Dendritic Cells: sensors of extreme antigen dilutions and role in immunity against *Salmonella typhimurium* infections

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I, the undersigned hereby declare that the contents of this thesis have been composed by myself and that the work described herein is entirely my own unless otherwise acknowledged.
Abstract

The intention of the work described in this thesis was to identify whether extremely high dilutions of antigen can induce changes in dendritic cell maturation and dendritic cell ability to initiate immune responses. Basic research performed trying to address whether such ultra-high, homeopathic, dilutions of antigen have a biological role is limited, while clinical investigations into this matter are inconclusive. Homeopathy is, however, gaining popularity, and is very often at the epicentre of intense scientific and medical debate. We hypothesised that dendritic cells, being an excellent APC, might be a good candidate cell to investigate this controversial topic. While dendritic cells are excellent APCs, capable of inducing T cell responses 100 times more effectively than other lymphocytes, there is mounting evidence suggesting that they might also be involved in bacterial dissemination during Salmonella infections. We investigated the role of dendritic cells in the development of protective immune responses against Salmonella typhimurium infections. Furthermore, we assessed whether the APC derived cytokine, IL-23, which shares the common p40 subunit with IL-12 p70, is required for the development of primary, memory as well as protective immune responses against Salmonella typhimurium. While IL-23 has been shown to be important for the maintenance of IL-17 responses by T cells, its role during Salmonella induced pathology remains elusive. Our work identified some interesting aspects of this cytokine in immunity against Salmonella typhimurium infections.
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List of abbreviations

Ab: Antibody
Ag: Antigen
AIDS: Acquired Immunodeficiency Syndrome
AMP: Antimicrobial Protein
APC: Antigen Presenting Cell
ATP: Adenosine Triphosphate
BPI: Bactericidal Protein Intermediate
CD: Cluster of Differentiation
CXCL: Chemokine Ligand
CCR: Chemokine Receptor
CT: Cholera Toxin
DC: Dendritic Cell
EAE: Experimental Autoimmune Encephalomyelitis
ER: Endoplasmic Reticulum
Flt3: FMS-like Tyrosine Kinase Receptor-3
Gr: Granulocyte
GM-CSF: Granulocyte Macrophage Colony Stimulating Factor
HIV: Human Immunodeficiency Virus
IFN: Interferon
IL: Interleukin
ICAM: Intracellular Adhesion Molecule
LFA: Lymphocyte Function-Associated Antigen
LPS: Lipopolysaccharide
MHC Major Histocompatibility Complex
MΦ: Macrophage
NET: Neutrophil Extracellular Trap
NRAMP: Natural Resistance-Associated Macrophage Protein
P.a: Propionibacterium Acnes
PAMP: Pathogen Associated Molecular Pattern
PRR: Pattern Recognition Receptor
REALM:
RNA: Ribonucleic Acid
SCID: Severe Combined Immunodeficiency
SPI: Salmonella Pathogenicity Island
S.t: Salmonella typhimurium
TAP: Transporter Associated Protein
TCR: T cell Receptor
TGF: Transforming Growth Factor
Th: T helper
TLR: Toll Like Receptor
TNF: Tumor Necrosis Factor
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Chapter 1
Introduction

1.1 The need for immunity

Competition and natural selection has driven species to inhabit a vast array of different niches, including ones within other organisms. While many examples of symbiosis are beneficial for both parties, such as in the case of commensal bacteria residing at the lumen of the intestinal tract (Yan and Polk, 2004), there are a number of organisms whose survival is detrimental to the health of their host, or even the community a host is a member of. The need to maintain physiological numbers of beneficial symbiotic organisms, and to defend against the invasion and colonisation by pathogenic organisms, while avoiding aggression against self-derived cells, has lead to the development of intricate chemical and cellular immune networks. These can be divided into two main categories; innate immunity and adaptive immunity. While both those “arms” of immunity have evolved to prevent from, and combat, pathogenic invasion, they have amongst others one fundamental difference; adaptive immunity has the unique ability to become “educated” and “remember” each specific pathogen.

1.1.1 Innate immunity

The evolutionary older form of immunity, innate immunity is characterised by being the first line of defence against pathogenic organisms. It includes (but is
not restricted to) physical barriers such as the skin, chemical, such as the acidic environment of the stomach or proteolitic enzymes, and cellular, such as macrophages (M\(\Phi\)s), neutrophils and dendritic cells (DCs) that can recognise a vast range of proteins, sugars and lipids from pathogens that share evolutionary traits, and either directly kill them or instruct other cells to perform this task.

1.1.2 First line of defence

Some of the primary functions of the skin are the isolation and provision of a mechanical barrier between the interior and the surrounding environment, thermoregulation, immunosurveillance and prevention from dehydration. The intestinal tract and the lung serve for the intake of nutrients and gas exchange respectively. In a normal person measuring 1.5 meters height, these three tissues expose together an area of around 400 m\(^2\) to the environment (Hooper and Macpherson; Mosteller, 1987; Roughton and Forster, 1957). As such, the rapid elimination of micro-organisms, dust particles, allergens and toxins that are encountered constantly is of prime importance. Ciliary action on the trachea actively removes dust particles and unwanted micro-organisms, while absorptive villus enterocytes residing at the gut surface prevent entry of pathogens through the epithelial layer (Negus, 1949; Srikanth and McCormick, 2008). Both gut and lungs are covered in mucus, which prevents tissue damage and provides an additional barrier against invading pathogens (Bergofsky, 1991; Srikanth and McCormick, 2008).
The keratinisation of the skin is important in providing immune protection as well as prevention of water loss. However, many pathogens such as bacteria, can produce proteases and thus penetrate the outer layer of the epidermis. Commensal bacteria living on the skin and gut contribute towards immunity by outcompeting unwanted pathogens. Furthermore, a number of anti-bacterial peptides and proteins (AMPs) are found on the surface of the skin, the gut lining, and alveolar spaces (Srikanth and McCormick, 2008). These proteases have been found to play important roles in direct pathogen elimination, recruitment of white blood cells to the sites of infection, as well as the maintenance of physiological levels of commensal bacteria (Niyonsaba et al., 2009; Schroder and Harder, 2006). Examples of AMPs include dermicidin, cathelicidin LL-37, psoriasin and RNase-7.

Dermicidin is an AMP produced under physiological conditions by eccrine gland cells on the skin and shows antimicrobial activity against *Staphylococcus aureus*, *Eneterococcus faecalis*, *Escherichia coli* as well as fungal pathogens such as *Candida albicans* (Harder and Schroder, 2005). The antimicrobial peptide cathelicidin (LL-37) is expressed in eccrine and duct cells in the skin as well as mast cells and respiratory epithelial cells in the lung and gut, under inflammatory conditions (Doss et al.; Harder and Schroder, 2005). It shows activity against *S. aureus* and *C. albicans* as well as viruses, while it is also chemotactic for neutrophils, monocytes, mast cells, eosinophils and T cells (Doss et al.; Harder and Schroder, 2005). Psoriasin, which is produced constitutively by keratinocytes in areas with high bacterial colonisation on the skin, such as hair follicles, is a potent *E. coli* bactericidal AMP (Bevins, 2005), while it is also believed to be important in maintaining healthy numbers of commensal bacteria and recruit neutrophils and T
cells to the sites of infection (Harder and Schroder, 2005). RNase 7 is an inducible AMP produced by keratinocytes and neutrophils residing at the epidermis. It is chemotactic for neutrophils, mast cells and dendritic cells (Harder and Schroder, 2005). β-defensins are found on the skin, in the lumen of the intestinal tract as well as the lungs. They are highly cationic, inducible peptides that have antimicrobial properties against bacteria, viruses and eukaryotes (Schutte and McCray, 2002). They attract mast cells, DCs, neutrophils, monocytes and T cells to the site of infection. Some forms are found to be constitutively expressed by skin keratinocytes while others are induced during inflammation in response to pro-inflammatory cytokines and bacterial cell wall components, and are produced by keratinocytes, epithelial cells, neutrophils and mast cells (Schutte and McCray, 2002).

Taken together, AMPs play a very important role not only in direct pathogen elimination, but also in initiating a cascade of events that leads to the recruitment of highly specialised cells to the site of infection.

While AMPs provide a formidable chemical barrier against pathogen invasion, cytokines, a group of specialised signalling molecules play a crucial role in co-ordinating immune responses against such invading pathogens. Innate and proinflammatory cytokines such as IL-1, IL-6, IL-12, type 1 interferons and TNF-α as well as chemokines such as CXCL8 (IL-8) are secreted by innate cells in response to AMPs, bacterial products, host cell death products as well as stress such as injury. They play a crucial role not only in the recruitment of innate and adaptive cells to the site of infection, but are also required for the activation and polarisation of cells of the adaptive immunity.
Tumour necrosis factor-α (TNF-α) is secreted in response to bacterial, viral and protozoan infections. It stimulates the hypothalamic thermoregulatory centre, resulting in fever which is thought to inhibit bacterial growth (Kluger, 1991; Small et al., 1986), it causes vasodilatation (Hollenberg et al., 1991), and actively enhances migration of cells through vascular endothelial cells to the periphery (Smart and Casale, 1994), it is a chemoattractant for neutrophils and stimulates phagocytosis (Roilides et al., 1998). IL-1β and IL-6, similarly to TNF-α, have also been found to act on the hypothalamic thermoregulatory center (Lesnikov et al., 1991; Morgan et al., 2004). IL-1 (IL-1α and IL-1β) is secreted by MΦs, fibroblasts and DCs. It increases the expression of adhesion factors on endothelial cells allowing for transmigration of leukocytes to the periphery (Dangerfield et al., 2005). Recent work has also shown that IL-1 may also play an important role in polarising adaptive immune responses (Chapter 1.4, 1.5). IL-6, similarly to TNF-α and IL-1, acts on the hypothalamus to cause fever (Sundgren-Andersson et al., 1998). Recent work has shown that this innate cytokine is also involved in polarising adaptive immune responses, discussed further on (Chapter 1.4, 1.5). IL-12 p70 is a heterodimeric cytokine secreted by innate cells such as MΦs and DCs, in response to viral, bacterial and protozoan infections. It plays a pivotal role in the polarisation of adaptive immune responses (discussed in Chapter 1.4, 1.5). IL-10 is an anti-inflammatory cytokine secreted by both innate and adaptive cells in response to bacterial, viral, protozoan and nematode infections. It inhibits the synthesis of pro-inflammatory cytokines such as TNF-α and regulates adaptive immune responses (Chapter 1.4, 1.5).
1.2 Cells of the innate immune system

Mechanical barriers as well as chemical defences provide formidable protection against the vast number of pathogenic organisms that vertebrates constantly encounter. Nevertheless, the “first line of defence” does not always ensure protection. A number of pathogens are able to colonise their hosts by exploiting naturally occurring tissue damage such as in the case of Clostridium tetani, a Gram-positive rod shaped anaerobic bacterium which is the causative agent of tetanus (Cooke, 2009). Alternatively, pathogens can invade their hosts by outcompeting commensal bacteria and avoid killing by AMPs such as in the case of Salmonella enterica, a Gram-negative rod shaped bacterium that causes typhoid fever (Pilonieta et al., 2009). Some pathogens possess methods that allow them to directly transverse mechanical barriers and evade detection such as in the case of the cercariae of Schistosoma mansoni, a trematode that causes schistosomiasis (McKerrow and Salter, 2002). Immediately after pathogen invasion, the host has a number of cellular and chemical means by which it deals with the foreign organism.

1.2.1 Dendritic cells

DCs reside at almost every tissue in the body. However, they are strategically enriched at sites of pathogen entry such as the skin, mucosal surfaces, airways, intestinal lining, female reproductive tract and uterus (Iwasaki, 2007). They constantly sample their environment by receptor mediated endocytosis, phagocytosis and macropinocytosis. These processes are well-characterised
methods of bacterial clearing by phagocytes in general (such as DCs, neutrophils and MΦs). However, recognition and internalisation of pathogens by DCs are the means by which they acquire antigen (Ag) in order to educate the adaptive immune system about the nature of the invading pathogen. Their phagocytic capacity in conjunction with their ability to educate cells of the adaptive arm of immunity places DCs at the interface between innate and adaptive immune responses. As such, work performed on DC biology aims to understand how this phagocyte translates “signals”, received in the periphery, to “message”, for the initiation of adaptive immune responses.

Upon pathogen or “danger” signal recognition through pattern recognition receptors (PRRs), DCs internalise pathogens or pathogen-associated products. The internalised pathogen is contained in the phagosome, which in turn is enriched with vacuoles containing lytic enzymes and reactive oxygen species, creating the lysosome (Luzio et al., 2007). Extracellular proteins are processed within the lysosome and transported via transporter associated proteins (TAP) for loading onto major histocompatibility complex class II molecules (MHC class II) (Mukherjee et al., 2001). While this process occurs, DCs migrate through the lymphatics to the respective draining lymph node or spleen (Faure-Andre et al., 2008). The migration process is characterised by the downregulation of CCR6 and upregulation of CCR7, which ligates to CCL21 and directs DCs towards secondary lymphoid organs and T cell areas (Page et al., 2002). During migration, DCs lose their phagocytic and endocytic capacity, load degraded, pathogen-derived Ag on to MHC molecules, upregulate co-stimulatory molecule expression on their surface, and secrete pro-inflammatory cytokines (Cella et al., 1997). Once they
reach secondary lymphoid organs, DCs transverse T cell areas where they present processed Ag, in the context of MHC, to naïve CD4⁺ T cells (Cella et al., 1997).

The role of DCs in immune response development is thought to mainly be the activation of naïve T cells or memory cells via MHC-T cell receptor (TCR) interactions, there is evidence, however, suggesting that DCs are also able to bind unprocessed Ag for presentation to B cells, playing an important role in direct initiation and regulation of antibody (Ab) responses (Wykes et al., 1998).

DCs also play a key role during the T cell negative selection process in the thymus, and establish T cell tolerance in the periphery (Hawiger et al., 2001; Proietto et al., 2008) (Discussed in more detail in Chapter 1.4). They regulate ongoing immune responses against pathogens by homing to infected sites and recruiting T cells, neutrophils and other leukocytes. Recirculating DCs have been reported to home to sites of inflammation sustaining infiltration of T cells (van Rijt et al., 2005). In addition, DCs can receive information from T or B cells via co-stimulatory molecules or cytokines, affecting their activation state and subsequently the way they prime T cells (Alpan et al., 2004; Bayry et al., 2005; Orabona et al., 2004). During ongoing infections, DCs establish cross-talks with resident and infiltrating immune cells, resulting in the fine-tuning and regulation of the ongoing immune response (Bennouna et al., 2003; Rimoldi et al., 2005). The fact that DCs occupy sites with different immunological challenges and needs, together with their ability to perform a vast range of tasks, has posed them as a very interesting candidate for targeted vaccination methods.
1.2.2 Macrophages

MΦs are bone marrow derived cells, and ontogenically similar to DCs since those two APCs are thought to share common precursors (Geissmann et al., 2010). Similarly to DCs, they can acquire Ag in the periphery, process that Ag and load it on MHC molecules and, consequently, present it to naïve CD4+ T cells for activation (Chang et al., 2005). However, they are thought to be less efficient at antigen presentation and activation of naïve T cells than DCs (Steinman and Witmer, 1978). Furthermore, bone marrow derived MΦs have been shown to preferentially migrate to sites of infection, where they release cytokines and recruit other lymphocytes, rather than to secondary lymphoid organs, where naïve T cells naturally reside (Zhao et al., 2006). Nevertheless, MΦs are excellent at eliminating pathogens as well as “debris” in the form of dead bacteria and dead neutrophils, which accumulate during infection and inflammation (Martin and Leibovich, 2005). As such, the role of MΦs in infection is thought to be immunomodulation through cytokine secretion, microbial killing and lymphocyte recruitment (Fujiwara and Kobayashi, 2005; Martinez-Pomares and Gordon, 2007; Seljelid and Eskeland, 1993).

Recent advances in MΦ biology have revealed some intriguing functions of this cell type that differentiate them from DCs. MΦs activated through cytokines associated with allergy, asthma and helminth infections (Taylor et al., 2005) (discussed in more detail in Chapter 1.4) have been found to play important roles in wound healing and muscle regeneration (Loke et al., 2007; Tidball and Wehling-Henricks, 2007). Such “alternatively” activated MΦs are characterised by the
expression of Ym1, REALMα and arginase (Hesse et al., 2001; Nair et al., 2003; Raes et al., 2002). The accumulation of these MΦs in response to platelet derived growth factors at injured sites is followed by the release of transforming growth factors which activate fibroblasts and keratinocytes to produce collagen (Wahl et al., 1989). Additionally, the hypoxic environment at injured sites, where MΦs accumulate, activates them to promote angiogenesis (Luft, 2008). The role of MΦs in muscle regeneration is poorly understood, however, it is clear that in the absence of infiltrating MΦs, myocytes regenerate slowly and incompletely (Tidball and Wehling-Henricks, 2007).

MΦs and DCs share many phenotypical and functional similarities, often blurring the image we hold of them as two distinct cell populations. Whether they are a single cell population that performs distinct tasks under different environmental stimuli, or two distinct populations is an intriguing question which advances in DC and MΦ biology might soon answer!

1.2.3 Neutrophils

Neutrophils constitute around 60-70% of circulating white blood cells in humans and in an adult they are produced at a rate of as many as $10^{11}$ cells daily (L.J Witts, 1967; Textbook of Haematology). Under physiological conditions they have a lifespan of 12 hours. They are one of the first white blood cell recruited to the site of infection and in many cases also the last one a pathogen will “see” before elimination. Neutrophils become activated by AMPs, complement factors such as C5, cytokines such as interleukin 8 (IL-8 or CXCL8) and IFN-γ (Interferon gamma),
as well as bacterial products such as N-formyl-peptides (Nathan, 2006). Upon activation, they migrate to the site of infection, a process that takes 15-45 minutes, and is mediated by the binding of L-selectin found on neutrophils to E- and P-selectin on endothelial cells (Burns et al., 1999). Upregulation of lymphocyte-function-associated antigen (LFA) on endothelial cells promotes binding to intracellular adhesion molecules (ICAMs) on neutrophils, establishing a tight adhesion that arrests migration and enables extravasation through chemotaxis, mediated mainly by IL-8 (DiVietro et al., 2001).

Phagocytosis, oxygen dependent and oxygen independent killing mechanisms, and neutrophil extracellular traps (NETs) are some of the methods that this cell type employs in order to achieve pathogen elimination (Nathan, 2006). Phagocytosis (first described by Metchnikoff in 1901) is mainly mediated through Fcγ Receptors (CD32 and CD16) and complement receptors CDR1 (CD35), and is the process by which neutrophils and other phagocytes, such as DCs and MΦs, engulf microorganisms into the phagosome (Witko-Sarsat et al., 2000). There, pathogens are exposed to antimicrobial peptides, proteolytic enzymes and reactive oxygen species (ROS). Once killing is completed, neutrophils go through apoptosis in a process that involves CD11b/CD18 and the activation of caspases (Nathan, 2006). The oxygen independent killing mechanism, is characterised by the degranulation of neutrophils (as well as MΦs and DCs) and the release of peptides such as defensins, cathelicidins and bactericidal permeability-increasing proteins (BPIs) (Yang et al., 2001a).
Oxygen-dependent killing is initiated with the assembly of the NADPH oxidase enzyme complex at the membrane of the phagosome. The oxidation of NADPH results in the formation of hydrogen peroxide, which is converted to hypochlorite (HOCl) (Hampton et al., 1998). Killing is achieved by the reduction of pathogen related proteins and nucleic acids by hypochlorite (Hampton et al., 1998).

An interesting and recently identified feature of neutrophils is their ability to form extracellular traps. These are extracellular structures composed of chromatin, which can bind to extracellular bacteria (Brinkmann and Zychlinsky, 2007). Upon IL-8 or TLR activation, neutrophils utilise the same cascade to the one described for the oxidative killing mechanisms. This pathway, however, can also lead to the rearrangement of chromatin in the nucleus, followed by the degradation of the nuclear envelope, resulting in the chromatin coming into direct contact with the cytoplasm (Brinkmann and Zychlinsky, 2007). When neutrophils get instructed to undergo apoptosis they release NETs to the surrounding environment creating a web that can bind to bacterial proteins and kill microbes directly (Brinkmann and Zychlinsky, 2007). The role of NETs in disease are yet unclear since it is a novel field of research, but it has been found that strains of S. pneumoniae capable of degrading NETs by DNAse activity can escape killing and are more pathogenic than strains lacking DNAse activity (Beiter et al., 2006).

Similarly to DCs and MΦ, neutrophils express PRRs and upon activation produce IFN-γ, TNF-α, IL-1, IL-6 as well as AMPs (Nathan, 2006). This enables the recruitment of DCs, mast cells, T cells, MΦs and more neutrophils to the site of infection. Furthermore, recruited cells come in direct contact with neutrophils,
getting information about the nature of the invading pathogen. Direct cell-cell contact of neutrophils with DCs has been described and is thought to play an important role in fine-tuning immune responses (Bennouna et al., 2003; Ludwig et al., 2006).

1.3 Dendritic cell, the main antigen presenting cell

DCs were first described in the skin in 1868 by Paul Langerhans. However, it took more than 100 years before they were characterised as DCs by Cohn and Steinman in 1973 (Steinman and Cohn, 1973) and as veiled cells (human immature DCs) by Balfour and Knight in 1982 (Balfour et al., 1982). Their function was not understood till mixed leukocyte reaction (MLR), T-dependent antibody responses, and graft rejection studies established them as a very potent antigen presenting cell (Inaba and Steinman, 1984; Inaba et al., 1983; Inaba et al., 1984; Steinman and Witmer, 1978), capable of inducing T cell responses 100 times more efficiently than MΦs or other leukocytes. Isolation of DCs from blood or secondary lymphoid organs was an arduous process since DCs constitute between 1-2% of total leukocytes. However, the generation of monoclonal antibodies against DC surface molecules, technological advances in flow cytometric techniques and the discovery of methods for the in vitro generation of DCs from bone marrow precursors in large numbers, provided the necessary tools to accelerate work on the function of these cells, and thus further our understanding of DC involvement in the initiation and regulation of immune responses (Crowley et al., 1989; Inaba et al., 1992; Nussenzweig et al., 1982).
1.3.1 Dendritic cell subsets

Dissecting immune responses against different pathogens, revealed functional differences between initially DCs and MΦs. Advances in DC biology revealed functional and ontogenical differences between DCs. Patterns linking expression levels of cluster of differentiation numbers (CD numbers) with tissue residence and function became apparent, leading to the emergence of DC subsets. While widely accepted as CD11c$^+$ - the integrin-α subunit thought to play a role in the adherance of monocytes to endothelium cells and phagocytosis of complement coated particles (Stewart et al., 1995) - MHC class II high, CD40 low and CD80 low, DCs were initially divided into two broad groups, DC1 and DC2. These subsets reflected their ability to drive Th1 or Th2 responses respectively (Rissoan et al., 1999). Attractive as this paradigm might have been due to its neat categorisation of DC subsets according to the type of adaptive immune responses they elicited, it was short lived. Further work using a wide range of surface markers showed that DCs exhibited more plasticity than that proposed by the DC1/DC2 identification system.

Today, murine DCs are typically identified by their levels of CD11c, MHC II, CD4, CD8a, CD11b and CD205 expression (Sato and Fujita, 2007). Different DC subsets have been identified including CD4$^+$CD8α$^+$, CD4$^+$CD8α$^-$, CD4$^+$CD8α$^-$ and CD11c$^+$B220$^+$ plasmacytoid DC (pDC) (Shortman and Liu, 2002). The T cell marker CD8 is expressed as an αα-homodimer by DCs rather than an αβ-heterodimer as is the case on T cells (Sato and Fujita, 2007). Both CD8α$^+$ and CD8α$^-$ DC subsets appear equally competent at driving T cell proliferation. However, CD8α$^-$ DCs
secrete greater quantities of IL-12 upon ligation of CD40 and are thought to be better at skewing responses towards a Th1 profile, whereas CD8α⁺ DCs, are considered to be better at driving T helper type 2 responses (Maldonado-Lopez et al., 1999; Pulendran et al., 1997; Pulendran et al., 1999). For a period of time CD8α⁺ and CD8α⁻ DCs were thought to be of either myeloid or lymphoid origin, respectively, however, we know today that both myeloid and lymphoid DC populations can give rise to CD8α⁺ or CD8α⁻ DCs (Chicha 2004, Manz 2001, Traver et al. 2000).

Characterisation of DC subsets has also revealed links between their preferential homing capacity and expression levels of surface markers. Ongoing research will certainly improve our understanding of DC function and homing capacity. However, we could divide DC subsets into two main populations (Figure 1.1), a) non-lymphoid tissue resident DCs and lymphoid migratory DCs, and b) plasmacytoid DC (pDC). Non lymphoid resident DCs are further subdivided into those present at sterile organs such as the heart and pancreas, filtrating sites, such as the kidney and liver as well those found in the skin and mucosal tissues such as lungs and gut (Naik et al., 2007). Lymphoid DCs, which have been studied extensively in the mouse model, and of which little is known in the human, are found in the spleen and lymph nodes (Naik et al., 2007). Plasmacytoid DCs are circulating blood DCs, but have been described to be present in mucosal surfaces, secondary lymphoid organs and gut (Naik et al., 2007).

Understanding to which tissues different DC subsets preferentially home to as well as what type of immune responses they bias might prove to be a very
useful tool for the generation of targeted vaccination techniques. However, it is worth bearing in mind that despite the fact that the nature of different DC subsets may bias the type of response elicited, it does not necessarily determine the resulting profile of the adaptive immune response developed (Manz et al., 2001; Wu et al., 2001).

1.3.2 Dendritic cell precursors

DC precursors originate in the bone marrow. So far three different types of DC precursors and progenitors have been identified and classified according to their cell surface markers. Hematopoietic stem and progenitor cell (HSPC), characterised by CD45⁺, Lin⁻ (Lineage), c-Kit⁺ (CD117) and Sea-1⁺ (Mouse haematopoietic Stem cell antigen 1) expression, is thought to be the precursor for tissue resident non-lymphoid DCs (Massberg et al., 2007; Wright et al., 2002). MΦ DC precursor (MDP) characterised by CD34⁺, Lin⁻, c-Kit intermediate and CD11b⁻, is thought to be involved in the generation of spleen resident DCs and MΦs, conventional DCs (cDCS), pDCs and monocytes, including Gr1⁺ (Granulocyte) inflammatory monocytes, that develop into TNF-α - and Inducible Nitric Oxide Synthase-Producing DCs (TIP DCs) during infection (Auffray et al., 2009; Fogg et al., 2006). Common DC progenitor (CDP) or clonal DC precursor (pro-DC), characterised by CD34⁺, Lin⁻, c-Kit intermediate and Flt-3⁺ expression, have been described to drive the generation of conventional and plasmacytoid DCs residing at secondary lymphoid organs (Onai et al., 2007). A detailed schematic of the different fates of DC precursors is found at Figure 1.1.
Despite the identification of different DC subsets and their implication in generating tissue specific DCs, increasing amount of evidence suggests that the fate of DC progenitors is equally dependent on chemokine, cytokine, and growth factor presence. For example DC precursors that do not express the receptor for GM-CSF or Flt3, are thought to develop into the DC subpopulation predicted according to their cell surface markers described above (Kingston et al., 2009). However, there is evidence suggesting that a common DC precursor expressing GMCSF ligand and Flt3 can develop into a cDC or a pDC in the presence of either GM-CSF or Flt3 ligand (Naik et al., 2007; Xu et al., 2007).

DC precursors exhibit a great deal of plasticity, while no master regulator responsible for the generation of distinct DC subtypes has yet been identified. Additionally, mounting evidence suggests that the cytokine and growth factor milieu in the bone marrow, blood or secondary lymphoid organs, as well as physiological or pathological conditions may play an important role in the fate of a DC precursor. Ultimately, DCs and their precursors have demonstrated remarkable functional plasticity and are able to differentiate into different types of DCs after encounters with various stimuli (Banchereau and Palucka, 2005).

1.3.3 Recognition of pathogens and “danger signals”

The concept of a pattern recognition receptor (PRR) was first envisaged by Charles Janeway in 1989, when he proposed that the host possesses a number of receptors that can recognise pathogenic components and trigger antimicrobial responses to a number of diverse infectious organisms (Janeway, 1989). While this hypothesis established the foundations of the discrimination of self from non-self,
it was later recognised that the host can also recognise host-derived “danger” signals (Matzinger, 1998). Innate recognition of pathogenic organisms and self-derived “danger” signals - usually intracellular components - is a crucial stage of immune development.

Antigen presenting cells have a large array of pattern recognition receptors (PRRs) that recognise evolutionary conserved molecular patterns (called pathogen associated molecular patterns or PAMPs) expressed by infectious organisms such as bacteria, viruses, helminths, yeast and protozoa, as well self-derived Ags. Examples of known PRRs include the toll like receptors (TLR’s, 1-11 in humans, 1-13 in mice), C-type lectins, CARD helicase receptors such as retinoic acid-inducible gene 1 (RIG-1) like receptors (RLRs), and nucleotide-binding oligomerisation domain (NOD) – like family receptors (NLRs), such as NOD-1 and inflammasomes (NALPs).

1.3.4 TLRs

1.3.4.α Bacterial recognition

Ligation of bacterial components such as LPS or peptidoclycan to TLR-4 or TLR-2, respectively, are known to induce classical activation of DCs (Michelsen et al., 2001), characterised by production of cytokines such as IL-12 p40 and p70, TNF-α and IL-6, as well as up-regulation of MHC class II and surface expression of co-stimulatory molecules. CpG unmethylated DNA, which is characteristic of bacterial but not eukaryotic DNA, has been shown to classically activate DCs in vitro by ligation to TLR-9 (Boonstra et al., 2003). Lipoproteins, such as OspA, an
outer surface lipoprotein from *Borrelia burgdoferi*, can bind to both TLR-2 and TLR-1 while bacterial flagellins bind to TLR-5 inducing classical DC activation (Alexopoulou et al., 2002).

### 1.3.4.β Viral recognition

Binding of single stranded RNA such as that from influenza virus or vesicular stomatitis virus (VSV) to TLR-7 induces type 1 interferons and IL-1 production by pDC (Lund et al., 2004). Double stranded (ds) synthetic polyriboinosinic:polyribocytidylic acid (polyI:C), the synthetic analogue of viral dsRNA or encephalomyocarditis virus (EMCV) binds to TLR-3 inducing DC activation and cross presentation (discussed in 1.3.7) for CD8+ T cell activation (Schulz et al., 2005).

