Size regulates particulate adjuvant induced IL-10 and IL-12p70 production

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Declaration of authorship

This report represents the sole work of the author except where stated and has not been submitted in whole or in part to any other university or institution as a part of a degree or other qualification.

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Seán McCluskey
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## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>AS</td>
<td>Adjuvant system</td>
</tr>
<tr>
<td>Adju-Phos</td>
<td>aluminium phosphate</td>
</tr>
<tr>
<td>Alum</td>
<td>aluminium hydroxide</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium persulphate</td>
</tr>
<tr>
<td>BMDCs</td>
<td>Bone marrow-derived dendritic cells</td>
</tr>
<tr>
<td>BCG</td>
<td>Bacille Calmette–Guérin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>Bregs</td>
<td>Regulatory B cells</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptors</td>
</tr>
<tr>
<td>CLRs</td>
<td>C-type lectin receptors</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element-binding protein</td>
</tr>
<tr>
<td>cDCs</td>
<td>conventional DCs</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CARD</td>
<td>caspase recruitment domain</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CpG</td>
<td>cytidine-phosphate-guanosine</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>cGAS</td>
<td>Cyclic GMP-AMP Synthase</td>
</tr>
<tr>
<td>ChIP-Seq</td>
<td>Chromatin Immunoprecipitation Sequencing</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DAMPs</td>
<td>Damage associated molecular patterns</td>
</tr>
<tr>
<td>DC-SIGN</td>
<td>DC-specific intercellular adhesion molecule-3-grabbing non-integrin</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence reagent</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>GF</td>
<td>Growth Factor</td>
</tr>
<tr>
<td>MSKs</td>
<td>Mitogen-and Stress-activated protein Kinases</td>
</tr>
<tr>
<td>MSU</td>
<td>Monosodium urate</td>
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<tr>
<td>MDP</td>
<td>Muramyl dipeptide</td>
</tr>
<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>Mo-DCs</td>
<td>Monocyte derived inflammatory DCs</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony-stimulating factor M-CSF</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary response gene 88</td>
</tr>
<tr>
<td>MβCD</td>
<td>Methyl-beta-cyclodextrin</td>
</tr>
<tr>
<td>MPL</td>
<td>3-O-desacyl-4’-monophosphoryl lipid A</td>
</tr>
<tr>
<td>nm</td>
<td>Nanometre</td>
</tr>
<tr>
<td>NLRs</td>
<td>Nod-like receptors</td>
</tr>
<tr>
<td>NAD</td>
<td>NACHT-associated domain</td>
</tr>
<tr>
<td>NLRC4</td>
<td>NLR family CARD domain-containing 4</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NLR family pyrin domain-containing 3</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide oligomerisation domain</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>OPD</td>
<td>O-Phenylenediamine dihydrochloride</td>
</tr>
<tr>
<td>OVA</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>OTII</td>
<td>OVA transgenic</td>
</tr>
<tr>
<td>O/W</td>
<td>Oil-in-water</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinases</td>
</tr>
<tr>
<td>PLA</td>
<td>Poly (lactic acid)</td>
</tr>
<tr>
<td>PLG</td>
<td>Poly (D L-lactide)-co-glycolide</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly (lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PGE2</td>
<td>Prostaglandin E2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Name</td>
</tr>
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<td>--------------</td>
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</tr>
<tr>
<td>poly I:C</td>
<td>Polyriboinosinic acid-polyribocytidylic acid</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>pDCs</td>
<td>Plasmacytoid DCs</td>
</tr>
<tr>
<td>PLGA</td>
<td>poly(lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PYD</td>
<td>Pyrin domain</td>
</tr>
<tr>
<td>PS</td>
<td>Polystyrene</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
</tr>
<tr>
<td>QS21</td>
<td><em>Quillaja saponaria</em> fraction 21</td>
</tr>
<tr>
<td>RIG</td>
<td>Retinoic acid-inducible gene</td>
</tr>
<tr>
<td>RLRs</td>
<td>Retinoic acid-inducible gene RIG-like receptors</td>
</tr>
<tr>
<td>RORγt</td>
<td>RAR-related orphan receptor gamma</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SARM</td>
<td>Sterile-alpha and Armadillo motif containing protein</td>
</tr>
<tr>
<td>Syk</td>
<td>Spleen tyrosine kinase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
</tr>
<tr>
<td>TLRs</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>TRIF</td>
<td>TIR-domain-containing adapter-inducing IFN-β</td>
</tr>
<tr>
<td>Tfh</td>
<td>Follicular T cell</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethyl benzidine</td>
</tr>
<tr>
<td>TH</td>
<td>Helper T cell</td>
</tr>
<tr>
<td>TIR</td>
<td>Toll/Interleukin-1 receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>Regulatory T cell</td>
</tr>
<tr>
<td>T-bet</td>
<td>T-box transcription factor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumour necrosis factor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>TRAF</td>
<td>TNF receptor-associated factor</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus like particles</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>W/O</td>
<td>Water-in-oil</td>
</tr>
<tr>
<td>µm</td>
<td>Micron</td>
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Abstract

Until recently, vaccines were largely comprised of whole cell killed and attenuated pathogens. However, over recent decades, there has been a shift towards subunit vaccine development, using purified antigens which although much safer are less immunogenic, requiring adjuvants to generate protective immunity. There is an urgent need to develop improved vaccines that generate potent Th1 and CD8+ responses for diseases including TB, malaria, HIV and for cancer. However, adjuvants such as alum and oil in water emulsions are strong inducers of humoral immunity but less effective at promoting cellular immunity.

The hypothesis underlying this project was that this limited capacity to drive cellular immunity was related to adjuvant induced production of the anti-inflammatory cytokine IL-10 and inhibition of the Th1 polarising cytokine IL-12p70 by dendritic cells (DCs) and macrophages. One key parameter dictating the effectiveness of particle based adjuvants is size but how size influences the induction of T cell polarising cytokines has not been elucidated. Using biodegradable poly (lactide-co-glycolide) (PLGA) particles between 100 nm and 30 µm in size, it was shown that 500 nm-2 µm particles potently enhanced IL-10 production by DCs and these DCs could inhibit T cell proliferation. These particles also potently enhanced IL-10 production in the draining lymph nodes following intramuscular injection. Moreover, deficiency in IL-10 resulted in enhanced Th1 responses after intra-muscular immunisation with OVA and 500 nm PLGA particles.

Particles of this specific size were shown to selectively interact with lipid rafts on the DC membrane and activate the kinase Syk, leading to p38 and CREB phosphorylation and enhanced transcription and secretion of IL-10. Remarkably it was found that this mechanism is more broadly employed by DCs in response to physical stresses. Specifically, 500 nm PLGA particles, osmotic stress, oxidative stress or disruption of actin polymerisation resulted in DCs becoming rounded, decreasing their membrane fluidity and enhancing their capacity for IL-10 production. Conversely, increasing membrane fluidity using local anaesthetics or causing cell swelling inhibited IL-10 production. Overall, a novel mechanism by which particulate adjuvants and mechanical signals enhance IL-10 production by DCs is proposed, highlighting the therapeutic potential of tailored biodegradable particles to block inflammation and promote tolerance.
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Chapter 1

Introduction
1.1 Vaccines

Infectious diseases remain the second leading cause of death worldwide after cardiovascular disease, but the leading cause of death in infants and children (1). Vaccines continue to be the safest, cheapest and most dependable strategy to counter infectious diseases. As of 2017, there are over 70 licensed vaccines (FDA approved), which prevent the pathogenesis associated with and reduce the incidence of around 30 viral and bacterial infections. It has been one of the most successful and efficient forms of protection against infectious diseases for well over 100 years. Through vaccination, smallpox has been completely eradicated worldwide while Polio has nearly been abolished. Childhood vaccination has also reduced mortality and morbidity associated with infectious diseases such as measles, mumps, rubella, diphtheria, tetanus and *Haemophilus influenzae* type b in adults and children (2). Vaccines have also been successful in targeting chronic diseases that can lead to cancers, with the hepatitis B and human papillomavirus (HPV) vaccines reducing the incidence rates of liver and cervical cancer respectively (3).

The fundamental idea underlying vaccination is to mimic natural infection and induce specific long-lasting protection against infection or disease while avoiding unwanted side effects and risk of developing the disease (4, 5). Vaccines act by initiating the innate immune response and activating Dendritic cells (DCs) and other antigen presenting cells (APCs) to induce protective adaptive immune response. Upon exposure to antigens, the APCs capture, process and present the resulting antigen epitope to T cells (6). Upon re-exposure to a particular pathogen, rapid activation of host memory T and B cells triggers an effective immune response, preventing disease.

Traditionally, early vaccines were developed using live-attenuated pathogens or whole killed pathogens with reduced pathogenicity to induce protective immunity, successfully eradicating disease (Smallpox) or dramatically reducing the number of cases worldwide (Polio) (7). Nevertheless, the need for new vaccines remains high as there are several diseases for which optimal vaccines do not exist (HIV, TB, and Malaria) and there is a desire to replace existing vaccines with less reactogenic formulations. The use of live attenuated or inactivated pathogens is not suitable for all diseases due to safety concerns regarding toxicity, the potential for reversion to virulence and limited efficacy in some cases (8, 9). For example, one virus strain in the attenuated oral polio virus vaccine has been associated with rare Poliomyelitis outbreaks, mainly in countries with low vaccine coverage (9, 10). BCG and measles vaccination is not
recommended for use in patients with impaired immunity. The RotaShield vaccine against rotavirus was withdrawn from the market due to unwanted side effects (11). In other cases, the inactivated pertussis vaccine was shown to induce strong reactivity at the injection site and a potential to induce high fever (12, 13). Furthermore, some vaccines do not provide lifelong protection (Tetanus, Diphtheria, and Pertussis), only generate partial protection (BCG for leprosy and TB), require booster vaccinations (pertussis, MMR and Polio) or are only effective against certain strains (annual influenza vaccine, pneumococcal and meningococcal vaccines).

In order to further impact global health, there is a need to develop effective vaccines against Human immunodeficiency virus (HIV), TB, and Malaria as well as cancers and autoimmune diseases. While the use of live attenuated vaccines has been highly effective they are not applicable to these diseases due to safety concerns (e.g. highly mutagenic HIV virus) (14). This has led to the development of the so-called subunit vaccines composed of a single or few well-characterised and highly purified antigens. Subunit vaccines can be based on highly purified recombinant proteins, polysaccharides or protein-polysaccharide conjugates rather than whole microorganisms.

While these subunit vaccines containing well defined purified antigens will improve safety profiles compared to live attenuated and whole killed pathogen vaccines, they are also less immunogenic due to the removal of microbial motifs such as TLR ligands. For example, live attenuated vaccines provide both the antigen and the PAMPs to activate APCs, subunit vaccines are typically unable to activate APCs on their own and induce protective immunity. Therefore, they require adjuvants to induce adequate immune responses. To date, there are only a few adjuvants in clinical use with limited abilities to induce a broad and robust Th1 response needed for many complex pathogens such as TB, malaria and HIV.

1.2 Innate Immunity

The immune system is categorised into two arms: innate and adaptive. The innate immune system acts as the first line of defence and provides the host with a rapid response for detecting infection by viral, microbial and fungal pathogens. The innate response is mediated and coordinated by cells including antigen presenting cells (APCs) particularly macrophages and DCs, neutrophils, natural killer cells and secondary soluble components secreted by these cells such as cytokines and complement proteins. These cells have the ability to quickly sense and
respond to pathogens. APCs can phagocytose and kill pathogens through detection of conserved molecular patterns. DCs are a very important cell type at coordinating protective immunity in response to infection or immunisation by linking the innate and adaptive immune response.

In contrast, the adaptive immune system involves a slower antigen-specific response mediated by B and T lymphocytes promoting the generation of memory cells which upon reinfection can mount a quicker and more effective immune response. It had long been believed that the innate immune response functions as a temporal defence system against infection until the adaptive immune response can be elicited. However, recent studies have demonstrated that innate immunity is essential for the effective induction of adaptive immunity. The ever-increasing understanding of how the innate and adaptive immune system function has been exploited to accelerate, enhance and manipulate the induction of vaccine-specific adaptive immune responses.

1.3 PRRs, PAMPs and DAMPs

Recognition of infection is triggered through binding of specific pathogen-associated molecular patterns (PAMPs) to pathogen recognition receptors (PRRs) on innate immune cells to initiate an immune response (15, 16). These PAMPS are a set of evolutionarily conserved motifs expressed either on the surface or intracellularly by pathogens and are crucial for their survival, for example Lipopolysaccharide (LPS) a component found on the cell wall of gram-negative bacteria. PRRs are the first line of defence in immunity and regulate the inflammatory response according to the type of threat they encounter (17). PRRs are expressed primarily by cells at the front line of the innate immune system such as Macrophages and DCs. In addition to PAMPs, PRRs can also respond to damage associated molecular patterns (DAMPS), endogenous molecules released by host necrotic cells (18).
Figure 1.1: DCs bridge the innate and adaptive immune responses

Immature DCs act as sentinels and survey the periphery where they efficiently phagocytose and process antigens into peptides. These antigenic peptides are then presented in the context of either MHC class I (MHCI) or MHC class II (MHCII) molecules to naïve T cells. Upon activation, DCs lose their endocytic ability but acquire an enhanced potential for antigen processing and presentation, facilitating the provision of signal 1 to T cells through peptide display on major histocompatibility complex (MHC) molecules and gain chemokine receptor expression (CCR7 and CXCR4) to enable migration to T-cell rich zones in draining lymph nodes. Activated DCs also express enhanced levels of co-stimulatory molecules (e.g. CD80 and CD86 which interact with CD28 on T cells or CD40 which interacts with CD40L on T cells) (Signal 2) and produce cytokines to determine the fate of the naïve T cells (signal 3) (19, 20). DC subsets can specialise in driving either CD4+ T cell or CD8+ T cell adaptive immune responses. PRRs (e.g. TLR4, NOD1/2 and inflammasome NLRs) can detect PAMPS and DAMPS within the environment to mediate cytokine release. TLR4 and NOD1/2 engagement activates signalling pathways (p38, ERK etc.) to modulate proinflammatory and anti-inflammatory cytokine release influencing T cell polarisation. NLRP3 activation is coordinated through multiple pathways. In the priming step, PRRs induce NF-κB-dependent induction of components of NLRP3 itself and pro-IL-1β. The second signal allows NLRP3 to oligomerise and form the inflammasome to process pro-IL-1β and pro-IL-18 into their active forms. The inflammasome can sense various danger signals that emerge during infections, tissue damage or metabolic imbalances to trigger formation of the inflammasome. This includes extracellular ATP, microbial toxins, monosodium urate crystals, alum and other lysosome-derived molecules, ROS and potassium efflux. Taken from (21).
PRRs are expressed on either the cell surface or in intracellular compartments of immune cells. PRRs consist of four main families: Toll-like receptors (TLRs) (Fig 1.2), retinoic acid-inducible gene RIG-like receptors (RLRs), Nucleotide-binding and oligomerisation domain NOD-like receptors (NLRs) and C-type lectin receptors (CLRs) (22-24) (Fig 1.4 and 1.6). Moreover, there are also various cytoplasmic DNA receptors that respond to cytoplasmic DNA after microbial infection, which belong to different protein families, including cyclic-di-GMP-AMP synthase (cGAS) and DNA-dependent activator of interferon-regulatory factors (DAI) (25, 26).

TLR activation is a hallmark of innate immune responses. The TLRs are the most studied family of PRRs, sensing a diverse range of PAMPs (27). The binding of PAMPs and/or DAMPs to PRRs activates intracellular signalling cascades, leading to the release of inflammatory and anti-inflammatory mediators including cytokines, chemokines, and type 1 interferons (IFNs) inducing innate immune response as well as the development of acquired immunity against a particular pathogen (28) (Fig 1.1 and 1.9). TLRs are located either on the surface or intracellular endosomal compartments (Fig 1.2). The intracellular localisation of TLRs is thought to be critical for ligand recognition as well as for preventing TLRs from encountering self-nucleic acids, which could potentially cause autoimmunity. For example, Endogenous TLR9 is not exposed to mammalian DNA and can only be activated by viral DNA ingested and acidified within endosomes (29).

One class of PRRs located on the cell surface, namely, C-type lectins (i.e., Dectin-1, 2 and Mincle), has the capacity to signal in a TLR-independent manner (30). They mainly recognise carbohydrate moieties on pathogens, including mannose, fucose and glucan structures. For example, fungal components (from Candida albicans and Aspergillus fumigatus) such as β-glucans are recognised by the C-type lectin dectin-1 to mediate Th1 and Th17 immune responses leading to the activation and recruitment of neutrophils and macrophages (31, 32). Another example is the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), which can recognise a wide range of pathogens, including HIV, Measles, Mycobacterium tuberculosis and Helicobacter pylori (24, 33).

NLRs and RLRs maintain immune surveillance in the cytoplasm. Of these, NLRs are a large group of cytoplasmic sensors that recognise not only microbial products but also endogenous danger signals associated with inflammation (34). The NLR family comprises more than
twenty cytosolic receptors in mammals, divided into different groups based on the N-terminal activation domains involved in signal transduction (NLRA, NLRB, NLRC and NLRP) (35).

Some NLRs have the ability to form an inflammasome, a tightly regulated multi protein complex mediating the maturation of the inactive precursor forms of interleukin (IL)-1β and IL-18 into active proinflammatory cytokines (36). The complex consists of a central NOD (or NACHT) domain, required for oligomerisation, a C-terminal series of leucine-rich repeats (LRRs) involved in agonist sensing or ligand binding and the variable N-terminal protein–protein interaction domain defined by the caspase recruitment domain (CARD) (NLRC), pyrin domain (PYD) (NLRP), acidic transactivating domain (NLRA), or baculovirus inhibitor repeat (BIR) (NLRB) (34, 37). For example, the NLR family member, NACHT, LRR, PYD domain containing protein 3 (NLRP3) can sense a wide variety of molecules and compounds, including monosodium urate (MSU), adenosine triphosphate (ATP) microbial toxins (nigericin and α-hemolysin), silica and aluminium adjuvants to mediate assembly of the inflammasome (38). Subsequently, NLRP3 oligomerise and the N terminal associates with the adaptor apoptosis-associated speck-like protein containing CARD (ASC) via its pyrin domains. ASC in turn recruit’s pro-caspase-1 through CARD-CARD interactions. Pro-caspase-1 clustering on the oligomerised NLRP3 results in cleavage of procaspase-1 into caspase-1 to catalyse the processing of pro-IL-18 and pro-IL-1β into IL-18 and IL-1β (35, 39) (Fig 1.1).

Furthermore, a range of NLRs function independently of inflammasomes, including NOD1, NOD2 and others (Fig 1.1 and 1.2). NOD1 senses diaminopimelic acid (DAP) expressed on most Gram-negative and specific Gram-positive bacteria while NOD2 recognises muramyl dipeptide (MDP), a ubiquitous component of all bacterial peptidoglycans to mediate proinflammatory cytokine production (34, 40). Additionally, NOD2 can interact with ATG16L1 and stimulate the formation of the autophagosome (41).

Finally, the RIG-I like receptors (RLRs) are a class of PRRs implicated in the recognition of and response to most RNA viruses. Retinoic acid inducible gene-1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) are intracellular sensors of dsRNA (from ds RNA viruses or dsRNA generated during replication of ssRNA viruses) leading to type I IFN gene expression (42, 43).
Following ligand binding, TLRs dimerise and recruit TIR domain-containing adaptor molecules and subsequently propagate intracellular signal transduction. These adaptor molecules include; myeloid differentiation primary response gene 88 (MyD88), TIR-domain containing adapter-inducing IFN-β (TRIF), TRIF-related adaptor molecule (TRAM) and MyD88 adaptor-like (Mal) (Fig 1.2) (44).

There is also evidence that specific adaptor molecules can negatively regulate TLR related immune responses. Sterile-alpha and Armadillo motif containing protein (SARM) can negatively regulate TRIF-dependent Toll-like receptor signalling (45). Likewise, the recently described B-cell adaptor for PI3K (BCAP) negatively regulates proinflammatory cytokine secretion and links TLR signalling to the PI3K signalling cascade (46). Most TLRs directly interact with MyD88 to trigger downstream signalling pathway, although TLR2 and TLR4 require the bridging adaptor Mal to associate with MyD88. TLR4 (and TLR3) also engage a MyD88-independent, TRIF-dependent pathway which requires the bridging adaptor TRAM to associate with TRIF (Fig 1.2).

Downstream signalling from TLR4 is driven by recruitment of the adaptor proteins, MyD88 and TRIF (44, 47). The early response relies on the MyD88-dependent pathway, leading to downstream activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and MAPKs (p38, ERK, JNK), which induce proinflammatory and anti-inflammatory responses (28) (Fig 1.1 and 1.2). The late response relies on engagement of the TRIF pathway following endocytic engagement of TLRs and subsequent activation of IFN regulatory factor 3 (IRF3) and ‘late’ NF-κB transcription factors triggering cytokine (IFN-β) and chemokine expression (IP-10) (48, 49) (Fig 1.1 and 1.2). Thus, MyD88- and TRIF-dependent pathways have overlapping functions to regulate both proinflammatory and adaptive immune responses (50).

Vaccine-induced immunity is heavily reliant on the activation of innate immune mechanisms, using either PAMPs or DAMPs to engage PRRs and subsequently condition the adaptive immune response. The PAMPs could for example be TLR agonists from the microbial cell surface. Additionally, adjuvants within the formulation could release endogenous DAMPs at the site of injection to trigger the innate response.
Figure 1.2 Mammalian Toll like receptors and downstream signalling pathways

TLRs are pattern recognition receptors with the ability to distinguish conserved pathogen-associated molecular patterns from self. TLR2 heterodimerises with TLR1 or TLR6 and recognises triacylated or diacylated forms of lipoproteins, respectively. TLR4 recognises LPS from enteric bacteria and a number of viral and bacterial proteins (e.g. Escherichia coli LPS and fusion proteins from Respiratory syncytial virus). TLR4 moves from the plasma membrane to the endosomes in order to switch signalling from MYD88 to TRIF. The only known protein ligand for human TLRs is bacterial flagellin, which is recognised by TLR5 (51). Intracellular receptors located in the endosomal compartments can recognise viral and intracellular bacterial nucleic acids, including unmethylated cytidine-phosphate-guanosine (CpG) motifs (TLR9), ssRNA (TLR7/8) and dsRNA (TLR3). Engagement of a PAMP with its corresponding TLR results in the recruitment of two main signalling adaptor molecules, MyD88 and TRIF. All TLRs use MyD88 except TLR3, which signals through TRIF. TLR4 is the only TLR known to use MyD88 and TRIF molecules. TLR2 and TLR4 also require MAL for recruitment of MyD88. Upon receptor ligation, adaptor molecules stimulate downstream pathways involving interaction between IL1R-associated kinases (IRAKs) and the TNF receptor-associated factors (TRAFs) leading to downstream phosphorylation and activation of MAPKs (p38, ERK1/2 and JNK) and other signalling molecules. This leads to translocation of transcription factors (CREB, AP1, NF-κB and IRF3/7), ultimately resulting in the expression of genes required for cell activation and immunity. Taken from (44).
1.3.1 MAPKs: p38 and ERK

Mitogen-activated protein kinases (MAPKs) are a conserved serine-threonine family of kinases that mediate intracellular signalling events, promoting cellular responses to a wide range of extracellular cues including growth factors (GF), hormones, cytokines and stress. The MAPK cascade has fundamental roles in many cellular processes including, cytokine production, proliferation, survival and differentiation acting as positive and negative regulators at multiple levels within the signalling cascade (52-54) (Fig 1.1 and 1.2).

TLR stimulation with PAMPs such as LPS activates several highly regulated MAPK signalling pathways crucial for synthesis of proinflammatory and anti-inflammatory cytokines (55). Any dysregulated signalling from PRRs leads to excessive proinflammatory cytokine production and host tissue damage in disease states including chronic inflammatory conditions, autoimmune disease and cancers (56-58).

The MAPK cascade consists of at least three protein kinases in series that leads to the activation of a multifunctional MAPK. MAPK pathways consist of a MAPK kinase kinase (MKKK) that phosphorylates and activates a MAPK kinase (M KK) that then activates the functional terminal MAPK by dual phosphorylation. Upstream of the kinase module are members of the Ras and Rho families of GTPases that relay signals from receptor complexes to the module (52, 53, 59, 60).

Among the most important kinases activated are the p38 mitogen-activated protein kinases. There are four members of the p38 MAPK family (p38α, p38β, p38γ and p38δ), with the p38α isoform the most abundant and best-characterised isoform (61). The p38 family plays a role in angiogenesis and cell proliferation and is critical for normal immune and inflammatory responses. The p38 MAPK pathway is a key regulator of pro-inflammatory cytokine biosynthesis at the transcriptional and translational level (61) (Fig 1.1 and 1.2). The extracellular signal-regulated kinase (ERK) family, consists of several isoforms: ERK1 to ERK8. In the ERK signalling pathway, ERK1 or ERK2 are especially important, regulating cytokine production, cell cycle, differentiation and apoptosis (58, 62, 63). The c-Jun N-terminal kinase (JNK) is also an important member of the MAPKs. JNKs have been shown to have important roles in a number of cell processes, including apoptosis, the development of multiple cell types of the immune system and cytokine production (62).
Activated MAPKs (ERK1/2 and p38) phosphorylate and activate Mitogen-and Stress-activated protein Kinases (MSKs) and various substrate proteins including transcription factors. For example, MSK1 and MSK2 can phosphorylate transcription factors such as cAMP response element-binding protein (CREB) and AP-1 (e.g. c-Fos and c-Jun) which subsequently bind to the IL-10 promoter to induce transcription (64, 65) (Fig 1.1 and 1.2).

ERK1/2 and p38 MAPKs are critical for both pro- and anti-inflammatory immune responses. They mediate DC production of cytokines which determine the polarisation of naive CD4+ T cells into Th1 (66, 67), Th2 (68), Th17 (69, 70), or regulatory T cell subsets (71, 72). For example, p38 has been shown to be essential for both IL-12 and IL-10 induction. In contrast, ERK is crucial for IL-10 induction, but mediates a negative feedback of IL-12 inhibition (through inhibition of the IL-12p40 subunit) (66, 73-77). Both p38 and ERK cooperate and cross talk with each other during TLR-induced IL-10 production. ERK- and p38-dependent IL-10 production has also been shown to be inhibited by IFN-γ (78).

1.3.2 Phosphatidylinositol 3-kinase

Phosphatidylinositol 3-kinase (PI3K) consists of a family of lipid kinases that play crucial roles in metabolism, rearrangement of the cytoskeleton, growth, proliferation, survival, apoptosis and immune cell effector functions (79-81). PI3Ks are divided into three classes (I, II, III) based on their structures and substrate specificities. Class I PI3Ks are further divided into class IA (p110α, β and γ) and class IB (p110γ) (81). Class I PI3Ks are heterodimers composed of a regulatory subunit (p85) and a catalytic subunit (p110) involved in receptor-mediated signalling pathways in the immune system (82).

TLR-induced activation of the PI3K pathway can differentially regulate IL-10 and IL-12 production in both human and mouse macrophages (83, 84). The PI3K pathway has an important role in the differentiation and activation of DCs, particularly the regulation of proinflammatory cytokines (IL-12, IL-6, and TNF-α) and partially regulating IL-10 secretion (85).

Class I PI3K signals to the serine/threonine kinase Akt, also termed PKB (protein kinase B) and can control pro-inflammatory cytokine production (81, 86, 87). Akt can also activate the mammalian target of rapamycin (mTOR) or inhibit glycogen synthase kinase-3β (GSK-3β), in
two separate pathways. Signalling through the PI3K/AKT/mTOR (mTORC1) pathway can enhance IL-10 production, leading to IL-12 inhibition in an IL-10 dependent manner (88). Furthermore, the PI3K/Akt/mTOR pathway has extensive roles in innate immune cells, including neutrophils, mast cells, monocytes, macrophages and myeloid as well as plasmacytoid dendritic cells (mDCs and pDCs, respectively) (89). Activation of PI3K/AKT can also inhibit GSK-3β, a multifunctional enzyme regulating cytokine production and other cellular functions. GSK3 enhances IL-12p70, IL-6, and TNF-α secretion, and inhibits IL-10 in *E. coli*–stimulated human monocyte-derived DCs (85). TLR4 mediated GSK3β activation has shown to suppress CREB mediated IL-10 production in response to *Leishmania donovani* infection (90). In contrast, IFN-γ can also trigger GSK-3β activation by antagonising the PI3K/AKT pathway. This leads to inhibition of TLR-induced IL-10 production through inhibition of the transcription factors, CREB and AP-1 (78, 90, 91) (Fig 1.4).

### 1.4 Dendritic cells and antigen presentation

DCs were first described by Steinman and Cohn in 1973 as cell population with a distinctive morphology (clearly distinct from macrophages) with a unique ability to activate and prime naïve T cells. Thus, they are the primary instigators of adaptive immunity, and function as the bridge between innate and adaptive immunity (92, 93). DCs are not the only immune cell capable of presenting antigens; other APCs including Macrophages and B cells also play significant roles.

DCs represent a crucial class of APCs which possess multiple PRRs capable of recognising various PAMPs and DAMPs and subsequently activate a specific adaptive immune response. DCs thus play important roles in triggering immune response against pathogens or initiating tolerance or autoimmunity against self-antigens during the steady state. Therefore, DCs are essential mediators for bridging and shaping the innate and adaptive immune response (94, 95). DCs can adopt two main states, either an immature tolerogenic state which samples the peripheral environment for antigens or a mature state that induces an effector or memory T cell response. A switch of DCs from tolerance maintenance to immune stimulation, referred to as maturation, may be initiated by PAMPS, DAMPs, cytokines or CD40/CD40L signalling (20).

DCs phagocytose and process antigen into peptides which are loaded onto MHC molecules and then transported to the cell surface to be presented to T cells. The origin of the antigens
determines whether the peptide is displayed on MHC class I or II. Extracellular pathogens will be endocytosed, degraded in lysosomes by pH dependent proteases and the peptides are delivered and loaded onto the MHC II complex and transported to the cell surface. In contrast, endogenous antigens (intracellular) such as viral proteins undergo proteasomal degradation in the cytosol and are transported to the endoplasmic reticulum where they are loaded onto MHC class I molecules (96). Finally, a third pathway exists called cross presentation. This mechanism allows the loading and presentation of exogenous peptides on MHC class I molecules (97).

In addition to the antigen presenting capabilities of DCs, a range of cytokines secreted by DCs influence T-cell differentiation (Fig 1.3b and 1.9). DCs producing IL-12 promote Th1 expansion in response to microbial stimuli such as *Toxoplasma gondii* or to LPS (98) while secretion of the cytokines IL-23 and IL-1β promotes Th17 differentiation (99, 100). In the case of Th2 differentiation, IL-10 and IL-33 play a crucial role (68, 101, 102). IL-4 is also important for Th2 differentiation but DCs are not believed to be the main source (103). Pathogens linked to a Th2 response can cause secretion of IL-4 (IL-5 and IL-13) rapidly by a variety of other innate cells (104, 105), but whether IL-4 has a direct effect on DC function remains unclear.

DCs represent a heterogeneous group of cells that differ in their origin, anatomic location, phenotype, surface markers and function but all are capable of activating naïve T cells (106) (Fig 1.3a and 1.3b). For instance, PRRs can be differentially expressed on DC subsets which influences their capacity to respond to a particular PAMP or DAMP. Murine and human DCs in steady state are predominantly classified as either plasmacytoid DCs (pDCs) or conventional (cDCs) differing in their function and phenotype such as surface markers (107) (Fig 1.3a and 1.3b).

pDCs represent a small subset of DCs and express a restricted set of receptors and low levels of major histocompatibility complex class II (MHCII), CD11c and costimulatory molecules in the steady state. They selectively express high levels of TLR7 and TLR9 to recognise viral nucleic acids or host-derived nucleic acids, leading to the production of large amounts of type I IFNs (108, 109) (Fig 1.3a). This is especially crucial for an effective antiviral response. They also link the innate and adaptive immune response by controlling the function of cDCs, T cells, B cells, and Natural Killer (NK) cells (110).
On the other hand, cDCs (also known as classical or myeloid) main function is to initiate and drive the adaptive response by capturing antigen in peripheral tissue and migrate to T cell zones of secondary lymphoid tissues. They have a short half-life (approximately 3-5 days) and are continuously replaced from bone marrow precursors, a process dependent on the cytokine Fms-like tyrosine kinase-3 ligand (Flt3L) whereas monocytes require macrophage colony-stimulating factor (M-CSF) (111). cDCs are found in lymphoid tissues including the thymus, spleen, lymph nodes, and bone marrow, but are also widely distributed among non-lymphoid tissues such as the skin, liver, kidney, intestinal tract and lung allowing them to constantly acquire tissue and blood antigens (112, 113).

In mice, cDCs are defined by expression of the transcription factor Zbtb46 and can be further separated into two main subsets based on expression of the transcription factors BATF3 or IRF4, whereas pDCs express the transcription factor E2-2 (114, 115). Batf3 expressing cDCs express CD8α in the spleen and CD103 or CD24 in the periphery, whereas IRF4+ cDCs express CD11b and are negative for CD8α. In contrast to CD8a and CD103 DCs, the population currently defined as CD11b+ cDCs is heterogeneous and remains less well characterised (116). CD11b+ cDCs are the most abundant cDCs in lymphoid organs except for the thymus and can also be found in nonlymphoid tissue (117).

The CD8+ and CD103+ cDCs specialise in mediating cross-presentation of exogenous antigens on MHC-I molecules to CD8+ T-cells, presenting glycolipid antigens in a CD1d manner and IL-12 secretion to promote Th1 responses (116). For instance, stimulating TLR receptors on the CD8a+ and CD103+ cDC lineage induces IL-12p70 production (98). In contrast, CD11b+ DCs are more efficient at driving CD4+ T cell responses, potentially because of their higher expression of MHC-II presentation machinery (118) and may be the dominant subtype involved in driving Th2 (119) or Th17 responses (120). CD11b+ DCs can also be characterised by their production of cytokines, such as IL-6, IL-23 and proinflammatory chemokines (116). Interestingly, both subtypes differ in their ability to acquire and phagocytose particular types of antigen. CD11b+ DCs preferentially acquire and transport soluble antigen, whereas CD103+ DCs are more efficient at dealing with particulate material in the lung of mice (121, 122).

DCs can also arise from monocytes in lymphoid and nonlymphoid organs, these monocyte derived inflammatory DCs (Mo-DCs) appear after inflammation or infection (112) (Fig 1.3b).
These DCs can be generated *in-vitro* from bone marrow cells using GM-CSF (Section 2.2.5) expressing high levels of CD11c and major histocompatibility complex class II (MHC II), capable of migrating to draining lymph nodes to activate T cells. They play an essential role in clearing pathogens by secreting large amounts of TNF-α and inducible nitric oxide synthase (iNOS) (19, 123). Mature Mo-DCs migrate to draining lymph nodes and activate CD4⁺ and CD8⁺ T cells. In terms of antigen presentation function, Mo-DCs are as active as cDCs, including cross presentation of proteins on MHCI *in vivo* (124). Although, Nussenzweig *et al* demonstrated that Mo-DCs were less efficient in presenting phagocytosed antigen than cDCs (125). Therefore, it remains unclear whether Mo-DCs significantly contribute to presenting phagocytosed antigen to CD8⁺ or CD4⁺ T cells. It has been suggested that their primary function is to act as innate immune effector cells that destroy phagocytosed antigen (126).

DCs are known to play a central role in vaccination due to their ability to induce and regulate T cell responses. Therefore, understanding how DCs respond to established and novel vaccine formulations is essential for designing vaccines with improved clinical efficacy and understanding how established vaccines work.

### 1.5 IL-10 and suppression of immune responses

Interleukin-10 (IL-10) is a pivotal anti-inflammatory cytokine that protects the host from excessive tissue damage during host defence to pathogens and acts as one of the key molecules critically involved in the development and maintenance of immune tolerance and homeostasis.

IL-10 was first called cytokine synthesis factor (CSIF). It was identified as a factor produced by Th2 cells with the ability to inhibit Th1 responses (127). Since this discovery, the list of cells which can produce IL-10 and information on the functions of this cytokine has greatly expanded. Even in recent years new roles for IL-10 have been reported. For example, IL-10 secreted by Tregs during the resolution phase of infection has been linked to the promotion of memory CD8⁺ T cells (128). In contrast, IL-10 restricts the magnitude of the effector and memory Th1 responses (and memory CD8 responses) during the early stages of an acute viral infection and modulates the balance between Th1 and Tfh cells (129).
Figure 1.3: Dendritic cell lineages have specialised functions

A) DCs form a heterogeneous population that have been classified into various groups according to their origin, location, patterns of migration and expression of PRRs.

B) Progenitors in the bone marrow, called macrophage and DC precursors (MDP), gives rise to pre-DCs and monocytes. pDCs differentiate in the bone marrow, while Pre-cDCs can also move into the blood and migrate to lymphoid and non-lymphoid organs, where they terminally differentiate into cDCs controlled by different transcription factors, including the CD8α+/CD103+ and CD11b+ subset. Different DC subsets carry out specific and specialised functions depending on environmental cues. They can respond differently to pathogen challenge through the secretion of T cell polarising cytokines.

Taken from (111).
The initial immune response to pathogens involves the rapid release of pro-inflammatory cytokines that trigger host defences. Over activation or excess inflammation can lead to host damage and autoimmunity. As a result, the immune system has evolved to trigger anti-inflammatory mechanisms simultaneously to limit pro-inflammatory cytokine production and restore homeostasis after infection or damage. IL-10 is a crucial multifunctional anti-inflammatory cytokine, primarily acting to limit excessive immune responses and resolve inflammation, thereby limiting chronic and acute inflammation and preventing autoimmunity (130, 131). For example, human genetic deficiencies and polymorphisms in IL-10 or its receptor can strengthen the inflammatory response to gut microbes but also lead to development of inflammatory bowel disease (ulcerative colitis and Crohn’s disease) (132-135). IL-10 deficient mice are much more prone to autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) as the immune response shifts to an amplified Th1 phenotype (136). IL-10 producing B cells were found to be required to prevent disease onset (137, 138).Viruses have evolved to manipulate the anti-inflammatory properties of IL-10 to limit host immune responses, leading to persistent infection (139, 140). Similarly, IL-10 producing DCs and T regulatory cells are upregulated during Schistosoma mansoni and Mycobacterium tuberculosis infection, inhibiting the effector T-cell response and reducing parasite control (141, 142).

Its importance is highlighted by the fact that the majority of innate and adaptive immune cells are capable of secreting IL-10 and responding to its secretion by expressing the IL-10 receptor (IL-10R) (143, 144). IL-10 can be secreted by macrophages, monocytes, DCs, mast cells, eosinophils and B cells (143). Additionally, various subsets of CD4+ and CD8+ T cells are a significant source of IL-10, especially Th2 and Treg cells, but it can also be produced by Th1, Th17 and CD8+ T cells (130, 143). Even non-immune cells are capable of producing IL-10 including keratinocytes, epithelial cells and tumour cells to evade the immune system (130, 145). APCs are one of the most important sources of IL-10 (146) (Fig 1.1). IL-10 has many suppressive mechanisms, including inhibiting DC maturation and function by restricting antigen presentation and downregulating MHC II and costimulatory molecule expression (147).

IL-10 can provide an autocrine feedback to limit or resolve inflammatory gene expression by macrophages and DCs activated by TLR agonists such as LPS, thus limiting the production of proinflammatory cytokines (TNF-α IL-6 and IL-12) and chemokines (MCP1, MCP5,
RANTES, IL-8, IP-10, and MIP-2) (148, 149). IL-10 can inhibit phagocytosis and microbial killing by limiting the production of reactive oxygen and nitrogen intermediates in response to IFN-γ (150). IL-10 also acts directly on IL-10R expressing T cells to suppress their functions (144), by inhibiting proliferation and production of IL-2, IFN-γ, IL-4, IL-5 and TNF-α (151, 152) and can promote anergy and tolerance by Treg cells (153, 154). In B cells, IL-10 prevents apoptosis, enhances proliferation and modulates immunoglobulin (Ig) class switching (155).

IL-10 has been suggested to drive Th2 cell differentiation through IRF4 activation in DCs (68). Studies using IL-10⁻⁻ DC demonstrated a shift in the balance between Th2 and Th1 response in a mouse model of allergic dermatitis (156). Novel therapies focusing on the induction or inhibition of IL-10 have been used to treat chronic inflammatory bowel disease, rheumatoid arthritis, psoriasis, asthma, systemic lupus erythematosus, multiple sclerosis, transplant rejection and cancer (155, 157, 158).

**1.5.1 IL-10 regulation**

IL-10 regulation has several layers, including changes in the chromatin structure, enhancement or silencing of IL-10 transcription and post-transcriptional regulatory mechanisms (159). Distinct mechanisms control the production of IL-10 in different cell types. IL-10 production in APCs is triggered by engagement of TLRs, intracellular PRRs including RIG-I and Nod2-like family receptors, C-type lectin signalling, or ligation of CD40 and Fc receptors (157) (Fig 1.4).

Once IL-10 is secreted, it binds to a heterodimeric receptor complex, consisting of IL-10R1 and IL-10R2 subunits on the cell surface, activating the Janus kinase (JAK1) and signal transducer and activator of transcription (STAT3) (JAK-STAT) pathway. STAT3 then activates IL-10 responsive genes such as suppressor of cytokine signalling 3 (SOCS3) to inhibit proinflammatory cytokines (160).
**Figure 1.4: IL-10 regulation in APCs**

A) IL-10 expression is induced by TLR (TLR2, 3, 4, 9) or non-TLR (e.g. dectin-1 or DC-SIGN) signalling in macrophages and DCs. Following engagement of TLRs and adaptor molecules (MyD88 and TRIF), ERK1/2, p38 and Nf-κB pathways and downstream signalling molecules (MSK1/2) result in transcription factor (e.g. CREB) binding to the IL-10 promoter and subsequent IL-10 production. In myeloid DCs. Non TLR signalling through c type lectin receptors such as Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) and RAF1 kinase augments TLR2 induced IL-10 production (prolongs and increases IL-10 transcription after TLR2 ligation) (161). Dectin-1 ligation results in recruitment and activation of Syk and ERK and IL-10 secretion. Macrophages also use NOD2 signalling to enhance IL-10 production by cross talking with TLR2 (162). Taken from (143).

B) Positive and negative feedback loops also regulate IL-10 in APCs. The p38 and ERK pathways are tightly regulated by IL-10 itself as well as IFN-γ (78). IL-10 through the IL-10 receptor upregulates Dual-specificity protein phosphatase 1 (DUSP1) which inhibits p38 (negative feedback) (163). However, IL-10 also activates tumour progression locus 2 (TPL2), upstream activator of ERK (positive feedback) (164). IFN-γ can inhibit the PI3K/AKT pathway, enabling release of GSK3 which block IL-10 transcription factors (CREB/AP1) Taken from (143).
1.5.2 IL-10 transcription factors

There are a number of transcription factors that regulate IL-10 expression (Fig. 1.5). The promoter region in human and murine IL-10 share a high degree of homology. The number of transcription factors involved in different cell types reflects the complexity of IL-10 regulation. The importance of different promoter elements varies depending on the cell type and stimuli. The transcription factors known to regulate IL-10 in APCs are CREB (165), c-maf (166), C/EBPβ (167), activating transcription factor 1 (ATF1) (143), specific protein (SP1) (168) and NF-κB (143, 169) (Fig 1.5).

CREB can regulate IL-10 transcription following p38 signalling after engagement of TLR4 and Mal (170, 171). Factors leading to elevated levels of secondary messenger cAMP activate cAMP-dependent protein kinase A (PKA) that in turn will phosphorylate CREB at Ser-133. Likewise, increased Ca²⁺ signalling activates a Ca²⁺/calmodulin-dependent protein kinase (CaMK) family member and phosphorylates CREB (172). Similarly, TLR interaction can trigger Ser-133 phosphorylation of CREB, and in association with agents that elevate cAMP, lead to increased CREB-mediated transcription. CREB has been associated with TLR dependent and independent IL-10 production in macrophages and DCs (165, 173, 174).

The main inflammatory transcription factors activated by TLRs are the NF-κB family. However, along with upregulation of pro-inflammatory cytokines including IL-6, IL-12, IL-1β and TNF-α, the NF-κB1 (p50) homodimer can enhance expression of IL-10 by binding to a κB site upstream of the IL-10 promoter (169). More recently, the transcription factor interferon regulatory factor 4 (IRF4) whose expression is regulated by NF-κB and TLR4 has been linked to upregulation of IL-10 in dendritic cells (68). Interestingly, IRF4 has also shown to inhibit IL-12 production and thereby control Th1 response (175, 176). While IRF4 has important roles in development of both innate and adaptive immune cells including DCs (177, 178), recent studies have only started to show how IRF4 can regulate the function of DCs including determining the cytokine secretion profile. Furthermore, the transcription factor DC-specific transcript (DC-SCRIPT; also known as ZNF366) which is specifically expressed in DCs has been shown to regulate IL-10 and subsequently IL-12p70 levels in human DCs (179, 180).

Post transcriptional regulation is also important by stabilising the IL-10 transcript. Like many transcripts, IL-10 mRNA is subject to rapid degradation after synthesis (181). IL-10 mRNA
contains clusters of adenosine and uridine rich elements (ARE) located on the 3’ untranslated region (UTR). These AREs are very important for determining RNA stability as they are targets for tristetraprolin (TTP), proteins that can induce rapid degradation of mRNA following binding to ARE regions (182). Furthermore, p38 activation has been reported to stabilise IL-10 mRNA in BMDMs by inhibiting the action of TTP, suggesting that p38 is involved in both IL-10 transcription and post transcriptional regulation to promote IL-10 secretion (183).

IL-10 mRNA stability can be positively or negatively regulated by small non-coding RNA sequences called microRNA. Several well-established microRNA, including miR98, miR-27, miR-106 and let7 negatively regulate IL-10 (184-187). While miR-4661 can upregulate IL-10 production by antagonising TTP (188).

Figure 1.5: Signalling molecules and transcription factors controlling IL-10 regulation by innate and adaptive immune cells
A host of signalling molecules (outer circle) and transcription factors (inner circle) have been found to regulate the expression of IL-10 both in APCs and T helper cells. DCs and macrophages rely on signalling molecules such as ERK, p38, Syk and NF-κB to activate transcription factors such as CREB, MAF, SP1, ATF1, NF-κB and C/EBpβ. T helper cells mainly rely on ERK and STAT signalling molecule to activate several transcription factors. Taken from (143).
1.5.3 Role of Syk and kinase mediators

Spleen tyrosine kinase (Syk), a non-receptor protein tyrosine kinase expressed in numerous cell types, particularly in immune cells has been associated with activation of both pro and anti-inflammatory responses (143, 189, 190).

Syk signalling was initially believed to be restricted to classical immunoreceptors such as B cell receptors (BCR), T cell receptors (TCR) and Fc Receptors (FcR) of the adaptive immune system (191). Recently, Syk has been shown to be required for several innate cell immune functions and certain non-immune functions, such as cytokine regulation, detection of fungi and tissue damage, cell migration, pathogen clearance, cell differentiation and proliferation, vascular development and bone metabolism (191-193).

Certain receptors (e.g. FcγRIIA) incorporate signalling adaptors containing a short, tyrosine-containing peptide sequence known as the immunoreceptor tyrosine-based activation motif (ITAM) on the cytoplasmic tail chain of the receptor. Others receptors (BCR and TCR) associate with transmembrane signalling adaptors containing ITAMs (191). Receptor ligation leads to signal transduction, initiated by phosphorylation of two ITAM tyrosine residues by Src family kinases (Fig 1.6). The phosphorylated ITAMs recruit and activate Syk leading to activation of downstream kinases such as PI3K (by binding to its p85a subunit) and NF-κB.

C-Type lectin receptors (CLRs) are a large family of proteins containing a carbohydrate-recognition domain that can recognise carbohydrate-based PAMPs (e.g. fungal β-glucan) and non-carbohydrate ligands, such as lipids and proteins. CLRs are primarily expressed on myeloid cells that mediate several immune responses, including phagocytosis, respiratory burst, autophagy, Th1 and Th17 polarising cytokines and anti-inflammatory cytokines (e.g. IL-10) (24, 33, 194). For instance, the CLR Dectin-1 recognises β-glucan expressed on the cell wall to mediate protection against Candida albicans and other fungi species. In addition to fungi, CLRs have a role in the defence against bacterial pathogens, including mycobacteria which are recognised by several CLRs such as Dectin-1, Mincle, CLECSF8 and Dectin-2 (24, 195). However, their role in vivo remains unclear as most CLRs appear redundant during infection, which may be due to compensation by other CLRs. CLRs have also been linked to controlling homeostasis, autoimmunity, allergy and involved in the recognition of dead cells and tumours (24).
Interestingly, some classes of receptors that do not contain conventional ITAMs, including CLRs (e.g. CLEC7A, also known as dectin-1) (196) and integrins (197, 198) can also activate Syk on innate immune cells (Fig 1.4). Syk is able to bind to hemi-ITAM motifs in dimerised C-type lectins (196, 199) (Fig 1.4 and 1.6). For instance, engagement of the fungal β-glucan receptor dectin-1 can promote synthesis of IL-2, IL-23 and IL-10 through recruitment of Syk kinase and CARD9 and activation of the transcription factor CREB (64, 200, 201). Additionally, Syk-coupled PRRs that signal through the ITAM-containing adaptor protein FcRγ, CLEC6A (also known as Dectin-2) and CLEC4E (also known as MINCLE) were recently identified (202). Although the exact roles are unclear, receptor activation induces CARD9 and NF-κB-mediated pro-inflammatory responses, promoting a strong Th17 adaptive immune response (191). Furthermore, FcR ligation in the presence of TLR signals in macrophages can also lead to ERK activation and augment IL-10 production (203).

Syk also has the ability to associate with receptors in an ITAM-independent manner (191). In response to stimulation by TLR ligands, Syk is phosphorylated and associates with TLRs, including TLR4 and TLR9 (204, 205). TLR4 ligation results in phosphorylation of tyrosine residues and subsequent recruitment of Syk to initiate responses through activation of various downstream signalling molecules (206, 207). Phosphorylated Syk associates with TLR4 and MyD88 in human monocytes after LPS treatment (206). Syk knockout BMDMs have been shown to secrete enhanced concentrations of proinflammatory cytokines (TNF-α, IL-6 and IL-1β) in response to TLR agonists, implying that the TLR signal might be indirectly and negatively regulated through Syk (208). In contrast, blocking Syk activity with the pharmacological agent piceatannol reduces IL-10, IL-12p40 and proinflammatory (TNF-α and IL-6) cytokine production in LPS stimulated human and mouse monocytic cells (206, 209).

Syk has also been shown to negatively regulate TLR4-mediated production of IFNβ and IL-10 and promotes inflammatory responses in DCs through PI3K-Akt and NF-κB signalling pathways (210). Therefore, the role of Syk in TLR-associated innate immunity remains unclear. Interestingly, Syk in B cells has now been linked to proliferation, as well as cytokine and antibody production independent of the BCR (211).

Recent studies have reported that Syk is also involved in pathogen-induced NLRP3 inflammasome activation and IL-1β secretion (212, 213) (Fig 1.6b). This response has been seen for anti-fungal host defence (214), allergic sensitisation (215), alum, MSU and hemozoin crystals (212, 216, 217). For example, Syk-deficient dendritic cells fail to respond to MSU
crystals (present in the joints of gout patients) (218). In neutrophils, LPS engagement of TLR4 recruits Syk and mediates inflammasome assembly and caspase-1 activation, explaining why the LPS signal alone is sufficient to stimulate IL-1β secretion by neutrophils (219). This could explain why neutrophils only need one signal while DCs and macrophages need two signals to activate the inflammasome.

Neutrophils can produce large amounts of IL-10 after microbial stimulation due to co-activation of MyD88 and Syk pathways (220). Simultaneous activation of TLR-MyD88 and CLR-Syk dependent pathways led to prolonged phosphorylation of p38 MAPK and PI3 kinases in neutrophils. Genetic deletion of Syk can attenuate antibacterial host defence in mice (221). *Mycobacterium tuberculosis* can activate Syk through a number of CLRs although how important this response is for host defence is unclear (202). The increased understanding of how Syk can modulate the immune response has led to the emergence of Syk inhibitors as promising therapeutic agents. For example, an oral SYK inhibitor Fostamatinib was tested in a phase III clinical trial in patients with rheumatoid arthritis (222).

Recently, it has been suggested that the adjuvanticity of alum (and MSU crystals) could be related to its engagement with cholesterol and other lipids on the plasma membrane, leading to lipid sorting and forming of lipid rafts. Furthermore, this induces receptor independent activation through the aggregation of immunoreceptor signalling motif (ITAM)-containing receptors, and subsequent Syk- and PI3K mediated activation (Fig 1.11)
Figure 1.6: Activation of Syk, downstream signalling molecules and cellular effects

A) Ligation of receptors leads to recruitment of Syk (ζ-chain associated protein kinase of Zap70) to the plasma membrane through binding of tandem SH2 domains on Syk (or Zap70) to phosphorylated tyrosine residues on the receptor. The tyrosine residues can be on ITAMs, either present on the cytoplasmic tail of the receptor itself or present in receptor associated transmembrane adaptors. Additionally, two hemITAMS on two different associated receptors can also activate Syk. Further, Syk can be activated in an ITAM independent manner. Taken from (191).

