig, M.C. and R.M. Steinman, *Contribution of dendritic cells to stimulation of the murine syngeneic mixed leukocyte reaction*. J Exp Med, 1980. **151**(5): p. 1196-212.
149. Steinman, R.M. and M.D. Witmer, *Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice*. Proc Natl Acad Sci U S A, 1978. **75**(10): p. 5132-6.
150. Inaba, K., M.D. Witmer, and R.M. Steinman, *Clustering of dendritic cells, helper T lymphocytes, and histocompatible B cells during primary antibody responses in vitro*. J Exp Med, 1984. **160**(3): p. 858-76.
151. Shortman, K. and S.H. Naik, *Steady-state and inflammatory dendritic-cell development*. Nat Rev Immunol, 2007. **7**(1): p. 19-30.
152. Grouard, G., et al., *The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand*. J Exp Med, 1997. **185**(6): p. 1101-11.
153. Liu, K., et al., *In vivo analysis of dendritic cell development and homeostasis*. Science, 2009. **324**(5925): p. 392-7.
154. Waskow, C., et al., *The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues*. Nat Immunol, 2008. **9**(6): p. 676-83.
155. Nicolas, C. and D. Schmitt, *[Langerhans cells: methods of identification]*. Pathol Biol (Paris), 1984. **32**(3): p. 199-208.
156. Valladeau, J., C. Dezutter-Dambuyant, and S. Saeland, *Langerin/CD207 sheds light on formation of Birbeck granules and their possible function in Langerhans cells*. Immunol Res, 2003. **28**(2): p. 93-107.
157. den Haan, J.M., S.M. Lehar, and M.J. Bevan, *CD8(+) but not CD8(-) dendritic cells cross-prime cytotoxic T cells in vivo*. J Exp Med, 2000. **192**(12): p. 1685-96.
158. Hochrein, H., et al., *Differential production of IL-12, IFN-alpha, and IFN-gamma by mouse dendritic cell subsets*. J Immunol, 2001. **166**(9): p. 5448-55.
159. Vremec, D., et al., *CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen*. J Immunol, 2000. **164**(6): p. 2978-86.
160. Annacker, O., et al., *Essential role for CD103 in the T cell-mediated regulation of experimental colitis*. J Exp Med, 2005. **202**(8): p. 1051-61.
161. Johansson-Lindbom, B., et al., *Functional specialization of gut CD103+ dendritic cells in the regulation of tissue-selective T cell homing*. J Exp Med, 2005. **202**(8): p. 1063-73.
162. Kilshaw, P.J., *Expression of the mucosal T cell integrin alpha M290 beta 7 by a major subpopulation of dendritic cells in mice*. Eur J Immunol, 1993. **23**(12): p. 3365-8.

163. Sung, S.S., et al., *A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins*. J Immunol, 2006. **176**(4): p. 2161-72.
164. Serbina, N.V., et al., *TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection*. Immunity, 2003. **19**(1): p. 59-70.
165. Kool, M., et al., *Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells* J Exp Med, 2007.
166. Izon, D., et al., *A common pathway for dendritic cell and early B cell development*. J Immunol, 2001. **167**(3): p. 1387-92.
167. Naik, S.H., et al., *Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo*. Nat Immunol, 2007. **8**(11): p. 1217-26.
168. Geissmann, F., et al., *Development of monocytes, macrophages, and dendritic cells*. Science, 2010. **327**(5966): p. 656-61.
169. Onai, N., et al., *Identification of clonogenic common Flt3+M-CSFR+ plasmacytoid and conventional dendritic cell progenitors in mouse bone marrow*. Nat Immunol, 2007. **8**(11): p. 1207-16.
170. Sarrazin, S., et al., *MafB restricts M-CSF-dependent myeloid commitment divisions of hematopoietic stem cells*. Cell, 2009. **138**(2): p. 300-13.
171. Auffray, C., M.H. Sieweke, and F. Geissmann, *Blood monocytes: development, heterogeneity, and relationship with dendritic cells*. Annu Rev Immunol, 2009. **27**: p. 669-92.
172. Bakri, Y., et al., *Balance of MafB and PU.1 specifies alternative macrophage or dendritic cell fate*. Blood, 2005. **105**(7): p. 2707-16.
173. Cisse, B., et al., *Transcription factor E2-2 is an essential and specific regulator of plasmacytoid dendritic cell development*. Cell, 2008. **135**(1): p. 37-48.
174. Kabashima, K., et al., *Intrinsic lymphotoxin-beta receptor requirement for homeostasis of lymphoid tissue dendritic cells*. Immunity, 2005. **22**(4): p. 439-50.
175. Romani, N., et al., *Presentation of exogenous protein antigens by dendritic cells to T cell clones. Intact protein is presented best by immature, epidermal Langerhans cells*. J Exp Med, 1989. **169**(3): p. 1169-78.
176. Schuler, G. and R.M. Steinman, *Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro*. J Exp Med, 1985. **161**(3): p. 526-46.
177. Austyn, J.M., J.W. Kupiec-Weglinski, and P.J. Morris, *Migration patterns of dendritic cells in the mouse*. Adv Exp Med Biol, 1988. **237**: p. 583-9.
178. Banchereau, J., et al., *Immunobiology of dendritic cells*. Annu Rev Immunol, 2000. **18**: p. 767-811.
179. Reis e Sousa, C., *Dendritic cells in a mature age*. Nat Rev Immunol, 2006. **6**(6): p. 476-83.
180. Liu, Y.J., *Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity*. Cell, 2001. **106**(3): p. 259-62.
181. Langenkamp, A., et al., *Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells*. Nat Immunol, 2000. **1**(4): p. 311-6.
182. Kalinski, P., et al., *T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal*. Immunol Today, 1999. **20**(12): p. 561-7.
183. Pulendran, B., *Variegation of the immune response with dendritic cells and pathogen recognition receptors*. J Immunol, 2005. **174**(5): p. 2457-65.

184. Albert, M.L., M. Jegathesan, and R.B. Darnell, *Dendritic cell maturation is required for the cross-tolerization of CD8⁺ T cells*. Nat Immunol, 2001. **2**(11): p. 1010-7.
185. Sporri, R. and C. Reis e Sousa, *Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4⁺ T cell populations lacking helper function*. Nat Immunol, 2005. **6**(2): p. 163-70.
186. Jensen, P.E., *Recent advances in antigen processing and presentation*. Nat Immunol, 2007. **8**(10): p. 1041-8.
187. Baumeister, W., et al., *The proteasome: paradigm of a self-compartmentalizing protease*. Cell, 1998. **92**(3): p. 367-80.
188. Hansen, T.H. and M. Bouvier, *MHC class I antigen presentation: learning from viral evasion strategies*. Nat Rev Immunol, 2009. **9**(7): p. 503-13.
189. Rock, K.L., et al., *Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules*. Cell, 1994. **78**(5): p. 761-71.
190. Kloetzel, P.M., *Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII*. Nat Immunol, 2004. **5**(7): p. 661-9.
191. Rock, K.L., I.A. York, and A.L. Goldberg, *Post-proteasomal antigen processing for major histocompatibility complex class I presentation*. Nat Immunol, 2004. **5**(7): p. 670-7.
192. Schmitt, L. and R. Tampe, *Structure and mechanism of ABC transporters*. Curr Opin Struct Biol, 2002. **12**(6): p. 754-60.
193. Sijts, A.J. and E.G. Pamer, *Enhanced intracellular dissociation of major histocompatibility complex class I-associated peptides: a mechanism for optimizing the spectrum of cell surface-presented cytotoxic T lymphocyte epitopes*. J Exp Med, 1997. **185**(8): p. 1403-11.
194. Howarth, M., et al., *Tapasin enhances MHC class I peptide presentation according to peptide half-life*. Proc Natl Acad Sci U S A, 2004. **101**(32): p. 11737-42.
195. Dick, T.P., et al., *Disulfide bond isomerization and the assembly of MHC class I-peptide complexes*. Immunity, 2002. **16**(1): p. 87-98.
196. Schubert, U., et al., *Rapid degradation of a large fraction of newly synthesized proteins by proteasomes*. Nature, 2000. **404**(6779): p. 770-4.
197. Vabulas, R.M. and F.U. Hartl, *Protein synthesis upon acute nutrient restriction relies on proteasome function*. Science, 2005. **310**(5756): p. 1960-3.
198. Ackerman, A.L. and P. Cresswell, *Cellular mechanisms governing cross-presentation of exogenous antigens*. Nat Immunol, 2004. **5**(7): p. 678-84.
199. Groothuis, T.A. and J. Neefjes, *The many roads to cross-presentation*. J Exp Med, 2005. **202**(10): p. 1313-8.
200. Shen, L., et al., *Important role of cathepsin S in generating peptides for TAP-independent MHC class I crosspresentation in vivo*. Immunity, 2004. **21**(2): p. 155-65.
201. Iyoda, T., et al., *The CD8⁺ dendritic cell subset selectively endocytoses dying cells in culture and in vivo*. J Exp Med, 2002. **195**(10): p. 1289-302.
202. Burgdorf, S., et al., *Distinct pathways of antigen uptake and intracellular routing in CD4 and CD8 T cell activation*. Science, 2007. **316**(5824): p. 612-6.
203. Kleijmeer, M.J., et al., *MHC class II compartments and the kinetics of antigen presentation in activated mouse spleen dendritic cells*. J Immunol, 1995. **154**(11): p. 5715-24.