### 1.3.5 Other PRRs

NLRs constitute a cytoplasmic PRR family, containing more than 20 members. The first NLRs to have been described are NOD-1, which recognises \(\gamma\)-d-glutamyl-meso-diaminopimelic acid on gram negative bacteria and NOD-2, which is a bacterial and single stranded RNA virus sensor, recognising muramyl dipeptide (MDP) (Lee and Min, 2007; Murray, 2009). Flagellin from *Salmonella* and *Legionella*, which as discussed earlier can bind to TLR-5, can also bind to NLRs, IPAF and NAIP5, activating the caspase-1 signaling pathway, which leads to the production of IL-1\(\beta\) and host cell death (Mariathasan et al., 2004; Zamboni et al., 2006). Extracellular ATP (adenosine triphosphate) and uric acid, both released
following stress in the form of physiological exhaustion, or during bacterial or viral-induced cell death, bind to NALP3, leading to the activation of caspase-1 as well as secretion of IL-1β and IL-18 (Mariathasan et al., 2006; Martinon et al., 2006), suggesting that NLRs might be important in sensing “danger” signals. Cytoplasmic CARD helicases, such as RIG-I and MDA-5 receptors, bind to poly I:C synthetic RNA and viral double stranded RNA (Thompson and Locarnini, 2007; Yoneyama et al., 2004), inducing type 1 interferon responses (Gitlin et al., 2006).

C-type lectin receptors, such as DC (DC)-specific ICAM-grabbing nonintegrin (DC-SIGN) (CD209) in humans and SIGN-R1 in mice, and DC-associated C-type lectin 1 (DECTIN-1) receptor are found on MΦs, DCs as well as B cells (Geijtenbeek and Gringhuis, 2009). They are extracellular receptors that bind a range of glycans (mannose, fucose) on viruses, bacteria, fungi, protozoa and helminths (Geijtenbeek and Gringhuis, 2009). C-type lectin binding promotes phagocytosis, endocytosis and antigen presentation by DCs (Bonifaz et al., 2004; Gazi and Martinez-Pomares, 2009; Zhang et al., 2005). They have also been found to be involved in secretion of type 1 interferons by APCs (Chen et al., 2008), driving allergic and type 2 responses (Barrett et al., 2009; Sato et al., 2006; Shreffler et al., 2006) as well as inducing IL-10, in response to probiotic (commensal) bacteria (Smits et al., 2005), possibly involved in regulation.

1.3.6 PRR interplay

Characterising the importance of individual PRRs in pathology and in pathogen recognition has proven useful in understanding how APCs recognise
pathogens and “danger” signals in the periphery, and subsequently how they become activated and drive immune responses. A single infectious organism, however, will simultaneously present a number of different PAMPs to PRRs on APCs. While, some PRRs work synergistically, amplifying APC activation, it is not uncommon for others to counter-regulate each other.

Ligation of TLR-4 via LPS has been shown to upregulate TLR-4, TLR-2 and TLR-9, possibly enhancing APC sensitivity to bacterial cell wall components and CpG unmethylated DNA (An et al., 2002; Gautier et al., 2005; Napolitani et al., 2005). On the other hand, ligation of TLR-2 has been found to alter DC responsiveness to TLR-4 and TLR-3, by blocking their ability to induce type 1 interferons, possibly through an IL-10 dependent manner, since TLR-2 ligation drives strong IL-10 responses by DCs (Re and Strominger, 2004). The importance of TLR interplay has also been investigated in pathologic conditions. Double, TLR-2 -/- TLR-9 -/-, deficient mice were shown to be much more susceptible to aerosol mycobacterial infections than the respective TLR-2 -/- or TLR-9 -/- single gene deficient mice, suggesting that TLR synergy is important for optimal immune response development (Bafica et al., 2005).

There is limited evidence that CARD helicases also interplay with TLRs. MDA-5 has been shown to be expressed and translocated to the cytosol upon IFN-β stimulation, a process which is facilitated upon TLR activation (Kang et al., 2002). NLRs such as NOD-1 and NOD-2 have been found to act synergistically with TLR-4 for the induction of TNF-α and IL-6, while activation of NODs allows for maturation of human DCs with sub-activating doses of LPS, suggesting the
synergy between NODs and TLR-4 allows for DC activation at lower bacterial concentrations.

Counter regulation between TLRs and other PRRs has also been reported. RIG-1 is negatively regulated by A20, an LPS-inducible regulator of TLR-4, suggesting that TLR-4 may indirectly inhibit signalling through RIG-1 (Lin et al., 2006). CLR’s have been shown to modulate TLR signalling. For example, DC-SIGN ligation by mycobacterial glycans in human DCs acts to modulate TLR-3, TLR-4 and TLR-5 signalling, by inducing IL-10 production, and concomitant reduction of IL-12p70 (Geijtenbeek et al., 2003; Gringhuis et al., 2007).

1.3.7 Antigen processing and presentation

Following pathogen recognition, APCs internalise pathogens and process them for presentation to T cells. Antigen processing and antigen presentation by APCs is a crucial part of T cell homeostasis and immune response development. Two distinct pathways of antigen processing are recognised today. The endogenous pathway, which results in presentation of cellular and viral peptides on MHC class I molecules, or human leukocyte antigen (HLA in humans). And the exogenous pathway, which results in presentation of extracellular derived antigen on MHC class II molecules.

Select cell types, in particular DCs, can use the exogenous pathway for presentation of extracellular derived Ag on MHC class I molecules in a process termed “cross-presentation” (Heath et al., 2004). Similarly DCs can utilise the endogenous pathway for presentation of intracellular derived Ag on MHC class II molecules (Nuchtern et al., 1990).
**Endogenous pathway**

Missfolded or worn out proteins are ubiquitininated and targeted for proteasomal degradation within the cytosol and endoplasmic reticulum. Proteasomes such as leucine aminopeptidase, puromycin-sensitive aminopeptidase and bleomycin hydrolase degrade proteins to 8 to 10 amino acid length in size (Towne et al., 2008; Towne et al., 2007). The transporter associated with antigen protein (TAP), which spans the membrane of the endoplasmic reticulum binds to the degraded peptides and transports them to the lumen of the E.R (Shepherd et al., 1993). There, further degradation takes place, reducing the peptides to the right size for MHC I loading. A series of chaperone proteins facilitate the folding of class I MHC molecules and its association with β2-microglobulin (Ortmann et al., 1994). The partially folded MHC I molecule associates with TAP, a process catalysed by tapasin, which loads the peptide on to the MHC I molecule and stabilising it. This induces the transport of the MHC I-peptide complex to the cell membrane via the Golgi complex (Banchereau et al., 2000).

**Exogenous pathway**

Extracellular bacteria or Ag are internalised into the phagosome, which after fusing with lysosomes, forms the phagolysosome (or late endosome) (Banchereau et al., 2000). Protein degradation and MHC II loading is performed within this compartment. Nascent MHC II is formed in the E.R by α and β chains as well as an invariant (Ii) chain, which serves to block the MHC docking site from endogenous and cellular peptide loading, as well as to transport the MHC II
molecule to the late endosome (Banchereau et al., 2000). The MHC-Ii complex is transported to endosomal vesicles where lysosomal proteases degrade the invariant chain leaving behind a small fragment called CLIP. HLA-DM, an MHC II like protein, removes CLIP and replaces it with a peptide from the phagolysosome (Watts, 2004). The Ag loaded MHC class II is then transported to the cell membrane for presentation to naive CD4+ T cells.

1.4 Adaptive immune responses

The importance of the adaptive arm of immunity has been clearly demonstrated in animal models such as those of thymectomised or severe combined immunodeficiency (SCID) mice, as well as in humans, such in the case of acquired immunodeficiency syndrome (AIDS) or hyper IgM syndrome. Humans and mice lacking T or B cells succumb much more rapidly than normal hosts to pathogenic infections. Moreover, they are prone to opportunistic infections by agents that are otherwise non-invasive, such as pulmonary infections with Cryptococcus neoformans, Aspergillus fumigatus and Histoplasma capsulatum as seen in HIV-1 infected humans (Antinori et al., 1996), and Pneumocystis carinii in both HIV-1 infected humans and SCID mice (Rapaka et al., 2007). It is thus clear that adaptive immune responses are integral to the effective elimination of pathogens by the host.
1.4.1 T cells

The activation and subsequent involvement of T cells in immune responses is a critical step towards the elimination of infectious organisms. Animal experiments addressing the importance of T cells in immunity have shown that T cell deficient mice are severely immunocompromised and, similarly to humans suffering from AIDS, succumb to infections caused by opportunistic pathogens, rapidly. T cells amplify immune responses by recruiting leukocytes to the sites of infection by activating MΦs, neutrophils and natural killer cells (NK cells), by providing help to B cells for Ab switching and clonal expansion, and by direct killing of infected cells.

T cell lineages include αβ T cells γδ T cells. αβ T cells are further divided into CD4⁺, CD8⁺ and NKT cells. These subtypes of T cells are selected for early, during thymic development, and show no plasticity. CD4⁺ αβ T cells are the most abundant T cell subset and responsible for most of the functions described below. CD8⁺ T cells are essential for eliminating viral and intracellular pathogen infected cells. γδ CD4⁺ T cells represent a very small proportion of the total T cell population. They home to mucosal and dermal tissues and are thought to be involved in localised immune responses. NKT cells show limited diversity of αβ TCR in comparison to CD4⁺ T cells, and recognise glycolipids presented on CD1d.

Groundbreaking work in T cell immunology by Mossman and Coffman in 1986 recognised distinct subpopulations of T cells according to their ability to secrete either IL-4 or IFN-γ (Mossman & Coffman, 1986). Soon after, it became apparent that T cell populations exert specific effector functions against the vast
range of pathogens encountered by the host. Today, we recognise the existence of 4 such subpopulations of T helper cells: T helper 1 (Th1), T helper 2 (Th2), inducible T regulatory cells (Treg), and T helper 17 cells (Th17) (Figure 1.2). Recent work has proposed the existence of two more subsets, T helper 9 and T helper 22 cells, for which, however, no master transcriptional regulator has yet been identified. Understanding how distinct T cell subsets combat pathogens and prevent or cause autoimmune disease may lead to improved vaccination, cancer therapy strategies as well as graft rejection and autoimmune disease prevention strategies (Mariotti et al., 2008a; Sredni et al., 1996; Weaver et al., 2005). It is worth bearing in mind that distinct pathogens may require a range of different T cell subsets to be eliminated. Furthermore, it is not uncommon during pathology for individual T cells to secrete a number of different cytokines, blurring the somewhat artificial division we ascribe to them.

1.4.2 Th1

Th1 responses are characterised by the secretion of IFN-γ and expression of the transcription factor T-bet by T cells (Szabo et al., 2000). Th1 responses are associated with clearing bacterial, viral, fungal and protozoan infections such as *Salmonella typhimurium*, hepatitis C virus, *Candida albicans* and *Leishmania major* (Cenci et al., 1997; Davis et al., 2008; Ferlin et al., 1998; Mittrucker et al., 2002). They are also involved in eliminating cancer cells, in graft rejection and pathology during autoimmune disorders such as multiple sclerosis (Disis and Park, 2009; Mariotti et al., 2008b; Selmaj et al., 1991). Th1 responses augment the infiltration of
MΦs, NK cells and neutrophils at the sites of infection. Responses against Th1 polarising pathogens are characterised by high levels of IFN-γ, lymphotoxin-α and IL-2.

IFN-γ is an important cytokine for the activation of MΦs, inducing NO synthesis and microbial killing, lymphocyte recruitment, virus infected cell-killing, enhancement of Ag uptake and presentation, and inhibition of cell division (Schroder et al., 2004). Lymphotoxin-α is thought to play a role in clearing viral infections through the activation of CD8+ T cells (Kumaraguru et al., 2001). IL-2 is important for the expansion of both CD4+ and CD8+ populations as well as the generation of memory CD8+ populations (Williams et al., 2006). CD4+ T cells play a major role in providing help to B cells for Ab switch via CD40-CD154 interactions (Lederman et al., 1992), and induction of memory CD8+ T cells through CD27 ligation and provision of IL-15 (Oh et al., 2008; Xiao et al., 2008). Furthermore, CD4+ and CD8+ T cells can directly kill lymphocytes, infected cells, as well as cancer cells, via the Fas-Fas ligand and perforin-granzyme route (Canaday et al., 2001; van den Broek et al., 1996; Zheng et al., 2007).

1.4.3 Th17

Early experiments on experimental autoimmune encephalomyelitis (EAE) attributed disease pathology to IFN-γ secreting CD4+ T cells (Miller and Karpus, 1994). Later work using either IL-12 blocking antibodies or IFN-γ deficient mice showed that IL-12 is necessary for EAE induction, whereas the absence of IFN-γ contributed to pathology (Krakowski and Owens, 1996; Leonard et al., 1995).
Strikingly, it was found that IL-12p35 deficient mice were susceptible to EAE, suggesting that a different IL-12 member than IL-12p70 was responsible for the induction of an autoreactive T cell repertoire (Gran et al., 2002). IL-23 (composed of IL-12 p40 and IL-12 p19) was later shown to be responsible for the induction of EAE pathology. Furthermore, mounting evidence suggested that IL-23 was also responsible for the induction of IL-17A (IL-17) responses, suggesting that CD4\(^+\) IL-17 producing cells are responsible for disease pathology (Cua et al., 2003; Park et al., 2005) in the EAE setting. Today we know that Th1 CD4\(^+\) T cells and CD8\(^+\) T cells are in fact also necessary for establishing disease pathology in the EAE setting. Nevertheless, the link of IL-17 to this autoimmune disease model provided the impetus for addressing the role of Th17 cells in immunity.

The generation of Th17 cells in mice is dependent on the presence of TGF-β and IL-6 (Mangan et al., 2006; Veldhoen et al., 2006), while human studies have found that Th17 differentiation is dependent on IL-1β plus IL-6 or IL-1β plus IL-23 (Acosta-Rodriguez et al., 2007a; Wilson et al., 2007), and is under the control of the transcriptional factor ROR\(\gamma\)T (Ivanov et al., 2006). Th17 responses are described by the secretion of IL-17, IL-21 and IL-22.

IL-17 is thought to play a role in the induction of proinflammatory cytokines and chemokines as well as participating in the disruption of endothelial cell tight junctions at the blood-brain barrier (Kebir et al., 2007; Khader et al., 2007). IL-21 acts on T cells to further expand Th17 populations (Korn et al., 2007), while IL-22 is thought to play a role in promoting microbial killing by inducing the secretion of AMPs (described in Chapter 1.1.2) (Liang et al., 2006). Recent work on the EAE
model has shown that IL-17 secreting T cells home to inflamed tissues with established lesions (O'Connor et al., 2008), while work on the *Mycobacterium tuberculosis* disease model has suggested that perhaps Th17 cells may be important in recruiting CD4⁺ T cells to infected sites (Bettelli et al., 2008; Khader et al., 2007). Ongoing research is aiming to understand the role of this relatively novel cytokine in protective immunity and autoimmune disease.

### 1.4.4 Th2

Th2 responses are characterised by the production of IL-4 by T cells and the expression of the transcription factor GATA-3 (Zheng and Flavell, 1997). Th2 responses have been described in the resolution of helminth infections and pathology associated with allergic inflammation and asthma (Else and Finkelman, 1998; Ngoc et al., 2005). Evidence suggests that skewing transplant recipients from Th1 to Th2 responses can prolong graft survival (Qin et al., 2001), however, Th2 responses have also been shown to induce chronic graft rejection (Mhoyan et al., 2003). Th2 responses are characterised by the secretion of IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 (IL-17 E), IL-33 and amphiregulin by T cells (Zhu and Paul, 2008).

IL-4 is a positive feedback cytokine for Th2 differentiation. It also promotes B cells to class switch to IgE (Kopf et al., 1993), which binds to basophils and mast cells, resulting in the secretion of active mediators such as serotonin and histamine (Prussin and Metcalfe, 2003). IL-5 is a powerful chemoattractant for eosinophils (Coffman et al., 1989). IL-13 is involved in the expulsion of helminths (Wynn, 2003), while amphiregulin is an epithelial cell growth factor that has been shown to also be involved in the expulsion of *Trichuris muris* (Zaiss et al., 2006). IL-10 inhibits
DC maturation, as well as directly inhibiting Th1 differentiation (Fiorentino et al., 1989). IL-25 acts as an IL-4, IL-5 and IL-13 positive regulator, and is secreted by non-T or B cells at mucosal sites (Fallon et al., 2006).

1.4.5 Regulatory T cells

The notion that regulation is involved in immunity was introduced in the 1970s (Katz, 1972). It was not, however, until 1995 that the revolution in immune regulation began, with work by Sakaguchi who demonstrated that IL-2 receptor (CD25) depleted T cells transferred into athymic mice resulted in autoimmune disorders (Sakaguchi et al., 1995). Today, we have identified a number of different regulatory T cell populations that can be broadly divided into naturally occurring T regs, which are of thymic origin, and inducible T regs, which are generated in the periphery. In addition, regulatory populations have been further subdivided according to their cytokine-secretion profiles into Treg and Th3 populations. Work on scurfy mice and X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome (IPEX) on humans recognised that forkhead/winged-helix protein (Foxp3) is the master transcriptional regulator for the generation of regulatory T cells (Brunkow et al., 2001; Fontenot et al., 2003; Wildin et al., 2001). TGF-β, in the absence of other proinflammatory cytokines, is thought to be the primary inducer of regulatory T cells (Chen et al., 2003). Naturally occurring T regs modulate immune responses by killing autoreactive T cell clones that escape into the periphery via the Fas/Fas ligand and granzyme/perforin routes, while inducible T regs modulate ongoing immune responses by killing effector
lymphocytes (Banz et al., 2002; Gondek et al., 2005; Weber et al., 2006). Additionally, Tregs modulate the ability of APCs to activate T cells by secreting cytokines such as IL-10 and by cell-cell interactions involving CTLA-4/CD80 that downregulates costimulatory molecule expression on DCs (Fehervari and Sakaguchi, 2004; Wing et al., 2008).

1.4.6 B cells

B lymphocytes are best known for their antibody producing capacity, making them the prime cell providing humoral immunity. B cell precursors are of bone marrow origin. They recirculate in the blood and reside in germinal centres, where, with the provision of T cell help, they clonally expand and switch antibody isotype production (Allman et al., 2004). Many infectious models as well as human conditions such as hyper IgM syndrome, have established the importance of humoral responses in improved prognosis (Lougaris et al., 2005). Antibodies bind to pathogens such as bacteria and can initiate the complement pathway or opsonise phagocytosis by DCs and MΦs (Kelly et al., 2005; May and Frank, 1973). In mucosal tissues such as the gut, IgA Ab can bind to pathogenic bacteria, impairing their ability to attach to epithelial cells, while it also impedes bacterial motility required for host invasion (Vaerman, 1984).

Although, the consensus is that the main contribution of B cells in immunity is Ab production, without which disease clearance is severely impaired, B cells have been described to also play important roles in Ag presentation and provision of help T cells. B cell deficient models, addressing the contribution of this cell type
in initiating and sustaining immune responses, have shown that B cells enhance T cell numbers and immune response development (Kleindienst and Brocker, 2005), infection settings, such as the *Salmonella typhimurium* model in mice, have shown that full clearance of the pathogen is achieved only when both T and B cells are present, while pathology can not be overcome in the absence of B cells, whether antibody is present or not (Mastroeni et al., 2000a). Naive B cells are weak antigen presenting cells, however when clonally expanded, they exhibit excellent antigen presenting capacity, possibly contributing to the rapid clearance of pathogens later in infection, or even during early stages of concomitant invasions by the same pathogen (Ugrinovic et al., 2003). Furthermore, B cells are essential for the recovery from experimental autoimmune encephalomyelitis (Lyons et al., 2008). Provision of the IL-10 regulatory cytokine by autoreactive B cells is essential for EAE recovery (Fillatreau et al., 2002). B cells have recently been described to be crucial for the generation of memory T cells both in mice and humans by providing them with the right survival signals, most probably through the ligation of CD40 to CD154 (Crawford et al., 2006; Morales-Aza et al., 2009; Whitmire et al., 2009). Although their importance as an Ab-producing cell is immense, often overshadowing other key roles they may play during infection or autoimmune disease, it is obvious that B cells have a wide range of immunologically important functions during both primary and memory responses.

### 1.5 Dendritic cells bridge the innate with the adaptive immune responses

Pathogen derived components and host derived “danger” signals are recognised and taken up by DCs in the periphery. DCs then migrate to secondary
lymphatic tissues where they present parts of the internalised material in the context of MHC class II to naïve CD4⁺ T cells (Reis e Sousa and Germain, 1999). A crucial factor deciding the fate of immune response development (discussed in the previous chapter - Chapter 1.4) during pathogen invasion is the context in which DCs present Ag to T cells. The signal DCs receive in the periphery via PRR ligation becomes translated into a message for T cells in the form of MHC-antigen presentation, co-stimulatory molecule upregulation and cytokine secretion.

Different pathogens leave an “imprint” on DCs, which following maturation will initiate pathogen-specific T cell responses (Banchereau and Steinman, 1998; de Jong et al., 2002; MacDonald et al., 2002b; Mangan et al., 2006).

The activation and polarisation of naïve CD4⁺ T cells is determined by three signals provided by DCs in secondary lymphoid tissues. The first signal is provided via the presentation of short peptides (epitopes) that originate from pathogenic proteins acquired in the periphery by DCs on MHC molecules, which are bound by the appropriate TCR of naïve CD4⁺ T cells. The second signal is thought to be co-stimulation mediated primarily via CD28 on the T cell and CD80/CD86 on the APC, in whose absence T cells become anergic (Kapsenberg, 2003). The third signal provided by DCs, is a combination of cytokines and surface molecules on DCs that determine the polarisation state of the T cell (Kalinski et al., 1999; Kapsenberg, 2003).

**Signal 2**

The best characterised co-stimulatory molecules on DCs are two members of the B7 family, CD80 and CD86, which bind to CD28 and CTLA-4 (Cytotoxic T
Lymphocyte Antigen-5 or CD152) found on T cells. CD28 is expressed constitutively on T cells while CTLA-4 is upregulated upon activation (Carreno and Collins, 2002; Wang and Chen, 2004). Ligation of CD80 and CD86 to CD28 provides a powerful signal to T cells, which, in conjunction with the signal provided by TCR ligation to peptide-loaded MHC (signal 1) induces the upregulation of CD25 (IL-2 receptor) on T cells as well as the production of IL-2, resulting in T cell entry into the cycle and subsequent proliferation (Alegre et al., 2001; Carreno and Collins, 2002; Greenwald et al., 2005). Ligation of CD152 to CD80 or CD86, however, is thought to transmit an inhibitory signal to T cells (Carreno and Collins, 2002; Wang and Chen, 2004).

In addition to signal transduction via CD28 to T cells, some evidence suggests that CD80 and CD86 ligation to CD28 provides a retrograde signal to the APC (Khan et al., 2007). Recent work has revealed five new members of the B7 family, including inducible costimulator (ICOS) ligand, PD-L1, PD-L2, B7-H3 and B7-H4 (Greenwald et al., 2005). All of these have been shown to be expressed on APCs and provide a potential new mechanism for regulating T cell activation and tolerance.

Signal 3

The subset naïve CD4+ T cells develop into is, to a great extent, determined by the molecular signals provided during antigen presentation by APCs known as “signal 3” (Kapsenberg, 2003). The cytokine milieu naïve CD4+ T cells are found in, together with surface molecules expressed on APCs instruct T cells to become polarised towards the various different profiles described earlier (Chapter 1.4).
APC-derived IL-12 p70 polarises CD4+ T cell responses towards a Th1, IFN-γ secreting profile (Orabona et al., 2004; Van Gool et al., 1996). Nevertheless, the requirement for this cytokine has been found to not be restricted to DCs, since in the absence of DC derived IL-12, other cell sources of IL-12 p70 are sufficient to drive Th1 responses (MacDonald and Pearce, 2002; Soares et al., 2007). IL-23 secreted by APCs has been found to play a crucial role in the generation of IL-17 as well as IFN-γ responses, however it is yet unknown whether it is required for the instruction of CD4+ T cells to become IL-17 or IFN-γ producers or the activation of previously polarised CD4+ T cells (Aggarwal et al., 2003; Langrish et al., 2005). IL-6 secretion by APCs is thought to be important for activating T cells by inhibiting T regulatory cell-mediated responses (Pasare and Medzhitov, 2003). Regulatory T cell response development is known to be dependent on the presence of TGF-β, while IL-6 in conjunction with TGF-β is known to instruct CD4+ T cells to become IL-17 producers (Mangan et al., 2006). As such, IL-6 may be involved in averting the activation of naïve CD4+ T cells away from an undesirable regulatory phenotype, by directing them towards a Th17 one (Bettelli et al., 2006; Stockinger et al., 2007; Veldhoen et al., 2006). Low surface expression of co-stimulatory molecules on DCs coupled with reduced or absent IL-12 p70 production and elevated secretion of IL-10 and can lead to a T helper 2 (Th2) phenotype (Jankovic et al., 2004). IL-10, which is secreted by APCs as well as activated CD4+ T cells acts in a retrograde fashion back on APCs to inhibit MHC class II expression, CD86 expression, IL-12, TNF-α and IL-1β secretion (Liu et al., 2004; Steinbrink et al., 1997), a process thought to play an important role for controlling inflammation.
In addition to cytokines, a range of surface molecules are also involved in the provision of signal 3 to naïve T cells. CD40 is a cell surface receptor on APCs, which ligates to CD154 on T cells, a process thought to be essential for immune regulation as well as activation and function of APCs (Grewal and Flavell, 1998; van Kooten and Banchereau, 2000). Ligation of CD40 to CD154 has been shown to be crucial for the development of optimal Th1 as well as Th2 immune responses (al-Ramadi et al., 2006; MacDonald et al., 2002a; MacDonald et al., 2002b).

In addition to CD40, signal 3 can also be provided via OX40L. Expression of OX40L by DCs, as well as other APCs, has been shown to be induced following activation, while its expression can be enhanced following CD40 signalling (Croft, 2003; Gramaglia et al., 1998; Murata et al., 2000). OX40L associates with OX40 (CD134), on T cells, which is induced following TCR/CD3 signalling (Gramaglia et al., 1998). Interestingly, OX40L has been shown to be particularly important for the generation of Th2, but not Th1 responses (Jenkins et al., 2007).

### 1.6 Salmonella

*Salmonella* is a facultative Gram negative bacteria that infects humans and animals through the faecal-oral route. *Salmonella enterica* serovar Typhi (*S. typhi*) causes typhoid fever in humans and accounts for approximately 16 million reported cases a year and 600,000 deaths globally. *Salmonella enteritidis* servovar Enterica and Typhimurium (*S. enterica, S. typhimurium*) are common contaminants of raw meat, and cause between 200 million and 1.3 billion cases of intestinal disease and approximately 3 million deaths a year (Coburn et al., 2007). As such, *Salmonella enterica* pose a serious commercial problem for the food industry.
Vaccination efforts against *Salmonella* started as early as the 19th century. By 1957 it was recognised that vaccination with sub-lethal doses of live bacteria conferred protection in humans (Hobson, 1957). Adoptive transfer of immune serum improved prognosis, however, found to be inadequate for full protection. Subsequent studies addressed the requirement of T cells for the generation of protective immune responses (Killar and Eisenstein, 1985; Kotlarski et al., 1989), and soon after it was established that both humoral and cellular immune responses were essential for pathogen elimination (Mastroeni et al., 1993a).

**1.6.1 Salmonella pathogenicity factors, immune evasion and pathology**

The innate and adaptive arm of the immune system play crucial roles in both controlling and eliminating pathogen invasion and spreading. Equally, *Salmonella* possess a large range of immune evasion and survival methods, enabling it to overcome an otherwise very hostile environment. Responses against *Salmonella* infection can be divided into two categories: pathogen associated immune responses, that are directed against the pathogen, and virulence-associated pro-inflammatory responses, that are caused and exploited by the pathogen. The most important pathogenicity determinants are the *Salmonella* Pathogenicity Islands (SPI), SPI-1 and SPI-2, encoding a type III secretion system (T3SS) (Galan and Curtiss, 1989; Mills et al., 1995), which injects effector bacterial proteins into either host cells, through a process called translocation, or into the extracellular space, a process known as secretion.
1.6.2 SPI-1

Although initially recognised for their role in host invasion (Jepson et al., 2001), T3SS products are now known to be involved in a number of bacterial-host interactions that result in cell infiltration and the induction of cell death by the activation of caspase-1 (Coburn et al., 2007). When purified SipA, which is encoded by SPI-1, is added to intestinal epithelial monolayers, it induces the production of pathogen elicited epithelial chemoattractant (PEEC)(Lee et al., 2000), resulting in the direct recruitment of polymorphonuclear cells at the site of infection. Neutrophil infiltration to the gut lumen is linked to the disruption of the intestinal wall, crypt abscess formation, epithelial necrosis, oedema and fluid secretion (Clarke and Gyles, 1987; Finlay et al., 1989; Giannella et al., 1973; McGovern and Slavutin, 1979). SipB, which is also encoded by SPI-1, is translocated into the cytosol of host cells, where it binds caspase-1, leading to the release of pro-inflammatory cytokines IL-1β and IL-18 (Hersh et al., 1999), and inducing cell death by apoptosis and necrosis, a process termed pyroptosis. Although, pyroptosis, was initially believed to be essential for pathogen spreading from the intestinal lumen to systemic sites (Monack et al., 2000), studies using caspase-1 deficient mice showed that they are more susceptible to infection, suggesting that pyroptosis might be beneficial to the host (Lara-Tejero et al., 2006). SopE, encoded by SPI-1 is involved in the induction of bacterial uptake by phagocytes (Hardt et al., 1998), and inducing early inflammatory responses in the epithelium. Complete deletion of the SPI-1 gene results in delayed inflammatory responses against Salmonella, however systemic responses remain unaltered (Zeng et al., 2003).
The molecular products encoded by SPI-1 genes is known to be transcribed during early stages of invasion of the intestinal site, however, it is yet unclear whether the SPI-1 – encoded molecular apparatus is critical for invasiveness. SPI-1 is, however, linked to early inflammation and infiltration of polymorphonuclear cells at the site of bacterial invasion. Perhaps early inflammation which can lead to destruction of the gut lumen architecture, allows for faster translocation of pathogens to systemic sites.