B) ITAM phosphorylation by SRC kinases leads to recruitment and activation of Syk, leading to downstream signalling and activation of other kinase mediators, including PI3K (triggers p85a subunit), NLRP3 inflammasome, p38, ERK, AKT, NF-κB. Binding of Syk associated binding partners activates downstream signalling pathways, leading to various cellular responses. Taken from (191).
1.6 Role of the plasma membrane and actin in cell signalling

The plasma membrane is a complex structure containing proteins, lipids and carbohydrates that initiates biological processes through communicating with other cells or responding to external stimuli. It also creates a boundary between the extracellular and intracellular environment and compartmentalises areas within the cytoplasm to localise certain cell functions.

Membrane fluidity is one important physical feature of these biomembranes as it can control many cell functions. Changes in the biophysical properties of the cell membrane, including membrane fluidity can dramatically alter cell function such as proliferation and apoptosis through changes in the activity or localisation of membrane proteins (e.g. ion channels, signalling receptors and enzymes) (223). The pharmacological action of many drugs is through changes in fluidity including local anaesthetics (224) and non-steroidal anti-inflammatory drugs (225). It has also been reported that plasma membrane fluidity can modulate MAPK signalling pathways such as ERK and JNK (226). Furthermore, anticancer agents induce apoptosis through changes in the membrane fluidity of tumour cells (227).

Lipids are one of the major components of the plasma membrane and are essential for regulating plasma membrane fluidity. The lipid cholesterol is an essential component of the plasma membrane that gives the membrane its mechanical stability (planar steroid ring) as well as being a major component of lipid rafts to contribute to the normal functions of the cells. Changes in the cholesterol content can alter membrane fluidity, cell thickness, structure and stiffness and physically affect binding and functional properties of incorporated receptors and other proteins (228).

Evidence suggests that cholesterol distribution in the membrane is heterogenous and concentrated in lipid rafts. Lipid rafts are dynamic microdomains (<200 nm) within the plasma membrane which are enriched with tightly packed sphingolipids, glycosphingolipids and cholesterol, phospholipids with saturated acyl chains, glycosylphosphatidylinositol (GPI)-linked proteins as well as being enriched with signalling proteins such as innate and adaptive immune receptors (TLRs, Fc receptors, cytokine receptors, BCR and TCR), GF receptors, Src family kinases and MAPKs (229-232). These rafts have the potential to form larger domains (>300 nm) upon clustering induced by protein–protein and protein–lipid interactions (230).

Rafts can be viewed as a signalling platform which brings all required components together to initiate a signalling cascade. It is now accepted that receptors localise in lipid raft clusters upon
ligand binding, triggering signalling pathways to facilitate cell activation (233, 234). Receptors cross-link in small raft domains which leads to aggregation into stable micron sized raft domains allowing receptors to encounter their secondary messenger molecules and initiate the downstream signalling cascade (Fig 1.7A). For example, the dectin-1 receptor triggers signal transduction via its cytoplasmic hemi-ITAM to activate Syk and PLCγ1/2 in APCs leading to Th1- or Th17-type antifungal T cell responses (Fig 1.6) (235). Prior to receptor ligation, rafts are too small to form stable platforms and recruit receptors with ITAM motifs to initiate signalling (Fig 1.7A). Upon stimulation with curdlan, a dectin-1 agonist, more stable and larger rafts form and receptors translocate into these cholesterol rich lipid rafts regions on the cell surface. This also occurs during MHC antigen presentation, receptors are recruited into stable larger rafts, allowing crosslinking of raft associated receptors to initiate downstream signalling pathways (236).

Lipid rafts are accountable for a wide range of cellular functions including signal transduction relating to growth, proliferation and innate and adaptive immune receptor signalling (234, 237, 238), endo/exocytosis, migration (239), cell adhesion (240), cell to cell contacts (241), organisation of the cytoskeleton (242) and plasma membrane protein sorting (243). Many pathogens exploit receptors localised in rafts to enter cells, including Plasmodium falciparum (244) and Candida albicans which targets the dectin-1 lipid raft platform to infect cells (245). Depletion of cholesterol attenuates the functionality of lipid rafts as proteins dissociate from the rafts while increasing fluidity of the membrane (246). In contrast, an increase in lipid raft formation will also increase the rigidity and stiffness (decrease fluidity) of the plasma membrane (228).

Caveolae are ~60–80 nm cholesterol-enriched membrane invaginations that are usually smaller than lipid rafts and enriched with caveolin proteins (e.g. cholesterol-binding protein caveolin-1) which confers its distinct shape (lipid rafts lack caveolin). They can be viewed as a subset of lipid rafts used to compartmentalise numerous signalling processes (247) or used in a form of non-clathrin coated endocytosis (248) (Fig 1.7C).

Gangliosides (e.g. GM1), a class of glycosphingolipids, are another major component of lipid rafts (249-251). On the plasma membrane, gangliosides are involved in cell-cell recognition and adhesion and regulate signal transduction within raft domains. Cholera toxin (CT) binds to the membrane lipid ganglioside GM1 at the cell surface to gain entry to intestinal epithelial
cells, increasing cAMP levels that lead to water loss and diarrhoea (249). Therefore, the ganglioside GM1 is a marker to identify lipid rafts and fluorescent cholera toxin B subunit (CtB) that binds to the GM1 lipid receptor has been extensively used to visualise rafts in vitro (252).

Actin is an ATPase that exists in either monomeric or filamentous states. The actin cytoskeleton consists of a network of polymerising and depolymerising filaments which allow for changes in cell shape, mobility, adhesion, stiffness, cytokinesis, cell polarity, intracellular transport, phagocytosis (Fig 1.7C), apoptosis, receptor clustering and mediate signalling coming from the membrane/external forces (253, 254). For example, NLRC4 inflammasome dependent changes in actin polymerisation reduce intracellular Salmonella burden in macrophages (255). The NLRC4 mediates reactive oxygen species (ROS) release to inhibit bacterial replication and limits bacterial uptake through mechanical stiffening of the cell via actin polymerisation. Furthermore, this stiffening also reduces movement to control bacterial dissemination in tissues.

The semi-flexible polymers of filamentous actin (F-actin) are assembled from monomeric actin subunits (G-actin). Cells are constantly assembling, maintaining and disassembling F-actin fibres. Actin monomers complexed with profilin are added to the barbed end of actin filaments (polymerisation) in its ATP-bound state and removed from the other pointed end (depolymerisation) in its ADP state, giving rise to a process known as actin filament treadmilling (256). Profilin maintains a steady pool of actin monomers by promoting the exchange of ADP for ATP on actin, which replenishes the pool of ATP-actin monomers ready for polymerisation on the growing barbed-end, while preventing addition to the pointed end (257).

Polymerisation is controlled by a large set of regulatory proteins, e.g. Small activated GTPases of the Rho superfamily (Cdc42, Rac, and Rho) which can remodel actin for different functions and control the formation of filopodia, lamellipodia (both projections at the leading edge of cells), and stress fibres. For example, Cdc42 in its active state recruits WASP (Rac recruits WAVE family proteins) to the plasma membrane which subsequently activates the actin-related protein 2/3 (Arp2/3) complex to initiate actin polymerisation to form filopodia (or lamellipodia with Rac) branched filaments (258, 259) (Fig 1.7B). Rho mainly activate the formation of stress fibres, focal adhesions and Myosin II through Rho-associated kinase.
(ROCK)1/2 or polymerisation by activating the formin family of proteins (e.g. mDia1). ROCK increases the activity of actin cytoskeleton regulator myosin II light chain (MLC) by phosphorylating the myosin- MLC phosphatase, inactivating it and thereby preventing dephosphorylation of MLC leading to increased association with actin filaments to enhance contractibility and stress fibre formation (Fig 1.7B) (260, 261).

Other nucleators of actin assembly have also been identified, including Formins (also activated by Rho), and Spire (other examples include, Cobl, VopL/VopF and Sca2) (262). Filament nucleators are generally unrelated but, with the exception of formins, they all use the WASP-homology 2 (WH2) domain for interaction with monomeric actin (263). Their mechanisms of nucleation also vary greatly. As mentioned above, the WASP/WAVE family of proteins are necessary to activate the Arp2/3 but also contain WH2 domains. Therefore, the WASP/WAVE family of proteins also aid nucleation by delivering actin subunits at the barbed end for polymerisation. Formins, characterised by their formin homology (FH) domains nucleate the formation of linear unbranched filaments by interacting directly with and stabilising actin polymerisation intermediates (dimers and trimers) (262). Formins use the FH2 domain to initiate actin assembly by interacting and stabilising actin to induce nucleation while the FH1 domain binds to profilin to accelerate formin-mediated filament elongation (262). While Spire, assembles filaments through the recruitment and organisation of up to four actin monomers into a prenucleation complex that produces unbranched filaments (264). Moreover, actin elongation can be enhanced by the WH2 containing Ena/VASP (enabled/vasodilator stimulated phosphoprotein) family proteins which can recruit and bind profilin-bound G-actin to elongate filaments at the barbed end, protect from capping proteins such as CapZ (bind actin barbed ends to prevent elongation) and inhibit Arp2/3-mediated actin filament branching (265, 266).

The addition of actin polymers to actin filaments in close contact with the membrane pushes the cellular membrane forward allowing the cell to move. In contrast, depolymerisation is controlled by ADF/cofilin, myosin and Rho-family GTPase-activating proteins (GAPs) that facilitate GTP hydrolysis (267). Cdc42, Rac and Rho can also prevent depolymerisation by activating LIMK (LIM domain kinase), a kinase that in turn phosphorylates and inhibits cofilin during nucleation. Rho, Rac, and Cdc42 also affect gene transcription through signal transduction pathways not involving the actin cytoskeleton. For example, Rho GTPases can activate PI3K, JNK and p38 MAP kinase pathways that results in the transcriptional regulation of genes associated with cell functions such as proliferation (268-270).
Filopodia and lamellipodia are usually found on the leading edge of migrating cells, generating a protrusion force through actin polymerisation (271). Stress fibres generate their force using the motor activity of associated myosin II bundles. They contain bundles of actin filaments, crosslinked with α-actinin and myosin II, activated by ROCK to produce the contraction force (272). These stress fibres are essential for cell adhesion to the extracellular matrix (ECM) through anchoring focal adhesions, which link the ECM on the outside of the cell, through integrin receptors, to the actin cytoskeleton inside the cell. Stress fibres are also involved in migration (at cell to generate contractile forces) and in mediating changes in cell morphology by sensing mechanical signals from the environment (273).

Physical or mechanical signals can have a significant influence on the activity of MAPKs (e.g. p38) and transcription factors. This is usually mediated through changes in the stiffness of the ECM which can alter a cells shape and has been well studied in macrophages where in addition to the molecular markers and cytokine secretion profile, the cell morphology can also be used to distinguish between M1 and M2 phenotypes (274). Macrophages adopt a circular and flattened morphology in response to soluble factors such as LPS and IFN-γ. However, in the presence of the M2-inducing cytokines IL-4 and IL-13, they exhibit a much more elongated morphology (274). In vivo, cells sense mechanical signals from neighbouring cells or ECM and adjust the stiffness of the cytoskeleton, leading to a signalling response that can induce proliferation, apoptosis and migration (275). This is often mediated through changes in cell morphology and the actin cytoskeleton, for instance F-actin accumulation has been linked to proliferation and survival (270) through regulation of the transcription factors YAP and TAZ of the hippo signalling pathway in response to stress (276). F-actin accumulation underneath the cell membrane is crucial for cell shape, migration, phagocytosis and division (267, 277). Membrane phosphoinositides on the membrane such as PI(4,5)P₂ and PI(3,4,5)P₃ have been linked to assembly of actin networks (278). The actin nucleation promotion factors, WAVE and WASP, can facilitate actin polymerisation via the Arp2/3 complex upon binding PI(4,5)P₂. In contrast, actin-capping protein, ADF/Cofilin, and the G-actin–binding protein profilin are all inhibited by binding PI(4,5)P₂.

As mentioned above, many lipid raft functions (e.g. signalling, trafficking, adhesion, migration, and growth) require them to interact with the actin cytoskeleton (Fig 1.7A). Certain components that regulate actin polymerisation or stabilise microtubules are associated with membrane rafts (e.g. Src family kinases and small Rho GTPases) (242). The reorganisation of lipid domains also depends on the actin cytoskeleton and Src kinase activity (279). Thus, the
association of the actin cytoskeleton with the plasma membrane is fundamental to its structure and function. How particulate adjuvants might interact with the plasma membrane of DCs and macrophages to influence raft formation, actin distribution and cell morphology remains unclear and will be investigated here.
Fig 1.7: Formation of lipid rafts during phagocytosis and actin polymerisation signalling pathways

A) Lipid rafts on the plasma membrane are enriched in tightly packed sphingolipids and cholesterol. Upon ligand binding on the cell surface, larger and more stable rafts form which aggregates raft associated ITAM containing signalling molecules, leading to the recruitment and activation of Syk, which in turn recruits PI3K. Syk and PI3K activation leads to actin cytoskeleton rearrangement to strengthen signalling responses. Active Syk initiates signalling pathways including PI3K/AKT, ERK, PLCγ, p38 and NFκB to generate a downstream signalling effect, such as cytokine production, phagocytosis and growth (280).

B) GEFs and GAPs are upstream regulators of Cdc42, Rac, and Rho activity. Cdc42 and Rac stimulate actin polymerisation by activating the Arp2/3 complex through their GTP-dependent association with the Arp2/3 activating scaffold proteins, WASP and WAVE, respectively. Rather than activating Arp2/3, Rho promotes actomyosin contractility through activation of ROCK1/2 pathway or actin polymerisation by activating formins (281).

C) Particles can be taken up by cells through various pathways depending on their size. Particles between 50 and 80 nm are taken into the cell through caveolin-mediated endocytosis. <150 nm by clathrin-mediated endocytosis which require the formation of coated pits by the assembly of clathrin. Clathrin- and caveolin-independent endocytosis can also occur with particles < 100 nm but remains poorly understood. 0.5 to 5 µm particles are taken up non-specifically by micropinocytosis using actin protrusions that collapse onto and fuse with the membrane and particles larger than 0.5 µm are taken up by phagocytosis via actin assembly through Rho-family GTPases that surrounds the large particle (282, 283).
1.7 Regulation of cell-mediated immunity by the IL-12 family

The IL-12 family of cytokines are crucial for the induction of both a Th1 and Th17 cellular immune responses (284). The family is unique in having the only heterodimeric cytokines as well as mediating a vast number of biological functions. The IL-12 family consists of proinflammatory (IL-12 and IL-23), immune regulatory (IL-27) and anti-inflammatory (IL-35) cytokines (285, 286) (Fig 1.8). Each member is a heterodimer made up two subunits, some of which are shared among the different members of the IL-12 family. Despite sharing many structural features, cytokines of the IL-12 family mediate a vast number of different functional effects.

Interleukin-12 (IL-12) was the first member of the IL-12 family of cytokines to be identified. IL-12 is secreted in response to pathogenic organisms, including Gram- positive and Gram-negative bacteria, parasites, viruses, and fungi. IL-12 has multiple biological functions and importantly, it bridges the early innate response and subsequent antigen-specific adaptive immunity. IL-12 is primarily involved in Th1 cell polarisation and promoting IFN-γ production in T cells and natural killer cells. IL-12 is preferentially secreted by APCs, including DCs in response to PAMPs, leading to polarisation of naïve T cells into IFN-γ producing Th1 cells (287-291). A positive feedback loop is also in play, whereby IL-12 induced IFN-γ can prime APCs for additional IL-12 production (292). In contrast, IL-10 production can inhibit secretion of IL-12 (293, 294). Type 1 IFN can also regulate the expression of IL-12 (295). Finally interaction of T cells with APCs via CD40-CD40L interaction can enhance IL-12 secretion (296).

IL-12p70 is the biologically active form, a heterodimer composed of two subunits, p35 and p40 which are shared with IL-35 and IL-23, respectively (297) (Fig 1.8). The expression of both subunits in the same cell is essential to form the active heterodimer (298). However, generation of each subunit is independently regulated. The p35 gene is constitutively expressed in various cell types and tissues, however free p35 is not secreted without the p40 subunit (299). Expression of p40 is restricted to cells producing IL-12, including activated monocytes, macrophages, neutrophils and DCs. Unlike p35, the p40 subunit is also secreted as monomer and homodimer, antagonising IL-12 activity in mice (not observed in humans) (300).

The generation of p40 is predominantly regulated at a transcriptional level, induced upon engagement of most TLRs (301), regulation of p35 is more complicated, as regulation occurs at a transcriptional (including the use of alternative transcription start sites) and post
transcriptional level (73, 302). Upon LPS stimulation, signalling pathways involving ERK, p38 and PI3K can regulate IL-12p35 and IL-12p40 (73, 85, 88, 303-306). Furthermore, transcription factors like NF-κB and interferon regulatory factors (IRFs), through binding to complementary response elements have been shown to contribute to IFN-γ priming and LPS induction of p40 and p35 gene expression (307). Very recent studies have also demonstrated that JAK1/STAT3 activation can directly inhibit IL-12p35 in DCs (308).

IL-12p70 has a number of key functions: it is the most important cytokine at promoting naïve CD4+ cell differentiation into Th1 cells and enhances the generation and activity of cytotoxic T lymphocytes (CTLs). IL-12 increases macrophage antimicrobial activity. Finally, it can mediate upregulation of adhesion molecule expression on Th1 cells and influence T cell trafficking (292, 299).

IL-23, like IL-12 is a proinflammatory cytokine also secreted by APCs and takes part in polarising the immune responses. In contrast to IL-12, IL-23 can polarise Th17 responses by stabilising IL-17 expression (309, 310). IL-23 shares its IL-12p40 subunit with IL-12, but also comprises a unique IL-12p19 subunit (311) (Fig 1.8).

IL-27 is mainly a regulatory cytokine but it also has some proinflammatory functions. Initially, IL-27 like IL-12 was implicated in the development of Th1 responses, but only early in the initiation of polarisation (312). More recently, IL-27 has been shown to mediate suppressive effects on T cells during pro-inflammatory conditions, induce IL-10, and to promote specialised T regulatory cell responses (313). Finally, IL-35 is a potent inhibitory cytokine produced by Treg cells, contributing to their regulatory functions. For instance, IL-35 can inhibit proliferation in T cells (285, 314).

To conclude, both IL-12 and IL-10 plays a critical role in either the promotion or regulation of DC maturation and function. Therefore, adjuvants which have the ability to either enhance or suppress these cytokines are crucial in designing vaccines aimed at enhancing a particular response whether that would be a Th1, Th2 or even an anti-inflammatory Treg response.
All IL-12 family members are heterodimers. IL-12p70 is composed of P40 and p35 subunits, while IL-23 is composed of IL-12p40 and IL-12p19. IL-27 is composed of a p28 subunit and the product of Epstein-Barr virus–induced gene 3 (Ebi3). IL-35 is a heterodimeric cytokine composed of the p35 subunit and the p40-related protein EBi3. Receptor ligation activates JAK-STAT pathways. Taken from (285).

1.8 T-Cell Responses and Adaptive Immunity

While the innate immune system generates a swift immune response, the adaptive immune system develops a slower antigen-specific response to ensure clearance of the pathogen. Both the innate and adaptive immune responses are essential to provide the host with protection. Both responses are heavily linked to each other with DCs coordinating the type of adaptive immune response generated. Adaptive immunity is mainly mediated by T and B lymphocytes and memory cells to provide protective and long-lasting immunity. Antigen-specific memory cells are activated, ensuring that upon reinfection with the same pathogen, the host will react more quickly to clear the pathogen in order to prevent disease.

Activation of T cells generates the cell mediated response, providing defence against intracellular pathogens. B cells develop into antibody producing plasma cells resulting in humoral immune response, which protects against extracellular pathogens and their toxins.
1.8.1 T helper cells
T cells play a crucial effector role during immunity by orchestrating the immune responses against pathogenic microorganisms. T helper cells have a number of diverse functions depending on the subset, including activating B cells to secrete antibodies, antibody class switching, enhancing and maintaining CD8+ T cell responses and regulating macrophage function and suppressing immune responses. The majority of T cells are characterised by the presence of a heterodimeric αβ receptor, known as the T cell receptor (TCR) in association with CD3 molecules on the cell surface. T cells are classified into various subtypes depending on functions and co-receptor expression such as CD4 or CD8. DCs are essential for activating and differentiating naïve T cells via release of cytokines and other cofactors (315) (Fig 1.3 and 1.9). They present antigen through MHC molecules and present them to T cells via the T cell receptor (TCR). CD4+ cells recognise antigens presented on MHCII, while CD8+ recognise antigen on MHCI. Once activated, these cells differentiate to become effector and/or memory cells of specialised phenotypes.

Upon encountering antigens, naïve CD4+ T cells can differentiate into different T helper (Th) cell subsets and inducible regulatory T cells. Th cell subsets have specialised functions to deal with the diverse threats posed by infections but are also linked to development of autoimmune and allergic diseases. T cells can differentiate into Th1, Th2, Th17, T regulatory cells (Treg), T follicular helper (Tfh) and Th9 cells depending on cytokines released by APCs or other cytokines in the environment triggered by a specific pathogen. Each subset has a distinct biological function, including driving cell-mediated immunity for intracellular pathogens (Th1) or extracellular pathogens (Th17), inflammation (Th1 and Th17), humoral immunity (Th2), tolerance (Treg), germinal centre (GC) formation (Tfh) or parasitic helminth infections (Th9) (316).

1.8.2 Th1 cells
Th1 cells play a key role in the immune response against intracellular pathogens, including Mycobacterium tuberculosis, human immunodeficiency virus and Plasmodium falciparum. As mentioned above, IL-12 and IFN-γ produced by APCs and NK cells are the main mediators for inducing a Th1 response (317). Th1 cells are characterised by upregulation of the transcription factor t-bet, leading to increased IFN-γ and a decrease in IL-4 production (repressing Th2 responses) to drive cell mediated immunity (289) (Fig 1.9). IFN-γ activates mononuclear
phagocytes, NK cells and cytotoxic T cells and promotes killing of intracellular pathogens and virally infected or cancerous cells. In mice, IFN-γ cause B cells to class switch and secrete immunoglobulin (Ig) G2a (or the equivalent IgG2c in other mouse strains) (318). Therefore, Th1 cells provide protection against a variety of intracellular pathogens and tumours. A major problem facing a number of both licensed and experimental vaccines is their inefficiency at driving robust Th1 responses.

Th17 cells have an important function in combating extracellular bacterial and fungal infections, particularly at mucosal surfaces through secretion of IL-17A (IL-17F and IL-22). Th17 cells, characterised by the expression of the transcription factor RORγt cells have been implicated in the pathogenesis of autoimmune diseases, including rheumatoid arthritis and multiple sclerosis (319) (Fig 1.9).

1.8.3 Th2 cells
The type 2 T helper (Th2) subset is required for immunity against extracellular pathogens, including helminth infections (320, 321) but can also cause allergic diseases such as atopic asthma (315). IL-4 induces the activation of STAT6 which subsequently upregulates expression of GATA-binding protein (GATA 3), the key transcription factor involved in Th2 lineage differentiation which also inhibits polarisation to Th1 cells (322, 323). Th2 cells secrete the signature cytokine IL-4 which also strengthens the Th2 response through a positive feedback mechanism. Th2 cells mediate immune response through secretion of IL-4, IL-5 and IL-13, which are the hallmarks of allergic disease and helminth infection (Fig 1.9). These cytokines lead to antibody class switching to immunoglobulin E (IgE) and immunoglobulin G1 (IgG1) to neutralise foreign organisms and drive eosinophil recruitment, goblet cell mucus production, smooth muscle contraction and B-cell expansion (324). Unlike the Th1 response, the Th2 response is often associated with humoral responses during which high levels of pathogen-specific immunoglobulin are generated to neutralise foreign organisms (318). For example, the hallmark of allergic disease and helminth infection is induction of a CD4+ Th2 response, characterised by secretion of cytokines such as IL-4 and IL-13 (324).

1.8.4 T regulatory cells
In contrast to other Th cells, Treg cells have a central role in controlling tolerance against self-antigens and maintaining immune homeostasis (balance between immune activation and
tolerance). Their importance is highlighted in that defects in Tregs can lead to the development of autoimmune diseases (325). Treg development is dependent on STAT5 signalling and their master transcription factor is forkhead box P3 (FoxP3) (326) (Fig 1.9). Regulatory T cells are primarily classified as CD4+ CD25+ FoxP3+ T cells and divided into two subgroups, naturally occurring Treg (nTreg) and inducible Treg (iTreg) (327). However, FoxP3 independent Treg subtypes also exist, including class 1 Treg (Tr1) and Th3 cells which are involved in immune suppression by secreting IL-10 and TGF-β respectively (327). nTreg develop in the thymus during the TCR affinity selection process where they recognise self-antigens presented on MHC molecules in a TGF-β rich microenvironment (328). iTregs develop in the periphery from naïve CD4+ T cells under tolerogenic conditions (low co-stimulatory expression on APCs and a TGF-β, IL-15 and IL-2 cytokine environment) (329). Tregs are characterised by their IL-10, transforming growth factor (TGF)-β and IL-35 secretion capacities as well as the upregulation of the immunosuppressive cytotoxic T-lymphocyte antigen-4 (CTLA-4) surface protein, a CD28 family member that binds CD80/CD86, sending a negative signal to APCs (315, 318). Interestingly, a recent study has indicated that IL-10 derived from CD4+ Treg cells was necessary for the maturation of memory CD8+ T cells following acute infection with lymphocytic choriomeningitis virus (LCMV) (128).

1.8.5 CD8+ T cells

CD8+ T cells are CTLs whose main function is to directly kill tumour cells and virally infected cells. This is mediated by triggering death receptors (FasL) and secreting perforin and granzymes, which cause apoptosis of target cells (330) as well as IFN-γ secretion to mediate effector functions (Fig 1.9). CD8+ T cells recognise peptide-MHCI molecules on the cell surface to discriminate between healthy or infected cells. Additionally, IL-2 and IL-12 secretion drives effector CTL differentiation. This leads to increased numbers of short-lived effector CD8+ T cells and long-lived CD8+ T cells that contribute to a memory population (331, 332).

CTLs can be subdivided into cytotoxic T cells type 1 (Tc1) and type 2 (Tc2), supporting a Th1 or Th2 response respectively. Tc1 and Tc2 cells are equally cytotoxic: IL-12 promotes a Tc1 phenotype while IL-4 a Tc2 phenotype. Tc1 cells produce IL-2 and IFN-γ to support the Th1 response whereas Tc2 cells produce IL-4, IL-5, IL-10 and to a lesser extent IFN-γ, and aid a Th2 cell response (332).
Therapeutic vaccines designed for the elimination of cancer cells or virally infected cells depend on antigen-specific CD8+ T cells that generate cytotoxic T lymphocytes (CTLs) (333). Unfortunately, in vivo induction of a robust cytotoxic CD8+ T cells (CTL) response with non-living vaccines remains a challenge which requires innovative solutions.

**Figure 1.9: Differentiation of T helper cells into effector subsets and functions**

Naïve T cells activated by DCs can differentiate into a number of distinct effector subsets. The process is dependent on the cytokines present in the local environment, referred to as polarising cytokines. Effector CD4+ Th subsets are characterised by their cytokine expression profile and transcription factor usage. Taken from (334).

### 1.9 B cells and the humoral immune response

The humoral arm of the adaptive response is mediated by B cells and the specific antibodies they produce. Naïve B cells can recognise antigens through their B cell receptor (BCR) and upregulate co-stimulatory molecules, thus acting as an APC. However, their primary role is to generate antibodies to neutralise pathogens (335).
Short lived plasma cells that make IgM are generated during the primary immune response with no class switching involved. However, while both IgM and IgD are secreted quickly, they have low affinity for their target. IgM can form pentamers which can simultaneously bind to ten antigens to increase avidity as well as activate the complement system (336).

Effective B cell responses to infectious pathogens or immunisation generally require the assistance of CD4+ helper T cells (337). For example, Follicular helper T cells (Tfh cells) compose a heterogeneous subset of CD4+ T cells found within and in proximity to germinal centres in secondary lymphoid organs characterised by the expression of inducible costimulator (ICOS) and programmed death (PD)-1. Tfh cells express the chemokine receptor CXCR5, which directs them to B-cell follicles via gradients of the chemokine CXCL13. Tfh cells are involved in promotion of germinal centre responses which are needed for B cell class switching, efficient somatic hypermutation and generation of memory B cells and long lived plasma cells (338). Tfh cells secrete IL-10 and IL-21 to enhance growth and differentiation of CD40L-stimulated GC B cells, inducing secretion of all Ig isotypes (339) (Fig 1.9).

Following activation within the GCs, long lived B cells can produce different classes of antibodies with higher affinity, including isotypes such as IgG, IgA and IgE. IgG can be split into 4 subclasses, IgG1, IgG2, IgG3, and IgG4 in humans (IgG1, IgG2a or IgG2c IgG2b, and IgG3 in mice). IgG is monomeric and the most abundant antibody isotype in the blood. It is involved in the neutralisation and opsonisation of pathogens, as well as activating the complement system (340). IgA is synthesised as a monomer, which forms dimers at mucosal sites such as the gastrointestinal and respiratory tracts to neutralise pathogens within mucosal epithelial linings (340). IgE provides immunity against helminths but can also mediate type I hypersensitivity (asthma) and can bind to Fc receptors on Mast cells. Cross linking of IgE with antigen on mast cells leads to their degranulation, a process which initiates the release of pro-inflammatory mediators (341).

It is becoming increasingly clear that B cells do more than make antibodies such as modulating the immune responses. Regulatory B (Breg) cells are immunosuppressive cells that support immunological tolerance, through the production of IL-10, IL-35, and TGF-β, thereby inhibiting excessive inflammation, expansion of T cells and promoting anergy (342, 343). Interestingly, IL-10 secreting regulatory B cells have also been shown to be highly sensitive to Syk inhibition (344).
1.10 Adjuvants

The goal of vaccination is to induce protective immunity but in some cases, this can only be achieved by addition of adjuvants (Table 1.1). Adjuvants are used in vaccines to enhance and modulate the immunogenicity of a vaccine antigen. Many licensed vaccines consist of whole or inactivated pathogens that can promote a strong protective immune response since they contain naturally occurring adjuvants. However, there is a move toward vaccines based on purified antigens (subunit vaccines) which although safer are generally more tolerogenic with limited immunogenicity. Therefore, adjuvants are added to enhance immune responses towards the antigens and induce strong and long-lasting protection against infection.

There have already been some very successful subunit vaccines developed for use in humans, with alum adjuvanted vaccines being particularly successful for enhancing humoral responses although this adjuvant is a poor inducer of T cell responses. Therefore, new vaccines need to target not only a robust antibody response but also a strong T helper and cytotoxic T lymphocyte (CTL) response.

Only six new adjuvants have been used in licensed vaccines in the last 20 years (Table 1.2). Alum was first licensed in 1926 after it was demonstrated to induce increased antibody titres in guinea pigs when injected with soluble toxoid. Since then, both the USA and Europe have licensed other vaccine adjuvants. These include the squalene-based oil-in-water emulsion MF59, consisting of the naturally occurring squalene oil and of non-ionic surfactants Tween 80 and Span 85 emulsified in uniform particles of ~160 nm in size. MF59 has been used in influenza vaccines (FLUAD™) (345) and currently, is in clinical trials for vaccines against infectious diseases such as herpes simplex virus (HSV), HBV, and HIV (Table 1.2). Another squalene-based oil-in-water emulsion, namely adjuvant system 03 (AS03), has been used in the pandemic H5N1 influenza vaccine (Q-Pan H5N1) and the H1N1 influenza vaccine Pandemrix. AS03 has been shown to promote higher antigen-specific antibody responses compared with aluminium hydroxide (346). Virosomes are reconstituted membranes of an enveloped virus, consisting of a spherical phospholipid cell membrane bilayer carrying antigen either bound to the surface or encapsulated within the lumen. A combination of aluminium hydroxide and monophospholipid (MPL) A, an LPS analogue, (AS04) was approved for use in vaccines against hepatitis B virus (HBV) and human papilloma virus (HPV) (Table 1.2). Finally, AS01 is a liposome-based adjuvant formulation, comprised of MPL and...
the saponin QS-21 (347). The malaria vaccine, RTS,S, consists of a portion of the circumsporozoite protein of *Plasmodium falciparum* fused to hepatitis B surface antigen virus-like particles and AS01.

Despite the use of adjuvants clinically (and the many adjuvants under development) their mechanisms of action have yet to be fully elucidated. However, research conducted over the past decade is revealing the mechanisms responsible (Fig 1.10). There are several types of adjuvants with differing modes of action. These include mineral salts, oil emulsions, immune stimulating complexes (ISCOM), bacterial derivatives (TLR agonists), carbohydrate adjuvants, liposomes and virosomes, cytokines (IL-1, IFN-γ, IL-12 and GM-CSF), virus like particles and polymeric micro and nano particle adjuvants.
<table>
<thead>
<tr>
<th>Benefit</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Reduce antigen dose</strong></td>
<td>Increase global antigen supply in case of an epidemic and increase safety profile of vaccines</td>
</tr>
<tr>
<td>Broader antibody response and increased functional antibody titres</td>
<td>Needed for complex pathogens which display antigenic drift and strain variation (Malaria, HIV and Influenza)</td>
</tr>
<tr>
<td>More rapid response to pathogens</td>
<td>Reduce number of doses needed, important for pandemic flu outbreaks</td>
</tr>
<tr>
<td><strong>Effective T cell responses</strong></td>
<td>Important for vaccines against pathogens that are controlled by cellular immune responses, including those causing malaria, tuberculosis and HIV</td>
</tr>
<tr>
<td>Vaccines for elderly, young and immunocompromised</td>
<td>Overcome immune senescence</td>
</tr>
<tr>
<td><strong>Therapeutic vaccines</strong></td>
<td>Vaccines against various cancers and viruses (HPV)</td>
</tr>
</tbody>
</table>

**Table 1.1: potential benefits of using new adjuvants**
<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Composition</th>
<th>Description</th>
<th>Manufacturer</th>
<th>Licensed vaccine for use in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alum</td>
<td>Mineral salt-aluminium hydroxide</td>
<td>Improves humoral immune responses and antigen stability. Antigens are adsorbed to the surface.</td>
<td>Various</td>
<td>Pertussis, Tetanus, Diphtheria <em>Haemophilus influenzae</em> type b Pneumococcus Hepatitis A and B Papilloma virus</td>
</tr>
<tr>
<td>MF59</td>
<td>Oil in water emulsion</td>
<td>Improves humoral and cell-mediated immunity. Increases APC recruitment and activation. Promotes antigen depot, delivery, uptake and migration of cells to lymph nodes.</td>
<td>Novartis/GSK</td>
<td>Influenza (FLUAD)</td>
</tr>
<tr>
<td>AS03</td>
<td>Oil in water emulsion</td>
<td>Improves humoral and cell-mediated immunity Promotes cytokine production and recruitment of innate cells.</td>
<td>GSK</td>
<td>Pandemic H1N1 influenza vaccine Pandemrix and Arepanrix</td>
</tr>
<tr>
<td>Virosomes</td>
<td>Liposome</td>
<td>Carrier capabilities increases uptake and presentation by APCs Improves humoral and cell-mediated immunity.</td>
<td>Berna Biotech</td>
<td>Influenza (Inflexal V) Hepatitis A virus (Epaxal)</td>
</tr>
<tr>
<td>AS04</td>
<td>alum co-absorbed with TLR4 agonist monophosphoryl lipid A (MPL)</td>
<td>Improves humoral and cell-mediated immunity Enhances DC maturation and presentation.</td>
<td>GSK</td>
<td>Cervical cancer vaccine Cervarix against Papilloma virus</td>
</tr>
<tr>
<td>AS01</td>
<td>MPL and QS21</td>
<td>Enhances antibody titres, Th1 type immunity and CD8 T cell-mediated immunity.</td>
<td>GSK</td>
<td>Malaria vaccine, RTS,S (Mosquirix)</td>
</tr>
</tbody>
</table>

Table 1.2: Licensed vaccine adjuvants
Several mechanisms have been proposed through which adjuvants mediate their activity. Particulate vaccines can form a depot at the site of injection, slowly realizing antigen. Particulate vaccines can also modulate cytokine and chemokine release at the site of injection leading to immune cell recruitment (APCs, NK, neutrophils, monocytes and eosinophils) which also secrete chemokines and attract other immune cells (A). Inflammasome activation has also been implicated as a mechanism for some adjuvants. Activation of the inflammasome leads to the production of the proinflammatory cytokines IL-1β and IL-18 (B). Many adjuvants can act as ligands for PRRs on the cell surface (TLRs and CLRs) and intracellular receptors (NLR and RLRs) that activate an innate immune response. Receptor signalling can then activate transcription factors that induce the production of cytokines and chemokines that help direct a particular immune response, such as a Th1 or Th2 type response, as well as influence the immune cells that are recruited to the site of injection (C). Some adjuvants can influence maturation of APCs, increasing antigen processing, presentation and migration to draining lymph nodes. APCs then interact with antigen-specific B and T cells to activate the adaptive immune response (D). Other mechanisms include improving targeting and delivery to facilitate transport of antigens to APCs (D). Taken from (348).
1.10.1 **Novel and next generation adjuvants**

The number of licensed adjuvants for human vaccines remains limited and most formulations still use aluminium-based adjuvants. Many successful vaccines target pathogens that can be tackled by neutralising and opsonising antibodies while development of vaccines against other infectious diseases, where protection relies on strong cell-mediated immunity including HIV, malaria, tuberculosis and therapeutic cancer vaccines has proven challenging. There have been some promising results with adjuvants in preclinical and clinical trials, including, immunostimulatory adjuvants, combination vaccine adjuvants and particulate adjuvants. Most the adjuvants in clinical development are particulates (e.g. aluminum salts, emulsions, virosomes). Ligands of innate immune receptors have been also used as soluble compounds (e.g. Lipid A analogues) or in combination with particulates (e.g. AS01, AS04, CAF01) to boost immunogenicity of vaccine formulations.

1.10.2 **Immunostimulatory adjuvants**

Immune-stimulatory vaccine adjuvants provide a powerful approach to modulate the immune response (Table 1.3). These pathogen derived immunostimulatory components can activate APCs through a wide range of different innate sensors, including TLRs (e.g. the LPS derivative MPLA or CpG) (349) and other cell surface receptors such as CD40 (350). Consequently, their capacity to present vaccine antigens to T cells and generate protective immunity is enhanced.

However, the application of some TLR ligands (e.g. LPS) has been limited due to the possible side effects, including systemic inflammation and toxicity in humans (351). Therefore, there has been a drive to remove the toxic components but retain the ability to stimulate innate immunity. The TLR4 agonist 3-O-desacyl-4’-monophosphoryl lipid A (MPL) is a detoxified derivative of the LPS isolated from the Gram-negative bacterium *Salmonella minnesota* (R595 strain) (352). Hydrolysis of the bioactive lipid A core results in MPL which is considerably less toxic yet maintains immunostimulatory activity (353). In contrast to LPS which signals through MyD88 and TRIF to activate NF-κB and IRF3/7, respectively, MPL signals only through TRIF dependent pathways (49, 354, 355). Importantly, TRIF activation is necessary and sufficient for effective adjuvant activity of TLR4 agonists (356, 357) including the induction of Th1 responses (352, 358, 359).
There are numerous TLR agonists in pre-clinical and clinical trials for use as vaccine adjuvants (Table 1.3). For example, the TLR9 agonists, CpG oligonucleotides are in a number of vaccine clinical trials for infections such as hepatitis B and malaria vaccines and cancers (360). CpG enhances type I IFNs, the upregulation of costimulatory and MHCII molecules and antigen presentation on DCs, enhances Th1 responses and pro-inflammatory cytokines (360, 361).

Small molecule TLR agonists for TLR7 (imiquimod) and TLR7/8 (resiquimod) were shown to improve the immunogenicity of vaccine adjuvants through type I IFNs, enhanced DC activation as well as cellular immunity in a number of clinical trials for chronic viral infections such as hepatitis B (362, 363) and cancer (364). Interestingly, TLR7/8 and TLR9 agonist were combined in an oil in water emulsion vaccine formulation against HIV. The TLR agonists cooperate to enhance antibody responses against the HIV-1 envelope protein gp140 in Rhesus Macaques (365). Furthermore, vaccines, such as BCG (live attenuated vaccine for TB), influvac (inactivated subunit vaccine for influenza) and Typhim Vi (subunit vaccine for typhoid fever) contain TLR agonists that can induce DC maturation and enhance cellular immunity (366).

In addition to TLRs, there are potentially a number of other innate pathways that could be targeted. CD40 activation has been shown to enhance IL-12 and upregulate co-stimulatory molecules in APCs, leading to CD4+ and CD8+ T cell responses (367). In contrast, the absence of CD40 has been shown to promote tolerance (368). Anti-CD40 combined with TLR agonists can act synergistically to enhance the cellular immune response (350, 369, 370). Furthermore, Polyriboinosinic acid-polyribocytidylic acid (poly I:C) mimics viral dsRNA and is a promising candidate for a vaccine adjuvant against intracellular pathogens (371) (Table 1.3). Poly I:C binds to TLR3 as well as the intracellular innate receptor, retinoic acid inducible gene I (RIG-I) and Melanoma Differentiation-Associated protein 5 (MDA-5) to enhance CMI and type I interferons (372-374). RIG-I (and MDA-5) plays a crucial role in initiating innate antiviral immune responses by sensing viral RNAs (375, 376). However, the major draw-back of stability and toxicity issues needs to be addressed before proceeding to clinical application of dsRNAs, using particulate adjuvants to delivery and stabilise poly I:C has been suggested (372). Furthermore, new analogs have been produced to improve safety and efficacy, including poly I:C stabilised with poly-L-lysine and carboxymethylcellulose (Poly IC:LC) (377) and Poly I:C12U (Ampligen®), a synthetically modified version of Poly I:C (378), being the two...
most widely studied. Ampligen was recently investigated as an adjuvant for an intranasal H5N1 Influenza virus vaccine (379).

Bacterial flagellin is recognised by TLR5 and by cytosolic NLRs, specifically NOD-, LRR- and caspase recruitment domain (CARD)-containing 4 (NLRC4) (380, 381). Flagellin is a known immunomodulator and adjuvant (382, 383) (Table 1.3). Flagellin has a major advantage, in being a protein that can be engineered to incorporate antigens creating a fusion protein allowing co-delivery of TLR5 agonist and antigen to APCs (349). Some examples include influenza hemagglutinin (HA) and matrix protein 2 ectodomain (M2e) (384), *Yersinia pestis* (F1 antigen) (385), and *Helicobacter pylori* (FlaA flagellin) (386) with a flagellin/hemagglutinin-based vaccine (VAX128) and a flagellin (*Salmonella typhimurium*)/M2e vaccine (VAX102) both in clinical trials for influenza (384, 387).

### 1.10.3 Combination vaccine adjuvants

The vast progress made in biotechnology, molecular biology, synthetic chemistry and immunology have led to the development of well-defined PAMP based adjuvants. Combination vaccine adjuvants use established adjuvants and combine them with immunostimulatory agents. The aim is to enhance and tailor the immune response to a specific antigen.

GlaxoSmithKline (GSK) has developed combination adjuvants called Adjuvant Systems (AS). Adjuvant combinations such as AS01, AS03 and AS04, which contain several components specifically combined to enhance and modulate the immune responses. There are all composed of a mixture of established adjuvants (alum, emulsions, liposomes) and immunomodulators. AS04 is the furthest advanced, now licensed to be used in vaccines for cervical cancer. AS04 is composed of MPL adsorbed to aluminium salts (388). Clinical trials are ongoing for a number of vaccines against Malaria and cancers incorporating AS into the formulation (Table 1.2). MPL is approved as a component of the adjuvant system AS04 (358) (Table 1.2). A HPV vaccine has been formulated with virus-like particles of the L1 protein of HPV-16 and HPV-18, and AS04 (389). AS04 is also included in a hepatitis B virus vaccine (Fendrix) (390) and in a vaccine against herpes simplex 2 virus which is in phase III clinical trials (391). The success of MPL has led to the development of new generation of TLR4 agonists, such as aminoalkyl glucosaminide phosphates (AGPs) and a synthetic LPS mimetic RC-529.
Both MPL and *Quillaja saponaria* fraction 21 (QS21) (a derivative of Quil-A) are present in AS01 and AS02, which are liposome- and emulsion-based formulations, respectively (392). Quil-A is a saponin, a detergent derived from the plant *Quillaja saponaria Molina* (393), which has been shown to have potent immunostimulatory properties (394). However, the use of Quil has been hampered by its toxicity, so QS21, a less toxic immunostimulatory fraction Quil-A was purified which can be used at lower doses (395). QS21 has the ability to enhance antigen presentation by APCs, class switch to IgG2a and induces a robust CTL and Th1 response (396).

While QS21 is effective on its own, it is effective at lower doses when combined with other adjuvants such as MPL in the AS01 and AS02 formulations (388, 392) (Table 1.3). Recently, AS01 containing QS-21 has been used in the world's first licensed malaria vaccine (and the first licensed parasitic vaccine) called RTS,S (Mosquirix) (397, 398). The vaccine uses AS01 in combination with a pre-erythrocytic antigen of *Plasmodium falciparum* and a viral envelope protein of the hepatitis B virus (HBsAg) (399). It prevented a substantial number of cases of clinical malaria over a 3–4-year period in young infants and children when administered with or without a booster dose (400-402). The conclusion was that while on its own it would be unable to prevent disease, the vaccine has the potential to make a substantial contribution to malaria control when used in combination with other effective control measures, especially in areas of high transmission (400, 401).
1.10.4 Particulate vaccine adjuvants

Particulate adjuvants exist as microscopic particles that were originally used as delivery systems for antigens. However, as more particulate adjuvants are studied it has now become clear that many of these adjuvants possess immunostimulatory properties. They generally do not contain specific agonists for PRRs yet they are still capable of modulating the innate and adaptive immune responses. The exact mechanisms that these adjuvants employ are still not fully understood (403, 404), although several theories have been proposed (Fig 1.10, 1.11 and 1.12). This include depot formation, inducing the release of DAMPs (e.g. extracellular ATP), controlled antigen release, facilitating antigen uptake by DCs, antigen protection, enhanced presentation and cross presentation, immunomodulation and/or inflammasome activation (405-407). Several types of particulate adjuvants have been described, including emulsions, immuno-stimulating complexes (ISCOMs), mineral salts and biodegradable micro-particles such as poly (lactic-co-glycolic acid) (PLGA) (Table 1.4). The reason why many particulate adjuvants are unable to elicit a Th1 response while enhancing Th2 responses remains unclear. It has been suggested that adjuvants like alum can enhance IL-10 production, leading to the suppression of a Th1 response (408, 409).
<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Class</th>
<th>Function and description</th>
<th>Clinical phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG</td>
<td>TLR9 agonist</td>
<td>Enhances antibody titre, Th1 type immunity and CD8 T cell-mediated immunity</td>
<td>Phase 3</td>
</tr>
<tr>
<td>Flagellin</td>
<td>TLR5 agonist</td>
<td>Flagellin linked to antigen. Enhances antibody titre, mixed Th1 and Th2 type immunity.</td>
<td>Phase 1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Induces DC maturation</td>
<td></td>
</tr>
<tr>
<td>PolyI:C</td>
<td>TLR3 agonist</td>
<td>Double-stranded RNA analogues that enhance antibody titre, Th1 type immunity and CD8 T cell-mediated immunity. Increased IL-12, type I IFN, MHCII expression and cross presentation in DCs.</td>
<td>Phase 1</td>
</tr>
<tr>
<td></td>
<td>RIG-I activation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS02</td>
<td>Combination</td>
<td>MPL, QS21 and oil-in-water (O/W) emulsion Enhances antibody titre and Th1 type immunity.</td>
<td>Phase 3</td>
</tr>
<tr>
<td>ISCOMs and</td>
<td>Combination</td>
<td>Saponin and phospholipid Enhance antibody titre, Th1 and Th2 type immunity and CD8 T cell-mediated immunity. Targeting, antigen uptake and activation of DCs</td>
<td>Phase 2</td>
</tr>
<tr>
<td>ISCOMATRIX</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.3: Classes of clinically tested vaccine adjuvants**
1.10.4.1 **Emulsions**

Emulsions consist of a two-phase system that requires a surfactant for stabilisation. Two different types of emulsions have been developed: water-in-oil (W/O) and oil-in-water (O/W).

W/O emulsions consist of water droplets dispersed in a continuous oil phase. Incomplete Freund’s adjuvant (IFA) was the first emulsion of this kind developed, consisting of a paraffin oil based emulsion whose potency can be increased by the addition of heat-killed *Mycobacteria* (Complete Freund’s adjuvant (CFA)). Unfortunately, despite being highly effective, W/O emulsions were discontinued in humans due to their reactogenic nature, including formation of granulomas and lesions both at the site of injection and systemically (410, 411).

O/W emulsions comprising a continuous water phase containing oil droplets have an improved safety profile and have been successfully incorporated into licensed influenza vaccines. The most successful and well-known emulsion MF59; a nanoemulsion (<250 nm) originally licensed in 1997 has been effective at enhancing the response of older and at-risk people to influenza vaccines (412-414) (Table 1.2 and 1.4). It became the first new adjuvant for human use after alum, characterised by induction of high antigen-specific antibody responses as well as enabling antigen dose sparing. Following intramuscular injection, the adjuvant drives local cell death causes release of extracellular ATP driving the immune response (415), augments antigen uptake and upregulates activation markers on DCs (MHCII, CD86, CD83 and CCR7) to activate CD4⁺ T cells (416) and secretion of proinflammatory cytokines and chemokines to promote migration of immune cells to the site of injection (recruited cells including monocytes and granulocytes) and draining lymph nodes (417). Furthermore, the antibody responses generated by MF59 are stronger and show a more balanced IgG1:IgG2a profile than those obtained using alum (418, 419). MF59 adjuvanted vaccines for Herpes simplex virus (HSV) (420), Human immunodeficiency virus (HIV) (421), HBV (422) and Cytomegalovirus (CMV) have been tested (423). Other O/W emulsion examples include AS03 (Section 1.8) used for influenza vaccines.
1.10.4.2 Immunostimulatory complexes

Immunostimulatory complexes (ISCOMs) are another promising lipid-based adjuvant formation first described in 1984 (424). Classical ISCOMs form stable 40 nm spherical and ring-like structures spontaneously when antigens are mixed with cholesterol, phospholipid and QS-21 saponin. Their stability is due to the strong affinity between saponin and cholesterol (425). The second type of ISCOM is called ISCOMATRIX, which does not incorporate antigen during the formulation process. This allows flexibility in terms of the incorporation of a number of different antigens (e.g. non-hydrophobic). However, some issues remain as only lipophilic antigens can be incorporated in ISCOMs and while ISCOMs (and ISCOMATRIX) reduce QS-21 dose, safety and toxicity are still a concern, including a higher risk for injection site pain and swelling (426-429).

The ISCOM represents a unique particulate adjuvant as it combines the advantages of using a particulate carrier system which facilitates enhanced uptake by DCs and activation of DCs with the presence of an in-built adjuvant (Quil A) (430). They also destabilise the endosomal membrane, inducing more efficient delivery of the antigen into the cytoplasm (431). ISCOMs were designed to combine aspects of a virus, such as its size and surface protein orientation with the powerful adjuvanticity of saponins. ISCOMs can simultaneously induce a strong and long-lasting antibody response in addition to a potent T cell response by both parenteral and mucosal routes. They specifically enhance MHC-I presentation and cytokine production leading to induction of a specific CD8+ cytotoxic T cell response (432, 433).

1.10.4.3 Liposomes and virosomes

Other particulate vaccine adjuvants include liposomes and virosomes, which have been used as delivery systems for antigens, nucleic acids and drugs (434). A key advantage of liposomes and virosomes is their versatility and plasticity, with the composition, charge, size, entrapment and location of antigens, immunomodulators and adjuvants all able to be manipulated and changed in order to influence the type of immune response induced (435, 436).

Liposomes are double or multi-layered biodegradable delivery vesicles (<100 nm to several microns in size), made up of phospholipids mimicking the natural phospholipid bilayer membrane of a cell. This allows the liposome to be taken up directly into the cell by endocytosis (437, 438). The versatility of the system allows the incorporation of antigens,
immunomodulators or targeting ligands to aid delivery into the phospholipid layer (435, 439). Numerous studies have shown that co-administration of antigen with cationic liposomes induces stronger antigen-specific immune responses as compared with neutral/anionic liposomes (440, 441). However, the mechanism behind their adjuvanticity is still unclear. For example, the cationic liposome CAF01 is formed by N, N-dimethyl-N, N’-dioctadecylammonium (DDA) mixed with the synthetic mycobacterial immunomodulator α,α’-trehalose 6,6’-dibeheneate (TDB) inserted into the lipid bilayers (442). The adjuvant activity of CAF01 is characterised by robust Th1 and Th17 responses, IgG1 and IgG2 antibody titres (440). It forms a depot at the site of injection (443) and activates the inflammasome via the Syk/Card pathway (444). When subcutaneously injected with pathogen derived antigens, CAF01 has been shown to promote specific long lasting T cell response against *Mycobacterium tuberculosis* (mTB) (445) as well as HIV (446), *Chlamydia trachomatis* (447), influenza (448), and malaria (442) (Table 1.4). As mentioned in section 1.9.3, The RTS,S malaria vaccine contains liposomes which incorporate the immunostimulatory molecules QS21 and MPL, other liposome based vaccines against influenza, hepatitis A, Tuberculosis and non-small lung cell cancer have been either approved for human use or currently in clinical trials (436).