204. Nijman, H.W., et al., *Antigen capture and major histocompatibility class II compartments of freshly isolated and cultured human blood dendritic cells*. J Exp Med, 1995. **182**(1): p. 163-74.
205. Vyas, J.M., A.G. Van der Veen, and H.L. Ploegh, *The known unknowns of antigen processing and presentation*. Nat Rev Immunol, 2008. **8**(8): p. 607-18.
206. Cresswell, P., *Invariant chain structure and MHC class II function*. Cell, 1996. **84**(4): p. 505-7.
207. Jensen, P.E., et al., *Peptide exchange in MHC molecules*. Immunol Rev, 1999. **172**: p. 229-38.
208. Pierre, P. and I. Mellman, *Developmental regulation of invariant chain proteolysis controls MHC class II trafficking in mouse dendritic cells*. Cell, 1998. **93**(7): p. 1135-45.
209. Ohmura-Hoshino, M., et al., *Inhibition of MHC class II expression and immune responses by c-MIR*. J Immunol, 2006. **177**(1): p. 341-54.
210. Shin, J.S., et al., *Surface expression of MHC class II in dendritic cells is controlled by regulated ubiquitination*. Nature, 2006. **444**(7115): p. 115-8.
211. Blander, J.M. and R. Medzhitov, *Toll-dependent selection of microbial antigens for presentation by dendritic cells*. Nature, 2006. **440**(7085): p. 808-12.
212. Schmid, D., M. Pypaert, and C. Munz, *Antigen-loading compartments for major histocompatibility complex class II molecules continuously receive input from autophagosomes*. Immunity, 2007. **26**(1): p. 79-92.
213. Lee, H.K., et al., *Autophagy-dependent viral recognition by plasmacytoid dendritic cells*. Science, 2007. **315**(5817): p. 1398-401.
214. Sanjuan, M.A., et al., *Toll-like receptor signalling in macrophages links the autophagy pathway to phagocytosis*. Nature, 2007. **450**(7173): p. 1253-7.
215. Barral, D.C. and M.B. Brenner, *CD1 antigen presentation: how it works*. Nat Rev Immunol, 2007. **7**(12): p. 929-41.
216. Beckman, E.M., et al., *Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells*. Nature, 1994. **372**(6507): p. 691-4.
217. Tomura, M., et al., *A novel function of Valpha14+CD4+NKT cells: stimulation of IL-12 production by antigen-presenting cells in the innate immune system*. J Immunol, 1999. **163**(1): p. 93-101.
218. Vincent, M.S., et al., *CD1-dependent dendritic cell instruction*. Nat Immunol, 2002. **3**(12): p. 1163-8.
219. Kawano, T., et al., *CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides*. Science, 1997. **278**(5343): p. 1626-9.
220. Godfrey, D.I. and M. Kronenberg, *Going both ways: immune regulation via CD1d-dependent NKT cells*. J Clin Invest, 2004. **114**(10): p. 1379-88.
221. Nakagawa, R., et al., *Essential role of bystander cytotoxic CD122+CD8+ T cells for the antitumor immunity induced in the liver of mice by alpha-galactosylceramide*. J Immunol, 2004. **172**(11): p. 6550-7.
222. Nishimura, T., et al., *The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes*. Int Immunol, 2000. **12**(7): p. 987-94.
223. Dustin, M.L., et al., *A novel adaptor protein orchestrates receptor patterning and cytoskeletal polarity in T-cell contacts*. Cell, 1998. **94**(5): p. 667-77.
224. Kapsenberg, M.L., *Dendritic-cell control of pathogen-driven T-cell polarization*. Nat Rev Immunol, 2003. **3**(12): p. 984-93.

225. Kadowaki, N., et al., *Natural interferon alpha/beta-producing cells link innate and adaptive immunity*. J Exp Med, 2000. **192**(2): p. 219-26.
226. Salomon, B. and J.A. Bluestone, *LFA-1 interaction with ICAM-1 and ICAM-2 regulates Th2 cytokine production*. J Immunol, 1998. **161**(10): p. 5138-42.
227. Napolitani, G., et al., *Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells*. Nat Immunol, 2005. **6**(8): p. 769-76.
228. Skokos, D. and M.C. Nussenzweig, *CD8- DCs induce IL-12-independent Th1 differentiation through Delta 4 Notch-like ligand in response to bacterial LPS*. J Exp Med, 2007. **204**(7): p. 1525-31.
229. MacDonald, A.S., et al., *CD8- dendritic cell activation status plays an integral role in influencing Th2 response development*. J Immunol, 2001. **167**(4): p. 1982-8.
230. Pearce, E.J., et al., *Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni*. J Exp Med, 1991. **173**(1): p. 159-66.
231. Krawczyk, C.M., J. Sun, and E.J. Pearce, *Th2 differentiation is unaffected by Jagged2 expression on dendritic cells*. J Immunol, 2008. **180**(12): p. 7931-7.
232. Joffre, O., et al., *Inflammatory signals in dendritic cell activation and the induction of adaptive immunity*. Immunol Rev, 2009. **227**(1): p. 234-47.
233. Cao, X., et al., *Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance*. Immunity, 2007. **27**(4): p. 635-46.
234. Cooper, M.D., R.D. Peterson, and R.A. Good, *Delineation of the thymic and bursal lymphoid systems*. Nature, 1965 (205): p. 143-146.
235. Takahama, Y., *Journey through the thymus: stromal guides for T-cell development and selection*. Nat Rev Immunol, 2006. **6**(2): p. 127-35.
236. Mosmann, T.R., et al., *Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins* J Immunol, 1986. **136**(7): p. 2348-57
237. Wierenga, E.A., et al., *Evidence for compartmentalization of functional subsets of CD2+ T lymphocytes in atopic patients*. J Immunol, 1990. **144**(12): p. 4651-6.
238. Martin-Fontecha, A., et al., *Induced recruitment of NK cells to lymph nodes provides IFN-gamma for T(H)1 priming*. Nat Immunol, 2004. **5**(12): p. 1260-5.
239. Szabo, S.J., et al., *A novel transcription factor, T-bet, directs Th1 lineage commitment*. Cell, 2000. **100**(6): p. 655-69.
240. Thieu, V.T., et al., *Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination*. Immunity, 2008. **29**(5): p. 679-90.
241. Djuretic, I.M., et al., *Transcription factors T-bet and Runx3 cooperate to activate Ifng and silence Il4 in T helper type 1 cells*. Nat Immunol, 2007. **8**(2): p. 145-53.
242. Naoe, Y., et al., *Repression of interleukin-4 in T helper type 1 cells by Runx/Cbfbeta binding to the Il4 silencer*. J Exp Med, 2007. **204**(8): p. 1749-55.
243. Kano, S., et al., *The contribution of transcription factor IRF1 to the interferon-gamma-interleukin 12 signaling axis and TH1 versus TH-17 differentiation of CD4+ T cells*. Nat Immunol, 2008. **9**(1): p. 34-41.

244. Schijns, V.E., et al., *Mice lacking IL-12 develop polarized Th1 cells during viral infection*. J Immunol, 1998. **160**(8): p. 3958-64.
245. Jankovic, D., et al., *In the absence of IL-12, CD4(+) T cell responses to intracellular pathogens fail to default to a Th2 pattern and are host protective in an IL-10(-/-) setting*. Immunity, 2002. **16**(3): p. 429-39.
246. Amsen, D., A. Antov, and R.A. Flavell, *The different faces of Notch in T-helper-cell differentiation*. Nat Rev Immunol, 2009. **9**(2): p. 116-24.
247. Trembleau, S., et al., *Pancreas-infiltrating Th1 cells and diabetes develop in IL-12-deficient nonobese diabetic mice*. J Immunol, 1999. **163**(5): p. 2960-8.
248. Amsen, D., et al., *Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells*. Cell, 2004. **117**(4): p. 515-26.
249. Bray, S.J., *Notch signalling: a simple pathway becomes complex*. Nat Rev Mol Cell Biol, 2006. **7**(9): p. 678-89.
250. Maekawa, Y., et al., *Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells*. Immunity, 2003. **19**(4): p. 549-59.
251. Minter, L.M., et al., *Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21*. Nat Immunol, 2005. **6**(7): p. 680-8.
252. Cummings, H.E., R. Tuladhar, and A.R. Satoskar, *Cytokines and their STATs in cutaneous and visceral leishmaniasis*. J Biomed Biotechnol, 2010. **2010**: p. 294389.
253. Ouyang, W., et al., *Stat6-independent GATA-3 autoactivation directs IL-4-independent Th2 development and commitment*. Immunity, 2000. **12**(1): p. 27-37.
254. Jankovic, D., et al., *Single cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in vitro development of CD4+ lymphocytes with a Th2 cytokine profile*. J Immunol, 2000. **164**(6): p. 3047-55.
255. Angkasekwinai, P., et al., *Interleukin 25 promotes the initiation of proallergic type 2 responses*. J Exp Med, 2007. **204**(7): p. 1509-17.
256. Fallon, P.G., et al., *Identification of an interleukin (IL)-25-dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth expulsion*. J Exp Med, 2006. **203**(4): p. 1105-16.
257. Fort, M.M., et al., *IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo*. Immunity, 2001. **15**(6): p. 985-95.
258. Wang, Y.H., et al., *IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells*. J Exp Med, 2007. **204**(8): p. 1837-47.
259. Schmitz, J., et al., *IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines*. Immunity, 2005. **23**(5): p. 479-90.
260. Zhou, B., et al., *Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice*. Nat Immunol, 2005. **6**(10): p. 1047-53.
261. Ho, I.C., et al., *The proto-oncogene c-maf is responsible for tissue-specific expression of interleukin-4*. Cell, 1996. **85**(7): p. 973-83.
262. Zheng, W. and R.A. Flavell, *The transcription factor GATA-3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells*. Cell, 1997. **89**(4): p. 587-96.
263. Ansel, K.M., et al., *Regulation of Th2 differentiation and Il4 locus accessibility*. Annu Rev Immunol, 2006. **24**: p. 607-56.

264. Farrar, J.D., et al., *An instructive component in T helper cell type 2 (Th2) development mediated by GATA-3*. J Exp Med, 2001. **193**(5): p. 643-50.
265. Li, B., et al., *Regulation of IL-4 expression by the transcription factor JunB during T helper cell differentiation*. EMBO J, 1999. **18**(2): p. 420-32.
266. Rengarajan, J., et al., *Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression*. J Exp Med, 2002. **195**(8): p. 1003-12.
267. Yang, X.O., et al., *Requirement for the basic helix-loop-helix transcription factor Dec2 in initial TH2 lineage commitment*. Nat Immunol, 2009. **10**(12): p. 1260-6.
268. Amsen, D., et al., *Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch*. Immunity, 2007. **27**(1): p. 89-99.
269. Fang, T.C., et al., *Notch directly regulates Gata3 expression during T helper 2 cell differentiation*. Immunity, 2007. **27**(1): p. 100-10.
270. Langrish, C.L., et al., *IL-23 drives a pathogenic T cell population that induces autoimmune inflammation*. J Exp Med, 2005. **201**(2): p. 233-40.
271. Dong, C., *TH17 cells in development: an updated view of their molecular identity and genetic programming*. Nat Rev Immunol, 2008. **8**(5): p. 337-48.
272. Bettelli, E., et al., *Loss of T-bet, but not STAT1, prevents the development of experimental autoimmune encephalomyelitis*. J Exp Med, 2004. **200**(1): p. 79-87.
273. Aggarwal, S., et al., *Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17*. J Biol Chem, 2003. **278**(3): p. 1910-4.
274. Parham, C., et al., *A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R*. J Immunol, 2002. **168**(11): p. 5699-708.
275. Park, H., et al., *A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17*. Nat Immunol, 2005. **6**(11): p. 1133-41.
276. Harrington, L.E., et al., *Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages*. Nat Immunol, 2005. **6**(11): p. 1123-32.
277. Ivanov, II, et al., *The orphan nuclear receptor RORgamma directs the differentiation program of proinflammatory IL-17+ T helper cells*. Cell, 2006. **126**(6): p. 1121-33.
278. Mathur, A.N., et al., *Stat3 and Stat4 direct development of IL-17-secreting Th cells*. J Immunol, 2007. **178**(8): p. 4901-7.
279. Yang, X.O., et al., *STAT3 regulates cytokine-mediated generation of inflammatory helper T cells*. J Biol Chem, 2007. **282**(13): p. 9358-63.
280. Chen, Z., A. Laurence, and J.J. O'Shea, *Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation*. Semin Immunol, 2007. **19**(6): p. 400-8.
281. Bustle, A., et al., *The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4*. Nat Immunol, 2007. **8**(9): p. 958-66.
282. Manel, N., D. Unutmaz, and D.R. Littman, *The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgamma*. Nat Immunol, 2008. **9**(6): p. 641-9.
283. Volpe, E., et al., *A critical function for transforming growth factor-beta, interleukin 23 and proinflammatory cytokines in driving and modulating human T(H)-17 responses*. Nat Immunol, 2008. **9**(6): p. 650-7.