1.6.3 SPI-2

The molecular apparatus encoded by SPI-2 is essential for intracellular parasitism and virulence. Strains lacking SPI-2 genes are attenuated by up to five orders of magnitude compared to wild type strains, as identified by oral and intraperitoneal administration of these strains to naïve mice (Shea et al., 1996). Early studies on SPI-2 deficiencies described poor survival of Salmonella within MΦs, suggesting that proteins encoded by this pathogenicity island are interfering with the proteolytic pathways involved in intracellular killing mechanisms by phagocytic cells (Ochman et al., 1996). Interestingly, SPI-2 products induce the production of IL-10, prostaglandins and prostacyclins in MΦs (Uchiya et al., 2004; Uchiya and Nikai, 2004). These findings suggest that Salmonella may be actively inducing a regulatory microenvironment within the cells they invade.
1.6.4 Antigenic hindrance and survival of *Salmonella*

When a single bacterium translocates from the intestinal lumen into the lamina propria, and subsequently gets internalised by phagocytic cells, it changes its gene expression profile in order to survive detection and elimination by the host. *Salmonella* transitions from an extracellular phase (STEX) to an intracellular phase (STIN), a process that enables it to grow outside and inside host cells, respectively (Alaniz et al., 2006). FliC, a potent T cell stimulating subunit protein found predominantly on the flagellum (Strindelius et al., 2004), is also expressed on the bacterial surface. STEX bacteria can potently stimulate FliC specific T cells, however, intracellular bacteria fail to do so (Cummings et al., 2005). When *Salmonella* transitions from STEX to STIN phase it localises FliC to intracellular compartments rather than the bacterial membrane (Alaniz et al., 2006). In addition, intracellular *Salmonella* reduces FliC expression levels in order to avoid detection by T cells, while, simultaneously, it actively downregulates co-stimulatory expression by APCs (Cummings et al., 2005). PhoP is a gene involved in the regulation of the remodelling of the bacterial surface. During the transition from extracellular to intracellular compartments, PhoP becomes active and affects LPS recognition by the host by adding fatty acids, phosphoethanolamine and 4-amino-4-deoxy-L-arabinose, as well as acetylating the O-antigen (Gunn, 2001). Additionally, PhoP activity has been shown to decrease bacterial membrane permeability, allowing for survival in late endosomal and lysosomal compartments in the host (Murata et al., 2007). *Salmonella* can also inhibit the phagosome-lysosome fusion required for bacterial degradation (Tobar et al., 2004), thereby impeding antigen processing and presentation. While *Salmonella* can inhibit
recognition, degrading and presentation of Ag on APCs, it has also been shown to inhibit T cell activation via a direct, contact-dependent mechanism (van der Velden et al., 2005). Taken together, these findings demonstrate some of the challenges the host has to overcome in order to elicit an effective immune response against this pathogen.

1.6.5 Experimental model

The mouse model of *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) is commonly used in order to dissect the different aspects of immunity involved in the clearance of this pathogen. *Salmonella typhimurium* causes a systemic infection in mice akin to that of *Salmonella typhi* in humans. In particular, mice lacking the natural resistance-associated macrophage protein (NRAMP) gene that codes for iron transporter proteins, required for iron depletion of the phagolysosome within phagocytes, are highly susceptible to infection. NRAMP deficient mice such as BALB/c and C57BL/6 succumb to infections with virulent *Salmonella typhimurium* strains (Collins et al., 2002). However, those mice exhibit full protection when immunised with attenuated strains, prior to challenge with a virulent strain (Mastroeni et al., 1992). Common *Salmonella typhimurium* strains used to study how immunity and protection are conferred, are mutated at sites coding for SPI-1 or SPI-2 pathogenicity island genes (Niedergang et al., 2000), as well as genes that encode for aromatic amino acid synthesis (AroA) (Clarke and Gyles, 1987). While bacterial strains lacking virulence factors have thrown insight into the interplay of bacterial proteins and cells of the immune system, AroA
strains are better at characterising immune response development and protection, since they are antigenically identical to wild type strains.

1.6.6 Bacterial invasion of the intestinal tract

*Salmonella* has been described to invade two morphologically distinct sites at the distal ileum, the follicular associated epithelium, where Peyer’s patches are found, and the epithelium overlying villi (Carter and Collins, 1974). Bacteria preferentially invade and destroy M cells near Peyer’s patches (Jones et al., 1994; Mowat, 2003), creating gaps in the intestinal architecture and allowing adjacent cell infection (Jepson and Clark, 2001) (Figure 1.3). In addition, CD11b+ CD8α+ lymphoid DCs and CD11b+ CD8α- DCs, residing at interfollicular regions may be actively capturing and transporting *Salmonella* to the mesenteric lymph nodes (Iwasaki and Kelsall, 2001; Salazar-Gonzalez et al., 2006). Furthermore, CX3CR1+ DCs have also been reported to extend dendrites into the lumen and actively capture Ag and bacteria at columnar villus epithelium sites (Niess et al., 2005) (Figure 1.3), a process involving signalling through TLRs (Chieppa et al., 2006), however their role in direct immune response initiation is yet unclear. Bacteria reach Peyer’s patches through active transport of internalised *Salmonella* by DCs or M cells, or by pyroptosis of MΦs and DCs (Figure 1.3), which release bacteria to the interstitium and allow bacterial spreading through the lymphatics to secondary lymphoid organs.
1.6.7 DCs and Macrophages in S. typhimurium infections

DCs and MΦs recognise lipopolysaccharide (LPS) on the cell wall of *Salmonella* through TLR-2. Extracellular flagellins are recognized by TLR-5, while internalized flagellins by the Nod-like receptor Ipaf (Franchi et al., 2006; Miao et al., 2006). DCs are the most abundant APCs in Peyer’s patches, outnumbering MΦs (Hopkins et al., 2000), and as such are most likely to encounter the pathogen early. Furthermore, DCs, but not MΦs, infected *in vitro* with *Salmonella* express CCR7 and, when transferred to naïve animals, migrate to lymph nodes, while infected MΦs do not (Zhao et al., 2006). Although MΦs have been shown to internalise and kill bacteria at a faster rate than DCs (Delamarre et al., 2005), they also serve as a site for bacterial replication (Salcedo et al., 2001). *Salmonella* do not replicate within bone marrow derived DCs, and survival of the bacteria within late endosomal compartments in those cells is not dependent on the expression of SPI-2 genes by the pathogen, as is in the case of MΦs (Jantsch et al., 2003; Salcedo et al., 2001). The preferential survival of *Salmonella* within DCs might be important for the activation of the antigen presenting molecular apparatus. It has been reported that antigen presentation by MΦs but not DCs is impaired when *Salmonella* are deficient in virulence factors such as SPI-2, suggesting that DCs do not require active translocation of pathogen derived products in order to become activated (Niedergang et al., 2000). Human studies addressing the involvement of dendritic cells and MΦs in salmonellosis, reported that MΦs are more potent inducers of cytokine production, suggesting that they may have a greater role in recruitment of lymphocytes at the site of infection (Pietila et al., 2005). On the other hand, infected
DCs induce stronger T cell responses than MΦs, and as such they initiate immune responses against *Salmonella* more effectively (Kalupahana et al., 2005). While MΦs are not required for the generation of primary CTL CD8⁺ responses against this pathogen (Wijburg et al., 2002), both DCs and MΦs are capable of inducing the generation of memory CD8⁺ T cell populations (Pozzi et al., 2005).

### 1.6.8 Immune responses against *S. typhimurium*

Early stages of *S. typhimurium* infection are characterised by the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-24, TNF-α, IL-23 and IL-12p70 by APCs, fibroblasts and epithelial cells, resulting in infiltration of polymorphonuclear cells (PMNs) at the site of infection. Studies using GR1⁺ depletion methods have demonstrated that neutrophil infiltration can be both detrimental due to tissue damage, as well as beneficial to the host, depending on the type of pathology studied.

The presence of T cells is absolutely crucial for immunity against *S. typhimurium*, however, initial disease suppression is mediated by the innate arm immunity (Hormaeche et al., 1990). Upon activation, APCs migrate to secondary lymphoid organs and up-regulate CD80, CD86 and CD40. During this process DCs, degrade internalised bacteria and process Ag on MHC class II and MHC class I molecules for presentation to CD4⁺ and CD8⁺ T cells respectively, while they also secrete cytokines such as TNF-α, IL-12, IL-6, IL-23 and IL-1β. TNF-α released by DCs and MΦs during *Salmonella* infection enhances up-regulation of CD40, thereby promoting T cell activation (Pozzi et al., 2005).
Absence of TNF-α during primary or secondary infections, results in poor formation of granulomatous abscesses, characterised by early and high mortality rates in susceptible mice, irrespectively of their immunisation profile (Mastroeni et al., 1993b). Absence of TNF-α during later stages of infection also affects granuloma formation and allows for bacterial growth in both susceptible and resistant mice (Mastroeni et al., 1991; Mastroeni et al., 1995; Mastroeni et al., 1992). IL-12 is thought to be involved in controlling bacterial growth during *S. typhimurium* infections by promoting T cells to become polarised towards a T helper 1 phenotype and secrete IFN-γ (Mastroeni et al., 1998). Neutralising IL-12 during *S. typhimurium* infection results in deficient granuloma formation and impaired IFN-γ responses, in turn, affecting antigen presentation, nitric oxide synthase activity in MΦs and generalised increased mortality in susceptible mice (Mastroeni et al., 1996; Mastroeni et al., 1998). MΦs harbouring bacteria require IFN-γ in order to control and eliminate the pathogen, since depletion of this cytokine results in re-activation of latent *S. typhimurium* bacteria found to reside within MΦs (Monack et al., 2004). In the absence of T cells, early IFN-γ has been shown to be secreted by NK cells, nevertheless it is insufficient for controlling disease progression, suggesting that the importance of CD4+ T cells in clearing salmonellosis extends beyond their ability to secrete cytokine (Ramarathinam et al., 1993).

Naïve CD4+ T cells become activated in Peyer’s patches as early as 3 hours after oral infection (McSorley et al., 2002), however those T cells are restricted to the gut mucosa and do not recirculate to infected spleens or livers (McSorley et al.,
Following the initial activation of T cells in the gut mucosa, CD8+ and CD4+ T cells are activated by APCs in secondary lymphoid organs and are distributed to systemic sites of infection, where they either release cytokine, aiding leukocyte recruitment, or are involved in direct killing of infected cells (Kirby et al., 2004; Lo et al., 1999). While both CD8+ and CD4+ are crucial for pathogen clearance, γδ T cells may contribute but are not essential for improved prognosis, since they have been found to be dispensable in Salmonella infections, (Mixter et al., 1994; Weintraub et al., 1997). CD8+ T cells are required for either direct bacterial killing through the release of granulysin (Stenger et al., 1998), or for the elimination of infected cells (Lo et al., 1999). Although CD8+ T cells have been shown to be dispensable during primary immune responses, they are required during secondary challenges, since their absence results in poor bacterial clearing irrespectively of whether mice have been immunised or not (Lo et al., 1999). Activation of CD4+ T cells induces IFN-γ and TNF-α production, direct killing of infected cells, as well as activation of B cells. CD4+ T cells are required for the generation of memory CD8+ T cells as well as the generation of B cell clones that act as potent antigen presenting cells and Ab producers (Mittrucker et al., 1999; Shedlock and Shen, 2003). B cell role in amelioration of Salmonella infections has been widely attributed to their ability to produce Ab (Mastroeni et al., 1993a). Ab-coated bacteria are recognised by Fc receptors on phagocytes, and targeted directly to the phagolysosome for degradation, bypassing microbial evasive mechanisms (Tobar et al., 2004). B cells, nevertheless, can also present Ag and provide CD4+ T cells with help for the induction of CD4+ and CD8+ T cell memory responses, thus
contributing towards immunity at different stages of infection (Crawford et al., 2006; Mastroeni, 2002; Mastroeni and Sheppard, 2004; Mastroeni et al., 2000a).

1.7 Aims

DCs are the main APC, capable of driving immune responses 100 times more efficiently than other lymphocytes. We hypothesised that this unique capacity of DCs to translate message received in the periphery into signal for the initiation of immune responses, posed them as a very good candidate to investigate the highly controversial and dividing topic in the medical and scientific community of the efficacy of ultra-high dilutions of antigen.

Our understanding of the interactions between Salmonella bacteria and the host they invade has dramatically increased over the past 20 years. The different aspects of the adaptive arm of immunity required to confer protection against this pathogen have been well characterised. However, the contribution of the innate arm of immunity, and more specifically that of DCs, towards the generation of protective immune responses remains elusive. The experimental work performed in this thesis attempts to reveal possible innate factors that may be contributing to the generation of effective adaptive immune responses against S. typhimurium. Specifically, we investigated DC role in the generation of S. typhimurium-specific immunity. Furthermore, we addressed the role of the innate cytokine, IL-23, in the generation of adaptive immune responses against Salmonella.

The specific questions addressed in this thesis are:

1) Can ultra-high dilutions of Ag influence DC maturation (Chapter 3)
2) Can ultra-high dilutions of Ag influence immune response development by DCs (Chapter 3)

3) Can immune responses elicited by DC transferred into naïve mice alter immunopathology caused by *S. typhimurium* infections? (Chapter 4)

4) Does IL-23 influence immune response development against *Salmonella* infections? (Chapter 5)

5) Does IL-23 affect the generation of protective immunity against *S. typhimurium* infections? (Chapter 5)
Figure 1.1. DC progenitors and and subsets. DC develop from haemopoietic stem cell progenitors (HSCs) which give rise to lymphoid precursors (LP) and myeloid precursors (MP).

LPs develop into plasmacytoid DCs (pDC) and conventional DC precursors (Pre-cDCs), which circulate in the blood. Pre-cDCs can develop into conventional DCs that home to lymphoid tissues or DCs that home to mucosal sites, such as the intestinal lamina propria.

MPs develop into macrophage dendritic cell precursors (MDPs) which give rise to recirculating monocytes. These can develop into MΦs, myeloid-derived suppressor cells, TiP Dcs as well as Langerhans cells (skin DCs) and microglia (brain DCs)
Figure 1.2. Overview of CD4+ T helper cell subsets. Functions, transcription factors, as well as cytokines both produced by and crucial for the determination of different T helper cell subsets. Adapted from (Zhu and Paul, 2008)
Figure 1.3. *Salmonella* invasion and spreading. *Salmonella* can invade its host by a) transversing epithelial cells b) by invading M cells or c) by direct bacterial capture by DCs that extend dendrites into the intestinal lumen. Lamina propria DCs internalise *Salmonella* bacteria that exit M cells. These DCs can go through pyroptosis, thereby releasing bacteria into the lamina propria, which can then migrate as free bacteria through lymphatics to secondary lymphoid organs. Alternatively, lamina propria DCs carrying bacteria may migrate to secondary lymphoid organs, thereby contributing to bacterial dissemination. CXC3CR1 DCs have been described to extend dendrites into the intestinal lumen, a process also thought to contribute towards bacterial invasion and dissemination.
Chapter 2

Materials and Methods

2.1 Animals and reagents

WT C57BL/6 and p19+/- mice were bred and maintained in the Ann Walker Animal Facility or The March Animal Facility, University of Edinburgh. All mice were maintained in specific pathogen free conditions. Home Office personal and project license guidance was followed for all experiments. Six-to-twelve week old mice were used as bone marrow donors for DC culture. The antigens used for DC stimulation were chosen for physiological relevance and for correlation with previously published work. Heat-killed Propionibacterium acnes (P. acnes) was supplied by Professor Ian Poxton (University of Edinburgh), heat-killed Salmonella typhimurium strains SL1344 and SL3261 were supplied by Dr. Maurice Gallagher (University of Edinburgh) and heat-killed, purified and sonicated S. typhimurium was supplied by Professor David Gray (University of Edinburgh). Live, attenuated SL3261 and virulent SL1344 strains of S. typhimurium were grown in house from stocks kindly provided by Professor Gray and Dr. Maurice Gallagher, respectively. Concentrations of antigens used were based on experiments previously carried out in-house. Concentrations of live Salmonella infections were determined in-house as described in chapter 4.
2.2 Preparation of High and Ultra-high dilutions of antigen

In order to ensure minimal levels of contamination, high and ultra-high dilutions of heat-killed *S. typhimurium* SL 1344 strain, *P. acnes* or Cholera Toxin (Sigma Aldrich, Poole, USA) were prepared in-house using low endotoxin water (Sigma Aldrich). The different antigens were diluted in 10 ml of water in a 50 ml Falcon tube (CELLSTAR, Germany) at a concentration typically used for stimulating dendritic cells: stimulatory concentrations of the different antigens were determined by their ability to phenotypically activate DCs and/or the ability of DCs to induce immune response development in naïve animals (5µg/ml P.a, 5µg/ml S.t or 5µg/ml CT). The first dilution was named a 1CH dilution. The diluted sample was then shaken vigorously manually or automatically using a custom made DYNAMAT machine (DYNAMAT, Labotec, Brussels, Belgium) for a hundred repetitions. 100 µl of the 1CH dilution of antigen were then transferred to 10 ml of water and the process was repeated. The procedure was repeated up to 30 times resulting in a 30CH dilution of the original concentration (nCH = 10^[kn^2-2]) of 1CH, where 1CH = stimulatory concentration of antigen). Control (water) dilutions were prepared identically as described above, except that the starting 1CH sample was generated by adding 100 µl of water to 10 ml of water. The purchased water (Sigma) as well as the dilutions generated from it was tested for the presence of contaminants using Gas Ion exchange mass spectrometry (Figure 2.3). No contaminants were found either in the purchased water or in our ultra-high dilutions (Figure 2.3).

All high and ultra-high dilutions of antigen were generated in pure water which lacks minerals, amino acids and nutrients, and has much lower
osmolarity than the RPMI 1640 (GIBCO, Life Technologies, Paisley, UK) media DCs are normally incubated in. In order to normalise the osmotic and nutrient conditions of the high and ultra-high dilutions of antigen, RPMI 1640 (GIBCO) in powder form was used to make DC incubation medium at twice the concentration standardly used in the lab to incubate DCs. The media was prepared by adding half the amount of water than that recommended by the company’s specifications, and twice the recommended concentration of supplements; 20% FCS (Harlan, Loughborough, UK), 100 units/ml Penicillin/Streptomycin (GIBCO), 4mM L-glutamine and 10ng/ml GM-CSF (Peprotech, Rocky Hill, NJ, USA). This 2 * concentrated media was then filter sterilised by passing it through a 0.2µm Millex syringe filter (Millipore Corp., Bedford, USA) and mixed at 1/1 with the ultra-high dilutions of antigen.

2.3 Dendritic Cell Culture

Bone marrow-derived DC culture was performed as previously described (Lutz et al., 1999). Tibia and femurs were removed from sacrificed C57BL/6 mice. Attached muscle tissue was removed using forceps and 70 % ethanol soaked tissue. The bones were immediately placed in low endotoxin Dulbecco’s Phosphate Buffered saline (PBS) (Sigma Aldrich). PBS medium was discarded under sterile conditions and bones were washed in 70 % ethanol for 30 seconds before washing them three more times in PBS. Using razor blades sterilised in 70 % ethanol, the tips of both tibia and femurs were cut off. Bone marrow was flushed from the bones by injecting PBS through a 25G needle (Becton Dickinson, Mountain View, USA). To ensure that cell clusters were dispersed,
liberated bone marrow was then passed through an 18G needle (Becton Dickinson). Cells were seeded into standard bacteriological Petri dishes (Philip Harris Scientific, Cheshire, UK) at $2 \times 10^5$ cells/ml in 10 ml of RPMI 1640 (Sigma) media supplemented with 10% low endotoxin FCS (Harlan), 100 U/ml Penicillin-Streptomycin (Gibco), 2mM L-glutamine (Gibco) and 20ng/ml recombinant GM-CSF (Peprotech). Cells were incubated at 37 °C and 5% CO$_2$. On day 3, 10 ml of additional medium was added to each plate, while on days 6 and 8, 9 ml medium was carefully removed from the plate and replaced gently with 10ml of fresh medium. On day 11, DCs were harvested by gentle expulsion of medium over the dish to gather all semi-adherent DCs, counted using a haemocytometer, then re-plated in 24-well plates (Corning Incorporated, Corning, NY) in 1 ml of medium at a concentration of $1 \times 10^6$ cells/ml.

DCs were then incubated for 24 hours in fresh medium, high or ultra-high dilutions of antigen. Stimulatory concentrations of the different antigens (described above) were then added for a further 24 hours. After stimulation, DCs were harvested by gentle washing, centrifuged at 200 g for 5 minutes, and the supernatants stored at -20°C for subsequent ELISA analysis. Harvested cells were resuspended in 1 ml of media and assessed for phenotypic activation by Flow cytometry.

2.4 ELISAs

All ELISA antibodies, reagents and protocols had been previously optimised in house. Table 2.1 contains the list of antibodies, coating buffers,
recombinant cytokine standards and detection substrates. In brief, 96 well F96 MaxiSorp Nunc-immunoplates (Nalgene Nunc International, Hereford, UK) were incubated at 4°C overnight with coating antibodies. Between each step, plates were washed 4-8 times with PBS containing 0.1% Tween. The following day, plates were blocked for two hours at room temperature in PBS containing 10% FCS or 1% BSA (TNF-α only). Samples and recombinant cytokine standards were added in duplicate and incubated overnight at 4°C. Standard curves were generated by doubling dilutions of recombinant cytokines. Biotinylated detection antibodies were added for two hours at 37°C and peroxidase-streptavidin (Kirkegaard and Perry Laboratories, Maryland, USA) was added for 30 minutes at 37°C. ELISAs were developed using TMB Microwell Perox (Kirkegaard and Perry Laboratories) with the reaction stopped with 0.18M H₂SO₄ or ABTS (Kirkegaard and Perry Laboratories). Plates developed using TMB were read at 450nm wavelength, while plates developed in ABTS were read at 405 nm wavelength, using Multiscan Ascent® Labsystems equipment and software. TMB substrate was used for detection of lower levels of cytokine given its superior resolution, while ABTS was allocated for more robust cytokine production. Absolute concentrations were derived from optical densities using two-site binding (hyperbola) equations on the standard curve using Prism software. Only the linear phase of the equation was used in order to determine the values of individual experimental samples.

Ab ELISAs were performed using 96 well F96 MaxiSorp Nunc-immunoplates (Nalgene Nunc International, Hereford, UK). Wells were coated
overnight with purified sonicated S. typhimurium in PBS (Gibco). Serum from either non-immunised animals (Chapter 4 experiments) or previously immunised animals (Chapter 5 experiments) was used as standard.

2.5 Flow cytometry

Approximately 2*10^5 cells/200 µl were added to a V-bottomed 96 well FACS plate (Greiner Bio-One GmbH, Frickenhausen, Germany), incubated with αFcR block (2.4G2, 1µg/well) for 20 minutes at 4°C. Antibodies against cell surface markers described in Table 2.2 were then added for a further 30 minutes at 4°C. Samples were acquired using FACSCalibur (Becton Dickinson) and CellQuest software and analysed using FlowJo software (Treestar inc, Oregon, USA).

Live cells were gated using forward versus side scatter (Figure 2.1.A). Unstained controls were first used to calibrate for auto-fluorescence. All samples were then acquired on the FACSCalibur including isotype controls for each sample. Gating on fluorescence was determined by gating out 98% of the isotype control for each sample. DC purity was assessed by CD11c staining, CD4+ T cell purity was determined by CD4 staining and B cell purity was assessed by CD19 staining (Figure 2.2). DC, T cell and B cell purity was considered to be acceptable when cells constituted > 90% of the total amount of cells harvested.
2.6 T cell and B cell purifications

4*10^7 splenocytes from each individual mouse were collected and pooled within their own groups. Cells were centrifuged at 300g for 5 minutes and resuspended in PBS (Sigma Aldrich) supplemented with 2% FCS (Harlan) at a concentration of 1*10^8 cells/900µl. 100µl of anti CD4 beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) were added and the cells were kept at 4°C for 30 minutes. The cells were centrifuged at 300g for 5 minutes washed with PBS (Sigma Aldrich) supplemented with 2% FCS (Harlan) and resuspended in 500µls PBS (Sigma Aldrich) supplemented with 2% FCS. CD4^+ positive selection was performed by washing LD columns (Miltenyi Biotech) with PBS (Sigma Aldrich) supplemented with 2% FCS (Harlan) once before adding the 500µl of CD4-bead coated splenocytes. The LD (Miltenyi) columns were washed 3 times with 3ml PBS (Sigma Aldrich) supplemented with 2% FCS (Harlan). Another 5mls of PBS (Sigma Aldrich) supplemented with 2% FCS (Harlan) was added, the column was removed from the magnet and the captured cells were washed into a fresh Falcon tube by applying pressure with a plunger.

B cell sorting was performed by positive selection applying the same method as that for T cells, with the distinction that CD19^+ coated beads were used instead of CD4^+. Purity was determined as described above (Figure 2.2).

2.7 Dendritic Cell transfer Assays

DCs were harvested after being exposed to antigen, and resuspended in 5*10^6 cells/ml in low endotoxin PBS. Mice were injected with 50 µl of the cell
suspension either subcutaneously or into the footpad of each hind leg using a 25G needle and a 1 ml syringe. Mice were kept for a week in the animal facilities of the School of Biological Sciences at the University of Edinburgh before being sacrificed for further analysis. For some experiments, following the same injection protocol, mice were kept for 3 days or 5 weeks in the animal facilities of the School of Biological Sciences at the University of Edinburgh before being orally infected with the virulent SL1344 strain of *S. typhimurium* (see below).

### 2.8 Bacterial cultures and Infections

*S. typhimurium* strain SL3261 was grown overnight from stock in 20 ml of liquid agar broth anaerobically at 37 °C. 1 ml of the cultured bacteria was transferred into 9 ml of PBS. Yield was determined by serially diluting the 1/10 concentrate of the original culture ten fold and plating onto agar-coated Petri dishes. Bacterial yields varied between 1*10^7 CFU/ml and 1*10^8 CFU/ml. Mice were immunised i.p. with 100 µl of the 1/10 concentrate.

*S. typhimurium* strain SL1344 was grown overnight from stock kindly provided by Dr. Maurice Gallagher (University of Edinburgh) in 5 ml of liquid agar broth anaerobically at 37 °C. Gram staining was performed the following day to ensure absence of contamination. Cells were washed in PBS and yield was determined by serially diluting ten fold and plating onto agar-coated Petri dishes. Bacterial yields varied between 1*10^10 CFU/ml to 5*10^10 CFU/ml of liquid agar. Bacteria were resuspended at 2*10^8 CFU/100µl in PBS and administered orally by gavage.
2.9 Assessment of morbidity and serum harvest

For the immunisation procedures with attenuated SL3261 strain mice were weighed at the day of infection and every 2 days after the infections were preformed for the first 14 days in order to assess primary immunopathology caused by avirulent SL3261 infection. Mice were bled every 7 days in order to perform antibody serum analysis. Blood was left to clot for 24 hours at 4 °C before centrifuging at 600g for 10 minutes and serum collected for antibody analysis was stored at -20 °C.

For challenge infection with SL1344 virulent *S. typhimurium* strain, mice were weighed at the day of infection and every day post infection at 24 hour intervals. Mice were bled at the end point of each experiment (Day 3 or 4 depending on morbidity). Blood was left to clot for 24 hours at 4 °C before centrifuging at 600g for 10 minutes. Serum was collected and stored at -20 °C till antibody and cytokine analysis by ELISA were performed. Ab titres (using standards and negative controls) (Figure 4.12) were determined by calculating how many times greater was the absorbance value from serum from experimental groups than that from non-immunised negative standards. The values were determined from the linear phase of a two-site binding hyperbola from the experimental groups.

2.10 Infection recall responses

Mice were sacrificed one week or 10 weeks post immunisations with the avirulent SL3261 strains, or 4 days after challenge with the virulent SL1344
strain. Spleens and mesenteric lymph nodes were removed and processed under sterile conditions into single cell suspensions using sterile 5 ml syringe plungers and cell strainers (Falcon). Spleens were collected in order to assess systemic responses while mesenteric lymph nodes were collected in order to assess localised responses at the primary invasion and infection site. Splenocytes were centrifuged for 5 minutes at 300g and resuspended in 3 ml RBC lysis buffer (Sigma) for 2 minutes to remove any red blood cells. Cells were washed twice in DMEM (prepared in-house), were then resuspended at 1*10^7 cells/ml in DMEM supplemented with 5% FCS (Harlan), 5mM penicillin streptomycin (GIBCO), 2mM L-glutamine and 2-mercaptoethanol (2ME). The cells were plated at 200µl/well in a 96 flat-bottomed tissue culture plate (Corning Inc.) for 3 days at 37 °C and 5% CO₂. Lymph node cells (5*10^6 cells/ml, 200µl/well) were plated in a 96 round-bottomed tissue culture plate (Corning Inc.) for 3 days at 37 °C and 5% CO₂. Mesenteric lymph nodes yield smaller number of cells compare to spleens. As such the number of cells used to address recall MLN responses is smaller than that used for spleens. Since the number of working cells is smaller, round bottom plates were used in order to ensure cell-cell contact. Flat bottom plates were used for splenocyte responses, due to the fact that spleens yield more cells and as such more cells can be added in each well. A larger number of cells allows for cell-cell contact even in a flat bottom plate.

2.11 Recall responses following DC transfer
Mice were sacrificed either one week or five weeks after being injected with differently activated DCs. Spleens and inguinal lymph nodes were removed and processed under sterile conditions into single cell suspensions using sterile 5 ml syringe plungers and cell strainers (Falcon). Splenocytes were centrifuged for 5 minutes at 300g and resuspended in 3 ml RBC lysis buffer (Sigma) for 2 minutes to remove any red blood cells. Cells were washed 2 times in DMEM (Sigma), were then resuspended at 1*10⁷ cells/ml in X-vivo serum free media (Cambrex Bio Science, MD) supplemented with 2mM L-glutamine and 2-mercaptoethanol (2ME). X-vivo media was chosen for these recall response assays because it lacks serum. Since DCs were originally grown in medium supplemented with foetal calf serum, it was imperative that we avoided any response against serum proteins, which might alter the picture of the immune responses against the antigens investigated. Spleen cells from infected mice were resuspended at 1*10⁷ cells/ml in X-vivo supplemented with 2 ME and L-glutamine and 10 units/ml gentamicin (Gibco) and were plated at 200µl/well in a 96 flat-bottomed tissue culture plate (Corning Inc.). Lymph node cells (5*10⁶ cells/ml, 200µl/well) were plated in a 96 round-bottomed tissue culture plate (Corning Inc.) for 3 days at 37 °C and 5% CO₂.