Virosomes (otherwise known as virus-like particles) are nanoparticles designed to mimic the outer surface of a virus consisting of reconstituted viral envelope incorporating membrane lipids and viral glycoproteins. Virosomes are devoid of any genetic material (RNA or DNA) and hence do not possess any virulent properties (449, 450). Virosomes can be shaped to incorporate antigens and PAMPs (e.g. TLR agonists) and target them to specific cells. The virosome structure facilitates repetitive, high density display of epitopes and antigen presentation via both MHCI and MHCII, inducing both CD8+ and CD4+ T cell responses (451, 452). A number of approved virosomal vaccines are in use against influenza and hepatitis (453) (Table 1.4). In the case of the influenza vaccine, virosomes derived from influenza virus contain hemagglutinin (HA) protein (359).
<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Example</th>
<th>Proposed mode of action</th>
<th>Licensed for use in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biodegradable particles</td>
<td>PLGA and PLA</td>
<td>Dependent on shape, size, charge or modification.</td>
<td>PLGA is a licensed drug delivery system.</td>
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<tr>
<td></td>
<td></td>
<td>Can target DCs for delivery of antigen.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antigen depot for controlled antigen release.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Additional effects when combined with immunomodulators.</td>
<td></td>
</tr>
<tr>
<td>Mineral Salts</td>
<td>Alum</td>
<td>Improves humoral immune responses</td>
<td>Pertussis, Tetanus, Diphtheria <em>Haemophilus influenzae</em> type b Pneumococcus Hepatitis A and B Papilloma virus</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antigen stability and depot.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antigens are adsorbed to the surface</td>
<td></td>
</tr>
<tr>
<td>Emulsions</td>
<td>IFA, ASO3 and MF59</td>
<td>Improves humoral and Th2 immune response</td>
<td>Influenza flu vaccine in EU</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antigen depot</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Increase antigen uptake and APC maturation</td>
<td></td>
</tr>
<tr>
<td>ISCOMS</td>
<td>ISCOMs and ISCOMATRIX</td>
<td>Phagocyted to activate DCs leading to both humoral and T cell responses</td>
<td>Phase II clinical trials against influenza, HPV and HCV</td>
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<tr>
<td>Liposomes</td>
<td>CAF01 (Cationic Liposome)</td>
<td>Antigen can be incorporated onto the surface to facilitate targeting and uptake by APCs</td>
<td>Used as components of virus like particles (VLPs), phase I clinical trials Phase I TB vaccination trials</td>
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<td></td>
<td></td>
<td>Recruitment of monocytes and depot at injection site</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Immunomodulators incorporated enhance immunogenicity</td>
<td></td>
</tr>
<tr>
<td>Virosomes</td>
<td>Virosomes and virus-like particles</td>
<td>Enhanced antibody, Th1/Th2 and memory response</td>
<td>Influenza (Inflexal V) Hepatitis A virus (Epaxal)</td>
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</tbody>
</table>

Table 1.4: Particulate vaccine adjuvants
1.10.5 Aluminium adjuvants

The first adjuvants used in human vaccines were aluminium salts and these remain the most widely used adjuvants in humans (454). Aluminium salts exist in various forms, including aluminium potassium phosphate and aluminium hydroxide (Alhydrogel/alum) being used in licensed vaccine preparations (455, 456), each have a distinct physical and chemical composition. Currently, aluminium salts have been incorporated into numerous licensed vaccine formulations for Human Papillomavirus, Hepatitis A and B virus, DTaP (Diphtheria–Tetanus–Pertussis combination), Polio, *Haemophilus influenzae* B virus (Hib) and Pneumococcus (430, 457). Any potential new adjuvants have major hurdles to overcome due to alums ease of use, safety record and low cost.

Nevertheless, despite its extensive and continuous use, its mechanisms of action have only recently been revealed but remain incompletely understood (458, 459). It is known that aluminum-based adjuvants are potent drivers of humoral immunity and elicit a strong and persistent Th2 -biased response but fail to induce strong CMI particularly Th1, Th17 and cytotoxic T cells. This is characterised by the production of interleukin IL-4, IL-5, IL-13 and the induction of IgE and IgG1 (454, 459-461). Therefore, while the adjuvant is effective against diseases where neutralising antibodies are required for protection it is less effective at promoting cell mediated immunity.

Originally, it was proposed that alum acted as a “depot” allowing for slow sustained release of the antigen into the body (454, 462) (Fig 1.12b). However, recent data has indicated that the depot is not required for adjuvanticity (463). For instance, fibrinogen-deficient mice are unable to form a depot yet retain normal CD4+/CD8+ T cell and IgG1 titres levels compared to wild type mice when immunised with alum (464). Similarly, early surgical removal of the site of injection does not significantly impact the ability of aluminium salts to induce antibody responses (463).

It is currently thought that effective targeting of APCs is required for adjuvant effects. Depleting CD11c+ DCs abrogated the immune response associated with alum, as lower OVA specific IgG1 and IgE levels were detected (465). This can facilitate efficient antigen uptake and processing by DCs, with localised cytokine release and upregulation of costimulatory molecules promoting T-cell activation (389). It has also been suggested that the cytotoxicity of
alum is responsible for cell recruitment. Alum can be endocytosed by phagocytic cells to cause necrosis and subsequent release endogenous danger signals (DAMPs), including IL-33, IL-1α and HMGB-1 (466). Other alarmins released including uric acid (465) and host DNA (467) can directly activate the inflammasome and IRF3 pathways and subsequent secretion of IL-1β and IL-18 (468, 469) (Fig 1.10 and 1.11). Additionally, host DNA can activate IRF3 independent pathways, activating Tfh cells and class switching to IgE and IgG1 in B cells (470). However, controversy still surrounds how significant a role the NLRP3 inflammasome plays in the adjuvanticity of alum (471, 472). Some studies have reported that NLRP3 is crucial for the immunostimulatory and humoral responses (469, 472) while more recent reports indicate that alum adjuvanticity remains unaffected when comparing WT and NLRP3−/− mice (473).

Finally, it has been demonstrated that alum is not actually recognised by a specific receptor but rather directly interacts with plasma membrane lipids (e.g. cholesterol), triggering lipid membrane sorting. The aggregation of lipid rafts triggers receptor independent recruitment and activation of Syk and PI3K, leading to PGE2, IL-1β and IL-18 secretion (280, 474) (Fig 1.11). Alum, as well as other sterile particulates, such as uric acid crystals can also activate Syk in DCs to release IL-2, needed for optimal humoral and antigen-specific CD4+ T cell responses (475). The PI3K signalling cascade has also been linked to poor induction of cell mediated immunity, as activation of this signalling pathway can inhibit IL-12p70 in DCs (476). This receptor independent activation mechanism is also seen using other particulates such as MSU (477-479) (Fig 1.11).
Figure 1.11: Signalling cascade activated by alum

Alum can bind lipids (e.g. cholesterol) on the surface of APCs to trigger signalling cascades and initiation of an immune response (1). This activates Syk, PI3K and p38 (2) leading to NLRP3 independent PGE$_2$ release and inhibition of IL-12 (3 and 4). Simultaneously, alum can be internalised by the cell (5), lysosomal damage (6) is induced causing the release of enzymes like cathepsin B (7) into the cytoplasm leading to activation of the NLRP3 inflammasome for secretion of IL-1β (8). Alum can also cause cell death leading to the release of uric acid crystals and dsDNA which can also activate the NLRP3 inflammasome and IRF3/NF-κB transcription factor respectively to activate inflammatory DCs (9 and 10), enhanced proinflammatory cytokines and Th2 responses. Taken from (458).
1.1 Biodegradable PLGA particles

A variety of biodegradable polymers, including poly (lactide-co-glycolide) (PLG), polymethylmethacrylate (PMMA) and poly (lactide) (PLA) have been formulated to act as an adjuvant or used as a delivery system in vaccine studies. All have excellent biodegradability and biocompatibility profiles making them safe and well tolerated for use in humans. However, poly(lactic-co-glycolic acid) (PLGA) particles remain the most popular and extensively studied to try and improve the potency and safety of subunit vaccines (proteins, peptides, lipopeptides, viruses or plasmid DNA). The adjuvanticity of PLGA microparticles was first demonstrated over 20 years ago (480-482) (Fig 1.13b).

PLGA particles are prime candidates for future vaccine adjuvant development. The major advantage for PLGA (and PLA) is that the polymer is Food and Drugs Administration (FDA)-approved for use in humans (e.g. bone implants, sutures and drug delivery) (483, 484). PLGA is a polymer ester of two hydroxyl acids, lactic and glycolic acids at varying proportions. PLGA copolymers can be prepared at different ratios allowing their half-life to change from days to years depending on their composition (e.g. PLGA 50:50 identifies a copolymer consisted of 50% lactic acid and 50% glycolic acid). PLGA is a well-tolerated polymer that degrades into lactic acid and glycolic acid through hydrolysis of its ester linkages, which are normal metabolites in the body (485) (Fig. 1.13c). Glycolic acid and lactic acid enter the tricarboxylic acid cycle (TCA) and are metabolised and subsequently eliminated from the body as carbon dioxide and water (486). Although not currently licensed for use as adjuvants, the processing and manufacturing facilities are already in place for biodegradable micro-particles to make this transition (487).

PLGA (and PLA) have remained a popular choice of adjuvant as they can be easily formed with different particle sizes, surface chemistry, release characteristics and facilitate the incorporation of immune stimulating agents such as TLR agonists or rapamycin (488) (Fig 1.13A). Antigens can be either encapsulated within the particles or absorbed (or conjugated) on the surface, with PLGA acting as a carrier for controlled delivery to the target site as well as providing protection for the antigen (489) (Fig 1.12a). This facilitates efficient targeting and uptake of antigen by APCs to stimulate potent T cell responses. For instance, targeted delivery of antigen to DCs using PLGA has been a very attractive approach in attempting to enhance specific T cell responses and increase antibody titres compared to antigen/adjuvant alone (490).
A major advantage of the uptake of antigen loaded PLGA particles is that it results in MHCI cross presentation or MHCII presentation (405, 491, 492) (Fig 1.12d). Additionally, if co-delivered with TLR agonists it can improve processing, presentation and priming of CD4+ and CD8+ T cells by DCs (493, 494). For instance, PLGA microparticles have been used to carry antigen derived from various pathogens including *Plasmodium vivax* with mono-phosphoryl lipid A as adjuvant in a candidate malaria vaccine (495). Recently, PLG microparticles combined with the a immunostimulatory agent (defensin peptide) and the HIV antigen gp41 elicited a strong long lasting antibody response when administered intranasally in mice (496). PLGA microparticles encapsulated or absorbed with CpG (and attenuated Anthrax) or MF59 (and gp120 from HIV-1) allow for persistent exposure and delivery of antigen and adjuvant to immune cells leading to improved immunogenicity and protection if challenged with lethal dose of antigen (Anthrax) (497, 498). Finally, PLGA particles has been used for the passive delivery of drugs to treat various cancers (499).

While mainly used as a delivery system, PLGA particles have displayed intrinsic adjuvant properties capable of modulating immunity (487, 500). PLGA particles have exhibited strong adjuvant properties; they can act as a depot, slowly releasing the antigen and enhance antigen presentation as shown by increased *in vitro* and *in vivo* CD4+ T-cell proliferation (501) (Fig 1.12b). Mucosal vaccination studies have also demonstrated that PLGA particles can aid uptake by M-cells in the MALT and protect the antigen from acidic pH condition of the stomach and intestine (502).

A study comparing alum and PLGA microparticles (1-15 µm) loaded with tetanus toxoid injected s.c in mice, showed enhanced immune responses (measured by anti-TT IgG titres) compared to those generated by alum (503). PLG particles 1-80 µm in size, encapsulating weakly immunogenic malarial peptides can elicit strong and sustained proliferative T cell responses as well as antigen-specific antibody responses when compared with incomplete Freund's adjuvant (IFA) (504). Finally, mice immunised (s.c) with 250 nm PLGA nanoparticles elicited prolonged IgG1 antibody titres compared to liposomes (200 nm) and alum. Again, immunisation with PLGA correlated to the highest number of activated IFN-γ-expressing CD8+ tetramer-specific T cells and higher frequency of effector-like memory T cells, leading to an effective clearance of intracellular bacteria (505).

Interestingly, recent developments in adjuvant design has led to PLGA nanoparticles being modified with other particulate adjuvants such as chitosan, to improve efficacy (506). One
group has designed nanoparticles consisting of a 1,3-β-glucan functionalised chitosan shell, and poly(lactide)co-glycolide core which can stimulate reactive oxygen and nitrogen species production, pro-inflammatory cytokine secretion (IL-12p70, TNF-α and IFN-γ), and enhanced delivery inside human alveolar-like macrophages (507). Another example is combining PLA particles with cationic polymers to enhance cell mediated immunity against HBsAg (508).

Finally, particulate adjuvants have been used to induce durable and specific tolerance against a specific antigen. This is especially useful for treating inflammatory disease such as Rheumatoid arthritis. PLGA particles have been combined with rapamycin to effectively treat EAE in mice (488).
Figure 1.12: Mechanisms by which PLGA and other particles alter the immune response

A) Particle size influences how antigens are delivered. Nanoparticles can directly penetrate tissue and directly access draining lymph nodes.

B) Depot effect, which allows for a slow sustained release of antigen while keeping the antigen stable and maintains conformational integrity.

C) TLR agonists (e.g. CpG) displayed on PLGA particles can activate PRRs influencing cytokine profile that modulate the immune response (e.g. Th1, Th2 or Treg).

D) Exogenous uptake of antigen allows for cross presentation onto MHCI and activation of a CTL response.

Taken from (435).
Figure 1.13: PLGA particles can be modified by changing size and surface chemistry

A) PLGA can be highly modified with various functional groups. Vaccine antigens or other adjuvants can be attached through adsorption (hydrophobic interaction), conjugation (chemically cross-linked to the surface of a nanoparticle) or encapsulation (antigen released when particle has degraded in vivo or taken up by cells) (509). Modification on the surface can be used to target certain cell types (e.g. CD11c for DCs), moieties (Mannose glycoproteins bind to mannose receptors on APCs). Danger signals, peptides, antibodies and small molecules can be incorporated onto the surface to modulate the immune response in vivo.

B) Scanning electron microscopy (SEM) of 250 nm PLGA nanoparticles.

C) PLGA co-polymers are aliphatic polyesters composed of varying proportions of lactic and glycolic acids. Upon encountering aqueous media, PLGA is hydrolysed into lactic and glycolic acid monomers, which are normal tolerated metabolites in the human body.
1.12 Particle size: from nano to micron scale

The charge, composition, shape, size, and route of delivery of various particles influence efficiency of particle uptake and the type of immune response induced (509, 510). Although many particulate adjuvants have been compared based on their composition, comparing adjuvants based on size is an area that has been somewhat neglected, especially considering that the majority of adjuvants and vaccine studies use particles in the one micron range (300 nm-10 μm) (511). For example, Alhydrogel and AdjuPhos aggregates range between 0.5-10 μm (512). PLGA and other particulates have mainly been used within this range as well (513, 514). Furthermore, particulates of around 1 μm in diameter have been suggested to be the optimal for phagocytosis and the stimulation of immune responses (515). There is a lack of consensus within the vaccine community regarding the best size for polymeric particles for triggering a particular response (516). This could be attributed to several factors, including LPS contamination of the formulations. Furthermore, very few studies have compared particle size across a broad nano- and micro-scale and how they can induce different innate and adaptive immune responses. Of those studies that have examined a broader spectrum of sizes, there has been inconsistency regarding particle composition, antigen and route of administration.

It has been generally assumed that nanoparticles are better than microparticles for targeted drug delivery, due to their better biodistribution and ability to cross biological tissue and barriers (517, 518). It remains unclear whether nano or and micro-scale particles are more efficient at inducing a Th1 or Th2 adaptive immune response. It has been argued that different sized particles can mimic the size of pathogens, including bacteria, viruses, fungi and helminths to generate appropriate immune responses (Fig. 1.14) (511). For example, small particles (20–200 nm) are taken up via endocytosis by DCs and evoke virus-like responses and larger particles (<0.5 μm) are phagocyted by macrophages and lead to responses normally elicited by bacteria (519, 520).

There are advantages and disadvantages of using either nano or micron sized particles. For example, the uptake efficiency of larger particles by APCs is lower while nano sized particles have inferior loading efficiencies and may release their antigen before reaching any APCs. Although nanoparticles facilitate enhanced uptake and enhanced protein adsorption, micron sized particles enable prolonged antigen release (520, 521).
A study where ovalbumin (OVA) antigen was conjugated onto solid polystyrene beads of different size (i.e. 20, 40, 100 and 500 nm, and 1 and 2µm) demonstrated that 40 nm particles were optimal for inducing both antibody (IgG1) and cellular immune responses (IFN-γ) when intradermally administered to mice (522, 523). Mice vaccinated (i.p) with 300 nm sized PLG particles mixed with OVA produced the strongest CTL response and IgG2a titres favouring Th1-type immune responses compared to particles greater than 300 nm in size (1,7 and 17µm) (524). Another study demonstrated that subcutaneous administration of 230 nm OVA-loaded lecithin/glyceryl monostearate based nanoparticles were superior to 708 nm particles, in terms of antibody titres, OVA-specific cytotoxic T lymphocyte activity, inhibition of tumour growth and internalisation by various APCs (525). Similarly, another study showed that 200–600 nm nanoparticles encapsulated with hepatitis B surface antigen (HBsAg) injected intra muscularly produced the strongest IFN-γ cell mediated response, upregulation of MHCI and IgG2a titres. On the other hand, immunisation (i.m) with 2-8 µm microparticles generated the highest IL-4 and MHCII expression which are crucial for a Th2 response. However, the same study showed that larger 2–8 µm sized particles produced higher antibody titres than 200 or 400 nm particles (526). In contrast, vaccination with tetanus toxoid (TT) adsorbed onto PLGA particles showed that particles of 100 and 500 nm induced significantly higher antibody titres than larger particles (>1000 nm) after oral or intranasal (i.n) administration (527).

Liposome adjuvanticity has also been shown to be influenced by size, affecting vesicle trafficking to lymph nodes, antigen uptake, processing by APCs and differential T-cell activation (435, 528) with an increase in IgG2a titres and shift in Th1/Th2 bias for larger-sized vesicles (comparing 100 nm to 300 nm liposomes) (520). Furthermore, hemozoin particles measuring between 50 nm and 200 nm exhibited a stronger adjuvant effect compared with larger (2–20 µm) and smaller (<50 nm) particles (515).

The optimum size range for vaccination is likely to depend on the application but it has been proposed that particles measuring between 200 nm and 1 µm are optimal for phagocytosis and stimulation of the immune response (515, 529). However, particles within this size range have also been shown to promote regulatory cells in vitro. OVA-encapsulated in PLGA NP (400 nm) enhanced the induction of FoxP3+ in activated T-cells via a TGF-β and retinoic acid dependent mechanism by enhancing retinaldehyde dehydrogenase enzyme (RALDH) expression in lymph node-derived DCs (530).
The unclear findings and contradictions surrounding the effects of particle size on the immune response can be attributed to the absence of a full mechanistic understanding and lack of consistency in particle preparation. Hence, there is a need to fully elucidate the adjuvanticity of both nano- and micro-particles and to determine the mechanisms by which particles can generate distinct immune responses based on their size and composition.

**Figure 1.14: Particulate adjuvants have been engineered to mimic the size of different pathogens**

When designing vaccines, particle size can be used to mimic microbes such as bacteria (> 1000 nm), viruses (<1000 nm), fungi and helminths (>10 μm) which could promote tailored immune responses against a particular pathogen but without the associated risks (531). Particles resembling the dimensions of viruses are processed like viruses and induce a strong CD8+ T-cell response, whereas microparticles, being closer to the size of bacteria, induce a stronger humoral response. Size also determines how efficient uptake is by APCs, draining to lymphatic vessels and sustainability of antigen release.
1.13 Aims and Objectives

Previous results using PS particles show that size plays an important role in determining the type of innate and adaptive immune response that is generated following vaccination in mice. Therefore, vaccine formulations could be tailored to a size that best facilitates the induction of cellular immunity. The balance between the Th1 polarising cytokine IL-12 and the immunosuppressive IL-10 can be decisive in the outcome of immune responses. Previous reports from our lab showed that alum can inhibit IL-12 so addressing particulate adjuvant modulation of the IL-10/IL-12 balance is essential for adjuvant optimisation. PLGA particles are an attractive alternative as the size of the particles can be changed (from nano to micron scale) as well as already being licensed for use in humans. The mechanism by which particulate adjuvants can enhance IL-10 and inhibit IL-12 must also be addressed. A better understanding of both the adjuvanticity and mechanisms employed by particles and how size influences this response is critical for the rational design of new and approved vaccines. Thus, it is hypothesised that the size of particulate adjuvants can influence the IL-10/IL-12 balance, a key determinant for inducing effective cell mediated immunity during vaccination.

Therefore, this project aims to achieve the following objectives:

- To determine the role of particle size in PLGA particles induced modulation of IL-10, and IL-12p70 in dendritic cells, macrophages and in vivo.

- To examine the ability of different sized particles (100 nm, 500 nm, 2 µm, 10 µm and 30 µm) to modulate CD4+ T cell responses in DC-T cell co-cultures.

- To address whether adjuvant induced IL-10 compromises antigen-specific cellular immunity (IFN-γ and CD8+ T cell response).

- To examine the mechanism and pathways involved in regulating both the IL-10 and IL-12p70 responses to PLGA particles and determine whether IL-10 induction is a common mechanism employed by DCs under physical stresses (e.g. osmotic, oxidative and actin disruption).
Chapter 2

Materials and Methods
2.1 Materials
All materials are from Sigma-Aldrich unless otherwise stated.

2.1.1 General cell culture materials

**Complete RPMI 1640 medium**
Roswell Park Memorial Institute (RPMI) 1640 medium (Biosera) was supplemented with 2 millimolar (mM) L-Glutamine (Gibco), 50 units/ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco) and 8% (v/v) heat-inactivated (56 °C for 30 min) and filter sterilised foetal calf serum (FCS) (Biosera).

**Complete RPMI 1640 T cell Medium**
RPMI 1640 medium was supplemented with 0.04mM β-mercaptoethanol (Gibco), 0.88mM sodium pyruvate (Gibco), 0.88 mM L-Glutamine (Gibco), 4.4 units/ml penicillin (Gibco), 4.4 μg/ml streptomycin (Gibco), 0.88% (v/v) 100x MEM Non-Essential Amino Acids (Gibco), 0.35% (v/v) 100X MEM Vitamins (Gibco) and 10% (v/v) heat-inactivated (56 °C for 30 min) and filter sterilised FCS (Biosera).

**Complete DMEM culture medium (cDMEM)**
Dulbecco’s Modified Eagle Medium (DMEM) (Biosera) was supplemented with 2 mM L-Glutamine, 50 units/ml penicillin, 50 μg/ml streptomycin and 8% (v/v) heat-inactivated (56 °C for 30 min) and filter sterilised FCS.

**0.88% ammonium chloride (NH₄Cl) red blood cell lysis solution**
8.8 g ammonium chloride was dissolved in 1 litre (L) of endotoxin-free water (H₂O) (Baxter) and filter sterilised with a 0.22 μm syringe-driven filter (Millipore).

**MACS buffer**
500 ml PBS, 2mM EDTA and 0.5% fetal bovine serum
### 2.1.2 Cell culture treatments

**Table 2.1: TLR and C-type lectin agonists and *ex vivo* restimulation assay reagents**

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<th>TLR ligand or immunomodulator</th>
<th>Target/function</th>
<th>Supplier</th>
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<tbody>
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<td>Enzo Life Sciences</td>
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<tr>
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<tr>
<td>Jasplakinolide</td>
<td>F-actin</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>WGP Soluble</td>
<td>Dectin-1</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Ruxolitinib</td>
<td>JAK1/2</td>
<td>Invivogen</td>
</tr>
<tr>
<td>Rho Kinase inhibitor</td>
<td>Rho-associated kinase (ROCK)</td>
<td>Merck</td>
</tr>
<tr>
<td>Benzyl Alcohol</td>
<td>Local Anaesthetic</td>
<td>Sigma</td>
</tr>
<tr>
<td>2% w/v lidocaine Hydrochloride</td>
<td>Local Anaesthetic</td>
<td>Braun</td>
</tr>
<tr>
<td>YVAD</td>
<td>Caspase-1 inhibitor</td>
<td>Invivogen</td>
</tr>
</tbody>
</table>
2.1.3 **Real-time PCR materials**

**RNA isolation**

High Pure RNA Isolation Kit (Roche, IN 46250-0414, USA) was used per manufacturer’s instructions.

<p>| Table 2.3. Reverse Transcription reagents |</p>
<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>dNTPs</td>
<td>Promega</td>
<td>2.5mM/NTP</td>
</tr>
<tr>
<td>Reverse Transcriptase buffer</td>
<td>Promega</td>
<td>-</td>
</tr>
<tr>
<td>Random primers 5′-NNNNNNN-3′/Random Hexamers</td>
<td>MWG Biotech</td>
<td>1 µg/µl</td>
</tr>
<tr>
<td>Ribonuclease Inhibitor (RNaseOUT)</td>
<td>Invitrogen</td>
<td>40 U/µl</td>
</tr>
<tr>
<td>M-MLV Reverse Transcriptase</td>
<td>Promega</td>
<td>200 U/µl</td>
</tr>
</tbody>
</table>

**Real-time PCR reagents**

Kapa SYBR® Fast qPCR Kit (Kapa Biosystems) using the ROX low reference dye was used in combination with nuclease-free H2O.

Primers (MWG Biotech) were directed against mouse genes and designed to be intron-spanning to avoid amplification of genomic DNA. Dissociation curve analysis was performed after each real-time qPCR to exclude non-specific products.

**Table 2.4 Real-time primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>5′-AGCGCCTGTCATCGATTTC-3′</td>
<td>5′-GACACCTTGTTGGAGCTTAT-3′</td>
</tr>
<tr>
<td>IL-12p35</td>
<td>5′-ATCACACGGGAACAAACCA-3′</td>
<td>5′-ATCACACGGGAACAAACCA-3′</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>5′-GTGTAACCAGAAAGGTGTTT-3′</td>
<td>5′-TCGGACCCTGCAGGGACAC-3′</td>
</tr>
<tr>
<td>β-actin</td>
<td>5′-CTGGGCTCTTTCTTTGGGT-3′</td>
<td>5′-GCACGTCAGGGTCAGGTC-3′</td>
</tr>
<tr>
<td>IL-6</td>
<td>5′-GGTTCTCTGGGAATTCAGTGAA-3′</td>
<td>5′-AAGTGACTCATCGTTTCATACA-3′</td>
</tr>
<tr>
<td>TNFa</td>
<td>5′-CATCTTCTCAAAATTCAGTGACAA-3′</td>
<td>5′-CCTCCACTTGGTGTTTCTG-3′</td>
</tr>
</tbody>
</table>
2.1.4 Particulate adjuvants

Spherical polystyrene and PLGA micro- and nano-particles were sourced from Phosphorex, Inc (Ma 01748, USA).

Alhydrogel (alum) was sourced from Brenntag Biosector (Frederikssund, Denmark).

Fluorescent labelled PLGA particles were sourced from Phosphorex, Inc (Ma 01748, USA).

2.1.5 Western blot materials

**Table 2.5 – Buffers and Solutions**

<table>
<thead>
<tr>
<th>Buffer Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x SDS Running buffer</td>
<td>144 g Glycine, 30 g Tris Base and 10 g SDS. Made up to a final volume of 1 L with dH2O</td>
</tr>
<tr>
<td>1x Transfer Buffer</td>
<td>2.25 g Tris Base, 10.5 g Glycine, 1 g SDS and 200 ml Methanol. Made up to a final volume of 1 L with dH2O</td>
</tr>
<tr>
<td>Cell lysis buffer (100 ml)</td>
<td>Ripa Buffer- 0.87g NaCl, 5 ml 1M Tris pH 7.5, 1 ml of 10% SDS, 1 ml NP40 and 0.5g sodium deoxycholate</td>
</tr>
<tr>
<td>10x TBST (Tris-buffered saline containing Tween-20)</td>
<td>80 g NaCl, 2 g KCL, 30 g Tris Base and 10 ml Tween-20. Made up to a final volume of 1 L with dH2O and brought to pH 7.4</td>
</tr>
<tr>
<td>Blocking Buffer</td>
<td>1x TBST with 5% Bovine Serum Albumin (BSA) or 5% non-fat dry milk</td>
</tr>
<tr>
<td>Wash Buffer</td>
<td>1x TBST</td>
</tr>
<tr>
<td>Sample Buffer</td>
<td>Laemmli 2x concentrate (Sigma)</td>
</tr>
<tr>
<td>Stripping Buffer (100 ml)</td>
<td>20 ml 10% SDS, 12.5 ml Tris Base pH 6.8 0.5 M, 67.5 ml dH2O and 800 µl β-mercaptopethanol</td>
</tr>
</tbody>
</table>

Phosphatase inhibitor cocktail 3 (Sigma) and Protease inhibitor cocktail (Sigma) were added to RIPA buffer before lysis of cells.
Table 2.6 – Composition of resolving and stacking gels.

<table>
<thead>
<tr>
<th></th>
<th>Volume 10% Resolving Gel</th>
<th>Volume 6% Stacking Gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td>3.15 ml</td>
<td>2.65 ml</td>
</tr>
<tr>
<td>30% Acrylamide/ Bis-</td>
<td>2.665 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>acrylamide solution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5M Tris Base pH 8.8</td>
<td>2 ml</td>
<td>-</td>
</tr>
<tr>
<td>0.5M Tris Base pH 6.8</td>
<td>-</td>
<td>1.25 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>80 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>10% Ammonium persulfate (APS)</td>
<td>80 µl</td>
<td>50 µl</td>
</tr>
<tr>
<td>Tetramethylethylenediamine (TEMED)</td>
<td>8 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

Page ruler prestained protein ladder (Thermo scientific) ladder was used.
Table 2.7 – Antibodies for western blot analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
<th>Dilution</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-mouse Phospho-Syk (Tyr352) mAb</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% BSA in TBST</td>
</tr>
<tr>
<td>Total Syk Rabbit anti-mouse mAb</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% non-fat dry milk in TBST</td>
</tr>
<tr>
<td>Goat Anti-rabbit IgG, HRP-Linked Antibody</td>
<td>Cell Signalling</td>
<td>1/5000</td>
<td>5% BSA in TBST for Phospho proteins</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>5% non-fat dry milk in TBST for total proteins</td>
</tr>
<tr>
<td>Rabbit anti-mouse Phospho-p38 MAPK(Thr180/Tyr182) (28B10) mAb</td>
<td>Cell Signalling</td>
<td>1/2000</td>
<td>5% BSA in TBST</td>
</tr>
<tr>
<td>Total p38 MAPK Rabbit anti-mouse mAb</td>
<td>Cell Signalling</td>
<td>1/2000</td>
<td>5% non-fat dry milk in TBST</td>
</tr>
<tr>
<td>Rabbit anti-mouse Phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) mAb</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% BSA in TBST</td>
</tr>
<tr>
<td>Total p44/42 MAPK (ERK1/2) mAb</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% non-fat dry milk in TBST</td>
</tr>
<tr>
<td>Rabbit anti-mouse Phospho-Akt (Ser473)</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% BSA in TBST</td>
</tr>
<tr>
<td>Total Akt Rabbit anti-mouse mAb</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% non-fat dry milk in TBST</td>
</tr>
<tr>
<td>Rabbit anti-mouse Phospho CREB (Ser133) mAb</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% BSA in TBST</td>
</tr>
<tr>
<td>Total CREB (48H2) Rabbit anti-mouse mAb</td>
<td>Cell Signalling</td>
<td>1/1000</td>
<td>5% non-fat dry milk in TBST</td>
</tr>
</tbody>
</table>

Immobilon PVDF 0.2 µm Transfer Membrane was used to transfer proteins in semi-dry conditions and protein was detected using Immobilon Western Chemiluminescent HRP substrate (Merck Millipore) per the manufacturer’s protocol.
2.1.6 Enzyme-linked immunosorbent assay (ELISA) materials

ELISA antibodies
Cytokine (Table 2.9) antibodies were used at various concentrations depending on individual kits. Reagent diluents varied depending on individual ELISA kits (Table 2.10). Horseradish-peroxidase (HRP)-conjugated streptavidin concentrations are outlined in Table 2.11.

ELISA substrates
Two different substrates were used. A 20mg o-Phenylenediamine dihydrochloride (OPD) tablet was dissolved in 50 ml of phosphate citrate buffer containing 14μl H$_2$O$_2$. OPD was used for all ELISAs, except for IL-12p70 which was developed with Tetramethyl benzidine (TMB) (Millipore).

Table 2.8 – Buffers and Solutions

<table>
<thead>
<tr>
<th>Buffer Solution</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x PBS</td>
<td>400 g NaCl, 58 g Na$_2$HPO$_4$, 10 g KH$_2$PO$_4$ and 10 g KCl</td>
</tr>
<tr>
<td></td>
<td>Made up to a final volume of 5 L with dH$_2$O and brought to pH 7.2.</td>
</tr>
<tr>
<td>1% Bovine Serum Albumin (BSA)</td>
<td>10 g BSA, 1 L dH$_2$O</td>
</tr>
<tr>
<td>Sodium Carbonate buffer</td>
<td>8.4g NaHCO$_3$ and 3.56 g Na$_2$CO$_3$.</td>
</tr>
<tr>
<td></td>
<td>Made up to a final volume of 5 L with dH$_2$O and pH 9.5</td>
</tr>
<tr>
<td>Phosphate citrate buffer</td>
<td>10.19 g C$_6$H$_8$O$_7$ and 14.6 g Na$_2$HPO$_4$</td>
</tr>
<tr>
<td></td>
<td>Made up to a final volume of 1 L with dH$_2$O and pH 5.0.</td>
</tr>
<tr>
<td>0.05% PBS-Tween (Wash buffer)</td>
<td>8995 ml dH$_2$O, 1000 ml 10x PBS and 5 ml Tween 20.</td>
</tr>
<tr>
<td>Stop solution</td>
<td>1 M H$_2$SO$_4$</td>
</tr>
<tr>
<td>IL-1β and IFN-γ Reagent Diluent</td>
<td>0.1% BSA, 0.05% Tween 20 in Tris buffered Saline (20 mM Trizma base and</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl) and brought to pH 7.2</td>
</tr>
</tbody>
</table>
Table 2.9 – Antibodies used to measure cytokine concentrations by ELISA.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
<th>Capture Ab concentrations (in PBS)</th>
<th>Blocking solution</th>
<th>Top working Standard</th>
<th>Detection Ab concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10</td>
<td>Biolegend</td>
<td>1/200</td>
<td>1% BSA</td>
<td>2000 pg/ml</td>
<td>1/200</td>
</tr>
<tr>
<td>IL-10 (Human)</td>
<td>R&amp;D Systems</td>
<td>1/120</td>
<td>1% BSA</td>
<td>2000 pg/ml</td>
<td>1/60</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>R&amp;D Systems</td>
<td>4 µg/ml</td>
<td>1% BSA</td>
<td>2500 pg/ml</td>
<td>0.4 µg/ml</td>
</tr>
<tr>
<td>IL-1β</td>
<td>R&amp;D Systems</td>
<td>4 µg/ml</td>
<td>1% BSA</td>
<td>1000 pg/ml</td>
<td>1.5 µg/ml</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Biolegend</td>
<td>1/200</td>
<td>1% BSA</td>
<td>1000 pg/ml</td>
<td>1/200</td>
</tr>
<tr>
<td>TNF- α</td>
<td>R&amp;D Systems</td>
<td>0.8 µg/ml</td>
<td>1% BSA</td>
<td>2000 pg/ml</td>
<td>0.05 µg/ml</td>
</tr>
<tr>
<td>IL-17</td>
<td>Biolegend</td>
<td>1/200 (in Sodium Carbonate buffer)</td>
<td>1% BSA</td>
<td>1000 pg/ml</td>
<td>1/200</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>R&amp;D Systems</td>
<td>4 µg/ml</td>
<td>1% BSA</td>
<td>2000 pg/ml</td>
<td>0.6 µg/ml</td>
</tr>
<tr>
<td>IL-4</td>
<td>BD Pharmingen</td>
<td>4 µg/ml</td>
<td>10% Milk</td>
<td>2500 pg/ml</td>
<td>1 µg/ml</td>
</tr>
<tr>
<td>IL-6</td>
<td>Biolegend</td>
<td>1/200 (in Sodium Carbonate buffer)</td>
<td>1% BSA</td>
<td>2000 pg/ml</td>
<td>1/200</td>
</tr>
</tbody>
</table>

All capture antibodies were diluted in PBS, except for IL-17 and IL-6 which were diluted in Sodium Carbonate buffer.
Table 2.10 - Reagent diluents specified by individual suppliers of ELISA antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Diluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 (Mouse and human)</td>
<td>1% BSA</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>1% BSA</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Reagent diluent</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1% BSA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1% BSA</td>
</tr>
<tr>
<td>IL-17</td>
<td>1% BSA</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Reagent diluent</td>
</tr>
<tr>
<td>IL-4</td>
<td>PBS</td>
</tr>
<tr>
<td>IL-6</td>
<td>1% BSA</td>
</tr>
</tbody>
</table>

The reagent diluent recipe is listed in Table 2.8

Table 2.11-HRP-conjugated streptavidin concentrations

<table>
<thead>
<tr>
<th>Antibody</th>
<th>HRP-conjugated streptavidin concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 (Mouse)</td>
<td>1/1000</td>
</tr>
<tr>
<td>IL-10 (Human)</td>
<td>1/40</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>1/40</td>
</tr>
<tr>
<td>IL-1β</td>
<td>1/40</td>
</tr>
<tr>
<td>IL-1α</td>
<td>1/1000</td>
</tr>
<tr>
<td>TNF-α</td>
<td>1/40</td>
</tr>
<tr>
<td>IL-17</td>
<td>1/1000</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1/40</td>
</tr>
<tr>
<td>IL-4</td>
<td>1/750</td>
</tr>
<tr>
<td>IL-6</td>
<td>1/1000</td>
</tr>
</tbody>
</table>
### 2.1.7 Flow cytometry materials

#### Table 2.12 – Buffers and Solutions

<table>
<thead>
<tr>
<th>Buffer Solutions</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>FACS buffer</td>
<td>1x PBS was supplemented with 0.1% sodium azide and 2% heat-inactivated and filter sterilised FCS</td>
</tr>
<tr>
<td>4% Paraformaldehyde (PFA)</td>
<td>40 g PFA in 1L sodium phosphate buffer (13.4 g Na₂HPO₄, 6.9 g NaH₂PO₄, 1 L dH₂O). Heat for 30 to 45 min at 60 °C to dissolve PFA and adjust pH to 7</td>
</tr>
<tr>
<td>Fixation Buffer</td>
<td>2% PFA in PBS</td>
</tr>
<tr>
<td>Permeabilisation buffer</td>
<td>0.1% Saponin, 0.1% BSA, 1 mM CaCl₂ and 1 mM MgSO₄ in PBS</td>
</tr>
<tr>
<td>Annexin V binding buffer 10x</td>
<td>28.3 g HEPES, 87.7 g NaCl, 3.7 g KCl, 0.96 g MgCl₂ and 2.66 g CaCl₂ dissolved in 1 L dH₂O</td>
</tr>
</tbody>
</table>

LIVE/DEAD® Fixable Dead Cell Stain Kit (Invitrogen) was used to gate out dead cells in all experiments.

#### Fluorescent antibodies used to assess DC maturation by FACS

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11c</td>
<td>PE-Cy7</td>
<td>0.5 µg/ml (0.5 µg)</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>CD40</td>
<td>APC</td>
<td>0.15 µg/ml (0.15 µg)</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>CD80</td>
<td>FITC</td>
<td>0.2 µg/ml (0.2 µg)</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>CD86</td>
<td>PE</td>
<td>0.1 µg/ml (0.1 µg)</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>MHCcII</td>
<td>efluor 450</td>
<td>0.2 µg/ml (0.2 µg)</td>
<td>eBiosciences</td>
</tr>
<tr>
<td>CD16/CD32</td>
<td>N/A</td>
<td>2.5 µg/ml (0.25 µg)</td>
<td>BD Bioscience</td>
</tr>
</tbody>
</table>

#### Fluorescent antibodies used to assess cell death in BMDCs

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>Annexin V</td>
<td>FITC</td>
<td>1 µg/ml</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>PI</td>
<td>N/A</td>
<td>1 µg/ml</td>
<td>Sigma</td>
</tr>
</tbody>
</table>
## Fluorescent antibodies used to assess muscle cell infiltration

### Innate immune cell panel

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>PE-Cy7</td>
<td>0.1 µl/sample</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>MHCII</td>
<td>APC</td>
<td>0.5 µl/sample</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD45</td>
<td>APC-Cy7</td>
<td>0.5 µl/sample</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>CD11c</td>
<td>BV605</td>
<td>0.5 µl/sample</td>
<td>Biolegend</td>
</tr>
<tr>
<td>Gr-1</td>
<td>PE</td>
<td>0.2 µl/sample</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Ly6C</td>
<td>FITC</td>
<td>0.5 µl/sample</td>
<td>BD Biosciences</td>
</tr>
<tr>
<td>F4/80</td>
<td>A700</td>
<td>0.2 µl/sample</td>
<td>AbD Serotec</td>
</tr>
</tbody>
</table>

### Adaptive immune cells panel

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>PerCP-Cy5.5</td>
<td>0.5 µl/sample</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD4</td>
<td>APC</td>
<td>0.5 µl/sample</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD3</td>
<td>FITC</td>
<td>0.5 µl/sample</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD8</td>
<td>PE-Cy5</td>
<td>0.5 µl/sample</td>
<td>eBioscience</td>
</tr>
</tbody>
</table>

### Tetramer staining panel for splenocytes, lymph nodes and muscle cells

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>BV650</td>
<td>0.5 µl/sample</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD4</td>
<td>A780</td>
<td>1 µl/sample</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD8</td>
<td>PEA750</td>
<td>5 µl/sample</td>
<td>BioRad</td>
</tr>
<tr>
<td>CD44</td>
<td>FITC</td>
<td>5 µl/sample</td>
<td>BD Bioscience</td>
</tr>
<tr>
<td>B220</td>
<td>A700</td>
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<td>BD Bioscience</td>
</tr>
<tr>
<td>F4/80</td>
<td>A700</td>
<td>1 µl/sample</td>
<td>BioRad</td>
</tr>
<tr>
<td>CD11c</td>
<td>BV605</td>
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<td>Biolegend</td>
</tr>
<tr>
<td>CD16/CD32</td>
<td>N/A</td>
<td>2.5 µg/ml (0.25 µg)</td>
<td>BD Bioscience</td>
</tr>
</tbody>
</table>
### Description | Fluorochrome | MHC Allele | Sequence | Supplier
---|---|---|---|---
Ovalbumin | PE | H-2K (b) MHCcl | SIINFEKL | MBL

**Fluorescent antibodies used to assess DC-OVA transgenic (OTII) T cell co culture assay**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorochrome</th>
<th>Concentration</th>
<th>Supplier</th>
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</thead>
<tbody>
<tr>
<td>CD3</td>
<td>V450</td>
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<tr>
<td>CD4</td>
<td>APC efluor 780</td>
<td>0.2 µl/sample</td>
<td>eBioscience</td>
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<tr>
<td>IFN-γ</td>
<td>PE</td>
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<td>BD Pharamingen</td>
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<td>APC</td>
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<td>Invitrogen</td>
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<td>IL-17</td>
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<td>IL-4</td>
<td>PE-Cy7</td>
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<td>eBioscience</td>
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<tr>
<td>CD16/CD32</td>
<td>N/A</td>
<td>2.5 µg/ml (0.25 µg)</td>
<td>BD Bioscience</td>
</tr>
</tbody>
</table>

2.1.8 **Confocal microscopy materials**

100 nm, 500 nm and 10 µm Degradex fluorescent PLGA particles (green); excitation/emission: 460/500 nm

**Live cell imaging**

DNA stain: Hoechst 33342 (Thermofisher Scientific)

Vybrant Alexa fluor 594 Lipid raft labelling kit (Thermofisher Scientific).

**Fixed cell imaging**

DNA stain: DAPI (Sigma)

F-actin stain: Alexa fluor 647 Phalloidin (Thermofisher Scientific)

4% Paraformaldehyde (PFA)

Dako mounting medium
2.2 Methods

2.2.1 Mice
Female and Male C57BL/6 mice were obtained from Harlan Olac (Bicester, United Kingdom) and were used at 8-16 weeks old.

IL-10−/− mice were obtained from Jackson Laboratories (Maine, United States of America) and bred in the TCD Bioresources Unit.

OTII transgenic mice were purchased from Jackson Laboratories and bred in the TCD Bioresources Unit.

NLRP3−/− breeding pairs were provided by the late Prof. Jurg Tschopp (Department of Biochemistry, University of Lausanne, Switzerland) and bred in the TCD Bioresources Unit.

Cathepsin S−/− mice were obtained from Professor Christopher Scott, Queens University Belfast.

Animals were maintained according to the regulations of the Health Products Regulatory Authority (HPRA). Animal studies were approved by the TCD Animal Research Ethics Committee (Ethical Approval Number 091210) and were performed under the appropriate licence (Licence Number AE191364/P079).

2.2.2 Cell culture
Cells were cultured at 37 °C in an atmosphere maintained at 95% humidity and 5% CO₂

2.2.2.1 Cell viability and counting
Cell viability was determined using a trypan blue exclusion method. Samples were diluted by adding to 90 μl of trypan blue to 10 μl of the cell suspension. 10 μl of this solution was loaded onto a KOVA Glasstic cell counter slide with grids (Hycor Biomedical Inc.). Cells were viewed under a light microscope and cell viability was assessed by dye exclusion. The number of cells/ml was ascertained using the following formula:
number of cells/ml = cell number x 10^4 cells/ml x dilution factor

2.2.3 Culture of J558 granulocyte-macrophage colony-stimulating factor (GM-CSF)-expressing cell line

To obtain GM-CSF for growing BMDCs, the murine gene for GM-CSF was cloned into a mammalian expression vector (containing geneticin resistance gene) and transfected into the plasmacytoma line X63-AgS (J558 cells). Firstly, J558 cells (1x10^6 cells/ml) were grown in cRPMI supplemented the antibiotic Geneticin (1 mg/ml, Gibco) for 2 passages in T175 flasks (Grenier BioOne). Following the second passage, the J588 cells were washed with cRPMI and reseeded (1x10^6 cells/ml) in the absence of geneticin. The cells were seeded at a lower density (2.5x10^5 cells/ml) for all the passages that followed. Following each passage, supernatants were collected until passage 9, subsequently all the supernatants were pooled and the concentration of GM-CSF was quantified by ELISA. GM-CSF was then used to culture murine BMDCs.

2.2.4 Production of M-CSF conditioned medium from L929 cells

The murine gene encoding M-CSF was cloned into a mammalian expression vector and transfected into the murine aneuploid fibrosarcoma cell line L929. L929 cells were seeded at 0.5x10^6/ml, grown in cRPMI supplemented with ciprofloxacin (10 µg/ml) in T175 flasks (Grenier BioOne) and cultured for 7 days. Following this, the supernatants containing M-CSF were harvested and filter sterilised. The M-CSF can be frozen at -20º for up to 2 months or -80 ºC for longer storage. The cells were rinsed with 10 ml sterile Ca^2+/Mg^2+ free PBS, after which 10 ml of trypsin solution was added to the cell layer and incubated for 5 min at 37 ºC. Afterwards, the suspension was pipetted up and down using a 5 ml pipette until all the cells are detached. The cells were subsequently transferred to a sterile 50 ml falcon tube, centrifuged (400g, 5 min) and the supernatant discarded. The cells were resuspended in cRPMI supplemented with ciprofloxacin (10 µg/ml) and re-seeded at 0.5 x 10^6 cells/ml and the process repeated. Typically, BMDMs are differentiated using 20-30% of L929 conditioned medium. The exact amount of L929 conditioned medium was empirically determined for each batch by differentiating BMDMs in presence of different concentrations of the conditioned medium starting at 40% and working down.
2.2.5 Isolation and culture of BMDCs

Murine bone marrow-derived dendritic cells (BMDCs) were prepared based on protocols from Lutz et al (532). Briefly, female or male BMDCs from a C57BL/6 background were euthanised and their femurs and tibias were dissected from any surrounding muscle tissue. The bone marrow was flushed out with cRPMI medium using a 27 G needle. The cell aggregates were broken up using a 19 G needle and the cell suspension was pelleted by centrifugation (400g, 5 min at room temperature). The pellet was then resuspended in 1 ml of 0.88% ammonium chloride red blood cell lysis buffer for 2 min. Cells were then washed in cRPMI, centrifuged (400g, 5 min, room temperature) and resuspended in 10 ml of cRPMI. Cells were counted as outlined in in Section 2.2.2.1 and seeded in T175 flasks (Greiner BioOne) at 4x10^5 cells/ml in cRPMI medium, supplemented with 20 ng/ml of GM-CSF (obtained from the GM-CSF expressing J558 cell line). 37 ml of cells were added to the flask and incubated at 37 °C in a humidified atmosphere (5% CO₂). After 3 days, 30 ml of fresh medium containing 20 ng/ml of GM-CSF was added to the flask. On day 6, the cell culture supernatant containing all non-adherent cells (e.g. granulocytes) was removed and 30 ml of cRPMI containing 20 ng/ml GM-CSF was added. After 2 days, a further 30 ml of complete RPMI with 20 ng/ml GM-CSF was added. Finally, two days later, the loosely adherent cells were harvested by gentle repeat pipetting and counted. Cells were plated in cRPMI supplemented with 10 ng/ml GM-CSF in 96-well round bottom plates at a concentration of 0.625x10^6 cells/ml (200 μl/well) or in 12/24-well plates at a concentration of 1x10^6 cells/ml (1 ml/well); cells were used the following day for experiments. The specific treatments and conditions for restimulation of BMDCs are outlined in each experimental figure legend.

2.2.6 Generation of Bone Marrow Derived Macrophages

Bone marrow cells were flushed from murine bone marrow as described in section 2.2.2.3. Cells were seeded at an initial concentration of 1x10^6 cells/ml in complete DMEM supplemented with 20% of L929 conditioned medium containing M-CSF in T-175 flasks. The culture of this L929 cell line is described in Section 2.2.2.3. 35 ml of cells were added to the flask and incubated at 37 °C in a humidified atmosphere (5% CO₂). On day 4, 30 ml of complete DMEM supplemented with 20% L929 conditioned medium was added to the flask. On day 7, cells were lifted with ice cold PBS, scraped and pelleted by centrifugation (400g, 5 min, room temperature). Cells were subsequently resuspended in 10 ml of complete DMEM medium and cell numbers were determined as described in Section 2.2.2.1. Cells were plated at a
concentration of 1x10^6 cells/ml in 1 ml of complete DMEM supplemented with 10% L929 conditioned medium. Cells were stimulated the next day and the specific treatments and conditions for stimulation are outlined in each experimental figure legend. Cells were lysed for RNA extractions at indicated time points.

### 2.2.7 Isolation and culture of human monocytes

This study was approved by the research ethics committee of the School of Biochemistry and Immunology, Trinity College Dublin and is in accordance with the Declaration of Helsinki. Buffy coats from anonymous healthy donors were obtained with permission from the Irish Blood Transfusion Service, St. James’s Hospital, Dublin. PBMC isolation was performed by dilution of blood in pyrogen-free PBS (GIBCO) and differential density centrifugation over Ficoll-Paque density gradient media (GE Healthcare). After centrifugation, plasma was collected from supernatants and used for platelet removal and later for preparation of Percoll gradients. Platelets were removed from PBMC fraction by sedimentation through autologous plasma. Monocytes isolation was performed by Percoll (Sigma) density centrifugation. The isolated monocyte pellets were then washed with PBS/EDTA 1% (GIBCO). Washed monocytes were resuspended in RPMI 1640 culture medium (GIBCO) supplemented with 10% inactivated FBS and 1% penicillin/streptomycin. Monocytes (1x10^6 cells/ml) were plated and allowed to adhere for 1 h at 37 °C. The non-adherent cells were washed with PBS to ensure maximum purity.

### 2.2.8 Isolation and culture of splenocytes and draining lymph nodes

Following endpoint of experiment, female and male C57BL/6 mice were euthanised by CO2 and their spleens, inguinal and popliteal lymph nodes were isolated. The spleens and lymph nodes were homogenised and passed through a 70 μm cell strainer (BD) with complete RPMI 1640 medium to obtain a single cell suspension for each sample. Each cell suspension was centrifuged (1200rpm, 5 min) and supernatant discarded. Splenocytes (and lymph nodes if needed) were resuspended in 1 ml of 0.88% ammonium chloride (destroying red blood cells) for 2 min before washing with 10 ml complete RPMI 1640 medium. Each sample was centrifuged again (1200rpm, 5 min), supernatant decanted and resuspended in 5 ml cRPMI for splenocytes or 1 ml cRPMI for lymph nodes and counted. Splenocytes were plated at 2x10^6
cells/ml and lymph nodes cells at 1x10^6 cells/ml in 200 µl T cell medium in 96-well round bottom plates. Plated samples were stimulated ex-vivo with several different treatments, as outlined in each experimental figure legend. The cells were then incubated for 24 or 72 h at 37 °C in a humidified atmosphere (5% CO₂). Supernatants were collected either 24 or 72 h later (indicated in figure legend) for analysis of cytokine levels by ELISA.

