T cell polarisation by dendritic cells: a role for Notch ligands?

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A thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh

2009

Declaration

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 acknowledged otherwise.

Alan G. F. Worsley Date

Abstract

The intention of the work described in this thesis was to identify whether the Notch signalling pathway is utilized by antigen presenting cells in order to influence $CD4^+$ adaptive immune responses. The notion that Notch proteins may be involved in polarising $CD4^+$ T cells is relatively recent and most of the work that had been done in this area so far has concentrated on the consequences of Notch signalling within T cells. In contrast, the work that I have done has focussed on Notch ligand expression by antigen presenting cells and addresses the question whether Notch signalling is a redundant, necessary or irrelevant tool in the arsenal of antigen presentation.

Acknowledgements

I have received a great deal of help from many individuals during the course of this study, but several are noteworthy of special attention.

First and foremost, I would like to thank my principal supervisor Dr. Andrew MacDonald for providing invaluable support, advice and encouragement, as well an enormous amount of optimism and enthusiasm. Whether experiments went well, or the going became tough, his patience, understanding and support made this thesis possible.

Special thanks also go to Drs Georgia Perona-Wright, Stephen Jenkins and Tom Barr, whose input and guidance have helped form the bedrock of my understanding and appreciation of science.

I would also like to thank Dimitris Zienkiewicz and Alex Pythian-Adams for discussions, help with experiments and for keeping everything fun. I also greatly appreciate the contributions of Stephanie Long and Lowrie Griffiths for assistance with experiments and discussion of ideas. It has been a great pleasure working with everyone in the Immunology and Infection Research group.

Finally, I would like to thank my family. Although you were half a world away, you helped me every day.

Thank you so much,

Alan

Dedication

This thesis is dedicated to my mother,

I miss you

Abbreviations

AAM∅	alternative activated macrophage
Ab	Antibody
Ag	Antigen
APC	Antigen presenting cell
BCR	B cell receptor
BM	Bone marrow
BM-DC	Bone marrow-derived dendritic cell
CBF1	Centromere binding factor 1
CCR	Chemokine
cDNA	Complementary deoxyribonucleic acid
CD	Cluster of differentiation
CLR	C-type lectin receptor
CSL	CBF1/Su(H)/Lag1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic cell
DC-SIGN	DC-specific ICAM-3 grabbing non-integrin
DNA	Deoxyribonucleic acid
DSL	Delta/Serrate/Lag
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorter

FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
G	Gravitational force
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMF	Geometric mean fluorescence
HES	Hairy-enhancer of split
HRP	Horseradish peroxidase
IκB	Inhibitor of κB
ICOS	Inducible co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
Lnfg	Lunatic Fringe
M∅	Macrophage
M-CSF	Macrophage-colony stimulating factor
MAML	Mastermind-like protein
MAP	Mitogen activated protein
MLN	Mesenteric lymph node
mg	Milligram
MHC	Major histocompatibility complex
ml	Millilitre
MLR	Mixed leukocyte reaction
MyD88	Myeloid differentiation factor 88

MZB	Marginal zone B cells
NFκB	Nuclear factor κB
N^{ICD}	Notch intracellular domain
NK	Natural killer cell
NO	Nitrous oxide
ng	Nanogram
Ova	Chicken egg ovalbumin
Pa	*Propionibacterium acnes*
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PRR	Pathogen recognition recptor
PBS	Phosphate buffered saline
pDC	plasmacytoid dendritic cell
PGE_2	Prostaglandin E_2
RANTES	Regulated on activation normal T cell expressed and secreted
RBJκ	Recombination binding protein - Jκ
RELMα	Resistin-like molecule alpha
RIG-1	Retinoic acid-inducible gene 1
RLR	RIG-1-like receptor
RNA	Ribonucleic acid
SEA	Soluble Egg Antigen
St	*Salmonella typhimurium*
STAT	Signal transducer and activator of transcription

Su(H)	Suppressor of Hairless
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIRAP	Toll-IL-1 receptor domain-containing adaptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cell
TRIF	Toll-IL-1 receptor domain-containing adaptor IFN-β
°C	Degrees centigrade
μg	Microgram

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Chapter 1

Introduction

1.1 Bridging Innate and Adaptive Immunity

To successfully combat disease an organism must employ a measured and effective defensive mechanism. In mammals, it is critical that our immune response is capable of controlling infection and yet maintain the well-being of the host. This is accomplished by the cooperation of the innate immune system and the pathogen-specific response elicited by the adaptive immune system. During immune challenge, antigen presenting cells (APCs) such as dendritic cells (DCs) and macrophages (MØs), can direct $CD4^+$ T cells into a wide range of fates, including Th1, Th2, Th17 and regulatory T cells and thus influence the outcome of the entire adaptive immune response (Zhu and Paul, 2008). Understanding the molecular mediators involved in this crucial juncture between innate and adaptive immunity has been a focus of immunological research for decades (Germain, 2004; Medzhitov and Janeway, 1997).

The innate immune system comprises a wide variety of mechanisms immediately available for combating infectious diseases. It represents the front line of host defence, involving physical barriers presented by epithelial layers, chemical defences including antibacterial peptides, complement and lytic enzymes, as well as biological responses from innate immune cells patrolling the periphery (Fleer and Krediet, 2007). Importantly, however, innate responses are not thought to lead to any lasting immunity, nor are they specific for any particular pathogen. The defensive arsenal employed by the innate immune system is effective in combating many pathogens, however this system is constrained by relying on a limited and invariable

repertoire of pattern recognition receptors (PRRs) (Akira *et al.*, 2006). The active innate cell types tasked with seeking, recognising and destroying pathogens are phagocytic cells, predominantly composed of MØs and neutrophils. By using a combination of antimicrobial peptides, Nitric oxide (NO) and lysosomes containing lowered pH and lytic enzymes, MØs and neutrophils can eliminate pathogens without the aid of the adaptive immune system (Aderem and Underhill, 1999; Dale *et al.*, 2008).

It is only when innate host defences are impeded, avoided, or overwhelmed that the adaptive immune system is needed. In contrast to innate immunity, an adaptive immune response is specific for particular antigens (Ags) and is also capable of instilling immunological memory, so that prior infections trigger stronger and more immediate responses to subsequent infections (Ahmed and Gray, 1996; Dutton *et al.*, 1998; Swain *et al.*, 1996). The key aspect of the adaptive immune system is the inherent flexibility in its capacity to directly target foreign agents. This is made possible by the specialised antigen receptors on the surface of lymphocytes, which are generated by highly mutagenic and variable recombination events, ensuring that individual lymphocytes produce numerous copies of a single antigen receptor with a unique binding site (Bassing *et al.*, 2002). Once primed, these lymphocytes undergo differentiation into pathogen-specific effector cells, in addition to memory cells, which allow the immune system to "remember" previous infectious encounters (Kallies, 2008).

It is at the interface of these two defence systems that APCs play their part in the fight against infection. By first assisting in the recognition of invading organisms, and then directing the lymphocyte response to that pathogen, APCs play a critical

role in the determination of the overall tone of the entire immune response (Banchereau and Steinman, 1998; Kapsenberg, 2003). Depending upon the pathogen-associated molecular patterns (PAMP) stimulus, APCs are primed for microbicidal activity or antigen presentation (Janeway and Medzhitov, 2002). A main premise of my work is ascertaining how these cells communicate the information necessary between innate recognition and the initiation and coordination of the adaptive immune response.

1.2 Antigen Presenting Cells

A central theme of immunology in the early 1970s was understanding the mechanism of 'immunogenicity', the process by which Ag provokes an immune response (Steinman, 2007). A key component of this process was shown to be "immune response genes", primarily major histocompatability complex (MHC) (Vyas *et al*., 2008). In humans, the genes encoding the antigen presenting proteins of the MHC, known as human leukocyte antigen (HLA) are found on chromosome 6 and are recognised as the most variable region in the genome (Blackwell *et al*., 2009). For MHC class I these genes include HLA-A, -B, -C, -E, -F and –G, while class II genes are comprised by HLA-DR, -DQ, -DM and –DP. The primary function of MHC I is to present antigens derived from intracellular processes, such as viral infections or intracellular bacteria, while MHC II exhibits antigens sampled from the extra-cellular milieu (**Figure 1.1**). In the case of MHC I, intracellular peptides are generated during catabolism of endogenous proteins within the cytoplasm. The generation of these peptides enlists specific proteases which degrade and process proteins into eight to ten amino acid fragments (Rock *et al*., 2004; York *et al*., 1999).

The proteases involved are typical of the normal turnover and degradation of proteins including the ubitquitin-proteasome system and aminopeptidases in both the cytoplasm and endoplasmic reticulum such as leucine aminopeptidase (Beninga *et al*., 1998), puromycin-senstive aminopeptidase (Stoltze *et al*., 2000), bleomycin hydrolase (Stoltze *et al*., 2000), tripeptidyl peptidase II (TPPII) (Seifert *et al*., 2003; Tanioka *et al*., 2003) and L-RAP (Tanioka *et al*., 2003). These aminopeptidases are required for trimming the precursor peptides released from the proteasome as these peptides typically contain an (N) terminal extension of several amino acids (Kloetzel, 2004).

Following degradation, processing and trimming, intracellularly derived peptides are then transported to the endoplasmic reticulum by the transporter associated with antigen processing (TAP) (Kloetzel, 2004). Further trimming takes place by ER-resident aminopeptidases to produce peptides of the appropriate size to bind and stabilise MHC I molecules (Strehl *et al*., 2005). In this event, the MHC I heavy chain first assembles with β_2-microglobulin and then with the peptide-loading complex (Jensen, 2007). Several of the proteins involved in peptide loading and stable MHC complexes including tapasin, TAP1, TAP2, β_2-microglobulin and proteasome components are upregulated upon exposure to IFN-γ (Peaper and Cresswell, 2008). Once MHC I has been properly folded and peptide-bound it is transported to outer the cell membrane.via the Golgi complex where antigen can be presented to $CD8^+$ T cells (Jensen, 2007).

In contrast to the MHC I pathway, MHC II antigen presentation focuses on potential extracellular pathogens by sampling antigens via the endocytic pathway (Jensen, 2007). Extracellular antigens are placed within the phagosome which then

fuses with lysosomes to form a phagolysosome. It is within this compartment that extracellular antigens first interact with nascent MHC II molecules (Bryant and Ploegh, 2004). MHC II is formed by α and β chains as well as an invariant chain within the endoplasmic reticulum (ER) (Lamb and Cresswell, 1992; Marks *et al.*, 1990). The invariant chain occupies the peptide-binding site preventing premature peptide loading. After fusion with the endosomal vesicles, lysosomal proteases gradually degrade the invariant chain leaving behind an internal segment (CLIP) (van Niel *et al.*, 2008). Importantly, the generation of CLIP by cathepsin S-mediated proteolysis removes the previous targeting information embedded in the cytoplasmic domain of the invariant chain, as well as liberating the MHC II-αβ dimers (van Niel *et al.*, 2008). Once CLIP has been replaced by exogenous antigen with the aid of the MHC-like molecule HLA-DM, the MHC-peptide complex is thought to be transported to cell surface by transforming the MHC II-containing vesicles into tubular structures directed towards the site of T cell interaction (Boes *et al.*, 2003; Vyas *et al.*, 2007). Once on the cell surface, peptide loaded MHC II is ready to present antigen to $CD4^+$ T cells.

Most cells have the capacity to present peptides on MHC I molecules, which is essential for identification of virus-infected cells by cytotoxic T lymphocytes, whereas the cells which express MHC II are far more exclusive. Select cell types, particularly DCs, are less stringent in their differentiation between endogenous and exogenous Ags and both sources of Ags can be presented by MHC I (Heath *et al.*, 2004). The ability to process and present exogenous Ag on MHC I is referred to as 'cross presentation' (Heath *et al.*, 2004).

Cells which are capable of expressing Ag upon MHC II receptors can be broadly defined as APCs, prime examples of which include DCs, MØs and B cells (Jensen, 2007). Other cell types can be induced to express MHC II during infection including eosinophils (Shi, 2004), mast cells (Mekori and Metcalfe, 1999; Stelekati *et al*., 2007) and even γδ T cells (Scotet *et al*., 2008). Although under certain conditions all of these cell types may carry out the process of antigen presentation, the primary APCs are considered to be DCs, MØs and B cells (Jensen, 2007; Vyas *et al*., 2008). The designation of DCs as 'professional' APCs is due to their constitutive expression of MHC II, Ag acquisition via phagocytosis, expression of pathogen recognition receptors (PRRs) and the capacity to influence $CD4^+$ T cell differentiation through both co-stimulatory expression as well as cytokines (Janeway and Medzhitov, 2002).

1.2.1 B cells

B lymphocytes are understood best for their role in humoral immunity. By differentiating into memory cells or plasma cells and secreting antigen-specific antibodies, B cells act as the first line of defence for adaptive immunity and provide the mechanism by which most vaccines work (Chen and Jensen, 2008; Gray *et al.*, 2007; Parker, 1993). However, the capacity for B cells to function as APCs, providing both co-stimulatory molecules and cytokines, while influencing T cell differentiation *in vivo* should not be neglected. Historically, B cells have been considered poorer APCs due to *in vitro* studies demonstrating inefficient presentation of Ag that was not specific for their B cell receptor (BCR) (Sallusto and Lanzavecchia, 1994). Although B cells provided with Ag that is recognisable by their

BCR present Ag as efficiently as DCs (Sallusto and Lanzavecchia, 1994), the fact that there are very few antigen-specific B cells during the beginning of infection appears to rule out a substantial role for B cells as the initiators of T cell priming. Furthermore, other work has shown that B cell-deficient mice can be fully capable of inducing T cell responsiveness (Chen and Jensen, 2008; Epstein *et al.*, 1995; Vella *et al.*, 1996). These results have led to the perception that B cells are not as proficient at inducing naïve T cell differentiation as DCs and that, instead, they are much better at displaying Ag to previously expanded T cell populations (Gray *et al.*, 2007).

1.2.2 Macrophages

DCs and MØs are ubiquitously distributed cells that fulfil significant roles in the immune system. Both are bone marrow-derived with potent phagocytic properties and the capacity to induce T cell polarization (Banchereau and Steinman, 1998). During inflammation MØs have three major functions; phagocytosis, antigen presentation and immunomodulation via cytokine production (Fujiwara and Kobayashi, 2005). Although MØs participate in the induction of an immune response through antigen presentation and the consequent polarisation of naïve T cells, evidence suggests that their primary function is in combating infectious organisms through the induction of antimicrobial molecules (Martinez-Pomares and Gordon, 2007; Seljelid and Eskeland, 1993).

Recently MØ activation has been shown to be plastic, rapid and fully reversible depending on the stimulus, indicating that in addition to combating inflammation, MØs also participate in its resolution (Benoit *et al.*, 2008; Fujiwara and Kobayashi, 2005). MØs have also been found in most tissues and rapidly

redeploy to sites of infection or inflammation (Pozzi *et al*., 2005). Early studies implied that MØs were responsible for the stimulation of primary immune responses. However, at the time it was not known that DCs could acquire and cross-present Ag from other cells (Askonas *et al*., 1968; Pozzi *et al*., 2005). More recently, it has been shown by adoptive transfer that MØs can prime naïve CD8$^+$ T cells to proliferate and mature into both effector and memory cells (Pozzi *et al*., 2005).