2.12 Statistical analysis

All data are represented as means. Error bars, wherever visible, represent standard error of the mean. The Student’s unpaired t-test was used to determine whether means between groups differed significantly. Statistical significance was assigned to data returning a ‘P value’ of less than 0.05. Two-Way ANOVA
analysis was performed to determine significance in time-dependent experiments. Statistical significance for data analysed by Two-Way ANOVA was assigned for a ‘P value’ of less than 0.01. Data for which no statistical significance has been measured were obtained from samples with an n=1. More specifically, DC cytokine secretion (Chapter 1: Figures 3.1, 3.2, 3.3, 3.4, 3.5, 3.6) was determined by stimulating DCs in single wells, while cytokine analysis was performed in triplicate wells on the ELISA plate. As such, error bars denote technical variation on the ELISA plate and not biological variation. Cytokine secretion by purified T cells (Chapter 5: Figures 5.3, 5.5, 5.6, 5.9, 5.10) was determined by pooling splenocytes from mice of each experimental group. Supernatants were analysed in triplicate wells on an ELISA plate. Error bars denote technical variation. Biological variation could not be statistically measured since cytokine levels secreted by splenocytes from individual mice are lacking.

Experiments of a more controversial nature, such as those performed investigating the effect of ultra-high dilution of antigen on the ability of DCs to direct immune response development, were performed up to 7 times under identical experimental conditions. For the purpose of obtaining a more robust statistical picture of the conferred result we performed meta analysis on 7 repeat experiments. The results are summarised in Chapter 3.2.5.

Table 2.1 ELISA antibodies and reagents

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<th>Capture Antibody</th>
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Table 2.2 Flow cytometry antibodies

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Figure 2.1. DC cell gating and purity assessment. Cultured cells were assessed for CD11c expression. DC were commonly >90% CD11c⁺.

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Figure 2.2 Representative staining of purified CD4⁺ and CD19⁺ cells following processing of spleens or lymph nodes into single cell suspensions and addition of magnetic beads as described in 2.6. Cells were analysed for CD4⁺ or CD19⁺ staining prior and after selection.
Figure 2.3. Gas-Ion mass spectrometry analysis of water. Two different batches of water purchased from Sigma Aldrich were tested for the presence of contaminants. The respective water dilutions (30CH H$_2$O) as well as P.a (30CH P.a) dilutions were also tested for the presence of contaminants. A single peak was found in all samples, reflecting H$_2$O. The absence of any other peaks suggests that no other substance was found in the water tested.
Chapter 3

The effect of ultra-high dilution of antigen on DC function and immune response development

Abstract

Homeopathy is considered as a complementary medical discipline, which is gaining rapid popularity in the western world. In Europe and the United States, an average of 25% of the population has at some point sought homeopathic treatment or advice. Nevertheless, there is no real empirical evidence supporting that it has a measurable biological effect. A number of homeopathic medications use substances diluted many times beyond Avogadro’s number, making it impossible to explain how any biological effect of such dilutions could be brought about in the first place. Basic scientific research addressing this subject is minimal. To date, no studies exist that have investigated whether such low or ultra-low dilutions of antigen might influence immune response development. Dendritic cells are the surveyors of the immune system. They constantly sample their surrounding microenvironment through pinocytosis and phagocytosis and alert the immune system at the first sign of danger. With the aid of cytokine protein and co-stimulatory molecule expression DCs direct naïve CD4+ T cells towards a range of phenotypes, including Treg, Th1, Th2, and Th17 phenotype. As, such we hypothesised that DCs are an excellent candidate cell to test
whether extreme dilutions of antigen exert a biological effect. We addressed whether ultra-high dilutions of antigen could confer a measurable effect on DC activation, and whether DCs treated with such extreme Ag dilutions displayed altered ability to influence immune response development. We found that ultra-high dilutions of antigen have no measurable effect on DC activation state. However, DCs that have been treated with such dilutions may display altered function, inducing exaggerated immune responses to certain antigens.

3.1.1 A short history of homeopathy

The principles of homeopathy were introduced in early 19th century by Samuel Hahnemann. They were based on the idea that substances causing symptoms similar to these observed in distinct pathological conditions had a therapeutic potency towards the corresponding pathology. The experiments leading to this conclusion, involved self-administration of various substances, ranging from elements such as sulphur to tree extracts such as quinine – a common anti-malaria drug at the time. He recorded the symptoms caused by those substances and compared them to symptoms caused by pathological conditions. He proposed that serially diluting his samples and administering them to patients with pathological conditions exhibiting symptoms similar to those conferred by the various substances he used, had a therapeutic effect (S. Hahnemann, Materia Medica Pura: 1827).

During the 19th century, medicine used very crude therapeutic methods, such as blood letting and purging (1890) usually resulted in deterioration rather
than improvement of the patients' condition (Bloodletting Over the Centuries, Gilbert R. Seigworth, 1980). Substances such as mercury and arsenic, which are today known to be toxic and harmful were included in the American pharmacopoeia and commonly prescribed for conditions such as psoriasis (Farber, 1992). Hahnemann’s proposed methods of treatment appeared to be a huge improvement over the existing ones. Homeopathy gained vast popularity in Europe as well as in America. A large number of hospitals practising the new form of medicine were established while an increasing number of medical practitioners trained in homeopathy. The popularity of the practice of homeopathy started to decline at the end of the 19th century, around the time when scientific method and rigor found application in medical practice. Scientific research led to the rapid improvement in therapeutics, which left homeopathy in the margin. Revival came in the 1970s when herbal and alternative medicine gained popularity. The proportion of the public seeking alternative medical advice outside the NHS has been dramatically increasing in the last 10 years, posing a need for more rigorous scientific research in this field (Thomas and Coleman, 2004; Thomas et al., 2003).

3.1.2 Principles of homeopathic remedies

Substances, ranging from crude extracts such as that of the tree *Rus Toxicodendrum* to simple compounds such as Sulphur are base materials for the preparation of remedies. At high concentrations these substances can have toxic or allergic effects causing a variety of symptoms, which homeopathic practitioners claim resemble symptoms naturally occurring in pathologic
conditions. These substances are serially diluted 10 or 100 fold at a time in water, alcohol or even milk in a process called potensisation (Witt et al., 2006). Between each successful dilution the samples are shaken vigorously, a process called dynamisation (Sunila et al., 2009). Different schools of thought exist describing potensisation and dynamisation methods, resulting in a variety of protocols used worldwide. The number of shaking repetitions and the nature of the diluent can therefore vary according to the protocol used. What remains constant between protocols is the final concentration of the solute. The resulting dilution can vary from a 1000 fold dilution of the original concentration up to $10^{2000}$ dilution of the original concentration.

### 3.1.3 Homeopathic dilutions; an absurd concept

The amount of a substance that contains as many atoms, molecules, ions, or other elementary units as the number of atoms in 12 grams of carbon is called the Avogadro’s number. This number is $6.0225 \times 10^{23}$, which is the amount of elementary units in a mole of any substance. Any dilution generated by 12 one hundred-fold dilutions is equal to $1 \times 10^{-24}$ of the starting solution. A one molar starting solution would therefore be completely diluted out, meaning that not a single molecule of the solute should remain in solution. A dilution of 30 CH (30 times 100 fold dilutions) is equal to $10^{-60}$ concentration of the original solution. To attempt to put this concept into context, a 40 CH dilution could in theory dilute the whole universe down to a single proton since the universe is constituted from approximately $10^{80}$ protons ($1.575 \times 10^{79}$ by Arthur Eddington's best estimate in his "The Philosophy of Physical Science" lecture in 1939). Some
homeopathic dilutions are much greater than 40CH. Given the fact that most homeopathic preparations theoretically contain no substance other than the diluent, the biggest challenge of any data interpretation is explaining why any measurable effect should exist in the first place. The most common and logical scientific argument is that since there is no active ingredient in the solution, there should be no effect. Nevertheless, this does not prevent the widespread use and application of homeopathic techniques throughout the world. Thus, whether or not homeopathic treatments exert a scientifically measurable biological effect is still a matter of controversy and socioeconomic significance, and as such investigating the matter in a controlled scrutinised fashion is necessary.

3.1.4 Clinical Research and The placebo effect

There is no scientifically approved explanation as to why homeopathic preparations might exert a real effect. Over time, many clinical trials have been performed in order to assess the efficacy of homeopathic remedies (Kleijnen et al., 1991; Linde et al., 1997; Shang et al., 2005). However, the amount of clinical research that has been performed in this field is limited, and quite often been criticised for not meeting the clinical research standards of “mainstream” medicine. One major argument medical practitioners use to explain the homeopathic effect is by attributing it to a placebo effect. The average consultation time in normal hospitals of the NHS takes on average 17 minutes, while consulting a homeopathic practitioner takes at least in the first couple of instances between one and two hours (Bikker et al., 2005; Richards et al., 2004;
Thompson and Weiss, 2006). It is well established that the patient-practitioner trust relationship is a major contributor towards the placebo effect (Price et al., 2006; Thompson and Weiss, 2006). As a result, many medics feel that prolonged consultation time offered by homeopathic practitioners can be one of the reasons why patients believe they are being treated with more care, therefore enhancing the placebo effect (Thompson and Weiss, 2006). The differences between therapeutic approach between homeopathic and conventional medicine make comparative clinical trial design hard, and as such the need for more reductionist approaches in basic research are necessary.

3.1.5 Basic research

Basic scientific research in homeopathy was highlighted to the world in 1988 when Jacques Beneviste published in Nature the effects of homeopathic dilutions of lung histamine and *Apis melifica*, a diluted bee venom, on the degranulation of basophils (Publication removed from records). His findings indicated that different dilutions of histamine or *Apis melifica* greater than Avogadro’s number induced either degranulation of basophils or inhibition of degranulation of basophils. His work was immediately heavily criticized, while his experiments were repeated unsuccessfully by a number of independent laboratories (Maddox et al., 1988). Within a year his work was universally discredited and Nature withdrew the initial publication and apologised to their readers. His initial experiments, however, drew the attention of curious scientists who re-addressed his question. Progress in immunology, the identification of cell surface markers together with the introduction of flow
cytometric techniques, enabled researchers to address Jacques Benveiste’s initial questions in a much more controlled and quantitative fashion. Multicentre experiments were performed and in 1999 inhibition of degranulation of basophils in response to homeopathic preparations of histamine was confirmed in a publication in Inflammation Research (Belon et al., 1999), followed by one more publication in the same journal in 2001 (Brown and Ennis, 2001). Although the basophil degranulation experiments were in the epicentre of the homeopathic debate (Benveniste et al., 1994; Pool, 1988; Wiegant, 1994), some limited amount of basic research published in non-homeopathic journals, addressing the same question in different models does exist. A Ruta graveolens extract (plant extract) was used at a $10^{-12}$ concentration to investigate its effect on lymphocyte and cancer cell survival and death signalling. The study reported that the Ruta extract selectively induces cell death in brain cancer cells but proliferation in normal peripheral blood lymphocytes (Pathak et al., 2003). An independent laboratory showed that a $10^{-400}$ concentration of Sabal serrulata (plant extract) had a significant effect on prostate cancer cell growth (MacLaughlin et al., 2006). A simple model utilizing inhibition of luminescence in the bacterium Vibrio fischeri, reported that an ultra-high dilution of 3,5-dichlorophenol had a statistically significant but minute effect on luminescence (Lee et al., 1993). No study, however has aimed to identify the effect of homeopathic preparations on immune response development. Given that dendritic cells are capable of amplifying immune responses by activating adaptive cells, we hypothesized that DCs are an appropriate cell type to investigate the homeopathic effect.
Aims

1) To investigate the effect of sub-optimal and very low concentrations of antigen on DC activation
2) To investigate the effect of ultra-low concentrations of antigen on DC activation
3) To investigate the effect of ultra-low concentrations of antigen on immune response development

3.2.1 Effect of low antigen concentrations on DC activation

Before investigating the effect of ultra-high dilutions of Ag on DC maturation and immune response development it was necessary to establish whether DCs are capable of recognising sub-optimal concentrations of Ag. Furthermore we wished to determine whether DCs that had been stimulated with sub-optimal concentrations of Ag, were able to respond to further activation with a previously determined stimulatory concentration of the same Ag.

It has been previously reported that DCs become refractory to stimulation when they repeatedly encounter activatory doses of antigen (Langenkamp et al., 2000). Although, this may have profound implications in the way immune responses are initiated or controlled during disease progression, little work has been done on the ability of low-antigen dose-treated
DCs to respond to stimulatory concentrations of the same antigen. We investigated how different pathogens and pathogenic compounds influenced DC activation. Furthermore, we addressed whether the activation state of DCs could be influenced by previous exposure to sub-optimal and very low concentrations of the same Ag. In order to achieve this, bone marrow derived DC were incubated for 24 hours in sub-optimal or very low concentrations of HK P. acnes (P.a), HK S. typhimurium (S.t) or Cholera Toxin (CT) antigen from Vibrio cholera bacteria. DCs were then stimulated in previously determined stimulatory doses of these antigens for a further 24 hours.

### 3.2.2 Effect of sub-optimal and very low concentrations of antigen on DC activation

DC incubated for 24 hours in the presence of a stimulatory dose of HK P. acnes (5µg/ml) secreted a significant amount of TNF-α, IL-6 and IL-12 p40 (Figure 3.1). After adding a further 5µg/ml of HK P. acnes, DCs secreted more IL-6 and IL-12 p40 but failed to secrete any more TNF-α (Figure 3.1). While stimulating DCs with 0.025µg/ml HK P. acnes had no effect on cytokine production, stimulating these DCs with a further 5µg/ml HK P. acnes induced secretion of similar levels of IL-6, TNF-α and IL-12 p40 to DC that were pre-incubated in media alone followed by a 5µg/ml P. acnes stimulation. Interestingly, DC pre-incubated with 0.025µg/ml HK P. acnes and stimulated with 5µg/ml HK P. acnes produced more IL-12 p70 than DC that were pre-incubated in media alone followed by a 5µg/ml P. acnes stimulation (Figure 3.1).
Pre-incubating DC in 0.0025fg/ml HK *P. acnes* had no effect on the amount of cytokine they secreted after being stimulated with 5µg/ml.

Cholera Toxin has been reported to upregulate IL-6 production by DC, and depending on the biological model it is used in it can act as a Th2 or T regulatory response inducer (Gagliardi et al., 2000; Lavelle et al., 2003). We investigated whether pre-treating DCs with low concentrationd of CT could alter their activation state after exposure to a stimulatory dose of CT. Stimulating DCs with 5µg/ml Cholera Toxin induced secretion of IL-6 alone (Figure 3.2). Interestingly, DCs that had been pre-treated with 5µg/ml CT for 24 hours and then stimulated with a further 5µg/ml CT secreted more IL-6 and a measurable amount of IL-12 p40 than DCs stimulated with a single dose of 5µg/ml CT. Strikingly, DCs incubated for 24 hours with as little as 0.025µg/ml CT secreted IL-6 and IL-12 p40, while adding 5µg/ml CT to these DCs for a further 24 hours, induced secretion of TNF-α and IL-12 p70 as well as IL-6 production, suggesting that activating DCs with different concentrations of CT may affect the type of response these polarise (Figure 3.2). Stimulating DCs that had been previously incubated for 24 hours with 0.0025fg/ml CT with 5µg/ml CT had no effect on cytokine secretion, and no effect on their ability to respond to a stimulatory dose of CT (Figure 3.2), compared to media pre-incubated, control DCs.

DCs incubated for 24 hours with 5µg/ml HK S.t secreted significant levels of IL-6, TNF-α, IL-12 p40 and IL-12 p70 (Figure 3.3). However, when these DCs were stimulated with a further 5µg/ml HK S.t they secreted less IL-6, TNF-
α and IL-12 p40 than DCs stimulated only once with 5µg/ml S.t (Figure 3.3), suggesting, similarly to HK P. acnes stimulation, that classically activated DCs are refractory to further stimulation. Incubating HK DCs with 0.025µg/ml had no effect on their ability to secrete cytokine, nevertheless, stimulating these DCs with 5µg/ml HK S.t induced similar cytokine level secretion as that observed when DCs where pre-incubated with 5µg/ml S.t followed by a 5µg/ml HK S.t stimulation, suggesting that HK S.t can render DCs refractory to stimulation at much lower concentrations than those that induce classical activation. DCs that had been pre-incubated in 0.0025fg/ml S.t and then stimulated with 5µg/ml secreted similar cytokine levels to DCs pre-incubated in media alone (Figure 3.3).

3.2.3 Effect of ultra-high dilutions of antigen on DC activation

We wished to address whether various antigens diluted beyond the Avogadro’s number had any measurable biological effect on DC activation. To this end we investigated whether ultra-high dilutions of antigen could induce a change in DC activation profile or condition DC to respond differently upon exposure to stimulatory concentrations of pathogens or pathogen derived compounds. DC were incubated in ultra-high dilutions of antigen or water alone for 48 hours or incubated in ultra-high dilutions of antigen or water for 24 hours before exposing them to a stimulatory dose of antigen for a further 24 hours. In order to control for any variability introduced due to contamination
during the dynamisation and dilution process, water was also diluted and dynamised identically to the ultra-high dilution antigen preparations.

In no occasion did DCs that had been incubated in ultra-high dilutions (30CH=10^{-58} of the starting 5\mu g/ml concentration) of P.a (Figure 3.4) showed a significant difference in IL-12 p40 and IL-12 p70, IL-6 or TNF-\alpha production in comparison to DC incubated with water controls. Similarly, pre-treating DC in ultra-high dilutions of \textit{P. acnes} did not condition them to secrete different amounts of TNF-\alpha, IL-6 IL-12 p70 or IL-12 p40 after addition of a stimulatory dose of \textit{P. acnes} (Figure 3.4). Not surprisingly DCs that had been treated with ultra-high dilutions of P.a had the same levels of MHC II molecule, CD40 and CD86 expression on their surface as DCs that had been treated with 30CH water dilutions (Figure 3.7.a). Similarly, DCs that had been pre-treated in 30CH P.a or 30CH H_2O showed no statistical difference in expression levels of MHC II molecules, CD40 or CD86 after being stimulated with 5\mu g/ml P.a (Figure 3.7.b). Overall, the cytokine and surface molecule expression data suggested that ultra-high dilutions of P.a had no effect either on DC activation or conditioning DCs to respond differently to stimulatory concentrations of P.a.

DCs that had been treated in 30CH \textit{S. typhimurium} (30CH St) or 30CH H_2O for 48 hours produced similar levels of IL-12 p40, IL-12 p70, IL-6 and TNF-\alpha (Figure 3.5). DCs that had been pre-treated in, control, ultra-high dilutions of water for 24 hours and then stimulated with 5\mu g/ml \textit{S. typhimurium} were classically activated, characterised by IL-12 p40 and p70 production, as well as IL-6 and TNF-\alpha (Figure 3.5). DCs that had been pre-treated in the ultra-high
preparations of HK S.t before being exposed to a stimulatory concentration of HK S.t displayed similar level of cytokine secretion to their controls (Figure 3.5). DCs that had been treated with ultra-high dilutions of S.t had the same levels of MHC II molecule, CD40 and CD86 expression on their surface as DCs that had been treated with 30CH water dilutions (Figure 3.7.a). DCs that had been treated with ultra-high dilutions of S.t had the same levels of MHC II molecule, CD40 and CD86 expression on their surface as DCs that had been treated with 30CH water dilutions (Figure 3.7.a). Similarly, DCs that had been pre-treated in 30CH S.t or 30CH H₂O showed no statistical difference in expression levels of MHC II molecules, CD40 or CD86 after being stimulated with 5µg/ml P.a (Figure 3.7.b), suggesting that ultra-high dilutions of HK S.t have no effect on DC activation or the ability of DC to respond differently to stimulatory concentrations of S.t.

CT induced secretion of IL-6 by DC and a detectable amount of IL-12 p40 (Figure 3.6). Pre-treating DCs with ultra-high dilutions of CT had no effect on the capacity of DCs to respond to a stimulatory dose of the same antigen (Figure 3.6). In accord with the experiments performed with P.a and S.t ultra-high dilutions of CT failed to induce any apparent change in expression levels of MHC II molecule, CD40 or CD86 (Figure 3.7.a). Furthermore, ultra-high dilutions of CT did not condition DCs to alter their surface molecule expression when compared to ultra-high dilutions of water pre-treated DC, after adding a stimulatory concentration of CT (Figure 3.7.b).
3.2.4 Assessing whether ultra-high dilutions of antigen can instruct DC to influence immune response development

In vitro DC incubation with ultra-high dilutions of different antigens had no apparent effect on their activation or in their ability to respond differently to stimulation with optimal concentrations of antigen. These results indicated that there was no biological effect of ultra-high dilutions. However, it is not uncommon for antigens to induce no apparent change on DC activation. Th2 polarising antigens which do not activate DCs in a classical way, very often fail to induce cytokine secretion or surface molecule expression changes on DCs (Kelsall et al., 2002). Nevertheless, such antigens are still capable of polarising Th2 responses via DCs. As such, in order to conclude whether ultra-high dilutions of antigen do not exert a biological effect on DCs, we assessed whether such dilutions of Ag confer a change in immune response development in an in vivo setting. To that end we pre-treated DCs with ultra-high dilutions of antigen for 24 hours, and then added a previously determined stimulatory dose of the same antigen for a further 24 hours. We then transferred those DCs into naïve mice and a week later assessed splenocyte recall responses.

P. acnes, a well studied pathogen, is known to drive T helper 1 responses. Heat-killed P. acnes has been shown to conventionally activate DCs, and can induce a T helper 1 response when injected into mice directly or delivered via DCs (Sher et al., 2003). We first assessed whether DCs treated in ultra-high dilutions of antigen were able to prime a measurable immune response in mice. DC were treated for 48 hours with 30CH H₂O or 30CH P.a and were then
transferred into naïve mice. Splenocytes from recipient mice were restimulated a week later in either media or with 1µg/ml HK P.a. IFN-γ and IL-10 production by splenocytes was significantly increased in both groups of mice upon the addition of 1µg/ml P.a (Figure 3.8), suggesting that P.a induces cytokine production by splenocytes, which is possibly of innate origin. There was no difference, however, in IFN-γ or IL-10 production by splenocytes between recipient mice that had received DCs treated with ultra-high dilution of P. acnes or water controls, suggesting that ultra-high dilutions of antigen do not prime P. acnes specific responses (Figure 3.8). This experiment was performed 7 times and in none of the experimental repeats did 30CH P.a treated DCs succeed in inducing a different splenocyte response than water controls.

Although 30CH P.a treated DCs failed to induce a different immune response in recipient mice than their water control treated DCs, it was important to examine whether 30CH P.a was able to condition DCs, altering the way they responded to antigen acquisition and subsequently immune response priming. We addressed this question by following the same DC transfer protocol but altering the in vitro DC treatment stages by exposing DCs to a stimulatory dose of P.a after 30CH P.a pre-treatment. DCs were incubated overnight in a 30CH water or 30CH P.a dilution, and were then exposed to 5µg/ml P. acnes for a further 24 hours. DCs were then transferred to naïve mice and spleen recall responses were assessed a week later. Surprisingly, IFN-γ production from spleens of recipient mice that received the 30CH P.a pre-treated - P. acnes pulsed DCs was significantly greater than that from mice receiving 30CH H₂O pre-
treated-*P. acnes* pulsed DCs (P<0.05) ([Figure 3.9](#)), suggesting that ultra-high dilutions of antigen condition DC ability to acquire antigen and induce immune responses. Recall IL-10 production was not affected ([Figure 3.9](#)), suggesting that the mechanism by which conditioned DCs act is not dependent on regulation, but rather a direct induction of inflammatory responses. Due to the extraordinary nature of the experiment and the results obtained, we repeated the same experiment under identical conditions 7 times. Statistical significance was achieved in only 3 out of the 7 experiments, while the same trend was observed in 6 out of 7 experiments. For that reason we decided to perform a meta analytical study discussed further on.

However interesting the results obtained by transferring 30CH P.a pre-treated - *P. acnes* pulsed DCs into naïve recipient mice, it was important to establish whether generating ultra-high dilutions of other antigenic stimuli, such as heat-killed *S. typhimurium*, could also exert a measurable effect on immune response development. Since the *S. typhimurium* model had not been previously used in our lab it was also important to investigate whether DCs pulsed in HK *S.typhimurium* and transferred into naïve mice could induce immune responses. DCs were exposed for 24 hours to three different doses of HK S.t (5µg/ml, 10µg/ml and 20µg/ml). They were then transferred subcutaneously to naïve mice and 7 days later spleens were removed and restimulated with either media or 1µg/ml S.t. *S. typhimurium* specific splenocyte IFN-γ was detected when DCs were exposed to the highest, 20µg/ml dose of S.t ([Figure 3.10](#)). Splenocyte IL-10 production decreased with increasing doses of DC exposure to HK S.t. Highest level of IL-10 production was detected when DCs had been exposed to the
lowest S.t concentration of 5µg/ml, while exposing DCs to 20µg/ml HK S.t failed to induce any more IL-10 than background (Figure 3.10). On the basis of this data the concentration of 20µg/ml HK S.t was selected as the optimal dose for the induction of Th1 responses via DCs.

Having established the concentration of HK *S. typhimurium* that induces optimal Th1 responses, we addressed the ability of 30CH S.t-treated DCs to affect immune response development. This was performed by incubating DCs for 24 hours in either 30CH H₂O or 30CH S.t before exposing them for a further 24 hours to 20µg/ml heat-killed S.t. The DCs were then transferred subcutaneously into naïve mice and 7 days later spleens were removed and splenocytes restimulated with either media or 1µg/ml HK S.t. DCs that had been exposed to 30CH H₂O or 30CH S.t before being exposed to a stimulatory concentration of S.t induced statistically similar splenocyte IFN-γ production in recipient mice (Figure 3.11). Surprisingly, S.t specific splenocyte IL-10 production by splenocytes of recipient mice that received DCs that had been pre-treated with 30CH S.t was significantly higher (P<0.05) than that secreted by splenocytes from recipient mice that had received DCs pre-treated with 30CH H₂O (Figure 3.11).

Given the extraordinary nature of the results obtained with ultra-high dilutions of *P. acnes* and *S. typhimurium*, we wished to re-address the question with a different antigen, this time in a blind fashion. There were two reasons behind this approach. First, we wanted to investigate the same question with an antigen for which the ultra-high dilution effect had not been previously investigated and as such we had no bias towards the “expected” result. And
second, we performed the experiment in a blind fashion to eliminate any bias introduced during handling. For that reason we addressed the question with the Th2/Treg driving antigen, Cholera Toxin.

BMDCs that had been treated with either water controls or an ultra-high dilution of CT did not differ in their ability to induce IL-5, IL-10, IFN-γ or IL-4 production by spleen cells of recipient mice upon CT restimulation (Figure 3.12).

The potential of ultra-high dilutions of Ag to condition DCs exposed to ultra-high dilutions of antigen was investigated by incubating 30CH CT or 30CH H₂O (control) pre-treated DCs with 5µg/ml CT for 24 hours, and then transferring these DC to naïve, recipient mice. Splenocytes from recipient mice were restimulated with 2 different doses of CT for 3 days, and the recall immune response conferred by the two groups of DCs was assessed by ELISA. Control recipient mice mounted a Th2 response characterised by IL-5 and IL-10 production by splenocytes, while CT specific IL-4 only emerged at the higher restimulation dose of 0.5µg/ml CT (Figure 3.13). Surprisingly, IL-5 and IL-4 production by splenocytes of recipient mice receiving 30CH CT pre-treated – CT pulsed DC was significantly higher than that observed by control mice (P<0.05). Interestingly 30CH CT-pre-treated – CT pulsed DCs induced an IFN-γ specific response in splenocytes from recipient mice that had been restimulated with the lower 50ng/ml CT concentration. At this restimulation concentration the 30CH CT-pre-treated – CT pulsed DC failed to induce a significantly larger amount of IL-4 or IL-10 than the 30CH H₂O (control) pre-treated DC group (Figure 3.13).
This suggests that IL-4/IL-10 and IFN-γ were possibly counter-regulating each other. In contrast, when splenocytes were restimulated with the higher concentration (500ng/ml) of CT the picture was reversed. Ultra-high dilution of CT pre-treated DC induced significantly more IL-4 and IL-10 than ultra-high dilution of water (control) pre-treated DC (Figure 3.13). At this restimulation concentration, no more IFN-γ was secreted by mice receiving ultra-high dilution of CT pre-treated DC than mice receiving ultra-high dilution of water pre-treated DC, further supporting the notion that the IL-4/IL-10 axis may be acting in a counter-regulatory mechanism towards IFN-γ (Figure 3.13).

3.2.5 Meta analysis reveals minute but statistically significant effect of ultra-high dilutions of antigen on immune response initiation by DCs

The nature of our investigation into the biological effect of ultra-high dilutions of antigen is highly unusual simply because we are working with dilutions that exceed Avogadro’s number. For that reason we repeated the experiment addressing the effects of ultra-high dilutions of P. acnes 7 times under identical conditions. Mean IFN-γ production from mice receiving 30CH P.a pre-treated – P.a stimulated DC was higher than the mean amount of IFN-γ secreted by controls in 6 experimental repeats. Statistical significance was achieved in only 3 of those experiments. In order to obtain a statistically relevant result from our experimental repeats we decided to perform meta analysis, (Figure 3.14). The results of the meta analysis suggest that, in our system, IFN-γ secretion by splenocytes from mice receiving 30CH P.a pre-treated – P.a
stimulated DCs was statistically higher than that induced in splenocytes from recipient mice of water control pre-treated – P.a stimulated DCs (Figure 3.12). The mean IFN-\(\gamma\) production from splenocytes of recipient mice that received 30CH P.a pre-treated – P.a stimulated DC was 9ng/ml higher than that of 30 CH water pre-treated - P.a stimulated DC. Both upper and lower bound values (variation) were above the null effect mark with 95% confidence.

3.3 Discussion

DCs respond differently to antigen stimulation when they have been previously “conditioned” by low concentrations of the same antigen. Ultra-high dilutions of antigen have no effect on DC activation. Nevertheless, ultra-high dilutions of antigen exert a significant, yet minute, effect on immune response priming by DCs. Conditioning of DCs by ultra-high dilutions of antigen is a novel way to study the biological relevance of homeopathic practice. However uncommon, our work has provided some interesting as well as surprising insight into this elusive field.