2.2.9 Isolation and culture of muscle tissue

Muscle tissue was isolated from injected leg and placed into 24-well plate. The muscle was minced with a clean pair of scissors until obtaining small pieces of tissue approximately 2 mm in size. 1 ml of digestion medium, containing 30 mg of type I collagenase (GIBCO) and 100 µg DNAse-1 (Roche) in RPMI with no FCS was added to each well. The digestion mixture was pipetted up and down 5-6 times and incubated at 37 °C and 5% CO₂ for 30 min with agitation. After incubation, the mixture was pipetted up and down for around 30 seconds until most of the tissue was disrupted. The cell suspension was filtered through a 100 µm sterile cell strainer and washed with 5 ml of PBS supplemented with 5 mM EDTA. A complete digestion of extracellular matrix and fibrous tissue will not be achieved. However, a longer incubation with the digestion medium will result in increased cell death and destruction of extracellular proteins affecting analysis of samples. After filtering, the samples were centrifuged (400g, 10 min, 4 °C), resuspended in 5 ml RPMI and counted using Trypan blue. This also determines if the cells are viable after digestion process.

2.2.10 Flow cytometry

All data were analysed using Flowjo™ software (Treestar, Oregon).

2.2.10.1 Measurement of cell death

Cell death was assessed using Annexin V and PI staining. For Annexin V staining, all washes, incubations and measurements were performed in 1x Annexin V binding buffer. PI staining was performed in PBS. BMDCs were stimulated for 24 h, then washed and incubated with 1 µg/ml Annexin V-FITC (200 µl). After 5 min, cells were washed and resuspended in 200 µl of binding buffer. Finally, PI was added directly to FACS tubes (1 µg/ml) immediately before acquiring the samples on the BD FACs Canto flow cytometer.
2.2.10.2 Measuring DC maturation

BMDCs (1x10^6 cells/ml) were plated on a 96-well tissue culture plate and stimulated with medium, LPS, CpG or PLGA particles. After 24 h, plates were centrifuged (400g, 5 min, 4°C) supernatants discarded and BMDCs were resuspended in 200 μl FACS buffer in FACS tubes (BD Falcon). The cells were again centrifuged (400g, 5 min, 4°C) and stained with Aqua LIVE/DEAD (Invitrogen) used at a 1:1000 dilution in PBS in a total volume of 200 μl/sample. After 30 min in the dark, cells were washed again and resuspended in 200 μl of FACS buffer supplemented with purified anti-mouse CD16/CD32 monoclonal antibody (dilution outlined in section 2.1.7) and incubated at 4 °C for 10 min. Cells were stained with Fluorochrome-labelled anti-CD11c and antibodies specific for the DC maturation markers CD80, CD86, CD40 and MHC class II (section 2.1.7) for 30 min on ice in the dark. Afterwards, cells were centrifuged (400g, 5 min, 4 °C) and washed twice with 200 μl FACs buffer before being analysed for immunofluorescence using a BD LSRFortessa flow cytometer.

2.2.10.3 DC and OTII co-culture and staining

BMDCs (2x10^4 cells/ml) were plated in a 96-well tissue culture plate and stimulated with medium, LPS, OVA protein (25 μg/ml) or different sized PLGA particles (0.1 mg/ml) alone or in combination with each other overnight. The following day, spleens and lymph nodes from an OTII mice were taken and processed as outlined in section 2.2.8. Cells were counted and T cells were isolated using a MACS protocol from Pan T cell isolation kit II (Miltenyi Biotec). Briefly, LS MACs columns (Miltenyi Biotec) were placed in the magnetic field of a MACS separator and washed with 3 ml of MACS buffer (Section 2.1.1). Cells were resuspended in 40 μl MACS buffer per 10^7 total cells, 10 μl Pan T cell Biotin-Antibody cocktail per 10^7 total cells were added, mixed and incubated for 5 min at 4°C. 30 μl of buffer was added per 10^7 total cells and then 20 μl of Anti-Biotin Microbeads per 10^7 total cells was added, mixed and incubated for an additional 10 min at 4°C. Cells were then added to the washed LS columns on the MACS separator and the flow through containing unlabelled cells was collected, representing the enriched T cells. The column was also washed (1x3 ml) with MACs buffer to collect additional T cells. The T cells were centrifuged, resuspended (1x10^6 cells/ml) in T cell media and incubated at 37 °C for 1 h.

T cells were centrifuged (400g, 5 min, 4°C) and resuspended in 1 ml PBS containing 5% (v/v) FCS. A 10 μm (2x) solution carboxyfluorescein succinimidyl ester (CFSE) was prepared by
adding 2 µl of a 5 mM stock to 1 ml of PBS and quickly added to 1 ml of thoroughly resuspended cells. Cells were mixed immediately on a vortex and incubated in the dark for 5 min at room temperature. Cells were then washed 3 times by adding 10 ml PBS containing 5% (v/v) FCS, centrifuged (400g, 5 min, 4 °C) and supernatant discarded. The BMDCs in 96-well plates were centrifuged and supernatant removed. Simultaneously, T cells were resuspended in T cell media, counted and 2x10⁶ T cells/well, in a final volume of 250 µl were added to the 96-well plates containing the BMDCs. The co-culture was left for 72 h.

After 72 h, cells were stained for surface markers and intracellular cytokines. Carefully, 50 µl of media from the co-culture was removed and 50 µl of Brefeldin A (50 µg/ml) in T cell media was added to wells to give a final concentration of 10 µg/ml. Brefeldin A disrupts the Golgi apparatus in cells, restricting the secretion of intracellular cytokines. The 96-well plates were returned to the incubator for 4 h.

The 96-well plates were centrifuged (400g, 5 min, 4 °C) and supernatants discarded. Cells were resuspended in 200 µl PBS containing 2 mM EDTA and triplicates pooled into the same FACS tube. Cells were washed with 1 ml PBS, centrifuged (400g, 5 min, 4 °C) and stained with Aqua LIVE/DEAD (Invitrogen) used at a 1:1000 dilution in PBS in a total volume of 200 µl/sample. After 30 min in the dark, cells were washed again and resuspended in 200 µl of FACS buffer supplemented with purified anti-mouse CD16/CD32 monoclonal antibody (dilution outlined in Table 2.) and incubated at 4 °C for 10 min. Cells were stained for T cells with Fluorochrome-labelled anti-CD3 and CD4 (dilutions outlined in Table 2.) for 30 min on ice in the dark.

1 ml of PBS was added to the cell suspension, centrifuged (400g, 5 min, 4 °C) and supernatant decanted. Cells were resuspended in 100 µl PBS and 100 µl of 2% PFA was added to fix the cells. The cells were vortexed and incubated at room temperature in the dark for 15 min. 1 ml of PBS was added and the cell suspension was centrifuged (400g, 5 min, 4 °C) and the supernatant decanted. The cells were resuspended in 50 µl permeabilisation buffer (Table 2.12) and incubated for 30 min at 4 °C in the dark. Cells were then stained with 50 µl permeabilisation buffer containing intracellular fluorochrome-labelled antibodies (0.3 µl/sample) and incubated in the dark at room temperature for 30 min. Cells were then washed twice by adding 1 ml PBS, centrifuging the cells (400g, 5 min, 4 °C) and discarding the supernatants. The cells were resuspended in 200 µl FACS buffer for analysis of immunofluorescence. Cells were analysed using a BD LSRFortessa flow cytometer.
2.2.10.4 **Analysis of immune cell infiltration after intramuscular immunisation**

After muscle cells were isolated and counted (section 2.2.8), they were resuspended in PBS without FCS to make up a suspension at 2x10^7 cells/ml. 50 µl of this suspension was added to a FACS tube (gives 1x10^6 cells/total). Cells were stained with Aqua LIVE/DEAD (Invitrogen) used at a 1:1000 dilution in PBS in a total volume of 100 µl/sample. After 30 min in the dark, cells were washed and resuspended in 200 µl of FACS buffer supplemented with purified anti-mouse CD16/CD32 monoclonal antibody (dilution outlined in Section 2.1.7) and incubated at 4 °C for 10 min. Cells were stained with Fluorochrome-labelled antibodies specific for either innate or adaptive cells (Section 2.1.7) by adding a total volume of 10 µl of antibodies to the cells and incubated for 30 min on ice in the dark. Cells were washed with 1 ml FACS-EDTA buffer (PBS, 2% FCS and 5 mM EDTA) and centrifuged (400g, 5 min, 4 °C). Cells were washed one more time with 2 ml PBS without FCS, centrifuged (400g, 5 min, 4 °C) and resuspended in 200 µl FACS buffer. Cells were analysed using a BD LSRFortessa flow cytometer.

2.2.10.5 **Tetramer staining**

Splenocytes, lymph node and muscle cells were processed as before (section 2.2.8 and 2.2.9), without putting cells on ice. Cells were then stained with an antibody panel to study Tetramer^+ CD8^+ T cells (section 2.1.7). Splenocytes (20x10^6 cells), lymph nodes (entire sample) and muscle (entire sample) were transferred to FACS tubes with lids, stained with an optimised volume of PE-labelled H-2k(b) chicken OVA 257-264 SIINFEKL tetramer at a final concentration of 15 µg/ml (2.5 µl/sample) in T cell media for 2 h at 37 °C with 95% humidity and 5% CO_2, with gentle shaking. Briefly, without washing, cells were incubated with Fc block (anti-CD16/CD32) and the antibody panel (section 2.2.14) prepared in FACS buffer for 30 min at 37 °C and 5% CO_2 with shaking. Cells were washed with 1 ml PBS and pelleted via centrifugation at 1200 rpm for 5 min. Supernatants were removed and cells resuspended in 500 µl viability stain (Live/Dead aqua) for 20 min at room temperature, protected from the light. Cells were washed again in PBS and fixed in 2% PFA for 20 min at room temperature protected from the light. Cells were washed and resuspended in 200 µl FACS buffer. Cells were then analysed using a BD LSRFortessa flow cytometer.
2.2.11 Real-time PCR

RNA isolation
Total RNA from cells was isolated using a High Pure RNA Isolation Kit (Roche) following the manufacturer’s instructions. Following stimulations and incubating for appropriate time points, cells were lysed, added to a column and incubated with DNase I to digest any contaminating DNA present. After three washing steps, RNA was eluted into nuclease-free microcentrifuge tubes. 1 µl of RNA sample was added to a Nanodrop spectrophotometer and the absorbance read at 260 nm and the 260/280 nm ratio to measure the quality and concentration of RNA. RNA was then stored at -80 °C for future use.

Reverse transcription
Isolated RNA was used to produce complementary DNA (cDNA). The RNA concentration of each sample was adjusted to 200 ng by addition of H₂O up to a volume of 5 µl. cDNA was synthesised using 5 µl of master mix added to each sample (Table 2.3), including random hexamers to act as random primer, Reverse transcriptase (RT) enzyme to generate cDNA and RNaseOUT to inhibit ribonuclease. To exclude the possibility of DNA contamination a random sample without RT enzyme (replaced with nuclease-free H₂O) was used as a control with each RT-PCR cycle. The PCR cycle settings used are described in Table 2.13 below, after the cycle finished 20 µl of nuclease-free water was added to dilute the cDNA sample and stored at -20 °C.

Table 2.13 – cDNA synthesis thermocycler program

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Time (mins)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>10</td>
</tr>
<tr>
<td>42</td>
<td>60</td>
</tr>
<tr>
<td>95</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

Quantitative Real-Time qPCR
qPCR was performed with previously made cDNA and forward and reverse primers (5pmol/µl) using the Kapa SYBR® Fast qPCR Kit (Section 2.1.6). In addition to the no RT control, cDNA was replaced with nuclease free H₂O to exclude genomic DNA contamination in the samples or reagents. Samples were run on an Applied Biosystems 7500 Fast system for 40 cycles of 2 min at 95 °C followed by 3 seconds at 95 °C, and finally by one cycle of 30 seconds at 60 °C.
Dissociation curve analysis was performed after a completed real-time qPCR to exclude non-specific products. The change in gene expression was normalised to β-actin expression from the corresponding sample.

2.2.12 Western Blot

Protein extraction from supernatants
BMDCs (1x10^6 cells/ml) were plated in 1 ml of cRPMI supplemented with GM-CSF (10 ng/ml) in a 12-well round bottom tissue culture plate. Cells were then stimulated at various time points and pelleted by centrifugation (1200rpm, 5 min at 4 °C). The supernatants were removed and cells washed with PBS. After centrifuging again and removing the PBS, the cell pellet was lysed with 100 μl RIPA lysis buffer (Table 2.5) containing protease (1% v/v) and phosphatase inhibitors (1% v/v) on ice for 30 min, making sure to vortex every 10 min. The resulting cell lysate was transferred to an Eppendorf and centrifuged at 13,000g for 10 min at 4 °C. The supernatant containing total protein was collected and transferred to a new Eppendorf and immersed in liquid nitrogen to snap freeze. Samples were then stored at -80 °C for later use or kept on ice for immediate homogenisation.

Protein concentration assay
The BCA protein assay kit (Pierce, Thermo Scientific) was used to measure protein concentration. The procedure was performed following the manufacturer’s protocol. BSA protein standards ranging from 0-2000 μg/ml were prepared and 25 μl of each standard and unknown replicate were pipetted in triplicate onto a 96-well flat bottom tissue culture plate (Greiner BioOne). Subsequently, equal concentrations of each sample were prepared.

SDS-PAGE
10 μl of sample was mixed with 10 μl of 2x laemaelli sample buffer (approximately 20 μg of protein), boiled for 5 min at 95 °C. Gels were prepared (Table 2), 20 μl of sample was loaded into each well in addition to 5 μl Page ruler prestained protein ladder (Thermo scientific). Once the samples were loaded, the gel was run at 100 volts (V) for 90 min in running buffer (Table 2.5).
Transfer of proteins onto nitrocellulose membrane

Proteins were transferred to a PVDF membrane using a semi-dry blotter (Cleaver Scientific Ltd). The gel was removed from the glass plates and incubated in transfer buffer (Table 2.5) for 10 min. The PVDF membrane was activated in methanol (incubate for 5-10 sec), washed in water (2 min) and then soaked in transfer buffer (5 min). The PVDF membrane was then placed onto a sheet of filter paper that had been soaked in transfer buffer and the gel was carefully placed over the PVDF membrane. An additional sheet of moist filter paper was placed on top of the PVDF membrane and gel and finally any air bubbles were removed. The proteins were transferred at 50 milliamperes (mA) per gel for 75 min.

Immunodetection of proteins

The blotted membrane was removed from the transfer apparatus and immediately placed in blocking buffer (Table 2.5). The blot was incubated for 1 h at room temperature with gentle shaking. The membrane was then probed with appropriate dilution of primary antibody (1/1000) in blocking buffer incubated overnight at 4 °C with gentle shaking. The membrane was washed 3 times with 20 ml wash buffer (Table 2.5) for 5 min each. The membrane was then incubated with recommended dilution of HRP-conjugated secondary antibody (1/5000) in blocking buffer at room temperature for 1 h with gentle shaking. The membrane was washed again 3 times for 5 min each. Finally, the membrane was developed using freshly prepared Immobilon Western Chemiluminescent HRP substrate (Merck Millipore). 1 ml of this detection substrate was added to the membrane for 5 min, afterward excess reagent was removed. The protein was then detected using Bio-Rad ChemiDoc imaging system and analysed using Bio-Rad image lab 5.0.

2.2.13 Enzyme-linked immunosorbent assay (ELISA)

Measurement of cytokine secretion

96-well high binding ELISA plates (Grenier Bio-one) were coated with 40 μl of rat anti-mouse capture antibody diluted in PBS per well (dilutions outlined Table 2.9) and were incubated overnight at 4 °C. The ELISA plates were washed with PBS/Tween 4 times and incubated with 100 μl per well of appropriate blocking solution (refer to Table 2.9) for 1 h at room temperature. The plates were washed again 4 times and 40 μl of supernatants or standards (top working standards outlined in table 2.9) serially diluted in reagent diluent (Table 2.10) were added to the plates and incubated for 2 h at room temperature. Plates were subsequently washed 4 times
with PBS/Tween before adding biotinylated goat anti-mouse detection antibody diluted in reagent diluent (40 μl/well) and incubated for appropriate times at room temperature in the dark (see below). After washing, HRP-conjugated streptavidin or avidin diluted in appropriate reagent diluent (40 μl/well) (Table 2.11) was added to the plates and incubated in the dark as indicated below. Finally, the plates were washed and o-Phenylenediamine dihydrochloride (OPD) substrate dissolved in phosphate citrate buffer (0.4 mg/ml OPD) and hydrogen peroxide (0.7 μl hydrogen peroxide per 1 mg of OPD) was added to the solution and allowed to develop for required time in the dark. For IL-12p70 ELISA 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate was added instead. The enzyme reaction was stopped by the addition of 1 M H₂SO₄ (40 μl/well). The OD values were determined by measurement of absorbance at 492 nm (OPD) or 450 nm (TMB) using a Versa Max microtitre plate reader. The standards generated a standard curve which was then used to determine the cytokine concentration of the unknown replicates.

**Individual ELISA kit protocols**

**R&D kits**
Blocking solution: 1% (w/v) BSA in PBS, 1 h incubation  
Detection antibody: 2 h incubation  
Streptavidin-HRP: 1/40, 30 min incubation

**Biolegend**
Blocking solution: 1% (w/v) BSA, 1 h incubation  
Detection antibody: 1 h incubation  
Avidin-HRP: 1/1000, 20 min incubation

**Becton Dickenson (BD)**
Blocking solution: 10% (w/v) milk in PBS, 1 h incubation  
Detection antibody: 1 h incubation  
Xtravidin-HRP: 1/750, 30 min incubation
2.2.14 Confocal Microscopy

Live cell imaging
BMDCs (0.5x10^6 cells/ml) were plated in cRPMI on 35 mm glass bottom tissue dishes. Non-adherent cells were removed on the following day and cells were treated with latrunculin B (0.5 µm) for 1 h or 10 mM of MβCD (x2) for 20 min and left in 2 mM MβCD or left untreated. Cells were washed several times with ice cold PBS and stained with Hoechst 33342 (5 µg/ml) in cRPMI containing either latrunculin B, MβCD or medium alone for 30 min. Cells were washed again and resuspended in 0.5 ml cRPMI. Fluorescent PLGA particles (100 µg/ml) were added to the cells and viewed using a Point Scanning Confocal Microscope with a heated stage and CO₂ chamber (Olympus FV100 LSM Confocal Microscope).

Lipid raft staining
BMDCs (0.5x10^6 cells/ml) were plated in cRPMI on 35 mm glass bottom tissue dishes overnight, non-adherent cells were removed and cells were stained with Hoechst 33342 (5 µg/ml) for 30 min at 37 °C. The cells were washed with ice cold PBS and stimulated with either 100 nm, 500 nm or 10 µm green fluorescent PLGA particles (100 µg/ml) and left for 15, 30 or 45 min. The cells were fixed with 1% PFA for 2 min and washed with ice cold PBS. The cells were labelled with chilled cholera toxin subunit B (CtB) labelled with Alexa Fluor 594 (1 µg/ml) and incubated for 10 min at 4 °C. After this incubation, the cells were gently washed several times with chilled PBS. The CtB labelled lipid rafts were cross linked with anti-cholera toxin subunit B antibody (anti-CtB), rabbit serum for 15 min at 4 °C. After this incubation, the cells were gently washed several times with ice cold PBS. The cells were resuspended in mounting medium and visualised by fluorescence microscopy using Leica microscope.

As mentioned in the introduction (section 1.6), CtB binds to the pentasaccharide chain of plasma membrane ganglioside GM1, which selectively concentrates into lipid rafts. It should be noted that in order to visualise the lipid rafts, an antibody is needed against CtB to crosslink the labelled rafts into distinct patches on the membrane, otherwise the raft domains would be too small to visualise by confocal microscopy.
**F-actin staining**

BMDCs (0.5x10^6 cells/ml) were plated in cRPMI on 35 mm glass bottom tissue dishes overnight, non-adherent cells were removed the following day and 0.5 ml cRPMI was added to wells. Fluorescent (green) or plain 100 nm, 500 nm or 10 μm PLGA particles (100 μg/ml) were added to cells for indicated timepoints. Cells were washed with PBS and fixed in 2% PFA for 10 min at room temperature. Cells were washed again and permeabilised with 0.1% Triton-x and incubated for 5 min at room temperature. Following incubation, cells were washed and blocked with 1% BSA in PBS (500 μl). After 60 min, cells were stained with Alexa fluor 647 Phalloidin (0.5 μM) in 1% BSA for 30 min.

Cells were washed and stained with DAPI (1 μg/ml) for 10 min at room temperature, cells were washed again and 2 drops of Dako mounting media was added to dish and stored at 4 °C until ready to image. Cells were viewed using Point Scanning Confocal Microscope (Olympus FV100 LSM Confocal Microscope).

**2.2.15 Statistical analysis**

Statistical analysis was performed using Graphpad Prism 6 software. The means for three or more groups were compared by one-way ANOVA. Where significant differences were found, the Tukey multiple comparisons test was used to identify differences between individual groups. The means for two groups were compared using an unpaired Student’s t test. For human monocytes, a paired non-parametric T test was used.
Chapter 3

Investigating the modulation of IL-10 and IL-12 production by particulate vaccine adjuvants
3.1 Introduction

The majority of non-living vaccines on the market, especially those adjuvanted with alum elicit their protection by inducing neutralising antibodies (533). In contrast, vaccines for complex infections such as HIV-1/AIDS, tuberculosis, malaria, as well as therapeutic cancer vaccines are required to induce a robust and durable Th1 cell response (533). Many different strategies have been used to enhance cell-mediated immunity to subunit vaccine antigens, but most have failed to elicit a sufficient response or meet exacting safety standards.

A feature shared by many licensed non-living vaccines is their inability to promote effective Th1 responses. Alum is a key example, while it can provoke a strong Th2 response it is less effective against pathogens that require Th1–cell-mediated immunity, but the reasons for this are not well understood (454). Therefore, there is a need to increase our understanding of how vaccine adjuvants modulate immune responses. This could allow the tailored design of vaccines which selectively drive specific types of adaptive immune responses. Some novel recent strategies have been shown to enhance cell-mediated immunity. For example, combining alum with the TLR4 agonist MPLA (AS04) can improve Th1 responses and the adjuvant has been used in a number of vaccine formulation including a vaccine for cervical cancer (359). As of 2015, The RTS,S candidate Malaria vaccine which uses a combination of MPL, QS21 and liposomes (AS01) is seen as an additional method along with bed nets and drugs to control outbreaks.

IL-10 is a key cytokine involved in the anti-inflammatory response and maintaining immune homeostasis in the body. It has the ability to restrict both Th1 and Th17 responses, both through inhibition of IL-12 and promotion of either Treg or Th2 responses (534). IL-10 has also been associated with the development and differentiation of Th2 response (102). Data from our lab have indicated that alum drives an IL-10 response both in vitro and in vivo to restrict both Th1 and Th17 responses and mediate regulatory effects during vaccination (Oleszycka, McCluskey et al, manuscript under revision). Wild type and IL-10−/− mice were immunised with alum and Ova. As expected immunisation with OVA and alum did not result in significant antigen-specific IFN-γ or IL-17 production in wild type mice (Oleszycka PhD thesis, 2013). However, deficiency in IL-10 led to spontaneous and antigen-specific secretion of IFN-γ by peritoneal exudate cells and splenocytes.
In contrast, IL-12 is the most crucial component in polarising to a Th1 type immune response. Bioactive IL-12p70, made up of IL-12p40 and IL-12p35 subunits is preferentially produced by APCs such as dendritic cells. Due to its crucial role in mediating cell mediated immunity by initiating and maintaining Th1 responses, expression of both IL-12 subunits is tightly regulated in DCs (535). Data from our lab indicates that alum specifically inhibits IL-12p35 in DCs to reduce IL-12p70 levels (476).

Micro- and nano-particles generated from a range of materials have been extensively studied as potential vaccine adjuvants, including investigations into the influence of size, shape, charge and chemical composition on adjuvanticity. Several studies have addressed the importance of size in particulate vaccines to modulate and enhance certain immune responses (520). However, most these studies have relied on antibodies as the readout, yet no study has provided a link between particle size and the induction of IL-10 and IL-12p70. Our lab has previously shown that micro- and nano-particles can influence the innate and subsequent adaptive immune response that follows. PS particles were initially chosen as model particles to analysis the relationship between size and IL-10/IL-12p70 induction. PS particles of 50-100 nm, 500 nm-1 μm, 10 μm, 30 μm and 100 μm in diameter were chosen to mimic the size of different pathogens. These sizes also cover the range licensed adjuvants use as well both smaller and larger sizes to establish whether they can mediate a different response (511). Afterwards, whether the effects of PS particles translate to PLGA (using 100 nm, 500 nm, 2 μm, 10 μm and 30 μm) was examined.

Modulation of DCs responses by adjuvants is now becoming a major focus in vaccine research due to their superior ability to present antigen to naïve T cells and consequently induce protective immune responses. IL-10 and bioactive IL-12 (IL-12p70) secreted by DCs are key factors determining the effectiveness of new adjuvants aimed at enhancing cell mediated immunity and potentially explaining how licensed vaccines elicit their protection. Thus, the aim of the study was to determine how particulate adjuvants and their size affect these responses. Depending on their physicochemical characteristics, particulate adjuvants could trigger immunoregulatory mechanisms, which actively block induction of cellular immune responses.
3.2 Results

3.2.1 Different sized PS particles elicit distinct cytokine profiles in Dendritic cells.

The first question addressed was how particle size influences cytokine secretion in dendritic cells. The cytokines secreted by APCs are influential in determining type of T helper cell response induced.

In order to determine the effect of particle size on DC cytokine production, BMDCs were co-stimulated with LPS (5 ng/ml) and PS particles across a range of sizes at concentrations from 0.063-1 mg/ml. After 24 h, supernatants were collected and analysed by ELISA for IL-12p70, IL-10 and the proinflammatory cytokines IL-1β and IL-1α. PS particles regardless of size did not induce cytokine secretion without the presence of LPS. Surprisingly, LPS-induced secretion of all cytokines measured was altered by the presence of PS particles, with size being the major determining factor. LPS has been shown to induce significant production of IL-12p70 and IL-10 by DCs. However, when LPS was added to cells together with 10 µm PS particles, IL-12p70 secretion was significantly inhibited even at lower particle concentrations (Fig 3.1). In contrast 1 and 10 µm PS particles enhanced LPS-induced IL-10 secretion when compared to LPS on its own with the 10 µm PS particles being the most potent (Fig 3.1). Finally, BMDCs preferentially secreted either IL-1β or IL-1α depending on PS particle size. PS particles alone did not drive IL-1β release from BMDCs but 10 µm PS particles alone could drive IL-1α release. When co-incubated with LPS (5 ng/ml), 50 nm and 10 µm PS particles were capable of driving IL-1β and IL-1α secretion (Fig 3.2). However, at lower particle doses 10 µm PS particles were more potent stimuli than 50 nm particles for IL-1β production but in the case of IL-1α secretion responses were more comparable.
Figure: 3.1. Polystyrene (PS) particles enhance IL-10 and inhibit IL-12p70 secretion by in DCs in a size dependent manner.

BMDCs from C57BL/6 mice (6.25x10^5 cells/ml) were stimulated with LPS (5 ng/ml) or PS particles of different sizes at concentrations from 0.063-1 mg/ml alone or together for 24 h. Concentrations of IL-10 and IL-12p70 were measured in supernatants by ELISA. PS + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.2: PS particles enhance IL-1β and IL-1α secretion by DCs in a size dependent manner

BMDCs from C57BL/6 mice (6.25x10^5 cells/ml) were stimulated with LPS (5 ng/ml) or different PS particles of different sizes at concentrations from 0.063-1 mg/ml alone or together for 24 h. Concentrations of IL-1β and IL-1α were measured in supernatants by ELISA. PS + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
3.2.2 PLGA particle-induced cytokine modulation is size dependent

Having established size-dependent modulation of LPS-induced cytokine production by PS particles, whether this pattern would translate to PLGA particles of similar sizes was investigated. PLGA particles have the advantage of being biodegradable and biocompatible and available in many different sizes. Unfortunately, 50 nm PLGA particles were not commercially available and therefore 100 nm particles were used instead, the smallest size available. Otherwise all sizes were comparable to those used for the PS particle experiments.

In order to determine the effect of different PLGA particle sizes (100 nm, 500 nm, 10 µm and 30 µm) on cytokine production by dendritic cells, varying concentrations of LPS (5, 1 and 0.2 ng/ml) were co-incubated with PLGA particles at decreasing concentrations (1, 0.5, 0.25, 0.125 and 0.063 mg/ml) to determine the optimal concentrations for each. After 24 h supernatants were analysed by ELISA for the Th1 polarising cytokine IL-12p70, inflammatory cytokines (IL-1β and IL-1α) and the anti-inflammatory IL-10. As in the case of PS particles, PLGA particles alone did not induce cytokine secretion in non LPS treated DCs.

Interestingly, while the correlation between particle size and cytokine secretion was the same for some sizes, there were also differences indicating that size but also particle composition can modulate DC responses. As reported with PS particles, PLGA particles could inhibit LPS-induced IL-12p70 secretion but the inhibitory size range was broader, going from only 10 µm in the case of PS particles to between 500 nm and 10 µm in the case of PLGA particles (Fig 3.3). Moreover, the IL-10 secretion profile was similar to that seen with PS particles but at low particle concentrations only 500 nm PLGA particles had the ability to significantly promote IL-10 secretion in DCs using LPS concentration as low as 0.2 ng/ml (Fig 3.4). This contrasts with the PS particles, where 10 µm PS particles could drive the strongest IL-10 response (although particles in the 500 nm-1 µm range were also able to significantly promote IL-10 secretion).

Finally, there were also considerable differences in potency between different sized PLGA particles regarding the induction of IL-1β and IL-1α secretion by DCs. PLGA particles between 100 nm and 10 µm in size were able to drive IL-1β and/or IL-1α production but the 10 µm particles were the strongest at driving both. Therefore, a similar profile was observed with the
10 µm PLGA and PS particles, indicating that particles of this size potently drive inflammation independent of composition (Fig 3.5 and 3.6).

Figure 3.3: PLGA particles of between 500 nm and 10 µm in size inhibit IL-12p70 secretion by DCs

BMDCs from C57BL/6 (6.25x10⁵ cells/ml) mice were stimulated with LPS (5, 1 and 0.2 ng/ml) or PLGA particles at different sizes (100 nm, 500 nm, 10 µm and 30 µm) at concentrations from 0.063-1 mg/ml alone or together for 24 h. Concentrations of IL-12p70 were measured in supernatants by ELISA. PLGA + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.4: 500 nm PLGA particles most potently promote IL-10 secretion by DCs

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with LPS (5, 1 and 0.2 ng/ml) or PLGA particles of different sizes (100 nm, 500 nm, 10 µm and 30 µm) at concentrations from 0.063-1 mg/ml alone or together for 24 h. Concentrations of IL-10 were measured in supernatants by ELISA. PLGA + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.5: PLGA particles of between 100 nm and 10 µm in size increase IL-1β secretion by DCs with 10 µm particles being most potent.

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with LPS (5, 1 and 0.2 ng/ml) or different sizes of PLGA particles (100 nm, 500 nm, 10 µm and 30 µm) at concentrations from 0.063-1 mg/ml alone or together for 24 h. Concentrations of IL-1β were measured in supernatants by ELISA. PLGA + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.6: PLGA particles of between 100 nm and 10 µm in size increase IL-1α secretion by DCs with 10 µm particles being most potent.

BMDCs from C57BL/6 (6.25x10⁵ cells/ml) mice were stimulated with LPS (5, 1 and 0.2 ng/ml) or different sizes of PLGA particles (100 nm, 500 nm, 10 µm and 30 µm) at concentrations from 0.063-1 mg/ml alone or together for 24 h. Concentrations of IL-1α were measured in supernatants by ELISA. PLGA + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
3.2.3 **PLGA particles selectively inhibit IL-12p35 expression in a size-dependent manner**

Having demonstrated that PLGA particles selectively inhibit IL-12p70 secretion within a narrow size range (500 nm-10 µm), the effect of PLGA particles on IL-12p35, IL-12p40 and IL-10 expression was investigated. This was to determine whether IL-10, IL-12p35 and IL-12p40 are transcriptionally or post transcriptionally regulated by particles. Additionally, IL-12p35 and IL-12p40 expression are regulated by distinct pathways (535). 500 nm PLGA particles were used as these were most potent in enhancing IL-10 and suppressing IL-12p70 secretion.

BMDCs (1x10^6 cells/ml) were stimulated with LPS alone or in the presence of 500 nm PLGA particles for 6, 12 and 24 h. Expression of IL-10, IL-12p35, IL-12p40 and β-actin was determined by real-time PCR. LPS-induced IL-10 expression peaked at 12 h. Remarkably, when LPS was combined with 500 nm PLGA particles, IL-10 mRNA expression was increased 10-fold when compared to LPS alone (Fig 3.7). This confirms that PLGA particle induced IL-10 is mainly regulated at the transcriptional level.

IL-12p35 and IL-12p40 mRNA expression peaked 6 h after stimulation with LPS alone. However, only IL-12p35 expression was down-regulated by 500 nm PLGA particles co-incubated with LPS after 6 h (Fig 3.7). IL-12p40 expression was not significantly affected by addition of PLGA particles, which indicates that the inhibition of IL-12p35 is not a nonspecific effect (Fig 3.7).
Figure 3.7: 500 nm PLGA particles enhance LPS induced IL-10 mRNA and selectively inhibit IL-12p35 expression in DCs

BMDCs (1x10^6 cells/ml) were stimulated for indicated times with medium, LPS (5 ng/ml), 500 nm PLGA particles or 500 nm PLGA particles and LPS (5 ng/ml) together. Total RNA was isolated and the mRNA levels of IL-10, IL-12p35 and IL-12p40 were determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells. Results are expressed as the mean ±SD of samples in triplicate and are representative of three independent experiments. LPS + adjuvant + LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
3.2.4 The ability of PLGA particles to promote IL-10 production is not restricted to BMDCs

In order to evaluate whether the ability of PLGA particles to enhance IL-10 expression and secretion was restricted to dendritic cells, the effects of PLGA particles across a range of sizes was analysed in bone marrow derived macrophages (BMDMs). Macrophages are known to be one of the main producers of IL-10 during the innate immune response and may also be influenced by particulate adjuvants. BMDMs primarily produce IL-12p40 but very little IL-12p35, hence they are not a good model to examine IL-12p70 production.

Firstly, BMDMs were stimulated with LPS alone (100, 10 and 1 ng/ml) or LPS with different sizes of PLGA particles at the same concentration (250 µg/ml). After 24 h, supernatants were collected and analysed for cytokine secretion. Furthermore, BMDMs (1x10^6 cells/ml) were also stimulated with 500 nm PLGA particles and LPS (10 and 1 ng/ml) alone or together. mRNA was then isolated and analysed for an increase in IL-10 by RT-PCR.

Similar to what was observed in BMDCs, only BMDMs incubated with 500 nm PLGA particles exhibited a significant increase in IL-10 secretion over LPS alone (Fig 3.8B, C and D). Outside this size range, PLGA particles were unable to increase IL-10 above the levels induced by LPS alone. Furthermore, IL-10 transcript levels were measured in BMDMs to confirm that 500 nm particles can significantly enhance IL-10 mRNA after 12 h and that any increase is regulated at the transcriptional level (Fig 3.8E and F). As before, PLGA particles alone were unable to promote IL-10 production (Fig 3.8A).
Figure 3.8: 500 nm PLGA particles can selectively enhance IL-10 transcription and secretion in BMDMs

BMDMs (1x10^6 cells/ml) were treated with 100 (B), 10 (C) or 1 ng/ml (D) LPS and different sizes of PLGA particles (100 nm, 500 nm, 10 µm or 30 µm) at a concentration of 250 µg/ml alone (A) or together for 24 h. Supernatants were collected and IL-10 levels were determined by ELISA (A, B, C and D). BMDMs (1x10^6 cells/ml) were also stimulated with LPS (10 or 1 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 12 h. Total RNA was isolated and the mRNA levels of IL-10 was measured using quantitative real-time PCR (E and F). Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells. Results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. LPS v adjuvant + LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
3.2.5 Alum enhances IL-10 expression and secretion by DCs and macrophages

Having established that PS and PLGA particles of specific sizes can enhance IL-10 secretion, the ability of other particulate vaccine adjuvants to increase IL-10 was also investigated. Alum can inhibit IL-12p70 secretion in DCs (476) but whether alum can also induce or enhance IL-10 production was addressed.

BMDCs were stimulated with alum at concentrations from 0.5-200 μg/ml in the presence of LPS or the TLR2 agonist zymosan and IL-10 secretion was measured 24 h later by ELISA. As expected, alum alone did not promote IL-10 secretion by DCs. However, at a concentration of 50 μg/ml, alum enhanced LPS-induced IL-10 secretion, while zymosan-induced IL-10 production was further increased by lower concentrations of alum (0.5-10 μg/ml) (Fig 3.9A).

Having demonstrated that alum can enhance IL-10 secretion, the effect of alum on IL-10 mRNA expression was investigated. DCs were stimulated with LPS alone or in the presence of alum for 3, 6 and 12h and expression of IL-10 and β-actin was determined by real-time PCR. Similar to 500 nm PLGA particles, alum significantly increased IL-10 mRNA expression which peaked after 6 h (Fig. 3.9B).

To determine the optimal concentration of alum to increase IL-10 mRNA expression, DCs were stimulated with LPS alone or in the presence of a range of alum concentrations. Cells were stimulated for 6 h and IL-10 and β-actin mRNA levels were determined by real-time PCR. In accordance with results obtained by ELISA, alum significantly enhanced IL-10 expression at a concentration of 50 μg/ml (Fig 3.9C).

Having established that alum enhanced IL-10 in BMDCs, whether this effect was selective or if the adjuvant could also promote IL-10 in BMDMs was tested. Similar to what was observed in BMDCs, BMDMs incubated with different concentrations of alum showed a significant increase in IL-10 secretion over LPS alone (10, 1 or 0.1 ng/ml) (Fig 3.10A). However, only lower concentrations of alum (below 10 μg/ml) could enhance IL-10 secretion in BMDMs, as it has been shown that macrophages are more susceptible to necrotic cell death by alum (Ewa Oleszycka, PhD 2013). Finally, IL-10 transcript levels were measured in BMDMs stimulated with LPS alone (1 and 0.1 ng/ml) or together with alum (10 or 20 μg/ml) for 6 or 12 h. As expected, alum enhanced IL-10 mRNA expression, which as in the case of BMDCs peaked after 6 h (Fig 3.10B).
Figure 3.9: Alum can enhance IL-10 production by Dendritic cells.

DCs from C57BL/6 mice were stimulated with LPS (5 ng/ml) or zymosan (1 μg/ml) alone or together with Alhydrogel (0.5-50 μg/ml). Concentrations of IL-10 were determined in 24 h supernatants by ELISA (A). DCs were incubated with LPS (5 ng/ml) alone or together with alum (50 μg/ml). The expression of IL-10 mRNA was determined in cells after 3, 6 and 12 h by real-time PCR (B). DCs were incubated with LPS (5 ng/ml) alone or with alum at concentrations from 5-50 μg/ml. The expression of IL-10 mRNA was determined in cells after 6 h by real-time PCR (C). Alum + LPS v LPS only. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. All results are representative of two independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate. Experiment performed by Dr Ewa Oleszycka.
Figure 3.10: Alum can enhance IL-10 production by Macrophages.

Macrophages from C57BL/6 mice were stimulated with LPS (10, 1 or 0.1 ng/ml) alone or together with Alhydrogel (100-0.5 μg/ml). Concentrations of IL-10 were determined in 24 h supernatants by ELISA (A). Macrophages were incubated with LPS (10, 1 or 0.1 ng/ml) alone or together with alum (20 and 10 μg/ml). The expression of IL-10 mRNA was determined in cells after 6 and 12 h by real-time PCR (B). Alum + LPS v LPS only. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. All results are representative of two independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
3.2.6 PLGA particles alone do not promote the upregulation of costimulatory molecules on the surface of BMDCs or induce IL-6 secretion.

The activation of DCs is an essential requirement for effective adjuvanticity. DC maturation involves the upregulation of surface markers including CD40, CD80, CD86 and MHCII. Many reported effects of particulate adjuvants have been due to endotoxin contamination and not the direct effects of the adjuvants themselves. Accordingly, the ability of PLGA particles to induce DC maturation and upregulate IL-6 secretion in DCs were assessed. Any contamination in the PLGA preparation would potentially correspond with an increase in DC maturation markers and IL-6 secretion in BMDCs.

BMDCs were incubated with medium or different sized PLGA particles (100 nm, 500 nm, 2 µm, 10 µm or 30 µm) at 0.25 mg/ml. Additionally, the TLR agonists LPS (5 ng/ml) and CpG (4 µg/ml) were used as positive controls to upregulate maturation markers. DCs were incubated with each stimulus for 24 h and surface marker expression was analysed by flow cytometry. Cells were gated on single live CD11c⁺ cells and analysed for upregulation of surface markers, with CD11c⁺ used as the pan-DC marker in these experiments. As expected, both LPS and CpG enhanced surface expression of CD40, CD80 and CD86, with an increased proportion of cells expressing MHCII rather than an overall increase in surface expression of this marker. None of the PLGA particle sizes tested significantly enhanced surface marker expression (Fig 3.11A).

IL-6 is one of the best readouts for endotoxin contaminations in DCs. LPS concentrations as low as 10pg/ml can induce secretion of IL-6 in DCs (536). Therefore, BMDCs and BMDMs from C57BL/6 mice were incubated with different sized PLGA particles (100 nm, 500 nm, 2 µm, 10 µm or 30 µm) at 0.25 mg/ml, LPS at varying concentrations (5, 1, 0.2 ng/ml) or CpG (4 µg/ml). Alum acted as a negative control, unable to elicit IL-6 secretion. PLGA particles of all sizes and alum failed to elicit the secretion of IL-6 in BMDCs or BMDMs (Fig 3.11B). In contrast, LPS and CpG induced a strong increase in IL-6 secretion (Fig 3.11B). This, suggested that all the PLGA particles used are endotoxin free, and any modulatory effects are directly due to the particles themselves.
Figure 3.11: PLGA particles regardless of size do not enhance maturation or IL-6 secretion.

A) BMDCs were left untreated or incubated with the indicated sizes of PLGA particles (0.25 mg/ml), LPS (5 ng/ml) and CpG (4μg/ml) for 24 h. CD11c+ cells were analysed for expression of CD40, CD80, CD86 and MHCII by flow cytometry. Immunofluorescence is shown for treated cells (blue) compared to untreated cells (red). Data are representative of two independent experiments.

B) BMDCs and BMDMs from C57BL/6 mice were incubated with medium or the indicated sizes of PLGA particles (0.25 mg/ml), LPS (5, 1 and 0.2 ng/ml), CpG (4 μg/ml) or alum (50 μg/ml) for 24 h. Levels of IL-6 in supernatants were determined by ELISA. Results are expressed as the mean ± SD of triplicate samples and are representative of three independent experiments. medium v adjuvant/TLR agonists. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
3.2.7 PLGA particle mediated cytokine modulation in BMDC is not due to toxicity in vitro

Vaccine adjuvants such as alum are known to induce cytotoxicity and local inflammation at the injection site, which may contribute to their adjuvanticity (515, 537). Therefore, the cytotoxicity of PLGA particles on BMDCs was assessed and whether they exhibit a similar toxicity profile to other particulate adjuvants was investigated. In tandem, 50 nm PS particles were tested, which have been known to induce cell death in vitro and in vivo (Sharp, PhD thesis 2010) as was alum which causes necrosis in cells when phagocytosed.

To determine whether PLGA particles can induce apoptosis or necrosis, Annexin V and PI were used. Propidium iodide (PI) can bind nucleic acids in necrotic cells. One of the early changes in apoptotic cells is translocation of phosphatidylserine (PS) to the outer surface of the plasma membrane, which can be detected by flow cytometry with the use of fluorochrome-conjugated annexin V. In order to determine whether PLGA particles can induce necrosis or apoptosis, PI and FITC-conjugated annexin V (AnnV-FITC) was used. BMDCs were incubated with alum or actinomycin D/cycloheximide, which were used as positive controls to induce necrosis and apoptosis respectively.

BMDCs were incubated with medium, increasing concentrations of PLGA particles, PS particles and alum (1-250 µg/ml) and different particle sizes (50 nm, 100 nm, 500 nm, 10 µm and 30 µm). After 24 h (or 4 h for controls), DCs were collected and stained for CD11c, annexin V and PI and analysed by flow cytometry to distinguish between apoptotic and necrotic cell death. As expected, alum induced only necrosis, while actinomycin D and cycloheximide triggered apoptosis in BMDCs after 4 and 24 h (Fig 3.12B). BMDCs incubated with most of the PLGA particles did not induce apoptosis or necrosis after 24 h (Fig 3.12A). At higher concentrations, there was an increase in apoptosis with the 10 µm PLGA particles which could be mediating IL-1β production, although this was not significant (Fig 3.12A). To further confirm these results, supernatants of each stimulation will be used to measure cell death using an LDH assay in the future.

Interestingly, BMDCs incubated with 50 nm PS particles only induced apoptosis (Fig 3.12B), a profile that was not seen in the 100 nm PLGA particles. This could be why 50 nm PS particles strongly enhance IL-1β secretion while in the case of 100 nm PLGA particles there was no significant increase in IL-1β production. With nanoparticles, a difference of 50 nm could
potentially change its cytotoxic profile. Unfortunately, 50 nm PLGA particles were not available to address this issue.

A.

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**Figure 3.12:** PLGA particles are not highly toxic to dendritic cells.

A) BMDCs from C57BL/6 mice were stimulated with medium or the indicated concentrations of PLGA particles (0.063-1 mg/ml) at different sizes (100 nm, 500 nm, 10 µm and 30 µm). After 24 h, cell death was assessed by Annexin V and PI staining on CD11c⁺ dendritic cells. Cells were analysed by flow cytometry with representative results from one experiment shown. Results are representative of two independent experiments.
B) BMDCs were incubated with medium, alum (1-250 µg/ml) or PS particles (0.063-1 mg/ml) overnight (o/n), actinomycin D (5 µM) or cycloheximide (5 µg/ml) for either 4 h or left o/n. Cells were stained with CD11c, annexin V-FITC and PI. Cell death was assessed by flow cytometry. Results were analysed to measure annexin V positive apoptotic or PI positive necrotic cells and graphed. Results are representative of two independent experiments. Error bars show mean ± SD of per experimental group tested in triplicate. Medium v treatment. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
3.2.8 **PLGA particles can modulate the potential of DCs to drive T cell proliferation and cytokine production in a size dependent manner.**

A DC-OVA transgenic (OTII) T cell *in-vitro* co culture assay was used to determine how PLGA particle size regulates DC induced T cell proliferation, IFN-γ, IL-4, IL-10 and IL-17 production. OTII TCR-transgenic mice were used which only have MHC class II-restricted, ovalbumin-specific CD4+ T cells. BMDCs generated from C57BL/6 mice were incubated with either endotoxin free OVA protein (25 µg/ml), LPS (5 ng/ml) or different sizes of PLGA particles (0.1 mg/ml) alone or in combination with each other overnight to allow the uptake, processing and presentation of OVA. DCs were then washed and incubated with OTII T cells at a ratio of 1:10 (1x10⁴ DCs: 1x10⁵ OTII T cells) and left for 72 h. T cells were analysed by flow cytometry for proliferation by CFSE staining and cytokine production. DCs incubated with medium, LPS (1 ng/ml) only, OVA (25 µg/ml) only or OVA (25 µg/ml) and LPS (1 ng/ml) were used as controls.

All combinations of LPS, OVA and different sized particles were also added to T cells in the absence of DCs, to rule out any nonspecific effects of the PLGA particles and OVA on T cells. As expected, there was no increase in proliferation or cytokine production (data not shown) with any of the stimulations. Therefore, any differences were due to the particle modulation of DCs which subsequently regulate T cell responses.

OTII T cells were stained with CFSE (5 µM) before co-culturing with BMDCs, after 72 h any new generation of proliferating cells will be represented by individual peaks on the histogram plot (proliferation measured by CFSE dilution). Incubation of DCs with 500 nm PLGA particles results in increased levels of IL-10 secretion (Fig 3.4), and here it was found that DCs incubated with these particles down regulate CD3+CD4+ T cell proliferation (Fig 3.13 and 3.17). In contrast, incubation of DCs with antigen and any of the other PLGA particle sizes resulted in a significant increase in T cell proliferation. This particle-mediated enhancement was observed when DCs were incubated with OVA and PLGA particles alone or in the presence of LPS (Fig 3.13 and 3.17). PLGA particles alone or in combination with LPS (1 ng/ml) were unable to promote proliferation demonstrating that DCs require OVA antigen to be processed and presented on MHCII to influence T cell expansion.
CD3^+CD4^+ T cells were also stained for IL-10, IL-4, IL-17 and IFN-γ to assess whether polarisation to a specific T helper cell response occurred. Using the same stimulations and controls as before, cells were stained after 72 h incubation. Similar to the proliferation results, exposure of DCs to OVA and PLGA particles of all sizes other than 500 nm resulted in increased IFN-γ production by OTII T cells compared to OVA alone (Fig 3.14 and 3.17). Incubation with 100 nm particles and OVA enhances gamma production compared to OVA alone but this is not the case with 500 nm particles. Furthermore, when PLGA particles and OVA were mixed with LPS (Fig 3.14 and 3.17), only 500 nm PLGA particles could block the LPS induced effect on IFN-γ production by OTII T cells. Interestingly, exposure of DCs to the larger particles (10 µm and 30 µm) increased the percentage of IL-4 expressing T cells, while no significant difference in IL-4 expression was observed in DCs incubated with the 100 nm PLGA particles when compared to the controls (Fig 3.15 and 3.17). Furthermore, DCs incubated with OVA and the 500 nm PLGA particles decreased IL-4 production mimicking their ability to inhibit IFN-γ (Fig 3.15 and 3.17).

As 500 nm PLGA particles can enhance IL-10 responses in DCs, there was also a possibility that this anti-inflammatory cytokine could promote Treg cells and thus a corresponding increase in IL-10 production from T cells. However, no increase in T cell IL-10 production was observed using any of the PLGA particle sizes assessed (Fig 3.16 and 3.17). Although IL-10 is associated with a Treg response, other cytokines such as TGF-β, IL-15 and IL-2 are more important at promoting the differentiation of Treg cells. Additionally, none of the PLGA particle sizes tested modulated the ability of DCs to promote Th17 responses (data not shown).