Although less well understood than their proinflammatory role during acute microbial infection, it is becoming increasingly clear that macrophages can also display an 'alternative' activation state triggered by Th2 cytokines such as IL-4 and IL-13 (Gordon, 2003). These alternatively activated macrophages (AAM∅s) have been suggested to be involved in both wound healing and tissue remodelling during Th2 infections and allergy (Loke *et al.*, 2002; Nair *et al.*, 2005; Sandler *et al.*, 2003). Importantly, reliable molecular indicators for AAM∅s have been identified including Ym1, RELMα and arginase 1 (Hesse *et al.*, 2001; Nair *et al.*, 2005; Raes *et al.*, 2002a). However, the origins of these cells, as well as the mediators other than Th2 cytokines necessary for their induction, remain largely unknown (Loke *et al.*, 2007).

1.2.3 Dendritic Cells

Steinman and Cohn identified distinct morphological features, including 'dendrites', within spleen cells prepared from mouse peripheral lymphoid organs in 1973 and named these cells DCs (Steinman and Cohn, 1973). Concurrent work by Knight and others illustrated the trafficking of veiled cells (later described as DCs) and their interaction with lymphocytes as activators and as the carriers of contact

sensitizers (Balfour *et al*., 1985; Knight *et al*., 1982). Unfortunately, purifying this new cell type from the body to enable further characterisation proved to be difficult, both due to their low frequency (1-2% of the total leukocytes) and a lack of available identification markers (Steinman and Cohn, 1973). With the advent of reliable *in vitro* culture techniques, a wide array of functional and developmental studies were then able to be carried out, and it became clear that multiple and heterogenous subsets of DCs exist (Gluckman *et al*., 1997; Liu, 2001; Lutz *et al*., 1999; O'Garra and Trinchieri, 2004; Sato and Fujita, 2007; Shortman and Liu, 2002; Wilson and O'Neill, 2003).

DC progenitors in the bone marrow give rise to circulating precursors that migrate towards tissues, where they reside in an immature state in search of pathogens or danger signals (Banchereau *et al*., 2000; Matzinger, 1998). In an immature state DCs retain a heightened capacity for endocytosis and phagocytosis but have low expression of both MHC and co-stimulatory molecules (Sato and Fujita, 2007). Following capture of Ag, DCs migrate towards lymphoid tissue and proceed to initiate naïve T cell immune responses from Ag-specific T cells (Banchereau *et al*., 2000). During the migration process, DCs also acquire a mature phenotype, including changes in morphology, increased MHC and costimulatory marker expression, loss of endocytic capacity and phagocytic receptors and the activation of Ag-processing machinery (Reis, 2006). Functionally mature DCs then migrate to the lymph nodes, facilitated by the expression of chemokine receptor CCR7, whose ligands are produced by the cells of the lymphatic vessels or in the T cell areas of secondary lymphoid tissue (Gunn *et al*., 1999; Kellermann *et al*., 1999; Sallusto *et al*., 1999).

The tissue in which DCs reside appears to influence the subset into which they develop, which may play a role in their comparative capacity to be either phagocytic and migratory or present Ag and polarise T cells. Within lymphoid tissues, follicular DCs and germinal center DCs participate in activation and selection of B cells, or as strong APCs for resident T cells, respectively (Sato and Fujita, 2007). Thymic DCs, meanwhile, are suggested to be involved in the negative selection of T cells (Gallegos and Bevan, 2006). DCs present within the epidermis are unusually slow in their migration towards lymphatic tissue (Valladeau *et al.*, 1999) and, as a result, are thought to play a largely immunoregulatory role, as they arrive after immune responses are underway (Kissenpfennig *et al.*, 2005; Randolph *et al.*, 2008).

The basic definition of murine DCs is typically confined to cells expressing CD11c, MHC II, and a combination of CD4, CD8α, CD11b and CD205 (Sato and Fujita, 2007). Murine DCs display a great deal of heterogeneity in this regard, with subsets including $CD4^{+}CD8\alpha^{-}$, $CD4^{-}CD8\alpha^{+}$, $CD4^{-}CD8\alpha^{-}$ (DN) 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 αβ-heterodimer as is the case on T cells (Sato and Fujita, 2007). Although both $CD8\alpha^{+}$ and $CD8\alpha^{-}$ DC subsets appear equally competent at driving T cell proliferation, in the past, $CD8\alpha^{+}$ DCs were considered the 'lymphoid' population which could be induced to secrete greater quantities of IL-12, whereas $CD8\alpha^{-}$ DCs were thought to be the 'myeloid' population, which skew T cell responses towards Th2 (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1997; Pulendran *et al.*, 1999). However, this perception is now thought to have been misconceived, given that expression of CD8α does not

necessarily delineate the haematopoietic origin of the DC precursor and that the nature of the DC precursor may bias, but does not determine, the phenotype of the resulting DC (Manz *et al*., 2001; Wu *et al*., 2001b). Ultimately, DCs and their precursors have demonstrated remarkable functional plasticity and are able to differentiate into different types of DC after encounters with various stimuli (Banchereau and Palucka, 2005).

DCs have traditionally been considered to be the most proficient APC, as evidenced by mixed leukocyte reactions (MLR), which were used at the time as a model for graft rejection (Inaba and Steinman, 1984; Nussenzweig *et al*., 1980; Steinman and Witmer, 1978). The MLR utilizes the genetic incompatibility between T cells and MHC-expressing cells, which is quite frequent given the extent of polymorphism within MHC, leading to a T cell response (Steinman, 2007). These studies demonstrated that DCs were approximately 100 times more proficient at driving T cell proliferation than total spleen cells, despite accounting for only 1% of the splenic population (Steinman and Witmer, 1978). Subsequent work showed that, by upregulating co-stimulatory molecules and migrating to T cell areas of lymphoid organs, DCs are able to activate and expand antigen-specific naïve T cells (Banchereau and Steinman, 1998).

1.3 Recognising Pathogens

One of the conundrums of immunology in the 1970s and 1980s was how the adaptive immune system could discriminate between self and non-self (Matzinger, 1998). It was recognised by Charles Janeway that one of the keys tasks of the innate immune system during the initial encounter with a pathogen is to discriminate

between non-infectious self and infectious non-self (Janeway, 1989; Janeway and Medzhitov, 2002). This hypothesis posited that, in addition to their role in bacterial killing and phagocytosis, APCs can discern the nature of the stimulus by expressing PRRs able to recognise evolutionarily conserved PAMPs (Janeway, 1989). In the intervening years, it has become clear that these various PRRs are crucial for linking the innate immune system to the appropriate activation of the adaptive immune system and provide a bridge between innate recognition and the engagement of acquired immunity (Fleer and Krediet, 2007).

1.3.1 *Toll-like* Receptors

Following Janeway's remarkably prescient postulation, a major breakthrough in innate recognition occurred when human homologues of the evolutionarily conserved set of *Drosophila* receptors, known as 'Toll' receptors, were discovered (Medzhitov *et al*., 1997). These Toll receptors were crucial both for dorsal-ventral development in fly embryos (Belvin and Anderson, 1996) as well as, importantly, antifungal defence (Lemaitre *et al*., 1996). The human homologue to the *Drosophila* Toll receptor was called the *Toll-like* receptor (TLR), and was later found to be the receptor recognising lipopolysaccharide (LPS), which is now designated TLR4 (Poltorak *et al*., 1998).

TLRs are type1 membrane proteins characterized by an ectodomain capable of PAMP recognition as well as a cytoplasmic Toll/IL-1 receptor (TIR) region required for downstream signalling (Fleer and Krediet, 2007). To date there are 11 different human TLRs and 13 TLRs in mice, each recognizing a unique set of microbial agents (Janeway and Medzhitov, 2002; Kawai and Akira, 2007; Pasare and

Medzhitov, 2004b). TLRs can be categorised into several groups depending on the types of PAMPs they recognise. TLRs 1, 2, 4 and 6 all recognise lipids components. For example, as mentioned above, TLR4 recognises the LPS component of Gram-negative bacteria such as *Salmonella typhimurium* (Pasare and Medzhitov, 2004a). Many TLRs form heterodimers with either other TLRs, or non-TLR proteins such as CD36 for increased PAMP recognition capacity. TLR2, for example, is able to form heterodimers with TLR1 or TLR6 in order to discern lipoproteins or Gram-positive bacteria, petidoglycans and zymosan (Akira *et al*., 2006). TLR5 and TLR11 recognise protein ligands including bacterial flagellin (Kawai and Akira, 2007). TLRs 3, 7, 8 and 9, meanwhile, detect foreign nucleic acids and are localized intracellularly, typically in endosomes (Kawai and Akira, 2007). TLR3 has been shown to recognise double-stranded RNA, TLR7 and TLR8 detect single-stranded RNA as well as imidazoquinoline-like molecules, while TLR9 is activated by CpG DNA (Akira *et al*., 2006). Thus, merely through the expression of TLRs alone, there is a great deal of diversity in the range of organisms that are recognisable by innate immune cells.

1.3.2 Other PRRs

In addition to TLRs, currently the most extensively studied of PRRs, a range of other molecules exist that may fulfil a similar pattern-recognition function. The retinoic acid-inducible gene 1 (RIG-1) like receptors (RLRs) bind viral double-stranded RNA (Thompson and Locarnini, 2007). Similar to TLRs, signalling through RLRs can induce the production of proinflammatory cytokines and type I interferons by innate immune cells, through the transcription factor NF-κB (Kaisho and Tanaka,

2008; Saito and Gale, 2008). Furthermore, the nucleotide-binding oligomerization domain (NOD) -like family of receptors function as PRRs within the cytoplasm, detecting bacterial-associated products such as peptidoglycans (Creagh and O'Neill, 2006; van Vliet *et al*., 2007). Finally, C-type lectin receptors (CLRs), which normally function as cell-cell adhesion molecules, have also been shown to recognise pathogens and facilitate antigen presentation (van Vliet *et al*., 2007). CLRs such as dendritic cell-specific intercellular adhesion molecule-3-grabbing non- integrin (DC-SIGN) and dendritic cell-associated C-type lectin 1 (DECTIN-1) are specific for glycans, including mannose structures and Lewis antigens, which are expressed by a variety of pathogens including viruses, bacteria and even the parasitic helminth *Schistosoma mansoni* (Meyer et al., 2005; Saunders et al., 2008; van Liempt et al., 2007).

1.3.3 TLR Signalling

After recognising their respective PAMPs, TLRs recruit adaptor proteins capable of perpetuating the pathogen recognition signal (**Figure 1.2**). Adaptor proteins utilised in this fashion include myeloid differentiation factor 88 (MyD88), TIR domain-containing adaptor protein (TIRAP), TIR-domain-containing adaptor-inducing interferon-β (TRIF) and translocating chain-associating membrane (TRAM). MyD88 is a universal adaptor shared by all TLRs except TLR3, signalling through which ultimately leads to translocation of NF-κb to the nucleus, as well as the activation of MAP kinases (MAPK) (Underhill and Ozinsky, 2002). Importantly, TLR engagement induces the production and release of inflammatory signals by

innate immune cells including IL-1β, TNFα, IL-6, IL-10 and IL-12, chemokines and antimicrobial peptides (Kumagai *et al*., 2008).

The association of TLRs with MyD88 stimulates the recruitment of members of the IL-1 receptor-associated kinases (IRAK), which then disassociate from MyD88 and interact with TNF receptor associated factor 6 (TRAF6). This interaction activates TGF-β activated kinase 1 (TAK1), a kinase in the MAPK family which, in turn, activates the IκB kinase (IKK) complex responsible for targeting the degradation of IκB and the subsequent translocation of NF-κB to the nucleus (Kawai and Akira, 2007). MyD88-deficient mice show a failure to activate NF-κB and induce inflammatory cytokines in response to TLR2, 5, 7 and 9 specific PAMPs. However, evidence suggests that a MyD88 independent pathway exists in TLR3 and TLR4 signalling. In these cases, TRIF was identified as an essential adaptor of the MyD88 independent pathway (Oshiumi *et al*., 2003; Yamamoto *et al*., 2002). TRIF-deficient mice display defective IFN-γ induction after LPS and poly IC stimulation (Hoebe *et al*., 2003; Yamamoto *et al*., 2003). NF-κB as well as MAPK activation is completely abolished in TRIF and MyD88 double deficient mice following LPS stimulation (Yamamoto *et al*., 2003).

However, the specificity of these responses is incompletely understood. It is thought that the association of these different intracellular signalling pathways, along with heterodimerisation and synergy between PRRs, cooperate to determine the appropriate host response (Kumagai *et al*., 2008). The extent to which a pathogen activates a variety of separate PRRs ultimately determines APC maturation state, achieving a specifically tailored activation status, capable of orchestrating the overall adaptive immune response (Napolitani *et al*., 2005).

Specific pairing or sets of different PRRs have been proposed to mediate host immunity. Dectin-1 and TLR-2 have been proposed to collaborate in the host response to fungal pathogens, and the activation of both pathways works synergistically in order to drive optimal cytokine, chemokine and co-stimulatory marker production by DCs primarily, but also by other APCs (Dennehy *et al.*, 2008). It seems evident that pathogens activate a cascade of different PRRs and it is the synergy and collaboration of these pathways that ultimately leads to a specific maturation profile.

1.4 APC Maturation

The ultimate aim of APC activation is to translate environmental signals into a definitive pathogen-specific response and to dictate the fate of T cells capable of responding to that pathogen. Following stimulation in response to pathogens or danger signals, APCs generally lose their responsiveness to subsequent pathogen stimuli, while upregulating production of certain cytokines, co-stimulatory molecules and chemokine receptors (such as CCR7), in order to migrate towards the lymphoid tissue (Banchereau and Steinman, 1998; Kapsenberg, 2003). Following such migration, the activation and polarisation of naive $CD4^+$ T cells is thought to be determined by three signals provided by the APC. The first signal is provided by peptide presented in the context of MHC II, which is bound by the appropriate T cell receptor- (TCR) bearing $CD4^+$ cell. The second signal is co-stimulation provided by the APC, primarily mediated via CD28 on the T cell and CD80/CD86 on the APC, in whose absence T cells become anergic (Kapsenberg, 2003). Finally, 'signal 3' represents the polarising signal provided by the APC, typically thought of as a

combination of cytokines and other molecules that could influence Th1, Th2, Th17 (or other) development (Kalinski *et al.*, 1999; Kapsenberg, 2003).

It is quite important to separate two concepts with regards to DC maturation. As detailed by Reis e Sousa (2006), there is phenotypic maturation, as defined by the upregulation of MHC II and to co-stimulatory markers, as well as a functional maturation, wherein APCs acquire the ability to induce immunogenic T cell responses (as opposed to tolerance) (Reis, 2006). A functionally-mature APC utilises its arsenal of T cell polarising signals, as dictated by the nature of the stimulus encountered, in order to drive a pathogen-specific response. Characterising the scope of this arsenal in relation to different pathogens has become an underlying goal for understanding the interface between innate and adaptive immunity.

1.4.1 Signal 2

The second signal provided to CD4$^+$ T cells during antigen presentation provides a crucial stimulus-driving effector function versus T cell anergy. Broadly, co-stimulatory molecules are defined as signals induced by ligation of membrane-bound molecules that either synergize with or modify the signal provided through TCR-MHC engagement (Croft, 2003). Different profiles of activation marker expression can result in distinct APC phenotypes capable of directing different outcomes of T cell differentiation (as well as T cell tolerance) (Kapsenberg, 2003). Augmented expression of cell surface co-stimulatory molecules on APCs is one of the most significant early developments of early immune system activation (Carreno and Collins, 2002; Collins *et al.*, 2005; Greenwald *et al.*, 2005). The best characterised co-stimulatory molecules consist of two B7 family members CD80

(B7-1) and CD86 (B7-2), which bind to the same two receptors, CD28 and CTLA-4 (CD152). CD28 is constitutively expressed on naïve T cells while the expression of CTLA-4, considered an inhibitory signalling mechanism, is upregulated on the T cell surface following activation (Carreno and Collins, 2002; Wang and Chen, 2004). Signalling through CD28 provides a potent signal in conjunction with an activated TCR, resulting in the induction of IL-2 and expression of CD25, and entry into the cell cycle (Alegre *et al.*, 2001; Carreno and Collins, 2002; Greenwald *et al.*, 2005). CD86 or CD80 binding to CD28 provides an important additional biochemical signal that enhances and prolongs those transduced by the TCR and its CD3 complex (Crow, 2006). There is some evidence that CD80 and CD86 may also play a role in retrograde signalling to APC, which can result in the induction of innate-effector function in M∅s (Khan *et al.*, 2007). Recently, five new members of the B7 family have also been discovered, 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.