3.3.1 DCs encountering low concentrations of antigen are differentially activated by stimulatory concentrations of the same antigen

We found that exposing DCs to a stimulatory concentration (5µg/ml) of HK S.t twice over a period of 48 hours, results in an inability to secrete more cytokine than that produced by DCs encountering HK S.t just once (Figure 3.3). On the other hand DCs encountering 5µg/ml of P.a or CT twice, secreted more
IL-6 and IL-12 p40 than DCs stimulated only once with the respective antigen (Figure 3.1, 3.2). The contrast between the way repetitive S.t and P.a stimulation influence the ability of DCs to secrete cytokine suggests that there are intrinsic differences between these two Th1 driving pathogens and the way they influence DC activation. Whether, during pathogen invasion and spreading, a single DC can repetitively encounter pathogens or antigenic molecules is unclear, nevertheless not entirely improbable. In vitro assessment of the activation state of DCs after repetitive exposure to pathogens could throw some light into how this cell type might be influenced by different pathogens at distinct stages during disease pathology. It would be worth investigating whether DCs that are being stimulated with the same antigen repetitively go through apoptosis or necrosis, and whether they do so at a different rate than DCs that have been activated with antigen only once. Simple PI (propidium iodide) versus annexin-5 staining could reveal whether the type and rate of cell death differs between DCs that have been stimulated repetitively with antigen prior to activation and DCs that have been only activated with a stimulatory concentration of antigen once. Further characterisation of DCs by anti-CD80, CD86 and CD40 staining should give us a better picture of their activation state. Additionally, it would be interesting to investigate how these DCs initiate immune responses. Transferring DCs that have encountered stimulatory concentrations of antigen twice, into naïve mice, could give us some useful information about how disease progression could affect the way DCs polarise responses against various pathogens. Using antigens for which animal disease models are readily available could enable direct comparison between the
immune profile established during disease progression and that established after transferring differently activated DCs.

Stimulating DCs with 0.025µg/ml S.t failed to induce any cytokine secretion, however when these DCs were then activated with 5µg/ml S.t, they secreted similar levels of cytokine to DCs that had been stimulated with 5µg/ml S.t twice (Figure 3.3). Whether *Salmonella* antigen can pose DCs refractory to stimulation at much lower concentration than that required to classically activate DCs is unclear, however it would be worth investigating. Transferring DCs that have received a high+high or low+high concentration of S.t to naïve animals and assessing the immune response developed would give an indication as to whether a low concentration of HK S.t can indeed have a refractory to further stimulation effect on DCs.

Surprisingly, stimulating DCs with 0.025µg/ml CT followed by a further stimulation with 5µg/ml CT induced secretion of IL-12p70, IL-6 and TNF-α (Figure 3.2). It would be worth investigating whether DCs that have been exposed to these concentrations of antigen upregulate CD40, CD80 and CD86 and whether they initiate Th1 responses in recipient mice. Nevertheless, this result poses a question about the way CT influences DCs and consequently the way CT influences immune response development. While in our hands CT behaved as a Th2 inducing antigen (Figure 3.13), the emergence of TNF-α and IL-12 p70 after a low+high CT stimulation of DCs would suggest that these DCs might be better at polarising CT specific Th1 responses. It would be worth investigating whether in an *in vivo* setting, the antigen concentration, as well as
the route of exposure to CT can have an impact on the type of immune response developed.

DCs that had been pre-treated in a 30CH dilution of P.a did not respond differently to a stimulatory dose of *P. acnes* than DCs that had been pre-treated in 30CH H₂O. The production of proinflammatory cytokines by DCs, such as IL-6 and TNF-α and cytokines often associated with polarisation of Th1 responses such as IL-12 p40 and IL-12 p70 was not significantly altered by pre-treating DCs in ultra-high dilutions of P.a (*Figure 3.4*). Similarly we found no difference in surface molecule expression between DCs that had been treated or pre-treated in ultra-high dilution of P.a and DCs that had been treated or pre-treated in ultra-high dilution of water (*Figure 3.7.a, 3.7.b*). No measurable effect was detected in the ability of ultra-high dilution preparations of either Cholera Toxin or *S. typhimurium* to alter surface molecule expression and cytokine secretion by DCs (*Figure 3.5, 3.6, 3.7.a, 3.7.b*). Not surprisingly, these findings suggested that ultra-high dilutions of antigen conferred no biological effect on DCs.

### 3.3.2 Immune response development

We demonstrated that ultra-high dilution preparations of heat-killed bacteria or antigen can condition DCs to prime different immune responses than those primed by untreated DCs. The recall immune response to heat-killed P.a detected in the spleens of recipient mice that had received DCs exposed to P.a was characterised by IFN-γ and IL-10 production (*Figure 3.9*). Strikingly, IFN-γ production by splenocytes of mice that had received 30CH P.a – pre-treated DCs
was greater than that produced by splenocytes of control mice. The induction of the exaggerated Th1 response by the 30CH P.a pre-treated DCs compared to control DCs was not due to an inhibition of an IL-10 dependent regulatory mechanism, since IL-10 production in the spleen of recipient mice was identical in both control and experimental groups of mice (Figure 3.9). Given the fact that we observed, no apparent effect of ultra-high dilution of antigen on DC activation state (Figure 3.4, 3.5, 3.6, 3.7), it is hard to discern the reason why such dilutions of antigen may influence immune response development by DCs. Nevertheless, the in vivo DC transfer experiments would suggest that ultra-high dilutions of P.a should have a biological effect on DC activation state. In order to identify possible factors that might be contributing towards the observed difference between the ability of 30CH P.a – pre-treated DCs and 30CH H₂O - pre-treated DCs to drive P.a specific IFN-γ responses in naïve, recipient mice, we would need to perform gene array analysis of both DC groups. Although, gene arrays would not explain how such extreme dilutions of antigen that exceed Avogadro’s number exert their effect, they might explain whether such extreme antigen dilutions have indeed a direct biological effect on DC activation state.

In order to investigate whether ultra-high dilutions of a different Th1 polarising antigen could condition the way DCs drive immune responses, we repeated the DC transfer experiments using heat-killed Salmonella typhimurium as the antigen in question. We investigated whether transferring DCs that had been pre-treated with ultra-high diluted HK S.t could have an effect on immune response development. Once again the results were intriguing. Recall responses were characterised by significantly more IL-10 production from the spleens of
recipient mice that had received 30CH S.t pre-treated – S.t pulsed DCs than control, 30CH H_2O pre-treated - S.t pulsed DCs (Figure 3.11). There was no difference detected in splenocyte IFN-γ levels between the two groups of mice. Interestingly, there was a contrast in the way 30CH P.a and 30CH S.t influenced immune response development via DCs stimulated with the respective antigens. While 30CH P.a induced an exaggerated IFN-γ response by splenocytes of recipient mice, 30CH S.t induced an exaggerated IL-10 response, suggesting extreme dilutions of different Th1 polarising antigens have distinct ways by which they influence DCs to drive immune responses. Alternatively, the observed difference in the way ultra-high dilutions of different antigens condition DCs to drive immune responses might be influenced by the concentration of antigen used to stimulate DCs and not by the ultra-high dilution of antigen. As we observed in Figure 3.10 increasing the concentration of S.t DCs were stimulated with, shifted the response from an IL-10 specific one to an IFN-γ specific one. Whether identical ultra-high dilutions of antigen might be exerting their effect in a number of ways, depending on the stimulatory dose of antigen used to activate DCs with, is open to investigation. In order to explain whether ultra-high dilutions of P.a or S.t condition DCs in distinct ways or similar ways depending on antigen concentration, we would need to perform the DC transfer experiments using DCs that have been stimulated with a range of concentrations of S.t and P.a.

Our observations using the Th1 polarising antigens P.a and S.t were, indeed, very intriguing, however controversial. We decided to perform the same
DC transfer experiments in a blind fashion, using a different, Th2 polarising antigen. By performing the experiment in a blind fashion using an antigen for which we did not have an “expected” result, we hoped to eliminate any kind of bias introduced during the preparation of the dilutions, or handling bias. Strikingly, Th2 response induction by CT-pulsed DCs was also altered when the transferred DCs had been pre-treated with a 30CH CT dilution prior to incubating them with a stimulatory dose of antigen (Figure 3.13). CT specific recall responses in the spleens of recipient mice that had received the 30CH CT pre-treated – CT pulsed DCs were characterised by increased IL-4 and IL-5 compared to the water control pre-treated – CT pulsed DCs. IL-5 production by splenocytes of recipient mice was detected irrespectively of the restimulation dose of CT used. CT specific IL-4, however, was only detected upon the higher restimulation dose and was significantly elevated by splenocytes of recipient mice that had received 30CH CT pre-treated – CT pulsed DCs compared to controls. In contrast, IFN-γ specific recall response was significantly higher in splenocytes of recipient mice that had received 30CH CT pre-treated – CT pulsed DCs compared to 30CH water pre-treated – CT pulsed DCs, when the lower (50ng/ml CT) CT restimulation concentration was used (Figure 3.13). It is possible that the observed switch from an IFN-γ to an IL-4 response is due to the fact that Th1 and Th2 are known to counter regulate each other. Nevertheless, it is interesting that depending on the restimulation concentration of CT we observe a significant effect on the type of response developed. In light of the results obtained in Figure 3.2 where DCs responded differently to CT depending on dose and frequency of stimulation, it would certainly be worth
investigating whether the DC stimulation dose as well as the splenocyte restimulation dose can have an effect on the type of the immune response developed. Furthermore, it would be interesting to examine whether extreme dilutions of CT can affect the way such responses are polarised.

Ultra-high dilutions of different antigens altered immune response development \textit{in vivo} in distinct ways. We addressed how ultra-high dilutions of three different antigens affect the ability of DCs to initiate immune responses against their respective antigens. It would be interesting to investigate whether the effect an extreme dilution of an antigen has on the ability of DCs to drive immune responses is specific to the antigen itself or whether it can exhibit “cross-reactivity”. Commonly prescribed homeopathic preparations against diseases are usually of a different nature than the agents that are actually causing pathology. To assess whether cross reactivity between homeopathic preparations and pathogens occurs we would need to repeat the DC transfer experiments using a number of different combinations of homeopathic preparations and antigenic stimuli.

Our investigations into the very controversial field of homeopathy warranted extreme scrutiny. As seen in \textbf{Figure 2.3}, we assessed the purity of the water utilised to generate the extreme dilutions of antigen by gas-ion exchange mass spectrometry. The reason behind this was to eliminate any possibility of contamination that might affect DC activation. Furthermore, in order to eliminate handling bias, we performed a transfer experiment in a blind fashion. Last, we performed a meta-analytical study of 7 independent, identical DC
transfer experiments. Meta analysis revealed a significant effect of 30CH P.a on the ability of DCs to drive IFN-γ responses (Figure 3.14).

Although we did not succeed in identifying any possible reasons as to why preparations of antigen that has been diluted beyond Avogadro’s number should exert a measurable biological effect, our meta analytical results suggest that more work needs to be done into this elusive field.
Figure 3.1 Effect of low-dose HK P.a on the ability of DCs to secrete cytokine.

DC were either incubated in media alone for 24 hours and then activated with 5µg/ml of HK Pa for a further 24 hours, or incubated with 5µg/ml HK P.a for 24 hours followed by another 5µg/ml P.a for a further 24 hours, or incubated with 0.025 µg/ml of HK P.a for 24 hours and then activated with 5µg/ml P.a for 24 hours, or incubated with 0.0025 µg/ml HK P.a for 24 hours, or incubated with 0.0025 fg/ml HK P.a for 24 hours followed by a further 24 hour incubation with 5µg/ml P.a. Each bar is represented by mean and standard error mean (SEM) from 3 replicates on the ELISA plates. Data are representative of 3 individual experiments.
Figure 3.2 Effect of low-dose CT on the ability of DCs to secrete cytokine. DCs were either incubated in media alone for 24 hours and then activated with 5µg/ml of CT for a further 24 hours, or incubated with 5µg/ml CT for 24 hours followed by another 5µg/ml CT for a further 24 hours, or incubated with 0.025 µg/ml of CT for 24 hours and then activated with 5µg/ml CT for 24 hours, or incubated with 0.0025 fg/ml CT for 24 hours followed by a further 24 hour incubation with 5µg/ml CT. Cytokine was measured by ELISA. Each bar is represented by mean and standard error mean (SEM) from 3 replicates on the ELISA plates. Data are representative of 3 individual experiments.
**Figure 3.3 Effect of low-dose HK SL1344 on ability of DC to secrete cytokine.**

DC were either incubated in media alone for 24 hours and then activated with 5µg/ml of HK SL1344 for a further 24 hours, or incubated with 5µg/ml HK SL1344 for 24 hours followed by another 5µg/ml SL1344 for a further 24 hours (cells not washed in between stimulations), or incubated with 0.025 µg/ml of HK SL1344 for 24 hours and then activated with 5µg/ml SL1344 for 24 hours, or incubated with 0.0025 fg/ml HK SL1344 for 24 hours followed by a further 24 hour incubation with 5µg/ml SL1344. Cytokine was measured by ELISA. Experiment is representative of 3 individual experiments. Each bar is represented by mean and standard error mean (SEM) from 3 replicates on the ELISA plates. Data are representative of 3 individual experiments.
Figure 3.4. Pre-treating DC with ultra-high dilutions of \textit{P. acnes} does not affect cytokine production upon exposure to a stimulatory dose of \textit{P. acnes}. DC were incubated for 24 hours in 30CH H$_2$O or 30CH \textit{P. acnes}. Pre-treated DCs were then exposed to a stimulatory dose of 5µg/ml heat-killed \textit{P. acnes} for a further 18 hours. Cytokine was measured by ELISA. Bars are represented by mean and standard error mean of 3 replicate wells on the ELISA plate. Data representative of 7 individual experiments.
Figure 3.5. Pre-treating DC with ultra-high dilutions of HK S. typhimurium does not affect their ability to respond to a stimulatory dose of HK S. typhimurium. DC were incubated for 24 hours in 30CH H₂O or 30CH S. typhimurium (St). Pre-treated DCs were then exposed to a stimulatory dose of 5µg/ml heat-killed S. typhimurium for a further 18 hours. Cytokine was measured by ELISA. Bars are represented by mean and standard error mean of 3 replicate wells on the ELISA plate. Data representative of 7 individual experiments.
Figure 3.6. Pre-treating DC with ultra-high dilutions of Cholera toxin does not affect their ability to respond to a stimulatory dose of CT. DC were incubated for 24 hours in 30CH H₂O or 30CH CT. Pre-treated DC were then exposed to a stimulatory dose of 5µg/ml CT for a further 18 hours. Cytokine was measured by ELISA. Bars are represented by mean and standard error mean of 3 replicate wells on the ELISA plate. Data representative of 7 individual experiments.
Figure 3.7.a. Incubating DC with ultra-high dilutions of different antigens does not alter expression levels of surface markers of activation. DC were incubated either in 30CH H₂O, 30CH Pa, 30CH CT or 30CH St for 48 hours. Cells were analysed for expression of MHC II, CD80 and CD40 expression. Graphs are represented by 30CH H₂O (black line) over 30CH antigen (blue line). Data representative of 7 individual experiments.
Figure 3.7.b. Pre-treating DC with ultra-high dilutions of different antigens does not alter expression levels of surface markers of activation after exposing those DC to stimulatory concentrations of the respective antigens. DC were incubated either in 30CH H₂O, 30CH Pa, 30CH CT or 30CH St for overnight. A stimulatory concentration of 5µg/ml of the respective antigen was then added for a further 24 hours. Cells were analysed for expression of MHC II, CD80 and CD40 expression. Graphs are represented by 30CH H₂O (black line) over 30CH antigen (blue line). Data are presentative of 7 individual experiments.
Figure 3.8. 30CH P. acnes treated DC do not induce a stronger IFN-γ response in splenocytes of recipient mice than 30CH H2O treated ones. DC were pre-treated with 30CH H2O or 30CH P. acnes for 42 hours. They were then transferred subcutaneously into naïve mice and 7 days later spleens were removed, processed into single cell suspensions and restimulated with either media or 1µg/ml P. acnes. IFN-γ and IL-10 was measured by ELISA. Bars represent mean and standard error mean of 4 mice per group. Data are representative of seven individual experiments. Statistics were performed using Students t test. * = P<0.05
Figure 3.9. Splenocyte cytokine production by mice receiving 30CH P.a pre-treated DC. DC were treated with 30CH H₂O or 30CH P.a for 24 hours. They were then incubated in media or 5µg/ml Hk P.a for a further 24 hours. DCs were then transferred subcutaneously into naïve mice and 7 days later spleens were removed, processed into single cell suspensions and restimulated with either media or 1µg/ml HK P.a. IFN-γ and IL-10 was measured by ELISA. Bars represent mean and standard error mean of 4 mice per group. Data are representative of seven individual experiments. Statistics were performed using Students t test. * = P<0.05
Figure 3.10. Splenocyte cytokine production by mice receiving DCs exposed to increasing doses of HK S.t. DCs were incubated in media for 24 hours. 5µg/ml, 10µg/ml, or 20µg/ml HK S.t was then added for a further 24 hours. DCs were transferred subcutaneously into naïve mice and 7 days later spleens were removed, processed into single cell suspensions and restimulated with either media (open bars) or 1 µg/ml HK S.t (closed bars). Splenocyte IFN-γ and IL-10 was measured by ELISA. Error bars represent standard mean and standard error mean of 4 mice per group. Experiment is representative of two individual experiments. Statistics were performed using Students t test. * = P<0.05
Figure 3.11. Splenocyte cytokine production by mice receiving 30CH S.t pre-treated DC. DC were treated with 30CH H₂O or 30CH S.t for 24 hours. They were then incubated in media or 20µg/ml HK S.t for 24 hours. DCs were transferred subcutaneously into naïve mice and 7 days later spleens were removed, processed into single cell suspensions and restimulated with either media (open bars) or 1µg/ml HK S.t (closed bars). Splenocyte supernatant IFN-γ and IL-10 was measured by ELISA. Error bars represent standard error mean of 4 mice per group. Experiment is representative of three individual experiments. Statistics were performed using Students t test. * = P<0.05
Figure 3.12. 30CH CT treatment of DC does not induce any different recall responses than 30CH H₂O treatment. DC were pre-treated with 30CH H₂O or 30CH CT for 24 hours. They were then transferred subcutaneously into naïve mice and 7 days later spleens were removed, processed into single cell suspensions and restimulated with either media, 50ng/ml CT or 0.5µg/ml. IL-4, IL-5, IL-10 and IFN-γ were measured by ELISA. Bars represent mean and standard error mean from 3 mice per group. Experiment is representative of two individual experiments. Experiment performed in a “blind” fashion. Statistics were performed using Students t test. * = P<0.05
Figure 3.13. Induction of a Th2 response by ultra-high dilution of CT treated DC. DC were pre-treated with 30CHH₂O or 30CH CT for 24 hours. They were then incubated in media or 5μg/ml CT for a further 18 hours. DC were then transferred subcutaneously into naïve mice and 7 days later spleens were removed, processed into single cell suspensions and restimulated in either media, 50ng/ml CT or 0.5 μg/ml CT. IL-4, IL-5, IL-10 and IFN-γ were measured by ELISA. Bars represent mean and standard error mean from 3 mice per group. Experiment is representative of two individual experiments. Experiment performed in a “blind” fashion. Statistics were performed using Students t test. * = P<0.05
Figure 3.14. Meta analysis of 7 independent experiments addressing the effect of ultra-high dilution of antigen on immune response development reveals minute but statistically significant biological effect of such extreme dilutions on the ability of DCs to drive immune responses. Y axis represents the effect size in ng/ml of IFN-γ secreted by splenocytes of recipient mice that had received 30CH P.a pre-treated P.a stimulated DC over 30CH water pre-treated P.a stimulated DC. Y axis = 0 denotes a null effect on immune response development by ultra-high dilution of Ag pre-treated DC. Y axis < 0 denotes an inhibitory effect on IFN-γ priming by 30CH P.a pre-treated DCs and Y axis > 0 denotes a positive effect on IFN-γ priming by 30CH P.a pre-treated DCs. X axis: Groups 1-7 represent 7 independent experiments performed under identical conditions. Group 8 represents the statistical sum of all 7 experiments, after performing meta analysis. Squares represent the mean effect size of IFN-γ secretion by splenocytes from recipient mice that had received 30CH P.a pre-treated DCs over mice that had received 30CH H2O pre-treated DCs. Error bars represent the variation within each experiment. Degree of freedom=9.0, Q statistics=8.720, Summary Mean=9.095, Lower Bound of 95% CI=3.606, Upper Bound of 95% CI=14.584
Chapter 4

Can vaccination with dendritic cells alter the course of *Salmonella typhimurium* infections?

Abstract

DCs are required for the initiation of adaptive immune responses. Recent work addressing the role of DCs in immune response development against *Salmonella* infections has shown, interestingly, that DCs are also responsible for bacterial dissemination. We decided to use the murine *S. typhimurium* disease model in order to investigate this intricate relationship between DCs and *Salmonella* bacteria. We wished to address whether the adaptive immune responses elicited by DCs in naïve mice could affect *S. typhimurium*-induced immunopathology. We approached this question by vaccinating naïve animals with DCs that had been previously exposed to stimulatory concentrations of heat-killed *S. typhimurium*. We then addressed whether primary or memory responses induced by this vaccination regime could alter immunopathology caused by live virulent SL1344 *S. typhimurium* infections. Memory responses in vaccinated-infected mice were characterised by significantly elevated serum and splenocyte IFN-γ responses as well as type 1, and strikingly, type 2 Ab levels. Additionally, we found that memory responses in vaccinated animals were characterised by reduced serum TNF-α and slower weight loss patterns.
4.1 Introduction

*Salmonella enterica* servovar Typhimurium is a Gram-negative facultative intra-cellular anaerobic bacterium. It is one of around 2500 serovars identified, grouped into 6 sub-species (Ochman and Groisman, 1994). In humans *Salmonella* infections can be divided into two groups according to the clinical symptoms observed; typhoid and enterocolitis. Typhoid is a systemic infection in humans cause by serovars Typhi, Paratyphi and Sendai, while most other serovars cause enterocolitis/diarrhea and bacteremia (Fierer and Guiney, 2001). *Salmonella enterica* serovar Typhimurium (or *Salmonella typhimurium*) causes enterocolitis in humans. However, it has proven to be a very good model for the study of typhoid fever, since, similarly with the human *S.typhi*, it causes systemic infection in mice, characterised by extensive dissemination of the pathogen to secondary lymphoid organs as well as sterile sites such as the heart.

4.1.1 Pathogen invasion

*Salmonella* infects humans and animals via the oral route, usually due to contaminated food and water. The initial uptake and transfer of *Salmonella typhimurium* from the gut lumen to Peyer’s patches involves an interplay between the host and the pathogen. Invading bacteria survive the acidic environment of the stomach and colonise the ileum and cecum, by outcompeting commensal bacteria (Baumler et al., 1997; Jones et al., 1994). M cells and DCs have been identified as crucial factors for the invasion and translocation of the pathogen from the lumen to Peyer’s patches (Jones et al.,
1994; Rescigno et al., 2001a; Rescigno et al., 2001b). Within Peyer’s patches CCR6\(^+\) dendritic cells instruct resident T cells to become activated (Rescigno, 2006; Salazar-Gonzalez et al., 2006), while bacteria are disseminated through CD18\(^+\) macrophages to the spleen and liver (Vazquez-Torres et al., 1999). There, the bacteria are internalised and reside within polymorphonuclear cells (PMNs) (GR1\(^+\) cells), DCs (CD11c\(^+\), MHC class II\(^+\) cells), and B cells (Liang-Takasaki et al., 1983; Souwer et al., 2009; Warren et al., 2002; Yrlid et al., 2001a).

### 4.1.2 Host immunity and clearance

Understanding the underlying mechanisms that enable the host to clear any infection, is key for the development of effective ways to either generate vaccination strategies or treatment regimes. The animal model (mouse) of *Salmonella enterica* serovar Typhimurium provides us with a useful tool to better understand typhoid fever in humans. The NRAMP gene, which encodes for a bivalent metal pump that is recruited to the *Salmonella*-containing phagosome is crucial for the early clearance of the pathogen (Vidal et al., 1993), and as such NRAMP deficient mice are highly susceptible to *Salmonella* infections. No such gene has been identified in humans (Dunstan et al., 2001), however the clinical symptoms of *Salmonella typhimurium* in mice lacking NRAMP and *Salmonella typhi* in humans are very similar, characterised by rapid bacterial dissemination and dehydration.

Early host defences against *Salmonella* require reactive oxygen intermediates. These are generated intracellularly via a process initiated by NADPH oxidase (Mastroeni et al., 2000b). This form of oxidative killing is
regulated by TNF-α (Vazquez-Torres et al., 2001). Similarly TNF-α plays an important role in the formation of granulomatous lesions, which are involved in containing the pathogen, as it has been demonstrated in mice treated with anti-TNF-α antibodies (Mastroeni et al., 1995). Macrophages, recruited to the infected sites are responsible for the formation of such granulomas, while their provision of pro-inflammatory cytokines such as IL-12 and IL-18 enables NK T-cell killing of infected cells. Late responses against *Salmonella* involve both cellular as well as humoral immunity (Mastroeni et al., 1993a). It has been shown that CD4⁺, CD8⁺ T cells, B cells, as well as the presence of Ab are required for protective immunity *(Chapter 1.6)* (Barr et al., 2009; Mastroeni and Menager, 2003).

### 4.1.3 Vaccination strategies against *S. typhimurium*

Attempts to prevent salmonellosis by implementation of hygiene methods have proven ineffective, while antibiotic treatment, although effective, is problematic due to the emergence of multi-drug resistant *Salmonella* strains (Mastroeni et al., 2001).

Vaccination is a powerful tool for the prevention of infectious diseases. Three different vaccination approaches are today available against *Salmonella* infections; a) Whole-cell killed vaccines, b) Subunit vaccines and c) live attenuated vaccines (Mastroeni et al., 2001).

Whole-cell killed vaccines are generated by either heat-killing or acetone treating *Salmonella* bacteria and administering the preparations parenterally (Mastroeni et al., 2001). Whole-cell killed vaccines induce good antibody
responses in humans, however they have proven to provide poor cell mediated immunity (Mastroeni et al., 2001). Nevertheless, this vaccination regime confers >70% protection in humans, however, it is less effective in NRAMP deficient mice (Engels et al., 1998; Harrison et al., 1997; Levine et al., 1989). Despite their relatively high protective efficacy in humans, whole-cell killed vaccines are reactogenic, inducing adverse effects, and as such are not used commercially (Collins, 1974; Engels et al., 1998).

Salmonella subunit vaccines are generated by purifying immunogenic proteins from bacterial cultures (Mastroeni et al., 2001). Although they confer worse protection against Salmonella than whole-cell vaccines, they induce less or no adverse effects (Engels et al., 1998). There is one commercially available subunit vaccine against Salmonella recommended by the World Health Organisation (WHO), the Vi (virulence) capsular polysaccharide vaccine (ViCPS). It is made from purified Vi capsular polysaccharide from the Ty2 Salmonella typhi strain and confers between 55 and 70% protection (Engels et al., 1998). When it is bound to protein carriers its protective efficacy can be, however, increased (Mastroeni et al., 2001). Other subunit vaccines have been tested experimentally, such as ones based on detoxified LPS, cell extracts, porins and O-polysaccharides, however, these vaccines have been proven to be less efficacious (Mastroeni et al., 2001).

The availability of the complete genome sequences for S.typhi and S. typhimurium, in conjunction with advances in genetic modification, allowed for extensive research in the efficacy of live-attenuated Salmonella strains as vaccines. One commercially available live-attenuated vaccine, which is
recommended by the WHO, Ty21a, is an rpoS (RNA polymerase, sigma S - sigma 38 - factor) mutant derivative of the wild type Ty2 S. typhi strain. Ty21a, has been found to confer comparable protection levels to the ViCPS vaccine (Yang et al., 2001b). Many different attenuated strains are currently used in animal infection models as well as in clinical trials. The Ty800 strain, which was generated by deleting the PhoP/PhoQ genes (Chapter 1.6.4) of the Ty2 S. typhi strain has been shown to confer both humoral and cellular immunity in humans (Hohmann et al., 1996). The AroA attenuated strain (Chapter 1.6), which is widely used in animal models, is an auxotroph mutant of the SL1344 wild type strain and confers full protective immunity in NRAMP deficient mice (Harrison et al., 1997).

DCs, which are known to be involved in the generation of adaptive immune responses, are one of the first cell type that encounters Salmonella bacteria. However, little is known about the contribution of this cell type in the generation of protective immune responses against S. typhimurium infections. As such, we wished to investigate whether DCs are indeed able to induce protective responses in naïve animals.

4.1.4 Aims

1) To establish immunopathological parameters induced by S. typhimurium infections in NRAMP mice.
2) To investigate whether primary and memory immune responses generated by HK *Salmonella*-pulsed DCs in recipient mice alter immunopathology caused by *S. typhimurium* infections.

4.2 Results

We wished to assess whether immune responses elicited by DCs transferred into naïve mice were sufficient to alter immunopathology following infection with virulent SL1344 strain *S. typhimurium*. However, before we could proceed to these investigations we needed to establish the pathological parameters of different infective doses of *S. typhimurium* infection in C57BL/6 (NRAMP) mice.

4.2.1 *Salmonella typhimurium* infection – pathology in C57BL/6 mice

Previous work on *S. typhimurium* infections has shown that different strains of mice have different natural resistance to *S. typhimurium*. Disease progression and mortality is dependent on route of infection, infective dose as well as the strain of *S. typhimurium* used. In order to keep continuity between our previous DC transfer and infection experiments we decided to use C57BL/6 (NRAMP) mice for all infection experiments.

Immunopathology caused by *S. typhimurium* strain SL1344 was assessed by orally infecting mice with increasing doses of *S. typhimurium* and measuring weight changes during infection as well as serum levels of cytokine, splenomegaly and bacterial loads in the spleens and livers of infected mice. The
method of infection was chosen in order to keep in line with the naturally occurring route of infection (i.e. oral).

Mice were infected with 100µl of either 1*10⁶, 1*10⁷ or 1*10⁸ CFU SL1344/100µl PBS. Mouse weights were recorded at the day of infection and at day 4 after infection (Figure 4.1.A). Since individual mice were not marked, the pattern of weight loss for each mouse within a group could not be plotted. Standard variation within each group was therefore deduced by combining the standard variations at day 0 and day 3 of infections according to J.A Nissim’s mathematical formula (J.A.Nissim).

There was no significant difference in weight loss between any of the groups of mice receiving the different doses of S. typhimurium (Figure 4.1.A). All infected mice exhibited spleen enlargement (Figure 4.1.B). There was no correlation, however, found between infective dose and splenomegaly.