284. Bettelli, E., et al., *Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells*. Nature, 2006. **441**(7090): p. 235-8.
285. Mangan, P.R., et al., *Transforming growth factor-beta induces development of the T(H)17 lineage*. Nature, 2006. **441**(7090): p. 231-4.
286. Veldhoen, M., et al., *TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells*. Immunity, 2006. **24**(2): p. 179-89.
287. Okuda, Y., et al., *IL-6 plays a crucial role in the induction phase of myelin oligodendrocyte glucoprotein 35-55 induced experimental autoimmune encephalomyelitis*. J Neuroimmunol, 1999. **101**(2): p. 188-96.
288. Das, J., et al., *Transforming growth factor beta is dispensable for the molecular orchestration of Th17 cell differentiation*. J Exp Med, 2009. **206**(11): p. 2407-16.
289. Santarlasci, V., et al., *TGF-beta indirectly favors the development of human Th17 cells by inhibiting Th1 cells*. Eur J Immunol, 2009. **39**(1): p. 207-15.
290. Sutton, C., et al., *A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis*. J Exp Med, 2006. **203**(7): p. 1685-91.
291. Sutton, C.E., et al., *Interleukin-1 and IL-23 induce innate IL-17 production from gamma delta T cells, amplifying Th17 responses and autoimmunity*. Immunity, 2009. **31**(2): p. 331-41.
292. Chung, Y., et al., *Critical regulation of early Th17 cell differentiation by interleukin-1 signaling*. Immunity, 2009. **30**(4): p. 576-87.
293. Bauquet, A.T., et al., *The costimulatory molecule ICOS regulates the expression of c-Maf and IL-21 in the development of follicular T helper cells and TH-17 cells*. Nat Immunol, 2009. **10**(2): p. 167-75.
294. Murphy, C.A., et al., *Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation*. J Exp Med, 2003. **198**(12): p. 1951-7.
295. Murphy, K.M. and S.L. Reiner, *The lineage decisions of helper T cells*. Nat Rev Immunol, 2002. **2**(12): p. 933-44.
296. Ye, P., et al., *Interleukin-17 and lung host defense against Klebsiella pneumoniae infection*. Am J Respir Cell Mol Biol, 2001. **25**(3): p. 335-40.
297. Ye, P., et al., *Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense*. J Exp Med, 2001. **194**(4): p. 519-27.
298. da Matta Guedes, P.M., et al., *IL-17 produced during Trypanosoma cruzi infection plays a central role in regulating parasite-induced myocarditis*. PLoS Negl Trop Dis, 2010. **4**(2): p. e604.
299. Lin, L., et al., *Th1-Th17 cells mediate protective adaptive immunity against Staphylococcus aureus and Candida albicans infection in mice*. PLoS Pathog, 2009. **5**(12): p. e1000703.
300. Zelante, T., et al., *IL-23 and the Th17 pathway promote inflammation and impair antifungal immune resistance*. Eur J Immunol, 2007. **37**(10): p. 2695-706.
301. Gershon, R.K. and K. Kondo, *Cell interactions in the induction of tolerance: the role of thymic lymphocytes*. Immunology, 1970. **18**(5): p. 723-37.
302. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single*

- mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
303. Kanangat, S., et al., *Disease in the scurfy (sf) mouse is associated with overexpression of cytokine genes.* Eur J Immunol, 1996. **26**(1): p. 161-5.
 304. Kim, J.M., J.P. Rasmussen, and A.Y. Rudensky, *Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice.* Nat Immunol, 2007. **8**(2): p. 191-7.
 305. Curotto de Lafaille, M.A. and J.J. Lafaille, *Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor?* Immunity, 2009. **30**(5): p. 626-35.
 306. Josefowicz, S.Z. and A. Rudensky, *Control of regulatory T cell lineage commitment and maintenance.* Immunity, 2009. **30**(5): p. 616-25.
 307. Liu, Y., et al., *A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells.* Nat Immunol, 2008. **9**(6): p. 632-40.
 308. Shevach, E.M., et al., *The lifestyle of naturally occurring CD4+ CD25+ Foxp3+ regulatory T cells.* Immunol Rev, 2006. **212**: p. 60-73.
 309. Chen, W., et al., *Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3.* J Exp Med, 2003. **198**(12): p. 1875-86.
 310. Li, M.O., S. Sanjabi, and R.A. Flavell, *Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms.* Immunity, 2006. **25**(3): p. 455-71.
 311. Marie, J.C., et al., *TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells.* J Exp Med, 2005. **201**(7): p. 1061-7.
 312. Fantini, M.C., et al., *Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7.* J Immunol, 2004. **172**(9): p. 5149-53.
 313. Belkaid, Y., *Regulatory T cells and infection: a dangerous necessity.* Nat Rev Immunol, 2007. **7**(11): p. 875-88.
 314. Roncarolo, M.G., et al., *Interleukin-10-secreting type 1 regulatory T cells in rodents and humans.* Immunol Rev, 2006. **212**: p. 28-50.
 315. Brunkow, M.E., et al., *Disruption of a new forkhead/winged-helix protein, scurfy, results in the fatal lymphoproliferative disorder of the scurfy mouse.* Nat Genet, 2001. **27**(1): p. 68-73.
 316. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
 317. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
 318. Zhou, L., M.M. Chong, and D.R. Littman, *Plasticity of CD4+ T cell lineage differentiation.* Immunity, 2009. **30**(5): p. 646-55.
 319. Coombes, J.L., et al., *A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism.* J Exp Med, 2007. **204**(8): p. 1757-64.
 320. Mucida, D., et al., *Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid.* Science, 2007. **317**(5835): p. 256-60.

321. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid*. J Exp Med, 2007. **204**(8): p. 1775-85.
322. Read, S., V. Malmstrom, and F. Powrie, *Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation*. J Exp Med, 2000. **192**(2): p. 295-302.
323. Takahashi, T., et al., *Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4*. J Exp Med, 2000. **192**(2): p. 303-10.
324. Sakaguchi, S., et al., *Regulatory T cells: how do they suppress immune responses?* Int Immunol, 2009. **21**(10): p. 1105-11.
325. Miller, J.F. and G.F. Mitchell, *Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes*. J Exp Med, 1968. **128**(4): p. 801-20.
326. Zhu, J. and W.E. Paul, *CD4 T cells: fates, functions, and faults*. Blood, 2008. **112**(5): p. 1557-69.
327. Breitfeld, D., et al., *Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production*. J Exp Med, 2000. **192**(11): p. 1545-52.
328. Schaerli, P., et al., *CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function*. J Exp Med, 2000. **192**(11): p. 1553-62.
329. King, I.L. and M. Mohrs, *IL-4-producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells*. J Exp Med, 2009. **206**(5): p. 1001-7.
330. Nurieva, R.I., et al., *Bcl6 mediates the development of T follicular helper cells*. Science, 2009. **325**(5943): p. 1001-5.
331. Yu, D., et al., *The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment*. Immunity, 2009. **31**(3): p. 457-68.
332. Nurieva, R.I., et al., *Generation of T follicular helper cells is mediated by interleukin-21 but independent of T helper 1, 2, or 17 cell lineages*. Immunity, 2008. **29**(1): p. 138-49.
333. Spolski, R. and W.J. Leonard, *IL-21 and T follicular helper cells*. Int Immunol, 2010. **22**(1): p. 7-12.
334. Zaretsky, A.G., et al., *T follicular helper cells differentiate from Th2 cells in response to helminth antigens*. J Exp Med, 2009. **206**(5): p. 991-9.
335. Vogelzang, A., et al., *A fundamental role for interleukin-21 in the generation of T follicular helper cells*. Immunity, 2008. **29**(1): p. 127-37.
336. Tsuji, M., et al., *Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches*. Science, 2009. **323**(5920): p. 1488-92.
337. Batista, F.D. and M.S. Neuberger, *Affinity dependence of the B cell response to antigen: a threshold, a ceiling, and the importance of off-rate*. Immunity, 1998. **8**(6): p. 751-9.
338. Okada, T., et al., *Antigen-engaged B cells undergo chemotaxis toward the T zone and form motile conjugates with helper T cells*. PLoS Biol, 2005. **3**(6): p. e150.
339. Ozaki, K., et al., *Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6*. J Immunol, 2004. **173**(9): p. 5361-71.