1.4.2 Signal 3

Which subset a naïve $CD4^+$ T cell will differentiate into is thought to be largely determined by the various molecular signals expressed by APCs during antigen presentation, sometimes termed ‘signal 3’ (Kapsenberg, 2003). The cytokines produced in the microenvironment in which naïve T helper cells are stimulated, are key to the development and regulation of the immune response. APC-derived IL-12 has a direct and critical role in the Th1 process, stimulating production

of IFN-γ by T cells (Kobayashi *et al*., 1989), although its provision by APC may not be absolutely necessary for Th1 induction in all cases (MacDonald and Pearce, 2002; Soares *et al*., 2007). IL-6 produced by DCs, M∅s and B cells has been thought to play a crucial role in T cell activation by inhibiting T regulatory cell-mediated suppression (Pasare and Medzhitov, 2003). More recently IL-6 is has been shown to play a role moderating between the generation of Th17 T cells and T regulatory cells (Bettelli *et al*., 2006; Stockinger *et al*., 2007; Veldhoen *et al*., 2006), although APCs have yet to be shown to be the definitive source for this cytokine. However, in conjunction with IL-10, IL-6 is involved in a wide range of actions including the inhibition of Th1 cell responses (Fickenscher *et al*., 2002; Groux and Powrie, 1999; Langer *et al*., 2004; Moore *et al*., 2001; Wolk *et al*., 2002). In DCs, IL-10 can inhibit MHC II, CD86 and CD54 expression and suppress IL-1, IL-12 and tumour necrosis factor-α (TNFα) transcription, which is thought to be crucial for its anti-inflammatory abilities (Liu *et al*., 2004; Steinbrink *et al*., 1997). TNFα is a highly pluripotent cytokine, but can be broadly considered a pro-inflammatory mediator and is considered a key player in processes such as septic shock (Wajant *et al*., 2003).

In addition to cytokines, a range of surface molecules may provide signal 3 for T cells. CD40 is a cell surface receptor whose association with T cell CD154 is thought to be essential for immune regulation as well as activation and function of APCs (Grewal and Flavell, 1998; van Kooten and Banchereau, 2000). DCs exposed to Th1 polarising Ag generally display increased CD40 expression, and ligation of CD40 with CD40L-expressing cells enhances their Th1-promoting capacity (de Jong *et al.*, 2002). During cross-talk with T cells, CD40-CD40L results in the sustained activation of NF-κB and other transcription factors. This interaction in turn drives

increased expression of other T cell-polarizing factors, such as IL-12 (Hilkens *et al.*, 1997; O'Sullivan and Thomas, 2002; Snijders *et al.*, 1998).

OX40 (CD134) is another co-stimulatory molecule from the TNFR super family which is not expressed on resting T cells, but can be induced between 12-24 hours after TCR/CD3 signalling (Gramaglia *et al.*, 1998). Expression of OX40L by DCs, as well as other APCs, has been shown to be induced following activation; and its expression can be enhanced following CD40 signalling (Croft, 2003; Gramaglia *et al.*, 1998; Murata *et al.*, 2000; Stuber *et al.*, 1995). Recently, OX40L has been shown to be particularly important for Th2, but not necessarily crucial for the generation of Th1 responses (Chen *et al.*, 1999; Jenkins *et al.*, 2007).

1.5 Effector T helper Cells

The purpose of antigen presentation is to activate T cells specific to the ongoing immune challenge, inducing proliferation and polarisation. The polarisation into specialised subsets of $CD4^+$ effector T cells (**Figure 1.3**) is critical for mounting an effective response to diverse types of infectious micro-organisms. Originally, two distinct T helper subsets, named T helper 1 (Th1) and T helper 2 (Th2), were identified by their distinct expression of cytokines and functions (Coffman and Mosmann, 1991; Mosmann *et al*., 1986). Later work established the $CD4^+$ T cell lineages of T regulatory cells (Chen *et al*., 1994) and, most recently, Th17 cells (Weaver *et al*., 2006). Th cells play critical roles in orchestrating adaptive immune responses through the secretion of cytokines and chemokines that function to both activate and/or recruit other cells types (Zhu and Paul, 2008).

1.5.1 Th1

Th1 CD4$^+$ effector cells are integral to the proinflammatory responses against intracellular pathogens such as bacterial or viral infections, as well as some protozoan and fungal infections. This is mainly carried out through the production of IFN-γ and other inflammatory cytokines, which in turn enhance the microbicidal activity of innate immune cells (Zhu and Paul, 2008). Th1 cells produce high levels of IFN-γ, lymphotoxin α and IL-2, along with expressing the transcription factors T-bet (Szabo *et al.*, 2000) and Stat1 (Lighvani *et al.*, 2001). IFN-γ production is important for activation of M∅s (Suzuki *et al.*, 1988), as well as the recruitment of lymphocytes and NK cells (Agnello *et al.*, 2003; Zhu and Paul, 2008). IL-2, as well as being a T cell growth factor, is crucial for stimulation of CD8$^+$ cells and the generation of Th1 memory (Darrah *et al.*, 2007).

1.5.2 Th2

Th2 cells mediate host defence against extracellular parasites, including helminths, and are also important for the induction of allergic responses (Mosmann *et al.*, 1986). T helper 2 (Th2) cells upregulate the transcription factor GATA3 and produce cytokines such as IL-4, IL-5, IL-9, IL-10, IL-13, IL-25 and IL-33 (Anthony *et al.*, 2007; Perrigoue *et al.*, 2008; Romagnani, 2000). The overall process mediating Th2 effector immunity is less well-understood than Th1, but involves the recruitment of eosinophils and mast cells, as well as class-switching to the release of IgE antibodies. IgE binds to the FcεRI on basophils and mast cells leading to the secretion of active mediators such as histamine and serotonin (Le Gros *et al.*, 1990; Swain *et al.*, 1990; Zhu and Paul, 2008). Th2 cytokines are also responsible for the

induction of goblet cell hyperplasia and mucin production, recruitment of alternatively activated macrophages (AAMacs), and increase epithelial cell turnover and muscle hyper-contractility, but inhibit the functions of phagocytic cells (Anthony *et al*., 2007; Perrigoue *et al*., 2008; Romagnani, 2000). IL-4 is one of the key Th2 mediators and is required for the maintenance of Th2 differentiation, as T cells from IL-4$^{-/-}$ mutant mice failed to produce Th2-derived cytokines (Kopf *et al*., 1993). IL-13 is produced by activated T cells and is the main effector cytokine for the expulsion of helminths, the induction of airway hypersensitivity and fibrosis (Wynn, 2003). IL-5 also plays an important role in the recruitment of eosinophils in addition to its effects on mast cells and Ig class switching (Coffman *et al*., 1989).

1.5.3 T Regulatory Cells

A third regulatory subset of CD4^{+} T cells (Treg) plays an essential role in the maintenance of a balanced response that is not harmful to the host while effectively resisting infection (Vignali *et al*., 2008). Treg cells are typically defined by high expression of the IL-2 receptor α chain (CD25), and transcription factor forkhead box P3 (Foxp3) (Askenasy *et al*., 2008). Regulatory T cells are thought to operate primarily at sites of inflammation, modulating immune reactions via cell-to-cell contact (Piccirillo *et al*., 2002). Using mechanisms such as perforin/granzyme, or Fas-ligand, Treg cells can directly kill effector cells either in the lymph nodes or in the target tissues; and thus deplete T cell effector functions (Banz *et al*., 2002; Gondek *et al*., 2005; Weber *et al*., 2006). Additionally, Treg cells can inhibit effector cell production or secretion of cytokines, such as IL-2, which are involved in the maintenance of an immune reaction (Piccirillo and Shevach, 2001; Thornton and

Shevach, 2000) as well as contributing their own immunomodulatory cytokines to the inflammatory environment (Cottrez and Groux, 2001; Zheng *et al*., 2004).

1.5.4 Th17

More recently, a new subset of effector $CD4^+$ T cells with an independent lineage from Th1 and Th2 has been discovered, named Th17 cells. Studies have shown that these cells likely play a critical role in the defence against certain microbial pathogens, such as extracellular bacteria and fungi (Weaver *et al*., 2006), as well as in cancer and autoimmunity (Langrish *et al*., 2005). Th17 cells are generated in the context of IL-6 and TGF-β and subsequently produce IL-17A, IL-17F, IL-21 and IL-22 in conjunction with the transcription factors ROR-γt (Ivanov *et al*., 2006; Yang *et al*., 2008) and STAT3 (Ouyang *et al*., 2008; Yang *et al*., 2007; Zhu and Paul, 2008). Although the precise function of Th17 cells in different disease settings remains unclear, there is increasing evidence that IL-17 promotes recruitment, activation, chemokine and cytokine production of both M∅s and, particularly, neutrophils (Dragon *et al*., 2008; Mills, 2008; Ye *et al*., 2001a; Ye *et al*., 2001b).

1.5.5 Diversity of subsets

It should be noted that the examples previously given of various $CD4^+$ T helper subsets represent the extreme cases in each category, and provide a somewhat simplistic overview of the known Th cell subsets. In addition to their effector functions, $CD4^+$ T lymphocytes must also regulate both innate and adaptive immune responses. Typically, this has been attributed to the cross-regulation between subsets,

such as the down-modulation of Th2 functions through the production of IFN-γ by Th1 cells, and conversely the dampening of Th1 responses by IL-10 secretion of Th2 cells (Abbas *et al.*, 1996; Coffman and Mosmann, 1991). That Th1 cells can, in some settings, produce IL-10 (Del Prete *et al.*, 1993), even in conjunction with IFN-γ secretion by the same Th1 cell (Jankovic *et al.*, 2007), demonstrates the extent to which T helper cell function can be modified as a consequence of an ongoing immune response. Indeed, the $CD4^+$ T cell response to any pathogen typically resolves in a 'spectrum' of T helper cells, marked both by the presence of multiple subsets, and the secretion of multiple cytokines within a subset. Furthermore, as research in this area continues, it is likely that our knowledge of different $CD4^+$ T cell subsets will increase. The recent proposal of a possible 'Th9' subset of $CD4^+$ T cells (Veldhoen *et al.*, 2008) is an illustration of how we are still expanding our knowledge of potential $CD4^+$ T cell fates and the plasticity of their functions.

1.6 Tissue microenvironment and complexities *in vivo*

Of course, a substantial amount of our knowledge of APC generated Th responses was derived from reductionist *in vitro* approaches, specifically designed to remove the complexities of an *in vivo* immune response. During live infections, interactions between APCs and T cells occur in the context of an entire network of cell types, all of which are capable of altering APC activation and the immune environment. The impact of tissue factors, such as thymic stromal lymphopoietin (TSLP) or IL-25, on the activation and function of DCs have only recently been investigated (Fort *et al.*, 2001; Ito *et al.*, 2005; Liu *et al.*, 2007; Wang *et al.*, 2007; Ziegler and Liu, 2006). Cytokine secretions by, or cell-to-cell interaction with,

epithelial cells, natural killer cells, basophils and others, all add an additional layer of complexity to the process by which DCs become activated. The fact that these environmental influences upon DC function are so poorly understood may, in part, explain our inability to identify mechanisms by which DCs drive Th2 responses. The investigation of these additional layers of complexity could be crucial for the discovery of these mechanisms.

1.7 Effector Immunity to Complex Pathogens

Once the context of the adaptive immune response has been established, T effector cells facilitate pathogen termination or removal. The exact mechanisms employed are dependent upon the specifics of the infectious agent, whether it is viral, bacterial, protozoal, fungal, helminth, or any other form of disease. However, the complexities involved in an ongoing infection are often difficult to replicate *in vitro*. Many pathogens possess methods of immune system evasion, such as *Salmonella* (Bueno *et al.*, 2007) or trypanosomes (Donelson *et al.*, 1998). Larger metazoan pathogens, such as the helminth *Schistosoma mansoni*, present an especially complex challenge to the immune system due to multiple life cycle stages within the host (Gryseels *et al.*, 2006; Pearce and MacDonald, 2002), a diverse array of potential Ags (Perona-Wright *et al.*, 2006), and even the sheer size of the parasite presents a mechanical disruption to the immune system and facilitates tissue damage (Wynn *et al.*, 2004). Ascertaining the impact of these additional complexities *in vivo* on the activation and function of APCs is essential for our understanding of the overall immune response to infection.

1.8 Schistosomiasis

There are an estimated 200 million people in the developing world who are chronically infected with trematodes of the genus *Schistosoma* resulting in as many as 200,000 deaths a year (Chitsulo *et al.*, 2000; Chitsulo *et al.*, 2004; Pearce and Freitas, 2008). Females lay hundreds to thousands of eggs per day once a male-female pair is established. These eggs are excreted in the urine or faeces and upon contact with water, hatch to release a free living stage called the miracidium (Gryseels *et al.*, 2006). The miracidia then locate and infect the intermediate host, freshwater snails, and multiply asexually into cercarial larvae (Gryseels *et al.*, 2006). Following 4-6 weeks of development, and at the cue of light, cercariae rupture out of the snail and swim in search of the definitive mammalian host. The cercariae attach to and penetrate the skin, and then migrate through the blood to the lungs before making their way to the portal vein of the liver (Gryseels *et al.*, 2006). Once in the vasculature, schistosomes mature (about 4-6 weeks post-infection) and mate, starting the lifecycle over again. Accumulation of the egg stage of the parasite within tissues eventually causes the immunopathology that can ultimately be fatal (Chitsulo *et al.*, 2004; Pearce and MacDonald, 2002).

1.8.1 Schistosomiasis and the Immune System

The initial stages of schistosome infection provoke a mixed and muted Th1/Th2 response from the host immune system. Absence of host IL-7 or $CD4^+$ cells both impairs schistosome growth and reduces egg burden (Davies *et al.*, 2001; Davies and McKerrow, 2003; Pearce and Freitas, 2008; Wolowczuk *et al.*, 1999). However, it is the production of eggs by female schistosomes, intended to pass through the body via the intestinal (*S. mansoni* and *S. japonicum*) or bladder (*S. haematobium*) lumen, which causes the most severe immunopathology during

schistosomiasis (Pearce and MacDonald, 2002). Following egg deposition, the overall immune response begins to categorically shift and by 8 weeks post-infection is strongly Th2 in character (Grzych *et al.*, 1991; Pearce *et al.*, 1991). The granulomatous inflammation and fibrosis around the parasite eggs lodged in the liver and intestines is the principle source of the cell-mediated immune reaction to *S. mansoni* (Pearce, 2005). From these eggs, proteolytic enzymes are secreted which provoke eosinophilic inflammatory reactions leading to fibrosis. The severity of pathology is dependent both on the extent of the infection and the intensity of the individual's immune response (Gryseels *et al.*, 2006).

The role of Th2 differentiation is something of a double-edged sword in the course of *S. mansoni* infection: mice that are deficient in either the production of IL-4 cytokine (Brunet *et al.*, 1997; Fallon *et al.*, 2000) or expression of IL-4 receptor (Herbert *et al.*, 2004) suffer increased mortality following egg production. However, it has been shown that the inflammation induced by the eggs and the resulting host-protective granulomas are dependent on Th2 differentiated $CD4^+$ T cells, while the root cause of fibrosis is due to the cytokine IL-13, itself produced in abundance by Th2 cells (Pearce, 2005; Wynn *et al.*, 2004).