Bacterial loads in the spleen and livers of infected mice correlated with increasing infective doses (Figure 4.2). We cannot infer to the statistical significance of this observation since the livers and spleens from individual mice within groups were pooled. However, livers from mice receiving 1*10⁶ CFU SL1344 had approximately 2 logs less mean bacterial loads per gram of tissue than those receiving 1*10⁸ CFU. The difference in the spleens was less pronounced with only one log difference in the bacterial burden between mice receiving the lowest, 1*10⁶ CFU, and highest 1*10⁸ CFU S. typhimurium (Figure 4.2). The intermediate infecting dose of 1*10⁷ CFU resulted in intermediate bacterial burdens in both the spleen and the liver.
Serum IFN-γ and TNF-α was measured at day 4 post infection. Serum IFN-γ levels peaked at the $10^7$ CFU/100µl PBS concentration (Figure 4.3). Both, the $10^6$ and $10^8$ CFU/100µl PBS bacterial concentrations induced significantly less serum IFN-γ than the $10^7$ CFU/10µl PBS concentration (P<0.05) (Figure 4.3). Interestingly, serum TNF-α levels showed a different picture. The highest infective dose of SL1344 ($10^8$ CFU/100µl PBS) induced significantly more TNF-α than the rest of the infective doses (P<0.05) (Figure 4.3).

4.2.2 Can primary immune responses induced by DCs exposed to heat-killed *S. typhimurium* alter the course of *S. typhimurium* infections?

First we established that heat-killed *S. typhimurium* induces DC activation (Chapter 3), which in turn can induce an immunological response in naïve mice. We wanted to examine whether the immune response developed as a result of transferring heat-killed *S. typhimurium*-pulsed DCs could alter pathology conferred during infection with live virulent SL1344 *S. typhimurium*. DCs were grown from bone marrow and stimulated with 10µg/ml HK S.t according to material and methods 2.3. DCs were injected in mice according to materials and methods section 2.7 in the footpads. 3 days later mice were infected orally with $2\times10^7$ CFU/100µl PBS. The infective dose was chosen in view of the serum IFN-γ and TNF-α observed in Figure 4.3. The fact that the highest infective dose induced an exaggerated TNF-α response as well as preventing the onset of an IFN-γ one, suggested that at this bacterial concentration the mice were perhaps exhibiting excess immunopathology, which might shadow any future
immunological observations we wished to make. Accordingly, we chose the $10^7$ CFU/100µl PBS infective dose.

No significant difference was observed between weight loss patterns of naïve mice, mice receiving unstimulated DCs or mice receiving heat-killed *S. typhimurium*-pulsed DCs (Figure 4.4), after infecting them with $2 \times 10^7$ CFU/100µl PBS. Similarly, liver bacterial burdens as well as splenomegaly were not affected by primary immune responses generated by *S. typhimurium*-pulsed DCs (Figure 4.5.A, 4.5.B), suggesting that primary immune response development by HK S.t pulsed DCs was insufficient to alter pathology. IFN-γ and TNF-α levels in the serum were statistically similar in all groups of infected mice (Figure 4.6). To further assess whether there was any difference in the immunological profile between the non-vaccinated and the different groups of DC-vaccinated mice, spleens were removed and splenocytes of individual mice were incubated in the presence of gentamycin, penicillin and streptomycin alone or in the presence of gentamycin, penicillin, streptomycin and heat-killed *S. typhimurium*. The reason exogenous *S. typhimurium* was added to the spleen cell cultures was to eliminate any difference in splenic bacterial burdens the infected mice may have between them. By adding 10µg/ml heat-killed *S. typhimurium* the amount of bacteria in the splenocytes was increased by approximately 2 logs, effectively decreasing the dose variability in the splenocyte cultures by 2 decimal places. There was no significant difference in the levels of IFN-γ, IL-17 or IL-10 produced by splenocytes between naïve mice, mice that received unstimulated DCs or mice that had received HK St-pulsed DCs (Figure 4.7).
4.2.3 Can immunological memory induction by DCs alter the course of *S. typhimurium* infections?

We wished to investigate whether memory responses conferred in recipient mice by DCs that had been pulsed with *S. typhimurium* altered the course of *S. typhimurium* infections. To this end we transferred HK S.t pulsed-DCs five weeks before infecting mice orally with $1.72 \times 10^7$ CFU SL1344.

No statistical difference in weight loss patterns during the first 5 days of infection was observed between mice receiving unstimulated DCs and mice receiving HK S.t-pulsed DCs (Figure 4.8). Nevertheless, mice that had received HK S.t-pulsed DCs lost significantly less weight than naïve mice, after being infected (P<0.01), suggesting that DC vaccination delayed the onset of pathology. No difference in liver bacterial burdens was observed between the different groups of mice (Figure 4.9.A). Strikingly, mice that had received S.t-pulsed DCs exhibited significantly less splenomegaly than naïve mice or mice receiving unstimulated DCs (P<0.05) (Figure 4.9.B), suggesting that the memory immune response elicited by HK S.t-pulsed DCs altered immunopathology caused by *S. typhimurium* infection. Moreover, serum IFN-γ levels were significantly higher in mice that had received HK S.t-pulsed DCs than that observed in naïve mice or mice that had received unstimulated DCs (P<0.05) (Figure 4.10). Serum TNF-α levels were significantly lower in mice that had received HK S.t-pulsed DCs than naïve-infected mice, further suggesting that memory immune responses elicited by St-pulsed DCs altered immunopathology during infection (Figure 4.10). Similarly to serum IFN-γ, splenocytes from mice
that had received S.t-pulsed DCs secreted significantly more IFN-γ than naïve-infected mice or mice that had received unstimulated DCs (P<0.05) (Figure 4.11). There was no statistically significant difference in the levels of IL-10 secreted by splenocytes from the different groups of infected mice, suggesting that the IFN-γ responses observed previously were not due to regulatory responses elicited by S. typhimurium (Figure 4.11). IL-17 secretion by splenocytes from mice that had received S.t-pulsed DCs was significantly higher than that secreted by splenocytes of mice that had received unstimulated DCs (P<0.05) (Figure 4.11).

Surprisingly, mice that had received S. typhimurium – pulsed DCs exhibited higher total antibody titers, at day 5 of infection, than mice that had received no DCs or unstimulated DCs (Figure 4.12), suggesting that DCs might be involved in whole antigen presentation to B cells. More specifically, IgG2b and IgG2c titers (both Th1 associated Abs), were higher in mice that had received S. typhimurium – pulsed DCs (Figure 4.12). This finding is consistent with the increased IFN-γ secreted by splenocytes from S. t-pulsed-DC recipient mice. Surprisingly, mice that had received S. typhimurium – pulsed DCs exhibited a significantly higher titer of IgG1 Ab, which is associated with Th2 responses (Figure 4.12). This finding suggests that S. typhimurium pulsed DCs may be inducing a mixed Th1/Th2 response. No significant difference in IgM titers was observed between any groups of infected mice (Figure 4.12).
4.3 Discussion

We established pathological parameters induced by various infective doses of *S. typhimurium* SL1344 strain in C57BL/6 mice. Our data clearly demonstrated that animals succumb to severe disease, characterised primarily by rapid weight loss, splenomegaly and increased levels of TNF-α in the serum (Figure 4.1, 4.3).

4.3.1 *S. typhimurium* infections cause severe immunopathology in C57BL/6 mice

Mice infected with virulent *S. typhimurium* deteriorate rapidly, many exhibiting terminal, as established by Home Office Regulations, weight loss patterns by day 3 of infection. In our experimental setting, mice frequently dropped below 20% of their initial weight, and in some cases down to 30% of their original weight, by losing 10% or more within a 24h period of time. It was evident that infected mice exhibited clear signs of severe dehydration, ruffled and wet fur, hunching, immobility and in more severe cases periorbital inflammation. The increased TNF-α found in the serum of infected mice (Figure 4.3), may be playing responsible for the weight loss patterns since it is known to cause vasodilatation and increased endothelial permeability to water (Worrall et al., 1997). Livers from infected mice were often characterised by white patching, which is characteristic of hepatic septicaemia, granuloma formation and lesions, we would, however, need to perform histological analysis to confirm these observations.
The purpose of our investigations into the effect different concentrations of live *S. typhimurium* SL1344 have on immunopathology C57BL/6 mice was to establish which infective dose would be optimal for further work performed using DCs as vaccines. We decided that $10^7$ CFU/100µl PBS was an appropriate infective dose since at this concentration mice exhibited the highest serum IFN-γ responses in conjunction to moderate serum TNF-α levels (Figure 4.3).

### 4.3.2 Primary immune responses elicited by DCs are insufficient to alter immunopathology caused by *S. typhimurium* infections

Transferring *S. typhimurium* pulsed DCs into naïve recipient mice 3 days before infecting them did not alter disease pathology, suggesting that the primary immune response conferred by bone marrow derived DCs is insufficient to protect from salmonellosis (Figure 4.4, 4.5, 4.6). Weight loss patterns from mice that had received DCs pulsed with heat-killed *S. typhimurium* were statistically similar to those observed in infected mice that had not received DCs or mice that had received unstimulated DCs (Figure 4.4). Similarly, neither liver bacterial burdens nor spleen enlargement was affected by this vaccination protocol (Figure 4.5.A, 4.5.B). Furthermore, neither serum cytokine levels nor cytokine levels determined by splenocyte recall responses seemed to be affected by transferring HK S.t pulsed-DCs to naïve mice 3 days prior to infecting (Figure 4.6, 4.7), strongly suggesting that the immune responses elicited by DCs were inadequate to alter immunopathology.
4.3.3 Memory responses elicited by DCs reduce immunopathology caused by *S. typhimurium* infections.

Interestingly, mice that had received DCs stimulated with HK *S. typhimurium* SL1344 5 weeks prior to being infected, exhibited slower weight loss patterns, reduced splenomegaly and serum TNF-α as well as elevated levels of IFN-γ and IL-17 than naïve-infected mice (Figure 4.8, 4.9, 4.10, 4.11).

TNF-α has been previously shown to be involved in *S. typhimurium* induced granuloma formation (Mastroeni et al., 1992), which is essential for preventing bacterial spreading. In contrast, TNF-α is also responsible for vasodilatation and increased water permeability of endothelial cells (Worrall et al., 1997). HK S.t-DC vaccinated mice exhibited slower patters of weight loss as well as significantly less serum TNF-α compared to naïve-infected animals, an observation that supports the notion that perhaps excess TNF-α is detrimental to the well-being of the animals (Figure 4.8, 4.10). We could try and verify this hypothesis by providing animals, that have previously been immunised with live-attenuated *Salmonella* strains, with recombinant TNF-α and observe whether, such fully protected animals, do indeed experience TNF-α dependent dehydration. TNF-α is known to be important for the containment of *Salmonella* within granulomas (Mastroeni et al., 1992). As such it might be worth investigating whether there are either differences in the number of granulomas or differences in the cell infiltrate of each granuloma between HK S.t-pulsed DC-vaccinated mice and naïve mice, following infection. This would allow us to determine whether the different levels of serum TNF-α we detected between
different groups of mice in our experimental model correlate with bacterial containment. In order to exclude the possibility that factors other than TNF-α might be contributing towards granuloma formation in our experimental model, we would need to include TNF-α depleted groups of mice.

We found that mice that had been vaccinated with S.t-pulsed DCs did no exhibit splenomegaly, compared to mice that had not been vaccinated or had been vaccinated with unstimulated DCs (Figure 4.9.B). It has been previously reported that the presence of TNF-α correlates with splenomegaly (Mastroeni et al., 1992), however, the mechanisms that govern this observation are unknown. TNF-α is known to be a chemoattractant for leukocytes (Mannel and Echtenacher, 2000), while it also induces dysfunctional endothelial permeability (Worrall et al., 1997), both factors that could contribute towards spleen enlargement. Flow cytometric analysis of the numbers of cells as well as the type of cells infiltrating the spleens of infected animals might give us an answer as to the factors contributing towards spleen enlargement.

DCs that had been pulsed with H.K S.t induced *Salmonella*-specific IFN-γ and IL-17 in splenocytes of recipient, infected, mice (Figure 4.11). Intracellular staining of splenocytes, might give us a better picture about which cell populations are responsible for the increased IFN-γ and IL-17 we observed in the S.t-pulsed DC vaccinated mice compared to naïve and unstimulated DC transferred mice. Analysing for the presence of intracellular IFN-γ in CD4+ and CD8+ T cell populations would give us useful information about the ability of DCs to drive both CD4+ and CD8+ T cell responses in this experimental setting.
Previous work has shown that IFN-γ contribute towards the ability of MΦs to contain *Salmonella*. As such, it would be worth investigating whether the differences in IFN-γ observed between the various groups of mice also correlate with altered ability to contain the bacteria within MΦs.

Surprisingly, IgG2b and IgG2c was significantly elevated in the serum of St-DC vaccinated mice, as compared to naïve mice, or mice that had received unstimulated DCs, strongly suggesting that DC vaccinations conferred humoral immunity in recipient mice (*Figure 4,12*). We cannot rule out the possibility that free, *S. typhimurium*, Ag, which might have been present in the supernatant of the *in vitro* S.t DC stimulation assay, was transferred to recipient mice, thereby inducing Ab responses. Previous work performed in our laboratory has shown that supernatant from *in vitro* *P. acnes*-DC stimulation assays is insufficient to induce *P. acnes*-specific cytokine recall responses in recipient mice. We would, however, need to test this in the *Salmonella* system, as well as testing whether such supernatant can induce humoral responses.

It has previously been reported that DCs are able to confer humoral responses (Wykes et al., 1998). We showed that, interestingly, S.t pulsed-DCs induce type 1 and, strikingly, type 2 Ab responses (*Figure 4.12*). Whether the induction of type 2 Abs in our DC transfer model is a due to the culture conditions of the BMDCs, which were grown in foetal calf serum, is open to investigation. Unstimulated DCs, however, failed to induce type 2 Abs, which would support the notion that the BMDC culture conditions are not responsible for the induction of type 2 responses (*Figure 4.12*). We would need to test
whether in our system HK *Salmonella* induces IL-4, IL-5 and IL-13 cytokine secretion. This would give us an indication whether HK *Salmonella* elicits a mixed Th1/Th2 response. Mixed responses are not uncommon in pathological conditions. The *Salmonella* flagellar protein FliC, has been previously shown to elicit Th2 or Th1 responses depending on whether it is found in soluble form or attached to bacteria, respectively (Cunningham et al., 2004). It would be interesting to investigate whether a mixed Th1/Th2 response is beneficial to the host or the pathogen in our experimental model. Identifying which party in this pathogen-host interaction is the beneficiary of such mixed responses might allow us to re-evaluate the way we design *Salmonella*-specific vaccines.

It has previously been reported that, in contrast to humans, live attenuated vaccines confer better protection in NRAMP deficient mice than whole-cell killed vaccines (Engels et al., 1998; Harrison et al., 1997). Nevertheless, the reasons for this are unknown. As we discussed in Chapter 1.6, when *Salmonella* bacteria transition from an extracellular to an intracellular phase, they modify cell surface protein expression, and at the same time induce the secretion of proteins required for their survival within intracellular compartments (Chapter 1.6.4). Whole cell vaccines are made from *in vitro* *Salmonella* cultures, which correspond to the extracellular phase of the bacterial life cycle. Whether proteins, which are secreted during the intracellular phase are immunogenic or not is uncertain, however, an interesting question worth investigating. Designing whole cell killed vaccines from *Salmonella* bacteria purified from infected animals rather than from *in vitro* cultures might give us a
better indication as to whether intracellular and extracellular *Salmonella* bacteria present different Ags to DCs and subsequently to T cells.

Acetone treated whole-cell killed vaccines have been shown to be better at conferring cellular and humoral immune responses in NRAMP deficient mice than whole-cell heat-killed vaccines (Diena et al., 1973). This may not be surprising, since heat-killing bacteria will most certainly result in excessive protein denaturing and degradation, which can affect both MHC presentation of peptides as well as B cell antibody responses. Given that the difference in the immunogenic capability of acetone-treated versus heat-killed vaccines has already been established (Diena et al., 1973), it would be very interesting to identify which proteins are denatured and degraded in the heat-killed preparations compared to the acetone treated preparations. This might provide us with useful information about which *Salmonella* derived Ags are immunogenic, in effect providing us with useful information on how to approach subunit vaccine design.

The DC vaccination experiments provided us with useful information about the role of this cell type in immunity against *S. typhimurium*. The advantage of this reductionist approach of the DC vaccination system in contrast to whole animal vaccination was that it provided us with detailed information about the specific role of DCs in immune response development against *S. typhimurium*. Further work, involving investigations of different subunit vaccines or even live attenuated vaccines, utilising this DC transfer system, might provide us with useful information about the intricate relationship of this APC with *Salmonella*. With the recent introduction of the
transgenic CD11c/diphtheria toxin receptor mouse, which allows for short term ablation of DCs in vivo, it is certain that more information will soon become available about the role of DCs in immunity against *Salmonella.*
Figure 4.1. Weight loss and splenomegaly. Increasing doses of *S. typhimurium* infection do not affect splenomegaly in C57BL/6 mice. Mice were either administered orally 100µl PBS or infected orally with $1 \times 10^6$, $1 \times 10^7$ or $1 \times 10^8$ CFU SL1344/100µl PBS (5 mice per group). Graph A represents mean and standard error mean percentage weight change at day 4 of infection for 5 mice per group. Graph B represents splenomegaly expressed as spleen to total mouse-body weight ratio at day 4 of infection. Bars represent mean and standard error mean (SEM) of 5 mice per group. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 4.2. Increasing the infective dose of live *S. typhimurium* correlates with increasing bacterial loads in the spleens and livers of infected C57BL/6 mice. Mice were either administered orally 100µl PBS or infected orally with $1 \times 10^6$, $1 \times 10^7$ or $1 \times 10^8$ CFU SL1344/100µl PBS (5 mice per group). Animals were sacrificed and CFUs were determined at day 4 of infection. Bars represent pooled livers and pooled spleens from 5 animals per group. Liver and Spleen CFU titres were determined by plating serial dilutions of either liver or spleen homogenates onto agar plates. Data are representative of two independent experiments.
Figure 4.3. Increasing the infective dose of live *S. typhimurium* correlates with increased TNF-α levels but not IFN-γ levels in the serum of C57BL/6 mice at day 4 of infection. Mice were either administered orally 100μl PBS or infected orally with 1*10^6, 1*10^7 or 1*10^8 CFU SL1344/100μl PBS (5 mice per group). Serum was collected at day 4 of infection and IFN-γ and TNF-α were measured by ELISA. Bars represent mean and standard error mean (SEM) of 5 mice per group and 2 wells per group on the ELISA plate per mouse. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 4.4. Primary immune response induced by HK St-pulsed DCs does not affect rate of weight loss in mice infected with virulent SL1344 S. typhimurium. Mice were either naïve, transferred sub cutaneously (s.c) with unstimulated DCs, or DCs pulsed with 10µg/ml heat killed S. typhimurium. 3 days later all mice were infected orally with 2*10⁷ CFU of S. typhimurium. Weights recorded daily. Graph represents average percentage loss and SEM for 5 mice per group. Data representative of two independent experiments.
Figure 4.5. Primary immune response induced by HK St-pulsed DCs does not affect liver bacterial loads or splenomegaly of mice infected with virulent SL1344 *S. typhimurium*. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed *S. typhimurium*. 3 days later they were infected orally with 2*10^7 CFU of *S. typhimurium* (SL1344) for a further 4 days. A) Bacterial titers were determined by serially diluting individual liver homogenates. B) Splenomegaly (white bars uninfected mice, closed bars infected mice) expressed as spleen to total mouse-body weight ratio at day 4 of infection. Bars represent mean and standard error mean of 5 mice per group. Data are representative of 2 independent experiments. Statistics were performed using a Students t test. *P<0.05
Figure 4.6. Primary immune response induced by HK St-pulsed DCs does not affect IFN-γ or TNF-α levels in the serum of mice infected with virulent SL1344 S. typhimurium. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed S. typhimurium SL1344, 3 days later they were infected orally with 2*10^7 CFU of S. typhimurium (SL1344) for a further 4 days. Serum was taken at day 4 of infection and cytokine was measure by ELISA. Bars represent mean and standard error mean of 5 mice per group and 2 replicate wells on the ELISA plate per mouse. Data are representative of 2 independent experiments. Statistics were performed using a Students t test. *=P<0.05
Figure 4.7. Primary immune response induced by HK St-pulsed DCs does not affect recall splenocyte responses in mice infected with virulent SL1344 S. typhimurium. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed S. typhimurium. 3 days later they were infected orally with 2*10^7 CFU of S. typhimurium (SL1344) for a further 4 days. Spleens were processed into single cell suspensions at day 4 of infection and cytokine was measure by ELISA. Bars represent mean and standard error mean (SEM) of 5 mice per group and duplicate wells on ELISA plates per mouse. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 4.8. Memory responses induced by HK St-pulsed DCs prevent weight loss induced by SL1344 S. typhimurium infection. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed S. typhimurium SL1344. 5 weeks later they were infected orally with 1.72*10^7 CFU of S. typhimurium (SL1344) for a further 5 days. Weights recorded daily. Graph represents average percentage weight loss plus standard error mean of 5 mice per group. Data representative of 3 independent experiments. Statistics performed using Two Way ANOVA *= P > 0.01
Figure 4.9 Memory responses induced by HK St-pulsed DCs alter immunopathology in mice infected with virulent SL1344 S. typhimurium. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed S. typhimurium SL1344. 5 weeks later they were infected orally with 1.72*10^7 CFU of S. typhimurom (SL1344) for a further 5 days. A) Bacterial titers were determined by serially diluting individual liver homogenates. B) Splenomegaly (white bars uninfected mice, closed bars infected mice) expressed as spleen to total mouse body weight ratio at day 5 of infection. Bars represent mean and standard error mean of 5 mice per group. Data are representative of 3 independent experiments. Statistics were performed using a Students t test. *=P<0.05
Figure 4.10 Memory responses induced by HK St-pulsed DCs are characterised by elevated IFN-γ but no change in TNF-α in the serum of mice infected with virulent *S. typhimurium*. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed *S. typhimurium* SL1344. 5 weeks later they were infected orally with 1.72*10^7 CFU of *S. typhimurium* (SL1344) for a further 5 days. Serum was collected at day 5 of infection and cytokine was measure by ELISA. Bars represent mean and standard error mean of 5 mice per group and 3 replicate wells on the ELISA plate per mouse. Data are representative of 3 independent experiments. Statistics were performed using a Students t test. *=P<0.05
Figure 4.11. Memory responses induced by HK St-pulsed DCs affect recall splenocyte responses in mice infected with virulent SL1344 S. typhimurium. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed S. typhimurium SL1344. 5 weeks later they were infected orally with 1.72*10^7 CFU of S. typhimurium (SL1344) for a further 5 days. Spleens were processed into single cell suspensions at day 5 of infection and cytokine was measure by ELISA. Bars represent mean and standard error mean (SEM) of 5 mice per group and duplicate wells on ELISA plates per mouse. Data are representative of 3 independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 4.12. HK St-pulsed DCs induce type 1 antibody responses. Mice were either naïve, transferred s.c with unstimulated DCs or DCs pulsed with 10µg/ml heat killed S. typhimurium SL1344. 5 weeks later they were infected orally with 1.72*10^7 CFU of S. typhimuroid (SL1344) for a further 5 days. Serum was collected at day 5 of infection and antibody titers were determined by ELISA. Bars represent mean fold increase and standard error mean of antibody titer over non immune serum. Data are representative of 3 independent experiments. Statistics were performed using Students t test. * = P<0.05
Chapter 5

The role of IL-23 in immunity against *Salmonella typhimurium*

Abstract

IL-23 is a heterodimeric cytokine composed of two subunits, p19 and p40. While both IL-12 and IL-23 share the common subunit p40 they contain distinct p35 and p19 subunits respectively, and are thought to play distinct roles in activating T cells. IL-12 has been described to be a key factor in combating bacterial and viral infections by inducing T helper 1 cells to secrete IFN-γ. IL-23 has recently been described to be a major cytokine inducing IL-17 secretion from activated T cells. It is thought to be important for the pathogenesis of organ-specific autoimmune diseases, such as experimental autoimmune encephalomyelitis and rheumatoid arthritis, as well as for the clearance of bacterial and fungal pathogens. We addressed how IL-23 affects primary, memory and secondary challenge responses against *Salmonella typhimurium*. We found that IL-23 is not only critical for the development of memory T helper 17 cells, but that it is also required for the activation or survival of memory IFN-γ producing Th1 cells at infected loci.

5.1 Introduction

Dendritic cells (DCs) are professional antigen presenting cells that, together with macrophages and B cells, are thought to orchestrate immune responses. It is widely accepted that the cytokine milieu DCs are found in, the
type of antigen that they internalise and their homing capacity are all critical factors that influence their activation state, which in turn determines the polarisation state of T cells.

Recent developments in immunology have reshaped our oversimplified view of Th1 and Th2 CD4$^+$ T cell responses. New additions are introduced at a fast pace and today we can identify responses including $T_{reg}$, Th0, Th1, Th2, Th3 and Th17. While T cell responses are required for disease resolution, it is the APCs that are responsible for initiating the distinct immune profiles of the adaptive arm of immunity. Together with the expression of co-stimulatory molecules, APC-derived cytokines such as IL12p70, IL-10, IL-6, TNF-α, type 1 interferons and IL-1β are important factors that can be involved in determining the fate of T cell immune responses. Novel DC derived cytokines such as IL-23 and IL-27 are now also being identified as important regulators of immune responses.

IL-23 is composed of two polypeptide subunits; p19, unique to IL-23, and p40, which is common to IL-12 (Oppmann et al., 2000). Since its discovery in 2000 (Oppmann et al., 2000), IL-23 has been the subject of intense investigation. The initial study by Oppmann et al. that identified this molecule showed that IL-23 shares a common subunit with IL-12p70, which binds to the common IL-12Rβ1 chain and induces proliferation of CD4$^+$ CD45Rb$^{low}$ T cells, activates STAT-4 and subsequently induces IFN-γ, and activates CD45RO memory T cells (Oppmann et al., 2000). IL-23 was later described to signal through both IL-12Rβ1
chain and the IL-23 receptor activating both STAT-4 and STAT-3, respectively (Parham et al., 2002).

5.1.1 Pathogen induced IL-23 secretion by APC

Differently stimulated DCs tune T cells towards different effector phenotypes that are important for both combating a wide range of pathogens as well as immune regulation. IL-23 secretion by DCs can also be triggered by distinct pathogens and stimulatory agents which are not normally involved in the induction of Th1 polarising cytokines such as IL-12. Gram negative and Gram positive commensal bacteria, as well as their cell wall components in isolation, have been found to stimulate DCs to induce different levels of IL-12 and IL-23 (Smits et al., 2004), suggesting that differential TLR-2 and TLR-4 ligation provide distinct activation thresholds that lead to the induction of IL-23. Activation of DCs though TLR-3 and TLR-7 simultaneously can also lead to a preferential IL-23 production by DCs when they are infected with Theiler's murine encephalomyelitis virus (TMEV) (Al-Salleeh and Petro, 2007). Flagellin from members of the *Bordetella* species such as *B. pertussis* (which is known to induce inflammation through TLR-5 ligation (Lopez-Boado et al., 2005)), drives IL-23 but not IL-12 production by DCs (Fedele et al., 2005). The hyphae from *Candida albicans*, which is known to be able to stimulate DCs through the mannose receptor as well as TLRs, is a potent IL-23 inducer and an inhibitor of IL-12 (Acosta-Rodriguez et al., 2007b). Similarly, prostagladin E2 has also been shown to be a potent IL-23 inducer (Sheibanie et al., 2004). Together, these findings indicate that IL-23 production is dependent on the nature of the
stimulatory agent the DC encounters and the combination of PAMP recognition by PRRs. The range of the different IL-23-driving agents as well as the specificity with which these stimulate the production of IL-23 by APCs, strongly suggests that there is a distinct role for IL-23 in immune regulation, pathology and possibly immune protection.

5.1.2 IL-23 involvement in autoimmune disease

The introduction of the Th1-Th2 model provided by Mosmann and Coffman in the 80s (Mosmann et al., 1986) attempted to explain how different CD4⁺T cell subsets play a critical role in immunity to infection, and pathogenesis of autoimmune disease and asthma. IFN-γ producing CD4⁺ T cells have been known to be the most important T cell subset for aiding clearance of viral, bacterial and protozoa infections (Flynn et al., 1993; Suzuki et al., 1988; Yu et al., 1996), as well as the causative agent of immunopathology during organ specific autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE) - a mouse model for human multiple sclerosis (MS) - and rheumatoid arthritis (RA).

Inconsistencies, however, with the idea that Th1 cells are crucial for autoimmune disease pathology started arising when it was first suggested that IFN-γ may not in fact be responsible for clinical severity of EAE, as demonstrated in IFN-γ deficient mice (Ferber et al., 1996). Indeed, some studies suggested that IFN-γ may actually confer resistance to EAE (Krakowski and Owens, 1996), and be protective against collagen induced RA (Vermeire et al.,
1997). Similarly, mice lacking the IL-12Rβ2, the p35 specific receptor, also showed exacerbated pathology associated with EAE (Zhang et al., 2003).

Following these studies indicating that the IL-12 - IFN-γ paradigm could no longer explain the onset of pathology in certain organ specific autoimmune diseases, it was shown that IL-23, not IL-12, was responsible for autoimmune disease in EAE (Cua et al., 2003). Moreover, while it had initially been described to be able to drive IFN-γ production by CD4+ T cells, IL-23 was also found to promote a distinct population of IL-17 producing CD4+ cells that were required for the induction of EAE (Aggarwal et al., 2003; Langrish et al., 2005). These studies demonstrated the importance of IL-23 in autoimmune disease as well as identifying it as a key molecule involved in the induction of IL-17 responses. Recent work on the autoimmune disease model of EAE has shown that IL-17 secreting CD4+ T cells can not home to non-inflamed tissue, but are readily recruited once lesions have been established (O’Connor et al., 2008), a finding suggesting that perhaps IL-17 secreting CD4+ T cells are not involved in the initiation but rather the propagation of autoimmune disease pathology.

5.1.3 IL-23 involvement in immunity against pathogen invasion

The role of IL-23 in bacterial or viral, or even helminth, infections is still somewhat elusive. Studies using bacterial infection models such as that of the Gram negative bacterium Klebsiella pneumoniae have identified that IL-23 is important for mounting protective immunity against this pathogen (Happel et al., 2005). Mice lacking IL-23 were severely impaired in their ability to induce IL-
17, and showed increased susceptibility to *Klebsiella* infections. Nevertheless, when IL-23 deficient mice were infected with the Gram positive bacterium *M. tuberculosis*, they exhibited normal IFN-γ responses, and unaltered protective immunity against this pathogen (Khader et al., 2005). IL-17 deficient mice have also been shown to be protected against *Mycobacterium bovis* Bacille Calmette-Guerin infections (Umemura et al., 2007). These two studies suggest that IL-23 and IL-17 are not required for protective immunity against this pathogen.