340. Watanabe, T., et al., *Striking alteration of some populations of T/B cells in systemic lupus erythematosus: relationship to expression of CD62L or some chemokine receptors*. *Lupus*, 2008. **17**(1): p. 26-33.
341. Murphy, E., et al., *Reversibility of T helper 1 and 2 populations is lost after long-term stimulation*. *J Exp Med*, 1996. **183**(3): p. 901-13.
342. Voo, K.S., et al., *Identification of IL-17-producing FOXP3+ regulatory T cells in humans*. *Proc Natl Acad Sci U S A*, 2009. **106**(12): p. 4793-8.
343. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgamma function*. *Nature*, 2008. **453**(7192): p. 236-40.
344. Osorio, F., et al., *DC activated via dectin-1 convert Treg into IL-17 producers*. *Eur J Immunol*, 2008. **38**(12): p. 3274-81.
345. Wei, G., et al., *Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells*. *Immunity*, 2009. **30**(1): p. 155-67.
346. Krawczyk, C.M., H. Shen, and E.J. Pearce, *Functional plasticity in memory T helper cell responses*. *J Immunol*, 2007. **178**(7): p. 4080-8.
347. Tiselius, A. and E.A. Kabat, *Electrophoresis of Immune Serum*. *Science*, 1938. **87**(2262): p. 416-417.
348. LeBien, T.W. and T.F. Tedder, *B lymphocytes: how they develop and function*. *Blood*, 2008. **112**(5): p. 1570-80.
349. Dedeoglu, F., et al., *Induction of activation-induced cytidine deaminase gene expression by IL-4 and CD40 ligation is dependent on STAT6 and NFkappaB*. *Int Immunol*, 2004. **16**(3): p. 395-404.
350. Barrington, R.A., et al., *CD21/CD19 coreceptor signaling promotes B cell survival during primary immune responses*. *J Immunol*, 2005. **175**(5): p. 2859-67.
351. Rajewsky, K., *Clonal selection and learning in the antibody system*. *Nature*, 1996. **381**(6585): p. 751-8.
352. Norvell, A., L. Mandik, and J.G. Monroe, *Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis*. *J Immunol*, 1995. **154**(9): p. 4404-13.
353. Radbruch, A., et al., *Competence and competition: the challenge of becoming a long-lived plasma cell*. *Nat Rev Immunol*, 2006. **6**(10): p. 741-50.
354. Allman, D., et al., *Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation*. *J Immunol*, 2001. **167**(12): p. 6834-40.
355. Allman, D. and S. Pillai, *Peripheral B cell subsets*. *Curr Opin Immunol*, 2008. **20**(2): p. 149-57.
356. Hardy, R.R. and K. Hayakawa, *B cell development pathways*. *Annu Rev Immunol*, 2001. **19**: p. 595-621.
357. Hayakawa, K., et al., *The "Ly-1 B" cell subpopulation in normal immunodeficient, and autoimmune mice*. *J Exp Med*, 1983. **157**(1): p. 202-18.
358. Snapper, C.M., et al., *Distinct types of T-cell help for the induction of a humoral immune response to Streptococcus pneumoniae*. *Trends Immunol*, 2001. **22**(6): p. 308-11.
359. Haas, K.M., et al., *B-1a and B-1b cells exhibit distinct developmental requirements and have unique functional roles in innate and adaptive immunity to S. pneumoniae*. *Immunity*, 2005. **23**(1): p. 7-18.
360. Martin, F. and J.F. Kearney, *Marginal-zone B cells*. *Nat Rev Immunol*, 2002. **2**(5): p. 323-35.

361. Attanavanich, K. and J.F. Kearney, *Marginal zone, but not follicular B cells, are potent activators of naive CD4 T cells*. J Immunol, 2004. **172**(2): p. 803-11.
362. Song, H. and J. Cerny, *Functional heterogeneity of marginal zone B cells revealed by their ability to generate both early antibody-forming cells and germinal centers with hypermutation and memory in response to a T-dependent antigen*. J Exp Med, 2003. **198**(12): p. 1923-35.
363. Cariappa, A., et al., *Perisinusoidal B cells in the bone marrow participate in T-independent responses to blood-borne microbes*. Immunity, 2005. **23**(4): p. 397-407.
364. Cyster, J.G., *Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs*. Annu Rev Immunol, 2005. **23**: p. 127-59.
365. Garside, P., et al., *Visualization of specific B and T lymphocyte interactions in the lymph node*. Science, 1998. **281**(5373): p. 96-9.
366. MacLennan, I.C., et al., *Extrafollicular antibody responses*. Immunol Rev, 2003. **194**: p. 8-18.
367. MacLennan, I.C., *Germinal centers*. Annu Rev Immunol, 1994. **12**: p. 117-39.
368. Tarlinton, D., *B-cell memory: are subsets necessary?* Nat Rev Immunol, 2006. **6**(10): p. 785-90.
369. Haberman, A.M. and M.J. Shlomchik, *Reassessing the function of immune-complex retention by follicular dendritic cells*. Nat Rev Immunol, 2003. **3**(9): p. 757-64.
370. Han, S., et al., *Cellular interaction in germinal centers. Roles of CD40 ligand and B7-2 in established germinal centers*. J Immunol, 1995. **155**(2): p. 556-67.
371. Kawabe, T., et al., *The immune responses in CD40-deficient mice: impaired immunoglobulin class switching and germinal center formation*. Immunity, 1994. **1**(3): p. 167-78.
372. Ozaki, K., et al., *A critical role for IL-21 in regulating immunoglobulin production*. Science, 2002. **298**(5598): p. 1630-4.
373. Vinuesa, C.G., et al., *Follicular B helper T cells in antibody responses and autoimmunity*. Nat Rev Immunol, 2005. **5**(11): p. 853-65.
374. Manz, R.A., et al., *Maintenance of serum antibody levels*. Annu Rev Immunol, 2005. **23**: p. 367-86.
375. Zinkernagel, R.M., *What is missing in immunology to understand immunity?* Nat Immunol, 2000. **1**(3): p. 181-5.
376. Ramon, G., *Sur la toxine et sur l'anatoxine diphtériques*. Ann. Inst. Pasteur 1924. **38**: p. 1-10.
377. Glenny, A., et al., *Immunological Notes XVII to XXIV*. J Pathol, 1926. **29**: p. 31-40.
378. O'Hagan, D.T. and E. De Gregorio, *The path to a successful vaccine adjuvant--'the long and winding road'*. Drug Discov Today, 2009. **14**(11-12): p. 541-51.
379. Gupta, R.K. and G.R. Siber, *Adjuvants for human vaccines--current status, problems and future prospects*. Vaccine, 1995. **13**(14): p. 1263-76.
380. Lemaire, G., et al., *Natural and synthetic trehalose diesters as immunomodulators*. Med Res Rev, 1986. **6**(3): p. 243-74.
381. Schoenen, H., et al., *Cutting edge: Mincle is essential for recognition and adjuvant activity of the mycobacterial cord factor and its synthetic analog trehalose-dibehenate*. J Immunol, 2010. **184**(6): p. 2756-60.
382. Lemesre, J.L., et al., *Protection against experimental visceral leishmaniasis infection in dogs immunized with purified excreted secreted antigens of Leishmania infantum promastigotes*. Vaccine, 2005. **23**(22): p. 2825-40.

383. Weiner, G.J., et al., *Immunostimulatory oligodeoxynucleotides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization*. Proc Natl Acad Sci U S A, 1997. **94**(20): p. 10833-7.
384. Bevan, M.J., *Helping the CD8(+) T-cell response*. Nat Rev Immunol, 2004. **4**(8): p. 595-602.
385. Harandi, A.M. and J. Holmgren, *CpG DNA as a potent inducer of mucosal immunity: implications for immunoprophylaxis and immunotherapy of mucosal infections*. Curr Opin Investig Drugs, 2004. **5**(2): p. 141-5.
386. Reed, S.G., et al., *New horizons in adjuvants for vaccine development*. Trends Immunol, 2009. **30**(1): p. 23-32.
387. Pichichero, M.E., *Improving vaccine delivery using novel adjuvant systems*. Hum Vaccin, 2008. **4**(4): p. 262-70.
388. Hurtado, P.R., et al., *CpG oligodeoxynucleotide stimulates production of anti-neutrophil cytoplasmic antibodies in ANCA associated vasculitis*. BMC Immunol, 2008. **9**: p. 34.
389. Shimazu, R., et al., *MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4*. J Exp Med, 1999. **189**(11): p. 1777-82.
390. Johnson, A.G., S. Gaines, and M. Landy, *Studies on the O antigen of Salmonella typhosa. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide*. J Exp Med, 1956. **103**(2): p. 225-46.
391. Mueller, M., et al., *Aggregates are the biologically active units of endotoxin*. J Biol Chem, 2004. **279**(25): p. 26307-13.
392. Kundi, M., *New hepatitis B vaccine formulated with an improved adjuvant system*. Expert Rev Vaccines, 2007. **6**(2): p. 133-40.
393. Alderson, M.R., et al., *TLR4 agonists as immunomodulatory agents*. J Endotoxin Res, 2006. **12**(5): p. 313-9.
394. Gorden, K.B., et al., *Synthetic TLR agonists reveal functional differences between human TLR7 and TLR8*. J Immunol, 2005. **174**(3): p. 1259-68.
395. Barr, I.G. and G.F. Mitchell, *ISCOMs (immunostimulating complexes): the first decade*. Immunol Cell Biol, 1996. **74**(1): p. 8-25.
396. Espinet, R.G., *Nouveau vaccin antiaphteux a complexe glucoviral*. Gac. Vet. (5). Aires Gac.Vet, 1951. **13**: p. 268-270.
397. Dalsgaard, K., *Saponin adjuvants. 3. Isolation of a substance from Quillaja saponaria Molina with adjuvant activity in food-and-mouth disease vaccines*. Arch Gesamte Virusforsch, 1974. **44**(3): p. 243-54.
398. Kensil, C.R., et al., *Separation and characterization of saponins with adjuvant activity from Quillaja saponaria Molina cortex*. J Immunol, 1991. **146**(2): p. 431-7.
399. Kensil, C.R., *Saponins as vaccine adjuvants*. Crit Rev Ther Drug Carrier Syst, 1996. **13**(1-2): p. 1-55.
400. Newman, M.J., et al., *Induction of cross-reactive cytotoxic T-lymphocyte responses specific for HIV-1 gp120 using saponin adjuvant (QS-21) supplemented subunit vaccine formulations*. Vaccine, 1997. **15**(9): p. 1001-7.
401. Sasaki, S., et al., *Induction of systemic and mucosal immune responses to human immunodeficiency virus type 1 by a DNA vaccine formulated with QS-21 saponin adjuvant via intramuscular and intranasal routes*. J Virol, 1998. **72**(6): p. 4931-9.
402. Simmons, C.P., et al., *MHC class I-restricted cytotoxic lymphocyte responses induced by enterotoxin-based mucosal adjuvants*. J Immunol, 1999. **163**(12): p. 6502-10.