The experiment was repeated using IL-10^-/- DCs to see if either the inhibition of proliferation or IFN-γ induced by 500 nm PLGA could be reversed. Stimulating IL-10^-/- DCs with OVA and LPS resulted in similar proliferation and IFN-γ levels when compared to WT DCs. The capacity of 500 nm PLGA particles to inhibit OTII T cell proliferation was partially reversed when IL-10^-/- DCs were used (Fig 3.18), indicating IL-10 has a significant role in preventing proliferation. However, IFN-γ levels were not significantly reversed (Fig 3.19), this may be due to the ability of the 500 nm PLGA particles to also inhibit IL-12p70 secretion, which is known to drive IFN-γ in T cells.
Figure 3.13: Exposure of DCs to 500 nm PLGA particles inhibits their capacity to promote OTII T cell proliferation

BMDCs (2x10^4 cells/ml) were stimulated with medium, LPS (1 ng/ml), OVA protein (25 µg/ml) or different sized PLGA particles (0.1 mg/ml) alone or in combination with each other overnight. The following day, OTII T cells were isolated, stained with CFSE (5 µM) and co-cultured (2x10^5 T cells/well) with BMDCs for 72 h. Cells were then counted, stained and analysed for proliferation by flow cytometry. T cells were gated as CD3^+CD4^+, only including single and live cells (Aqua LIVE/DEAD negative). CD3^+CD4^+ T cells were then analysed for CFSE dilution on histograms to measure the extent of proliferation. Results are representative of two independent experiments.
Figure 3.14: Exposure of DCs to 500 nm PLGA particles inhibits their capacity to drive IFN-γ expression in OTII T cells

BMDCs (2x10⁴ cells/ml) were stimulated with medium, LPS (1 ng/ml), OVA protein (25 µg/ml) or different sized PLGA particles (100 nm, 500 nm, 10 µm and 30 µm) at a concentration of 0.1 mg/ml alone or in combination with each other overnight. OTII T cells were isolated, counted and co-cultured (2x10⁵ T cells/well) with BMDCs for 72 h. After harvesting, cells were again counted and stained for surface markers and intracellular cytokines by flow cytometry. T cells were gated as CD3⁺CD4⁺, only including single and live cells (Aqua LIVE/DEAD negative). CD3⁺CD4⁺ T cells were then analysed for IFN-γ production. Results are representative of two independent experiments.
Figure 3.15: Exposure of DCs to 10 µm or 30 µm PLGA particles enhances their capacity to promote IL-4 production in OTII T cells

BMDCs (2x10^4 cells/ml) were stimulated with medium, LPS (1 ng/ml), OVA protein (25 µg/ml) or different sized PLGA particles (100 nm, 500 nm, 10 µm and 30 µm) at a concentration of 0.1 mg/ml alone or in combination with each other overnight. OTII T cells were isolated, counted and co-cultured (2x10^5 T cells/well) with BMDCs for 72 h. After harvesting, cells were again counted and stained for surface markers and intracellular cytokines by flow cytometry. T cells were gated as CD3^+CD4^+, only including single and live cells (Aqua LIVE/DEAD negative). CD3^+CD4^+ T cells were then analysed for IL-4 production. Results are representative of two independent experiments.
Figure 3.16: Exposure of DCs to PLGA particles does not enhance their capacity to promote IL-10 expression in OTII T cells

BMDCs (2x10^4 cells/ml) were stimulated with medium, LPS (1 ng/ml), OVA protein (25 μg/ml) or different sized PLGA particles (100 nm, 500 nm, 10 μm and 30 μm) at a concentration of 0.1 mg/ml alone or in combination with each other overnight. OTII T cells were isolated, counted and co-cultured (2x10^5 T cells/well) with BMDCs for 72 h. After, cells were again counted and stained for surface markers and intracellular cytokines by flow cytometry. T cells were gated as CD3+CD4+, only including single and live cells (Aqua LIVE/DEAD negative). CD3+CD4+ T cells were then analysed for IL-10 production. Results are representative of two independent experiments.
Figure 3.17: Exposure of DCs to 500 nm PLGA particles inhibits their capacity to promote proliferation and IFN-γ expression in OTII T cells

Wild type BMDCs (2x10⁴ cells/ml) were stimulated with medium, LPS (1 ng/ml), OVA protein (25 µg/ml) or different sized PLGA particles (100 nm, 500 nm, 10 µm and 30 µm) at a concentration of 0.1 mg/ml alone or in combination with each other overnight. OTII T cells were isolated, counted and co-cultured (2x10⁵ T cells/well) with BMDCs for 72 h. After, cells were again counted and stained for surface markers and intracellular cytokines by flow cytometry. T cells were gated as CD3⁺CD4⁺, only including single and live cells (Aqua LIVE/DEAD negative). Graphs represent the percentage of proliferating OTII T cells (A), IFN-γ⁺ OTII T cells (B), IL-4⁺ OTII T cells (C) or IL-10⁺ OTII T cells (D) based on number of live CD3⁺CD4⁺ T cells. OVA v PLGA. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Error bars show means ± SEM for 2 or 3 independent experiments.
Figure 3.18: PLGA particle induced inhibition of OTII T cell proliferation is partially reversed using IL-10⁻/⁻ BMDCs

Wild type or IL-10⁻/⁻ BMDCs (2x10⁴ cells/ml) were stimulated with LPS (1 ng/ml) and OVA protein (25 µg/ml) only or also with different sized PLGA particles (0.1 mg/ml) overnight. The following day, OTII T cells were isolated, stained with CFSE (5 µM) and co-cultured (2x10⁵ T cells/well) with either wild type or IL-10⁻/⁻ BMDCs for 72 h. Cells were then counted, stained and analysed for proliferation by flow cytometry. T cells were gated as CD3⁺CD4⁺, only including single and live cells (Aqua LIVE/DEAD negative). CD3⁺CD4⁺ T cells were then analysed for CFSE dilution on histograms to measure the extent of proliferation. Results are representative of two or three independent experiments. OVA v PLGA or WT v IL-10⁻/⁻. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Error bars show means ± SEM for 2 or 3 independent experiments.
Figure 3.19: PLGA particle induced inhibition of OTII T cell IFN-γ expression remains unchanged using IL-10−/− BMDCs

BMDCs (2x10^4 cells/ml) were stimulated with LPS (1 ng/ml) and OVA protein (25 µg/ml) only or also with different sized PLGA particles (0.1 mg/ml) overnight. The following day, OTII T cells were isolated, stained with CFSE (5 µM) and co-cultured (2x10^5 T cells/well) with either wild type or IL-10−/− BMDCs for 72 h. After harvesting, cells were again counted and stained for surface markers and intracellular cytokines by flow cytometry. T cells were gated as CD3+CD4+, only including single and live cells (Aqua LIVE/DEAD negative). CD3+CD4+ T cells were then analysed for IFN-γ production. Results are representative of two or three independent experiments. OVA v PLGA or WT v IL-10−/−. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Error bars show means ± SEM for 2 or 3 independent experiments.
The capacity of PLGA particles to enhance DC IL-10 production peaks around the 500 nm size range

Having established that PLGA particles of 500 nm in size promote IL-10 responses in DCs, whether this was specific to one size or occurred across a specific range of particle sizes was examined. In terms of size, the difference between 500 nm and 10 µm PLGA particles is substantial so an additional group with 2 µm PLGA particles was included.

BMDCs stimulated with LPS (5, 1 and 0.2 ng/ml) were co-incubated with PLGA particles of different sizes (100 nm, 500 nm, 2 µm, 10 µm or 30 µm) at a range of concentrations for 24 h. Secretion of IL-10, IL-12p70 and IL-1β was determined by ELISA. In addition to the IL-10 promoting effect of 500 nm PLGA particles, 2 µm PLGA particles were also able to significantly enhance IL-10 secretion (Fig 3.20), thus confirming that particle induced IL-10 occurs over a specific size range while outside this range, particles do not enhance IL-10 secretion.

In parallel, secretion of IL-12p70 was assessed to address whether the particle size range capable of promoting IL-10 corresponded with that capable of inhibiting IL-12. All PLGA particles within the 500 nm-10 µm range have the ability to inhibit IL-12p70 secretion by DCs (Fig 3.21).

Moreover, 2 µm PLGA particles like 10 µm particles can increase IL-1β secretion by DCs. The data shows that the 500 nm and 2 µm PLGA particles display different properties despite being very similar in size (Fig 3.22).

Finally, whether 2 µm PLGA particles induced a similar effect to 500 nm PLGA particles in the DC-OTII co-culture assay was investigated. As in the case of 500 nm PLGA particles, incubation of DCs with 2 µm PLGA particles in the presence of OVA and LPS could also inhibit OTII T cell proliferation (Fig 3.18) and IFN-γ production (Fig 3.19). However, reversal of T cell proliferation was not as strong when using IL-10+ DCs when compared to the 500 nm PLGA particles (Fig 3.18). This may be due to the different immunomodulatory properties of the 2 µm PLGA particles, as they can significantly enhance IL-1β levels in BMDCs while 500 nm PLGA particles lack this ability (Fig 3.22). As expected, the decrease in IFN-γ levels remained unchanged when 2 µm particles were incubated with IL-10+ DCs (Fig 3.19).
Figure 3.20: Figure 3.18: The capacity of PLGA particles to induce IL-10 secretion by DCs is restricted to a narrow size range

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with 5 (B), 1 (C) and 0.2 ng/ml (D) LPS or different sizes of PLGA particles (100 nm, 500 nm, 2 µm, 10 µm and 30 µm) at concentration of 250 µg/ml alone (A) or together for 24 h. Concentrations of IL-10 were measured in supernatants by ELISA. PLGA + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.21: PLGA particles of between 500 nm and 10 µm in size can inhibit IL-12p70 secretion by DCs

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with 5 (B), 1 (C) and 0.2 ng/ml (D) LPS or different sizes of PLGA particles (100 nm, 500 nm, 2 µm, 10 µm and 30 µm) at concentration of 250 µg/ml alone (A) or together for 24 h. Concentrations of IL-12p70 were measured in supernatants by ELISA. PLGA + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.22: PLGA particles of between 500 nm and 10 μm in size can enhance IL-1β secretion by DCs.

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with 5 (B), 1 (C) and 0.2 ng/ml (D) LPS or different sizes of PLGA particles (100 nm, 500 nm, 2 μm, 10 μm and 30 μm) at concentration of 250 μg/ml alone (A) or together for 24 h. Concentrations of IL-1β were measured in supernatants by ELISA. PLGA + LPS v LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
3.2.10 **Size dependent PLGA particle-induced cytokine modulation in DCs is also seen in combination with alternative TLR ligands.**

Having established the size range in which PLGA particles modulate cytokine responses in BMDCs and BMDMs, whether this effect was specific to the TLR4 ligand (LPS) was investigated. BMDCs were stimulated with different TLR agonists including the TLR7/8 agonist R848, TLR 1/2 agonist PAM3CSK4 and the TLR9 agonist CpG. The TLRs 1 and 2 like TLR 4 are found on the cell surface while both R848 and CpG have their receptors in intracellular endocytic compartments (Fig 1.2). Each of these receptors have also been targeted by new vaccine adjuvants (29).

In order to determine the effect of using different TLR agonists on PLGA induced cytokine modulation, BMDCs were co-stimulated with different concentrations of either R848 (10, 1 or 0.1 µg/ml), Pam3CSK4 (500, 100, 1 or 0.1 ng/ml) or CpG (5, 1 or 0.1 µg/ml) alone or together with PLGA particles across a range of sizes (0.25 mg/ml). After 24 h, supernatants were collected and analysed by ELISA for IL-12p70, IL-10 and the proinflammatory cytokine IL-1β. As before, PLGA particles regardless of size did not induce cytokine secretion without the presence of a TLR agonist.

Both the combination of PLGA particles with the TLR7/8 agonist R848 (Fig 3.23) and TLR 1/2 agonist Pam3CSK4 (Fig 3.24) induced a similar cytokine pattern to particles + LPS, where 500 nm-2 µm PLGA particles strongly enhanced IL-10 secretion and 500 nm-10 µm PLGA particles inhibited IL-12p70 production. However, only the combination of particles and the TLR 1/2 agonist could drive IL-1β production (Fig 3.24), indicating that this effect could depend on activation on the cell surface only.

Surprisingly, BMDCs stimulated with CpG and different sizes of PLGA particles had an opposite effect on IL-10 secretion, as 500 nm and 2 µm PLGA particles inhibited CpG-induced IL-10 secretion (Fig 3.25). Although, 500 nm-10 µm PLGA particles inhibited IL-12p70 production, indicating a shared mechanism for all the TLR agonists (Fig 3.23, 3.24 and 3.25).
Figure 3.23: Size dependent modulation of DC cytokine secretion by PLGA particles in the presence of the TLR7/8 agonist R848

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with R848 (10, 1 or 0.1 µg/ml) or different sizes of PLGA (100 nm, 500 nm, 2 µm 10 µm and 30 µm) at concentration of 250 µg/ml alone or together for 24 h. Concentrations of IL-10, IL-12p70 and IL-1β were measured in supernatants by ELISA. PLGA + R848 v R848. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.24: Size dependent modulation of DC cytokine secretion by PLGA particles in the presence of the TLR2/1 agonist Pam3CSK4

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with Pam3CSK4 (500, 100, 1 or 0.1 ng/ml) or different sizes of PLGA (100 nm, 500 nm, 2 µm 10 µm and 30 µm) at concentration of 250 µg/ml alone or together for 24 h. Concentrations of IL-10, IL-12p70 and IL-1β were measured in supernatants by ELISA. PLGA + Pam3CSK4 v Pam3CSK4. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.25: Size dependent modulation of DC cytokine secretion by PLGA particles in the presence of the TLR9 agonist CpG.

BMDCs from C57BL/6 (6.25x10^5 cells/ml) mice were stimulated with CpG (5, 1 or 0.1 µg/ml) or different sizes of PLGA (100 nm, 500 nm, 2 µm 10 µm and 30 µm) at concentration of 250 µg/ml alone or together for 24 h. Concentrations of IL-10, IL-12p70 and IL-1β were measured in supernatants by ELISA. PLGA + CpG v CpG. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
3.2.11 The size-dependent ability of PLGA particles and alum to enhance IL-10 translates to human monocytes

Whether the ability of certain sizes of PLGA particles and alum to drive IL-10 seen in murine BMDCs and BMDMs could translate to a human context was investigated. A key issue in vaccine development is whether novel adjuvants tested in animals have the same immunostimulatory potential, efficacy, toxicity and pharmacokinetics in humans. If the effects described in mice also translate to humans, this could help justify further development of the adjuvant and potential movement into early clinical trials in addition to helping explain how licensed adjuvants (e.g. alum) work in humans.

To determine the effect of different PLGA particle sizes (100 nm, 500 nm, 2 µm, 10 µm and 30 µm) on IL-10 production by human monocytes, varying concentrations of LPS (10, 1 and 0.1 ng/ml) were co-incubated with PLGA particles (0.25 mg/ml) for 6 individual donors. After 24h supernatants were analysed by ELISA for IL-10. PLGA particles alone did not induce IL-10 secretion in non LPS treated monocytes (Fig 3.26). As with murine DCs and Macrophages, the capacity of PLGA particles to enhance IL-10 productions peaks between 500 nm-2 µm (Fig 3.26).

It also remains unclear whether alum has similar effects in terms of cytokine production/modulatory properties on human and mouse antigen-presenting cells. Like BMDCs and BMDMs, human monocytes co-incubated with LPS (10, 1 and 0.1 ng/ml) and alum (20 µg/ml) exhibited elevated IL-10 production in human monocytes from 6 individual donors when compared to LPS alone (Fig 3.27).
Figure 3.26: PLGA particles between 500 nm and 10 µm in size enhance IL-10 secretion by human monocytes

Human monocytes isolated from 6 donors (1x10^6 cells/ml) were stimulated with 10 (B), 1 (C) or 0.1 ng/ml LPS (D) or different sizes of PLGA (100 nm, 500 nm, 2 µm, 10 µm and 30 µm) at concentration of 250 µg/ml alone (A) or together for 24 h. Concentrations of IL-10 were measured in supernatants by ELISA. Results are representative of three independent experiments. PLGA + LPS v LPS. A paired non-parametric T test where *p<0.05, **p<0.01, ***p<0.001. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 3.27: Alum enhances IL-10 secretion in human monocytes

Human monocytes isolated from 6 donors (6.25x10^5 cells/ml) were stimulated with LPS (10, 1 and 0.1 ng/ml) or alum (20 µg/ml) alone or together for 24 h. Concentrations of IL-10 were measured in supernatants by ELISA. Results are representative of three independent experiments. PLGA + LPS v LPS. A paired non-parametric T test where *p<0.05, **p<0.01, ***p<0.001. Error bars show means ± SD for each experimental group tested individually in triplicate.
3.2.12 Intramuscular injection of PLGA particles promotes innate and adaptive immune cell recruitment into the injection site

Cell recruitment is an important characteristic of effective adjuvants. However, many adjuvant studies have been using intra peritoneum (i.p) and subcutaneous (s.c) routes of injection to analyse cell recruitment and inflammatory profiles of various adjuvants. Most human vaccines are administered intramuscularly. Therefore, an intramuscular (i.m) model was used to investigate cell infiltration and its kinetics into the site of injection.

To determine whether injection of PLGA particles can promote the recruitment of a distinct repertoire of innate immune and adaptive immune cells into the muscle after injection, mice were injected intramuscularly with 500 nm PLGA particles or PBS. 24 and 72 h after injection, muscle tissue was isolated containing the site of injection, treated with collagenase to break down the muscle fibres and stained for markers of both innate (neutrophils, monocytes, dendritic cells) and adaptive (B cell, CD4+ and CD8+ T cells) cells (Fig 3.28). Firstly, acquired cells were gated using forward and side scatter parameters, to exclude any debris and only include single cells, followed by exclusion of dead cells using the Aqua LIVE/DEAD stain. Cells were stained for CD11b+ and other surface markers for immune cells (Fig 3.28).

Intramuscular injection of 500 nm PLGA particles causes a significant increase in the infiltration of neutrophils, monocytes, DCs and B cells in terms of percentage and total number that peaks after 24 h (Fig 3.29 and 3.30). By 72 h after injection the particles were unable to sustain the recruitment of innate and adaptive cells in the muscle, with only CD19+ B cells remaining at a higher level than in PBS injected mice (Fig 3.29 and 3.30).
Figure 3.28: Gating strategy for innate and adaptive cell infiltration in the muscle.

Muscle cells from C57BL/6 mice were isolated 24 h post PBS or PLGA particle injection and were stained with Aqua LIVE/DEAD and antibodies against CD11b, F4/80, Gr-1, CD11c, Ly6C, CD11b, CD4, CD8 and CD19. Cells were acquired using a BDFortessa flow cytometer.
Figure 3.29: Characterisation of innate and adaptive cell infiltration after i.m injection of PLGA particles

Mice were sacrificed 24 h or 72 h following intramuscular injection and muscle tissue incorporating the site of injection was isolated and cells liberated using collagenase and DNAase. Cells were counted, stained and analysed by flow cytometry on live single CD11b+ cells for the presence of infiltrating neutrophils (F4/80lowGR-1_{high}Ly6C_{low}), DCs (GR-1_{low}CD11c_{high}MHCII_{high}), monocytes (CD11c_{low}MHCII_{low}Ly6C_{high}F4/80_{low}), Macrophages (CD11c_{low}MHCII_{low}Ly6C_{high}F4/80_{high}) and eosinophils (F4/80_{high}Ly6C_{low}). Cells were also stained for infiltrating adaptive immune cells, including B cells (CD19+) and CD3+CD4+ T Cells or CD3+CD8+ T cells. Results are representative of two independent experiments with 3-5 mice per experimental group.
Figure 3.30: i.m injection of PLGA particles causes innate and adaptive immune cells to infiltrate the site of injection after 24 h.

C57BL/6 mice were injected intramuscularly with PBS or 500 nm PLGA particles (1mg/mouse). Mice were sacrificed 24 or 72 h following injection and the muscle was isolated. Cells were counted, stained and analysed by flow cytometry for the presence of infiltrating neutrophils, monocytes, dendritic cells, B cells and CD11b⁺ immune cells. PBS v PLGA. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SEM for 3-5 mice per experimental group tested in triplicate.
3.2.13 Injection of 500 nm PLGA particles and alum can promote IL-10 production and inhibit Th1 associated responses in vivo

Having established that PLGA particles of 500 nm in size can modulate IL-10 production in vitro, the ability of PLGA particles to promote IL-10 responses in vivo was investigated. Previous findings in the lab using alum (Ewa Oleszycka, PhD 2014) have indicated that the IL-10 enhancement observed in vitro with DCs and macrophages was also seen following injection of mice. For example, i.p injection of alum was able to enhance IL-10 production in the draining mediastinal lymph nodes, while subcutaneous injection of alum primed cells to secrete IL-10 in the spleen. Furthermore, subcutaneous injection of PS and PLGA particles in the 1 µm size range increased IL-10 production in the spleen and lymph nodes (Fiona Sharp, PhD Thesis). However, most licensed vaccines are administered by the intramuscular route. Therefore, to determine if the immunomodulatory effects observed with PLGA particles can be translated in vivo using a clinically relevant route, mice were injected with 500 nm PLGA (1mg) or PBS intramuscularly (i.m) and sacrificed either 24 h or 72 h post injection.

Both stimuli for innate pathogen recognition receptors (LPS, CpG and HK E. coli) and T cells (anti-CD3 alone or together with PMA) were used to determine if 500 nm PLGA particle injection primed for enhanced IL-10 production. Cells from the draining lymph nodes (Inguinal and Popliteal nodes) and spleen were isolated, plated and restimulated ex vivo anti-CD3 or anti-CD3 in combination with phorbol 12-myristate 13-acetate (PMA) for 72 h to activate T cells or LPS, heat-killed Escherichia coli (HK E. coli) and CpG for 24 h to activate innate immune cells such as macrophages and/or DCs as well as B cells. Supernatants were collected and IL-10 was measured. Interestingly, injection of 500 nm PLGA particles primed cells in the inguinal nodes to secrete enhanced IL-10 when restimulated with anti-CD3 and PMA, LPS, HK E. coli or CpG 72 h post injection (Fig 3.31). No significant differences in IL-10 secretion were observed in either the popliteal lymph nodes or spleen (Fig 3.31).

Additionally, cytokines specific for T cell subsets were also analysed. Specifically, IFN-γ, IL-4, and IL-17 were measured to characterise the ability of PLGA particles to drive secretion of Th1, Th2 or Th17 cell associated cytokines responses respectively. However, no significant modulatory effects were observed for IFN-γ, IL-4, and IL-17 in the inguinal nodes (Fig 3.32) as well as the spleen and popliteal nodes (data not shown). Although, there was an increase
Having established the timepoint at which priming for IL-10 production peaks after injection of 500 nm PLGA particles, this experiment was repeated with injection of other PLGA particle sizes (100 nm, 10 μm and 30 μm) to see if particle induced priming for IL-10 production in vivo is size dependent. PLGA particles (0.5mg/leg) were injected intramuscularly and cells from the inguinal lymph nodes were isolated and restimulated ex vivo as before. As seen in vitro, 500 nm PLGA particles most potently primed inguinal lymph node cells to secrete IL-10 when restimulated with HK E. coli, CpG, CD3 and CD3 together with PMA (Fig 3.33). No differences in the IL-10 response was detected in the splenocytes (data not shown). Furthermore, the ability of i.m. alum injection to prime cells for enhanced IL-10 production was investigated. Similar to 500 nm PLGA particles, i.m. injection of alum also primes draining lymph node cells to secrete elevated concentrations of IL-10 (Fig 3.34).

Moreover, to determine the effect of IL-10 on the capacity of i.m. injected 500 nm PLGA particles to promote Th1 responses in a vaccination context was investigated. 100 nm PLGA particles were used to compare to the 500 nm PLGA particles, as they did not enhance IL-10 production in vitro or in vivo. WT and IL-10−/− mice were immunised i.m with PBS, OVA alone, 500 nm or 100 nm PLGA particles co-administered with OVA, boosted after 14 days and sacrificed on day 21 post immunisation. Splenocytes and ILNs were isolated and restimulated for 72 h with OVA ex vivo and antigen-specific IFN-γ and IL-10 concentrations were determined (Fig 3.35). Vaccination of WT mice with antigen and either 100 nm or 500 nm PLGA particles did not significantly enhance antigen-specific IFN-γ responses in splenocytes or nodes. However, in the absence of IL-10, injection of OVA and 500 nm PLGA particles promoted antigen-specific Th1 responses. This also occurs when IL-10−/− mice are immunised with OVA and alum in contrast to the case in WT mice (Oleszycka, McCluskey et al, in revision). Interestingly, there was no clear correlation between particle size and the induction of antigen-specific IL-10 responses as injection of the 100 nm PLGA particles promoted a stronger antigen-specific IL-10 response in the spleen and nodes than 500 nm particles.

Our lab has shown that alum (538) and 50 nm PS particles (Katie O’ Grady, PhD) drive antigen-specific CD8+ T cell responses following i.m injection, although, 50 nm PS particles were much stronger than alum at inducing antigen-specific CD8+ T cells responses and antigen-specific
cytokine production (IFN-γ and IL-10) following *ex vivo* re-stimulation (Fig 3.35). Whether these results seen in the case of PS particles translate to the same sizes of PLGA particles and the role IL-10 in this process was investigated.

Wild-type and IL-10-deficient mice injected intramuscularly with PBS, OVA or OVA with 50 nm PS, 100 nm PLGA or 500 nm PLGA particles on day 0 (prime) and day 14 (boost). Mice were sacrificed on day 21 and spleens, inguinal lymph nodes and muscle were isolated, processed and cells were analysed for OVA-specific CD8+ T cell responses using tetramer staining for flow cytometry and the gating strategy as outlined (Fig 3.36). Briefly, debris, doublets, dead cells, F4/80+, B220+ and CD11c+ cells were excluded. CD3+, CD8+ and CD44+ were included as an indication of activated effector T cells, antigen-specific T cells were detected using an ova-specific H-2K (b) MHCcl tetramer (Fig 3.36).

As expected, 50 nm PS were capable of driving a strong antigen-specific CD8+ T cell response in the spleen, nodes and muscle (Fig 3.37 and 3.38). There was not the same increased frequency of tetramer+ CD8 T cells when WT mice were immunised with 100 nm or 500 nm PLGA particles (Fig 3.37 and 3.38). However, when IL-10−/− mice were used, there was a significant increase in tetramer+ CD8+ T cells with 100 nm PLGA particles and while it was not significant, there was a higher percentage of tetramer+ CD8+ T cells in the mice injected with 500 nm PLGA particles. Interestingly, there was not an increase in antigen-specific CD8+ T cells when IL-10−/− mice were immunised with OVA and 50 nm PS particles.
Figure 3.31: i.m injection of 500 nm PLGA particles primes inguinal lymph node cells for enhanced IL-10 secretion

C57BL/6 mice were injected intramuscularly with PBS only or 500 nm PLGA particles (1mg/mouse) and sacrificed 24 or 72 h following injection. The inguinal and popliteal draining lymph nodes and spleen were isolated and plated. Splenocytes (2x10^6 cell/ml) and lymph node cells (1x10^6 cells/ml) were stimulated with medium, LPS (1 μg/ml), heat-killed *E. coli* (MOI 1:10) or CpG (5 μg/ml) for 24 h or anti-CD3 (200 ng/ml) alone or anti-CD3 with PMA (25 ng/ml) for 3 days. Supernatants were collected and analysed for IL-10 by ELISA. PBS v 500 nm PLGA (24 or 72 h). One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SEM for 4-5 mice per experimental group tested in triplicate.
Figure 3.32: i.m injection of 500 nm PLGA particles does not prime inguinal lymph node cells for enhanced IFN-γ, IL-4 or IL-17 secretion

C57BL/6 mice were injected intramuscularly with PBS only or 500 nm PLGA particles (1mg/mouse) and sacrificed 24 or 72 h following injection. The inguinal lymph nodes were isolated, plated (1x10^6 cells/ml) and stimulated with medium, LPS (1 μg/ml), heat-killed *E. coli* (MOI 1:10) or CpG (5 μg/ml) for 24 h or anti-CD3 (200 ng/ml) alone or anti-CD3 with PMA (25 ng/ml) for 3 days. Supernatants were collected and analysed for IFN-γ (A), IL-4 (B), and IL-17 (C) by ELISA. PBS v 500 nm PLGA (24 or 72 h). One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SEM for 4-5 mice per experimental group tested in triplicate.
Figure 3.33: 500 nm PLGA particles most potently prime inguinal lymph nodes to promote IL-10 secretion following i.m injection.

C57BL/6 mice were injected intramuscularly with PBS only or different sizes of PLGA particles (100 nm, 500 nm, 2 μm, 10 μm and 30 μm; 1mg/mouse) and sacrificed 72 h following injection. The inguinal lymph nodes were isolated, plated (1x10^6 cells/ml) and stimulated with, LPS (1 μg/ml), heat-killed *E. coli* (MOI 1:10) or CpG (5 μg/ml) for 24 h or anti-CD3 (200 ng/ml) alone or anti-CD3 with PMA (25 ng/ml) for 3 days. Supernatants were collected and analysed for IL-10 by ELISA. PBS v PLGA particles. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SEM for 4 mice per experimental group tested in triplicate.
**Figure 3.34: i.m injection of alum primes inguinal lymph node cells for enhanced IL-10 secretion**

C57BL/6 mice were injected intramuscularly with PBS only or alum (1mg/mouse) and sacrificed 72 h following injection. The inguinal lymph nodes were isolated, plated (1x10^6 cells/ml) and stimulated with medium, LPS (1 μg/ml), heat-killed *E. coli* (MOI 1:10) or CpG (5 μg/ml) for 24 h or anti-CD3 (200 ng/ml) alone or anti-CD3 with PMA (25 ng/ml) for 3 days. Supernatants were collected and analysed for IL-10 by ELISA. PBS v alum. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SEM for 4-5 mice per experimental group tested in triplicate.
Figure 3.35: In the absence of IL-10, injection of OVA and 500 nm PLGA particles promotes antigen-specific Th1 responses.

C57BL/6 WT and IL-10⁻/⁻ mice were immunised i.m. on day 0 with PBS or OVA (10 µg/mouse) either alone, or mixed with 100 nm PLGA, 500 nm PLGA or 50 nm PS particles (0.5 mg/leg). The mice were boosted with these same treatments on day 14 and were sacrificed on day 21. Spleen (2×10⁶ cells/ml) and inguinal lymph node cells (1×10⁶ cells/ml) were restimulated ex vivo with either medium alone, OVA (200 µg/ml or 20 µg/ml), heat-killed E. coli, ConA or anti-CD3 for 3 days. Supernatants were collected and analysed for IL-10 (A) or IFN-γ (B) by ELISA. OVA v OVA + particle or WT v IL-10⁻/⁻. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SEM for 4 mice per experimental group tested in triplicate.
Fig 3.36: Gating strategy for antigen-specific CD8+ T tetramer staining

Debris, doublets, dead cells (Aqua LIVE/DEAD+), F4/80+, B220+ and CD11c+ cells were excluded. CD3, CD8 and CD44 were gated on as an indication of activated effector T cells. Antigen-specific T cells were detected using ova-specific H-2K (b) MHCcl tetramer. Cells were acquired using BDFortessa flow cytometry.
Fig 3.37: IL-10 negatively regulates the induction of an antigen-specific CD8+ T cell response following i.m. injection with antigen and PLGA nanoparticles

Mice (C57BL/6 and IL-10-/-) were immunised i.m. with PBS, OVA alone (10 µg/mouse) or OVA in combination with 50 nm PS, 100 nm PLGA or 500 nm PLGA particles (0.5mg/leg) on days 0 and 14. Mice were sacrificed on day 21 and Spleens (A), Nodes (B) and Muscle (C) were isolated, processed and stained. The frequency of tetramer+ CD8+ T cells was examined by flow cytometry. Results are from one experiment. Representative FACS plots from 1 of 4 mice per experimental group are shown.
Fig 3.38: IL-10 negatively regulates the induction of an antigen-specific CD8+ T cell response following i.m. injection with antigen and PLGA nanoparticles

Mice (C57BL/6 and IL-10⁻/⁻) were immunised i.m. with PBS, OVA alone (10 µg/mouse) or OVA in combination with 50 nm PS, 100 nm PLGA or 500 nm PLGA particles (0.5mg/leg) on days 0 and 14. Mice were sacrificed on day 21 and Spleen (A), Nodes (B), Muscle (C) were isolated, processed and stained. Graphs represent the percentage of CD44+ tetramer+ cells based on number of live CD3+ CD8+ T cells and number of CD44+ tetramer+ cells per gram of tissue. Results are from one experiment. Error bars show means ± SEM for 4 mice per experimental group tested in triplicate. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
3.3 Discussion

This study aimed to investigate whether particulate vaccine adjuvants can mediate an immunosuppressive effect, making them inefficient at driving effective Th1 responses. For example, the principal vaccine adjuvant used in humans is alum, which can drive robust humoral responses but is not optimal for promoting effective cell mediated immunity needed for diseases such as HIV, TB and Malaria. Importantly, the majority of these adjuvants are within a narrow size range (1-20 µm) which could be an underlying reason for this phenomenon. By altering the size of adjuvants, one can therefore potentially affect their ability to drive cell mediated immunity.

Previous studies have stressed the importance of particle size in dictating particle-induced immunity. However, there are significant discrepancies in this field with some studies reporting that nano-particles and micro-particles induce Th1- and Th2-type responses respectively, whilst conversely other studies have reported the opposite. It was reported that nanoparticles were efficiently taken up by APCs leading to higher cellular response in comparison to microparticles (2–8 μm) (526) while Wenderof et al showed higher antibody titres using PLGA microparticles (~1 μm) over nanoparticles (110 nm) with two different antigens via three different routes of administration – intraperitoneal, intramuscular and intranasal (520, 539). Such inconsistencies may originate from the intrinsic differences in the materials as well as the difficulty in preparing particles with a narrow monodisperse and reproducible size distribution. There are advantages and disadvantages to using different sizes, nanoparticles are favoured for drug delivery and uptake as they can easily penetrate biological barriers while microparticles can sustain or delay release of antigen for longer periods. Yet the optimum size for generating a specific type of adaptive immune response remains unclear.

The activation of the innate immune system by particulate adjuvants is now regarded as the most important step for determining the type of immune response that develops. Dendritic cells are key in dictating the type of adaptive response that will develop in response to a particular antigen and adjuvant formulation. DCs are professional APCs with the unique ability to present antigen to naïve T cells. Particulate adjuvants can have a major influence on DC responses to antigens (540, 541). Combining an antigen with an adjuvant can enhance antigen uptake, target delivery to APCs or certain sites in the body, sustain the release of an antigen, release immunostimulatory DAMPs and even modulate cytokine secretion by DCs which can control the type and magnitude of the adaptive immune response induced.
Biodegradable polymers made from aliphatic polyesters such as poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) are commonly used. In particular, poly(lactide-co-glycolide) (PLGA) has been the most extensively investigated using either nano- or microparticles due to their inherent advantages. The co-polymers have the advantage of protecting and sustaining the release of an encapsulated therapeutic drug or protein over a period of days to several weeks. Furthermore, encapsulated proteins were demonstrated to improve efficiency of antigen uptake by APCs, leading to subsequent processing and presentation via both MHC class I and MHC class II pathways. Importantly, PLGA particles have been shown to be efficiently phagocytised by DCs and macrophages in vitro and in vivo, and exert immunostimulatory effects on DCs (542). Therefore, PLGA particles are considered to be a potent immunological adjuvant but how size influences the immune response remains unclear. PLGA particles can have many characteristics fine-tuned such as size to potentially improve subsequent humoral responses or antigen-specific cellular immunity.

It has been proposed that smaller particles have a larger surface area with increased inflammatory potential (543). However, our lab has demonstrated that injecting either 50 nm or 10 µm PS particles triggers an increase in inflammatory cells infiltrating the injection site. Interestingly, PS particles in the 1µm size range failed to generate a similar inflammatory response while depleting resident macrophage populations (Claire Hearnden, PhD 2014). The hypothesis underlying the current work was that particulate adjuvants within this size range promote anti-inflammatory responses, by increasing IL-10 secretion in APCs while simultaneously inhibiting IL-12, a cytokine needed to polarise naïve T cells to a Th1 type response. Interestingly, many studies using biodegradable particles have used an approximate 1µm size to optimise their vaccine components, neglecting optimising the particle size itself.

IL-10 has the capacity to limit Th1 responses while promoting Th2 differentiation (68). Concurrently, the absence of IL-12 production is a prerequisite for inflammatory DCs to be able to induce Th2 cell immunity. For instance, retroviral overexpression of IL-12 abolishes DC-driven Th2 cell development and subsequent asthma (544). Therefore, a combination of secreting excess IL-10 and downregulating IL-12 will lead to a poor induction of effector T cell responses.

The majority of licensed vaccines in humans favour a humoral/Th2 biased response. Even when adjuvants such as alum have been combined with other immunomodulatory molecules such as TLR agonists they were still limited in their ability to elicit protective cellular immune
responses. For example, alum has been combined with the TLR agonist MPL to improve humoral and cell mediated immunity but so far is only licensed for a cervical cancer vaccine. PLGA has been combined with a whole host of immune stimulating compounds, including TLR agonists, other adjuvants (chitosan) and immunomodulatory compounds with some mild success at enhancing cell mediated immunity (493, 501). In this regard, there is still an unfulfilled need for a safe and potent adjuvant for non-living vaccines capable of promoting cell mediated immunity. Therefore, this study aimed to address possible immunosuppressive effects of PLGA particles and alum and whether changing the size of these particles can reverse these responses to allow for more effective cell mediated immunity.

Firstly, by using PS particles covering the nano and micron scale, the ability to modulate TLR agonist-induced cytokine production in vitro was determined. Although PS particles are not approved for use in humans, as a material PS is widely used to elucidate the influence of particle size on the induction of immune responses (545-547). Particle sizes were chosen to represent sizes used in vaccines (1-10 µm) as well as smaller (50, 100 and 500 nm) and larger particles (30 and 100 µm) (511). This size range also covers the size ranges from virus to bacteria as well as some fungi and protozoa. Using only adjuvants within a narrow (suboptimal) size range could be a leading cause as to why many subunit vaccines trigger limited Th1 responses. Therefore, going outside this size range could potentially positively modulate the immune response. For example, the nano scale would represent the size of many viruses which require strong CTL and Th1 responses to provide protective immunity, while larger parasites (10-100 µm) require a very strong Th2 response. Interestingly, only PS particles in the 1-10 µm range were able to significantly increase IL-10 production in BMDCs. Furthermore, only injection (i.p) with 1 µm or 10 µm PS particles significantly upregulated IL-10 mRNA expression in PECs 3 h post injection in C57BL/6 mice (Hearnden, PhD 2014). In agreement with previous data from our lab using alum, PS particles also impaired IL-12p70 production, a response that again was size restricted with only 10 µm particles capable of modulating IL-12 production. Furthermore, this confirmed that IL-12p70 inhibition is not restricted to one type of particulate adjuvant.

Having established a size pattern with PS particles for modulating DC cytokine production, whether this could be translated to adjuvants which are more likely to be used in humans was investigated. As discussed, PLGA particles were chosen as the polymer is well tolerated in humans. A similar pattern emerged with PLGA particles as 500 nm-2 µm particles were
capable of promoting a strong IL-10 response, although it was a narrower range than in the case of PS particles. Interestingly, it has been demonstrated that PLGA particles with an average diameter of 250 nm-500 nm were able to induce tolerance (501), although the size of the particles was never stated to be a factor nor was whether IL-10 was responsible. Other particulates are also capable of driving this robust IL-10 response, it is not restricted to just PS and PLGA particles. alum, which has an average size of around 1-10 µm, was also able to significantly promote IL-10 secretion in BMDCs in vitro and in vivo at the injection site and draining lymph nodes (Oleszycka, McCluskey et al, in revision). Since many cell types can express and secrete IL-10 (372), IL-10 reporter mice were used to identify the cell types (data not shown) which indicated that DCs, Macrophages and neutrophils secreted the highest levels of IL-10 following injection over the control mice. Alum also strongly promoted IL-10 transcription and secretion in BMDMs, demonstrating that the DCs and macrophages in vitro data corresponds with in vivo data. This also shows that alum can directly modulate APCs, as the effects of alum and macrophages could be due to secondary effects resulting from DAMP release following alum injection (470, 548).

Inflammation has a very important role for determining the effectiveness of subunit vaccines, especially for alum and other particulate adjuvants (549). Our lab previously suggested that nanoparticles may induce harmful inflammation in the host, leading to a regulatory response that negatively influences the ensuing adaptive immune response. Conversely, large particles are recognised by the host and drive limited inflammation leading to an adaptive immune response that protects the host. This theory proved partially true, with the 50 nm PS particles driving the strongest IL-1β and IL-1α responses. However, for large particles only PS at a certain size can enhance IL-1β and IL-1α. In contrast to 50 nm PS particles, 100 nm PLGA particles were unable to promote IL-1β and IL-1α to the same extent. Several reasons could be responsible for this; the particle composition is different or else a difference of only 50 nm when using nanoparticles could dramatically change the response. For instance, 40 nm PS particles assessed as nano vaccines against tumours were more effective in terms of uptake and inducing humoral immunity and CD8+ responses than 100 nm, 500 nm, 1, and 2µm PS particles (523). IL-1β and IL-1α secretion by DCs was increased by 10 µm PLGA and PS particles which confirms a general effect of particles of this size regardless of composition. Furthermore, in-vivo data from our lab shows when comparing a broad range of particle sizes, that IL-1β is only present in the peritoneal exudate and serum of mice injected with 50 nm and 10 µm PS particles after 3 h (Hearnden, PhD 2014). This demonstrates a correlation between our in vitro and in
vivo while also showing that cytokine response can be influenced by the size of the particles used.

The particle size range for suppressing IL-12p70 secretion by DCs was broader. All PLGA particle sizes between 500 nm-10 µm demonstrated an ability to impair IL-12p70 production. Thus, this demonstrates that widely used vaccine adjuvants such as alum and particulates of a comparable size are potent inhibitors of TLR agonist-induced IL-12 secretion. The most widely used adjuvants in both preclinical and clinical use have multiple inhibitory effects on DCs by enhancing IL-10 and suppressing bioactive IL-12 secretion which surprisingly can be influenced by the size of the particles.

The bioactive IL-12p70 is composed of two subunits, IL-12p35 and IL-12p40. Both subunits are tightly regulated by separate pathways to prevent excessive Th1 responses. PLGA particles only inhibited expression of the IL-12p35 subunit. Interestingly, alum also only inhibits IL-12p35, but not IL-12p40 expression suggesting that many particulate adjuvants may have a selective inhibitory effect on Th1 responses through suppression of IL-12p35. It should be highlighted that some particulate adjuvants do not inhibit IL-12 secretion (e.g. chitosan), while combining particulates with certain TLR agonists (e.g. CpG) can drive a potent cell mediated response (476). It would be interesting to combine PLGA particles (e.g. 100 nm) which don’t inhibit IL-12 with CpG or other TLR agonist (e.g. MPL) to see if they also drive a robust Th1 response.

Many other cell types are capable of secreting IL-10 (143). Macrophages are one of the main producers of IL-10 in the innate immune system and like DCs play a key role in immune surveillance. Activation is often triggered by the same typical inflammatory stimuli such as LPS that induce the release of proinflammatory cytokines (147). IL-10 produced by macrophages is especially needed for immune homeostasis, tissue remodelling and wound repair. Whether the immunoregulatory effects of 500 nm PLGA particles can also be translated to BMDMs was examined. Confirming that this response was not just limited to DCs, only 500 nm PLGA particles were capable of significantly enhancing IL-10. Differentiated macrophages can be been broadly classified as M1 (inflammatory and Th1 response) or M2 (Th2 and immune regulatory), it would be intriguing to see if 500 nm PLGA particles can upregulate the IL-10-induced M2c subtype (550).

In order for DCs to initiate T cell immunity they must undergo activation by a process referred to as maturation, which is highly important for the initiation of adaptive immune responses.
The maturation of DCs is associated with increased expression of several cell surface markers, including the co-stimulatory molecules CD40, CD80, CD83, CD86 in addition to, MHC class I, and MHC class II (551). It is well known that DC maturation can be induced by inflammatory factors such as LPS, bacterial DNA, or cytokines such as TNF-α. It is possible that the interaction between DC and particulates induces the upregulation of co-stimulatory markers too. To address the question of whether PLGA particles could activate DCs, the ability of PLGA to induce DC maturation was studied. PLGA particles were unable to significantly enhance the expression of any co-stimulatory molecules on the surface of BMDCs. However, other adjuvants such as chitosan have shown the ability to induce maturation in BMDCs (552). In contrast, all PLGA particles tested here were unable to induce maturation. Incubation of BMDCs with alum like PLGA is insufficient to enhance surface expression of MHCII and other co-stimulatory molecules while other groups have stated that host DNA released in response to injection of aluminium adjuvants enhances MHCII mediated antigen presentation to prolong CD4 T cell interaction (467). This suggests that while particles are unable to activate DCs directly in vitro, they could potentially induce DC maturation indirectly in vivo.

IL-6 is a sensitive readout for LPS contamination after particle preparation. For example, an LPS concentration as low as 10pg/ml can promote IL-6 secretion by BMDCs (536). A lot of immunomodulatory effects stated for novel adjuvants can potentially be attributed to contaminating endotoxin instead of the particles themselves. All sizes of PLGA particles failed to induce secretion of IL-6 in both DCs and macrophages which along with their failure to promote DC maturation in vitro indicates that the preparations used were free of endotoxin such as LPS and any immunoregulatory effects were directly due to the particles themselves.

It has been reported that alum, which is regarded as a safe adjuvant, kills cells by necrosis in vitro, induces local cell death and releases endogenous danger signals at the site of injection (465). Other particulate adjuvants can also release danger signals and increase IL-1β concentrations (515, 549). Furthermore, by injecting particulates, necrosis is often observed around the site of injection. Interestingly, the PLGA particles tested here were unable to induce either necrosis or apoptosis. Alum as has been reported before can induce necrosis in BMDCs, a process which causes rapid release of DAMPS due to a loss of membrane integrity and cell swelling (553). In contrast, 50 nm PS particles were able to promote apoptosis which may be linked to the increased IL-1β secretion associated with these PS particles. While necrosis is associated with release of DAMPs, apoptosis is regarded as non-inflammatory, due to the fact that membrane integrity is maintained and the cell cannot release DAMPs (553). It is possible
that small differences in particle size can exert major effects as 100 nm PLGA particles did not trigger cell death while 50 nm PS particles can significantly induce apoptosis in BMDCs. However, it cannot be discounted that these differences are due to the different polymers used or other parameters such as surface charge. How 10 µm PLGA and PS particles drive an inflammatory response remains unclear, one explanation might be that DCs have trouble taking up the particles leading to frustrated phagocytosis while smaller particles will be easily endocytosed or phagocytosed and larger 30 µm particles are simply too large.

Particulates of a certain size (e.g. 500 nm PLGA and alum) could prevent development of a Th1 response through increased IL-10 and inhibition of IL-12p70. Consequently, the size of the particulate has proved to be an important factor for modulating immune responses via differential interactions with APCs. Having demonstrated that 500 nm PLGA particles can induce a unique response, whether this effect was specific for this one size or applied across a wider size range size was investigated. Therefore, 2 µm PLGA particles were chosen based on the polydispersity of the 500 nm and 10 µm PLGA particles. A small difference in size could potentially have a major effect on adjuvanticity. Gutierrez et al. demonstrated that vaccination with 1,000 nm-sized BSA-loaded PLGA particles elicited a higher serum IgG response than 500 or 200 nm-sized particles (554). Furthermore, the 500 nm particles elicited significantly higher serum IgG responses compared to 200 nm particles when administered intranasally, but no differences were observed by the subcutaneous or oral route. This suggests that the biodistribution of nano- and microparticles and the particle-related immune response can be regulated by controlling or changing the size of the particles. Here, both 500 nm and 2 µm PLGA particles were capable of enhancing IL-10, giving a distinct cut off for when particles will not promote IL-10 as well as confirming the effect is not restricted to one size. Yet, the 500 nm and 2 µm particles still had different qualities, as the 2 µm particles proved to be more inflammatory by increasing IL-1β levels, comparable to the 10 µm particles. As mentioned earlier, all particle sizes between 500 nm and 10 µm will suppress IL-12p70 secretion in LPS stimulated BMDCs.

An issue that needs to be addressed is size/weight in relation to the PLGA particles. In all experiments, the number of particles added was fixed by weight (e.g. 250 µg/ml) but since the diameters of the particles range from 100 nm to 30 µm, the molarity and number of particles present vary significantly. For example, using particles by concentrations you are comparing mass of particles being added to the cells. But because particles have different sizes and consequently volume (that is affected by the radius) and weight (since they are made from the
same material), you will have a lot more 100 nm PLGA particles compared to 30 μm PLGA particles which will affect surface area presented to cells. Nanomaterials are generally described as being more immunostimulatory than micron-sized particles of the same material, when tested at the same weight (555). However, the same might not be true when relating to surface area, as cells directly interact with the surface area presented to them. Our lab has already demonstrated that by using surface area (m²/g) relationships to normalise the dose of particles (using Brunauer, Emmett and Teller (BET) analysis), any differences observed in terms of inflammatory profile generated by hydroxyapatite (HA) particles were due to differences in size rather than surface area presented to dendritic cells (555). Therefore, PLGA particle dose will need to be normalised to ensure that differences in PLGA particle size rather than the surface area presented to APCs is responsible for the anti-inflammatory profile. The surface area of all the particles (\(A=4\pi r^2\)) will be calculated and then particles tested using the same area instead of concentration. This could also be done using volume (\(V=4/3\pi r^3\)) to estimate the number of particles being added to cells. Using surface area relationships or number of particles to normalise the dose, it will be demonstrated that any differences observed were due to size rather than surface area presented or the number of particles present.

These results indicate that DC treatment with different sized PLGA (or PS) particles has potential to act as a tool for immunomodulation by directing autologous T-cell responses. Nano particles (< 200 nm) resemble the size of viruses which require a robust Th1 response characterised by the secretion of IFN-\(\gamma\). In contrast, large 10 μm and 30 μm PLGA particles are comparable in size to schistosome, hookworm and large parasites which require a characteristic IL-4 dependent Th2 response. For example, intra-dermal administration of 40 nm carboxylated PS particles conjugated to OVA was found to induce the greatest IFN-\(\gamma\) CD8\(^+\) responses when comparing T cell responses induced by vaccination with particles from 20 nm to 2 μm in diameter (523). These results were also duplicated in studies on sheep (556, 557). Similarly, 200–600 nm nanoparticles encapsulating hepatitis B surface antigen (HBsAg) injected intramuscularly produced the strongest IFN-\(\gamma\) response, upregulation of MHCI and IgG2a titres whereas 2-8 μm PLA particles induced IL-4 responses (558), again indicating a Th1/Th2 bias between nano- and micro-particles. Furthermore, mice vaccinated with 300 nm sized PLG particles and OVA produced the strongest CTL response and IgG2a titres suggesting Th1-type immune responses compared to particles greater than 300 nm in size (1,7 and 17 μm) (524). Finally, a study demonstrated that 230 nm OVA-loaded lecithin/glyceryl monostearate
based nanoparticles injected subcutaneously were superior to 708 nm particles, in terms of antibody titres, OVA-specific cytotoxic T lymphocyte activity, inhibition of tumour growth and internalisation by various APCs (525). Therefore, size has proven very important in determining the effectiveness of promoting either a Th1 or Th2 response.

Limited knowledge is available on how PLGA and other particulates modulate the function of DCs and subsequent effects on CD4+ T-cell differentiation and activation. T cell proliferation is a key sign of their activation by DCs. PLGA nanoparticles have been described to enhance T cell activation upon nasal application. PLGA particles have been shown to enhance antigen presentation by DCs, as shown by increased in vitro and in vivo CD4+ T-cell proliferation (501, 530). Interestingly, all PLGA particle sizes other than the 500 nm PLGA could induce proliferation in OTII cells.

When DCs were treated with PLGA particles in the presence of the antigen (OVA), autologous CD3+CD4+ T cells co-cultured with these DCs showed differential expression levels of both IFN-γ and IL-4 depending on the size of the particles. IFN-γ is known as a cytokine representative for Th1 response while IL-4 is a Th2 associated cytokine. DCs treated with PLGA (particle size not stated) particles and co-cultured with T cells enhanced production of the Th1 associated cytokine, IFN-γ (559). They also stated that the biomaterial effects on DC phenotypes was due to the biomaterial themselves and not endotoxin associated with these materials (559). Remarkably, using 100 nm PLGA particles in the DC-OTII co-culture assay shifted the response in favour of IFN-γ. On the other hand, both 10 µm and 30 µm triggered a more biased IL-4 response suggesting Th2 polarisation. Mimicking the size of a particular pathogen with polymeric particles can generate the desired immune response which is most effective at clearing the pathogen. However, 500 nm PLGA particles show a decrease in both IFN-γ and IL-4 expression indicating that particles around this size range are favouring a more tolerogenic response. If both the IFN-γ and IL-4 levels are observed concurrently, the ratio shifts towards IL-4 and thus a Th2 response for the large micron sized particles (10 µm and 30 µm). In contrast, 100 nm PLGA particles favour IFN-γ, indicating that they could be the favoured size in adjuvants to promote a Th1 response.

It would be conceivable that the enhanced IL-10 secreted by DCs was responsible for a decrease in proliferation of the T cells (560). Therefore, IL-10−/− and wild type DCs were utilised in the DC-OTII co-culture system to see if a lack of IL-10 can reverse suppression of
proliferation and IFN-γ production by 500 nm PLGA particles. The lack of IL-10 produced by DCs led to partial reversal of proliferation, yet there was no change in IFN-γ production. This could be due to the ability of the 500 nm PLGA particles to also inhibit IL-12p70 production which is known to be a key regulator in priming T cell IFN-γ production (561).

Tregs are often associated with increased secretion of IL-10. Nasal administration and intramuscular injection of 400 nm PLGA nanoparticles was found to induce an immunoregulatory response as shown by enhanced FoxP3+ expression (501, 530). The OVA-encapsulated PLGA NP enhanced the induction of FoxP3+ T-cells via a TGF-β and RA dependent mechanism by increasing retinaldehyde dehydrogenase enzyme (RALDH) expression. As PLGA particles can enhance IL-10 production by DCs, it came as a surprise that there wasn’t a similar increase in IL-10 producing T cells. Alum injected i.p was also unable to induce the expansion of Foxp3+ regulatory T cells (Oleszycka, McCluskey et al, in revision). Finally, 250–500 nm TMC-TPP (N-trimethyl chitosan tripolyphosphate) nanoparticles and 50 nm gold nanoparticles have demonstrated an ability to induce Th17 polarisation (501, 562). However, in this study PLGA particles were unable to promote IL-17 production in T cells after 3 days.

It has been reported that monocytes are recruited to the skeletal muscle following injection with alum and can be targeted by alum to induce an immune response (416). Alum can induce pro-inflammatory cytokine secretion (e.g. IL-1β), enhance inflammatory cell recruitment to the injection site and activate uptake and presentation in human monocytes (409, 459). However, whether alum can concurrently increase IL-10 secretion in human monocytes has never been demonstrated, although incubation of human PBMCs with alum has been reported to increase IL-10 secretion (409). Therefore, demonstrating that alum strongly enhances IL-10 secretion in both primary human and mouse APCs, may explain why alum is ineffective at driving strong cell mediated immunity in both human and mouse vaccine studies. Additionally, studies on the effects of PLGA particles on human APCs have only addressed changes in uptake and pro-inflammatory responses (563). Similar to mouse BMDCs and BMDMs, 500 nm PLGA particles induced the strongest IL-10 response in human monocytes, indicating a mechanism that is shared between human and mouse APCs.