1.8.2 SEA

A fundamental and as yet unanswered immunological question, is how DCs respond to Th2-inducing pathogens such as *S. mansoni*. Furthermore, it is currently unclear how such DCs then proceed to co-ordinate $CD4^+$ T cell responses (MacDonald and Maizels, 2008). However, addressing this question is a daunting task, considering that all the life cycle stages of *S. mansoni* secrete a multitude of potential targets for immune recognition (Perona-Wright *et al.*, 2006). Of the various

components produced by *S. mansoni*, soluble egg Ag (SEA) is the best characterised. SEA has been a particular focus of ongoing studies due to its ability to act both as an adjuvant (Okano *et al.*, 1999; Okano *et al.*, 2001) and for its remarkable capability to promote Th2 responses (Pearce and MacDonald, 2002; Perona-Wright *et al.*, 2006). In contrast to bacterially derived stimuli, SEA induces very little DC upregulation of MHC II and no significant upregulation of cytokines or co-stimulatory markers such as CD80, CD86, CD54, CD40 or OX-40L (MacDonald *et al.*, 2001). However, despite the lack of 'classical' signs of activation, SEA-pulsed DCs have proven to be potent initiators of SEA specific Th2 responses either following transfer into naïve mice (MacDonald *et al.*, 2001), or through co-culture with ovalbumin-specific TCR transgenic T cells *in vitro* (Artis *et al.*, 2005; Jankovic *et al.*, 2004; Kane *et al.*, 2008). It remains unclear the mechanisms by which DCs are able to prime SEA-specific Th2 responses, but the fact that SEA-pulsed DCs alone are sufficient to drive Th2 polarisation speaks both to the effectiveness of DCs as APCs and the immunogenicity of SEA.

The lack of any known 'signal 3' candidates for the induction of Th2 responses by DCs stands in stark contrast to the ever-increasing understanding of how DCs drive Th1 differentiation (MacDonald and Maizels, 2008). One explanation is that Th2 induction is merely a default pathway, the proscribed outcome in the absence of IL-12. However, during infections where Th2 responses are paramount for successful pathogen clearance, it would seem unlikely that host survival should depend entirely upon a default process. Identification of mechanisms by which DCs drive Th2 responses would answer many of the critical questions driving current

immunological research. A central theme of this thesis is determining whether Notch signalling may act as one of these mechanisms.

1.9 Notch Background

Notch receptors and ligands provide an evolutionarily-ancient metazoan mechanism for signalling between neighbouring cells (Artavanis-Tsakonas *et al.*, 1999). First discovered in 1917, its name was derived from the notched wing phenotype apparent in partial loss of function *Drosophila* mutants (Mohr, 1919; Morgan, 1917). However, the field of Notch signalling would later be defined by its role as a 'neurogenic' gene, named for the mutant embryos displaying excessive neuronal differentiation at the expense of the epidermis (Lehman *et al.*, 1983; Poulson, 1937). It was these studies that laid the foundation for further investigations establishing the core components of the Notch signalling apparatus. The Notch signalling pathway later became renowned for its extensive versatility. Over the years, analysis of genetic deficiency in both vertebrates and invertebrates has demonstrated the extraordinary extent to which metazoan development relies on Notch signalling, being utilized for exchanging amplification signals, determining molecular differences and even inducing apoptosis (Artavanis-Tsakonas *et al.*, 1999).

Notch-like proteins have been characterized in species as diverse as *Caenorhabditis elegans*, insects, sea urchins, mice and humans and, in all cases so far, mutations in the Notch receptor invariably result in developmental abnormalities that are usually lethal (Artavanis-Tsakonas *et al.*, 1999). The first mammalian Notch homolog (Notch1) was discovered as a partner in a recurrent chromosomal translocation in a rare subtype of human T-cell acute lymphoblastic leukaemia

(Ellisen *et al.*, 1991). In development, Notch signalling is thought to act as a tool used to direct cell fate in neurogenesis, differentiation of the epidermis and hematopoiesis (Artavanis-Tsakonas *et al.*, 1999; Miele and Osborne, 1999). As a result of its fundamental role in a wide array of functions, Notch has been nicknamed by Miele and Osbourne, the "arbiter of differentiation and death" (Miele and Osborne, 1999).

1.9.1 Elements of Notch Signalling

The primary components of Notch signalling include ligands, receptors and a transcription factor of the CBF1/Su(H)/Lag1 (CSL) family (**Figure 1.4**). Each of the Notch receptors and ligands are conserved, single pass transmembrane proteins that are expressed on the cell surface. In mammals there are 4 types of receptor, Notch 1-4, and two distinct families of ligand, Delta (1, 3 and 4) and Jagged (1 and 2) (**Table 1.1**). The large extracellular domain of the Notch receptors and ligands contain multiple tandem EGF-like repeats as well as Notch/LIN 12 repeats. The intracellular region (N^{ICD}) contains 6 tandem ankyrin repeats, a glutamine-rich domain and a PEST sequence (Artavanis-Tsakonas *et al.*, 1999).

One of the most fascinating aspects of the Notch pathway is its unique mechanism of signalling. Upon activation by Notch ligand, the intracellular domain of the Notch receptor is cleaved by presenilin-1 and the Notch intracellular domain (N^{ICD}) is then transported to the nucleus. Rather than operating through a true cascade of signals, Notch utilizes a remarkably direct mechanism, wherein a portion of the receptor itself is directly involved in the nuclear activation of gene transcription (Lai, 2004). Once inside the nucleus, the N^{ICD} binds to the gene

repressor CSL/RBP-Jκ with the recruitment of additional proteins and modifies it to become an activator of gene transcription activating the Hairy-enhancer of split (HES) genes and other transcription factors (Robey and Bluestone, 2004). The simple elegance of this arrangement has enabled the Notch pathway to function in an extraordinarily broad range of biological systems.

For successful Notch signalling three distinct cleavage events must occur. The first is mediated by a furin-like protease just external to the transmembrane subunit during transit to the outer membrane (Maillard *et al.*, 2005). This creates a receptor with two noncovalently associated subunits (Maillard *et al.*, 2005). The other two cleavage events are a direct result of ligand binding. In mammals, the metalloprotease ADAM10 (Kuzbanian in Drosophila) has been implicated in separating the extracellular domain from the transmembrane region (Tian *et al.*, 2008), while a presenilin-containing multiprotein complex with γ−secretase activity releases the N^{ICD} (Fortini, 2002). Following translocation to the nucleus, the N^{ICD} binds the transcriptional activator CBF1/RBP-Jκ and recruits Mastermind-like proteins (MAMLs) through its ankyrin repeats, creating a large protein complex capable of transcriptional activation (Maillard *et al.*, 2004). Inhibition of either γ-secretase activity (Wolfe, 2001) or MAMLs ability to recruit other co-activators disables transcription of downstream targets (Maillard *et al.*, 2004; Weng *et al.*, 2003).

Additional components are also important in the regulation of Notch signalling including Fringe, Deltex (Dt), Numb and others (Artavanis-Tsakonas *et al.*, 1999; Maillard *et al.*, 2003). At the extracellular level, Notch signalling can be modified by the Fringe family of proteins, glycosyl transferases which add N-

acetylglucosamine to certain epidermal growth factor–like repeats of Notch receptors, promoting Notch signalling in response to Delta ligands and inhibiting Jagged-mediated Notch signalling (Haines and Irvine, 2003). The best studied of these proteins is Lunatic Fringe (Lnfg), which coordinates the timing and localization of Notch signalling during specific developmental processes, such as the somite segmentation clock during embryogenesis and is also important in thymopoiesis (Fleming *et al.*, 1997; Haines and Irvine, 2003; Visan *et al.*, 2006). Crucially, the glycolsylation of the extracellular domain of the Notch receptor by fringe proteins regulates the relative affinity for Notch ligands (Artavanis-Tsakonas *et al.*, 1999; Lai, 2004; Miele, 2006; Moloney *et al.*, 2000). In contrast, Deltex and Numb are both intracellular modulators of Notch signalling. It is believed that Numb acts upstream of Notch and prevents nuclear translocation of the N^{ICD} (Berdnik *et al.*, 2002) while in mammalian cells Deltex has been shown to have both positive and negative upon Notch signalling (Izon *et al.*, 2002; Matsuno *et al.*, 1995; Matsuno *et al.*, 1998; Ordentlich *et al.*, 1998). Within the nucleus, N^{ICD} can be targeted for degradation by proteins such as SEL-10 and other kinases (Wu *et al.*, 2001a). The vast majority of these Notch signalling proteins and other contributing factors are critical to the successful development of any metazoan organism.

1.9.2 Notch Signalling Mechanics

Although the basic core of the Notch signalling pathway is reasonably well understood, the mechanisms that regulate the transduction of that signal, or determine the downstream phenotype as a result of Notch signalling, is far more complex and less well characterized. Often, dramatic differences in signalling

between cells do not correlate with substantial differences in the expression levels of either Notch ligands or receptors (Maillard *et al.*, 2005). The relative levels of Notch, Delta and Jagged ligands between interacting cells regulate the polarity, intensity and duration of signalling, highlighting the importance of gene dosage (Lai, 2004). Further, the full involvement of how Notch ligands activate Notch receptors remains unclear. It is believed that Notch signalling is confined to neighbouring cells due to the membrane tethering of both receptors and ligands (Lai, 2004). However, there is some evidence that soluble forms of various Notch ligands exist and that they are able to activate the Notch pathway (Sun and Artavanis-Tsakonas, 1997).

A further complication with regards to Notch signalling pertains to the possibility of Notch receptors and ligands binding on the same cell (Artavanis-Tsakonas *et al.*, 1999). The mechanism underlying this *cis*-interaction between Notch proteins is not well understood. However, evidence suggests that it may act to inhibit Notch signalling with other cells (D'Souza *et al.*, 2008; Glittenberg *et al.*, 2006; Jacobsen *et al.*, 1998; Ladi *et al.*, 2005; Micchelli *et al.*, 1997; Sakamoto *et al.*, 2002). It is possible that *cis*-interactions would compete with *trans*-interactions for the same binding sites on Notch receptors (Glittenberg *et al.*, 2006).

Other complications include the prospect of CSL-independent mechanisms for Notch signalling, since analysis of mutant Notch receptors within mammalian cells has indicated that aberrant Notch signalling unable to activate a CSL-reporter gene was still able to mediate the downstream function of Notch signalling (Bush *et al.*, 2001). The existence of CSL-independent Notch signalling remains controversial but, it has been proposed that an alternate pathway for Notch signalling may involve

elements of the Wingless pathway (Axelrod *et al.*, 1996; Lai, 2004; Ramain *et al.*, 2001).

One of the most intriguing aspects of Notch signalling lies in trying to understand how two different ligand families can drive two separate signalling responses, as they share the same mechanism for transcriptional activation. It remains largely unclear how N^{ICD} could retain “ligand memory” given that both Jagged and Delta ligands should lead to essentially the same intracellular signal within cells. The very observation that different ligands have specific, and often non-overlapping expression patterns (Lindsell *et al.*, 1996), and are not always interchangeable (Huppert *et al.*, 1997; Jaleco *et al.*, 2001), in addition to enzymes that selectively alter Notch receptor specificity, suggests unique functions of these molecules (Amsen *et al.*, 2004; de La Coste and Freitas, 2006; Justice and Jan, 2002).

1.9.3 Notch and Immunity

Having noted that the *notch1* gene was expressed in the developing mammalian thymus (Weinmaster *et al.*, 1991, 1992), the first group to address the impact of Notch signalling in immunity investigated whether it was involved in the $CD4^+$ versus $CD8^+$ T cell lineage decision (Robey *et al.*, 1996). Constitutive expression of activated N^{ICD} within the thymus was found to encourage a bias towards $CD8^+$ T cell lineage fate (Robey *et al.*, 1996). In contrast, experiments using an inducible transgenic system, wherein a loxP flanked *notch1* gene was under the control of an interferon-inducible promoter, demonstrated that the absence of *notch1* 21 days after birth resulted in a dramatically reduced thymus size, as well as a

decrease in mature thymocytes (Radtke *et al.*, 1999). These studies confirmed the speculation that Notch signalling may play an integral role in the development of the mammalian immune system, and formed a basis on which long-standing questions on lymphocyte development and peripheral immunity could be addressed.

1.9.4 Notch in T cell development

The question of how progenitor cells with equivalent potential for lineage differentiation commit to different cell fates has been integral to our understanding of lymphocyte development (Allman *et al.*, 2002). Notch signalling has now been well established as a mechanism by which cell fate decisions can occur during lymphopoiesis (Maillard *et al.*, 2005). Multiple studies have also demonstrated the importance of Notch signalling within the T cell versus B cell lineage decision (Han *et al.*, 2002; Hozumi *et al.*, 2004; Jaleco *et al.*, 2001; Radtke *et al.*, 1999). Inactivation of the Notch1 receptor was shown to completely block T lineage development and promote differentiation into B cells (Radtke *et al.*, 1999). The inducible knockout of the transcription factor RBP-Jκ resulted in a similar phenotype (Han *et al.*, 2002). Conversely, mice reconstituted with bone marrow constitutively expressing Notch1 blocked B cell development and promoted the emergence of an immature T cell population (Pui *et al.*, 1999). The Notch2 receptor has been determined to be indispensable to the development of marginal zone B cells (MZB) and their precursors (Saito *et al.*, 2003). It is noteworthy that the complete blockade of T cell development induced by *notch1* deficiency suggests that other Notch receptors are unable to compensate for this loss. Why this is the case remains unclear.

Further studies have determined that the involvement of Notch signalling in lymphocytes extends beyond the T cell and B cell lineage decision. It is a contributing factor in the lineage choice between αβ versus γδ T cells (Tanigaki *et al.*, 2004), with reduced Notch1 activity favouring a γδ T cell fate (Washburn *et al.*, 1997) and is also crucial in the choice between $CD4^+$ and $CD8^+$ T cell development (Robey *et al.*, 1996). Notch signalling both initiates and sustains T-cell lineage programmes throughout their differentiation, and the presentation of Notch ligands by stromal cells is considered a key element during T cell development *in vitro* (Schmitt and Zuniga-Pflucker, 2002). Indeed, N^{ICD} is the only transcriptional regulator that demonstrably promotes T cell development when over-expressed, although on its own Notch signalling is insufficient in determining these lineage decisions (Rothenberg *et al.*, 2008).

1.9.5 Notch in DC and M∅ Differentiation

Notch signalling is also thought to be involved in the differentiation and development of haematopoietic cells other than lymphocytes, such as DCs and MØs (Cheng *et al.*, 2006; Cheng *et al.*, 2003; Kumano *et al.*, 2003; Ohishi *et al.*, 2001). DC differentiation is critically dependent on bone marrow microenvironment; including a complex network of cytokines, as well as direct physical interaction between haematopoietic progenitor and stem cells with bone marrow stroma (Cheng *et al.*, 2007). Although *notch1* deficiency does not appear to affect myeloid development (Radtke *et al.*, 2000; Radtke *et al.*, 1999), constitutive expression of N^{ICD} reportedly inhibits the differentiation of myeloid progenitors into DCs (Bigas *et al.*, 1998; Milner *et al.*, 1996; Schroeder and Just, 2000a). However, a different

approach to examining DC phenotype in murine *notch1* conditional knockouts concluded that DCs generated from Notch-deficient hematopoietic progenitor cells (HPCs) displayed reduced MHC II and co-stimulatory molecules, and concluded that Notch signalling was necessary, but not sufficient, for DC differentiation (Cheng *et al.*, 2003). Further work supported the suggestion that interaction between differentiating myeloid cells and the bone marrow stroma employs Notch molecules in order to determine cell fate. Expression of a peptide mimicking human Jagged1 by a stromal cell line was shown to influence the cell fate decision of granulocytic differentiation, as well as inhibiting DC differentiation (Li *et al.*, 1998). In a different study, soluble Jagged1 was able to induce maturation of monocyte-derived human DCs (Weijzen *et al.*, 2002). In contrast, addition of immobilized Delta1 to myeloid precursors in the presence of M-CSF resulted in apoptosis, whereas Delta1 and GM-CSF led to stable DC differentiation; suggesting that Delta1 drives precursor cells towards a DC fate and inhibits MØ differentiation (Ohishi *et al.*, 2000). The presence of several apparently contradictory reports on the impact of Notch signalling on DC differentiation highlights the difficulty of drawing firm conclusions from such studies. The combination of several distinct methods of investigation, with a signalling pathway renowned for it's sensitivity to dose and signal strength, likely contributes to this overall lack of understanding.