403. Scharton-Kersten, T., et al., *Transcutaneous immunization with bacterial ADP-ribosylating exotoxins, subunits, and unrelated adjuvants*. Infect Immun, 2000. **68**(9): p. 5306-13.
404. Sverremark, E. and C. Fernandez, *Immunogenicity of bacterial carbohydrates: cholera toxin modulates the immune response against dextran B512*. Immunology, 1997. **92**(1): p. 153-9.
405. Weltzin, R., et al., *Parenteral adjuvant activities of Escherichia coli heat-labile toxin and its B subunit for immunization of mice against gastric Helicobacter pylori infection*. Infect Immun, 2000. **68**(5): p. 2775-82.
406. Holmgren, J., N. Lycke, and C. Czerkinsky, *Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen vector systems*. Vaccine, 1993. **11**(12): p. 1179-84.
407. Takahashi, I., et al., *Mechanisms for mucosal immunogenicity and adjuvancy of Escherichia coli labile enterotoxin*. J Infect Dis, 1996. **173**(3): p. 627-35.
408. Tsuji, T., et al., *Humoral immunoresponse to varicella-zoster virus pernasally coadministered with Escherichia coli enterotoxin in mice*. Vaccine, 2000. **18**(19): p. 2049-54.
409. Williams, N.A., T.R. Hirst, and T.O. Nashar, *Immune modulation by the cholera-like enterotoxins: from adjuvant to therapeutic*. Immunol Today, 1999. **20**(2): p. 95-101.
410. Levine, M.M., et al., *New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development*. Microbiol Rev, 1983. **47**(4): p. 510-50.
411. Mutsch, M., et al., *Use of the inactivated intranasal influenza vaccine and the risk of Bell's palsy in Switzerland*. N Engl J Med, 2004. **350**(9): p. 896-903.
412. van Ginkel, F.W., et al., *Cutting edge: the mucosal adjuvant cholera toxin redirects vaccine proteins into olfactory tissues*. J Immunol, 2000. **165**(9): p. 4778-82.
413. Eriksson, A.M., K.M. Schon, and N.Y. Lycke, *The cholera toxin-derived CTA1-DD vaccine adjuvant administered intranasally does not cause inflammation or accumulate in the nervous tissues*. J Immunol, 2004. **173**(5): p. 3310-9.
414. Baudner, B.C., et al., *Enhancement of protective efficacy following intranasal immunization with vaccine plus a nontoxic LTK63 mutant delivered with nanoparticles*. Infect Immun, 2002. **70**(9): p. 4785-90.
415. Peppoloni, S., et al., *Mutants of the Escherichia coli heat-labile enterotoxin as safe and strong adjuvants for intranasal delivery of vaccines*. Expert Rev Vaccines, 2003. **2**(2): p. 285-93.
416. Pizza, M., et al., *Mucosal vaccines: non toxic derivatives of LT and CT as mucosal adjuvants*. Vaccine, 2001. **19**(17-19): p. 2534-41.
417. Stephenson, I., et al., *Phase I evaluation of intranasal trivalent inactivated influenza vaccine with nontoxigenic Escherichia coli enterotoxin and novel biovector as mucosal adjuvants, using adult volunteers*. J Virol, 2006. **80**(10): p. 4962-70.
418. Cohen, D., et al., *Safety and immunogenicity of two different lots of the oral, killed enterotoxigenic escherichia coli-cholera toxin B subunit vaccine in Israeli young adults*. Infect Immun, 2000. **68**(8): p. 4492-7.
419. Holmgren, J., et al., *An oral B subunit: whole cell vaccine against cholera*. Vaccine, 1992. **10**(13): p. 911-4.

420. Taylor, D.N., et al., *Two-year study of the protective efficacy of the oral whole cell plus recombinant B subunit cholera vaccine in Peru*. J Infect Dis, 2000. **181**(5): p. 1667-73.
421. WHO, *ETEC vaccines* 2009.
422. Wiedermann, G., et al., *Double-blind, randomized, placebo controlled pilot study evaluating efficacy and reactogenicity of an oral ETEC B-subunit-inactivated whole cell vaccine against travelers' diarrhea (preliminary report)*. J Travel Med, 2000. **7**(1): p. 27-9.
423. Le Moignic, P., *Les vaccins en emulsion dans les corps gras ou "lipo-vaccins"*. Comptes Rendus de la Societe de Biologie 1916: p. 201-203.
424. Ramon, G., *Procedes pour accroître la production des antitoxins*. Ann. Inst. Pasteur, 1926. **40**: p. 1-10.
425. Freund, J., J. Casals, and E.P. Hosmer, *Sensitization and antibody formation after injection of tubercle bacilli and paraffin oil*. Proc. Soc. Exp. Biol., 1937. **37**: p. 509-513.
426. Jansen, T., et al., *Structure- and oil type-based efficacy of emulsion adjuvants*. Vaccine, 2006. **24**(26): p. 5400-5.
427. Herbert, W.J., *The mode of action of mineral-oil emulsion adjuvants on antibody production in mice*. Immunology, 1968. **14**(3): p. 301-18.
428. Allison, A.C. and N.E. Byars, *An adjuvant formulation that selectively elicits the formation of antibodies of protective isotypes and of cell-mediated immunity*. J Immunol Methods, 1986. **95**(2): p. 157-68.
429. Ott, G., G.L. Barchfeld, and G. Van Nest, *Enhancement of humoral response against human influenza vaccine with the simple submicron oil/water emulsion adjuvant MF59*. Vaccine, 1995. **13**(16): p. 1557-62.
430. Seubert, A., et al., *The adjuvants aluminum hydroxide and MF59 induce monocyte and granulocyte chemoattractants and enhance monocyte differentiation toward dendritic cells*. J Immunol, 2008. **180**(8): p. 5402-12.
431. O'Hagan, D.T., *MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection*. Expert Rev Vaccines, 2007. **6**(5): p. 699-710.
432. Aucouturier, J., S. Ascarateil, and L. Dupuis, *The use of oil adjuvants in therapeutic vaccines*. Vaccine, 2006. **24 Suppl 2**: p. S2-44-5.
433. Scalzo, A.A., et al., *Induction of protective cytotoxic T cells to murine cytomegalovirus by using a nonapeptide and a human-compatible adjuvant (Montanide ISA 720)*. J Virol, 1995. **69**(2): p. 1306-9.
434. Wu, Y., et al., *Phase 1 trial of malaria transmission blocking vaccine candidates Pfs25 and Pvs25 formulated with montanide ISA 51*. PLoS One, 2008. **3**(7): p. e2636.
435. O'Neill, D.W., et al., *Comparison of the immunogenicity of Montanide ISA 51 adjuvant and cytokine-mature DC in a randomised controlled clinical trial of melanoma vaccine*. Journal of Clinical Oncology, 2009.
436. Horzinek, M. and M. Mussgay, *Studies on the substructure of togaviruses. I. Effect of urea, deoxycholate, and saponin on the Sindbis virion*. Arch Gesamte Virusforsch, 1971. **33**(3): p. 296-305.
437. Morein, B., et al., *Iscom, a novel structure for antigenic presentation of membrane proteins from enveloped viruses*. Nature, 1984. **308**(5958): p. 457-60.
438. Sanders, M.T., et al., *ISCOM-based vaccines: the second decade*. Immunol Cell Biol, 2005. **83**(2): p. 119-28.

439. Kersten, G.F., et al., *Incorporation of the major outer membrane protein of Neisseria gonorrhoeae in saponin-lipid complexes (iscoms): chemical analysis, some structural features, and comparison of their immunogenicity with three other antigen delivery systems*. Infect Immun, 1988. **56**(2): p. 432-8.
440. Lovgren, K. and B. Morein, *The requirement of lipids for the formation of immunostimulating complexes (iscoms)*. Biotechnol Appl Biochem, 1988. **10**(2): p. 161-72.
441. Pearce, M.J. and D. Drane, *ISCOMATRIX adjuvant for antigen delivery*. Adv Drug Deliv Rev, 2005. **57**(3): p. 465-74.
442. Watson, D.L., et al., *Interactions between immune-stimulating complexes (ISCOMs) and peritoneal mononuclear leucocytes*. Microbiol Immunol, 1992. **36**(2): p. 199-203.
443. Emery, D.L., J.S. Rothel, and P.R. Wood, *Influence of antigens and adjuvants on the production of gamma-interferon and antibody by ovine lymphocytes*. Immunol Cell Biol, 1990. **68** (Pt 2): p. 127-36.
444. Frazer, I.H., et al., *Phase 1 study of HPV16-specific immunotherapy with E6E7 fusion protein and ISCOMATRIX adjuvant in women with cervical intraepithelial neoplasia*. Vaccine, 2004. **23**(2): p. 172-81.
445. Davis, I.D., et al., *Recombinant NY-ESO-1 protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4(+) and CD8(+) T cell responses in humans*. Proc Natl Acad Sci U S A, 2004. **101**(29): p. 10697-702.
446. Clements, C.J. and E. Griffiths, *The global impact of vaccines containing aluminium adjuvants*. Vaccine, 2002. **20 Suppl 3**: p. S24-33.
447. WHO, *Immunological adjuvants. WHO technical report series no. 595. Geneva: WHO; 1976: p. 6-8. 1976.*
448. Chang, M., et al., *Degree of antigen adsorption in the vaccine or interstitial fluid and its effect on the antibody response in rabbits*. Vaccine, 2001. **19**(20-22): p. 2884-9.
449. Holt, L.B., *Developments in Diphtheria Prophylaxis*. Heinemann, London, united Kingdom, 1950.
450. Gupta, R.K., et al., *In vivo distribution of radioactivity in mice after injection of biodegradable polymer microspheres containing ¹⁴C-labeled tetanus toxoid*. Vaccine, 1996. **14**(15): p. 1412-6.
451. Jordan, M.B., et al., *Promotion of B cell immune responses via an alum-induced myeloid cell population*. Science, 2004. **304**(5678): p. 1808-10.
452. McKee, A.S., et al., *Gr1+IL-4-producing innate cells are induced in response to Th2 stimuli and suppress Th1-dependent antibody responses*. Int Immunol, 2008. **20**(5): p. 659-69.
453. Wang, H.B. and P.F. Weller, *Pivotal advance: eosinophils mediate early alum adjuvant-elicited B cell priming and IgM production*. J Leukoc Biol, 2008. **83**(4): p. 817-21.
454. Brewer, J.M., et al., *Neither interleukin-6 nor signalling via tumour necrosis factor receptor-1 contribute to the adjuvant activity of Alum and Freund's adjuvant*. Immunology, 1998. **93**(1): p. 41-8.
455. Brewer, J.M., et al., *Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling*. J Immunol, 1999. **163**(12): p. 6448-54.