Previously our lab has shown that alum enhanced IL-10 production in DCs is not restricted by the TLR ligand used, as priming with zymosan, a TLR2 and dectin-1 agonist in place of LPS
could also induce an increased IL-10 response in BMDCs treated with alum (Oleszycka, McCluskey et al, in revision). The IL-10 enhancement and inhibition of IL-12p70 by 500 nm PLGA particles was also not specific for the TLR4 ligand. Both surface (Pam3CSK4) and intracellular endocytic compartment (R848) TLR ligands were capable of enhancing IL-10 or inhibiting IL-12p70 with the same PLGA particle sizes as LPS in DCs. This also includes the ability of 2 µm-10 µm PLGA particles to enhance IL-1β production. Regardless of TLR ligand used or location of TLR receptor, PLGA particles modulate a similar cytokine pattern based on their size. Thus, confirming that the mechanism of action of PLGA particles is not to just hold the TLR agonist at the cell surface (or prevent TLR receptor internalisation) for a more robust response. However, CpG stimulated DCs in presence of PLGA particles did not elevate IL-10 (or IL-1β) production, this could be that the TLR9 ligand is activating a unique pathway leading to a different response. As stimulation with different TLR ligands can induce a distinct secretion pattern of cytokine in DCs (564, 565).

The inflammatory response at the injection site has proved to be very important for adjuvanticity by influencing the subsequent adaptive immune response that develops. Many particulate adjuvants induce a rapid innate immune response at the site of injection, with immune cells infiltrating after just 1 h and remaining for up to 24 h (465, 566, 567). However, many adjuvant studies in mice do not use clinically relevant routes (e.g. intramuscular injections) that are used in humans. PLGA particles were injected intramuscularly which enhanced local cellular infiltration of both innate (neutrophils, monocytes and DCs) and adaptive immune cells (B cells) after 24 and 72 h. By far the majority of cells infiltrating the muscle were B cells, although not investigated here, it would be interesting to examine whether these cells express regulatory markers (e.g. PD-L1) (342, 568) as well as whether they and other cell types secrete increased amounts of IL-10. A more comprehensive set of markers could also be included for innate and adaptive cells, including regulatory B cells which are known to be producers of large amounts of IL-10. Most innate and adaptive immune cells have shown the ability to secrete IL-10, demonstrating its importance in mediating homeostasis and other functions. It will be crucial to determine what cell types (e.g. B cells, APCs or neutrophils) are responsible for any increase in IL-10 secretion after i.m injection of alum and PLGA particles and how size can affect these responses.

In addition to local cellular infiltration, injection with 500 nm PLGA particles promoted enhanced IL-10 secretion in the inguinal lymph nodes after 3 days. No effects were seen on IL-
17, IFN-γ or IL-4 secretion. Thus, PLGA particles can prime both innate and adaptive immune cells to secrete increased IL-10 levels, when cells are restimulated ex vivo. No enhancement of cytokines was observed in the spleen illustrating a selective effect, which corresponds with other results in the lab using alum, PS and PLGA particles. Furthermore, when different PLGA particles were tested in vivo, 500 nm PLGA particles selectively enhanced IL-10 production ex vivo, corresponding to the in vitro data. Mice immunised with alum (i.p) showed a similar increase in IL-10 in the draining lymph nodes (Ewa Oleszycka, PhD 2014). Interestingly, alum injected intramuscularly (in absence of an antigen) increased IL-10 in the ILNs restimulated ex vivo, demonstrating the effects of alum characterised using intraperitoneal injection can be translated to other more clinically relevant routes and produces a similar response to 500 nm PLGA particles in vivo. This increased IL-10 could be detrimental to developing vaccine where a strong cell mediated response is required, as shown by the impact IL-10 has on the magnitude and quality of the Th1 response in vaccine-elicited protection (569). How alum and certain sizes of PLGA particles are selectively predisposing cells to secrete higher IL-10 levels is unknown. Although, as the source of this IL-10 is innate this could be due to the particulates innate ‘training’ of immune cells. It has been shown that cellular stressors including vaccination (e.g. BCG vaccine) can influence the epigenetic landscape of innate immune cells to change their phenotype leading to enhanced cytokine production upon re-exposure (570, 571). This theory is currently being explored in our lab, where alum exposure predisposes mouse and human monocytes and macrophages to secrete elevated IL-10 in a training model (section 5).

Blocking IL-10 has been shown to cause a shift from a Th2 to Th1 response. For instance, co-administration of an IL-10R blocking antibody and OVA promotes antigen-specific IFN-γ secretion, typical of a Th1 response (572). The increase in IL-10 following alum injection also has a detrimental effect in terms of developing a Th1 response. Unpublished data from our lab showed that absence of IL-10 signalling in mice immunised with OVA and alum leads to development of an OVA-specific Th1 response and antigen-specific production of IFN-γ, a response that is absent in wild type mice immunised with OVA and alum. However, the Th1 response was not reflected in the humoral responses, as no significant differences in antibody titres were observed between the IL-10+/− and wild type mice. Compared to using PLGA particles, IL-10 can also limit the Th1 adaptive response in mice immunised with 500 nm PLGA particles, while 100 nm PLGA particles showed no difference. Mice deficient in IL-10 lead to antigen-specific secretion of IFN-γ by splenocytes in mice immunised with 500 nm
PLGA particles and antigen only. Surprisingly, 100 nm PLGA particles did not enhance IFN-γ in wild type mice, despite showing an increase in the OTII assay. Additionally, the 50 nm PS particles and 100 nm PLGA particles did not induce the same response, as only 50 nm PLGA particles produced antigen-specific IFN-γ in splenocytes. Although not addressed in this project, it could be that a difference of 50 nm can change the particles properties, this is evident by the fact 50 nm PS particles strongly induce apoptosis in DCs compared to 100 nm PLGA particles.

Interestingly, injection of 100 nm or 500 nm PLGA particles did not result in enhanced antigen-specific IL-10 production by splenocytes when compared to the 50 nm PS particle control. However, enhanced production of IL-10 was observed when these cells were restimulated *ex vivo* with anti-CD3 which is indicative of priming. This indicated that while both PLGA particle sizes enhance the overall production of IL-10, only the IL-10 associated with 500 nm PLGA particles restricted Th1 responses, a result which also corresponds to the results obtained using alum. Thus, both alum and specific sizes of PLGA particles should be avoided when designing new adjuvants for vaccines targeting Th1 associated diseases and cancer. It should be noted that other effects of particulates such as IL-12 inhibition could also be a factor in determining the type of response generated.

Unpublished data from our lab indicates that using particles around the 50 nm size range are strongest at inducing antigen-specific CD8+ T cell responses *in vivo*. 100 nm and 500 nm PLGA particles were compared to 50 nm PS particles, to see if the ability to drive antigen-specific CD8+ responses translated to other materials and if the same size range was involved. However, neither PLGA particle size enhanced the induction of tetramer+ cells, unless IL-10+ mice were used. It’s been established that IL-10 can act both directly and indirectly on CD4+ and CD8+ T cells to inhibit their expansion, function, and memory formation (129). Changing the material/polymer used or only a small size differences could be responsible for the contrasting results between 100 nm PLGA and 50 nm PS particles. Currently, 50 nm PLGA particles are not available and so PLGA of this specific size cannot be directly compared to PS particles. Different mechanisms could be employed by the 50 nm PS particles as they are the only particulates able to strongly induce apoptosis in DCs *in vitro*. It has been shown in tumour models that cross-presentation of antigens released by apoptotic cancer cell results in specific CD8+ responses (573). Furthermore, injection of antigen and 50 nm PS particles enhanced antigen-specific IFN-γ and IL-10. In contrast, injection of 100 nm or 500 nm PLGA particles did not trigger comparable antigen-specific IFN-γ and IL-10 response, but this did enhance
nonspecific IL-10 production when cells re-stimulated ex vivo. When no IL-10 is available, injection of antigen and 100 nm PLGA particles can significantly induce tetramer+ CD8+ T cells, while injection of antigen and 500 nm PLGA particles also demonstrated a trend for increased tetramer+ cells in the spleen. The difference between 100 nm and 500 nm PLGA particles could be the ability of the 500 nm PLGA particles to inhibit IL-12 production, which is also a key regulator of CD8+ T-cell activation (574, 575).

Increased IL-10 can have negative impact on generating protective Th1 responses needed for vaccines against infections such as tuberculosis, malaria and HIV (569). Therefore, adjuvants such as alum and certain sizes of PLGA particles (500 nm-2 µm) should be avoided if developing new vaccines targeting these infections. However, their properties could also be exploited to treat inflammatory diseases. Biodegradable nanoparticles coated with autoimmune peptide-MHC complexes have Treg-expanding properties (576). Use of PLGA nanoparticles have shifted from vaccines to inducing immunological tolerance and improving the efficacy and safety of biologic drugs used to treat inflammatory diseases (577, 578). Based on our results, particulates in a certain size range have therapeutic potential to block inflammation and promote tolerance. Mice injected intravenously with PLGA particles (~ 500 nm) containing encapsulated OVA induced safe and effective tolerance by inhibiting Th2 responses and airway inflammation both prophylactically and therapeutically in a model for allergic airway inflammation (579). Injection of PLGA nanoparticles (300 nm) encapsulating type II collagen enhanced oral tolerance induction and protection against collagen-induced arthritis (580). Furthermore, injection of 200 nm PLGA particles combined with the immunomodulator rapamycin induced durable antigen-specific tolerance in EAE, a mouse model of hypersensitivity and Haemophilia A mice (488). Importantly, it has been demonstrated that 500 nm PLGA or PS particles conjugated to an encephalitogenic antigen (myelin proteolipid protein epitope) administered intravenously can induce tolerance to prevent the onset of EAE (581). Additionally, injection of 500 nm, negatively charged, carboxylated PS particles can reduce immune pathology in peritoneal inflammation, inflammatory bowel disease (IBD), EAE and cardiac and kidney reperfusion injury by inhibiting Inflammatory monocytes migrating to the site of inflammation (546). However, this is not consistent as other groups have not observed similar effects in the same models (e.g. EAE) (582). This could be due to the use of particles with different sizes (or charges), which as demonstrated in chapter 4 trigger alternative mechanisms based on their size. Interestingly, Getts et al (581) were using PLGA particles of 500 nm in size, which corresponds to the size range which elevates IL-10 production in DCs.
and macrophages (Section 3.2.2 and 3.2.4). This effect seems to be size-dependent, because intravenous injections of 500 nm PLGA particles coupled to myelin proteolipid protein are more effective at protecting from EAE than smaller or larger particles, by reducing CNS infiltration of Th1, Th17 cells and inflammatory monocytes/macrophages (583).

To conclude, this study showed that PLGA particles, PS particles or alum can modulate cytokine release by DCs and their capacity for T cell activation. The size of the particles has a major influence in determining these responses, with particles within a specific size range (500 nm-10 µm) showing stronger IL-10 enhancement and IL-12 suppression which have a detrimental effect on promoting Th1 responses. It also clarifies why alum and other particulates which have mainly been used around the 1 µm size range are relatively ineffective at driving strong cell mediated immunity in both human and mouse vaccine studies. By going outside this established size range, particles could potentially promote more effective cell mediated immunity (<100 nm) or enhanced Th2 responses (>10 µm). These are vital new factors that should be considered for the future development of any vaccine formulation incorporating particulate adjuvants.
Chapter 4

Investigating the mechanism underlying particulate adjuvant modulation of IL-10 and IL-12 production
4.1 Introduction

To advance the development of new vaccines, a move away from empirical approaches towards a mechanistic understanding of how adjuvants induce and direct immune responses is required. The mechanism behind the adjuvanticity of many particulate adjuvants remains unclear. Previously, particulate adjuvants were believed to form a depot for sustained release of antigen as well aiding delivery of antigen to APCs. It is now appreciated that the ability of particulate adjuvants to modulate innate immune responses is key to their adjuvant properties. Understanding the mechanism of action including the signalling pathways underlying adjuvanticity will provide the basis for developing improved adjuvants capable of promoting effective response against complex pathogens as well as identifying pathways to be avoided.

Adjuvant research has progressed very quickly over the past number of years leading to an increased understanding of how many particulate adjuvants modulate the immune response. For instance, the adjuvanticity of alum is now believed to mainly related to the release of DAMPs (IL-33, DNA and uric acid) (466) and inflammasome activation with the antigen depot being largely dispensable. Alum can also interact with lipids on the plasma membrane of innate cells, leading to receptor independent activation of signalling pathways such as Syk-PI3K signalling to further modulate the immune response through release of proinflammatory cytokines and mediators (IL-1β and PGE₂). There could also be other mechanisms and pathways directing the immune response. Therefore, it is now acknowledged that particulate adjuvants are capable of directly mediating immune response due to their intrinsic physicochemical properties.

There have been a few suggested mechanisms that biodegradable particulates employ to mediate their adjuvanticity, including a depot effect, protection of antigens, promoting antigen uptake and influencing cytokine secretion by APCs. Despite this, how these adjuvants modulate the immune response remains unclear. For example, most clinically used particulate adjuvants are inefficient at driving Th1 response while favouring antibody responses.

For adjuvants to induce protective immune responses they must first engage with the innate immune system. Many of these effects are at the level of APCs, in particular DCs, given their distinct capacity to initiate T cell activation. Having demonstrated that particulate adjuvants within a certain size range can induce IL-10 and inhibit IL-12 secretion, the mechanism modulating these two responses in DCs were investigated.
IL-10 has several different layers of regulation through different pathways. It has been reported that MAP kinase signalling pathways play an essential role in regulating IL-10 in APCs. For instance, the parasite *Leishmania infantum* relies on the activity of Syk, phosphatidylinositol-3 kinase and p38 to mediate IL-10 secretion in B cells (584). Alum has been demonstrated to activate the Syk-PI3K kinases independently of receptors through interaction with cholesterol on lipid rafts of membranes, leading to proinflammatory cytokine release. However, these pathways have never been linked to any anti-inflammatory responses mediated by particulate adjuvants such as alum or PLGA. The hypothesis underlying this work was that particulates can activate both pro- and anti-inflammatory pathways when interacting with the membrane in a size dependent manner and that by changing the size of the particles a detrimental IL-10 response could be avoided. Whether this mechanism is a generic response of DCs under certain stresses (e.g. osmotic and oxidative) or specific to engagement with particulates was also investigated.

Due to the role of IL-12 family cytokines in polarisation of adaptive immune responses, their expression is tightly regulated. It is known that alum is not an effective adjuvant for promoting Th1 and Th17 responses, but the reasons for this are not well understood. It is hypothesised here that alum can induce immunoregulatory mechanisms, which actively block induction of cellular immune responses. Bioactive IL-12p70 is made up of two subunits, p35 and p40 with each being differentially regulated. Secretion of IL-12 can be inhibited by various mechanisms, which are best characterised for cells activated by TLR ligands. Our lab has shown that alum can inhibit IL-12 transcription and secretion, an effect mediated through the PI3K pathway (476).

Therefore, this study sought to determine the mechanisms underlying particle-modulation of IL-10 and IL-12p70 secretion and whether the two responses are connected or independent. IL-10 has been shown to inhibit IL-12 through the mTOR and GSK3β pathways (88, 293). However, particulate adjuvant mediated modulation of IL-10 and IL-12 has received limited attention so the underlying mechanisms were addressed here.

Finally, The NLRP3 inflammasome has been shown to be activated by particulates (e.g. alum, PS and 1 µm PLGA particles) to increase IL-1β secretion (472, 549). Whether other PLGA particles that can also enhance IL-1β secretion share the same mechanism was investigated (e.g. 10 µm PLGA particles).
4.2 Results

4.2.1 PLGA particle induced IL-10 and IL-12p70 are modulated by two independent pathways in BMDCs

IL-10 is a potent immunosuppressive cytokine with the ability to regulate functions of many cells, including DCs. For instance, IL-10 is capable of inhibiting DC maturation and function (585) and suppressing IL-12 secretion by APCs (293), thereby compromising Th1 responses. Hence, whether PLGA induced IL-10 was responsible for the down regulation of IL-12p70 was investigated.

Both wild type and IL-10−/− BMDCs were stimulated with LPS (5 ng/ml) or different sized PLGA particles (100 nm, 500 nm, 10 µm and 30 µm) alone or in combination with each other. After 24 h, supernatants were collected and IL-12p70 and IL-10 (acting as a control in this experiment) levels were determined in supernatants by ELISA. As demonstrated in chapter 3 (section 3.2.2), PLGA particles of between 500 nm and 10 µm in diameter can inhibit IL-12p70 production in BMDCs stimulated with LPS (5 ng/ml). Particle mediated IL-12p70 inhibition was comparable in IL-10−/− and wild type BMDCs, indicating that IL-10 enhancement and IL-12p70 inhibition by PLGA particles are mediated by independent pathways (Fig 4.1).

Since particle-mediated enhancement in IL-10 secretion was dispensable for IL-12 inhibition, other pathways implicated in IL-12 inhibition including PI3K were investigated. PI3K can upregulate IL-10 through the mTOR signalling pathway, leading to the inhibition of IL-12 (88), or else the GSK3β pathway which directly regulates IL-12p35 expression (85). Otherwise IL-10 and the PI3K pathway could be acting in a synergistic manner. 500 nm PLGA particles were used as they drove the highest levels of IL-10 as well as having the ability to inhibit IL-12p70.

Therefore, wild type and IL-10−/− BMDCs were treated with wortmannin (100 nM), an inhibitor of PI3K for 1 h before stimulation. Both WT and IL-10−/− BMDCs were stimulated with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together. After 6 h, expression of IL-12p35 and IL-12p40 was determined by RT-PCR (Fig 4.2A) or after 24h, supernatants were collected to analyse IL-12p70 concentrations by ELISA (Fig 4.2B). Neither the lack of IL-10 nor the inhibition of the PI3K signalling pathway significantly compromised PLGA particle mediated inhibition of IL-12p70 secretion (or IL-12p35 mRNA expression).
Figure 4.1: The inhibition of IL-12p70 by PLGA particles is not IL-10-dependent.

BMDCs from C57BL/6 and IL-10-deficient mice were incubated with medium, LPS (5 ng/ml) alone or LPS with different sized PLGA particles at concentrations from 1-0.063 mg/ml. Supernatants were collected after 24 h and IL-12p70 and IL-10 concentrations were determined by ELISA. Results are representative of three independent experiments. Error bars show mean ± SD for each experimental group tested individually in triplicate. LPS v PLGA+LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
Figure 4.2: IL-10 and PI3K signalling do not synergise to mediate PLGA particle inhibition of IL-12p35 expression

A) BMDCs (1x10^6 cells/ml) were treated with medium or wortmannin (100 nM) for 1 h before stimulating cells with medium, LPS (5 ng/ml), 500 nm PLGA particles or 500 nm PLGA particles and LPS (5 ng/ml) together for 6 h. Total RNA was isolated and the expression of IL-12p35 and IL-12p40 was determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

B) BMDCs (6.25x10^5 cells/ml) were pre-treated with wortmannin (100 nM) or medium control for 1 h before stimulating cells with LPS (5 ng/ml) or 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected to measure IL-12p70 concentrations by ELISA. Results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. Wild Type v IL-10^-/-_. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
4.2.2 PLGA particle mediated enhancement of IL-1β is NLRP3 and cathepsin S dependent

The mechanism for increased IL-1β production by certain sizes of PLGA particles was also investigated. Our lab has demonstrated that alum and other particulates mediate their IL-1β responses through the NLRP3 inflammasome *in-vitro* (549, 586). Although recently, our lab has demonstrated that alum induced IL-1β is inflammasome independent *in vivo* (538). However, cathepsin S is required for alum induced IL-1β secretion *in vitro* and *in vivo* (538). Furthermore, NLRP3 deficiency has been shown to decrease IL-10 production in macrophages (587). Therefore, the role the NLRP3 inflammasome play in PLGA induced cytokine modulation was investigated.

Results from chapter 3 (Section 3.2.1 and 3.2.2) indicated that 10 µm PLGA and PS particles significantly enhanced IL-1β production compared to all other sizes. The mechanism employed by these particles along with the 500 nm PLGA particles was tested to determine if any increase in IL-1β secretion was due to the NLRP3 inflammasome.

NLRP3 and cathepsin S deficient BMDCs were compared to wild type BMDCs for IL-1β secretion. Both types of BMDCs were stimulated with LPS and 500 nm or 10 µm PLGA particles alone or together for 24 h. As expected all IL-1β production driven by 500 nm and 10 µm PLGA particles was dependent on both the NLRP3 inflammasome and cathepsin S protease (Fig 4.3) thus, confirming that PLGA particles like other particulate adjuvants mediate IL-1β secretion mainly through the NLRP3 inflammasome and cathepsin S *in vitro*. Additionally, the NLRP3 inflammasome was shown to have no role in mediating PLGA particle induced IL-10 secretion or inhibition of IL-12p70 (Fig 4.4A). Similarly, the caspase-1 inhibitor YVAD made no difference to IL-10 or IL-12p70 modulation by PLGA particles (Fig 4.4B).

Why the 10 µm PLGA and PS particles are superior in promoting IL-1β secretion over the other particle sizes remains unclear. It may have to do with enhanced cell death or frustrated phagocytosis which leads to cell swelling but this has yet to be confirmed (Section 3.2.7). To conclude, when combined with TLR agonists such as LPS, PLGA particles of certain sizes promotes NLRP3 and cathepsin S-dependent secretion of IL-1β by BMDCs.
Figure 4.3: PLGA particles promote IL-1β secretion by signalling through the NLRP3 inflammasome or cathepsin S protease

A) BMDCs from C57BL/6 and NLRP3<sup>−/−</sup> (6.25x10<sup>5</sup> cells/ml) mice were stimulated with LPS (5 ng/ml) or 10 μm PLGA particles at concentrations from 1-0.063 mg/ml alone or together for 24 h. Concentrations of IL-1β was measured in supernatants by ELISA.

B) BMDCs from C57BL/6 and NLRP3<sup>−/−</sup> (6.25x10<sup>5</sup> cells/ml) mice were stimulated with LPS (5 ng/ml) or 500 nm PLGA particles at concentrations from 1-0.063 mg/ml alone or together for 24 h. Concentrations of IL-1β was measured in supernatants by ELISA.

C) BMDCs from C57BL/6 and cathepsin S<sup>−/−</sup> (6.25x10<sup>5</sup> cells/ml) mice were stimulated with LPS (5 ng/ml) or 500 nm and 10 μm PLGA particles (0.25 mg/ml) alone or together for 24 h. Concentrations of IL-1β was measured in supernatants by ELISA.

Wild type v NLRP3<sup>−/−</sup> or cathepsin S<sup>−/−</sup>. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 4.4: PLGA particle induced IL-10 and IL-12p70 inhibition does not require the NLRP3 inflammasome or caspase-1

A) BMDCs from C57BL/6 and NLRP3⁻/⁻ (6.25x10⁵ cells/ml) mice were stimulated with LPS (5 ng/ml) or 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Concentrations of IL-10 and IL-12p70 were measured in supernatants by ELISA.

B) BMDCs (6.25x10⁵ cells/ml) were incubated with varying concentrations of the caspase 1 inhibitor YVAD for 1 h before stimulating with LPS (5 ng/ml), 500 nm or 2 µm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 and IL-12p70 concentrations were measured by ELISA.

Wild type v NLRP3⁻/⁻. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of two independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
4.2.3 Dissecting the mechanism by which PLGA particles promote IL-10

Macrophages and DCs produce IL-10 in response to TLR and C-type lectin receptor ligand binding (143). Here, the mechanisms involved in PLGA particle induced IL-10 were investigated. Dectin-1 is a well characterised c-type lectin receptor via which fungal pathogens promote IL-10 production in DCs (64). Furthermore, non-specific signalling through Fc receptors could play a role in particle driven IL-10 responses. Fc receptors have been shown to associate with Syk to mediate responses including cytokine secretion in APCs (588).

BMDCs were pre-incubated for 1 h with a range of concentrations of the dectin-1 antagonist, WGP soluble or Fc block (0.25 µg/ml) before stimulating with LPS (5 ng/ml) and 500 nm PLGA (250 µg/ml) alone or together for 24 h. In addition, BMDCs after treatment with WGP soluble were incubated with curdlan to act as a dectin-1 dependent positive control. Blocking dectin-1 or FC receptors on DCs had no effect on PLGA induced IL-10 (Fig 4.5) or IL-12p70 suppression (data not shown). The use of curdlan confirmed that WGP soluble was blocking the dectin-1 receptor as IL-10 levels were significantly reduced (Fig 4.5).
Figure 4.5: PLGA particle induced IL-10 is not mediated through the C-type lectin receptor dectin-1 or Fc receptors

A) BMDCs (6.25x10^5 cells/ml) were incubated with WGP soluble for 1 h before stimulating with LPS (5 ng/ml) or 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 concentrations were measured by ELISA.

B) BMDCs (6.25x10^5 cells/ml) were incubated with WGP soluble at various concentrations for 1 h before treating with curdlan (100 µg/ml). Supernatants were collected after 24 h and IL-10 concentrations were measured by ELISA.

C) BMDCs (6.25x10^5 cells/ml) were incubated with Fc block for 1 h before stimulating cells with LPS (5 ng/ml) or 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 concentrations were measured by ELISA.

treatment v inhibitor + treatment. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
4.2.4 The PI3K and ERK signalling pathways partially regulate PLGA particle induced IL-10 secretion in BMDCs

Many signalling pathways have been implicated in both IL-10 and IL-12 regulation, including class I PI3K, ERK and mTOR. The class I PI3K/AKT pathway has demonstrated to have key roles in the differentiation and activation of DCs, regulating proinflammatory cytokines (IL-12, IL-6, and TNF-α) and partially regulating IL-10 (85). Inhibitors for PI3K such has wortmannin have been widely used, however they have also been shown to non-specifically inhibit a whole host of other kinases (589). Therefore, the specific pan-class I PI3K inhibitor ZSTK474 was used, which has been shown to only inhibit class I PI3K isoforms (590).

Rapamycin is an inhibitor of the Ser/Thr protein kinase called mammalian target of rapamycin (mTOR) that regulates cell growth, metabolism and cytokines in response to environmental cues. mTOR is one of the pathways activated by the PI3K/AKT kinases, capable of producing IL-10 in innate cells (591).

BMDCs were pre-treated with varying concentrations of ZSTK474 or Rapamycin for 1 h prior to stimulating cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h and IL-10 and IL-12p70 concentrations in supernatants were determined by ELISA. Furthermore, BMDCs pre-treated with ZSTK474 (0.5 µM) for 1 h were stimulated with LPS and/or particles for 6 and 12 h and mRNA was then isolated and analysed for an increase in IL-10, IL-12p35 and IL-12p40 expression by RT-PCR.

ZSTK474 only partially inhibited IL-10 production driven by PLGA particles (Fig 4.6A) and was unable to reverse IL-12p70 (Fig 4.6A) or IL-12p35 (Fig 4.6B) suppression by PLGA particles. Similarly, inhibiting the mTOR pathway had no effect on IL-12p70 secretion nor influence IL-10 production (Fig 4.9A), indicating that the partial regulation of particle-driven IL-10 by PI3K is independent of the mTOR pathway and may involve other signalling pathways associated with PI3K such as GSK3β.

The MAP kinase ERK1/2 has also been shown to differentially regulate both IL-10 and IL-12 in APCs (66, 73-77). U0126, a highly selective inhibitor of both MEK1 and MEK2, upstream of the ERK pathway was used. BMDCs were pre-treated with different concentrations of U0126 for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml)
alone or together for 24 h to analyse IL-10 and IL-12p70 secretion by ELISA. In addition, BMDCs were pre-treated with U0126 (5 µM) for 1 h prior to stimulating for 6 and 12 h and IL-10, IL-12p35 and IL-12p40 expression was determined by RT-PCR.

The PLGA particle induced enhancement in IL-10 production was diminished but still evident when ERK was blocked by U0126 (Fig 4.7A and 4.7B). This is similar to the results obtained for blocking PI3K, indicating that both pathways partly mediate IL-10 induction by PLGA particles. In contrast, both p38 and Syk are essential for the enhancement of IL-10 by PLGA particles. Both IL-12p70 secretion (Fig 4.7A) and IL-12p35 expression (Fig 4.7B) was partly reversed if the ERK pathway was blocked. These findings indicate that ERK activation may be partly responsible for the PLGA particle inhibition of IL-12p70 but other pathways or responses are likely to be playing a more pronounced role.

The JAK1/STAT3 signalling pathway can directly inhibit the IL-12p35 subunit and subsequent IL-12p70 release in dendritic cells (308). Ruxolitinib which inhibits both JAK1 and JAK2 was utilised to see if it reverses PLGA inhibition of IL-12p70. BMDCs were pre-treated with different concentrations of Ruxolitinib for 1 h prior to stimulating with LPS (5 ng/ml) alone or together with 500 nm PLGA particles (250 µg/ml) for 24 h to analyse IL-10, IL-12p70 and TNF-α secretion by ELISA. Inhibiting the JAK1 and JAK2 pathways boosted the IL-10 response associated with 500 nm PLGA particles (Fig 4.9B), indicating an inhibitory role for this pathway. In contrast, it attenuated IL-12p70 as well as partially mediated TNF-α release in LPS stimulated BMDCs (Fig 4.9B), demonstrating that JAK1 and/or JAK2 are needed to promote IL-12p70 and TNF-α secretion in DCs.

Finally, the ability of PLGA particles to activate both AKT (downstream of PI3K) and ERK1/2 was investigated by western blot. BMDCs (1x10^6 cells/ml) were stimulated with either LPS (5 ng/ml) or 500 nm PLGA particles (250 µg/ml) alone or together for 15 and 30 min. Total protein was isolated and immunoblotted for phospho-Akt, phospho-ERK1/2 and total Akt and ERK1/2 in the cells. Unlike p38 (section 4.2.11), no differences in Akt phosphorylation were observed between cells treated with LPS alone or LPS and PLGA particles (Fig 4.8A). In the case of ERK, phosphorylation was sustained for longer in cells incubated with PLGA particles and LPS (Fig 4.8B). If Syk was inhibited for 1 h before stimulating the cells, both ERK1/2 and Akt phosphorylation disappears completely in DCs incubated with LPS alone or LPS and PLGA particles (Fig 4.8).
To conclude, both the PI3K and ERK pathways are implicated in IL-10 and IL-12p70 regulation by PLGA particles in DCs, however neither pathway is the principal immunomodulatory mechanism.
Figure 4.6: The inhibition of IL-12 by PLGA particles is not reversed by inhibiting class 1 PI3 kinase but the pathway partially mediates the IL-10 enhancement.

A) BMDCs (6.25x10⁵ cells/ml) were incubated with varying concentration of the class 1 PI3K inhibitor ZSTK474 for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 and IL-12p70 secretion was measured by ELISA.

B) BMDCs (1x10⁶ cells/ml) were pre-treated with ZSTK474 (0.5 µM) for 1 h before stimulating treated or non-treated cells with medium, LPS (5 ng/ml), 500 nm PLGA particles (250 µg/ml) or 500 nm PLGA particles and LPS together for 6 and 12 h. Total RNA was isolated and the expression of IL-10 (12 h), IL-12p35 and IL-12p40 (6 h) was measured using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

All results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. Treated v non-treated. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
Figure 4.7: ERK1/2 partially mediates PLGA particle induced modulation of IL-10 and IL-12p70

A) BMDCs (6.25x10^5 cells/ml) were incubated with varying concentration of the MEK/ERK inhibitor U0126 for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 and IL-12p70 concentrations were measured by ELISA.

B) BMDCs (1x10^6 cells/ml) were pre-treated with U0126 (5 µM) for 1 h before stimulating treated or non-treated cells with medium, LPS (5 ng/ml), 500 nm PLGA particles (250 µg/ml) or 500 nm PLGA particles and LPS together for 6 and 12 h. Total RNA was isolated and the expression of IL-10 (12 h), IL-12p35 and IL-12p40 (6 h) was measured using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

All results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. Treated v non-treated. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
Figure 4.8: 500 nm PLGA particles do not enhance AKT or ERK phosphorylation

BMDCs (1x10^6 cells/ml) were treated with piceatannol (20 µM) 1 h before stimulating treated and non-treated cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 15 and 30 min. Total protein was isolated and immunoblotted for phospho Akt and total Akt (A), phospho ERK and total ERK (B). Results are representative of two independent experiments.
Figure 4.9: The mTOR and JAK/STAT pathway are not involved in IL-10 or IL-12p70 modulation by PLGA particles

A) BMDCs (6.25x10^5 cells/ml) were incubated with varying concentrations of the mTOR inhibitor rapamycin for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 and IL-12p70 concentrations were measured by ELISA.

B) BMDCs (6.25x10^5 cells/ml) were incubated with varying concentrations of the JAK/STAT inhibitor Ruxolitinib for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10, IL-12p70 and TNFα concentrations were measured by ELISA.

All results are expressed as the mean ± SD of samples in triplicate and are representative of two independent experiments. LPS vs PLGA + LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
4.2.5 The size of PLGA particles influences the shape and size of DCs.

Having determined that different sized PLGA particles can differentially impact IL-10, IL-12p70 and IL-1β secretion, the underlying mechanisms were addressed. Firstly, whether exposure to particles can change the shape or granularity of the cells was determined. Recently, it has become clear that the shape of the cell can influence its properties including proliferation and macrophage polarity (274, 592). It is also known that the actin cytoskeleton can control macrophage shape to determine its polarisation state (274). Furthermore, external forces or soluble factors (e.g. cytokines) have been shown to mediate downstream signalling pathways through changes in the actin cytoskeleton (593, 594).

Therefore, BMDCs were incubated with a range of PLGA particle sizes (100 nm, 500 nm, 2 µm, 10 µm and 30 µm) and the forward and side scatter analysed to assess any changes in the size or granularity of the cell. Only exposure to the 500 nm and 2 µm PLGA particles resulted in a difference in either forward or side scatter (Fig 4.10). Incubation of cells with both particle sizes led to reduced forward scatter, indicating a decrease in size and a small increase in side scatter, indicating an increase in granularity (Fig 4.10). It was only these two sizes which could promote an enhanced IL-10 response in DCs.

To follow up on this, BMDCs were stained for F-actin using Phalloidin (red) to see how actin is distributed within the cell and if there were any alterations to cell shape that corresponded to the forward and side scatter data (Fig 4.10). BMDCs were incubated with 100 nm, 500 nm or 10 µm PLGA particles for 2 h, cells were fixed and subsequently stained with DAPI and Alexa Fluor 594 Phalloidin (red) and then analysed by confocal microscopy. F-actin accumulated around the membrane and periphery of cells incubated with 500 nm PLGA particles, within the cell following incubation with 100 nm PLGA particles while the distribution of actin in cells incubated with the 10 µm PLGA particles was comparable to controls (Fig 4.11). Furthermore, BMDCs incubated with fluorescent PLGA particles (green) for 3 h exhibited a more rounded phenotype compared to those incubated with either the 100 nm or 10 µm PLGA particles (Fig 4.12).
Figure 4.10: Incubation with 500 nm or 2 µm PLGA particles reduced the size and increased the granularity of BMDCs

BMDCs were left untreated or incubated with the indicated sizes of PLGA particles (0.25 mg/ml) for 24 h. Live CD11c+ cells were analysed for size and granularity by comparing forward and side scatter of the different treatments by flow cytometry. 100 nm PLGA (red) and 500 nm PLGA (blue) were compared for any differences (A) or medium samples (red) were compared to different PLGA sizes (blue) for changes in forward scatter (B). Data are representative of two independent experiments with each experimental group tested in duplicate.
Figure 4.11: The size of PLGA particles influences filamentous actin distribution within DCs.

BMDCs were incubated with either 100 nm, 500 nm or 10 µm PLGA particles (100 µg/ml) for 2 h (A) or else stimulated with 500 nm PLGA particles for 30 min (B). Cells were fixed and subsequently stained with DAPI and Alexa Fluor 594 Phalloidin (red) followed by analysis by confocal microscopy. Confocal images are representative of two independent experiments, scale 10 µm.
Figure 4.12: The size of PLGA particles influences filamentous actin distribution and shape of DCs.

BMDCs were incubated with 100 nm, 500 nm or 10 µm PLGA particles (100 µg/ml, green) for 3 h. Cells were fixed and subsequently stained with DAPI and Alexa Fluor 594 Phalloidin (red) and then analysed by confocal microscopy. Confocal images are representative from three independent experiments, scale 25 µm.
4.2.6 500 nm PLGA particles require cholesterol to induce IL-10 and enhance lipid raft formation.

Recently, it has been suggested that particles (alum and MSU crystals) can directly interact with cell membranes leading to lipid sorting and receptor independent activation of various kinases (280, 474). For instance, alum can interact with cholesterol-rich lipid rafts on the membrane of DCs, the subsequent raft formation and aggregation of ITAM-containing molecules activates the Syk-PI3K pathway and promotes the release of PGE$_2$, IL-18 and IL-1$\beta$.

While it has been shown that particles can interact with membranes to promote proinflammatory responses in cells, it has never been demonstrated that this receptor independent association with the membrane is also responsible for anti-inflammatory responses to particulates. The ability of PLGA particles of a specific size (500 nm) to induce raft formation and potentially leading to enhanced IL-10 production was investigated.

MβCD has been extensively used to deplete cholesterol, disrupt lipid raft formation and increase fluidity in the plasma membrane of cells (595). Therefore, BMDCs were treated with 10 mM MβCD twice for 20 min to remove cholesterol, then with 2 mM MβCD to maintain the depletion of cholesterol as DCs are capable of replenishing cholesterol on the surface of cells. BMDCs were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles (250 $\mu$g/ml) alone or together for 24 h. Cholesterol reduction led to a significant decrease in PLGA particle induced IL-10 secretion by DCs (Fig 4.13A) suggesting that it prevents 500 nm PLGA particles from binding to the membrane which results in raft formation to drive the IL-10 response.

Toxicity is an issue when using MβCD to reduce cholesterol on DCs. Therefore, to make sure that DCs remain viable after MβCD treatment. MβCD treated and non-treated cells were compared when stimulated with LPS and 24 h supernatants were analysed for TNF-$\alpha$ (Fig 4.13A). This was to make sure that treated cells had the same ability to produce cytokines and that any reduction in IL-10 was not due cell death, leaving fewer cells to secrete cytokines. As expected, no difference was observed in TNF-$\alpha$ secretion between treated and non-treated cells.

Moreover, treated and non-treated cells were also compared by stimulating with CpG, an agonist for intracellular TLR9. Similar to LPS, this was to confirm that the inhibitor did not exert a nonspecific effect on secretion of TNF-$\alpha$ as well as to make sure that IL-10 production
remained intact when induced via other signalling pathways. Moreover, no difference was observed between treated and non-treated cells (Fig. 4.13B).

Furthermore, BMDCs were treated with MβCD to reduce cholesterol before stimulating cells with LPS (5 ng/ml) and 500 nm PLGA alone or together. After 12 h, mRNA samples were collected and IL-10 expression was determined by RT-PCR. Reducing cholesterol levels significantly decreased IL-10 mRNA in PLGA particle-stimulated DCs (Fig 4.14A), thus, confirming a role for cholesterol in mediating PLGA particle-membrane interaction and subsequent IL-10 induction.

A potential role for cholesterol interaction in PLGA particle inhibition of IL-12p35 and p40 expression was also assessed. BMDCs (1x10^6 cells/ml) were treated with MβCD as before and then stimulated with LPS alone or in the presence of 500 nm PLGA particles. Expression of IL-12p35 and IL-12p40 was determined after 6 h by real-time PCR. Incubation of cells with 500 nm PLGA particles selectively inhibited IL-12p35, but reducing cholesterol had no impact on this inhibitory effect (Fig 4.14B and C).

Having established that removing cholesterol on the cell surface reduces the ability of 500 nm PLGA particles to promote the secretion of IL-10, whether this was due to increased lipid raft formation as the particles interact with the plasma membrane was investigated. Therefore, BMDCs were stained for lipid rafts using a labelled cholera toxin subunit B (CtB) that binds to the GM1 ganglioside, which is selectively located in lipid rafts and a CtB cross-linking antibody to help visualise the rafts by fluorescence microscopy.

BMDCs were labelled for lipid rafts and 500 nm PLGA particles were added to the cell and incubated in a confocal microscopy chamber (37 °C, 5% CO₂) for 60 min and single images taken every minute to monitor particle interaction with the membrane and the formation of lipid rafts. Incubation of cells with 500 nm PLGA particles promoted raft formation as the GM1 ganglioside became more concentrated in certain areas of the membrane (Fig 4.15A). The 500 nm PLGA particles attached to the cell membrane after 30 min (Fig 4.15B, video also available), but BMDCs phagocytose the GM1 ganglioside almost immediately when it is cross-linked with CtB (Fig 4.15B). Therefore, after BMDCs were stimulated with PLGA particles and labelled for lipid rafts, they were lightly fixed with 1% PFA for 2 min to prevent internalisation of the GM1 receptor (Section 2.2.14) and then visualised by confocal microscopy. BMDCs stimulated with 500 nm PLGA particles showed an increase in lipid raft
formation after 30 min (Fig 4.15C and D), which corresponds to when the particles attach to the membrane (Fig 4.15B and Fig 4.23A).

500 nm PLGA particles were compared to 100 nm and 10 µm PLGA particles to see if lipid raft formation is size dependent. Neither incubation of cells with 100 nm PLGA particles (Fig 4.16) or 10 µm PLGA particles (Fig 4.17) resulted in a significant increase in lipid raft formation when compared to the 500 nm PLGA particles. 100 nm PLGA particles were immediately taken up by BMDCs while 10 µm PLGA particles were too large to be phagocytosed.
Figure 4.13: The enhancing effect of 500 nm PLGA particles on IL-10 secretion requires interaction with cholesterol

BMDCs (6.25x10^5 cells/ml) were treated with 10 mM MβCD twice for 20 min and then incubated in 2 mM MβCD. Treated and non-treated were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) (A) or with the TLR9 agonist CpG (4 µg/ml) (B) for 24 h. Supernatants were collected and IL-10 and TNF-α levels determined by ELISA. Treatment v MβCD + treatment. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001. Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.
Figure 4.14: Cholesterol depletion inhibits PLGA particle induced IL-10 expression but does not reverse IL-12p35 inhibition.

BMDCs (1x10⁶ cells/ml) were treated with 10 mM MβCD twice for 20 min and then incubated in 2 mM MβCD. Treated and non-treated were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together for 6 (B and C) or 12 h (A). Total RNA was isolated and the expression of IL-10 (A), IL-12p35 (B) and IL-12p40 (C) was determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells. Results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. LPS + PLGA v LPS + PLGA + MβCD. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
Figure 4.15: PLGA particles of 500 nm-2 μm in size enhance lipid raft formation after interacting with the DC membrane

BMDCs were stained for lipid rafts (red), 500 nm PLGA particles (100 μg/ml) were then added to BMDCs and the cells placed in a confocal microscopy incubator for live cell imaging. Individual images were taken every minute up to 45 min (A and B). BMDCs were incubated with 500 nm PLGA (green) for indicated timepoints (C) or for 30 min (D), and subjected to immunofluorescence analysis with antibodies directed against lipid rafts (CtB, red) and nuclear stain Hoechst 33342. Representative confocal images from three independent experiments, scale 10 μm (A, B and D) or 25 μm (C).
Figure 4.16: 100 nm PLGA particles are rapidly taken up by DC and do not exhibit sustained interaction with the membrane.

BMDCs were incubated with 100 nm PLGA particles (green) for indicated timepoints, and subjected to immunofluorescence analysis to image lipid rafts using CtB (red) and a CtB cross-linking antibody and nuclear stain Hoechst 33342. Uptake of 100 nm PLGA particles was detected by confocal microscopy. Confocal images are representative of three independent experiments, scale bar 25 µm and 10 µm.
Figure 4.17: 10 µm PLGA particles are not phagocytosed by DCs.

BMDCs were incubated with 10 µm PLGA particles (green) for indicated timepoints, and subjected to immunofluorescence analysis to image lipid rafts using CtB (red) and a CtB cross-linking antibody and nuclear stain Hoechst 33342. Uptake of 10 µm PLGA particles was detected by confocal microscopy. Confocal images are representative of three independent experiments, scale bar 25 µm and 10 µm.
4.2.7 Disrupting actin polymerisation drives increased IL-10 and partially reverses PLGA particle modulation of IL-12p35.

Uptake of particulate adjuvants has been highlighted as important for their efficacy. For instance, uptake of alum and other particulate adjuvants has been linked to inflammasome activation and lysosomal rupture, features required for particulate induced IL-1β secretion by DCs (549, 596).

Actin polymerisation and rearrangement of the cytoskeleton is known to be essential in mediating phagocytosis (597), raft formation and sensing mechanical or physical signals (e.g. changes in cell geometry/shape) at the plasma membrane to trigger intracellular signalling cascades (275, 598). Therefore, the importance of phagocytosis and actin polymerisation in the context of PLGA particle mediated IL-10 enhancement and inhibition of IL-12p70 was examined.

Latrunculin B can inhibit actin polymerisation by binding G-actin monomers to prevent them from polymerising (599). Latrunculin B binds to G-actin 1:1 preventing this drug bound G-actin from polymerising. Effectively, they shift the pool of actin from F to the G-form. Many studies use latrunculin B to prevent phagocytosis in DCs and macrophages. In contrast, jasplakinolide promotes actin polymerisation by stimulating actin filament nucleation as well as stabilising F-actin (increases conversion of monomeric G-actin to filamentous F-actin), which can also inhibit phagocytosis (600). Latrunculin B treatment causes complete rounding up of mouse neuroblastoma and hamster fibroblasts (601). THP-1 cells treated with jasplakinolide also show a more rounded morphology (602). Jasplakinolide competes with phalloidin for binding to F-actin, so it is impossible to image cells by fluorescent confocal microscopy. However, BMDCs treated with either latrunculin B or jasplakinolide show a more rounded shape with fewer membrane projections when compared to untreated cells by phase contrast microscopy (data not shown). A similar morphology was seen in BMDCs incubated with 500 nm and 2 µm PLGA particles (Fig 4.10-4.12).

This rounding up may initiate downstream signalling pathway(s), as seen in the case of 500 nm PLGA particles. Interestingly, treatment with latrunculin B or jasplakinolide reduced osmotic swelling in cells treated with a hypotonic solution (section 4.2.9) (603). Furthermore, either latrunculin (602) or jasplakinolide (604, 605) can destabilise adhesion and prevent membrane protrusions forming in THP-1 cells, fibroblasts, endothelial and epithelial cell lines. LPS
activation increases the fluidity of the macrophage plasma membrane (606) and increases its elasticity which can be prevented by treating cells with latrunculin (594), while jasplakinolide can enhance clustering of raft associated proteins causing the membrane to become more rigid (less fluid) (607).

BMDCs were pre-treated with latrunculin B or jasplakinolide for 1 h at various concentrations before stimulating with LPS (5 ng/ml) and PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10, IL-12p70 and IL-1β concentrations were measured by ELISA (Fig 4.18-4.20). Additionally, BMDCs were treated with latrunculin B or Jasplakinolide (0.5 µM) for 1 h and stimulated with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 6 and 12 h. Expression of IL-10, IL-12p35 and IL-12p40 mRNA was then determined (Fig 4.18-4.20).

Interestingly, pre-treatment with either latrunculin B or jasplakinolide before stimulation of BMDCs with LPS led to elevated IL-10 secretion, as seen when 500 nm PLGA particles were added to BMDCs (Fig 4.18 and 4.19). Furthermore, inhibition of actin polymerisation using latrunculin B and jasplakinolide significantly increased IL-10 expression after 12 h in BMDCs stimulated with LPS alone and this effect was further amplified when LPS was combined with 500 nm PLGA particles (Fig 4.18B and 4.19B). This could suggest that sustaining the interaction of LPS at the membrane can enhance IL-10 production, an effect which may be exploited by particles. The effect also seems to be mediated by disruption of actin filament dynamics (leading to cell rounding) and not through a shift in G:F-actin levels, as both latrunculin B and jasplakinolide enhanced IL-10 production.

On the other hand, blocking actin polymerisation was unable to reverse PLGA particle induced IL-12p70 inhibition (Fig 4.18 and 4.20), and the inhibitors reduced LPS induced IL-12p70 secretion. Although expression of IL-12p35 was increased in inhibitor treated cells which were incubated with PLGA particles and LPS (Fig 4.18B and 4.20B), this was not reflected in terms of cytokine secretion. However, secretion of mature IL-12p70 which is made up of two subunits may depend on actin polymerisation and so the mRNA expression data may be more informative in terms of these experiments. Incubating BMDCs with jasplakinolide also enhanced IL-1β secretion in LPS activated cells to concentrations which were comparable to those seen in BMDCs incubated with LPS and 2 µm or 10 µm PLGA particles (Fig 4.21).
Lastly, as mentioned in section 1.6, Rho-associated kinase (ROCK), activated by RHO GTPases can mediate F-actin assembly (stress fibre formation) and cell contractility (258, 260). Therefore, BMDCs were incubated with varying concentrations of Rho kinase inhibitor for 1 h prior to stimulating cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 μg/ml) alone or together for 24 h. Supernatants were collected and IL-10 or TNF-α secretion was measured by ELISA. Furthermore, BMDCs were pre-treated with the Rho kinase inhibitor (1 μM) for 1 h before stimulating as before for 6 and 12 h. mRNA was then isolated and analysed to determine the expression of IL-10, IL-6 and TNF-α by RT-PCR.

Pre-treatment with the inhibitor resulted in increased IL-10 expression in DCs stimulated with either LPS alone or LPS combined with 500 nm PLGA particles (Fig 4.22A) while TNF-α expression and secretion was not significantly changed (Fig 4.22B). However, the Rho kinase inhibitor did not enhance IL-10 secretion to the same extent as either latrunculin B or jasplakinolide (Fig 4.18 and 4.19). Moreover, inhibitor treated and non-treated cells were compared by stimulating with CpG, to address whether responses to a different (TLR ligand) stimulus were similarly affected. IL-10 expression and secretion was also increased in CpG stimulated cells (Fig 4.22C), while TNF-α expression and secretion remained unchanged (Fig 4.22C). Similar to using latrunculin B and jasplakinolide, pre-treatment with Rho kinase inhibitor inhibited IL-12p70 secretion in LPS or CpG stimulated cells (data not shown).
Figure 4.18: Preventing actin polymerisation drives IL-10 expression and can reverse PLGA particle induced IL-12p35 inhibition

A) BMDCs (6.25x10^5 cells/ml) were incubated with varying concentration of latrunculin B for 1 h before stimulating with LPS (5 ng/ml) or 500 nm PLGA particles (250 μg/ml) alone or together for 24 h. Supernatants were collected with IL-10 and IL-12p70 concentrations were measured by ELISA.

B) BMDCs (1x10^6 cells/ml) were treated with latrunculin B (0.5 μM) for 1 h before stimulating cells with LPS (5 ng/ml), 500 nm PLGA particles or 500 nm PLGA particles and LPS (5 ng/ml) together for 6 and 12 h. Total RNA was isolated and IL-10 (12 h), IL-12p35 and IL-12p40 (6 h) mRNA determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

Results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
Figure 4.19: Inhibiting actin polymerisation enhances IL-10 mRNA expression

A) BMDCs (6.25x10^5 cells/ml) were incubated with varying concentration of jasplakinolide for 1 h before stimulating with LPS (5 ng/ml), 500 nm or 2 µm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 concentrations were measured by ELISA.

B) BMDCs (1x10^6 cells/ml) were treated with jasplakinolide (0.5 µM) for 1 h before stimulating cells with LPS (5 ng/ml), 500 nm PLGA particles or 500 nm PLGA particles and LPS (5 ng/ml) together for 12 h. Total RNA was isolated and IL-10 mRNA was determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

Results are expressed as the mean ± SD and are representative of three independent experiments. LPS v LPS + PLGA, LPS + inhibitor v LPS + PLGA + inhibitor. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
Figure 4.20: Inhibiting actin polymerisation can partially reverse PLGA particle induced IL-12p35 inhibition

A) BMDCs \((6.25 \times 10^5 \text{ cells/ml})\) were incubated with varying concentration of jasplakinolide for 1 h before stimulating with LPS \((5 \text{ ng/ml})\) or 500 nm PLGA particles \((250 \mu \text{g/ml})\) alone or together for 24 h. Supernatants were collected and IL-12p70 concentrations were measured by ELISA.

B) BMDCs \((1 \times 10^6 \text{ cells/ml})\) were treated with jasplakinolide \((0.5 \mu \text{M})\) for 1 h before stimulating cells with LPS \((5 \text{ ng/ml})\), 500 nm PLGA particles or 500 nm PLGA particles and LPS \((5 \text{ ng/ml})\) together for 6 h. Total RNA was isolated and IL-12p35 and IL-12p40 mRNA determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

Results are expressed as the mean ± SD and are representative of three independent experiments. LPS v LPS + PLGA, LPS + PLGA v LPS + PLGA + inhibitor. One-way ANOVA was used to determine statistical significance where \(*p<0.05\), \(**p<0.01\), \(***p<0.001\).
Figure 4.21: Preventing actin polymerisation increases IL-1β secretion by BMDCs

BMDCs (6.25x10^5 cells/ml) were incubated with varying concentration of jasplakinolide for 1 h before stimulating with LPS (5 ng/ml), 2 µm or 10 µm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-1β concentrations were measured by ELISA. Results are representative of three independent experiments. Error bars show mean ± SD for each experimental group tested individually in triplicate. LPS v jasplakinolide + LPS. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001
Figure 4.22: Inhibiting Rho kinase enhances TLR ligand and PLGA particle-induced IL-10 secretion and mRNA expression in BMDCs

BMDCs (6.25x10⁵ cells/ml) were incubated with varying concentrations of Rho kinase inhibitor for 1 h. Treated and non-treated cells were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together (A and B) or with the TLR9 agonist CpG (4 µg/ml) (C) for 24 h. Supernatants were collected and IL-10 or TNF-α concentrations determined by ELISA. BMDCs (1x10⁶ cells/ml) were treated with Rho Kinase inhibitor (1 µM) and then treated and non-treated cells were stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together (A and B) for 6 (TNF-α) or 12 h (IL-10). Total RNA was isolated and the expression of IL-10 (A) or TNF-α (B) was determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells. Results are expressed as the mean ± SD and are representative of two independent experiments. LPS + inhibitor v LPS, LPS + PLGA v LPS + PLGA + inhibitor. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
4.2.8 500 nm PLGA particles selectively exhibit a sustained interaction with the DC plasma membrane

Having demonstrated that exposure to 500 nm PLGA particles selectively changed the size and morphology of DCs (Fig 4.10-4.12) and induced lipid raft formation (Fig 4.15), whether this was due to direct interaction with the plasma membrane was investigated. As mentioned, other particulate adjuvants such as alum have also been shown to selectively interact with the membrane lipids to direct a pro-inflammatory response (474).