Using the same infectious model, however, a different group has shown that IL-23 production by DCs is required for optimal protection against *M. tuberculosis* (Wozniak et al., 2006). During the same study, it was additionally discovered that in the absence of IFN-γ, IL-17 alone was able to confer partial protection against the pathogen (Wozniak et al., 2006). In accord with previous work performed on this infection model, it was found that IL-23, independently of IL-12, was able to initiate IFN-γ responses, which conferred complete protection against *M. tuberculosis* (Wozniak et al., 2006). It has also been shown that IL-23 dependent memory Th17 responses are required for the rapid infiltration of IFN-γ secreting T cells at alveolar spaces during secondary *M. tuberculosis* infections, thus attributing an important role for IL-23 in Th1 CD4+ cell migration against tissue specific pathogens (Khader et al., 2007).

Supporting the notion that IL-23 can promote IFN-γ along with IL-17, a study by Kullberg and colleagues has demonstrated that inflammatory bowel disease (IBD) severity is heavily dependent on IL-23 (Kullberg et al., 2006), and that IL-23 alone is sufficient to induce the production of both IL-17 and IFN-γ by
CD4+ T cells. Colitis pathology in this *Helicobacter hepaticus* model was abrogated when both IL-23 and IL-12 were knocked out. However, IL-23 was found to be the main contributor towards disease severity. Subsequent work on IBD identified that IL-23 had no impact on systemic T cell inflammatory responses, but was essential for the induction of disease pathology in the intestine (Hue et al., 2006), suggesting that the effect of IL-23 and IL-17 may be localised to specific tissues.

A different aspect of the possible involvement of IL-23 in immunity was demonstrated in a *S. typhimurium* model, where it was demonstrated that IL-23 dependent IL-17 secretion induced T cells to produce IL-22. IL-22 was found to be responsible for microbial killing via the induction of defensin secretion by epithelial cells and keratinocytes (Schulz et al., 2008).

Outwith the realms of bacteria, studies concentrating on high pathology, Th1-skewed murine models of Schistosomiasis have identified IL-23 and IL-17 as important factors contributing towards immunopathology in certain mouse strains (Rutitzky and Stadecker, 2006; Shainheit et al., 2008). Neutralisation of either IL-23 or IL-17, but not IL-12p70 or IFN-γ, improved prognosis, suggesting a distinct role for IL-23/IL-17 responses in infection.

Although the role of IL-23 in bacterial or helminth infections remains currently unclear, there are strong indications that it is a crucial cytokine for conferring disease pathology, and possibly immune protection. Similarly to the IL-12 and IFN-γ paradigm, the impact of IL-23 and IL-17 appears to be highly dependent on the disease model and mouse strain used. However, the studies concentrating on *M. tuberculosis* and IBD seem to point in the direction that IL-23
may have a tissue specific, localised mode of action perhaps involved in the recruitment of other cells of the immune system. While IL-23 absence results in a systemic loss of IL-17 production by T cells, its effects are more pronounced at specific sites of infection.

5.1.4 IL-23-driven IL-17 is essential for lymphocyte recruitment

IL-17 has been implicated in the clearance of extracellular bacteria and fungi. Models using *K. pneumoniae, Bacteroides fragilis, Borrelia burgdorferi* and *M. tuberculosis*, have shown that IL-17 induces neutrophil recruitment, abscess formation, and chemokine secretion required for the rapid infiltration of lymphocytes to mucosal tissues (Chung et al., 2003; Infante-Duarte et al., 2000; Khader et al., 2007; Ye et al., 2001). Infection models using *M. tuberculosis* and *S. typhimurium* have shown that IL-23 dependent IL-17 secretion is involved in the infiltration of IFN-γ secreting CD4⁺ T cells to the site of infection (Khader et al., 2007; Schulz et al., 2008). IL-17 producing CD4⁺ T cells are thought to home to infected loci earlier than IFN-γ secreting cells. At the site of infection they induce the upregulation of chemokines and defensins such as β-defensin 2, and calgranulins (Siegemund et al., 2009), which are also known to be involved in lymphocyte recruitment. This results in the migration of effector cells to the sites of infection.

The link between IL-23 and IL-17 provided a very fertile matrix for the deeper understanding of the intricacies of immune responses and their effect on both “self” and invading organisms. However, a number of studies have
identified some unique roles of this cytokine that might help explain a number of the previous observations. IL-23 over-expression in mice was early on identified to cause neutrophilia and elevated levels of the pro-inflammatory cytokine TNF-α, and IL-1 (Wiekowski et al., 2001). It was later reported that IL-23 dependent IL-17 secretion is important for the recruitment of neutrophils to the site of inflammation and clearance of bacterial infections (Smith et al., 2007; Stark et al., 2005; Ye et al., 2001). Moreover the homeostatic control of neutrophil infiltration at the site of inflammation was found to be crucial for granulopoiesis and lesion formation, suggesting that IL-23 might be indirectly involved in these processes. The high pathology model of Schistomiasis has demonstrated that granulomas from IL-23 deficient mice show a decreased Gr-1+ cell recruitment, indicating that IL-23 may be important for normal granuloma formation (Rutitzky et al., 2008). These studies together suggest that IL-23 may be playing a key role in the recruitment of neutrophils and possibly other granulocytes to the site of infection of inflammation, and consequently promoting lesion and granuloma formation.

*S. typhimurium* is an intracellular bacterium that uses the fecal-oral route for infection. It invades hosts by attaching and transversing the epithelial lumen, invading APCs and translocating to spleens and livers where it replicates within macrophages (Yrlid et al., 2001b). Pathology is associated with high bacterial burdens in the spleens and livers, but it is not uncommon for this pathogen to also invade to a lesser degree sterile sites such as the heart. Effective immunity against *Salmonella* is characterized by Th1 responses involving both T and B cells and containment of the pathogen within granulomatous regions. Nevertheless,
the exact factors that limit bacterial replication and spreading are not yet identified. The pluripotent role of IL-23 in immunity posed the *S. typhimurium* model as a very attractive system to learn more about both the pathogen and this novel cytokine.

**AIMS**

1) To investigate the role of IL-23 in the induction and maintenance of CD4$^+$ T cell and B cell primary and memory responses, respectively, during *S. typhimurium* infections. To investigate the role of IL-23 in immunopathology caused by *S. typhimurium* infections.

2) To investigate the role of IL-23 in maintaining effective CD4$^+$ T cell and B cell responses against secondary challenge with *S. typhimurium* infections. To investigate the role of IL-23 in immunopathology caused by secondary exposure to *S. typhimurium*.

In order to investigate whether IL-23 is important for the induction and maintenance of primary immune responses, as well as following secondary infection, we addressed immunological and pathological parameters a week post primary infection (induction), 10 weeks post primary infection (maintenance/memory) and 4 days after secondary infection (challenge) of animals that had been given a primary infection 10 weeks previously.
To examine whether IL-23 contributed towards local and/or systemic immunity we analysed responses from both mesenteric lymph nodes (MLN)(local) and spleens (systemic).

5.2 IL-23 contribution towards the primary response against *S. typhimurium*

*Salmonella enterica* serovar Typhimurium is a Gram negative pathogen that is known to induce T helper 1 responses in both humans and mice associated with high level production of IL-12, TNF-α, IL-6 and IFN-γ. Protective immunity requires both the contribution of CD4+ T cells in the form of cytokine as well as B cells in the form of antibodies and as Ag presenting cells (Barr et al., 2009; Mastroeni and Menager, 2003). IgG2b and IgG2c are well-characterised Th1 associated antibodies produced during *S. typhimurium* infections, involved in protective immunity against this pathogen (Barr et al., 2009; Mastroeni and Menager, 2003).

In order to assess the contribution of IL-23 in the induction of immune responses against *S. typhimurium* we immunised i.p either C57BL/6 wild type (WT) or *p19*−/− mice with either PBS alone (control mice) or 1*10⁶ colony forming units (CFUs) of the attenuated SL3261 strain in PBS. Spleens and lymph nodes were processed, and single cell suspensions were restimulated *in vitro* with either media alone, or heat-killed (HK) avirulent SL3261. To determine whether IL-23 affected the ability of T cells to produce cytokine we purified CD4+ T cells from splenocytes of infected animals. Purified T cells were then stimulated with
increasing doses of HK, sonicated S. typhimurium in the presence of irradiated APCs (from irradiated splenocytes from a naïve syngeneic mouse). Cytokine production from whole splenocyte restimulation cultures, lymph node restimulation cultures as well as purified CD4+ T cell stimulation cultures was measured by ELISA.

5.2.1 Localised responses

MLN cells from WT and p19−/− mice that were immunised with 1*10^6 CFU SL3261 7 days previously, restimulated with HK SL1344 (Figure 5.1), produced statistically identical levels of IL-17, suggesting that during the primary stages of a S. typhimurium infection IL-23 does not contribute towards the localised IL-17 response. Interestingly, MLN cells from immunised p19−/− mice secreted significantly more IFN-γ than those from WT mice when restimulated with HK SL1344 (Figure 5.1). There was no significant difference between the amount of IL-10 secreted by WT and p19−/− lymph node cells when restimulated with SL1344 (Figure 5.1).

MLN cells from WT or p19−/− mice that had been immunised with 1*10^6 CFU of SL3261 failed to produce marked amounts of IFN-γ, IL-10 or IL-17 when cultured in media alone (Figure 5.1). As expected lymph node cells from naïve WT and p19−/− mice that received PBS injections did not produce any IFN-γ or IL-17 (Figure 5.1) after being stimulated with HK SL1344. However, MLN cells from naïve, PBS injected WT and p19−/− mice secreted a significant amount of IL-10 in response to stimulation with HK SL1344 (P<0.05) (Figure 5.1). This
suggests that HK salmonella can trigger IL-10 production in naïve mice, perhaps from innate sources, *in vitro*.

### 5.2.2 Systemic responses

Similar to what was seen with the local response (Figure 5.1), no statistical difference was observed between SL3261-immunised WT and SL3261-immunised p19⁻/⁻ splenocytes in their ability to secrete IL-17, when restimulated with HK SL1344 (Figure 5.2), suggesting that IL-23 is not required for the induction of IL-17 responses either systemically (Figure 5.2) or locally (Figure 5.1). However, in contrast to what was seen in the MLN, splenocytes from immunised p19⁻/⁻ mice secreted similar amounts of IFN-γ to those from WT mice, when restimulated with HK SL1344 (Figure 5.2), suggesting that the role of IL-23 in regulation of IFNg may be more important in sites proximal rather than distal to infection.

Interestingly, splenocytes from immunised p19⁻/⁻ mice secreted significantly less IL-10 than splenocytes from immunised WT mice when restimulated with SL1344 (Figure 5.2). Nevertheless, the reduced production of IL-10 by p19⁻/⁻ splenocytes had no impact on either IFN-γ or IL-17 secretion by these cells (Figure 5.2). Similar to what was seen with MLN cells, bulk splenocyte cultures from control, uninfected WT or p19⁻/⁻ mice that received PBS injections responded to HK salmonella by producing significant amounts of IL-10. In contrast to the MLN cells, they also produced detectable IFN-γ when exposed to HK bacteria for the first time. As expected, splenocytes from these
naïve animals did not secrete significant amounts of IFN-γ, IL-10 or IL-17 when cultured in media alone (Figure 5.2). Splenocytes from WT or p19−/− mice that were immunised with 1*10^6 CFU SL3261 also failed to secrete marked quantities of either IL-10 or IL-17 (Figure 5.2) when cultured in media alone, but did produce detectable IFN-γ in this setting.

5.2.3 IL-23 role in T cell polarisation during primary responses against S. typhimurium

In order to focus on the contribution of IL-23 in driving T cell responses specifically during primary S. typhimurium infections, we purified CD4+ T cells from pooled groups of splenocytes by positive selection using magnetic beads, and stimulated them in the presence of irradiated APCs and increasing doses of HK S. typhimurium.

Once purified from the bulk splenocytes, and in accord with lymph node cell responses, stimulation of purified splenic CD4+ T cells showed that there is a role for IL-23 in the regulation of IFN-γ in the S. typhimurium infection setting.

CD4+ T cells from WT mice that had been infected with SL3261 secreted increased levels of IFN-γ with increasing concentrations of HK S. typhimurium (Figure 5.3). CD4+ T cells from IL-23 deficient mice secreted significantly higher levels of IFN-γ than those from WT mice, irrespective of the concentration of Ag used for stimulation (Figure 5.3). Coincident with this, and reflecting the result from bulk cultures (Figure 5.2), IL-10 levels were also impaired in CD4+T cells isolated from p19−/− mice.
Not surprisingly, T cells from control, uninfected WT and p19\(^{-/-}\) mice that were injected with PBS failed to produce any IFN-\(\gamma\) regardless of the dose of antigen used to stimulate them with (Figure 5.3).

Underscoring the fact that IL-23 is not essential for the induction of a primary CD4\(^+\) T cell IL-17 response during the early stages of a \textit{S. typhimurium} infection, purified T cells from infected IL-23 deficient mice secreted IL-17 similarly to those isolated from WT infection. IL-17 secretion by CD4\(^+\)T cells from WT infected mice peaked at the lowest (1\(\mu\)g/ml) concentration of antigen used to stimulate them with, and decreased with increasing doses of antigen (Figure 5.3). In comparison, T cells from IL-23 deficient, PBS injected naïve mice, failed to secrete any detectable IL-17 (Figure 5.3). Somewhat surprisingly, and in contrast to what was seen with IL-23 deficient cells or bulk splenocyte cultures (Figure 5.2), CD4\(^+\) T cells purified from naïve WT mice produced detectable IL-17 in a dose-dependent manner following stimulation with HK.

T cells from both WT and p19\(^{-/-}\) mice that had been injected with SL3261 produced more IL-10 than their PBS-injected controls (Figure 5.3). Since the groups are pooled it is hard to convincingly infer to the statistical relevance this observation may have.

Interestingly, a measurable amount of IL-10 was detected in purified naïve CD4\(^+\) T cell cultures that were stimulated with HK salmonella. Together with the results from the whole naïve splenocyte stimulations (Figure 5.2), this indicates that HK salmonella can stimulate IL-10 production directly in naïve mice from a combination of innate cells and CD4\(^+\) T cells, but IFN-\(\gamma\) only from a non-CD4\(^+\) source.
No obvious differences were observed between WT and p19/− mice that had been injected with either PBS or SL3261 in their ability to produce IL-2 (Figure 5.3), perhaps due to the fact that IL-2 is not abundant during primary T cell responses.

5.2.4 IL-23 role in early B cell responses against *S. typhimurium*

Early B cell responses were characterised by significantly higher production of type 1 antibodies by immunised p19/− mice than WT immunised mice. Serum IgG2b and IgG2c as well as total immunoglobulin (Ig) were significantly higher in SL3261 immunised p19/− mice than SL3261 immunised WT mice (Figure 5.11, suggesting that the early increased IFN-γ secreted by p19/− mice may enhance type 1 antibody secretion by B cells. Furthermore, IgG2c responses in SL3261 immunised p19/− mice were characterised by different kinetics than those observed in immunised SL3261 WT mice as determined by interaction statistics using Two-way ANOVA analysis. Serum IgG1, IgG3 and IgM levels were statistically similar between SL3261 immunised WT and SL3261 immunised p19/− mice (Figure 5.11).

5.2.5 IL-23 role in maintenance of IL-17 responses during *S. typhimurium* infections

In order to assess the contribution of IL-23 in maintenance of memory responses against *S. typhimurium*, we immunised C57BL/6 or p19/− mice with either PBS alone (control mice) or with 1*10⁶ CFUs of the attenuated SL3261
strain in PBS. Ten weeks later spleens were processed, and single cell suspensions were cultured in either media alone, or with 10µg/ml HK avirulent SL3261 strain. We wanted to determine whether absence of IL-23 affected the ability of different memory cell populations to produce cytokine. We approached this by purifying by positive selection using magnetic beads T and B cells from pooled groups of splenocytes from infected or PBS injected control animals. CD4+ T cell populations were stimulated with increasing doses of HK, *S. typhimurium* in the presence of naïve irradiated APCs, while purified B cells were stimulated with HK SL3261. Cytokine production from whole splenocyte cultures, as well as T and B cell stimulation assays, was measured by ELISA.

In stark contrast to the lack of requirement for IL-23 in generation of the primary IL-17 response (Figures 5.1-5.3), IL-23 was a fundamental requirement for maintaining long-term IL-17 responses during *S. typhimurium* infections. Splenocytes from WT mice that had been infected 10 weeks previously with SL3261, restimulated with HK SL1344, secreted significantly higher levels of IL-17 compared to splenocytes from PBS-immunised WT controls (Figure 5.4). Interestingly, and in contrast to primary responses - during which splenocytes from immunised IL-23 deficient mice secreted significant levels of IL-17 (Figure 5.2) - splenocytes from *p19<sup>−/−</sup>* immunised mice failed to produce any measurable IL-17 (Figure 5.4). This suggests that, in the *S. typhimurium* infection setting, IL-23 is indispensable for either the homeostasis of IL-17 producing cells, or for the activation of memory cells to secrete IL-17 directly, or indirectly by instructing other cells to do so.
Similar to primary responses (Figure 5.2), in the memory phase, splenocytes from SL3261-immunised WT mice secreted similar levels of IFN-γ to SL3261-immunised p19⁻/⁻ mice, suggesting that IL-23 is not required for the maintenance of long term IFN-γ responses (Figure 5.4).

Surprisingly, splenocytes from SL3261-immunised WT mice secreted significantly more IL-2 than splenocytes from SL3261-immunised p19⁻/⁻ mice, irrespectively of whether these two groups were stimulated in media or with 10μg/ml HK SL1344, suggesting that IL-23 may be playing a role in T and B cell proliferation (Figure 5.4). Similarly to primary responses against S. typhimurium, splenocytes from WT mice produced significantly more IL-10 than splenocytes from p19⁻/⁻ mice when restimulated with HK SL1344 (Figure 5.4). This IL-10 production was not dependent on whether the mice were immunised or not, suggesting that the response was not antigen specific. As expected, splenocytes from either WT or p19⁻/⁻ mice that had received PBS injections failed to produce any measurable IL-17 or IL-2 after being stimulated with HK SL1344 (Figure 5.4). Splenocytes from both immunised WT and p19⁻/⁻ mice secreted significant levels of IFN-γ compared to uninfected controls when stimulated with HK SL1344 (Figure 5.4). No significant amount of IFN-γ was detected in supernatants from splenocytes of uninfected WT and p19⁻/⁻ mice stimulated with HK SL1344, or from splenocytes of infected WT and p19⁻/⁻ mice, stimulated in media alone (Figure 5.4).
5.2.6 IL-23 role in T cell activation and maintenance during late stages of *S. typhimurium* infections

Reflecting the results from the bulk splenocyte cultures, purified CD4+T cells from SL326-immunised WT mice secreted a significant amount of IL-17 compared to uninfected controls, while T cells from SL3261-immunised p19−/− mice failed to secrete any measurable IL-17 (Figure 5.5), suggesting that IL-23 is essential for either the activation of CD4+ IL-17 producing T cells, or for the provision of survival signals to CD4+ T cells that have been differentiated into IL-17 producing cells during the early phase of *S. typhimurium* infection.

In contrast to the responses observed at the primary phase of the infection, during which T cells from SL3261-immunised p19−/− mice produced significantly more IFN-γ than SL3261-infected WT T cells, memory responses showed a different picture. Purified CD4+T cells from SL3261-immunised WT mice produced similar levels of IFN-γ to T cells from SL3261-immunised p19−/− mice (Figure 5.5). Although, as seen in figure 5.5, there is a stimulatory dose dependency on IFN-γ secretion for both p19−/− and WT mice, we are unable to refer to the significance of this result since T cells were purified from pooled splenocytes. Additionally, the small differences in IFN-γ secretion between T cells from immunised p19−/− and immunised WT mice are not reflected at the splenocyte response level (Figure 5.4), making it hard to ascribe a biological significance to this observation. Nevertheless, any potential difference in the ability of WT and p19−/− T cells to secrete IFN-γ was not due to an IL-10 regulatory environment, as was seen in the primary phase of the infection, since
splenocytes from SL3261-infected WT mice secreted more IL-10 than splenocytes from SL3261-infected p19\(^{-/-}\) mice (Figure 5.4). Similar to the primary responses, T cells from either PBS injected WT or PBS injected p19\(^{-/-}\) mice failed to produce any measurable IFN-\(\gamma\) when restimulated with HK sonicated S. typhimurium (Figure 5.5).

T cells purified from immunised WT mice secreted a greater amount of IL-2 than T cells from immunised p19\(^{-/-}\) mice, suggesting that IL-23 might be involved in the ability of memory T cells to expand and proliferate (Figure 5.5).

### 5.2.7 IL-23 role in B cell responses during late stages of S. typhimurium infections

While early IgG2c, IgG2b and total Ig responses were significantly higher in immunised p19\(^{-/-}\) mice than WT immunised mice, only serum IgG2c was elevated in immunised p19\(^{-/-}\) mice compared to immunised WT mice at later stages of infection (day 51-day 67) (Figure 5.11). In terms of any possible non-conventional (i.e. other than Ab production) role for B cells in this setting, we found no significant difference in B cell production of IL-6 or IL-10 during memory responses, suggesting that the lack of IL-23 in Salmonella typhimurium infection does not affect this aspect of B cell activation (Figure 5.6).
5.2.8 IL-23 role in immune responses during secondary challenge with *S. typhimurium*

In order to better understand the role of IL-23 in systemic and localised responses against secondary challenge and protection against *S. typhimurium*, we infected previously immunised or naïve WT or p19<sup>-/-</sup> mice with 1*10<sup>8</sup> CFU SL1344 orally via gavage. Four days after challenge, MLNs and spleens were collected and processed into single cell suspensions and stimulated in the presence of HK SL1344 or in media alone. We also wished to specifically investigate whether IL-23 influenced the activation of T and B cells and their ability to secrete cytokine during secondary challenges. To this end we purified CD4<sup>+</sup> T and CD19<sup>+</sup> B cells by positive selection from splenocytes prior to culture. We stimulated T cells with HK, sonicated WT *S. typhimurium* in the presence of irradiated APCs and B cells in the presence of HK WT *S. typhimurium* alone.

5.2.9 Localised responses

Lymph node cells from WT and p19<sup>-/-</sup> mice that had been immunised with 1*10<sup>6</sup> CFU SL3261 followed by oral challenge with 1*10<sup>8</sup> CFU SL1344 10 weeks later, produced significantly more IFN-γ compared to non-immunised, challenged controls when restimulated with 10µg/ml HK SL1344 (*Figure 5.7*). Lymph node cells from SL3261-immunised - SL1344-challenged p19<sup>-/-</sup> mice secreted statistically similar levels of IFN-γ to lymph node cells from SL3261-immunised - SL1344-challenged WT mice (*Figure 5.7*). Lymph node cells from SL3261-immunised - SL1344-challenged p19<sup>-/-</sup> mice failed to secrete a
measurable amount of IL-17 or IL-2 when restimulated with 10µg/ml HK SL1344 (Figure 5.7), suggesting that IL-23 is crucial either in the maintenance or activation of effector cytokine responses required during secondary challenge responses.

Lymph node cells from SL3261-immunised - SL1344-challenged p19−/− mice secreted significantly less IL-10 than lymph node cells from SL3261-immunised - SL1344-challenged WT mice (Figure 5.7), suggesting that the reduced levels of IFN-γ, IL-17 and IL-2 secreted by p19−/− lymph node cells were not due to an IL-10 dependent regulation. In accord with primary responses against avirulent SL3261 S. typhimurium (Figure 5.1), lymph node cells from p19−/− mice that had been immunised with PBS followed by oral SL1344 challenge secreted significantly less IL-10 than lymph node cells than PBS-immunised SL1344-infected WT mice when restimulated with HK SL3261 (Figure 5.7). Although WT and p19−/− lymph node cells exhibited a different capacity to secrete IL-10, no statistical difference was observed between immunised and non-immunised animals for either group (Figure 5.7), suggesting that the IL-10 contributors of this cytokine could be innate cells.

MLN cells from WT or p19−/− mice that had been immunised with 1*10^6 CFU SL3261 and challenged with 1*10^8 SL1344 CFU failed to produce any measurable amount of IFN-γ or IL-17 when restimulated in media alone (Figure 5.7). Neither did lymph node cells from WT or p19−/− mice that had been immunised with PBS followed by oral SL1344 challenge secrete detectable IL-17, IL-10 or IL-2 (Figure 5.7) when stimulated in media alone.
5.2.10 Systemic responses

Similar to what was seen with localised MLN responses, splenocytes from WT mice that had been immunised with $1 \times 10^6$ CFU SL3261 followed by oral challenge with $1 \times 10^8$ CFU SL1344 10 weeks later produced significant levels of IFN-$\gamma$, IL-17 and IL-2 compared to non-immunised, challenged controls when stimulated with HK SL1344 (Figure 5.8). Surprisingly, and in contrast to lymph node responses (Figure 5.7), splenocytes from SL3261-immunised - SL1344 challenged p19$^{-/-}$ mice failed to secrete significantly more IFN-$\gamma$ than PBS immunised - SL1344-challenged control mice (Figure 5.8). This suggests that IL-23-dependent secondary challenge IFN-$\gamma$ responses are differentially induced in different tissues. In addition, splenocytes from SL3261-immunised - SL1344-challenged p19$^{-/-}$ mice failed to secrete a measurable amount of IL-17 or IL-2 when restimulated with HK SL1344 (Figure 5.8), suggesting that in this S. typhimurium infection model, IL-23 promotion of IL-17 maintenance and proliferation is systemic. In contrast to MLN responses, splenocytes from SL3261-immunised - SL1344-challenged WT mice secreted significantly more IL-10 than splenocytes from PBS-immunised - SL1344-challenged WT mice when stimulated with HK SL1344 (Figure 5.8).

5.2.11 IL-23 role in maintaining T cell responses during secondary challenge with S. typhimurium

In line with what was seen with bulk splenocyte cultures, purified CD4$^+$ T cells from WT mice that had been immunised with $1 \times 10^6$ CFU SL3261 followed
by an oral SL1344 challenge with $1 \times 10^8$ CFU secreted significantly more IFN-$\gamma$ than T cells from SL3261-immunised - SL1344-challenged p19$^{-/-}$ mice when stimulated with HK S. typhimurium (Figure 5.9). This suggests that, during challenge infection with S. typhimurium, IL-23 is required for either the maintenance or recruitment of IFN-$\gamma$ secreting T cells.

Both WT and p19$^{-/-}$ T cells from mice that had been immunised in PBS followed by oral challenge with SL1344 failed to produce a significant amount of IFN-$\gamma$.

CD4$^+$ T cells from WT mice that had been immunised with $1 \times 10^6$ CFU SL3261 followed by oral challenge with $1 \times 10^8$ CFU of SL1344, produced significant levels of IL-17 (Figure 5.9) compared to PBS-immunised SL1344-challenged controls. Similarly to MLN and bulk splenocyte responses, T cells from p19$^{-/-}$ mice that had been immunised with $1 \times 10^6$ CFU SL3261 followed by oral challenge with $1 \times 10^8$ CFU of SL1344, failed to produce any measurable IL-17 (Figure 5.9). In resemblance to the response observed by T cells from p19$^{-/-}$ mice that had been stimulated one week post immunisation with SL3261 (Figure 5.3), T cells from PBS-immunised - SL1344 challenged mice secreted significantly higher levels of IL-17 than T cells from p19$^{-/-}$ mice that had been immunised with SL3261 and infected with SL1344 (Figure 5.9, open boxes), further suggesting that IL-23 is not required for the induction of IL-17 during primary immune responses against either the virulent (SL1344) or avirulent (SL3261) strains of S. typhimurium.
Similarly to splenocyte responses, no significant difference was observed in the amount of IL-10 produced by WT or p19<sup>−/−</sup> T cells from mice that had been immunised with 1*10<sup>6</sup> CFU SL3261 followed by an oral challenge with 1*10<sup>8</sup> CFU SL1344 (Figure 5.9). Furthermore, both WT and p19<sup>−/−</sup> T cells from mice that had been immunised with PBS followed by an oral SL1344 challenge secreted a statistically identical amount of IL-10. This observation is at odds with the splenocyte responses, during which splenocytes from non-immunised and challenged p19<sup>−/−</sup> mice secreted more IL-10 than their WT controls (Figure 5.8), suggesting that the source of IL-10 in this <i>S. typhimurium</i> infection model is other than T cells alone.

As observed during memory responses, secondary challenge responses were characterised by a complete failure of T cells from p19<sup>−/−</sup> mice that had been immunised with PBS followed by an oral SL1344 challenge to secrete IL-2, in contrast to WT T cells which secreted significant amount of IL-2 (Figure 5.9).

### 5.2.12 IL-23 role in B cell responses during later stages of <i>S. typhimurium</i> infections

No significant difference was observed in serum levels of type 1 antibody between SL3261-immunised p19<sup>−/−</sup> and SL3261-immunised WT mice at day 3 post challenge with SL1344 (Figure 5.11). Similarly, we observed no significant difference in the ability of purified B cells from SL3261-immunised – SL1344 infected WT and SL3261 immunised – SL1344 infected p19<sup>−/−</sup> mice to secrete either IL-6 or IL-10 (Figure 5.10). The observed results could be explained by the
fact that 3 days might be a too short period for any differences in B cell activation to become apparent.

5.3 Conclusions

IL-23 has been previously reported to be involved in the maintenance but not the induction of T cell IL-17 responses (Bettelli et al., 2008; Khader et al., 2007). Our results would agree with this hypothesis. Further, the data presented in this chapter would also suggest that, during secondary challenge responses against *S. typhimurium*, IL-23 is required not only for the maintenance of IL-17 CD4+ T cell responses but also for either the provision of survival signals or recruitment of IFN-γ secreting CD4+ T cells to the sites of infection. The role of IL-23 in maintaining both IL-17 and IFN-γ responses pose it as a possible homeostatic and chemotactic cytokine during inflammation in general, and not restricted to IL-17 alone.