456. Brewer, J.M., et al., *In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production.* Eur J Immunol, 1996. **26**(9): p. 2062-6.
457. Li, H., S. Nookala, and F. Re, *Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release.* J Immunol, 2007. **178**(8): p. 5271-6.
458. Sokolovska, A., S.L. Hem, and H. HogenEsch, *Activation of dendritic cells and induction of CD4(+) T cell differentiation by aluminum-containing adjuvants.* Vaccine, 2007. **25**(23): p. 4575-85.
459. Gavin, A.L., et al., *Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling.* Science, 2006. **314**(5807): p. 1936-8.
460. Schnare, M., et al., *Toll-like receptors control activation of adaptive immune responses.* Nat Immunol, 2001. **2**(10): p. 947-50.
461. O'Hagan, D.T. and M. Singh, *Microparticles as vaccine adjuvants and delivery systems.* Expert Rev Vaccines, 2003. **2**(2): p. 269-83.
462. Challacombe, S.J., et al., *Enhanced secretory IgA and systemic IgG antibody responses after oral immunization with biodegradable microparticles containing antigen.* Immunology, 1992. **76**(1): p. 164-8.
463. Eldridge, J.H., et al., *New advances in vaccine delivery systems.* Semin Hematol, 1993. **30**(4 Suppl 4): p. 16-24; discussion 25.
464. O'Hagan, D.T., *Microparticles and polymers for the mucosal delivery of vaccines.* Adv Drug Deliv Rev, 1998. **34**(2-3): p. 305-320.
465. Shenderova, A., T.G. Burke, and S.P. Schwendeman, *The acidic microclimate in poly(lactide-co-glycolide) microspheres stabilizes camptothecins.* Pharm Res, 1999. **16**(2): p. 241-8.
466. Singh, M., et al., *A preliminary evaluation of alternative adjuvants to alum using a range of established and new generation vaccine antigens.* Vaccine, 2006. **24**(10): p. 1680-6.
467. Lavelle, E.C., et al., *The stability and immunogenicity of a protein antigen encapsulated in biodegradable microparticles based on blends of lactide polymers and polyethylene glycol.* Vaccine, 1999. **17**(6): p. 512-29.
468. Takahata, H., et al., *The distribution of protein associated with poly(DL-lactide co-glycolide) microparticles and its degradation in simulated body fluids.* J Control Release, 1998. **50**(1-3): p. 237-46.
469. Kazzaz, J., et al., *Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1.* J Control Release, 2000. **67**(2-3): p. 347-56.
470. Singh, M., et al., *Adsorption of a novel recombinant glycoprotein from HIV (Env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not.* Pharm Res, 2004. **21**(12): p. 2148-52.
471. Singh, M., et al., *Cationic microparticles: A potent delivery system for DNA vaccines.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 811-6.
472. O'Hagan, D.T., H. Jeffery, and S.S. Davis, *Long-term antibody responses in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microparticles.* Vaccine, 1993. **11**(9): p. 965-9.
473. Bachmann, M.F. and R.M. Zinkernagel, *Neutralizing antiviral B cell responses.* Annu Rev Immunol, 1997. **15**: p. 235-70.
474. Maloy, K.J., et al., *Induction of mucosal and systemic immune responses by immunization with ovalbumin entrapped in poly(lactide-co-glycolide) microparticles.* Immunology, 1994. **81**(4): p. 661-7.

475. Nixon, D.F., et al., *Synthetic peptides entrapped in microparticles can elicit cytotoxic T cell activity*. Vaccine, 1996. **14**(16): p. 1523-30.
476. Sun, H., K.G. Pollock, and J.M. Brewer, *Analysis of the role of vaccine adjuvants in modulating dendritic cell activation and antigen presentation in vitro*. Vaccine, 2003. **21**(9-10): p. 849-55.
477. Trial, P.H.C.
478. Scheerlinck, J.P. and D.L. Greenwood, *Virus-sized vaccine delivery systems*. Drug Discov Today, 2008. **13**(19-20): p. 882-7.
479. Wilschut, J., *Influenza vaccines: the virosome concept*. Immunol Lett, 2009. **122**(2): p. 118-21.
480. Gluck, R., C. Moser, and I.C. Metcalfe, *Influenza virosomes as an efficient system for adjuvanted vaccine delivery*. Expert Opin Biol Ther, 2004. **4**(7): p. 1139-45.
481. Grgacic, E.V. and D.A. Anderson, *Virus-like particles: passport to immune recognition*. Methods, 2006. **40**(1): p. 60-5.
482. Huckriede, A., et al., *The virosome concept for influenza vaccines*. Vaccine, 2005. **23** Suppl 1: p. S26-38.
483. Peek, L.J., C.R. Middaugh, and C. Berkland, *Nanotechnology in vaccine delivery*. Adv Drug Deliv Rev, 2008. **60**(8): p. 915-28.
484. Villa, L.L., et al., *Prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in young women: a randomised double-blind placebo-controlled multicentre phase II efficacy trial*. Lancet Oncol, 2005. **6**(5): p. 271-8.
485. Harper, D.M., et al., *Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial*. Lancet, 2004. **364**(9447): p. 1757-65.
486. Harper, D.M., et al., *Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial*. Lancet, 2006. **367**(9518): p. 1247-55.
487. Bosch, F.X. and S. de Sanjose, *Chapter 1: Human papillomavirus and cervical cancer--burden and assessment of causality*. J Natl Cancer Inst Monogr, 2003(31): p. 3-13.
488. Bangham, A.D., M.M. Standish, and N. Miller, *Cation permeability of phospholipid model membranes: effect of narcotics*. Nature, 1965. **208**(5017): p. 1295-7.
489. Gregoriadis, G. and A.C. Allison, *Entrapment of proteins in liposomes prevents allergic reactions in pre-immunised mice*. FEBS Lett, 1974. **45**(1): p. 71-4.
490. Gabizon, A.A., *Selective tumor localization and improved therapeutic index of anthracyclines encapsulated in long-circulating liposomes*. Cancer Res, 1992. **52**(4): p. 891-6.
491. Maruyama, K., et al., *Phosphatidyl polyglycerols prolong liposome circulation in vivo* International Journal of Pharmaceutics, 1994. **111**(1): p. 103-107.
492. Foged, C., et al., *Interaction of dendritic cells with antigen-containing liposomes: effect of bilayer composition*. Vaccine, 2004. **22**(15-16): p. 1903-13.
493. Fries, L.F., et al., *Liposomal malaria vaccine in humans: a safe and potent adjuvant strategy*. Proc Natl Acad Sci U S A, 1992. **89**(1): p. 358-62.
494. Palmer, M., et al., *Phase I study of the BLP25 (MUC1 peptide) liposomal vaccine for active specific immunotherapy in stage IIIB/IV non-small-cell lung cancer*. Clin Lung Cancer, 2001. **3**(1): p. 49-57; discussion 58.
495. Merck, *Stimuvax trial*. 2009.

496. Merck, *Stimuvax encephalitis SAE*. 2010.
497. Langley, J.M., et al., *Safety and immunogenicity of a Proteosome -trivalent inactivated influenza vaccine, given nasally to healthy adults*. *Vaccine*, 2006. **24**(10): p. 1601-8.
498. Chabot, S.M., et al., *TLR2 activation by proteosomes promotes uptake of particulate vaccines at mucosal surfaces*. *Vaccine*, 2007. **25**(29): p. 5348-58.
499. Massari, P., et al., *The role of porins in neisserial pathogenesis and immunity*. *Trends Microbiol*, 2003. **11**(2): p. 87-93.
500. Wetzler, L.M., *The role of Toll-like receptor 2 in microbial disease and immunity*. *Vaccine*, 2003. **21 Suppl 2**: p. S55-60.
501. Chabot, S., et al., *A novel intranasal Protollin-based measles vaccine induces mucosal and systemic neutralizing antibody responses and cell-mediated immunity in mice*. *Vaccine*, 2005. **23**(11): p. 1374-83.
502. Jones, T., et al., *Intranasal Protollin/FI-V vaccine elicits respiratory and serum antibody responses and protects mice against lethal aerosolized plague infection*. *Vaccine*, 2006. **24**(10): p. 1625-32.
503. Jones, T., et al., *Protollin: a novel adjuvant for intranasal vaccines*. *Vaccine*, 2004. **22**(27-28): p. 3691-7.
504. Treanor, J., et al., *Intranasal administration of a proteosome-influenza vaccine is well-tolerated and induces serum and nasal secretion influenza antibodies in healthy human subjects*. *Vaccine*, 2006. **24**(3): p. 254-62.
505. AS01 and GSK.
506. AS02 and GSK, *Malaria Trial results*.
507. AS03 and GSK, *Pandemrix vaccine*
508. Didierlaurent, A.M., et al., *AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity*. *J Immunol*, 2009. **183**(10): p. 6186-97.
509. AS04 and GSK, *Cervarix vaccine*.
510. Schiller, J.T., et al., *An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results*. *Vaccine*, 2008. **26 Suppl 10**: p. K53-61.
511. AS15 and GSK, *AS15 cancer vaccines*.
512. Verstraeten, T., et al., *Analysis of adverse events of potential autoimmune aetiology in a large integrated safety database of AS04 adjuvanted vaccines*. *Vaccine*, 2008. **26**(51): p. 6630-8.
513. Malyala, P., et al., *The potency of the adjuvant, CpG oligos, is enhanced by encapsulation in PLG microparticles* *J Pharm Sci*, 2007. **3**(97): p. 1155-1164.
514. Hunter, S.K., M.E. Andracki, and A.M. Krieg, *Biodegradable microspheres containing group B Streptococcus vaccine: immune response in mice*. *Am J Obstet Gynecol*, 2001. **185**(5): p. 1174-9.
515. Agger, E.M., et al., *Protective immunity to tuberculosis with Ag85B-ESAT-6 in a synthetic cationic adjuvant system IC31*. *Vaccine*, 2006. **24**(26): p. 5452-60.
516. Schellack, C., et al., *IC31, a novel adjuvant signaling via TLR9, induces potent cellular and humoral immune responses*. *Vaccine*, 2006. **24**(26): p. 5461-72.
517. Riedl, K., et al., *The novel adjuvant IC31 strongly improves influenza vaccine-specific cellular and humoral immune responses in young adult and aged mice*. *Vaccine*, 2008. **26**(27-28): p. 3461-8.
518. Intercell, *IC31*. 2010.