1.9.6 Notch in Peripheral Immunity

The initial suggestion that Notch signalling may be a direct mechanism by which APCs can influence T cell polarisation was proposed by Hoyne et al. (Hoyne *et al.*, 2000a). Splenic APCs were transfected with human *serrate1* (*jagged1*) by

retroviral mediated gene transfer and then used to immunise recipient mice (Hoyne *et al.*, 2000b). In this model, overexpression of *jagged1* by APCs suppressed the proliferation and cytokine production in $CD4^+$ T cells (Hoyne *et al.*, 2000b). Other direct evidence for an interaction between TCR and Notch signalling came from the observation that NICD1 over-expression in thymocytes reduced CD25 and CD69 expression; indicating that Notch signalling inhibited activation (Benson *et al.*, 2004; Izon *et al.*, 2001). Additional work has suggested that pre-exposure of T cells to *delta1*-expressing cell lines inhibits transplantation rejection (Wong *et al.*, 2003). However, the addition of Delta1 fusion protein to T cells stimulated with anti-CD3 and CD28 mAbs promoted Th1 differentiation (Maekawa *et al.*, 2003). In humans, overexpression of *jagged1* by B lymphocytes inhibited the classical response to Epstein Barr Virus (EBV) Ag and promoted differentiation of IL-10 producing T regulatory cells (Vigouroux *et al.*, 2003; Yvon *et al.*, 2003). Other studies have promoted a role for Notch signalling in T cell proliferation, demonstrating that both gain of function, and Notch inhibition experiments affected T cell expansion following TCR activation (Adler *et al.*, 2003; Palaga *et al.*, 2003). Thus, the role of Notch in peripheral T cell function is complex, and may be context-dependent.

Based on the growing knowledge of Notch ligand expression in the mature immune system, Amsen *et al*. (2004) hypothesized that, in addition to cytokine signalling and the surface expression of co-stimulatory factors, the Notch pathway may be a key factor in the differentiation of $CD4^+$ effector cells. Expression of the Notch ligand family Delta, by APCs, was proposed to prime Th1 differentiation, while the Jagged family induced Th2 differentiation *in vitro* (Amsen *et al.*, 2004). A subsequent *in vivo* study found that blocking signalling ability of Notch receptors 1-4

resulted in an impaired Th2, but not Th1, response (Tu *et al.*, 2005). Although evidence is emerging that Notch has a function in immunity beyond its developmental role, the exact nature of Notch signalling and the relative contribution of the two ligand families in the adaptive immune response is far from clear.

1.9.7 Notch and Human Disease

In addition to its evolutionarily conserved nature, unique signalling mechanism and surprisingly diverse array of functions, the Notch signalling pathway is also of keen interest due to its importance in several human diseases (**Table 1.3**). Due to its roles in cell differentiation and growth, as well as embryonic development and immunity aberrant Notch signalling has been implicated in cancer, neurological disorders and even autoimmunity. Notch receptors were recently identified as having oncogenic potential when a truncation of the *notch1* transcript which induced a constitutively-active Notch1-intracelular domain was found in a subset of T-cell lymphoblastic leukemias (Ellisen *et al.*, 1991). Further research indicated that constitutively active Notch1 or Notch3 signalling has a role in development of T-cell Acute Lymphoblastic Leukemia (T-ALL) in both animals and humans (Aster *et al.*, 2008; Chiaramonte *et al.*, 2005; Jundt *et al.*, 2008).

Notch signalling is also involved in human pathological conditions involving the vasculature including the congenital diseases CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy) and Alagille syndrome (AGS) (Roca and Adams, 2007). CADASIL is an inherited small vessel disease caused by mutations in the *notch3* gene, which leads to recurrent ischemic stroke and vascular dementia (Chabriat *et al.*, 1995; Dichgans *et al.*, 1998;

Opherk *et al.*, 2004). Mutations that cause the onset of disease invariably affect the quantity of cysteine residues within the EGF regions of the Notch3 extracellular domain (Dichgans *et al.*, 2000; Joutel *et al.*, 2000). The aberrant Notch3 extracellular domain then self-associates and its accumulation within the small arteries is thought to induce the degeneration of the vascular smooth muscle cells (Opherk *et al.*, 2009). AGS is a genetically heterogeneous disorder caused by mutations in the human gene *jagged1*, whose symptoms include abnormalities of the heart, eye, liver and skeleton (Li *et al.*, 1997; Oda *et al.*, 1997). Although it is not understood the exact role of Jagged1 in affected tissues, evidence suggests that within the liver Jagged1 controls hepatocyte growth factor (HGF), a critical protein for regulation of hepatic stem cells, and that this may be the mechanism by which liver disease occurs in AGS (Yuan *et al.*, 2006).

Multiple sclerosis (MS) is a human demyelinating disease wherein lesions are formed within the central nervous system, which are focal areas of myelin destruction (Mastronardi and Moscarello, 2005). It has also been found that elevated levels of Notch1 and Jagged1 are present in an MS brain and that the increase in these amounts were consistent with disease severity (Lubetzki and Stankoff, 2000). It is thought that the Notch1 signalling pathway may play a role in the timing and spatial regulation of myelination by oligodendrocytes and that the increased expression of both Notch1 receptor and the ligand Jagged1 may inhibit re-myelination (Genoud *et al.*, 2002).

The potential role of Notch signalling in human diseases such as these further exemplifies the need to understand the roles and functions of this dynamic signalling

pathway in both our understanding of basic biology and for the possible improvement of public health.

1.10 AIMS

Previous studies have examined the impact of Notch signalling on T cell differentiation from the perspective of signal reception. The experimental work performed in this thesis fundamentally focuses on determining the extent to which Notch proteins may be used by DCs as 'signal 3' to direct T cell polarisation, addressing whether Notch ligand provision by APCs is associated with specific $CD4^+$ T cell outcomes. Ultimately, determining whether Notch ligands are relevant in this context, or can act as markers for APC stimulation by diverse pathogens, may provide exciting new diagnostic or even therapeutic targets.

Hypothesis: That Notch ligands are utilized by antigen presenting cells, specifically dendritic cells, in the process of antigen presentation.

The specific questions addressed in this thesis are:

1) Is the expression of a distinct cohort of Notch ligands associated with pathogenic stimuli? (Chapter 3)

2) Do expression profiles of cytokines, co-stimulatory markers and Notch ligands by BM-DCs and BM-M∅s, in response to complex-pathogen derived Ags, accurately portray $CD4^+$ priming capacity? (Chapter 3)

3) Is the expression of Notch ligands by DCs required for their activation and function either *in vivo* or *in vitro*? (Chapter 4)

4) What other signals might influence Th2 priming by DCs? (Chapter 5)

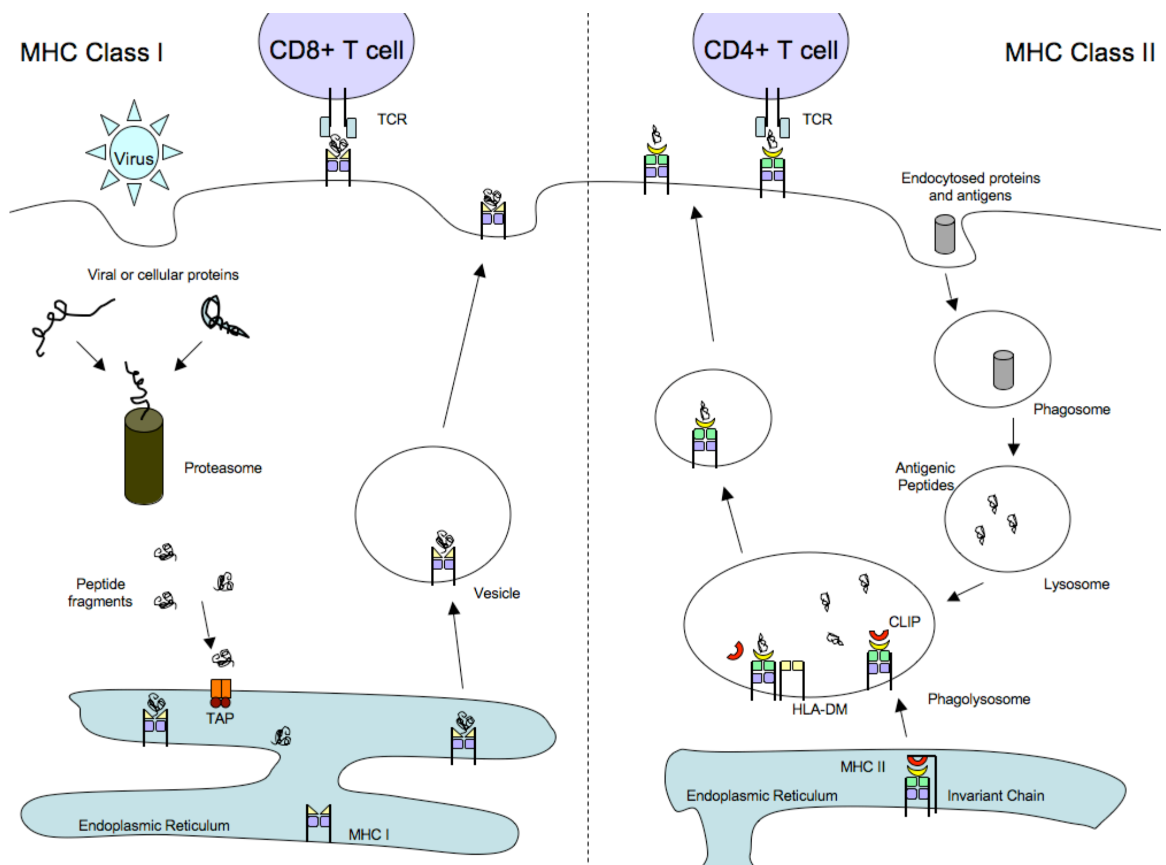


Figure 1.1 Peptide Loading and Presentation by MHC I and MHC II. MHC I is loaded with peptide within the ER following degradation of intracellular proteins by the proteasome. Peptide fragments are transported into the ER through the TAP transporter. Peptide-loaded MHC I is then transported directly to the cell surface where it presents antigen to CD8^{+} T cells. In contrast, MHC II is formed in the ER with the invariant chain preventing premature loading of peptide. The nascent MHC II is then transported to the phagolysosome containing degraded endocytosed proteins. The invariant chain is then degraded leaving a small fragment called CLIP, which continues to block the peptide-binding cleft. The CLIP fragment is then replaced with endocytosed peptide with the aid of HLA-DM. Stable MHC II is then transported to the cell surface where it presents antigen to CD4^{+} T cells.

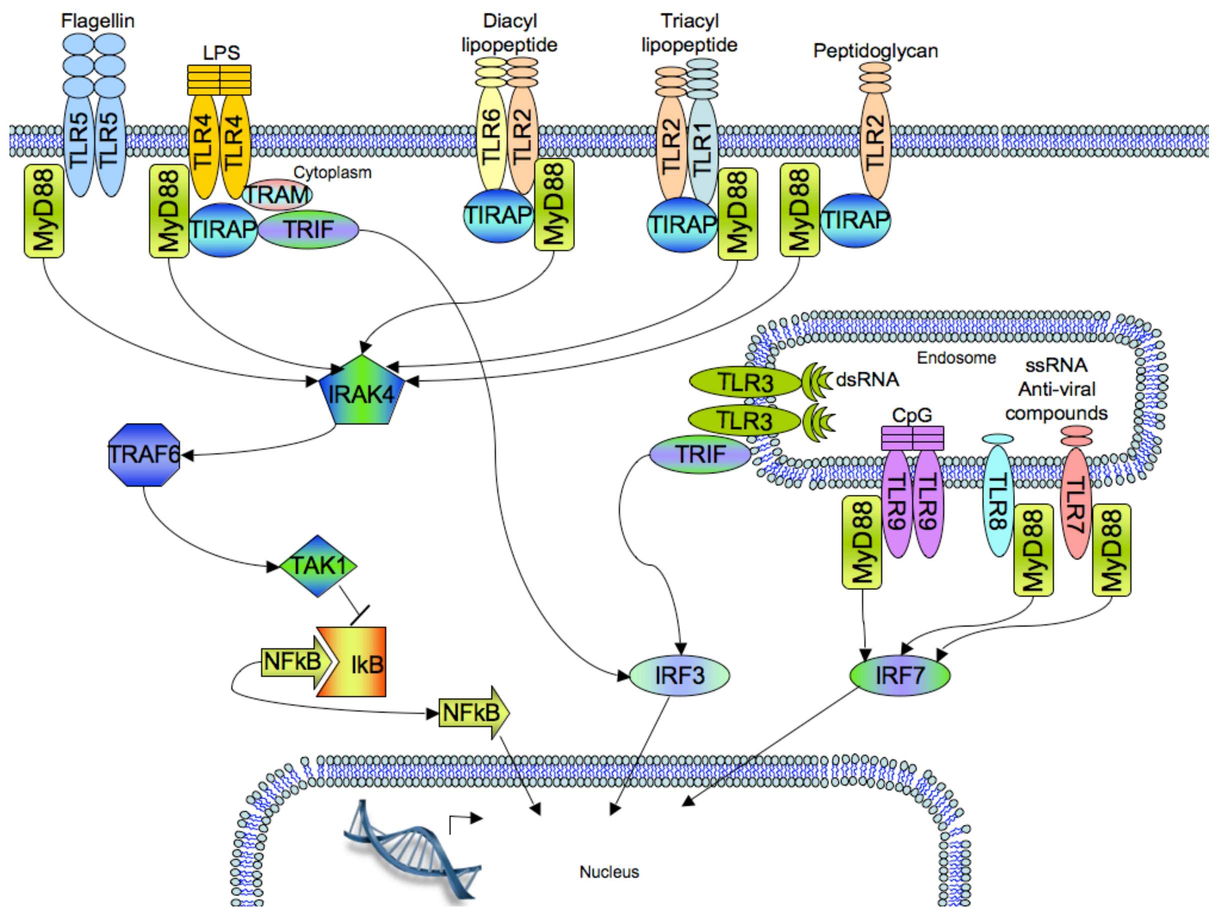


Figure 1.2 TLR signalling. TLRs use a variety of signalling mechanisms in order to activate downstream transcription factors. All TLRs, with the exception of TLR3 utilize the adaptor MyD88. MyD88 then binds IRAK4, which in turn activates TRAF6. TRAF6 then proceeds to phosphorylate IκB via TAK1 leading to its degradation and the release of NFκB. TLRs 7, 8 and 9 can leads to activation and nuclear translocation of IRF-7. A MyD88 independent signalling pathway involving the adaptor TRIF is utilised by TLRs 3 and 4 which can lead to the nuclear translocation of IRF3.