5.3.1 IL-23 is not required for the induction of IL-17 in a *S. typhimurium* primary infection

Primary IL-17 responses against *S. typhimurium* are not affected by the lack of IL-23, as observed in this model using p19−/− mice. Splenocytes, MLN cells and purified T cells from avirulent *S. typhimurium*- immunised p19−/− and WT mice secreted statistically similar levels of IL-17 (*Figure 5.1, 5.2, 5.3*). Previous reports trying to address cytokine requirements for the generation of IL-17 responses have established, in accord with our work, that IL-23 is not
essential for inducing T cell derived IL-17 production (Bettelli et al., 2008; Khader et al., 2007). Interestingly, we found that in both lymph node cells and splenic T cells, IFN-γ secretion during primary stages of infection was significantly elevated in p19–/– mice compared to WT mice (Figure 5.1, 5.3). Similarly, type 1 IFN-γ dependent antibody production was also significantly higher in serum from p19–/– compared to WT mice (Figure 5.11). There was no evidence of a statistical difference, however, in IFN-γ production by splenocytes from p19–/– and WT mice (Figure 5.2). This is at odds with the difference seen in splenic CD4+ T cell responses (Figure 5.3), suggesting that other cell sources of IFN-γ (such as NK or CD8+) in whole splenocyte restimulations might be contributing towards the total amount of IFN-γ observed. In order to conclude as to the reason for the differential IFN-γ secretion between p19–/– and WT mice in spleens and lymph nodes we would need to perform intracellular cytokine staining for different splenocyte populations. Nevertheless, the differential secretion of IFN-γ by p19–/– and WT mice observed between splenocyte and lymph node cell responses suggests distinct roles for IL-23 in tissue specific immunity. *Salmonella* invasion mechanisms are characterised by the invasion of Peyer’s patches, bacterial transport to MLNs and spleens and replication in MΦs in the spleens and livers. It would be interesting therefore to assess cytokine production comparing WT and p19KO mice in these other tissue sites. Perhaps IL-23 effect on IFN-γ production is dependent on the infection and replication site of *Salmonella.*
Some evidence has pointed out that IL-23 plays an inhibitory role on IFN-γ production by CD8+ cells, NK cells and, to a certain extent, CD4+ T cells in an in vitro and in vivo Listeria monocytogenes infection setting (Sieve at all, 2007). It would be interesting to know whether competition for the T cell IL-12β1 receptor, which is shared for signalling between IL-12 and IL-23, can have an effect on the capacity of T cells to secrete IFN-γ. While there is some evidence suggesting that IL-17 and IFN-γ secreting cells can counter-regulate each other (Cruz et al., 2006), this does not seem to be the case in this S. typhimurium setting since splenocytes, lymph node cells and T cells from IL-23 deficient mice secreted statistically similar levels of IL-17 to the respective cells from WT mice. This would suggest that it is improbable that the reduced IFN-γ secretion from WT cells is due to an IL-17 counter-regulatory effect.

We found that WT mice lost statistically more weight during the initial stage of immunisation than p19−/− mice (Figure 5.13A). Looking into other parameters of pathology such as bacteraemia and pro-inflammatory cytokine secretion in different tissues may reveal a more clear picture about the different immunopathological profile between p19−/− and WT mice and between different tissues. Differences between p19−/− and WT mice in the activation state of phagocyte intracellular compartments containing bacteria or even differences in the ability to contain this pathogen within granulomas cannot be excluded. Measuring bacterial burdens in spleens, livers and MLNs as well as bacteraemia in the blood or lymphatic circulation could answer whether there is a requirement for IL-23 in controlling early bacterial replication. It has been
previously reported that IL-23 can act directly on DCs to induce nitric oxide secretion (Bastos et al., 2005). It would therefore be interesting to find out whether IL-23 signaling to DCs or MΦs can impact *Salmonella* processing and survival within those cells. Measuring nitric oxide secretion by phagocytes as well as endosomal activity could reveal the possible involvement of IL-23 in phagocyte activation.

The fact that splenocytes from IL-23 deficient mice secreted less IL-10 than splenocytes from WT mice (Figure 5.2) raises some interesting questions about this disease model. Addressing disease pathology parameters in IL-23 deficient versus wild type mice could reveal whether the observed differential secretion of IL-10 and IFN-γ between WT and p19<sup>−/−</sup> mice is due to different immunological requirements for the control of localised tissue pathology. The fact that *S. typhimurium* preferentially occupies spleens and livers may be linked to the reduced splenocyte IL-10 in IL-23 deficient mice compared to WT mice. IL-23 is known to be important for the induction of G-CSF and subsequently for granuloma formation (Langrish et al., 2004; Stark et al., 2005). A possible inability of p19<sup>−/−</sup> mice to contain the pathogen within granulomas could result in an early, exaggerated compensatory T cell derived IFN-γ response in IL-23 deficient mice. Histology of infected sites such as livers and spleens would help understand whether IL-23 is involved in bacterial containment within granulomas. Measuring granuloma frequencies and cell infiltrates in hepatic and splenic granulomatous regions in infected p19<sup>−/−</sup> and WT mice might provide a link between IL-23, IFN-γ and IL-10 in tissue-specific disease
pathology and immunity. Furthermore, it would be informative to compare the number of IFN-γ producing T cells from WT and p19\(^{-/-}\) mice infiltrating different tissues. This could establish whether there is a bias for IFN-γ producing T cells to infiltrate spleens, livers or sterile tissue such as the heart, allowing for some inferences to be made as to whether inability to contain bacteria and immune pathology are linked.

5.3.2 IL-23 is indispensable for the maintenance of IL-17 producing CD4\(^{+}\) T cells during late stages of *Salmonella typhimurium* infection

IL-17 secretion by T cells and splenocytes was completely impaired at the 10\(^{th}\) week post infection in p19\(^{-/-}\) mice compared to wild type mice (Figure 5.4). As previously described, primary IL-17 responses in p19\(^{-/-}\) mice were normal compared to WT (Figure 5.1, 5.2, 5.3), suggesting that IL-23 is critical for the survival but not the induction of IL-17 CD4\(^{+}\) T cell responses. This is not entirely surprising since IL-23 has been previously reported to be acting on memory T cells and, more specifically, it has been described to promote maintenance of IL-17 producing cells (Stritesky et al., 2008). Nevertheless, staining for activation and memory markers such as CD45Rb, CD44 and CD62L versus intracellular staining for IL-17 and IFN-γ on CD4\(^{+}\) and CD8\(^{+}\)T cells from different tissues would give us an indication whether IL-17 secretion by T cells is abrogated in the absence of IL-23 or whether there is some correlation between activation and memory and the requirements for IL-23.
Memory responses in IL-23 deficient mice were characterised by impaired secretion of IL-2 and IL-17 compared to WT mice along with, in contrast to primary responses, normal secretion of IFN-γ. Intracellular staining of T cells at the 10th week post immunisation should reveal whether IL-23 impairment influences single IL-17 producers or its action is extended to single IFN-γ and double IL-17/IFN-γ producers. Furthermore, injecting exogenous IL-23 a short time (day-week) before collecting the T cells and performing the aforementioned intracellular staining should reveal whether IL-23 is involved in the activation or maintenance of distinct memory T cell populations.

Antibody secretion by WT and p19−/− during the late stages of S. typhimurium infections was characterised, in contrast to primary responses, by statistically identical titres of IgG2b (Figure 5.11). However, IgG2c serum titres were statistically different between WT and p19−/− mice, even at the later stages of the infection (Figure 5.11). It is not entirely clear whether IFN-γ alone, or another aspect of Th1 differentiated cells independent of IFN-γ, is responsible for driving antibody responses. Using IFN-γ depleting antibodies at different time points during infection might answer whether there is a stringent requirement of IFN-γ for the generation of type 1 Ab responses in our system. Additionally we could try depleting T cells in order to investigate whether the induction of Ab responses in this system is cytokine or T cell dependent.
5.3.3 IL-23 is required for the induction of tissue specific IFN-γ responses during secondary challenge responses against *Salmonella typhimurium* infections

Surprisingly, we found that during secondary challenge with virulent SL1344 IL-23 deficient mice had impaired IFN-γ responses in the spleens but not lymph nodes, compared to WT mice (Figure 5.7, 5.8). This observation was true for whole splenocyte responses as well as purified splenic CD4+ T cell responses (Figure 5.8, 5.9). In light of the primary responses, where we observed elevated T cell derived IFN-γ from p19−/− mice (Figure 5.1), it is clear that in the *S. typhimurium* infection model, IL-23 has an intricate and differential role in the induction of primary, memory and challenge IFN-γ responses. As discussed earlier (Chapter 5.3.1), pathological parameters such as rate and site of bacterial replication and infection may be imposing differential immunological challenges to different sites, and as such contributing towards the observed differences in tissue specific immune responses. IL-23 dependent IL-17 secreting cells are thought to infiltrate the sites of infection earlier than IFN-γ secreting cells in the *M. tuberculosis* setting (Khader et al., 2007). There, they are thought to induce chemokine receptor up-regulation, which results in the recruitment of IFN-γ secreting T cells (Khader et al., 2007). It is yet unclear whether, in our model, IL-17/IL-23 dependent T cell IFN-γ secretion is a tissue specific, migration related event or whether IL-23 is required for the homeostasis of all antigen specific IFN-γ secreting T cells. In order to verify whether IL-23 is required for activation or homeostasis of IFN-γ responses, we would need to
add exogenous IL-17 and IL-23 before or during secondary challenge responses, and observe whether IFN-γ responses are rescued or not in either case.

Strikingly, IFN-γ and CD4+T cell responses in the spleens of p19−/− mice were impaired during secondary infection challenge, compared to memory responses, during which they appeared to be normal. This suggests that IL-23 may be playing a role in providing survival signals to activated IFN-γ producing T cells. Whether this is done through a direct signalling of IL-23 to T cells or via IL-17 is unclear. It would be worth investigating whether exogenous addition of either IL-17 or IL-23 could rescue IFN-γ responses during challenge responses. Furthermore, comparing the effect of exogenous addition of IL-17 or adoptive transfer of IL-17-secreting T cells into IL-23 deficient mice on IFN-γ secretion during challenge should answer whether reduced IFN-γ production by T cells is due to IL-17 itself, or due to ‘help’ provided by IL-17 secreting T cells. Whether the reduced IFN-γ secretion by p19−/− mice during challenge responses could be attributed to a lack of IL-23 - IL-12β1 receptor signalling via STAT-4, which is required for the induction of IFN-γ responses is an interesting possibility. It would be worth investigating whether over expression of STAT-4 or addition of exogenous IL-12 p70 could rescue IFN-γ responses in this system.

Work on *M. tuberculosis* and *S. typhimurium* has revealed that IL-17 secreting T cells are important for the induction of tissue specific immune responses by recruiting neutrophils and macrophages to the site of infection (Khader et al., 2007) (Schulz et al., 2008). As such, it would be informative to compare both neutrophilia and the number of IFN-γ and IL-17 producing T cells
from WT and p19\(^{-/-}\) mice infiltrating different tissues during the course of primary infection as well as during secondary challenge. This could provide us with useful information about the involvement of IL-23 in some aspects of innate immunity and its effect on the recruitment and activation of cells of the adaptive arm of immunity.

Although initial studies on IL-23 and its involvement in IL-17 induction showed some interesting links between those two cytokines and autoimmune disease, recent work utilising both infection and autoimmune disease models has revealed some interesting immunological aspects of this IL-12 p70 homologue related to the IL-12 p70/IFN-\(\gamma\) axis. While some of the work presented in this chapter supports the consensus that IL-23 is required for IL-17 maintenance, but not induction, the implications of IL-17 responses in our hands and others using infection models seem to be localised to preferentially infected tissues. Importantly, whereas IL-12 p70 is clearly involved in the induction of IFN-\(\gamma\) effector responses, the work outlined in this chapter adds strength to the growing idea that IL-23 also plays an important role in providing survival signals and recruitment signals to IFN-\(\gamma\) secreting T cells.

Perhaps it would be interesting to repeat the experiments preformed for the purposes of this chapter by immunising mice via the oral route. Since IL-23 seems to be linked to localised responses, it might be useful to try and elicit immunity at the site of pathogen invasion. Such an approach might reveal more emphatically the role of IL-23 in localised responses.
The implication of IL-23, however, in lymphocyte recruitment provides good reasons for further investigations on this cytokine. Harnessing these recruiting functions of this APC derived cytokine might provide us with practical ways for disease treatment as well as vaccination delivery.
Figure 5.1. Mesenteric lymph node primary responses. IL-23 is not required for the induction of IL-17. WT (white bars) or p19−/− (grey bars) mice were immunised i.p with either PBS (3 mice/group) or 1*10^6 CFU SL3261 (5 mice per group). Seven days later lymph nodes were processed into single cell suspensions and restimulated for three days in either media or with 10µg/ml HK SL1344. IFN-γ, IL-17 and IL-10 production was measured by ELISA. Bars represent mean and standard error mean (SEM) of 3 ( naïve) or 5 (immunised) mice per group and duplicate wells on ELISA plates per mouse. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 5.2. Splenocyte primary responses. IL-23 is not required for the induction of IL-17. WT (white bars) or p19−/− (grey bars) mice were immunised i.p with either PBS (3 mice/group) or 1*10⁶ CFU SL3261 (5 mice per group). Seven days spleens were processed into single cell suspensions and restimulated for three days in either media or with 10µg/ml HK SL1344. IFN-γ, IL-17 and IL-10 production was measured by ELISA. Bars represent mean and standard error mean (SEM) of 3 (naïve) or 5 (immunised) mice per group and duplicate wells on ELISA plates per mouse. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 5.3. T cell primary responses. IL-23 deficiency correlates with increased IFN-γ secretion by T cells. WT (closed signs), or p19-/- (open signs) mice were immunised i.p with either PBS (squares) (3 mice/group) or 1*10^6 CFU SL3261 (circles) (5 mice/group). Seven days later T cells were purified from pooled splenocytes from individual groups of mice and stimulated for 3 days (1 day for IL-2) in the presence of irradiated APC with 1, 5, 10 or 25 µg/ml purified-sonicated C5. Gray rectangles represent irradiated APCs. IFN-γ, IL-17, IL-10 and IL-2 production by purified T cells was measured by ELISA. Geometrical shapes represent mean and standard error mean (SEM) of 3 replicate wells on ELISA plates per group. Data are representative of 2 independent experiments.
Figure 5.4. Splenocyte memory responses. IL-23 is required for the maintenance of IL-17 responses. WT (white bars) or p19\(^{-/-}\) (grey bars) mice were immunised i.p with either PBS (3 mice/group) or 1\(^{*}10^6\) CFU SL3261 (5 mice per group). 10 weeks later spleens were processed into single cell suspensions and restimulated for three days (1 day for IL-2) in either media or with 10\(\mu\)g/ml HK SL1344. IFN-\(\gamma\), IL-17, IL-10 and IL-2 production was measured by ELISA. Bars represent mean and standard error mean (SEM) of 3 (naïve) or 5 (immunised) mice per group and duplicate wells on ELISA plates per mouse. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 5.5. T cell memory responses. IL-23 is indispensable for the maintenance of IL-17 secretion by T cells. WT (closed signs), or p19^-/- (open signs) mice were immunised i.p with either PBS (squares) (3 mice/group) or 1*10^6 CFU SL3261 (circles) (5 mice/group). 10 weeks later T cells were purified from pooled splenocytes from individual groups of mice and stimulated for 3 days (1 day for IL-2) in the presence of irradiated APC with 1, 5 10 or 25 µg/ml purified-sonicated C5. Gray rectangles represent irradiated APCs. IFN-γ, IL-17, IL-10 and IL-2 production by purified T cells was measured by ELISA. Geometrical shapes represent mean and standard error mean (SEM) of 3 replicate wells on ELISA plates per group. Data are representative of 2 independent experiments.
Figure 5.6. B cell memory responses. IL-23 does not affect memory B cell pro-inflammatory and anti-inflammatory cytokine secretion. WT (closed signs), or p19−/− (open signs) mice were immunised i.p with either PBS (squares) (3 mice/group) or 1*10^6 CFU SL3261 (circles) (5 mice/group). 10 weeks later B cells were purified from pooled splenocytes from individual groups of mice and stimulated with increasing concentrations of HK SL3261. IL-6 and IL-10 production by purified B cells was measured by ELISA. Geometrical shapes represent mean and standard error mean (SEM) of 3 replicate wells on ELISA plates per group. Data are representative of two independent experiments.
Figure 5.7. Mesenteric lymph node challenge responses. IL-23 is required for the maintenance of IL-17 responses. WT (white bars) or p19<sup>−/−</sup> (grey bars) mice were immunised i.p with either PBS (3 mice/group) or 1*10<sup>6</sup> CFU SL3261 (5 mice per group). 10 weeks later all mice were orally challenged with 1*10<sup>8</sup> CFU SL1344. 3 days after challenge lymph nodes were processed into single cell suspensions and restimulated for three days (1 day for IL-2) in either media or with 10µg/ml HK SL1344. IFN-γ, IL-17, IL-10 and IL-2 production was measured by ELISA. Bars represent mean and standard error mean (SEM) of 3 (naïve) or 5 (immunised) mice per group and duplicate wells on ELISA plates per mouse. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 5.8. Splenocyte challenge responses. IL-23 is required for IFN-γ responses at the site of infection. WT (white bars) or p19⁻/⁻ (grey bars) mice were immunised i.p with either PBS (3 mice/group) or 1*10⁶ CFU SL3261 (5 mice per group). 10 weeks later all mice were orally challenged with 1*10⁸ CFU SL1344. 3 days after challenge spleens were processed into single cell suspensions and restimulated for three days (1 day for IL-2) in either media or with 10µg/ml HK SL1344. IFN-γ, IL-17, IL-10 and IL-2 cytokine production was measured by ELISA. Bars represent mean and standard error mean (SEM) of 3 or 5 mice per group and duplicate wells on ELISA plates per mouse. Data are representative of two independent experiments. Statistics were performed using Students t test. * = P<0.05
Figure 5.9. T cell challenge responses. IL-23 is required for CD4+ IFN-γ secretion at the site of infection. WT (closed signs), or p19−/− (open signs) mice were immunised i.p with either PBS (squares) (3 mice/group) or 1×10^6 CFU SL3261 (circles) (5 mice/group). 10 weeks later all mice were orally challenged with 1×10^8 CFU SL1344. 3 days after challenge T cells were purified from pooled splenocytes from individual groups of mice and stimulated for 3 days (1 day for IL-2) in the presence of irradiated APC with 1, 5 10 or 25 µg/ml purified-sonicated C5. Gray rectangles represent irradiated APCs. IFN-γ, IL-17, IL-10 and IL-2 production by purified T cells was measured by ELISA. Geometrical shapes represent mean and standard error mean (SEM) of 3 replicate wells on ELISA plates per group. Data are representative of 2 independent experiments.
Figure 5.10. B cell challenge responses. IL-23 does not affect B cell pro-inflammatory and anti-inflammatory cytokine secretion during secondary challenge with S. typhimurium. WT (closed signs), or p19-/- (open signs) mice were immunised i.p with either PBS (squares) (3 mice/group) or 1*10^6 CFU SL3261 (circles) (5 mice/group). 10 weeks later all mice were orally challenged with 1*10^8 CFU SL1344. 3 days after challenge B cells were purified from pooled splenocytes from individual groups of mice and stimulated with increasing concentrations of HK SL3261. IL-6 and IL-10 production by purified B cells was measured by ELISA. Geometrical shapes represent mean and standard error mean (SEM) of 3 replicate wells on ELISA plates per group. Data are representative of two independent experiments.
Figure 5.11. Antibody responses. IL-23 deficiency results in early, increased Th1 type antibody production by B cells. WT (closed squares), or p19−/− (open squares) mice were immunised with PBS. WT (closed circles) or p19−/− (open circles) mice were immunised with 1*10^6 CFU SL3261. Serum was collected from individual mice at day 0, 7, 10, 31, 51, 67, 66. All mice were challenged at day 69 post immunisation and serum was again collected at day 72 (last time point). Antibody was determined by ELISA. Geometrical shapes represent mean and standard error mean (SEM) for 5 mice per group and two replicate wells on the ELISA plate per mouse. Data are representative of 2 independent experiments. Statistics were performed using two-way ANOVA analysis.
Figure 5.12. Pathology. IL-23 deficiency does not affect immunopathology caused by primary or secondary SL1344 *Salmonella typhimurium* infection. WT (white bars) or p19⁻/⁻ (grey bars) mice were immunised i.p with either PBS (3 mice/group) or 1*10^6 CFU SL3261 (7 mice per group). 10 weeks later all mice were challenged orally with 1*10^8 CFU SL1344. Serum, spleens and livers were collected from individual mice at day 3 post challenge. Serum IFN-γ was determined by ELISA (A). Liver bacterial burdens were determined by serial dilutions of liver homogenate (B). Splenomegaly was determined by spleen weight/total mouse weight ratio (C). Bars represent mean and standard error mean (SEM) for 3 (naïve) or 7 (immunised) mice per group. Data are representative of 2 independent experiments. Statistics were performed using a Students t test. *=P<0.05
Figure 5.13. Pathology. IL-23 deficiency affects weight change during primary infection but not secondary challenge with *Salmonella typhimurium*. WT (blue squares) or p19⁻/⁻ (red triangles) mice were immunised i.p with 1*10⁶ CFU SL3261. Weight change was monitored at day 1, 3, 5, 7, 11 and 42 post immunisation (A). 10 weeks later WT non-immunised (black triangles), p19⁻/⁻ non-immunised (green rectangles), WT immunised (blue squares) and p19⁻/⁻ immunised (red triangles) mice were challenged orally with 1*10⁸ CFU SL1344. Weights were recorded daily for 3 days after challenge (B). Geometrical shapes represent mean and standard error mean from 3 (naïve) or 7 (immunised) mice per group. Data are representative of 2 independent experiments. Statistics were performed using Two-way ANOVA analysis.
Chapter 6

Final Discussion

6.1 General Discussion

Our work addressed the role of DCs in the generation of immunity against *S. typhimurium* infections. DCs are of crucial importance in the initiation of effective immune responses against pathogens, as well as the establishment of peripheral tolerance (Banchereau and Steinman, 1998; Mueller). Understanding how this cell type becomes activated by different antigens and pathogens, and how it subsequently drives immune responses against the vast range of pathogenic organisms may lead to the development of better vaccination strategies, as well as potential methods for controlling disease progression and autoimmune pathology (Banchereau et al., 2009; Figdor et al., 2004).

The ability of DCs to translate signal, received in the periphery, into message for the initiation of adaptive immune responses, posed them as an excellent candidate to investigate the highly controversial and dividing, among the medical and scientific community, topic of the biological role of ultra-high dilutions of Ag.

While the mechanisms of the adaptive arm of immunity required for protection against *Salmonella* are well characterised, the involvement of APCs in the initiation of protective immune responses against this pathogen are less understood. The central aim of this thesis was to reveal aspects of DC biology that might be playing a role in the generation of effective immune responses against *S. typhimurium* infections. We approached this by testing whether
immune responses developed by DCs, altered immunopathology in *Salmonella* infected animals. Furthermore, we investigated whether the novel APC derived cytokine, IL-23, affects the generation of protective immunity in mice.

6.2 Ultra-high dilutions of Ag alter immune response development by DCs

Our aim was to address the most controversial aspect of homeopathy, that of preparations that include no substance. As described in Chapter 3.1, homeopathy is an alternative medical discipline, which often uses preparations that are diluted to such extend that in theory the final solution should constitute of pure water. Our aim was to investigate whether such extreme dilutions of Ag have a biological effect on DCs, and whether DCs incubated in such extreme dilutions of Ag altered immune response development.

Our results were striking. We observed a measurable biological effect on immune response development conferred by DCs that had been pre-treated with ultra-high dilutions of antigen. The reason why in the absence of Ag any biological effect should be observed remains unclear. We did not succeed in determining a possible mode of action for such extreme dilutions. Although our meta analytical study, as well as experiments performed in a “blind” fashion, revealed a positive correlation of extreme Ag dilutions with cytokine production, our experiments would need to be performed by independent laboratories in order to obtain a robust statistical picture of our observations.

Nevertheless, our results suggested that there is a real possibility that extreme dilutions of Ag, which by our own mass-spectrometric investigations (Figure 2.3) were found to constitute of nothing but water, may exert a real
biological effect. In order to determine a mode of action for such dilutions we would need to identify what it is in our preparations that convenes the signal to DCs. It might therefore be interesting to know whether such extreme dilutions of antigen exhibit any measurable physical properties that might be different than those observed in pure, “untreated”, water.

6.3 Mice vaccinated with S.t-pulsed DCs exhibit less severe immunopathology compared to non vaccinated mice.

*Salmonella* infections are characterised by bacterial invasion through M cells residing at the intestinal tract, followed by dissemination to secondary lymphatics, a process facilitated by APCs (Salcedo et al., 2001). *Salmonella* has evolved a number of different mechanisms aimed at hindering immunogenic proteins from APCs, such as in the case of Flic and PhoP (Murata et al., 2007) (Chapter 1.6). Designing effective vaccines against this pathogen requires both understanding the underlying immune mechanisms that confer full protection, as well as understanding how different bacterial virulence factors and immunogenic molecules evade and induce immunity, respectively.

Our aim was to investigate the contribution of DCs in conferring effective adaptive immune responses against *S. typhimurium* infections. We found that *Salmonella* causes severe immunopathology in NRAMP deficient mice characterised by rapid weight loss and increased serum TNF-α (Figure 4.1, 4.2, 4.3) Interestingly, we found that memory immune responses induced by S.t-
pulsed DCs reduced immunopathology caused by *Salmonella* infections (Figure 4.8, Figure 4.9.B, 4.10).

Increased splenocyte IFN-γ, serum IgG2c and IgG2b (Figure 4.11, 4.12) in conjunction with reduced serum TNF-α (Figure 4.10) correlated with reduced splenomegaly and rate of weight loss (Figure 4.9B, 4.8). Our results suggest that, both cell mediated as well as humoral immune responses conferred by DCs reduce *S. typhimurium* induced immunopathology.

The experimental model of transferring DCs as an immunisation method against *Salmonella* is an attractive system that allows for the dissection of immune responses against this pathogen. Identifying the influence individual *Salmonella*-derived proteins have on maturation of different DC subsets, and on the potential of those subsets to generate effective immune responses against *Salmonella*, might enable us to both improve the design and the delivery methods of *Salmonella* vaccines.

### 6.4 IL-23 involvement in immunity

Disease models, such as the murine models for *M. tuberculosis* and *S. typhimurium* infections, suggest that IL-23 can partially rescue Th1 responses in the absence of IFN-γ and recruit IL-17 and IFN-γ secreting T cells to the site of infection (Khader et al., 2007; Khader et al., 2005; Schulz et al., 2008). The functional similarities of IL-23 and IL12p70 (Oppmann et al., 2000), its production by DCs (Smits et al., 2004), its distinct role in inducing proliferation of CD4⁺ CD45RBlow T cells (Oppmann et al., 2000), its importance in the
generation or maintenance of IL-17 responses (Khader et al., 2007) as well as its implication in autoimmune disease pathology (O'Connor et al., 2008) and immune responses against various pathogens (Happel et al., 2005) posed it as a very attractive molecule to study in order to further our understanding of immune response induction, development and regulation.

Our aim was to determine whether the APC-derived cytokine, IL-23, played a role in the induction and maintenance of CD4⁺ T cell and B cell primary and memory responses, respectively, during S. typhimurium infections. Additionally we wished examine whether IL-23 maintained effective CD4⁺ T cell and B cell responses against secondary challenge with S. typhimurium infections.

**6.5 IL-23 induces differential secretion of IFN-γ by spleens and lymph nodes during Salmonella infections**

We found that primary IL-17 responses against S. typhimurium are not affected by the lack of IL-23, (Figure 5.1, 5.2, 5.3). Interestingly IFN-γ secretion during primary stages of infection is significantly elevated in p19⁻/⁻ mice compared to WT mice in the mesenteric lymph nodes but not the spleens (Figure 5.1, 5.2). Given that the live attenuated SL3261 S. typhimurium was injected intraperitonealy, it is possible that IL-23 might be exerting its effect locally, at the site where the pathogen was introduced. Nevertheless, the increased IFN-γ secretion by p19 deficient mice is a surprising observation, since it is contrasting to the literature (Kullberg et al., 2006). Type 1 antibody levels in the serum of p19⁻/⁻ mice followed the same trend as IFN-γ responses (Figure
5.11), suggesting that the early, exaggerated IFN-γ response in those mice contributed towards elevated humoral responses. Live attenuated S.typhimurium infections induced mixed type 1 and type 2 responses characterised by IgG2c, IgG2b and IgG1 (Figure 5.11), further supporting the observation made in our DC transfer experiments, in which heat-killed S. typhimurium-pulsed DCs also induced mixed type 1 and type 2 Ab responses (Figure 4.12). IL-23 deficiency did not affect, however, IgG1 production, suggesting that this cytokine is not involved in the generation of Th2 type Abs. We found that WT mice lost statistically more weight during the initial stage of immunisation than p19−/− mice (Figure 5.13A). This is in accord with our observation that S.t-DC vaccinated animals, which were characterised by increased splenocyte and serum IFN-γ compared to naïve mice lost significantly less weight (Figure 4.8, 4.10, 4.11).

6.6 IL-23 is required for the maintenance of IL-17 responses during S. typhimurium infections

Memory responses in IL-23 deficient mice were characterised by the complete impairment of IL-17 secretion by T cells and splenocytes (Figure 5.4, 5.5). Our data confirm previous reports that have shown that IL-23 promotes maintenance of IL-17 producing cells (Stritesky et al., 2008). It is interesting that splenocytes from mice that had received DCs pulsed with heat-killed S. typhimurium 5 weeks prior to being infected with live S. typhimurium secreted IL-17, in comparison to mice that had received unstimulated DCs, which failed
to secrete any IL-17 after being infected (Figure 4.11). It is unclear whether BMDC transferred into naïve animals can persist for as long as 5 weeks, thereby sustaining an IL-17 producing population of T cells, nevertheless an interesting possibility. In the DC transfer experiments, memory responses were investigated at the 5th week post transfer, while in the live attenuated immunisation experiments memory responses were addressed at the 10th week post injection of live bacteria. The difference in the time post infection/injection might be accounting for the fact that IL-23 deficient mice were unable to elicit memory IL-17 responses. Perhaps investigating for the presence of IL-17 in the live bacterial immunisation experiments at earlier time points might give us a clearer picture about when IL-17 responses collapse in the absence of IL-23.

6.7 IL-23 is required for the induction of tissue specific IFN-γ responses during secondary challenge responses against *Salmonella typhimurium* infections

Surprisingly, and in stark contrast to primary immune responses, we found that during secondary challenge with SL1344, IL-23 deficient mice showed impaired IFN-γ responses in the spleens but not lymph nodes, compared to WT mice (Figure 5.7, 5.8). It is possible that preferential bacterial dissemination to and survival within different tissues is imposing differential immunological strain to different sites, hence contributing towards the observed differences in tissue specific immune responses.
6.8 In conclusion

The work presented in this thesis illustrates the contribution of DCs in the generation of effective immune responses against *S. typhimurium* infections, and the importance of the APC derived cytokine, IL-23, in localised immune responses. Further work addressing IL-23 involvement in localised immune responses against *S. typhimurium* and the role of DCs in generating such tissue specific responses may result in more effective vaccination strategies against this pathogen.


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