519. Lutz, M.B., et al., *An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow*. J Immunol Methods, 1999. **223**(1): p. 77-92.
520. Iyer, S., H. HogenEsch, and S.L. Hem, *Relationship between the degree of antigen adsorption to aluminum hydroxide adjuvant in interstitial fluid and antibody production*. Vaccine, 2003. **21**(11-12): p. 1219-23.
521. Romero Mendez, I.Z., et al., *Potentiation of the immune response to non-adsorbed antigens by aluminum-containing adjuvants*. Vaccine, 2007. **25**(5): p. 825-33.
522. Ulanova, M., et al., *The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism*. Infect Immun, 2001. **69**(2): p. 1151-9.
523. Mannhalter, J.W., et al., *Modulation of the human immune response by the non-toxic and non-pyrogenic adjuvant aluminium hydroxide: effect on antigen uptake and antigen presentation*. Clin Exp Immunol, 1985. **61**(1): p. 143-51.
524. Barksby, H.E., et al., *The expanding family of interleukin-1 cytokines and their role in destructive inflammatory disorders*. Clin Exp Immunol, 2007. **149**(2): p. 217-25.
525. Rubartelli, A., et al., *A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence*. EMBO J, 1990. **9**(5): p. 1503-10.
526. Gray, P.W., et al., *Two interleukin 1 genes in the mouse: cloning and expression of the cDNA for murine interleukin 1 beta*. J Immunol, 1986. **137**(11): p. 3644-8.
527. Lomedico, P.T., et al., *Cloning and expression of murine interleukin-1 cDNA in Escherichia coli*. Nature, 1984. **312**(5993): p. 458-62.
528. March, C.J., et al., *Cloning, sequence and expression of two distinct human interleukin-1 complementary DNAs*. Nature, 1985. **315**(6021): p. 641-7.
529. Carruth, L.M., S. Demczuk, and S.B. Mizel, *Involvement of a calpain-like protease in the processing of the murine interleukin 1 alpha precursor*. J Biol Chem, 1991. **266**(19): p. 12162-7.
530. Dinarello, C.A., *Interleukin-1 beta, interleukin-18, and the interleukin-1 beta converting enzyme*. Ann N Y Acad Sci, 1998. **856**: p. 1-11.
531. Keller, M., et al., *Active caspase-1 is a regulator of unconventional protein secretion*. Cell, 2008. **132**(5): p. 818-31.
532. Becker, C.E., E.M. Creagh, and L.A. O'Neill, *Rab39a binds caspase-1 and is required for caspase-1-dependent interleukin-1beta secretion*. J Biol Chem, 2009. **284**(50): p. 34531-7.
533. Pelegrin, P. and A. Surprenant, *Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor*. EMBO J, 2006. **25**(21): p. 5071-82.
534. Pelegrin, P. and A. Surprenant, *Pannexin-1 couples to maitotoxin- and nigericin-induced interleukin-1beta release through a dye uptake-independent pathway*. J Biol Chem, 2007. **282**(4): p. 2386-94.
535. Martinon, F., *Mechanisms of uric acid crystal-mediated autoinflammation*. Immunol Rev, 2010. **233**(1): p. 218-32.
536. Perregaux, D. and C.A. Gabel, *Interleukin-1 beta maturation and release in response to ATP and nigericin. Evidence that potassium depletion mediated by these agents is a necessary and common feature of their activity*. J Biol Chem, 1994. **269**(21): p. 15195-203.
537. Walev, I., et al., *Potassium regulates IL-1 beta processing via calcium-independent phospholipase A2*. J Immunol, 2000. **164**(10): p. 5120-4.

538. Meissner, F., K. Molawi, and A. Zychlinsky, *Superoxide dismutase 1 regulates caspase-1 and endotoxic shock*. Nat Immunol, 2008. **9**(8): p. 866-72.
539. van de Veerdonk, F.L., et al., *Reactive oxygen species-independent activation of the IL-1 β inflammasome in cells from patients with chronic granulomatous disease*. Proc Natl Acad Sci U S A, 2010. **107**(7): p. 3030-3.
540. Fodor, S., Z. Jakus, and A. Mocsai, *ITAM-based signaling beyond the adaptive immune response*. Immunol Lett, 2006. **104**(1-2): p. 29-37.
541. Auron, P.E., et al., *Nucleotide sequence of human monocyte interleukin 1 precursor cDNA*. Proc Natl Acad Sci U S A, 1984. **81**(24): p. 7907-11.
542. Dinarello, C.A., *IL-1: discoveries, controversies and future directions*. Eur J Immunol, 2010. **40**(3): p. 599-606.
543. Werman, A., et al., *The precursor form of IL-1 α is an intracrine proinflammatory activator of transcription*. Proc Natl Acad Sci U S A, 2004. **101**(8): p. 2434-9.
544. Kaplanski, G., et al., *Interleukin-1 induces interleukin-8 secretion from endothelial cells by a juxtacrine mechanism*. Blood, 1994. **84**(12): p. 4242-8.
545. Chen, C.J., et al., *Identification of a key pathway required for the sterile inflammatory response triggered by dying cells*. Nat Med, 2007. **13**(7): p. 851-6.
546. Mosca, F., et al., *Molecular and cellular signatures of human vaccine adjuvants*. Proc Natl Acad Sci U S A, 2008. **105**(30): p. 10501-6.
547. Fifis, T., et al., *Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors*. J Immunol, 2004. **173**(5): p. 3148-54.
548. Katare, Y.K., T. Muthukumaran, and A.K. Panda, *Influence of particle size, antigen load, dose and additional adjuvant on the immune response from antigen loaded PLA microparticles*. Int J Pharm, 2005. **301**(1-2): p. 149-60.
549. Mottram, P.L., et al., *Type 1 and 2 immunity following vaccination is influenced by nanoparticle size: formulation of a model vaccine for respiratory syncytial virus*. Mol Pharm, 2007. **4**(1): p. 73-84.
550. Fazilleau, N., et al., *The function of follicular helper T cells is regulated by the strength of T cell antigen receptor binding*. Nat Immunol, 2009. **10**(4): p. 375-84.
551. Goto, N. and K. Akama, *Histopathological studies of reactions in mice injected with aluminium-adsorbed tetanus toxoid*. Microbiology Immunology, 1982. **12**(26): p. 1121-1132.
552. Goto, N., et al., *Local tissue irritating effects and adjuvant activities of calcium phosphate and aluminium hydroxide with different physical properties*. Vaccine, 1997. **15**(12-13): p. 1364-71.
553. Kopf, M., et al., *Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component*. J Exp Med, 1998. **188**(10): p. 1895-906.
554. Fiorentino, D.F., M.W. Bond, and T.R. Mosmann, *Two types of mouse T helper cell. IV. Th2 clones secrete a factor that inhibits cytokine production by Th1 clones*. J Exp Med, 1989. **170**(6): p. 2081-95.
555. Moore, K.W., et al., *Interleukin-10 and the interleukin-10 receptor*. Annu Rev Immunol, 2001. **19**: p. 683-765.
556. O'Garra, A. and P. Vieira, *T(H)1 cells control themselves by producing interleukin-10*. Nat Rev Immunol, 2007. **7**(6): p. 425-8.
557. Gazzinelli, R.T., et al., *In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response*

- dependent on CD4⁺ T cells and accompanied by overproduction of IL-12, IFN- γ and TNF- α . J Immunol, 1996. **157**(2): p. 798-805.
558. Hunter, C.A., et al., *IL-10 is required to prevent immune hyperactivity during infection with Trypanosoma cruzi*. J Immunol, 1997. **158**(7): p. 3311-6.
 559. Berg, D.J., et al., *Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance*. J Clin Invest, 1995. **96**(5): p. 2339-47.
 560. Kuhn, R., et al., *Interleukin-10-deficient mice develop chronic enterocolitis*. Cell, 1993. **75**(2): p. 263-74.
 561. Ejrnaes, M., et al., *Resolution of a chronic viral infection after interleukin-10 receptor blockade*. J Exp Med, 2006. **203**(11): p. 2461-72.
 562. Roque, S., et al., *IL-10 underlies distinct susceptibility of BALB/c and C57BL/6 mice to Mycobacterium avium infection and influences efficacy of antibiotic therapy*. J Immunol, 2007. **178**(12): p. 8028-35.
 563. Castro, A.G., et al., *Anti-interleukin 10 receptor monoclonal antibody is an adjuvant for T helper cell type 1 responses to soluble antigen only in the presence of lipopolysaccharide*. J Exp Med, 2000. **192**(10): p. 1529-34.
 564. Anderson, C.F., et al., *CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis*. J Exp Med, 2007. **204**(2): p. 285-97.
 565. McGeachy, M.J., et al., *TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology*. Nat Immunol, 2007. **8**(12): p. 1390-7.
 566. Stumhofer, J.S., et al., *Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10*. Nat Immunol, 2007. **8**(12): p. 1363-71.
 567. Jankovic, D., et al., *Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection*. J Exp Med, 2007. **204**(2): p. 273-83.
 568. Romani, L., et al., *Neutrophil production of IL-12 and IL-10 in candidiasis and efficacy of IL-12 therapy in neutropenic mice*. J Immunol, 1997. **158**(11): p. 5349-56.
 569. Fillatreau, S., et al., *B cells regulate autoimmunity by provision of IL-10*. Nat Immunol, 2002. **3**(10): p. 944-50.
 570. Ben-Sasson, S.Z., et al., *IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation*. Proc Natl Acad Sci U S A, 2009. **106**(17): p. 7119-24.
 571. Ozaki, K., et al., *Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain*. Proc Natl Acad Sci U S A, 2000. **97**(21): p. 11439-44.
 572. Parrish-Novak, J., et al., *Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function*. Nature, 2000. **408**(6808): p. 57-63.
 573. Spolski, R. and W.J. Leonard, *Interleukin-21: basic biology and implications for cancer and autoimmunity*. Annu Rev Immunol, 2008. **26**: p. 57-79.
 574. King, C., *New insights into the differentiation and function of T follicular helper cells*. Nat Rev Immunol, 2009. **9**(11): p. 757-66.
 575. Coquet, J.M., et al., *IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production*. J Immunol, 2007. **178**(5): p. 2827-34.
 576. Kasaian, M.T., et al., *IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity*. Immunity, 2002. **16**(4): p. 559-69.