To assess whether 500 nm PLGA particles selectively interact with plasma membrane, fluorescent particles (green) were added to cells and incubated in a confocal microscopy chamber (37 °C, 5% CO₂) for 60 min and single images were taken every minute to monitor particle interaction with the membrane. 500 nm PLGA particles surrounded and attached to the DC plasma membrane, leading to a more rounded morphology (Fig 4.23A, video also available). Furthermore, DCs incubated with latrunculin B (1 µM) had a more rounded morphology compared to untreated cells, while a reduced number of PLGA particles interacted with these cells (Fig 4.23B, 4.24B and 4.25B). Incubation of DCs with MβCD prevented any particles interacting with the cell for a prolonged period and blocked particle induced cell rounding (Fig 4.23C).

100 nm and 10 µm PLGA particles were compared to 500 nm PLGA particles to assess whether 500 nm particles selectively interact with the membrane of cells. When 100 nm PLGA particles were added to BMDCs for live cell imaging, they were immediately taken up by the cell without any prolonged interaction with the plasma membrane (Fig 4.24A). In contrast, the 10 µm PLGA particles were too large to be phagocytosed by the cells (Fig 4.25A). When cholesterol levels were reduced with MβCD or actin polymerisation was inhibited with latrunculin B, BMDCs were still able to take up 100 nm PLGA particles, while there was no detectable uptake of 10 µm PLGA particles (Fig 4.24 and 4.25B and C). Thus, in contrast to 500 nm PLGA particles, 100 nm PLGA particles do not require actin polymerisation or lipid raft formation to be phagocytosed by BMDCs.

Finally, as alum can enhance IL-10 production in BMDCs (Fig 3.9), it was investigated if alum particles also exhibited sustained interaction with the plasma membrane. In a similar manner to 500 nm PLGA particles, alum exhibited sustained interaction with the DC plasma membrane when monitored by live-cell imaging (Fig 4.26).
Figure 4.23: 500 nm PLGA particles selectively interact with the DC plasma membrane in a manner dependent on lipid raft formation

BMDCs were treated with 1 μM latrunculin B (B), MβCD (C) or left untreated (A) for 1 h before staining with Hoechst 33342. 500 nm PLGA particles (green) were added to the cells and interactions monitored with live-cell imaging over 45 min. Cells were visualised by confocal microscopy with representative time-lapse images shown, scale bar 25 μm. Results are representative of two independent experiments (video also available).
Figure 4.24: BMDCs take up 100 nm PLGA particles independently of actin polymerisation or lipid raft formation.

BMDCs were treated with 1 µM latrunculin B (B), MβCD (C) or left untreated (A) for 1 h before staining with Hoechst 33342. 100 nm PLGA particles (green) were added to the cells and uptake monitored with live-cell imaging over 45 min. Cells were visualised by confocal microscopy with representative time-lapse images shown, scale bar 25 µm. Results are representative of two independent experiments (video also available).
**Figure 4.25: 10 µm PLGA particles are not phagocytosed by BMDCs**

BMDCs were treated with 1 µM latrunculin B (B), MβCD (C) or left untreated (A) for 1 h before staining with Hoechst 33342. 10 µm PLGA particles (green) were added to the cells and uptake monitored with live-cell imaging over 45 min. Cells were visualised by confocal microscopy with representative time-lapse images shown, scale bar 25 µm. Results are representative of two independent experiments (video also available).
Figure 4.26: Alum exhibits sustained interaction with the DC plasma membrane.
BMDCs were incubated with fluorescent alum (green, 20 μg/ml) and uptake and membrane interactions monitored with live-cell imaging over 45 min. Cells were visualised by confocal microscopy with representative time-lapse images shown, scale bar 10 μm. Results are representative of two independent experiments. Experiment performed by Dr. Ewa Oleszycka.
**4.2.9 Dendritic cell membrane fluidity is a key regulator of IL-10 production.**

As discussed in the introduction (section 1.6), fluidity is one of the most important biophysical characteristics of the cell. For example, changes in membrane fluidity (altering the physical properties of the membrane lipids) occur in response to environmental stresses such as temperature and osmotic stress and initiate downstream signalling pathways to activate stress associated gene activation (608).

As removal of cholesterol (increases fluidity) inhibited PLGA induced IL-10 production, and blocking actin polymerisation (decreases fluidity) increased the IL-10 response, it was proposed that 500 nm PLGA particles modulate DC membrane characteristics to drive the IL-10 response. Therefore, various agents were used to either increase or decrease cellular fluidity (or in other terms the rigidity) to confirm this. It has been demonstrated that MAP kinases are sensitive to changes in osmotic and oxidative stress which cause cell shrinkage, leading to Syk and p38 activation (609-611).

Sucrose is a cell impermeable substrate that is known to increase the osmotic pressure of the cell, which would increase cellular rigidity (decrease fluidity) without other metabolic effects in cells. Hyperosmotic media will cause an outward water flux from the cell and hence a decrease in membrane tension (decrease in fluidity) whereas hypoosmotic shock leads to cell swelling accompanied by an increase in membrane tension and fluidity (612-614). Therefore, a hypertonic solution (150, 75 or 12.5 mM sucrose in the culture medium) to decrease fluidity was used. BMDCs were incubated in a hypertonic solution for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10, TNF-α, IL-6 (data not shown) and IL-12p70 (data not shown) concentrations were measured by ELISA. Furthermore, BMDCs were pre-treated with sucrose (75 mM) for 1 h before stimulating as before for 6 and 12 h. mRNA was then isolated and IL-10, IL-6 and TNF-α mRNA assessed by RT-PCR.

Pre-treatment with sucrose led to increased IL-10 expression and secretion in cells stimulated with LPS alone or with LPS combined with 500 nm PLGA particles (Fig 4.27A) while TNF-α expression and secretion was inhibited (Fig 4.27B). Moreover, sucrose treated and non-treated cells were compared by stimulating with CpG, to determine if IL-10 responses to other TLR ligands were also enhanced. Indeed, CpG-induced IL-10 expression and secretion was also
increased (Fig 4.27A) while TNF-α and IL-6 (data not shown) expression and secretion remained unchanged (Fig 4.27B). IL-12p70 secretion was inhibited by sucrose in cells when stimulated with either LPS or CpG (data not shown).

Hydrogen peroxide (H₂O₂) through oxidative stress rigidifies the membrane lipid bilayer (decreasing fluidity) (615). H₂O₂ is spontaneously converted (catalysed by Fe²⁺ (Fenton reaction)) to the highly reactive hydroxyl radicals (HO⁻) that react instantaneously with any biological molecule from which it can abstract a hydrogen atom (616). Polyunsaturated phospholipids on the membrane are highly susceptible to oxidative stress which causes lipid peroxidation (degradation) (606). It has also been shown that H₂O₂ causes an increase in the number, but not the size of raft domains (615).

BMDCs were pre-treated with varying concentrations of H₂O₂ (10, 1 and 0.1 mM) for 1 h prior to stimulating cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h and secretion of IL-10, TNF-α and IL-12p70 (data not shown) secretion was determined by ELISA. Furthermore, BMDCs pre-treated with H₂O₂ (0.1 mM) for 1 h were stimulated with LPS and/or particles for 6 and 12 h and IL-10 and TNF-α expression was determined by RT-PCR. Like treating cells with sucrose, oxidative stress increased IL-10 expression and secretion in cells incubated with LPS alone or LPS combined with 500 nm PLGA particles (Fig 4.28A) while TNF-α expression and secretion remained unchanged (Fig 4.28B). Only 0.1 mM H₂O₂ was used as higher concentrations were toxic to cells (observed under microscope), leading to decreased IL-10 and TNF-α production in LPS and CpG treated cells (Fig 4.28A and B). There was a slight decrease in IL-10 and TNF-α secretion and expression in CpG treated cells (Fig 4.28A and B). IL-6 and IL-12p70 was also inhibited in H₂O₂ treated cells when stimulated with either LPS or CpG (data not shown). Therefore, the effect of H₂O₂ only applies to LPS stimulated DCs but not CpG activated cells.

Lidocaine is a local anaesthetic that disrupts lipid rafts without altering membrane cholesterol (617) and increases the fluidity of the membrane (224). Lidocaine (and MβCD) treated macrophages or epithelial cells can inhibit infection of pathogenic protozoa including Toxoplasma gondii by inhibiting the adhesion and internalisation of the parasites (618). By increasing the fluidity of the cells, it was expected that lidocaine would attenuate the IL-10 response, comparable to when cholesterol is removed from the cell membrane.

BMDCs were pre-treated with varying concentrations of lidocaine (1, 0.1 and 0.01 mg/ml) for 1 h prior to stimulating cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone
or together for 24 h. Supernatants were collected and IL-10 and TNF-α secretion was determined by ELISA. Furthermore, BMDCs were pre-treated with lidocaine (1 mg/ml) for 1 h before stimulating as before for 6 and 12 h and IL-10 or TNF-α expression assessed by RT-PCR. Lidocaine treatment significantly reduced 500 nm PLGA particle induced IL-10 secretion and expression in BMDCs (Fig 4.29A) while inhibiting TNF-α secretion and expression (Fig 4.29B). In contrast, IL-10 and TNF-α expression and secretion was downregulated in CpG stimulated cells (Fig 4.29A and B). Lidocaine treatment resulted in a decrease in IL-12p70 secretion and IL-6 expression in LPS or CpG treated cells (data not shown).

As mentioned above (section 4.2.9), swelling of cells (hypoosmotic shock) can also increase the fluidity of the membrane, which can be achieved by diluting medium with water, as cells are highly permeable to water causing them to swell. For this experiment, BMDCs were subjected to hypoosmotic stress by diluting medium with water in a final volume of 250 µl starting at a ratio of 10:1 (medium: H₂O) and increasing the volume of water used (5:1, 4:1, 3:1, 2:1). Cells were left for 2 h before stimulating with LPS alone (5 ng/ml) or together with 500 nm PLGA particles (250 µg/ml) for 24 h. Supernatants were collected and IL-10 and TNF-α concentrations measured by ELISA. Just as lidocaine inhibited IL-10 production, diluting the medium with water to increase cell swelling suppressed IL-10 production (Fig 4.30A). TNF-α levels remained unchanged in the hypoosmotic medium when cells were stimulated with LPS (Fig 4.30A). To reaffirm this, Benzyl alcohol (BA), an agent known to increase membrane fluidity was also used (619). Again, membrane fluidity was increased by adding various concentrations of BA for 1 h before stimulating cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. As expected, treating cells with BA (1 mM) significantly reduced IL-10 production while TNF-α production was unaffected in cells stimulated with LPS alone or LPS together with 500 nm PLGA particles (Fig 4.30B).

BMDCs treated to increase or decrease membrane fluidity were compared in terms of their shape and morphology. BMDCs were treated with latrunculin B (0.5 µm), sucrose (75 mM), MβCD (10 mM x 2 and then 2 mM) or lidocaine (1 mg/ml) for 1 h before stimulating cells with 500 nm PLGA particles (green) for 1 h or untreated. Cells were then fixed and subsequently stained with DAPI and Alexa Fluor 594 Phalloidin (red) and then analysed by confocal microscopy. Both latrunculin B and sucrose treated cells exhibited a more rounded morphology than controls (Fig 4.31), similar to BMDCs which had been incubated with 500 nm PLGA particles (Fig 4.12). In contrast, removal of cholesterol prevented cells displaying such a
phenotype after 2 h. Lidocaine treated cells detached from the slide and had their actin randomly distributed throughout the cell (Fig 4.31).

Having demonstrated that exposure to 500 nm-2 µm PLGA particles reduced cell size, whether other stresses could also promote cell shrinkage was investigated. Therefore, BMDCs were incubated with sucrose (75 mM), MβCD (10 mM x2 and left in 2 mM), latrunculin B (0.5 µM) or lidocaine (0.5 mg/ml) alone or together with 100 nm or 500 nm PLGA particles (0.25 mg/ml) and the forward scatter was analysed to determine changes in size. Incubation of cells with 100 nm PLGA particles had no impact on cell size, but incubation with 500 nm PLGA particles led to a decrease in forward scatter (Fig 4.10 and 4.32). Treatment of BMDCs with sucrose had a comparable effect on the forward scatter of cells to 500 nm PLGA particles (Fig 4.32). Increasing fluidity, either had no impact on forward scatter (lidocaine) or caused cells to increase in size (MβCD). Furthermore, increasing fluidity did not prevent 500 nm PLGA particle induced decrease in cell size (Fig 4.32). Instead, both treatments disrupt the initiation of downstream signalling pathways. Latrunculin B treatment increased cell forward scatter, but was still able to round up the cells, as previously demonstrated by confocal and phase contrast microscopy (Fig 4.23-4.25B).

Finally, BMDCs were pre-treated with the p38 inhibitor SB203580 before stimulating cells with sucrose or H2O2 in the presence or absence of LPS. As expected, blocking p38 abrogated both sucrose and H2O2 induced IL-10 (Fig 4.33A) indicating that, like 500 nm PLGA particles (section 4.2.11), IL-10 enhancement primarily depends on activation of the p38 kinase pathway. Furthermore, incubating BMDCs with sucrose enhanced p38 phosphorylation, even in the absence of LPS stimulation (Fig 4.33B).
Figure 4.27: Sucrose enhances IL-10 secretion by DCs

BMDCs (6.25x10^5 cells/ml) were incubated with varying concentrations of sucrose for 1 h. Treated and non-treated cells were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together or with the TLR9 agonist CpG (4 µg/ml) for 24 h. Supernatants were collected and IL-10 (A), TNF-α (B) concentrations determined by ELISA. BMDCs (1x10^6 cells/ml) were treated with sucrose (75 mM) or medium alone and then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together or with CpG (4 µg/ml) for 6 (TNF-α) or 12 h (IL-10). Total RNA was isolated and the expression of IL-10 (A) or TNF-α (B) was determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells. Results are expressed as the mean ± SD are representative of two independent experiments. LPS + sucrose v LPS, LPS + PLGA v LPS + PLGA + sucrose, *p<0.05, **p<0.01, ***p<0.001.
Figure 4.28: H\textsubscript{2}O\textsubscript{2} enhances IL-10 transcription and secretion in BMDCs
BMDCs (6.25x10\textsuperscript{5} cells/ml) were incubated with varying concentrations of H\textsubscript{2}O\textsubscript{2} for 1 h. Treated and non-treated were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together or with the TLR9 agonist CpG (4 µg/ml) for 24 h. Supernatants were collected and IL-10 (A) or TNF-α (B) concentrations determined by ELISA. BMDCs (1x10\textsuperscript{6} cells/ml) were treated with H\textsubscript{2}O\textsubscript{2} (0.1 mM) and then treated and non-treated cells were stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together or with CpG (4 µg/ml) for 6 (TNF-α) or 12 h (IL-10). Total RNA was isolated and the expression of IL-10 (A) or TNF-α (B) was determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells. Results expressed as the mean ± SD are representative of two independent experiments. LPS + H\textsubscript{2}O\textsubscript{2} v LPS, LPS + PLGA v LPS + PLGA + H\textsubscript{2}O\textsubscript{2}, *p<0.05, **p<0.01, ***p<0.001.
Figure 4.29: Lidocaine inhibits PLGA particle induced IL-10 in BMDCs

BMDCs (6.25x10^5 cells/ml) were incubated with varying concentrations of lidocaine for 1 h. Treated and non-treated were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together or with the TLR9 agonist CpG (4 µg/ml) for 24 h. Supernatants were collected and IL-10 (A) or TNF-α (B) concentrations were determined by ELISA. BMDCs (1x10^6 cells/ml) were treated with lidocaine (1 mg/ml) and then treated and non-treated cells were stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together or with CpG (4 µg/ml) for 6 (TNF-α) or 12 h (IL-10). Total RNA was isolated and the expression of IL-10 (A) or TNF-α (B) was determined using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells. Results expressed as the mean ± SD are representative of two independent experiments. LPS + lidocaine v LPS, LPS + PLGA v LPS + PLGA + lidocaine. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
A.

BMDCs (6.25x10^5 cells/ml) were subjected to hypoosmotic stress by diluting medium with H2O (10:1, 5:1, 4:1, 3:1, 2:1) at various ratios for 2 h. Cells were then stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together or for 24 h (A). BMDCs (1x10^6 cells/ml) were also treated with Benzyl alcohol for 1 h (50 mM-0.1 mM). Treated and non-treated cells were stimulated with LPS (5 ng/ml) and 500 nm PLGA particles alone or together for 24 h (B). Supernatants were collected and IL-10 and TNF-α concentrations determined by ELISA. Results are expressed as the mean ±SD are representative of two independent experiments. LPS + treatment v LPS, LPS+PLGA v LPS+PLGA+ treatment. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.

B.

Figure 4.30: Hypoosmotic stress suppresses IL-10 production by BMDCs
Figure 4.31: Changes in membrane fluidity affect filamentous actin distribution and shape of DCs BMDCs were treated with latrunculin B (0.5 \( \mu \text{M} \)), sucrose (75 mM), MβCD (10 mM x 2 and then 2 mM) or lidocaine (1 mg/ml) for 1 h before stimulating with 500 nm PLGA particles (100 \( \mu \text{g/ml} \), green) for 1 h. Cells were fixed and subsequently stained with DAPI and Alexa Fluor 594 Phalloidin (red) and then analysed by confocal microscopy. Confocal images are representative of three independent experiments, scale 25 \( \mu \text{m} \).
Figure 4.32: Exposure to sucrose and 500 nm PLGA particles reduces the size of BMDCs
BMDCs were treated with sucrose (75 mM), MβCD (10 mM x2 and 2 mM), latrunculin B (0.5 µM) or lidocaine (0.5 mg/ml) alone or with 100 nm or 500 nm PLGA particles (0.25 mg/ml) for 24 h. Live CD11c+ cells were analysed and their size determined by forward scatter by flow cytometry. Data are representative of two independent experiments tested in duplicate.
A. 

![Graph showing IL-10 production](image1)

**Figure 4.33: Sucrose induced IL-10 is p38 dependent in BMDCs**

A) BMDCs (6.25x10^5 cells/ml) were incubated with the p38 inhibitor SB203580 (5 µM) for 1 h before treated and non-treated cells were stimulated with sucrose (75 mM) or H_2O_2 (0.1 mM) in the presence or absence of LPS (5 ng/ml) for 24 h. Supernatants were collected and IL-10 concentration was determined by ELISA. LPS + sucrose/H_2O_2 v LPS, LPS + sucrose/H_2O_2 v LPS + SB203580 + sucrose/H_2O_2. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.

Results are representative of three independent experiments. Error bars show means ± SD for each experimental group tested individually in triplicate.

B) BMDCs (1x10^6 cells/ml) were stimulated with either sucrose (75 mM), H_2O (3:1), LPS (0.2 ng/ml) or left untreated for 30 and 60 min. Total protein was isolated and immunoblotted for phospho-p38 and total p38 to act as a loading control. Results are representative of two independent experiments.
4.2.10 The enhancement of IL-10 secretion by PLGA particles in BMDCs requires Syk

Spleen tyrosine kinase (Syk) is an important intracellular signalling molecule, coupling activated immunoreceptors to downstream signalling events to mediate a diverse number of cellular responses, including both proinflammatory IL-1β and anti-inflammatory IL-10 cytokine production (220, 478, 620).

Alum has been demonstrated to interact with lipid rafts on the membrane of cells, activating Syk in a receptor independent manner, triggering downstream signalling pathways and proinflammatory cytokine secretion (280, 474). However, this has not been demonstrated for other particulate adjuvants nor has Syk activation been linked to an anti-inflammatory response triggered by particulate adjuvants.

Firstly, the ability of PLGA particles to activate Syk was investigated. Syk has a number of phosphorylation sites that can result in either inhibition or activation. Phosphorylation of the tyrosine 525 and 526 sites (Tyr519/520 in mice) located in the activation loop of the Syk kinase domain is important for its function (621) and the tyrosine 352 (Tyr352) site is involved in the association with PLC-γ1, Akt and ERK (622). Therefore, the ability of PLGA particles to activate Syk was investigated. BMDCs (1x10⁶ cells/ml) were stimulated with either LPS (5 ng/ml) or 500 nm PLGA particles (250 µg/ml) alone or together for indicated time points (5, 15, and 30 min). Total protein was isolated and immunoblotted for phospho-Syk (Tyr352) and total Syk in the cells.

The exposure of DCs to 500 nm PLGA particles alone and in the presence of LPS enhanced Syk phosphorylation at early time points (5 and 15 min), while LPS alone was incapable of significantly activating Syk (Fig 4.34A). Additionally, 100 nm, 500 nm, 2 µm, 10 µm and 30 µm PLGA particles were compared for their ability to promote Syk activation. BMDCs were stimulated with PLGA particles alone or together with LPS or with curdlan (positive control) for 15 min and immunoblotted for Syk phosphorylation. Only 500 nm and 2 µm PLGA particles activated Syk, which corresponds to the sizes capable of enhancing IL-10 in BMDCs (Fig 4.35).

Subsequently, the role of cholesterol in mediating PLGA induced Syk activation was explored. Cholesterol was demonstrated (Section 4.2.6) to play a significant role in PLGA particle induced IL-10 responses in DCs. Cholesterol levels were reduced in BMDCs using MβCD as
before, BMDCs (1x10^6 cells/ml) were then stimulated with LPS and PLGA particles alone or together for 15 min. Cell lysates were prepared and immunoblotted to assess Syk phosphorylation. As before, 500 nm PLGA particles activated Syk, but this response disappeared when cells were depleted of cholesterol (Fig 4.34B). Thus, PLGA particles need cholesterol and subsequent raft formation on the cell surface of DCs to trigger Syk phosphorylation.

Pre-treating BMDCs with latrunculin B and then stimulating with LPS enhances IL-10 production, so whether inhibiting actin polymerisation also activates Syk was investigated. Latrunculin B treated and untreated BMDCs were stimulated with LPS alone (5 ng/ml) or together with 500 nm PLGA particles (250 µg/ml) or with curdlan (10 µg/ml) for 15 min. Cell lysates were prepared and immunoblotted for phospho-Syk. Inhibiting actin polymerisation did not activate Syk in LPS stimulated cells nor did it change Syk activation in 500 nm PLGA particle and LPS stimulated BMDCs (Fig 4.36).

The enhancement in IL-10 expression and secretion by 500 nm PLGA particles is suppressed following treatment with several different Syk inhibitors (Fig 4.37 and 4.38). Most small molecule Syk inhibitors, except for the substrate-site inhibitor piceatannol, target the ATP-binding site. Accordingly, both piceatannol and the ATP binding R788 Syk inhibitors were used to block Syk activation. Curdlan was used as a positive control to ensure that the inhibitors are used at an appropriate concentration to prevent non-specific inhibition of other kinases (Fig 4.37B and 4.38B). Curdlan is a dectin-1 agonist, which activates the Syk signalling pathway to promote IL-10 secretion (64).

BMDCs were stimulated with a range of concentrations of piceatannol or R788 for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 and IL-1β levels measured by ELISA. Piceatannol was able to significantly attenuate IL-10 and IL-1β production in PLGA stimulated DCs (Fig 4.37A). Concentrations between 20 µM and 5 µM significantly inhibited IL-10 secretion in both PLGA particle and curdlan treated DCs (Fig 4.37A and B).

Additionally, BMDCs were treated with piceatannol before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles alone or together for 12 h and IL-10 expression was determined by RT-PCR. Inhibiting Syk in PLGA particle stimulated DCs reduced IL-10 expression to a level comparable to DCs stimulated with LPS only (Fig 4.37C). These data indicate that the
enhancement in IL-10 production by PLGA particles is primarily mediated through the Syk pathway.

Furthermore, BMDCs were treated with the alternative Syk inhibitor, R788 for 1 h before stimulating cells as before for 24 h. Treating cells with R788 generated results consistent with those observed using piceatannol. R788 concentrations between 25 µM and 1 µM could prevent IL-10 secretion in PLGA particle and LPS treated DCs (Fig 4.38A) as well as in curdlan stimulated cells (Fig 4.38B), reaffirming that Syk plays a crucial role in mediating PLGA particle induced IL-10.
A. 

| 500 nm PLGA | + | + | + | + | + | + | + |
| LPS | + | + | + | + | + | + |

**Figure 4.34: PLGA particles activate Syk in DCs in a cholesterol dependent manner**

A) BMDCs (1x10^6 cells/ml) were stimulated with either LPS (5 ng/ml), 500 nm PLGA particles (500 µg/ml) alone or together for 5, 15 and 30 min. Total protein was isolated and immunoblotted for phospho-Syk and total Syk to act as a loading control. Results are representative of three independent experiments.

B) BMDCs (1x10^6 cells/ml) were treated with 10 mM MβCD twice for 20 min, then with 2 mM for 2 h before stimulation with LPS (5 ng/ml) or 500 nm PLGA particles (250 µg/ml) alone or together for 20 min. Total protein was isolated and immunoblotted for phospho-Syk and total Syk. Results are representative of two independent experiments.
Figure 4.35: Only PLGA particle sizes capable of driving IL-10 secretion in BMDCs activate Syk

BMDCs (1x10^6 cells/ml) were stimulated with either LPS (5 ng/ml), 100 nm, 500 nm, 2 µm, 10 µm or 30 µm PLGA particles (250 µg/ml) alone or together or curdlan (10 µg/ml) for 15 min. Total protein was isolated and immunoblotted for phospho-Syk and total Syk to act as a loading control. Results are representative of three independent experiments.
Figure 4.36: Inhibiting actin polymerisation does not affect Syk activation by PLGA particles in BMDCs

BMDCs (1x10^6 cells/ml) were treated with latrunculin B (0.5 µM) for 1 h before stimulating treated and non-treated cells with LPS (5 ng/ml), 500 nm PLGA particles (250 µg/ml) alone or together or Curdlan (10 µg/ml) for 10 min. Total protein was isolated and immunoblotted for phospho-Syk and total Syk. Results are representative of two independent experiments.
Figure 4.37: The induction of IL-10 by PLGA particles requires signalling through Syk

A) BMDCs (6.25x10^5 cells/ml) were incubated with piceatannol (20-1 µM) 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 and IL-1β concentrations were measured by ELISA.

B) BMDCs (6.25x10^5 cells/ml) were incubated with piceatannol (20-1 µM) 1 h before stimulating with medium or curdlan (10 µg/ml) for 24 h. Supernatants were collected and IL-10 concentrations measured by ELISA.

C) BMDCs (1x10^6 cells/ml) were pre-treated with piceatannol (20 µM) 1 h before stimulating treated or non-treated cells with medium, LPS (5 ng/ml), 500 nm PLGA particles or 500 nm PLGA particles and LPS (5 ng/ml) together for 12 h. Total RNA was isolated and the expression of IL-10 was measured using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

All results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. Treated v non-treated, *p<0.05, **p<0.01, ***p<0.001.
Figure 4.38: The Syk inhibitor R788 reduces PLGA particle induced IL-10 production in BMDCs

A) BMDCs (6.25x10⁵ cells/ml) were incubated with a range of concentrations of the Syk inhibitor R788 for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 concentrations measured by ELISA.

B) BMDCs (6.25x10⁵ cells/ml) were incubated with a range of concentrations of the Syk inhibitor R788 for 1 h before stimulating with medium or curdlan (10 µg/ml) for 24 h. Supernatants were collected and IL-10 concentrations measured by ELISA.

All results are expressed as the mean ± SD of samples in triplicate and are representative of two independent experiments. Treated v non-treated, *p<0.05, **p<0.01, ***p<0.001.
4.2.11 The p38 signalling pathway is required for the enhancement of IL-10 production by PLGA particles

Having confirmed that Syk, cholesterol and changes in membrane fluidity were crucial for promoting PLGA particle induced IL-10, the downstream signalling pathways activated by PLGA particles were further investigated. The mechanism that controls IL-10 production in DCs and macrophages in response to defined stimuli (e.g. LPS and zymosan) is thought to involve MAPKs, such as ERK, p38, mitogen and stress-activated protein kinases (64, 65). p38 plays a critical role in IL-10 production in response to TLR agonists as well being implicated in IL-12 regulation in APCs (143, 303). Furthermore, p38 is known to be activated in response to various stresses sensed by the cell, including osmotic and other external mechanical stresses (623, 624). Therefore, p38 was investigated for its role in modulating both IL-10 and IL-12p70 production by PLGA particles.

The ability of 500 nm PLGA particles to augment p38 activation was investigated by western blotting. BMDCs (1x10^6 cells/ml) were stimulated with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 15 and 30 min. Whole-cell lysates were prepared and analysed by immunoblotting with Abs specific for p38 phosphorylated at Thr180/Tyr182 and total p38, followed by ECL detection. Furthermore, Syk was also inhibited using piceatannol (20 µM) 1 h before stimulating cells. Incubation of DCs with PLGA particles increased p38 phosphorylation after 15 min (Fig 4.39). Additionally, inhibiting Syk blocked particle driven p38 phosphorylation, returning phosphorylation levels to those seen in cell treated with LPS only (Fig 4.39). It should also be noted that inhibiting Syk did not affect p38 activation in LPS treated DCs.

BMDCs were pre-treated with varying concentrations of the p38 inhibitor SB203580 for 1 h prior to stimulating cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Furthermore, BMDCs were pre-treated with SB203580 (5 µM) for 1 h before stimulating as before for 6 and 12 h. mRNA was then isolated and analysed for an increase in IL-10, IL-12p35 and IL-12p40 by RT-PCR.

Inhibition of p38 significantly reduced IL-10 secretion by DCs stimulated with LPS and PLGA particles together (Fig 4.40A and 4.40B). As in the case of inhibiting Syk, treatment with SB203580 prevented PLGA particles from enhancing IL-10 secretion by DCs, with IL-10 levels comparable to those in DCs incubated with LPS alone. In contrast, blocking p38 did not
reverse IL-12p70 or IL-12p35 inhibition (Fig 4.40A and 4.40B) indicating that the p38 pathway is not involved in regulating IL-12 by particles.

Previously it has been shown that disrupting actin polymerisation can activate p38 (625, 626). Hence, whether treating cells with latrunculin B to disrupt actin polymerisation could activate or enhance p38 activity in BMDCs was examined. Latrunculin B enhanced the activation of p38 in LPS stimulated cells, this was similar to when BMDCs are stimulated with 500 nm PLGA particles (Fig 4.41A) (there is also a small effect with the inhibitor alone). It was also revealed that latrunculin B treatment prolonged p38 activation in cells stimulated with LPS alone or together with 500 nm PLGA particles (Fig 4.41A). This could indicate that latrunculin B and 500 nm PLGA particles are inducing the same signalling pathways to increase IL-10, as cells pre-treated with latrunculin B prior to stimulation with LPS and 500 nm PLGA particles amplifies both the p38 response (Fig 4.41A) and IL-10 expression (Fig 4.18B). In contrast, removing cholesterol from the plasma membrane with MβCD prevented 500 nm PLGA particles from enhancing p38 activation (Fig 4.41B), indicating that the 500 nm PLGA particles require raft formation to promote p38 activation and IL-10 secretion.
Figure 4.39: 500 nm PLGA particles enhance p38 phosphorylation through the Syk signalling pathway

BMDCs (1x10^6 cells/ml) were treated with piceatannol (20 µM) 1 h before stimulating treated and non-treated cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 15 and 30 min. Total protein was isolated and immunoblotted for phospho p38 and total p38. Results are representative of two independent experiments.
A) BMDCs (6.25x10^5 cells/ml) were incubated with varying concentrations of the p38 inhibitor SB203580 for 1 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 and IL-12p70 concentrations were determined by ELISA.

B) BMDCs (1x10^6 cells/ml) were pre-treated with SB203580 (5 µM) for 1 h before stimulating treated or non-treated cells with medium, LPS (5 ng/ml), 500 nm PLGA particles or 500 nm PLGA particles and LPS (5 ng/ml) together for 6 or 12 h. Total RNA was isolated and the expression of IL-10 (12 h), IL-12p35 and IL-12p40 (6 h) was measured using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

All results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. Treated v non-treated. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
Figure 4.41: PLGA particle enhancement in IL-10 production fully depends on the p38 signalling pathway

BMDCs (1x10^6 cells/ml) were treated with latrunculin B (0.5 µM) for 1 h or MβCD (10 mM x2 and 2 mM) before stimulating treated and non-treated cells with LPS (0.2 ng/ml), 500 nm PLGA particles (250 µg/ml) alone or together for 30, 60 or 120 min. Total protein was isolated and immunoblotted for phospho-p38 and total p38. Results are representative of two independent experiments.
4.2.12  **Alum induced IL-10 production in BMDCs also requires Syk and p38 signalling**

Having demonstrated that 500 nm PLGA particles increase IL-10 production in DCs through the Syk and p38 signalling pathways, the capacity of other particulate adjuvants such as alum to activate the same pathway and modulate IL-10 was tested. Alum was chosen as it has already been demonstrated to enhance IL-10 production by BMDCs (Section 3.2.5) and activate Syk to promote PGE₂ and IL-1β production (474). Another signalling pathway, PI3K has already been implicated in alum induced IL-12 inhibition (476) and therefore its role in inducing IL-10 by alum was investigated.

BMDCs were treated with the PI3K inhibitor wortmannin (100 nM), Syk inhibitor piceatannol (20 µM) and p38 inhibitor SB203580 (5 µM) for 1 h prior to stimulating with alum (50 µg/ml) and LPS (10 ng/ml) alone or together. Supernatants were collected after 24 h and IL-10 secretion measured by ELISA. Additionally, mRNA samples were collected after 12 h using the same stimulations and analysed for IL-10 expression.

Alum increased both protein and transcript levels for IL-10 when incubated with LPS (Fig 4.42A and Fig 4.42B) after 24 and 12 h respectively. This response was inhibited by both the Syk and p38 inhibitors (Fig 4.42A and Fig 4.42B). Inhibiting PI3K did not significantly reduce alum induced IL-10 production. Thus, like 500 nm PLGA particles, alum can promote robust IL-10 responses in DCs which depend on the Syk-p38 signalling pathway. It would be interesting to look at other types of particles (e.g. silica) and compare different sizes (100 nm, 500 nm and 30 µm) to see if the effects observed with PLGA and alum are also applicable to other materials.
**Figure 4.42: Alum promotes IL-10 in a Syk and p38 dependent manner**

A) BMDCs (1x10^6 cells/ml) were pre-treated with wortmannin (100 nM), SB203580 (5 µM) or piceatannol (20 µM) 1 h before stimulating treated or non-treated cells with medium, LPS (5 ng/ml), alum (50 µg/ml) or alum and LPS together for 12 h. Total RNA was isolated and the expression of IL-10 was measured using quantitative real-time PCR. Data were normalised with respect to β-actin mRNA levels and results are expressed as fold induction over untreated cells.

B) BMDCs (6.25x10^5 cells/ml) were incubated with wortmannin (100 nM), SB203580 (5 µM) or piceatannol (20 µM) 1 h before stimulating treated or non-treated cells with LPS (5 ng/ml) and alum (50 µg/ml) alone or together for 24 h. Supernatants were collected and IL-10 concentrations were measured by ELISA.

All results are expressed as the mean ± SD of samples in triplicate and are representative of three independent experiments. Treated v non-treated. One-way ANOVA was used to determine statistical significance where *p<0.05, **p<0.01, ***p<0.001.
4.2.13 **Phosphorylation of the transcription factor CREB is enhanced in cells incubated with 500 nm PLGA particles**

Having investigated the signalling pathways primarily involved in PLGA particle induced IL-10, the transcription factors mediating this response were explored. The cAMP response element-binding protein (CREB) is one of the best characterised transcription factors mediating IL-10 production in macrophages and DCs (170). Other transcription factors may also be involved, including MAF, SP1, ATF1, NF-κB and C/EBPβ (143) CREB has been implicated as a key transcription factor that binds the IL-10 promoter in APCs (64, 170, 173).

First, whether PLGA particles had the capability to activate CREB was investigated by western blot. BMDCs were stimulated with 500 nm PLGA particles, LPS (5 ng/ml) alone or both together. Total protein was extracted and immunoblotted for phospho-CREB and total CREB. Enhanced CREB phosphorylation was observed in BMDCs co-incubated with PLGA particles and LPS compared to LPS on its own after 1 h (Fig 4.43A).

Having established that both Syk and p38 are required for PLGA particle induced IL-10, the requirement for these pathways in particle activation of CREB was tested. The Syk inhibitor piceatannol (20 µM) and p38 inhibitor SB203580 (5 µM) were added 1 h before DCs were stimulated with a lower dose of LPS (0.1 ng/ml) and PLGA particles (250 µg/ml). The LPS concentration was lowered so the synergy between LPS and PLGA particles would be easier to distinguish. LPS in combination with PLGA particles led to increased phosphorylation of CREB whereas; LPS at a lower dose (0.2 ng/ml) failed to phosphorylate CREB (Fig 4.43B), therefore, confirming that PLGA particles can enhance CREB phosphorylation. Moreover, inhibiting both Syk and p38 prevented PLGA particle-induced CREB phosphorylation (Fig 4.43B).

Finally, the role of cholesterol and actin polymerisation in particle activation of CREB was explored. The role of cholesterol in particle activation of Syk has been shown (Section 4.2.6), so the same method to deplete cholesterol was employed here. By reducing cholesterol levels on the surface of BMDCs, the ability of PLGA particles to phosphorylate and activate CREB was abolished while the response to LPS alone was unaffected (Fig 4.43C). Thereby, to further confirm the cholesterol-Syk-p38 pathway induced by 500 nm PLGA particles activates CREB to produce IL-10, CREB will need to be knocked down in a future experiment.
Disrupting actin polymerisation has been associated with increasing p38 kinase activity and subsequent IL-10 release so whether CREB activation was strengthened or prolonged in the presence of latrunculin B was examined. Latrunculin B treated (0.5 µM) and untreated BMDCs were stimulated with LPS (0.2 ng/ml) alone or together with 500 nm PLGA particles (250 µg/ml) for 60 and 120 min. Cell lysates were prepared and immunoblotted for CREB and examined for changes in the protein phosphorylation levels. Inhibiting actin polymerisation did not change or prolong CREB activation in cells stimulated with LPS alone or together with 500 nm PLGA particles (Fig 4.44), indicating that p38 is primarily controlling the increase in IL-10 when actin cytoskeleton is disrupted. Although, the response to PLGA particles alone was blocked at the earlier timepoint (60 min).
Figure 4.43: PLGA particles enhance the IL-10 transcription factor CREB through cholesterol, the Syk and p38 pathways.

A) BMDCs (1x10^6 cells/ml) were stimulated with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for indicated time points.

B) BMDCs (1x10^6 cells/ml) were treated with either SB203280 (5 µM) or piceatannol (20 µM) 1 h before stimulating treated and non-treated cells with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 60 min.

C) BMDCs (1x10^6 cells/ml) were treated with 10 mM MβCD twice for 20 min, then with 2 mM for 2 h before stimulating with LPS (5 ng/ml) and 500 nm PLGA particles (250 µg/ml) alone or together for 60 min.

Total protein was isolated and immunoblotted for phosphor-CREB and total CREB. Results are representative of two independent experiments.
Figure 4.44: 500 nm PLGA particle induced CREB activation is not affected by latrunculin B

BMDCs (1x10^6 cells/ml) were treated with latrunculin B (0.5 µM) for 1 h before stimulating treated and non-treated cells with LPS (5 ng/ml), 500 nm PLGA particles (250 µg/ml) alone or together for 60 or 120 min. Total protein was isolated and immunoblotted for phospho-CREB and total CREB. Results are representative of two independent experiments.
4.3 Discussion

Understanding the mechanisms of adjuvant action is essential for improving existing vaccines, rational design of new vaccines as well as identifying key pathways to be exploited or avoided if a certain response is required. The idea that particulate adjuvants can induce anti-inflammatory effects which in turn make them inefficient for developing effective cellular immune responses has not been widely investigated. The focus of this chapter was to elucidate the underlying mechanism by which particulate adjuvants induces the production of IL-10 and suppress IL-12 in BMDCs.

IL-10 can antagonise the expression of costimulatory molecules and pro-inflammatory cytokines which can inhibit APC function and affect T-cell development and differentiation (627). It is also well known that both IL-10 and IL-12 regulation can be strongly linked. For example, IL-10 can mediate suppression of IL-12 production in APCs through blocking or activating several transcription factors involved in regulating IL-12 production (e.g. p65 NF-κB, c-rel and c-Maf) (293, 628). Inhibition of IL-12 may be a prominent mechanism by which IL-10 regulates adaptive immune responses and limits immunopathology. For instance, It has been shown that both DCs and macrophages are potent producers of IL-10 following TLR ligand stimulation or CD40 ligation which impacts IL-12p70 secretion (146). Therefore, there was a strong possibility that any increase in IL-10 mediated by PLGA particles will lead to a subsequent decrease in IL-12p70 production. This was tested by comparing responses in wild type and IL-10−/− BMDC responses to PLGA particles. However, there was no link between increased IL-10 and the inhibition of IL-12, indicating that they are regulated by two different pathways.

The PI3K pathway is a negative regulator of TLR ligand-mediated IL-12 responses in DCs, in particular the IL-12p35 subunit through regulation of the mTOR and GSK3β pathways (85, 88). Potentially, there could be a combination of increased IL-10 along with activation of the PI3K signalling pathway mediating the suppression of IL-12p70 production in DCs. Surprisingly, IL-12 inhibition was not reversed using IL-10−/− BMDCs nor did simultaneously inhibiting the PI3K pathway make a difference. Thus, this definitively demonstrates that PLGA particle modulation of the IL-12 and IL-10 pathways operates via distinct pathways.

The NLRP3 inflammasome was not required for the modulation of IL-10 and IL-12p70 by PLGA particles but using NLRP3−/− BMDCs and a caspase-1 inhibitor, it was shown that PLGA particles of a certain size (10 µm) enhance secretion of IL-1β in a NLRP3 dependent manner.
However, recently the release of IL-1β by alum \textit{in vivo} has been shown to be inflammasome-independent. It has now been reported that a number of proteases, including cathepsin S are involved in IL-1β secretion following alum and monosodium urate crystal stimulation (538). IL-1β secretion induced by PLGA particles also partially relies on cathepsin S \textit{in vitro}. This suggests that cathepsin S along with other proteases also contribute to IL-1β release and the existence of a general pathway activated in DCs when they make contact with particulate adjuvants (538, 549). As mentioned above, particulates which are strong drivers of IL-1β could be mediating this through cell swelling too.

Since alum, PS and PLGA particles can significantly increase IL-10 secretion, an effect which was previously reported for calcium phosphate crystals (409) this may suggest a shared mechanism of IL-10 induction by particulates. The first objective was to determine if a specific receptor(s) on the surface of DCs or the uptake of the PLGA particles was involved in the induction of IL-10 or suppression of IL-12p70. It is currently unclear whether PLGA particles interact non-specifically with receptors on the cell surface. For example, it has been demonstrated that MSU crystals can directly bind to the FcγIII receptor (CD16) to activate Syk on neutrophils (629). Ligation of Fcγ receptors can lead to the phosphorylation of intracellular ITAMs which in turn recruits Syk kinase (192). On the other hand, alum and MSU crystals interact directly with lipids on the cell membrane of DCs to activate signalling pathways in an Fc receptor independent manner (280). Other receptors which could potentially be engaged by particulates include the C-type lectin receptor dectin-1 which activates Syk to promote IL-10 secretion in response to curdlan and zymosan in BMDMs (64). Syk-coupled to CLRs can act in cooperation with MyD88-coupled TLRs in DCs that culminates in the activation of p38, JNK and ERK MAPKs leading to induction of Th1/Th17 and IL-10 producing T cell responses (630). Having highlighted two potential groups of receptors which may mediate cytokine production by particulate adjuvants, both dectin-1 and Fc receptors on the cell surface were blocked before treating cells with 500 nm PLGA particles. However, blocking these receptors made no difference to either IL-10 or IL-12p70 modulation in DCs indicating that these pathways were not responsible for the particle induced modulatory effects.

Only the macrophage receptor with collagenous structure (MARCO) scavenger receptor has been shown to bind to PS nanoparticles and other unopsonised particles to aid phagocytosis. Blocking actin polymerisation with cytochalasin D slightly but significantly reduced particle-MARCO association when the cells were treated with 20 nm or 200 nm PS particles (631-633). However, our data demonstrated that particles of those sizes will be taken up by cells
independent of any actin dependent process or lipid raft formation. 100 nm PLGA particles were taken up by DCs in presence of latrunculin B and when cholesterol was removed using MβCD almost immediately after incubation with the cells. Additionally, i.v administration of 500 nm carboxylated PS or PLGA particles reduced the number of inflammatory monocytes trafficking into the site of inflammation, alleviated disease symptoms, and induced tissue repair in inflammatory disease models (546). The disease models examined included mouse models of West Nile virus encephalitis, EAE, peritonitis, colitis, and cardiac and kidney ischemia-reperfusion models. They implicate the MARCO receptor to mediate uptake of the particles which leads to apoptosis in the spleen (546). However, this was only shown for PS particles, as our data demonstrated in chapter 3, only 50 nm PS particles can induce apoptosis while all PLGA sizes are not as toxic to DCs. As will be discussed, 500 nm PLGA particles do not require uptake by the cells to drive their IL-10 response, rather it happens quickly at the cell surface. Although, the MARCO scavenger receptor will be blocked in a future experiment to confirm that it’s not involved in driving 500 nm PLGA induced IL-10.

Alterations to cell shape have long been associated with changes in cell function (634), which can be caused by physical cues such as changes in ECM (e.g. change in stiffness) and neighbouring cells or soluble factors (e.g. cytokines and chemokines) secreted during inflammation or injury (275, 593). Changing cell shape is important for mediating normal biological functions, such as tissue development, wound healing, cell division and motility (635). During tumour initiation and cancer cell metastasis, mechanical signals from the expanding tumour microenvironment, alter stiffness of ECM leading to cells with aggressive invasive properties (592, 636, 637).

Macrophages change their shape as they migrate to and probe sites of injury or inflammation, which depends on physical cues from the microenvironment (e.g. ECM stiffness) and soluble factors they encounter (593). BMDMs which exhibit a more rounded morphology is associated with a M1 like response, while cells with an elongated phenotype with more actin protrusions are in a M2 polarising state (274, 593). This has also been observed in human THP-1 macrophages, where M1 macrophages are smaller and more rounded then their M2 counterpart (638). These cells exhibited higher TNF-α production while smaller rounded cells had lower TNF-α production (639). Furthermore, the type of surface that macrophages encounter determines the level of cytokines produced and cell morphology. For instance, contact with both hydrophilic and anionic surfaces downregulated TNF-α, IL-1β and IL-6 and upregulated
IL-10 and resulted in a more rounded morphology (640-642). Tissues are composed of multiple cell types, various ECM proteins and other constituents, each with unique mechanical characteristics (643). Macrophages present in tissues can respond to various mechanical signals (593), when exposed to stiffer substrates they will display an inflammatory elongated phenotype (644). Such an environment is seen with atherosclerotic plaques which leads to macrophage activation and proliferation to aid disease progression. Furthermore, the type of biomaterial used for transplantation and wound healing is very important, as macrophages are among the first cells to interact with implanted biomaterials and regulate the host foreign body response (639).

While cell shape is important for inducing a specific response in macrophages, studies into how shape affects DCs responses remain incomplete. Here, we show that DCs incubated with 500 nm (and 2 µm) PLGA particles selectively exhibited a more rounded phenotype and exhibited a decreased cell size. This rounded phenotype has also been associated with a more differentiated state with enhanced transcription and secretion rates but lower DNA synthesis in hepatocytes (645). A rounded morphology is also induced by disrupting F-actin with cytochalasin D, decreasing DNA synthesis while enhancing its differentiated functions (646). This rounded shape was also observed here when BMDCs were incubated with latrunculin B and jasplakinolide to disrupt the actin cytoskeleton. Normal actin and myosin function is required for cell spreading, disrupting either results in the cells becoming more rounded and inhibits DNA synthesis (647). Disrupting F-actin assembly with cytochalasin D lowered both stiffness and spreading, while inhibiting myosin light chain kinase (Rho kinase pathway) inhibited spreading but increased cell stiffness. Therefore, despite having opposing effects on cell stiffness, they both induced a rounded phenotype in cells, suggesting that cell stiffness does not correlate with cell shape. Cell shape can also been manipulated during disease, as invasive breast cancer cells have a reduced the number of stress fibres (637), allowing the cells to become more motile while also displaying a more rounded phenotype (648).

However, it remains unclear how blocking actin polymerisation and uptake affects both IL-10 and IL-12p70 production in LPS and particle treated BMDCs. Both latrunculin B and cytochalasin D have been used to block uptake in cells, latrunculin B was unable to impair IL-10 or IL-12p40 production in zymosan stimulated BMDCs (200) while cytochalasin D had no effect on IL-10 production in *Staphylococcus aureus* treated macrophages (649). In contrast,
LPS treated Cdc42^-/- DCs, which have defect in actin organisation showed significantly reduced IL-12p70 secretion (650).

Latrunculin B and jasplakinolide both induced rounding in DCs, despite having opposing effects on actin polymerisation. Interestingly, disrupting the actin cytoskeleton with either agent or blocking the Rho kinase pathway enhanced IL-10 in LPS stimulated DCs. This was similar to DCs which show a more rounded morphology and enhanced IL-10 production following stimulation with 500 nm or 2 μm PLGA particles. This led to the idea that a general mechanism was being employed by DCs, through changes in morphology to enhance IL-10. Furthermore, disrupting the actin cytoskeleton increased PLGA particle induced IL-10 transcript levels indicating that more particles could be interacting with the membrane thereby activating Syk. It also indicated that phagocytosis of the particles was not needed to enhance the IL-10 response when DCs are stimulated with 500 nm PLGA particles. Using both latrunculin B and jasplakinolide indicated that disrupting actin and changing the morphology was enough to drive the IL-10 response while the G-actin: F-actin ratio didn't matter. This is can be seen with the Hippo pathway which plays a role in cell proliferation in response to mechanical signals. The pathway is insensitive to changes in G-actin: F-actin ratio. Rather, evidence suggests that F-actin structure and cell morphology regulate YAP/TAZ (nuclear transducers of the Hippo pathway) localisation and activity (651, 652).

It has been suggested that particle uptake can lead to cell death, release of DAMPs and inflammasome activation and subsequent IL-1β production. For example, uptake of PLGA particles, alum and chitosan can enhance IL-1β secretion (549, 653). The engulfed particles trigger lysosomal membrane damage, leading to NLRP3 activation via cathepsin B release (654). Particle uptake likely depends on both the cell type and the material, it has been suggested that particles (e.g. PLGA, liposomes and ISCOMs) ranging between 20 nm and 200 nm in size are more likely endocytosed for a better cellular immune response whereas larger particles such as those of 500 nm or 5 μm in size are primarily phagocytosed or micropinocytosed to promote humoral immunity (516). Uptake of particles by BMDCs greater than 10 μm in size is limited (549). Our lab has demonstrated that PS particles up to 10 μm in size can be taken up by BMDCs (549). Interestingly, disrupting actin polymerisation enhanced IL-1β secretion in LPS stimulated BMDCs, this may be due to the inability of the DCs to phagocytose the TLR4 receptor, indicating that IL-1β is mainly induced through the MyD88/MAL TLR4 pathway in DCs.
Finally, any agent used to disrupt actin polymerisation also decreased IL-12p70 secretion. However, this may be due to the inability of the IL-12p70 subunit to be properly released from the cell, as actin may be needed to form the bioactive IL-12p70 from its two subunits, IL-12p35 and IL-12p40. Latrunculin B, jasplakinolide and Rho kinase inhibitor partially reversed IL-12p35 levels inhibited by 500 nm PLGA particles. Disrupting actin polymerisation may block pathways other than uptake, including release or formation of IL-12p70 which is made up of its two subunits. Therefore, the expression data may be more informative on how uptake can modulate the response to particles. In contrast to the IL-10 pathway, the uptake of PLGA particles may play a role in suppressing IL-12p70 levels in DCs.

Alum and MSU crystals do not have specific receptors on the surface of DCs. Responses to MSU were intact in MyD88 and TRIF double knockout mice indicating that TLR signalling was not required (280, 655). The mechanism underlying how particulates or crystals interact with cells, particularly the initial contact with APCs remains unclear. However, direct interaction with the membrane and subsequent receptor independent activation of kinases remains a strong possibility (474). It has been suggested that alum does not enter DCs; it instead delivers soluble antigen across the plasma membrane in a non-phagocytic manner (474). Our confocal data demonstrates that both alum and 500 nm PLGA particles selectively interact with the membrane/cell surface of DCs for a prolonged time before eventually being taken up. This has been reported before as alum shows affinity for DC surface to activate the ITAM-Syk pathway without inducing phagocytosis (474). Since all of the confocal images are back projections, it’s not always clear that the particles are on the plasma membrane. Therefore, isosurface rendering of the Z stacks to create a 3-D image of the cell will be used to confirm that only 500 nm PLGA particles engage the plasma membrane surface for a prolonged period before being endocytosed.