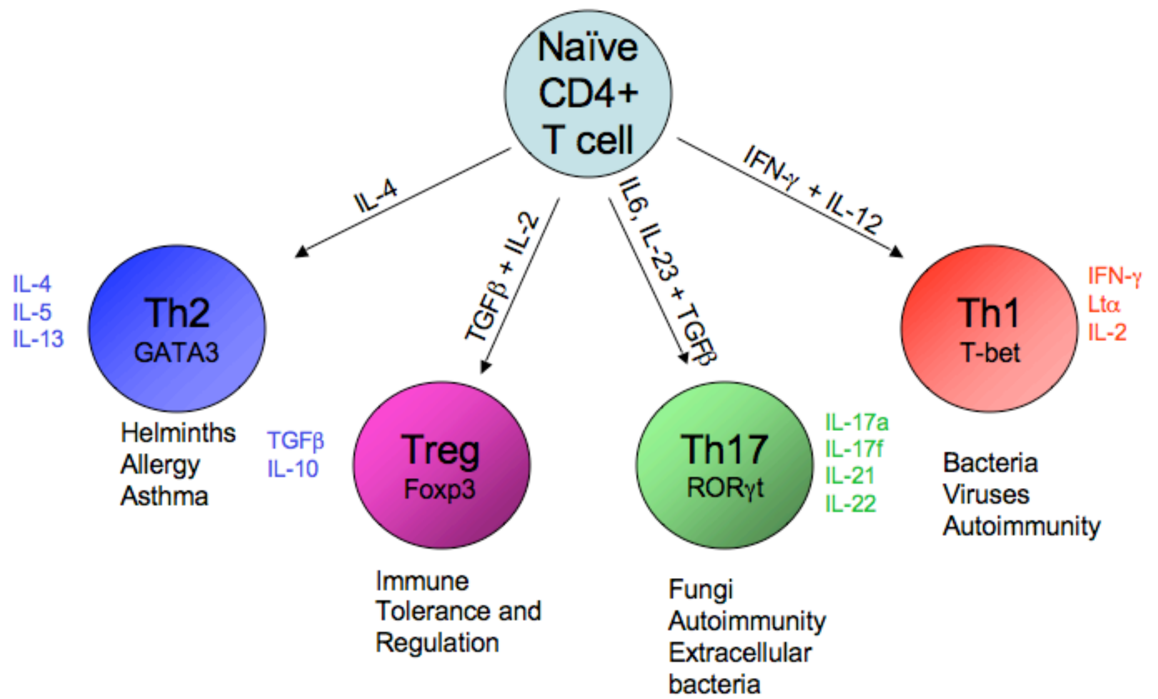


Figure 1.3 Overview of CD4$^+$ T helper cell subsets. Functions, transcription factors, as well as cytokines both produced by and crucial for the determination of different T helper cell subsets. Adapted from (Zhu and Paul, 2008)

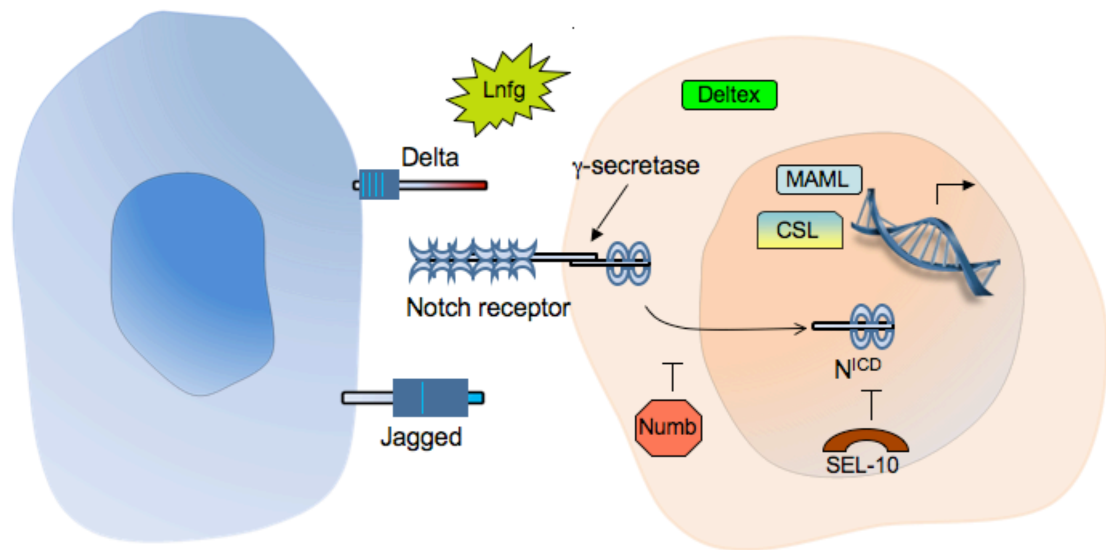


Figure 1.4 Components of the Notch signalling pathway. Mammals possess 4 receptors (Notch1-4) and five different ligands derived from two different families (*jagged1-2* and *delta 1, 3,* and *4*), all of which are expressed on the cell surface. Following ligand binding, a cleavage event mediated by presenilins with γ-secretase activity releases the Notch intracellular domain (N^{ICD}). The N^{ICD} is then translocated to the nucleus, where it binds to the transcriptional repressor CSL and, with the help of MAML proteins and other coactivators, initiates transcription. This pathway is regulated at several stages. Lnfg modifies the EGF domains of Notch receptors, altering their affinity for ligand binding. Deltex and Numb influence the translocation of the N^{ICD}, and SEL-10 can target the N^{ICD} for degradation from within the nucleus.

Component	*C. elegans*	*D. melanogaster*	Mammals
Ligand	DSL-1	Delta	Delta1 Delta3 Delta4
	LAG-2 APX-1	Serrate	Jagged1 Jagged2
Receptor	GLP-1 LIN12	Notch	Notch1 Notch2 Notch3 Notch4
Transcription Factor	LAG-1	Su(H)	CBF1/RBJκ

Table 1.1 Notch Components in different species - Adapted from (Lai, 2004)

Gene Deficiency	Phenotype	Reference
notch1$^{-/-}$	**Embryonic Lethal <E11.5** **Defective formation of hematopoietic stem cells**	**(Conlon *et al.*, 1995; Huppert *et al.*, 2000; Kumano *et al.*, 2003; Swiatek *et al.*, 1994)**
notch2$^{-/-}$	**Embryonic Lethal <E11.5**	**(Hamada *et al.*, 1999)**
notch3$^{-/-}$	**Normal embryonic development**	**(Krebs *et al.*, 2003)**
notch4$^{-/-}$	**Normal embryonic development**	**(Krebs *et al.*, 2000)**
jagged1$^{-/-}$	**Embryonic Lethal <E11.5** **Defect in vasculogenesis**	**(Xue *et al.*, 1999)**
jagged2$^{-/-}$	**Perinatal death due to craniofacial morphogenesis** **Also defects in limb, thymic development and γδ T cell differentiation**	**(Jiang *et al.*, 1998)**
delta1$^{-/-}$	**Embryonic Lethal E10-E12** **Defects in somite borders**	**(Hrabe de Angelis *et al.*, 1997)**
delta3$^{-/-}$	**Defects in somite borders and disruption of segmentation clock**	**(Dunwoodie *et al.*, 2002; Kusumi *et al.*, 1998)**
delta4$^{-/-}$	**Embryonic Lethal <E11.5** **Defect in vasculogenesis**	**(Gale *et al.*, 2004)**

Table 1.2 Notch gene deficiencies - Adapted from (Maillard *et al.*, 2005)

Common Human Notch Mutations	Subsequent Disease	Reference:
NOTCH1: activating mutations which lead to high levels of expression of a constitutively active intracellular domain	Activating mutations are found in >50% of T-cell acute lymphoblastic leukemias	(Ellisen *et al.*, 1991; Weng *et al.*, 2004)
NOTCH3: mutations affecting the number of cysteine residues in the EGF domain of Notch3	CADASIL - accumulation of Notch3 extracellular domain leading to disruption of the vasculature. Late-onset symptoms include stroke, migraine, dementia, and death	(Opherk *et al.*, 2009; Opherk *et al.*, 2004; Roca and Adams, 2007)
JAGGED1: several identified mutations found in the EGF domain, cysteine rich region and the DSL domain	Alagille Syndrome involves abnormailities in the liver, heart, skeleton, eye and facial features	(Oda *et al.*, 1997; Ropke *et al.*, 2003; Yuan *et al.*, 2006)

Table 1.3 Known human diseases related to Notch receptor and ligand mutations

Chapter 2

Materials and Methods

2.1 Animals and Reagents

WT C57BL/6 mice were bred and maintained in the Ann Walker Animal Facility, University of Edinburgh. Six – Eight week old mice were used as bone marrow donors for DC and M∅ culture. Numerous antigens were used for stimulation of the APCs in culture and 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), and heat-killed *Salmonella typhimurium* was supplied by Maurice Gallagher (University of Edinburgh), while SEA was prepared in house from schistosome eggs isolated from livers of *S. mansoni* infected C57BL/6 mice. LPS and PGE2 were obtained commercially (Sigma Aldrich, Poole, USA). Concentrations of antigens used were based on experiments previously carried out in-house, or taken from published papers.

2.1.2 SEA production

Generation of SEA requires isolation of schistosome eggs from livers of infected mice. Mice were infected percutaneously with 200 cercariae in a 200 ml volume of carbon-filtered water. 7 weeks later, livers were collected into PBS containing antibiotics and washed with 70% ethanol for 5 min before 3 washes with sterile PBS. Livers were then placed in a sterile Petri dish, and minced with sterile surgical blades. Minced livers were transferred to 50 ml tubes (4 livers per tube) then PBS was added to a total volume of 40 ml. To this solution, 5 ml of a 0.5% solution

of Collagenase D (Roche, Welwyn, UK) in PBS was added, as well as 0.5 ml 100X Penicillin-Streptomycin, 0.5 ml Polymyxin B Sulfate (Sigmab Aldritch), $5x10^6$ U diluted in 6 ml sterile water. After incubation overnight at 37° C on a rocker, supernatants were removed following centrifugation at 300 g for 5 min. After 2 washes with PBS, the pellet was removed, poured into a petri dish and then mashed with a monoject 30 cc syringe to break up the remaining fragments of liver. This was then resuspended in PBS and centrifuged for 5 min at 300 g. Pellets were resuspended in 10 ml PBS, then each layered over a Percoll gradient (20 ml 0.25 M sucrose, 10 ml Percoll in a 50 ml falcon, 5 ml of liver homogenate per gradient). After centrifugation at 300 g for 5 min, the upper layer was carefully removed with a pipette and discarded. Egg pellets were then pooled into one tube and counted. After centrifugation at 300 g for 5 min at 4° C, the supernatant was discarded and then the eggs were snap frozen before storing at -80° C.

2.2 Macrophage and Dendritic Cell Culture

Bone marrow-derived DC culture was performed essentially as previously described (Lutz et al. 1999). Femurs were extracted from killed mice and washed in 70% ethanol and PBS. Bone marrow was expelled with PBS using a syringe and 25G needle (Becton Dickinson, Mountain View, USA). DC culture medium was composed of RPMI-1641 medium (Sigma Aldrich), 100 units/mL penicillin-streptomyocin (GIBCO, Life Technologies, Paisley, UK), 10% FCS (Labtech International Ltd., Ringmer, East Sussex, UK), 2mM L-Glutamine (GIBCO) and 20 ng/ml GM-CSF (Peprotech, Rocky Hill, NJ, USA). Cells were seeded in standard bacteriological petri dishes (Philip Harris Scientific, Cheshire, UK) in 10 mL of

medium at a concentration of 2x10^5 cells/mL and cultured at 37°C, 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 10 mL of fresh medium. On day 11, DCs were harvested by gentle expulsion of medium over the dish to gather all semi-adherent DC, and then counted using a haematocytometer, and re-plated in 24-well plates (Corning Incorporated, Corning, NY) in 1 mL of medium at a concentration of 1x10^6 cells/mL.

M∅s were grown essentially as described (Fischer *et al.*, 1988). M∅ culture medium consisted of RPMI-1641 medium (Sigma Aldrich), 2mM L-Glutamine (GIBCO), 10% FCS (Labtech International Ltd.), and 20 ng/ml M-CSF (Peprotech). M∅s were seeded in 6-well non-tissue culture plates (Becton Dickinson Labware) in 4 mL of medium at a concentration of 1x10^6 cells/well. Cells were cultured at 37°C in 5% $C0_2$. On days 4 and 6, 2 mL of medium was removed from each well and replaced with 2 mL of fresh medium. M∅s were harvested on day 7 by the addition of the disodium salt of ethylenediamine tetraacetate (EDTA) solution as a calcium ion chelator (10 mM Glucose and 3 mM EDTA in PBS) to the wells after removal of the supernatant. Following incubation at 37° C for 15 min, adherent M∅s were removed from the bottom of the well, washed and resuspended in RPMI 1640 medium containing 10% FCS as described (Segura *et al.*, 1997).

DCs and M∅s were then exposed to an array of antigens, as stated in the text, and at specified time points after addition of the antigen, cells were harvested by gentle washing, centrifuged at 200 g for 5 min, and the supernatants stored at –20° C for ELISA analysis. Harvested cells were resuspended in 1 mL of RNAzol (AMS

Biotechnology, Oxam UK) and stored at –80°C and/or assessed for phenotypic activation by Flow cytometry.

2.3 ELISAs

All ELISA antibodies, reagents and protocols had been previously optimized in house. A list of antibodies, coating buffers, recombinant cytokine standards and detection substrate can be found in **Table 2.1**. Briefly, 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 (for TNFα only). Samples and recombinant cytokine standards were added in duplicate and incubated overnight at 4° C. Standard curves involved doubling dilutions of recombinant cytokines. Biotinylated detection antibodies were added for 2 hours at 37° C and peroxidase-streptavidin (Kirkegard and Perry Laboratories, Maryland, USA) was added for 30 min at 37°C. ELISAs were developed using TMB Microwell Perox (Kirkegaard and Perry Laboratories) with the reaction stopped with 0.18M H_2SO_4 or ABTS (Kirkegaard and Perry Laboratories). Plates developed using TMB were read at 450 nm, while plates developed in ABTS were read at 405 nm, 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 the two site binding (hyperbola) equation on the standard curve using Prism software. From this graph the sensitivity of the ELISA for each

experiment was determined by calculating the lowest point within the linear phase of the curve. Therefore the sensitivity measured was dependent upon the quality of the standard curve for each experiment. This result may differ from manufacturer's instructions as the observed sensitivity was calculated depending on the quality of each individual experiment.

2.4 Flow cytometry

Approximately $2x10^5$ cells in 200 μL medium were added to V-bottomed 96 well FACS plates (Costar), incubated with αFcR block (2.4G2, 1 μg/well) for 20 min on ice and then for 30 min with antibodies specific for phenotypic markers of interest at 4° C. Antibodies used are detailed in **Table 2.2**. Samples were acquired using FACSCalibur (Becton Dickinson) and Cell Quest software and analysed using FlowJo software.

Live cells were gated using forward scatter versus side scatter (**Figure 2.1A**). 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, while M∅ purity was assessed by F4/80 staining (**Figure 2.1**). DCs were considered of acceptable purity if they constituted > 90% $CD11c^+$ while M∅s were required to be at least 85% $F4/80^+$.