577. Nurieva, R., et al., *Essential autocrine regulation by IL-21 in the generation of inflammatory T cells*. *Nature*, 2007. **448**(7152): p. 480-3.
578. Chtanova, T., et al., *T follicular helper cells express a distinctive transcriptional profile, reflecting their role as non-Th1/Th2 effector cells that provide help for B cells*. *J Immunol*, 2004. **173**(1): p. 68-78.
579. O'Hagan, D.T., M. Singh, and J.B. Ulmer, *Microparticle-based technologies for vaccines*. *Methods*, 2006. **40**(1): p. 10-9.
580. Gutierrez, I., et al., *Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres*. *Vaccine*, 2002. **21**(1-2): p. 67-77.
581. Newman, K.D., et al., *Uptake of poly(D,L-lactic-co-glycolic acid) microspheres by antigen-presenting cells in vivo*. *J Biomed Mater Res*, 2002. **60**(3): p. 480-6.
582. Singh, M., et al., *Controlled release microparticles as a single dose hepatitis B vaccine: evaluation of immunogenicity in mice*. *Vaccine*, 1997. **15**(5): p. 475-81.
583. Matzinger, P., *The danger model: a renewed sense of self*. *Science*, 2002. **296**(5566): p. 301-5.
584. Shi, Y. and K.L. Rock, *Cell death releases endogenous adjuvants that selectively enhance immune surveillance of particulate antigens*. *Eur J Immunol*, 2002. **32**(1): p. 155-62.
585. Shi, Y., W. Zheng, and K.L. Rock, *Cell injury releases endogenous adjuvants that stimulate cytotoxic T cell responses*. *Proc Natl Acad Sci U S A*, 2000. **97**(26): p. 14590-5.
586. Iyer, S.S., et al., *Necrotic cells trigger a sterile inflammatory response through the Nlrp3 inflammasome*. *Proc Natl Acad Sci U S A*, 2009. **106**(48): p. 20388-93.
587. Li, H., A. Ambade, and F. Re, *Cutting edge: Necrosis activates the NLRP3 inflammasome*. *J Immunol*, 2009. **183**(3): p. 1528-32.
588. Willingham, S.B., et al., *Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC*. *Cell Host Microbe*, 2007. **2**(3): p. 147-59.
589. Brennan, M.A. and B.T. Cookson, *Salmonella induces macrophage death by caspase-1-dependent necrosis*. *Mol Microbiol*, 2000. **38**(1): p. 31-40.
590. Fink, S.L. and B.T. Cookson, *Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells*. *Infect Immun*, 2005. **73**(4): p. 1907-16.
591. Zimmermann, K.C., C. Bonzon, and D.R. Green, *The machinery of programmed cell death*. *Pharmacol Ther*, 2001. **92**(1): p. 57-70.
592. Zou, H., et al., *Apaf-1, a human protein homologous to C. elegans CED-4, participates in cytochrome c-dependent activation of caspase-3*. *Cell*, 1997. **90**(3): p. 405-13.
593. Kono, H. and K.L. Rock, *How dying cells alert the immune system to danger*. *Nat Rev Immunol*, 2008. **8**(4): p. 279-89.
594. Gallucci, S., M. Lolkema, and P. Matzinger, *Natural adjuvants: endogenous activators of dendritic cells*. *Nat Med*, 1999. **5**(11): p. 1249-55.
595. Sauter, B., et al., *Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells*. *J Exp Med*, 2000. **191**(3): p. 423-34.
596. Kerr, J.F., A.H. Wyllie, and A.R. Currie, *Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics*. *Br J Cancer*, 1972. **26**(4): p. 239-57.

597. Verhoven, B., R.A. Schlegel, and P. Williamson, *Mechanisms of phosphatidylserine exposure, a phagocyte recognition signal, on apoptotic T lymphocytes*. J Exp Med, 1995. **182**(5): p. 1597-601.
598. Edinger, A.L. and C.B. Thompson, *Death by design: apoptosis, necrosis and autophagy*. Curr Opin Cell Biol, 2004. **16**(6): p. 663-9.
599. Kroemer, G., et al., *Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009*. Cell Death Differ, 2009. **16**(1): p. 3-11.
600. Declercq, W., T. Vanden Berghe, and P. Vandenabeele, *RIP kinases at the crossroads of cell death and survival*. Cell, 2009. **138**(2): p. 229-32.
601. Festjens, N., et al., *RIP1, a kinase on the crossroads of a cell's decision to live or die*. Cell Death Differ, 2007. **14**(3): p. 400-10.
602. Cho, Y.S., et al., *Phosphorylation-driven assembly of the RIP1-RIP3 complex regulates programmed necrosis and virus-induced inflammation*. Cell, 2009. **137**(6): p. 1112-23.
603. Degterev, A., et al., *Identification of RIP1 kinase as a specific cellular target of necrostatins*. Nat Chem Biol, 2008. **4**(5): p. 313-21.
604. He, S., et al., *Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha*. Cell, 2009. **137**(6): p. 1100-11.
605. Holler, N., et al., *Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule*. Nat Immunol, 2000. **1**(6): p. 489-95.
606. Zhang, D.W., et al., *RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis*. Science, 2009. **325**(5938): p. 332-6.
607. Fujisawa, A., et al., *Disease-associated mutations in CLAS1 induce cathepsin B-dependent rapid cell death of human THP-1 monocytic cells*. Blood, 2007. **109**(7): p. 2903-11.
608. Hersh, D., et al., *The Salmonella invasin SipB induces macrophage apoptosis by binding to caspase-1*. Proc Natl Acad Sci U S A, 1999. **96**(5): p. 2396-401.
609. Monack, D.M., et al., *Salmonella exploits caspase-1 to colonize Peyer's patches in a murine typhoid model*. J Exp Med, 2000. **192**(2): p. 249-58.
610. Cervantes, J., et al., *Intracytosolic Listeria monocytogenes induces cell death through caspase-1 activation in murine macrophages*. Cell Microbiol, 2008. **10**(1): p. 41-52.
611. Jiang, W. and D.S. Pisetsky, *Expression of high mobility group protein 1 in the sera of patients and mice with systemic lupus erythematosus*. Ann Rheum Dis, 2008. **67**(5): p. 727-8.
612. Kokkola, R., et al., *RAGE is the major receptor for the proinflammatory activity of HMGB1 in rodent macrophages*. Scand J Immunol, 2005. **61**(1): p. 1-9.
613. Wang, H., et al., *HMG-1 as a late mediator of endotoxin lethality in mice*. Science, 1999. **285**(5425): p. 248-51.
614. Bianchi, M.E., *HMGB1 loves company*. J Leukoc Biol, 2009. **86**(3): p. 573-6.
615. Rovere-Querini, P., et al., *HMGB1 is an endogenous immune adjuvant released by necrotic cells*. EMBO Rep, 2004. **5**(8): p. 825-30.
616. Sha, Y., et al., *HMGB1 develops enhanced proinflammatory activity by binding to cytokines*. J Immunol, 2008. **180**(4): p. 2531-7.
617. Gonzalez-Navajas, J.M., et al., *TLR4 signaling in effector CD4+ T cells regulates TCR activation and experimental colitis in mice*. J Clin Invest, 2010. **120**(2): p. 570-81.

618. Khoruts, A., R.E. Osness, and M.K. Jenkins, *IL-1 acts on antigen-presenting cells to enhance the in vivo proliferation of antigen-stimulated naive CD4 T cells via a CD28-dependent mechanism that does not involve increased expression of CD28 ligands*. Eur J Immunol, 2004. **34**(4): p. 1085-90.
619. Pape, K.A., et al., *Inflammatory cytokines enhance the in vivo clonal expansion and differentiation of antigen-activated CD4+ T cells*. J Immunol, 1997. **159**(2): p. 591-8.
620. Bomford, R., *The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect on delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse of Freund's incomplete and complete adjuvants, alhydrogel, Corynebacterium parvum, Bordetella pertussis, muramyl dipeptide and saponin*. Clin Exp Immunol, 1980. **39**(2): p. 435-41.
621. Luthi, A.U., et al., *Suppression of interleukin-33 bioactivity through proteolysis by apoptotic caspases*. Immunity, 2009. **31**(1): p. 84-98.
622. Pollock, K.G., et al., *Interleukin-18 plays a role in both the alum-induced T helper 2 response and the T helper 1 response induced by alum-adsorbed interleukin-12*. Immunology, 2003. **108**(2): p. 137-43.
623. Linterman, M.A., et al., *IL-21 acts directly on B cells to regulate Bcl-6 expression and germinal center responses*. J Exp Med, 2010. **207**(2): p. 353-63.
624. Zotos, D., et al., *IL-21 regulates germinal center B cell differentiation and proliferation through a B cell-intrinsic mechanism*. J Exp Med, 2010. **207**(2): p. 365-78.
625. Brandt, K., et al., *Interleukin-21 inhibits dendritic cell activation and maturation*. Blood, 2003. **102**(12): p. 4090-8.
626. Jin, H., et al., *Distinct activation signals determine whether IL-21 induces B cell costimulation, growth arrest, or Bim-dependent apoptosis*. J Immunol, 2004. **173**(1): p. 657-65.
627. Spolski, R., et al., *IL-21 mediates suppressive effects via its induction of IL-10*. J Immunol, 2009. **182**(5): p. 2859-67.
628. Pearce, E.J., et al., *Th2 response polarization during infection with the helminth parasite Schistosoma mansoni*. Immunol Rev, 2004. **201**: p. 117-26.
629. Zhao, X., et al., *Vaginal submucosal dendritic cells, but not Langerhans cells, induce protective Th1 responses to herpes simplex virus-2*. J Exp Med, 2003. **197**(2): p. 153-62.
630. Brewer, J.M., et al., *Vesicle size influences the trafficking, processing, and presentation of antigens in lipid vesicles*. J Immunol, 2004. **173**(10): p. 6143-50.
631. Horisawa, E., et al., *Size-dependency of DL-lactide/glycolide copolymer particulates for intra-articular delivery system on phagocytosis in rat synovium*. Pharm Res, 2002. **19**(2): p. 132-9.
632. Eigenbrod, T., et al., *Cutting edge: critical role for mesothelial cells in necrosis-induced inflammation through the recognition of IL-1 alpha released from dying cells*. J Immunol, 2008. **181**(12): p. 8194-8.
633. Fulda, S., et al., *Cellular stress responses: cell survival and cell death*. Int J Cell Biol, 2010. **2010**: p. 214074.
634. Maung, A.A., et al., *Injury enhances resistance to Escherichia coli infection by boosting innate immune system function*. J Immunol, 2008. **180**(4): p. 2450-8.
635. Brody, D.T. and S.K. Durum, *Membrane IL-1: IL-1 alpha precursor binds to the plasma membrane via a lectin-like interaction*. J Immunol, 1989. **143**(4): p. 1183-7.

636. Rao, D.A., K.J. Tracey, and J.S. Pober, *IL-1alpha and IL-1beta are endogenous mediators linking cell injury to the adaptive alloimmune response*. J Immunol, 2007. **179**(10): p. 6536-46.

Appendix I