Alum and MSU crystals have been reported to directly engage lipids (e.g. cholesterol) on cell membranes, leading to lipid sorting which non-specifically aggregates ITAM containing receptors to recruit Syk and PI3K and leading to proinflammatory responses (280, 474). Recently, this response has also translated to human macrophages and DCs when stimulated with osteoarthritis-associated basic calcium phosphate crystals (656). Therefore, whether 500 nm PLGA particles selectively interact with membranes for a prolonged period to cause a lipid sorting event on the membrane of BMDCs and subsequent rounding of the cell was addressed.
Cholesterol is a major component of cell membranes, required to maintain appropriate fluidity and permeability and form part of lipid rafts which can mediate signal transduction and phagosome formation (657). Methyl-β-cyclodextrin (MβCD) removes plasma membrane cholesterol through formation of a soluble complex with cholesterol disrupting lipid raft formation (658). It should be noted that lipid rafts in immune cells are also rich in saturated fatty acids, sphingolipids and glycosylphosphatidylinositol anchored (GPI) proteins which could potentially interact with particles to facilitate signalling molecule recruitment (659). This may be the reason for only a partial decrease in the IL-10 response induced by PLGA particles when cholesterol is depleted but may also reflect that not all the cholesterol was removed due to the toxicity issue with MβCD. Additionally, immune cells can constantly produce new lipids such as cholesterol and deliver them to the cell surface. Removing cholesterol was unable to reverse PLGA particle mediated IL-12p35 inhibition, indicating that membrane interaction of PLGA particles is not involved in inhibiting IL-12.

Incubating BMDCs with either 100 nm, 500 nm or 10 µm PLGA particles and staining for lipid rafts highlighted the selective effects of 500 nm PLGA particles. Only 500 nm PLGA particles formed more lipid rafts in BMDCs as shown by confocal microscopy. These lipid rafts could be initiating a unique pathway only associated the 500 nm PLGA particles, like how alum can interact with the membrane to cause a lipid sorting event and downstream signalling (280). Subsequently, when cholesterol was removed from the cell surface, 500 nm PLGA particles were unable to interact with the plasma membrane to initiate a lipid sorting event. As the particles are unable to contact the membrane for a prolonged time, the IL-10 response is reduced. However, there was heterogeneity in BMDCs for raft formation when incubated with 500 nm PLGA particles. Lipid raft formation will need to be quantified by counting cells from several different experiments to calculate the percentage of cells that have increased raft formation. Also, BMDCs will be incubated for longer time periods with 500 nm PLGA particles to see if this reduces heterogeneity of the samples. Blocking actin polymerisation with latrunculin B reduced the number of particles interacting with the membrane but rounded the cells 500 nm PLGA particles. Thus, latrunculin B and 500 nm PLGA particles are having similar effects on IL-10 secretion, which is amplified when latrunculin B treated cells are stimulated with 500 nm PLGA particles at the transcript level. In contrast, DCs incubated with either 100 nm or 10 µm PLGA particles showed no significant difference in raft formation.

Different sized particles have been reported to have different uptake rates in macrophages (660), including 100 nm PLGA particles having faster uptake than larger particles (661). Here
we show that 100 nm PLGA particles were most efficiently taken up by cells compared with other sizes. Despite their ability to be easily taken up by DCs, 100 nm PLGA particles were unable to modulate cytokines secretion in vitro nor could they promote innate and antigen-specific adaptive immune responses in vivo. Although, antibody responses are enhanced with both 100 nm and 500 nm PLGA particles (data not shown).

A common feature of phagocytosis (for particles larger than 0.5 µm) and micropinocytosis (for particles less than 0.5 µm) is a reliance on the actin machinery (662, 663) (Fig 1.7C). Reports have suggested that actin is essential for different forms of particle phagocytosis. Yet, 100 nm PLGA particles were still phagocytosed when actin polymerisation was inhibited, indicating a pathway independent of actin machinery. It has also been shown that lipid rafts also play a role in the phagocytosis of PS microspheres (664), and that MβCD inhibits clathrin/caveolae-mediated endocytosis, clathrin/caveolae independent endocytosis and pinocytosis (665, 666). Removing cholesterol from the cell surface did not prevent uptake of the 100 nm PLGA particles, indicating that rafts also do not play a part in their phagocytosis. It has been shown that uptake of PS particles (< 25 nm) by HeLa cells occurs via a cholesterol-independent, non-clathrin- and non-caveolae-mediated non-degradative pathway that avoids endo/lysosomal trafficking (667), this may also be occurring with 100 nm PLGA particles. Therefore, this highlights that different endocytotic uptake mechanisms exist depending on the size of the particle. Finally, 10 µm PLGA particles were not phagocytosed by DCs after 2 h. However, after 24 h some DCs were found to contain small number of 10 µm particles (1 or 2 particles max) (data not shown).

Recent reports suggest that MSU crystals, silica and alum and can induce Syk dependent kinase signalling in DCs by directly engaging with cholesterol-rich cellular membranes of DCs in a receptor independent manner (280, 474, 478). ITAM-containing receptors will aggregate in cholesterol-rich lipid rafts on the cell surface which will subsequently recruit Syk which in turn can recruit PI3K to mediate downstream signalling pathways. However, particulate induced Syk activation has only been associated with promoting pro-inflammatory responses (IL-1β and PGE₂), even though the Syk pathway can also mediate IL-10 responses in response to bacterial and fungal stimulation (64, 220, 668). For the first time, this work shows that particulate adjuvants of a certain size can activate the Syk pathway to promote the release of the anti-inflammatory cytokine IL-10, thus indicating that the Syk pathway has the capability to activate pro- and anti-inflammatory responses simultaneously by particulate adjuvants. Syk may be a potential therapeutic target for treating human inflammatory disease, based on
successful results in arthritic mouse models (669, 670). While Syk inhibitors have been tested to treat rheumatic and other autoimmune diseases (670), they so far have either failed, only had moderate benefits over the placebo group or caused unwanted side effects in clinical trials (222, 671, 672), with no Syk inhibitor approved for use in patients yet. This could be related to their ability to block IL-10 production along with pro-inflammatory cytokines.

Moreover, PI3 kinase δ−/− mice administered with a Syk inhibitor, show that the pathway is important for recruitment of inflammatory DCs to the site of MSU crystal injection but was also required for the development of a Th2 response (479). Notably, Syk signalling in DCs is crucial to develop asthma and airway inflammation in OVA sensitised mice, a process that is driven by Th2 cells (673). Syk signalling can couple to the NLRP3 inflammasome to modulate IL-1β secretion (212, 214) and inhibiting Syk significantly inhibited particulate induced IL-1β secretion by BMDCs.

Only incubation of DCs with 500 nm and 2 µm PLGA particles phosphorylated and activated Syk, the same sizes which enhanced IL-10 production. 500 nm PLGA particles could activate Syk after only 5 min, indicating a rapid response by the BMDCs. However, if cholesterol was removed, PLGA particles no longer had the ability to activate Syk, indicating a link between the two. Furthermore, by targeting Syk using pharmacological inhibitors the particle mediated increase in IL-10 was abolished. As some Syk inhibitors (SykI, and BAY61-3606) may not be specific (204), two different Syk inhibitors which target different parts of the Syk protein (674) were used together with the positive control curdlan (10 µg/ml) which relies on Syk to promote IL-10 secretion (64). Piceatannol targets the Syk substrate binding site while R788 targets the ATP-binding site. R788 (Fostamatinib) has been used in clinical trials to treat rheumatoid arthritis, but results have been mixed (222, 675-677). It has been ineffective at treating active severe forms of the disease but significant improvements were seen in patients failing to respond to other treatments and with milder symptoms. As mentioned, this may be due to the dual role that Syk plays in promoting both pro and anti-inflammatory responses. Both inhibitors could prevent any increase in IL-10 mediated by 500 nm PLGA particles while also inhibiting curdlan induced IL-10 at the same inhibitor concentration. Thus, 500 nm PLGA particles can increase Syk activation in a cholesterol dependent manner to increase IL-10 expression in BMDCs.

Having established the involvement of Syk, the downstream signalling molecules involved in either IL-10 enhancement or IL-12 inhibition by PLGA particles were investigated. The
expression of IL-10 and IL-12 by APCs can be regulated by many signalling pathways, including PI3K, ERK and mTOR. PI3K/Akt can activate either the mTOR or GSK3β signalling pathway, playing pivotal roles in controlling IL-10 and IL-12 in APCs (678). For example, GSK3β activation has been demonstrated to suppress CREB mediated IL-10 production (90). PI3k/Akt activation inhibits GSK3β, preventing IL-10 suppression. Therefore, the class I PI3K inhibitor ZSTK474 allows the release and activation of GSK3β to inhibit IL-10. Thus, 500 nm PLGA particles could potentially enhance PI3K signalling to prevent GSK3β mediated inhibition of IL-10. Interestingly, suppressing PI3K with ZSTK474 only partially inhibited the PLGA particle induced IL-10 response. It has been demonstrated that rapamycin can decrease IL-10 expression and secretion in monocytes and DCs treated with LPS, indicating that mTOR positively regulates IL-10 while concurrently suppressing IL-12 (678-680). Surprisingly, mTOR played no role in either IL-10 or IL-12p70 regulation by 500 nm PLGA particles in BMDCs.

In contrast, while IL-12 is downregulated by PLGA particles of certain sizes (500 nm-10 µm) and alum, a role for PI3K has only been suggested in the case of alum (476). Results from our lab have shown that wortmannin treated BMDCs that are then stimulated with LPS exhibit enhanced IL-12 secretion. Furthermore PI3K⁺ splenic DCs demonstrate a similar pattern in response to *Staphylococcus aureus*, anti-CD40 or TLR agonists (681). Thus, highlighting a PI3K-mediated negative feedback mechanism for IL-12 production by DCs. However, no role for PI3K was found for PLGA particle induced IL-12p70 inhibition when the class I PI3K inhibitor ZSTK474 was used here. It would be interesting to use this inhibitor before treating DCs with alum to see if the same result is obtained as the non-specific PI3K inhibitor wortmannin was used in the previous report (476). In conclusion, the PI3K pathway was only partially responsible for PLGA induced IL-10 and the mTOR pathway was not involved.

TLR activation can also activate ERK1/2 which enhances IL-10 expression (143). IL-10 production downstream of Syk activation also requires signalling through the ERK pathway (668). ERK is also involved in the negative regulation of IL-12p40 (73). Interestingly, abrogation of either ERK or p38 activation in macrophages or DCs leads to a reduction, but not abrogation of IL-10 expression, which suggests that these two pathways might cooperate in TLR-induced IL-10 production (157). A similar trend was observed here with 500 nm PLGA particles: the enhanced IL-10 was only partially reduced by PI3K or ERK inhibitors but completed inhibited when p38 was blocked. Blocking ERK also partially reversed the PLGA particle mediated inhibition of IL-12. Finally, the JAK/STAT pathway has been implicated in
both IL-10 and IL-12p70 signalling pathways (308, 682). However, blocking the JAK/STAT pathways did not reverse IL-12p70 inhibition and enhanced IL-10 secretion in LPS treated DCs, implying that JAK/STAT are negative regulators for IL-10 secretion in DCs. In contrast, blocking the JAK/STAT pathway in BMDMs inhibits IL-10, indicating the pathway has opposite effects in DCs and macrophages (682).

MAP kinases such as p38 have proved to be essential for regulating IL-10 in TLR and C-type lectin receptor stimulated DCs and Macrophages (64, 157). IL-10 production can be compromised by inhibition of p38 signalling in LPS- or CpG-activated macrophages, DCs and human peripheral blood monocytes (143). For example, stimulation of macrophages with either a dectin-1 specific ligand (curdlan) or the combined dectin-1/TLR agonist zymosan (yeast cell wall extract) leads to Syk recruitment to the ITAM sequence in the cytoplasmic domain of dectin-1/TLR2. This leads to the activation of downstream signalling pathways including p38 and ERK1/2, eventually leading to phosphorylation of CREB on the IL-10 promoter (64). Inhibition of p38 can also increase IL-12 production in APCs upon stimulation with TLR agonists (303). Blocking p38 with the inhibitor SB203580 fully abolished PLGA particle induced IL-10 production, but was unable to reverse IL-12p70 (or IL-12p35) inhibition demonstrating that p38 only positively regulates IL-10 induction by PLGA particles. SB203580 returned IL-10 mRNA expression levels in PLGA particle stimulated cells back to those seen in cells treated with LPS alone, indicating that p38 is regulating IL-10 at the transcriptional level as p38 can also act post transcriptionally by stabilising IL-10 mRNA (183). Finally, p38 activation was increased in cells treated with PLGA particles and LPS to a greater degree than that seen in cells treated with LPS alone. This enhancement was abrogated when cholesterol was removed or Syk was blocked; indicating that membrane induced Syk activation mediates any further phosphorylation of p38 to increase IL-10.

The formation of lipid rafts has a major impact on the fluidity properties of the cells, as an increase in the amount of cholesterol relative to phospholipid has been shown to decrease fluidity in biological membranes (683). Cholesterol is needed to maintain the integrity of rafts to initiate downstream signalling pathways. Therefore, removing cholesterol from the cell surface will increase the membrane fluidity and disrupt raft formation (684).

Cells have adapted to appropriately respond to environmental stresses (685). Changes in plasma membrane fluidity activates stress-induced MAPK signalling pathways, supporting the importance of the plasma membrane in mechanotransduction (226, 686). It has been
demonstrated that changing fluidity in endothelial cells is enough to initiate signal transduction pathways, including ERK and JNK MAP Kinases (226). The p38 pathway is known for its role in various stress responses, including those to pro-inflammatory cytokines and osmotic shock (687). For example, hyperosmotic stress causes cell shrinkage which activates the p38 signalling cascade (688) to induce various responses including cell death (689). Hyperosmotic stress can be seen during inflammation and disease and has been used to treat sepsis (690, 691). Additionally, reactive oxygen species (ROS) are another well-known stressor that have been linked to activation of kinases, including ERK, JNK and p38 (692, 693). These stress responses have never been linked to IL-10 production.

Therefore, our hypothesis was that the PLGA particle sizes capable of driving IL-10 resulted from directly binding to and changing the fluidity properties of the DC plasma membrane, inducing a stress response to initiate a specific p38 dependent signalling cascade. Furthermore, it was hypothesised that a general mechanism was being employed by DCs to enhance IL-10 secretion in response to other common stresses. DCs will sense certain stresses (mechanical) to initiate a specific signalling pathway by decreasing membrane fluidity, leading to increased IL-10 production. This was evident as only 500 nm PLGA particles were capable of interacting with the plasma membrane for a prolonged period and activating stress associated kinase pathways, including Syk and p38 leading to IL-10 release. Both sucrose and hydrogen peroxide which induce osmotic and oxidative stress respectively also significantly enhanced IL-10 in a p38 dependent manner in LPS stimulated DCs, to the same levels as 500 nm PLGA particles. Furthermore, treatment with sucrose rounded the cells and caused cell shrinkage, like when 500 nm PLGA particles are incubated with DCs, indicating that a similar pathway was being activated.

Actin polymerisation is closely associated with the formation of rafts (242) and can translate external forces into certain biochemical signalling pathways (253). A similar stress response in cells could be occurring in response to disrupting actin polymerisation. This is highlighted by the finding that DCs treated with latrunculin B or jasplakinolide also exhibited enhanced and prolonged p38 activation leading to elevated IL-10 levels. No change in Syk activation occurred in LPS stimulated cells, indicating that Syk activation needs a mechanical signal (e.g. 500 nm PLGA particles) to get activated. Thus, this implicates p38 as the main regulator for stress induced IL-10.
In contrast, if the fluidity of the membrane was increased, it should inhibit IL-10 production. This was clarified using H$_2$O which causes cells to swell, benzyl alcohol, a known membrane fluidiser, MβCD and the local anaesthetic lidocaine. All three attenuated PLGA induced IL-10 production. It also highlights the profound and opposing effects of cell swelling (H$_2$O) and shrinkage (sucrose) on IL-10 production. It has been suggested that hypertonic (also known as hyperosmotic) solutions could be used to treat inflammatory disease by preventing NLRP3 inflammasome activation, while hypotonic solution which increases cell swelling are inflammatory through activation of the NLRP3 inflammasome, caspase-1 and release of mature IL-1β (694-696). Cell swelling has also been linked to activation or ERK, PI3K and JNK (697). Low osmolarity solutions and cell swelling can recruit macrophages in vivo and could be considered as a potential danger signal recognised by the immune system (696). This corresponds with our results as hyperosmotic solutions and other stressors enhanced the anti-inflammatory cytokine IL-10 which depends on the p38 pathway, while hypotonic solutions inhibited IL-10, which creates a more inflammatory environment. While not explored here, exposure of DCs to 10 µm PLGA particles promoted secretion of high concentrations of IL-1β, which may be due to their ability to swell the cell initiating the inflammatory cascade. It should also be noted that any agent that disturbs membrane fluidity also inhibits IL-12p70, the reason for this remains unknown but as mentioned it may be due to its complicated sub-unit secretion system.

Alum is capable of interacting with the membrane of cells to activate Syk, leading to secretion of proinflammatory cytokines and mediators (474). Whether the activation of Syk by alum also mediates p38 phosphorylation and subsequent IL-10 secretion was examined. The results here suggest that at 500 nm PLGA particles and alum share a common mechanism in BMDCs as blocking both Syk and p38 reduced the alum mediated increase in IL-10 production. PI3K has been shown to play a role in the inhibition of IL-12 by alum in BMDCs (476), however here it is shown that PI3K plays a limited role in alum driven IL-10 responses, suggesting that other pathways play a more dominant role.

There are a number of transcription factors that regulate IL-10 expression with the importance of different promoter elements varying depending on the cell type and stimuli. The main transcription factors known to regulate IL-10 in DCs and macrophages are CREB (165) and NF-κB (143, 169) with CREB regulating IL-10 transcription downstream of p38 after activation of TLR4 and Mal (170, 171). TLR ligation, increased cAMP or Ca$^{2+}$ can result in
CREB phosphorylation to initiate IL-10 transcription in DCs and macrophages (165, 172-174). Incubation of DCs with 500 nm PLGA particles led to enhanced CREB phosphorylation. Having established that the 500 nm PLGA particle mediated IL-10 required cholesterol, Syk and p38, the corresponding inhibitors were used to demonstrate the involvement of these pathways in particle driven CREB phosphorylation. Finally, blocking actin polymerisation with latrunculin B did not affect CREB activation in LPS stimulated cells. Like Syk, it seems CREB activation also requires a mechanical signal such as 500 nm PLGA particles to enhance activation.

The role of CREB and most of the other IL-10 promoting transcription factors have been well characterised in macrophages, as they produce IL-10 in much higher amounts when stimulated with TLR agonists (143). However, the importance of specific transcription factors in DCs is still unclear. Here, the results point to a role for CREB in PLGA particle induced IL-10 in DCs although the importance of CREB must be formally demonstrated. Therefore, CREB will need to be knocked down in DCs using siRNA to see if PLGA enhanced IL-10 is abrogated.

In conclusion, the results here suggest that 500 nm PLGA particles and alum share a common mechanism to induce IL-10 secretion. DCs membrane lipids act a pseudo receptor for these particles. By binding to cholesterol, lipid sorting ensues to trigger activation of Syk and its downstream signalling pathway chain of events (p38 and CREB) leading to the release of IL-10. A common IL-10 signalling pathway is also induced when cells come under certain physical stresses. Cells can sense these mechanical cues in their environment through their membrane, change shape to a more rounded morphology, induce lipid raft formation and convert these mechanical signals to a biochemical signal (Fig 5.1 and 5.3). Here, stresses such as 500 nm and 2 µm PLGA particles, oxidative stress, osmotic stress and disrupting actin polymerisation leads to elevated IL-10 release (Fig 5.3). In contrast, when the membrane fluidity is increased, these signals are not generated and IL-10 is not induced (Fig 5.3). On the other hand, IL-12 inhibition does not seem to be regulated by these membrane interactions but inhibiting actin polymerisation partially reverses IL-12p35 inhibition.
Certain sizes of PLGA particles and alum can interact with lipids including cholesterol on the surface of dendritic cells and macrophages which causes lipid raft formation (A). Once the cells sense this mechanical signal, they become more rounded, leading to activation of Syk and subsequent activation of downstream MAPKs (p38, ERK and PI3K) to activate IL-10 transcription factors (e.g. CREB) and subsequent IL-10 release from the cells (B). Other mechanical stresses such as osmotic and oxidative stress can also enhance IL-10 through this pathway while cell swelling and increase in membrane fluidity will prevent activation of the pathway (B).
Nanoparticles (<200 nm) are efficiently taken up by antigen-presenting cells (APCs) and can directly drain into lymphatic vessels. Results have demonstrated that nanoparticles (50-100 nm) induce a strong cytotoxic (CD8) T cell response in comparison with larger particles (>500 nm). Nanoparticles travel through lymphatic vessels to reach lymph nodes, where they can increase the probability of immune cell interaction, although the associated mechanism of action is unclear. In contrast, larger particles (500–2000 nm) will be trapped in the tissue at the injection site require uptake and transport by APCs to be delivered to lymph nodes. During this process, these particles will cause APCs to become rounded through membrane interactions which triggers enhanced IL-10 and suppressed IL-12p70 production as outlined in figure 5.1. This will lead to the attenuation of Th1 responses during priming by inhibiting proliferation and IFN-γ production.
Figure 5.3: Proposed model for how different PLGA particle sizes and cellular stresses induce changes in membrane fluidity to modulate DC driven immune responses

Certain PLGA particle sizes (500 nm-2000 nm) selectively interact with the plasma membrane of DCs for a prolonged period. DCs undergo a change in shape, fluidity and size leading to secretion of IL-10. Mechanical signals such as hyperosmotic and oxidative stress can also enhance IL-10 through this mechanism, while agents that cause hypoosmotic stress (H₂O) or increase membrane fluidity (e.g. local anaesthetics) will prevent induction of this IL-10 pathway. PLGA particles of this size could potentially be used to treat a variety of inflammatory diseases. In contrast, smaller PLGA particles (50-100 nm) are quickly phagocytosed by DCs and more efficient at inducing the antigen-specific cellular immune responses needed for vaccines against viruses and cancers.
Chapter 5

General Discussion
The concept of vaccine adjuvants was introduced almost a century ago by Gaston Ramon and Alexander Glenny who showed the value of administering antigens with insoluble or irritant substances (e.g. mineral salts, starches and saponins) to increase the yield of antisera (698). Since then, adjuvants have become key components in most vaccines to enhance and shape antigen-specific immune responses, particularly in subunit vaccines which are based on highly purified but poorly immunogenic antigens.

There are only a limited number of licensed adjuvants available for human vaccines, with most using aluminium-based formulations. They have been successful at targeting pathogens with a stable antigen repertoire and where only neutralising and/or opsonising antibodies are needed to provide protection. Alum is the most widely used in vaccines including those against tetanus, pertussis, diphtheria, HBV and *Haemophilus influenzae* type b. Yet, many infectious diseases and cancers require robust cell mediated immunity to induce protection. Designing vaccines against HIV, Malaria, TB and cancers has been challenging, as most licensed adjuvants are unable to promote effective cell mediated immunity. In line with this, aluminium-based adjuvants are potent drivers of humoral immunity and T-helper 2 (Th2) responses (699) but fail to induce strong CMI particularly Th1, Th17 and cytotoxic T cells (476). Consequently, there have been significant efforts to develop alternative adjuvants over recent decades. Recently, the adjuvant AS01 has been used in a Malaria vaccine called RTS,S which has shown an ability to promote strong CD4+ T cell-mediated immune responses and is currently in clinical trials to be incorporated in a vaccine against the Herpes zoster virus (347).

Understanding the mechanisms mediating adjuvanticity remains key to designing adjuvants that promote effective cellular immunity. Alum has had many proposed mechanisms, including the formation of a depot to slowly release antigen at the site of injection, which has now been challenged (463). Other proposed mechanisms include cell death at the site of injection and release of damage-associated molecular patterns including uric acid (465), host DNA (470) and IL-33 (466). Alum has been shown to activate the NLRP3 inflammasome *in vitro* and *in vivo* (469, 700). However, the contribution that the NLRP3 inflammasome makes to adjuvanticity remains controversial, as more recent studies found no role for NLRP3, IL-1β or IL-18 in alum-induced humoral immunity (470, 473, 566). Finally, a pathway which is a major focus of this study involves the direct binding of alum to the membrane of DCs to promote lipid raft formation that induces Syk and PI3K activation in a receptor-independent manner (474). Currently, the poor efficacy of alum at inducing cell mediated immunity particularly Th1 and CD8 responses is unclear. Our lab has suggested that a combination of enhancing the anti-
inflammatory cytokine IL-10 and inhibiting the Th1 polarising cytokine IL-12p70 could be contributing to this (476). IL-10 is a well characterised anti-inflammatory cytokine, capable of affecting the strength and quality of the cell mediated response (572). Stimulating BMDCs, BMDMs and human monocytes with alum significantly enhances IL-10 production and transcription. Furthermore, intramuscular injection of alum primes draining lymph node cells to secrete enhanced IL-10 following ex vivo restimulation. In the absence of IL-10 signalling, immunisation with OVA and alum leads to the induction of an OVA-specific Th1 response which is not seen in wild types.

Antigen presenting cells, particularly DCs are important targets for particulate adjuvants as they facilitate efficient antigen uptake and processing, with localised cytokine release and upregulation of costimulatory molecules determining the type of T-cell response. Thus, DCs are essential mediators of immunity and tolerance (92, 93). Given their key role, understanding how particulate adjuvants interact with and influence DC responses is critical for designing new vaccines aimed at inducing a particular response. For example, alums adjuvanticity is clearly mediated through targeting of DCs, as depleting CD11c+ DCs abrogated antigen-specific IgG1 and IgE following injection of aluminum hydroxide with OVA (465).

Most adjuvants have not been designed to target particular immune receptors or pathways as our understanding of their mode of action remains incomplete. Adjuvant selection should be based on a deep understanding of their mode of action and ability to trigger appropriate antigen-specific immune responses since an inadequate adjuvant may result in vaccine failure even with a strong candidate antigen. Unravelling the underlying mechanisms employed by particulate adjuvants may help to design more effective vaccines against Malaria, TB, HIV and various cancers. The objective of this thesis was to understand why widely used particulate adjuvants are unable to induce sufficiently potent cell mediated immunity and the importance of size in adjuvant induced cellular immunity.

Polymeric nano- and microparticles have emerged as novel adjuvant candidates. Biodegradable PLGA particles have been extensively studied as an attractive approach for controlled release of vaccine antigens, used as a delivery vehicle for drugs, proteins and various other macromolecules such as DNA and RNA (487, 500). They also protect the antigen from degradation and can be used to target APCs and facilitate the uptake of antigens to enhance immune responses. For example, they can deliver TLR agonists or immunomodulators (e.g. rapamycin) to APCs to induce a tolerogenic T cell response (488). In addition, they have been
shown to have intrinsic adjuvant activities alone or in combination with other immunostimulatory compounds (529, 701, 702), which we reaffirm here.

PLGA particles remain a strong alternative to alum, as they are already FDA approved for use in humans to aid drug delivery (483, 484) with currently 15 FDA-approved PLA/PLGA-based drug products available on the US market (FDA’s Regulatory Science Program for Generic PLA/PLGA-Based Drug Products). However, so far there has been disappointment incorporating PLGA particles into vaccine formulations for use in humans as no PLGA based vaccine has been clinically approved. Yet, PLGA particles remain an attractive option as they can be easily modified to change properties, including charge, shape, polymer MW and size which can impact on their capacity to promote antigen-specific immunity. Particle size will influence particle uptake, trafficking, circulation time, clearance and how particles interact with the environment (282, 510, 703). Here we focused on how changing the size of the particulates can influence their capacity to induce and direct both innate and adaptive immune responses.

There remains a lack of consensus on how adjuvant size influences the strength and quality of humoral and cell mediated immunity (526, 539, 554, 704). A number of reasons may explain this, as the characteristics of the material used are usually not consistent, which includes particle size distribution and/or uniformity of size, charge and surface modifications (516). Differences in experimental design such as vaccination route, antigen dose, immunisation scheme and mouse strain could have also contributed to the inconsistencies between studies. The principal readout for many studies is still antigen-specific antibodies, so it’s still unclear what size is best at inducing strong T cell responses. Our lab discovered that only 50 nm PS particles are capable of inducing antigen-specific CD8\(^+\) and Th1-type responses as well as the strongest humoral response (Claire Hearnden, PhD 2014) although, the precise mechanism by which 50 nm PS particles promote such responses has not been resolved.

Of interest, many particulate adjuvant studies use particles of around 1 µm in size. Early studies showed that efficient targeting of particulate antigen to APCs is a major factor contributing towards the generation of an adaptive immune response, which requires that the particle size should be between 1 and 10 µm (705, 706). Alum’s size has been reported to be within this size range. Nanoparticles (20–200 nm) are taken up via endocytosis by DCs to trigger a virus-like response while micron sized particles (e.g. 0.5 µm) are phagocytosed by macrophages and elicit responses associated with bacteria (519). It is possible that bacteria around this size range have evolved to trigger increased IL-10 production resulting from their direct interaction with
membranes of APCs. Interestingly, PS and PLGA particles within this size range (0.5-10 µm) are most potent at enhancing IL-10 secretion by murine DCs and macrophages and human monocytes, as well as inhibiting IL-12p70 production in DCs as was previously observed for alum. Incubation of DCs with 500 nm PLGA particles led to enhanced IL-10 but reduced IL-12p70 in the presence of a TLR4 (LPS), TLR7/8 (R848) or TLR1/2 (Pam3CSK4) agonist indicating that the modulatory effects were not due to a specific PRR pathway or location of the TLR.

Recent evidence has suggested that innate immune cells can promote a type of memory response. Innate immune training (also known as innate immune memory) is described as an enhanced innate host defense upon reinfection by the same or different pathogens (707). This memory response is orchestrated through epigenetic reprogramming of innate immune cells upon immune challenge. Mice vaccinated with the Bacille Calmette–Guérin (BCG) vaccine show protection against mycobacteria, but also against other infections (e.g. Salmonella typhimurium) (708). Furthermore, newborn children immunised with the BCG, measles or the polio vaccine has been shown to have non-specific protective effects against other infections and have significant better survival in early childhood (709). In this current study, it was demonstrated that intramuscular injection of alum or 500 nm PLGA particles in the absence of any antigen can prime inguinal lymph node cells to secrete enhanced IL-10 when restimulated ex vivo. How long this effect persists still needs to be investigated. An interesting hypothesis being explored in the lab is that alum and PLGA particles of a certain size (500 nm -2 µm) are training innate immune cells (e.g. monocytes and macrophages) to secrete higher levels of IL-10 upon restimulation. Evidence from our lab has shown that human monocytes and BMDMs trained with alum or 500 nm PLGA particles for 24 h and restimulated with LPS after 6 days show enhanced IL-10 secretion (Aoife Gorman, PhD). Therefore, 500 nm PLGA particles are potentially training innate cells to secrete elevated IL-10 by an unknown mechanism, which could also alleviate inflammation and/or induce tolerance. Thus, highlighting another pathway these PLGA particles could exploit to treat inflammatory diseases.

The next question was whether the particulate driven innate response has any impact on adaptive immunity. Unravelling the immunomodulatory properties of adjuvants and how they influence the development of CD4+ and CD8+ T cell responses is essential. DCs seem to be inducing a regulatory IL-10 response when stimulated with 500 nm PLGA particles to negatively influence the ensuing Th1 response. Limited knowledge is available on how PLGA microparticles and other particulates modulate the function of DCs and their subsequent effect
on CD4+ T-cell differentiation and activation. BMDCs stimulated with 500 nm-2 µm PLGA particles and OVA decreased the proliferation of OTII T cells, which partially relied on IL-10 produced by the DCs. Incubation of OTII T cells with DCs treated with particles also led to attenuated IFN-γ production, which although not proven here could be linked to the particles ability to inhibit IL-12p70 production. Intramuscular vaccination with OVA and 500 nm PLGA particles did not promote Th1 responses. However, in the absence of IL-10, a splenic antigen-specific Th1 response was induced, corresponding with results for alum. This effect was only demonstrated with 500 nm PLGA particles, as if mice were immunised with 100 nm PLGA particles and OVA in IL-10+/− mice, a Th1 response was not detected.

This contrasts with 50 nm PS particles which are a strong driver of antigen-specific IFN-γ, IL-10 and CD8+ T cells. This could be due to a difference in material and/or size but we were unable to directly confirm this as 50 nm PLGA particles are currently not commercially available. It seems 50 nm PS use a different mechanism to 100 nm PLGA particles as they were the only particulate tested capable of inducing high levels of apoptosis in DCs in vitro. It has been shown in tumour models that cross-presentation of antigens released by apoptotic cancer cell results in specific CD8+ responses (573).

On the other hand, 500 nm PLGA particles could potentially be exploited to induce tolerance and treat inflammatory diseases including arthritis, multiple sclerosis, asthma and IBD. For example, IL-10 responses have been shown to be dysregulated in rheumatoid arthritis synovial macrophages (710), while IL-10 therapy can play a protective role in in atherosclerosis (711), EAE (712) and many other inflammatory diseases (158). In recent years, the use of particulates has expanded from improving vaccine efficacy to treating inflammatory diseases. Remarkably these studies have focused on using particles in the 1 µm size range, which as shown in the current thesis promote the strongest IL-10 response, while concurrently inhibiting IL-12p70. Several studies have successfully used PLGA and PS particles with antigen only or in combination with immunomodulators in inflammatory models by blocking inflammation and promoting tolerance (488, 577, 578, 580). For instance, 500 nm PLGA particles conjugated to myelin oligodendrocyte antigen (MOG) can promote safe and effective tolerance to prevent the onset of EAE (581). PLGA particles encapsulating OVA downregulate Th2 responses and airway inflammation both prophylactically and therapeutically (579). Based on these studies and the results presented in this thesis, particles in the 500 nm-1 µm size range would be the most effective size to treat inflammatory diseases. Here we show for the first time that this may be due their ability to enhance IL-10 over other sizes. It would be interesting to see if the
efficacy of the tolerogenic treatments was affected if IL-10 or specific signalling components identified here were blocked. Based on these results, particulate adjuvants should avoid the 1 µm size range if a cellular immune response is desired. Particles of this size have instead emerged as a novel and effective treatment for inflammatory diseases (e.g. allergic asthma and autoimmunity) through induction of antigen-specific immune tolerance (488).

The therapeutic potential of the PLGA particles in driving tolerance and alleviating inflammation could be investigated in several inflammatory mouse models (EAE, colitis, sepsis and arthritis) to address whether the same pathway (Syk, p38 and CREB) is involved in the response and if 500 nm PLGA produce a stronger tolerogenic response over other sizes, highlighting a potential new pathway to target that alleviates inflammation.

Why only particles of a specific size range induced IL-10 was unclear before these studies. Early experiments showed that particles in the 1 µm size range can localise at the cell membrane (713) and deliver antigen to be presented via MHCII (714). It was proposed that alum does not need to be internalised to exert its immunostimulatory effects (280). Furthermore, Flach et al showed that alum selectively interacts with lipids on the membrane of DCs, that triggers a lipid sorting event to activate Syk and PI3K and subsequent inflammation (474). Interestingly, this mechanism has never been linked to an anti-inflammatory response, despite Syk mediating IL-10 production through dectin-1 activation (64, 668). If cholesterol which forms part of the lipid rafts was removed from the cell surface, it reduced the ability of 500 nm PLGA particles to promote the secretion of IL-10. Furthermore, only these PLGA particles triggered enhanced lipid raft formation as imaged by confocal microscopy. 500 nm PLGA particles selectively interacted with the plasma membrane for a prolonged period, leading to the rounding of DCs. On the other hand, 100 nm PLGA particles are immediately taken up by DCs and did not impact on the cell size and the 10 µm PLGA particles did not interact with the membrane for a prolonged period.

Macrophages can adopt different geometries in vivo (274). This is mediated through a diverse range of signals from their surrounding environment including neighbouring cells, ECM, soluble factors (274, 593) and other forces including biomaterial implantation (639, 715) and even the hypoxic tumour microenvironment (716). It has been observed that macrophages change shape depending on their polarisation state in vitro, where M2 cells exhibit an elongated shape compared with rounded M1 cells (274). A rounded morphology was also associated with a down regulation of inflammatory cytokines in response to certain biomaterials (e.g. chitosan
modified with quaternary ammonium salts and hyaluronic acid), as the smaller rounded THP-1 cells had lower TNF-α production (639). Despite this knowledge, no studies have looked at cell shape changes in DCs and how it affects their polarisation state in terms of cytokine secretion. Only incubation with the 500 nm-2 µm PLGA particles could reduce the size of DCs and cause the cells to round up, which corresponds to the sizes capable of enhancing IL-10, indicating that such a phenotype is associated with an anti-inflammatory response in DCs (Fig 5.2).

We found that abrogation or disruption of actin polymerisation with latrunculin B and jasplakinolide, as well as the ROCK pathway enhanced IL-10 in LPS stimulated DCs. The IL-10 levels were similar to those in DCs stimulated with 500 nm PLGA particles. Actin disruption also amplified the 500 nm PLGA particle induced IL-10 transcriptional response, indicating that more particles could be interacting with the membrane. A rounded morphology has been reported when F-actin is disrupted with cytochalasin D, decreasing DNA synthesis while enhancing its differentiated functions (e.g. enhanced transcription and secretion of cytokines) (646). Latrunculin B treatment causes complete rounding up of mouse neuroblastoma and hamster fibroblasts (601). When THP-1 cells are treated with jasplakinolide they also display a more rounded morphology (602). Inhibiting the ROCK pathway has been shown to block inflammatory cytokines (717). Thus, we suggested a critical role for actin and rounding of the cell to modulate the anti-inflammatory IL-10 production in DCs. This led to the idea that a general mechanism was being employed by DCs, through changes in morphology to enhance IL-10. Furthermore, it’s well known that uptake is blocked when actin polymerisation is disturbed and so it was also clarified that phagocytosis is not involved in the IL-10 response. This is in contrast to the mechanism associated with particulate induced inflammation, where uptake of particulate vaccine adjuvants by DCs activates the NLRP3 inflammasome (549). Moreover, particulate induced IL-12p35 inhibition was prevented by blocking actin polymerisation, the mechanism for which is still not well understood. Finally, the Imagestream flow cytometer which combines microscopy and flow cytometry in a single platform will be used to quantify changes in cell shape and F-actin accumulation. The advantage of using this system in comparison to fluorescent microscopy is its ability to analyse images from a large number of cells for statistical analysis (718). It could be used to further characterise cell shape and quantify F-actin accumulation around the plasma membrane when DCs are stimulated with 500 nm PLGA particles. The imageStream will provide detailed images of a large number
of DCs in a relatively short period of time and allows intensity and location of phalloidin as well as cell shape changes to be quantified.

For the first time, this work shows that particulate adjuvants of a certain size can activate the Syk pathway through lipid raft formation to promote the release of the anti-inflammatory cytokine IL-10, demonstrating that the Syk pathway has the capacity to activate pro- and anti-inflammatory responses simultaneously by particulate adjuvants. Syk can associate with receptors in an ITAM-dependent manner including C-type lectins (196) which enhance IL-10 during fungal infections (668) or in an ITAM-independent manner, such as in TLR signalling (191). Of note, Syk inhibitors have been used to treat inflammatory diseases but so far have failed in clinical trials, despite success in mouse models (669, 670). They either caused unwanted side effects or only moderate improvements over placebo groups (222, 671, 672). While Syk can induce the secretion of pro-inflammatory cytokines, it also strongly promotes the production of IL-10, a key anti-inflammatory cytokine that plays a critical role in controlling inflammation, which could cause unwanted side effects.

The p38 pathway has been shown to be essential for anti-inflammatory IL-10 production induced by LPS, viral proteins and fungal PAMPs (64, 719, 720). Fungal pathogen engagement with dectin-1 results in recruitment of Syk and phosphorylation of p38, leading to enhanced IL-10 production in macrophages. In this study, 500 nm PLGA particles were shown to associate with the plasma membrane to activate Syk in a receptor independent manner to enhance p38 phosphorylation and promote IL-10 release. When p38 is inhibited, IL-10 enhancement disappears, but if other kinases are blocked such as ERK, PI3K and mTOR, only a partial reduction was detected. Thus, this implicates p38 as the primary kinase regulating particulate induced IL-10. Interestingly, if actin polymerisation was inhibited with latrunculin B, p38 activation was enhanced and stayed activated for a longer period in LPS stimulated DCs. In contrast, if cholesterol was removed the 500 nm PLGA particles failed to enhance p38 phosphorylation.

The p38 MAP kinase is part of a family of stress kinases. Exposure of cells to suboptimal growth conditions or an environment that reduces cell viability can be considered stresses, including changes in nutrient levels, growth factors, damaging agents, and changes in the temperature, pH or osmolarity. Cells can sense various mechanical signals on their surface, transduce them into biochemical signals resulting in functional outcomes including migration, cytokine secretion (593), proliferation (270), apoptosis (689) and differentiation (721).
Growing evidence also suggest a possible link between mechanical stress and chronic inflammation, cancer and Alzheimer’s disease (722). Although, the initial events triggering a stress response are not completely understood. Physical stresses including temperature, oxidative and osmotic stresses will be sensed by the cell and subsequently activate a signalling cascade that involves p38 (685, 688, 723).

Therefore, we hypothesised that the process of 500 nm PLGA particles interacting with the membrane activates the p38 signalling pathway in a similar manner to when DCs sense other mechanical signal and stresses on the cell surface. While p38 is known to be a primary mediator of IL-10 signalling and a central kinase activated during mechanical stress, it has never been confirmed that cells exposed to these stresses activate the p38 pathway to enhance IL-10 production. It has even been suggested that a hypertonic solution could be used to treat inflammatory diseases (694). Here, for the first time it was demonstrated that stresses that decrease fluidity (oxidative and osmotic) will increase IL-10 production in DCs. In contrast, when the cell has its fluidity increased by removing cholesterol with MβCD or promoting cell swelling with H2O, this eliminated the PLGA particle-induced enhancement of IL-10 production. In fact, cell swelling induced by hypoosmotic stress has been shown to induce an inflammatory response through activation of the NLRP3 inflammasome, caspase-1 and release of mature IL-1β (694-696), a result we also confirmed when BMDCs were exposed to H2O, leading to increased IL-1β secretion. Although not explored in this thesis, this could be a potential mechanism modulating inflammation (IL-1β and IL-1α) caused by the c PLGA particles. To conclude, this thesis contributes to our understanding of how cells respond to mechanical stresses by implicating a p38 MAPK signalling cascade to enhance IL-10 secretion. It also suggests that a general mechanism exists when APCs come under certain stresses, including certain particle sizes, oxidative and osmotic stress.

The transcription factor CREB is one of the primary IL-10 transcriptional regulators mediated via p38 after engagement of TLR4 and Mal (170, 171). It was demonstrated that 500 nm PLGA particles promote CREB phosphorylation which relies on cholesterol, Syk and p38. If any of these pathways were inhibited, particle-induced CREB phosphorylation was attenuated. Furthermore, CREB is a downstream target of p38 MAPKs induced by mechanical stresses (685). Several experiments need to be performed to confirm CREB as the principal transcription factor driving the IL-10 response in DCs. It should be noted that the majority of studies have only characterised IL-10 transcription factors in macrophages (143), it remains
unclear which transcription factors are primarily involved in IL-10 production by DCs (Fig 1.5). Therefore, CREB could be knocked down in DCs using siRNA to see if PLGA enhanced IL-10 is abrogated. Chromatin Immunoprecipitation Sequencing (ChIP-Seq) could also utilised to confirm the binding of CREB to the IL-10 promoter regions when DCs are stimulated with PLGA particles.

Several other experiments will be needed to complete the IL-10 story. Whether sucrose like 500 nm PLGA particles can activate Syk or CREB will be examined. Furthermore, membrane fluidity in murine and human myeloid cells following exposure to 500 nm particles or sucrose could be measured using laurdan (724). Atomic force microscopy (AFM) could be used to measure the binding force and interaction of the PLGA particles with the membrane of the cell, although this is challenging when using PLGA particles, as surfaces are too weak for glueing on AFM tips or can dissolve rapidly in solution (474). To confirm that these IL-10 promoting effects are size and not material specific, other materials (e.g. silica and gold) of 500 nm in size could also be tested and in addition to IL-10 other anti-inflammatory cytokines such as TGF-β could be determined.

It is critical that the balance between pro and anti-inflammatory immune responses is tightly regulated to prevent excess inflammation and damage to the host in response to a pathogen. Why particle characteristics such as size can impact the type of immune response produced remains unclear. Is there an evolutionary reason why the size of the particles determines the cytokine response? This thesis highlights how mechanical signals influence the immune response through changes in membrane dynamics. How bacteria (around 500 nm-1 µm in size) interact with the membrane of cells could be just as important as the sensing of PAMPs for determining the type of immune response induced. For example, in vitro experiments using only a TLR agonist from a particular bacterium may produce a misleading readout for the type of response that is actually generated (e.g. level of IL-10 produced). Possible experiments would be to compare heat killed bacteria with outer membrane vesicles (OMVs, around 100 nm) from the same bacterium which are around 1 µm in size – our model may predict that at the same protein concentration, OMVs will be more inflammatory and drive less IL-10. A few papers have already shown that bacterial lysates are less inflammatory then their OMV counterparts (725). Interestingly, schistosoma mansoni eggs contain components (e.g. omega-1) that cause rounding in murine dendritic cells, suppress LPS-induced IL-12p40 and p70 secretion in DCs and they do not require MyD88/TRIF signalling to trigger Th2 responses.
Such components have also been linked to enhanced IL-10 production in DCs (727) and omega-1–pulsed DCs promote IL-10 responses *in vivo* (726). Therefore, a hypothesis being followed up in the lab is that the mechanism employed by 500 nm PLGA particles could also be used in a physiological context by helminth secretory components.

The potential mechanisms underlying the decrease in IL-12p35 expression and IL-12p70 secretion remain unclear. This can be attributed to the complex nature of how bioactive IL-12p70 is released from DCs. A partial reversal in IL-12p35 inhibition was seen when actin polymerisation was disrupted with either latrunculin B or jasplakinolide and if ERK kinase was inhibited. Increases in cAMP block IL-12p35 expression in human monocyte-derived DCs (728). Furthermore, the inhibitory effects of 500 nm PLGA particles on IL-12p35 expression could be mediated through Ca\(^{2+}\) influx or oscillations and subsequent NF-κBp65 (RelA) and cRel upregulation (562) or else PGE\(_2\) production signalling through the E prostanoid 2 (EP2) and EP4 receptors which subsequently inhibit IL-12p35 (729). Early experiments using the nonsteroidal anti-inflammatory drug indomethacin, which blocks cyclooxygenase enzymes (COX-1 and COX-2) prevented the inhibition of IL-12p70 by PLGA particles in BMDCs. COX-1 and COX-2 catalyse the conversion of arachidonic acid to the intermediate prostaglandin H2, which is then converted to a series of prostanoids (e.g. PGE\(_2\)) by cell-specific prostaglandin synthase. Furthermore, the PGE\(_2\) and EP2 receptor antagonist AH6809 also blocked IL-12p70 inhibition induced by PLGA particles. Therefore, PLGA particle-induced increases in cAMP, Ca\(^{2+}\) or PGE\(_2\) could potentially be contributing to the inhibitory effects.

To conclude, many vaccine formulations which incorporate particulates use particles in the 1 μm size range, which based on the current results may explain why they are incapable of promoting effective cellular immunity (Fig 5.2). As many of these studies only used antibodies as their readouts, simultaneous suppression of cellular responses was not always measured. Consistently, particles, including PS, PLGA and alum within the 1μm size range enhance IL-10 and inhibit IL-12p70 to hinder cellular immunity. This will have a major impact on the design of future CMI promoting vaccines as a specific size range needs to be avoided. In contrast, particles around the 1 μm size range could potentially and has already been used to improve treatments against inflammatory diseases through tolerance and/or dampening the inflammatory response.
The mechanism particulate adjuvants use including the underlying signalling pathways will provide the foundation for developing better adjuvants aimed at improving cellular immunity. There seems to be a general mechanism for particulates of a certain size, as we also confirmed that alum induced IL-10 is mediated by same pathway as PLGA particles (500 nm-2 µm). The cholesterol-Syk-p38-CREB pathway induced by these particulates would need to be avoided if designing adjuvants for vaccines against Malaria, TB, HIV and cancers. This could be achieved by not using particulates (~1 µm in size) capable of interacting with the membrane for a prolonged duration. This has already been proven in our lab using 50 nm PS particles which can induce the strongest antigen-specific CD8+ T cell response. Unfortunately, 50 nm PLGA particles were not available so we were unable to investigate if they promote a similar response. On the other hand, this same pathway could be targeted or else particles in the 1 µm size range could be used to treat inflammatory diseases through tolerance induction and/or suppressing inflammation, which has already been demonstrated in several papers. Although, the researchers were unaware they were already using the most appropriate size to promote immunosuppressive responses.

Furthermore, despite the importance of IL-10 in regulating the immune response, the mechanisms by which IL-10 is induced in DCs has not been fully resolved, as most studies have focused on macrophages. 500 nm PLGA particles were used as a tool here to highlight a common stress response in DCs when they sense mechanical signals in their environment through their membrane to promote IL-10. DCs can sense stresses, including PLGA particles (500 nm-2 µm), oxidative stress, osmotic stress and disruptions in actin polymerisation to trigger IL-10 production. Results in this thesis support the hypothesis that these stresses initiate a general pathway in DCs through changes in fluidity of the plasma membrane. DCs sense these physical signals, change shape to a more rounded morphology, induce lipid raft formation (decreases fluidity) and convert these mechanical cues to biochemical signals (Fig 5.1 and 5.3). In contrast, if fluidity was increased the particles were unable to initiate the stress pathway to increase IL-10 production in DC.s (Fig 5.1 and 5.3).
5.1 Future plans and experiments

- PLGA particle dose will be normalised using the Brunauer, Emmett and Teller (BET) surface area method to ensure that the distinct immune profiles generated by PLGA particles were not due to differences in surface area rather particle size is responsible for enhanced IL-10 production and IL-12p70 suppression.

- Quantify lipid raft formation by 500 nm PLGA particles. BMDCs will be counted from several experiments to calculate the percentage of cells that have increased raft formation in response to 500 nm PLGA particles. Enhanced raft formation will also be investigated when BMDCs are incubated with 500 nm PLGA particles for longer timepoints.

- The ImageStream flow cytometer will be used to characterise cell morphology, in order to see rounding of the cell, F-actin accumulation around the membrane of the cell and quantify the amount of F-actin formed (also quantify amount of F-actin by western blot).

- Confirm 500 nm PLGA particles selectively bind to membrane of the cell by using isosurface rendering of the Z stacks to create a 3-D images of the cell interacting with the PLGA particles.

- Do stresses that decrease membrane fluidity (e.g. sucrose and hydrogen peroxide) also activate Syk and CREB?

- Directly measure changes in membrane fluidity with laurdan following stimulation with PLGA particle sizes and stresses that increase or decrease fluidity (sucrose, lidocaine, MβCD etc).

- Is there a physiological context for the cholesterol-Syk-p38 pathway as well? For instance, do products secreted by live helminths (e.g. helminth-secreted excretory/secretory (ES) products such as *Schistosoma mansoni* soluble egg antigen) also induce rounding as they interact with the membrane of DCs to activate Syk and p38 and induce IL-10.
• Investigate whether PGE$_2$ plays a role in the suppression of IL-12p35 by PLGA particles in DCs.

• Does Flt3L-derived DCs (cultures based on FLT3L yield 3 subsets: pDCs, CD11b$^+$ cDCs, and an equivalent of the CD8α$^+$/CD103$^+$ cDC lineage) also enhance IL-10 in response to 500 nm PLGA particles.

• Investigate whether 500nm PLGA particles enhance the production of other anti-inflammatory cytokines in DCs and Macrophages (e.g. TGFβ).

• ChIP-Seq will be utilised to confirm whether 500 nm – 2 μm PLGA particles selectively enhances transcription factor binding to IL-10 promoters (e.g. CREB).

• Are PLGA particles in the 1 μm size range more effective than other sizes to treat inflammatory diseases in vivo through tolerance induction and/or suppressing inflammation (using different murine inflammatory models e.g. colitis, EAE, allergy and arthritis).

• Is the cholesterol-Syk-p38 pathway critical to induce tolerance in vivo and if so could the pathway be targeted for treatment of inflammatory diseases.
Chapter 6

References


polymerization as a key innate immune effector mechanism to control Salmonella infection. *Proc Natl Acad Sci U S A* 111: 17588-17593.


used prophylactic vaccines as an alternative for synthetically produced TLR ligands to mature monocyte-derived dendritic cells. *Blood* 116: 564-574.


550. Makita, N., Y. Hizukuri, K. Yamashiro, M. Murakawa, and Y. Hayashi. 2015. IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration. *Int Immunol* 27: 131-141.