2.5 RNA Extraction

0.2 mL of Chloroform was added per mL of RNAzol to cells and shaken for 30 sec. After 5 min incubation in ice, cells were centrifuged at 13,000 g at 4° C for 15 min. Afterwards, the aqueous phase was removed from the organic phase, 0.25 mL of isopropanol was added and allowed to incubate at room temperature for 5 min. After centrifugation at 13000 g for 15 min at 4° C, the pellet was washed with 1 mL of 75% ethanol. After a final 5 min spin at 6000 g, the pellet was dried under a heat lamp to remove any residual alcohol before resuspension in 30 μl of RNAse free water (Promega). RNA was converted to cDNA following the manufacturer's protocol using Promega Reverse Transcription System (Promega, Madison, USA). Briefly, 9 μl RNA and 1 μl Random Hexamer primers were heated for 10 min at 55° C. Once the RNA and primer mix had cooled to room temperature, a reverse transcription mix was added including $MgCl_2$ (5 mM), 10x Reverse Transcription buffer (1x), dNTP mixture (1 mM), RNAase inhibitor (1 u/μl), AMV-Reverse Transcriptase (0.75 u/ml) and RNAase free water. This reaction was incubated at 42° C for 1.5 hours, heated to 99° C for 5 min and cooled to 5° C. The resulting cDNA was then stored at –20° C. All RNA work was performed using pipettes and filter tips reserved for RNA use only (Axygen Scientific).

2.6 RNA extraction from tissue

Tissue samples isolated from naïve or *S. mansoni* infected mice were frozen in 500 μL of Trizol™ and stored at -80° C. For homogenisation, tissues were placed in a SafeLock Eppendorf (Qiagen) containing stainless steel beads (5 mm, Qiagen)

and vibrated using a Tissue Lyser (Qiagen) at 30 Hz for 2 min. RNA was then extracted as described above.

2.7 Quantitative PCR

PCR amplifications were performed in a 20 μl volume containing 10 μl of SYBR Green I mix (Invitrogen), 0.4 μl of both 10 μM reverse and forward primers and 7.6 μl of water. Reaction conditions optimised in the lab for RNA amplification were 94° C for 15 min, 94° C for 20 sec, 55° C - 65° C (annealing temp varied according to primer pair) for 20 sec, and 72° C for 20 sec. After 40 cycles, samples underwent melting curve analysis and were taken from 55° C to 94° C with an assay of SYBR Green fluorescence at every degree. Analysis and reaction was carried out using a Chromo4 Real Time Machine and Opticon Monitor software (GRI). When determining mRNA expression of a target gene from either DCs or M∅s three separate wells were cultured per treatment group and then each well was duplicated for Real Time PCR giving a total of six repeats per treatment group.

The following primers were purchased from Invitrogen unless otherwise stated:

Jagged1 Forward: GCAACGACCGTAATCGCATC

Reverse: TGCCTGAGTGAGAAGCCTTTTC

Provided by Dr. Png Loke

Jagged2 Forward: GTCGTCATTCCCTTTCAGTTCG

Reverse: AGTTCTCATCACAGCGTACTCG

Designed in house using www.ensembl.org and http://align.genome.jp/

DLL1 Forward: GCACTACTACGGAGAAGGTTGCTC

Reverse: TCACACCCTGGCAGACAGATTG

Provided by Dr. Png Loke

DLL3 Forward: GTAGTGAAACCTCTGGCTCCTTTG

Reverse: CCATTGAAGCAGGGTCCATCTG

Provided by Dr. Png Loke

DLL4 Forward: AGGTGCCACTTCGGTTACACAG

Reverse: CAATCACACACTCGTTCCTCTCTTC

Based on Amsen et al. (2004) (Amsen *et al.*, 2004)

18s Forward: GTAACCCGTTGAACCCCATT

Reverse: CCATCCAATCGGTAGTAGCG

Based on Schmittgen et al. (2000) (Schmittgen and Zakrajsek, 2000)

CD86 Forward: CACGAGCTTTGACAGGAACA

Reverse: TTAGGTTTCGGGTGACCTTG

Designed in house using www.ensembl.org and http://align.genome.jp/. Primers were validated both by running PCR reactions on a gel and by using a melting curve analysis following Quantitative Real Time PCR. PCR products were run on a 2% agarose gel and the size of the product was checked for conformity with the expected product size and no additional bands. Melting curve analysis involves steadily raising the temperature on the PCR product and reading the quantity of double-stranded DNA after each 1°C increase. The expected melting temperature can be calculated based upon product size and in this manner the product quality can be checked. Additionally any additional products, such as primer/dimer pairs, can be assessed by either melting curve analysis or by 2% agarose gel.

The units used for determining the quantity of PCR product were calculated using a standard, which accompanied each Real Time PCR run. This standard

consisted of a pool of cDNA that was then serially diluted with each tube containing a quarter of the quantity of cDNA of the tube preceding it. The top concentration was assigned the value of 100, with the next concentration of 25, the next 6.25 and so forth. This approach allows the Opticon™ software program to calculate the actual rate of amplification. In order to determine the value of mRNA expression of the gene of interest the average is taken from the repeats and then this is divided by the average value for the mRNA expression of 18s.

A different, and at the time of experiments, more common approach, involves calculating the difference between the cycle at threshold for the gene of interest versus the corresponding cycle at threshold for the internal reference. This result is quantified using the formula $2^{-\Delta\Delta cT}$ where the resulting quantity of mRNA for the gene of interest is represented as a fraction of the reference transcript. The advantage of this strategy is that a specific value can be attributed to each sample and thus cross-experimental comparisons can be easily made. However, this formula makes the assumption that the Taq polymerase is operating at 100% efficiency and that each cycle represents a complete duplication of material. By using a diluted standard as a reference, values can be used to take into consideration the real rate of amplification. When using Quantitative Real-Time PCR my question usually pertains to fold increase of expression under a variety of different conditions. The absolute quantity of transcript was less important than obtaining a more accurate approximation of the overall changes to mRNA expression and thus the diluted standard model was predominantly used when calculating mRNA expression.

2.8 DC/T cell Co-culture *in vitro*

DCs were grown as described above in **Chapter 2.2** and either stimulated overnight with various antigens (typically heat-killed *P. acnes* and SEA) or concurrently with the addition of T cells and OVA antigen (synthesized by Advanced Biotechnology Centre, Imperial College, London and a gift from Prof. D. Gray, University of Edinburgh, Edinburgh, UK). The following day, $CD4^+$ cells were extracted from spleens and lymph nodes of OT-II mice and purified as described in **Chapter 2.9.** A total of 2×10^4 DCs and 2×10^5 $CD4^+$ OT-II T cells were co-cultured in 96-well round bottom plates in 200 μl RPMI containing 100 units/mL penicillin-streptomycin, 2 mM L-Glutamine and 5 mM 2-Mercaptoethanol (Gibco). Either whole OVA protein (200 μg/ml) or $OVA_{323-339}$ peptide (10 ng/mL) was added per well as a source of antigen. In some experiments, the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) was included at a concentration of 5 mM to determine the effect of wholescale Notch signalling inhibition on the polarisation and proliferation of OT-II cells. Several different protocols were conducted to optimise proliferation and polarisation assays. To test whether we were observing optimal cytokine production, DC and OT-II $CD4^+$ cells were co-cultured for 5 days at 37° C and 5% CO_2, after which supernatants were stored for further analysis by ELISA, and cells were analysed by flow cytometry.

For optimal IL-4 intracellular staining, DCs were cultured with OT-II T cells and $OVA_{323-329}$ peptide at a 10:1 ratio of T cells:DCs together with 25 μg/ml SEA. Co-cultures were incubated for 3 days at 37 C, 5% CO_2 before harvest and then intracellular staining was carried out as described in **Chapter 2.10**

2.9 $CD4^+$ T cell Purification

$CD4^+$ T cells were purified using CD4 (L3T4) MicroBeads (Macs) as per manufacturer's instructions. Single cell suspensions of spleen and LN cells were prepared using sterile sefar nitex ribbon (Sefar Ltd., Bury, UK) and forceps in 35 mm petri dishes (Cell Star). Following centrifugation at 300 g, spleen cells were resuspended in 3 mL of Red Blood Cell Lysis Buffer (Sigma Aldrich) for 2 min and then washed in RPMI containing 10% FCS. Cells were resuspended at 10^7 cells per 90 μl to which 10 μl of Macs beads were added. These were incubated at 4°C for 15 min, washed and separated over a MACS LS separation column. Positively selected cells were retained on the magnetic column, which was removed from magnetic apparatus and washed with PBS. $CD4^+$ T cells were routinely > 85% pure by flow cytometry (**Figure 2.2**).

2.10 Intracellular Staining

Cells were stimulated with 20 ng/ml phorbol ester phorbol myristate acetate (PMA) and 1 mg/ml Ionomycin for a total of 4.5 hours. In some experiments, 50 mg/ml Brefeldin A (BFA) was also added 2.5 hours post PMA/ionomycin stimulation, while in other experiments, BFA was added at the same time. BFA, PMA and ionomycin were purchased from Sigma Aldritch. Following stimulation, cells were incubated with FcR block as previously described (**Chapter 2.4**) and were stained for 20 min on ice with APC-conjugated CD4 antibody. Samples were then fixed and permeabilized using a BD Cytofix/Cytoperm kit following manufacturer's instructions. Briefly, cells were resuspended in 100 μl BD Cytofix/Cytoperm and incubated overnight at 4° C. Cells were then stained for intracellular cytokines

(either IL-4 PE or IFN-γ FITC as detailed in **Table 2.2**), in the saponin-containing BD Perm/Wash™ buffer in the dark for 30 min at 4° C. Cells were washed twice in BD Perm/Wash™ and resuspended in staining buffer prior to acquisition and analysis.

2.11 CFSE Staining of T cells

Following purification of $CD4^+$ cells from spleens and lymph nodes, cells were washed and resuspended at a concentration of 10^7/mL in PBS containing 2% FCS (Sigma Aldrich). A dessicated 500 mg CFSE pellet (carboxyfluorescein diacetate succinimidyl ester, Molecular Probes, Oregon) was resuspended in 90 ml DMSO (Sigma Aldrich). An aliquot of this was diluted to a 100 mM working solution in PBS containing 2% FCS. CFSE was then added to cells to give a final concentration of 5 mM in solution, and dispersed thoroughly by inversion of the tube (Lyons and Parish, 1994). Samples were then incubated at 37°C, 5% CO_2 for 15 min, mixing every 5 min. Cells were washed and resuspended in 5 mL of RPMI prior to a further 30 min incubation at 37° C. Following a final wash with RPMI, cells were resuspended to a concentration appropriate for co-culture with DCs.

2.12 RNA inhibition

Stealth™ siRNA oligonucleotides and scrambled controls were purchased from Invitrogen using their own software for determining the most effective sequence for mRNA knockdown. siRNA oligonucleotides were stored at -20° C in the dark. Lipofectamine2000™ (Invitrogen) was used for transfection of siRNA oligonucleotides into recipient cells. Cells were transfected in 24-well plates as per

manufacturer's instructions. Briefly, 1 μl of Lipofectamine was diluted in 50 μl of serum free medium per culture well and allowed to mix for 5 min. Alternatively, HiPerFect Transfection Reagent (Qiagen) was used according to manufacturer's instructions. However, comparisons between the two separate transfection reagents demonstrated a comparatively poorer ability for HiPerFect to successfully transfect fluorescent oligonucletodies (data not shown). siRNA oligonucleotidess, or fluorescent nucleotides were added to the transfection reagent at a final concentration of 150 nMol and incubated for 20 min at room temperature before the combination was added to recipient cells. This concentration was determined by titration, measuring maximum transfection efficiency as assessed by fluorescent oligonucleotides without triggering DC phenotypic activation (data not shown).

Transfection efficiency was assessed by flow cytometry using fluorescent oligonucleotides, which fluoresced in the FITC channel **(Figure 4.1**). It is important to note that this measurement does not distinguish whether oligonucleotides are free within the cytoplasm. It is possible that the fluorescent oligonucleotides are sequestered into liposomes and, though detectable, are not necessarily representative of the quantity of freely available RNA interfering oligonucleotides.

siRNA sequences ordered from Invitrogen are as follows

Stealth CD86: GCACCAUGGGCUUGGCAAUCCUUAU

Control CD86: GCAGGUAUUCGACGGCCUAUCCUAU

Stealth Delta4: GGGAAGUACUGUGACCAGCCUAUAU

Control Delta4: GGGCAUGGUGUACCAUCCGAAAUAU

Stealth Jagged2: GCUGCUAUCACUCAGAGAGGAAAUA

Control Jagged2: GCUAUCUCACUGAGAGAAGACGAUA

2.13 *S. mansoni* infection of naïve mice

Mice were anesthetized before percutaneous infection with 75 cercariae. Mice were culled 7.5 weeks postinfection during the period of the acute Th2 response (Pearce and MacDonald, 2002). Spleen, mesenteric LN, liver and gut samples were then harvested for RNA extraction.

2.14 Assessment of DC or M∅ Priming Ability *in vivo*

3 x 10^5 DCs or M∅ activated overnight with SEA, St or Pa were injected i.p. into 6-10 week old naïve recipient mice. Cell suspensions were prepared from spleens removed 7 days later. Spleen cells were cultured in X-Vivo™ 15 serum free medium (Cambrex Bio Science, Wokingham, UK) containing 2 mM L-glutamine and 50 μM 2- Mercaptomethanol with or without SEA at a final concentration of 15 μg/ml, Pa at 1 μg/ml and St at 1 μg/ml. Supernatants were harvested after 72h for cytokine analysis by ELISA. Results did not differ significantly when transferred DC had been activated with SEA or Pa for 6 h rather than overnight (data not shown). Concentrations of Ag, number of cells transferred and time before cell and supernatant harvest has been optimized previously in the lab.

2.15 Construction of Foetal Liver Chimeras

jagged2 deficient BM was a generous gift from Dr. Caetano Reis e Sousa's laboratory (Cancer Research UK, London). The process by which they were derived

involved foetal livers from Ly5.2^{+} *jagged2*$^{-/-}$ x *jagged2*$^{+/-}$ matings, which were removed from day-14.5 embryos. *jagged2*$^{-/-}$ embryos were identified by PCR (Jiang *et al.*, 1998). Sub-lethally irradiated Ly5.1^{+} recipients were reconstituted with cells from *jagged2*$^{-/-}$ or *jagged2*$^{+/+}$ womb mates in a similar fashion to Washburn et al. (1997) (Washburn *et al.*, 1997). DC were grown from BM harvested from chimeras 8-20 weeks later. Donor origin was then verified by flow cytometry (**Figure 4.5**).

2.16 Statistical Analysis

The Student's unpaired *t*-test was used to determine whether means significantly differed in comparison to a standardised control value. Statistical significance was assigned to data returning a 'P value' of less than 0.05. *t*-tests were performed using Prism™ software.

All experimental results shown involving Quantitative Real Time PCR or ELISAs derive error bars mean and SEM from three separate culture wells. Samples are duplicated on ELISA plates and on Real Time PCR reactions, the average of these two values is taken as the reading per sample. Three independent samples for each condition then constitute the basis for related statistics. For *in vivo* animal experiments, each data point indicates an individual mouse and errors bars indicate mean + SEM from the individual mice. In cases where only one culture well for each sample was used, such as in early pilot experiments, no error bars are given.

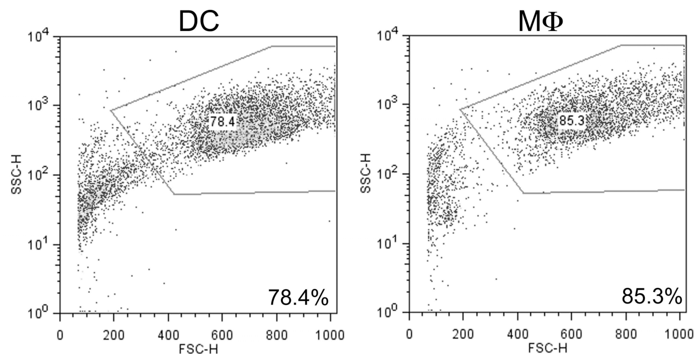
ELISA	Capture Antibody	Coating Buffer	Recombinant	Detection Antibody	Developing Substrate	Detection Limit
IL-4	11B11 (in house) [2 μg/ml]	PBS	Peprotech 1° well 10 ng/ml	24G2/BVD6 (in house) [0.25 μg/ml]	TMB	0.5 ng/ml
IL-5	TRFK5 (in house) [1.5 μg/ml]	PBS	Pharmingen 1° well 20 ng/ml	TRFK-4 (in house) [0.17 μg/ml]	ABTS	0.5 ng/ml
IL-6	MP5-20F3 (Phamingen) [2 μg/ml]	0.1M Na_2HPO_4, pH12	Peprotech 1° well 20 ng/ml	32C11 (Pharmingen) [0.2 μg/ml]	ABTS	2 ng/ml
IL-10	JES5-2A5 (Pharmingen) [2 μg/ml]	0.2M Na_2HPO_4	Pharmingen 1° well 25 ng/ml	SXC-1 (Pharmingen) [0.2 μg/ml]	TMB	1 ng/ml
IL-12p40	C15.6 (Pharmingen) [2 μg/ml]	0.2 M Na_2HPO_4	Peprotech 1° well 16 ng/ml	C17.8 (in house) [0.2 μg/ml]	ABTS	1 ng/ml
IL-12p70	9A5 (Pharmingen) [2 μg/ml]	0.2 M Na_2HPO_4	Peprotech 1° well 16 ng/ml	C17.8 (in house) [0.2 μg/ml]	TMB	1 ng/ml
IL-13	MAB413 (R&D) [2 μg/ml]	PBS/1% BSA/0.05% azide/5% sucrose	R&D 1° well 20 ng/ml	BAF413 (R&D) [0.1 μg/ml]	TMB	0.5 ng/ml
IL-17	TC11-18H10 (Pharmingen) [0.5 μg/ml]	0.1 M Na_2HPO_4 pH 9	Pharmingen 1° well 20 ng/ml	TC11-8H4.1 (Pharmingen) [0.25 μg/ml]	TMB	0.5 ng/ml
IFN-γ	R46A2 (in house) [2 μg/ml]	0.1M Na_2CO_3, pH 9.6	Peprotech 1° well 50 ng/ml	XMG1.2 (Pharmingen) [0.2 μg/ml]	ABTS	1 ng/ml
TNFα	Duo Set (RnD) [0.8 μg/ml]	PBS	(R&D) 1° well 4 ng/ml	(R&D) [150ng/ml]	TMB	0.2 ng/ml

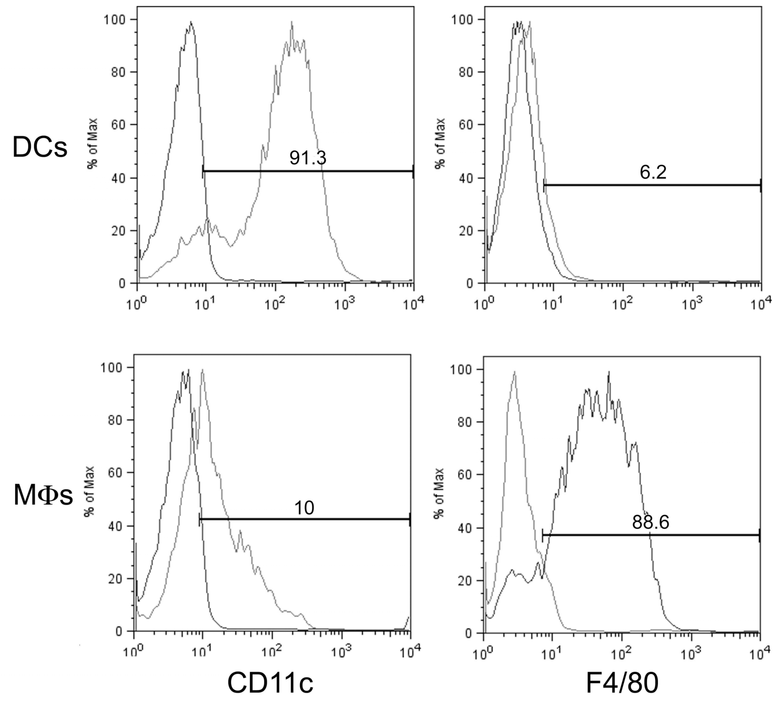
Table 2.1. ELISA Antibodies and reagents

Specificity	Fluoro-chrome	Clone	Isotype	Host	Dilution
MHC II (IA/IE)	FITC	M5114 (in house)	IgG2b	Rat	1:200
CD11c	APC	N418 (Pharmingen)	IgG	Armenian Hamster	1:200
F4/80	RPE – Alexa Fluor 647	CI:A3-1 (Serotec)	IgG2b	Rat	1:400
CD40	PE	3/23 (Pharmingen)	IgG2a k	Rat	1:100
CD80	PE	16/10A1 (Pharmingen)	IgG2 k	Armenian Hamster	1:100
CD86	PE	GL1 (Pharmingen)	IgG2a k	Rat	1:100
CD4	APC	RM4-5 (Pharmingen)	IgG2a k	Rat	1:400
IL-4	PE	11B11 (Pharmingen)	IgG1	Rat	1:100
IFN-g	FITC	XMG 1.2 (Pharmingen)	IgG1 k	Rat	1:100
Isotype	APC	RTK4530 (Pharmingen)	IgG2b, k	Rat	1:200
Isotype	FITC	RTK2071 (Pharmingen)	IgG1, k	Rat	1:200
Isotype	PE	HTK888 (Pharmingen)	IgG	Armenian Hamster	1:100

Table 2.2 List of antibodies used for Flow cytometry.

DC and MΦ gating





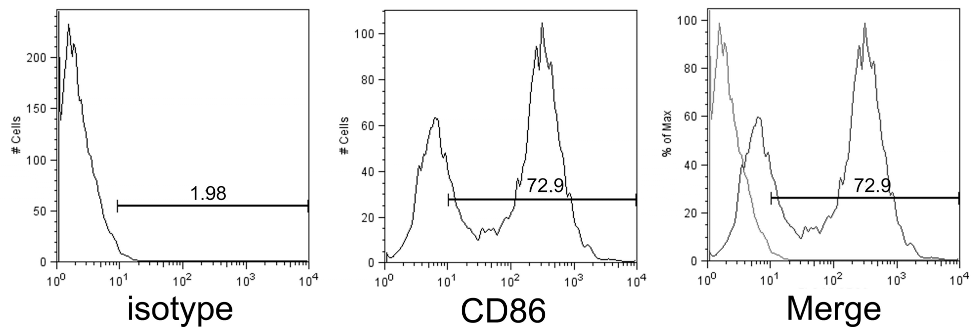


Figure 2.1 DC and M∅ cell gating and purity assessment. A) Live DCs and M∅s were gated based on Forward and Side scatter. B) Cultured cells were assessed for both F4/80 and CD11c expression. DCs were routinely >90% $CD11c^{+}$ and $F4/80^{low}$, while M∅ were >85% $F4/80^{high}$ and $CD11c^{low}$. C) Gates on individual stains were set to exclude 98% of the isotype control.

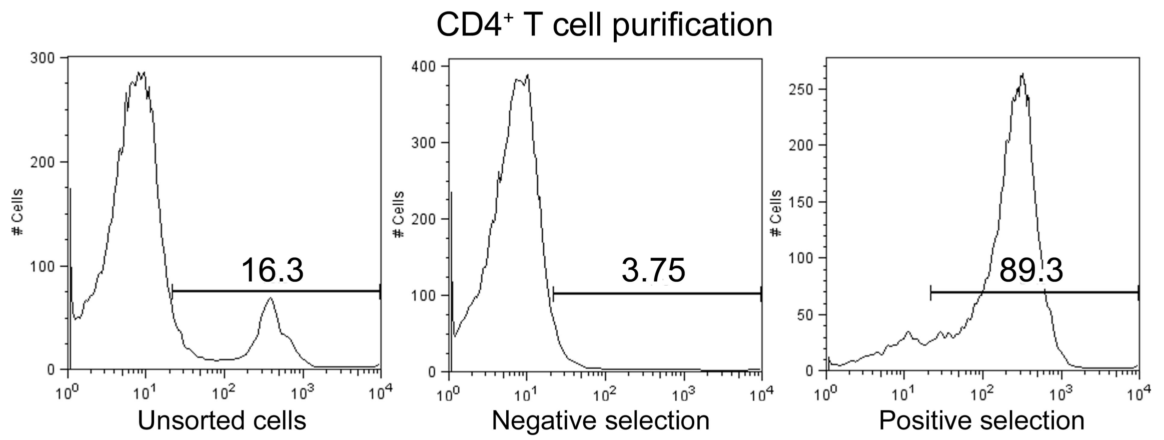


Figure 2.2 Representative staining of purified CD4$^+$ cells. Following manual disruption of either spleen or lymph node, and addition of magnetic beads as described in **2.9**, cells were analysed for CD4$^+$ staining prior to and after selection.

Chapter 3

Activation and function of DCs and MØs responding to diverse pathogens

3.1 Introduction

Notch ligands expressed by APCs have been proposed to play a role in the differentiation of $CD4^+$ T cells (Amsen *et al.*, 2004; Maekawa *et al.*, 2003; Maillard *et al.*, 2003) and may act as an additional indicator of APC maturation status. Currently, there are two well-characterised signals provided to naïve T cells by APCs. The first is the antigenic epitope presented by its MHC receptors to the TCR. The second are the co-stimulatory molecules CD80 and CD86, which bind to CD28 or CTLA-4 receptors on the T cell. However, it has been suggested that there is a "third signal" expressed by APCs, either cytokines or surface bound molecules, which predominantly influences T cell polarisation (Kalinski *et al.*, 1999; Valenzuela *et al.*, 2002) and that the Notch pathway could fulfil this function (Amsen *et al.*, 2004; Hoyne *et al.*, 2000a; Maekawa *et al.*, 2003).

Preliminary studies have investigated the effect of Notch signalling on T cells in the peripheral immune system (Eagar *et al.*, 2004; Hoyne *et al.*, 2001; Maekawa *et al.*, 2003; Maillard *et al.*, 2003). However, the current literature is as yet inconclusive as to the precise impact of distinct Notch ligands. Since the impact of signalling through Notch receptors on peripheral T cells was already being investigated (Amsen *et al.*, 2004; Maillard *et al.*, 2005), my research instead focussed on the relationship between APC maturation and the expression of Notch ligands. If Notch ligands are

expressed by APC to provide an additional signal to naive T cells as has been suggested (Adler *et al.*, 2003; Eagar *et al.*, 2004; Hoyne *et al.*, 2000a; Maillard *et al.*, 2003), and if these Notch ligands can drive distinct Th differentiation profiles (Amsen *et al.*, 2004; Maekawa *et al.*, 2003; Tu *et al.*, 2005), then it stands to reason that the expression of particular Notch ligands by APCs should be associated with specific activation profiles. The main hypothesis we examined was whether profiles of Notch ligand expression by APCs might reflect the nature of the stimulus given. Our intention was to investigate the patterns of Notch ligand expression by APC, in addition to more conventional markers of activation, to elucidate any association between specific Notch ligand expression by APCs and their Th polarisation potential.

APCs such as DCs and MØs influence the outcome of the entire adaptive immune response by directing $CD4^+$ T cells into a Th1, Th2, Th17 or regulatory T cell commitment (Zhu and Paul, 2008). Understanding the mechanisms underlying this process and assessing their subsequent downstream effect on the adaptive immune system has been a key question in immunology. In terms of T cell stimulating potential, DCs are often considered the "professional" APC, uniquely specialised to initiate T cell polarisation in the most efficient manner (Banchereau *et al.*, 2000; Steinman and Hemmi, 2006) while MØs are generally ascribed a primary role in microbicidal activity (Hope *et al.*, 2004; Yrlid *et al.*, 2000). However, it remains unclear exactly what DCs express that may make them so exceptional in their capacity to drive T cell responses. It has been shown that M∅s express lower levels of MHC II and the co-stimulatory molecules CD80 and CD86, both of which are further upregulated following exposure to IFN-γ (Yrlid *et al.*, 2000). Kalupahana

et al. (2005) demonstrated that while both bone marrow-derived DC and J774 M∅s were capable of nitric oxide secretion and induction of T cell proliferation *in vitro*, the subsequent expression of T-cell proliferation cytokines (such as IL-2), as well as T cell proliferation itself, as measured by thymidine incorporation, was 10-fold greater when Ag was presented by DCs (Kalupahana *et al.*, 2005). Although it has been demonstrated that MØs can stimulate primed $CD4^{+}$ T cells *in vitro* (Askonas *et al.*, 1968; Hsieh *et al.*, 1993a; Hsieh *et al.*, 1993b), direct evidence that MØs prime naïve T cells has proven more elusive. Much of the early evidence suggesting that DCs, but not MØs, were found to stimulate primary T cell responses were *in vitro* studies typically involving MLR (Inaba and Steinman, 1984; Steinman and Witmer, 1978). However, in the MLR assay T cell stimulation is due to polymorphisms of the MHC complex, which may not account for variability in innate Ag processing capability or non-MHC parameters of the activation status of either DCs or MØs.

In contrast, the work detailed in this chapter involved exposing bone-marrow derived DCs and MØs to biologically relevant pathogen preparations (as opposed to single TLR ligands or model antigens such as OVA protein) and then characterising cytokine production, co-stimulatory markers and Notch ligand expression. We then tested whether our expectation of APC ability based on this characterisation was an accurate prediction of DC or MØ ability to prime Ag specific responses *in vivo*. Bone-marrow derived DCs and MØs were chosen as the model cells for this study due to their demonstrable functional plasticity, and the ease with which a large quantity of immature cells can be cultured. Additionally, by culturing both DCs and MØs in this manner, both growth conditions and activation status can be rigidly controlled and held to be as equivalent as possible for the purposes of comparison.

In order to compare the activation profiles of both DCs and MØs in response to physiologically relevant Th1 antigens we used heat killed *Salmonella typhimurium*, heat killed *Propionibacterium acnes* and lipopolysaccharide (LPS). *S. typhimurium* and *P. acnes* were used as Ag because of their ability to activate DCs to promote a Th1 response (Balaram *et al.*, 2008; Matsui *et al.*, 1997), in addition to the physiological and pathogenic relevance of using whole bacterium. *S. typhimurium* is a Gram-negative bacterium, while *P. acnes*, in contrast, is a Gram-positive bacterium and a well established model for generating IFN-γ dependent immune responses (Balaram *et al.*, 2008; Matsui *et al.*, 1997). We reasoned that a comparison of DC activation by these two different bacteria might reveal subtle differences in Notch ligand expression, cytokine profile or co-stimulatory molecule expression. LPS, as a conventional control stimulus, is a cell wall component of Gram-negative bacteria which activates APCs predominantly through TLR4, resulting in increased MHC II, IL-1, IL-12 and TNFα (Eisenbarth *et al.*, 2002; Medzhitov, 2001). LPS was also the Th1 stimulant used by Amsen et al. (2004) (Amsen *et al.*, 2004) and so provides a reference control.

Although the mechanisms by which bacterial pathogens drive Th1 responses are reasonably well understood (Kalupahana *et al.*, 2005; Sundquist *et al.*, 2003), our knowledge of how Th2 responses are induced by APCs is much less robust. Th2 responses are critical for protection against pathogens such as helminths, yet it remains unclear which Th2-specific mechanisms are utilized by DCs in this process (MacDonald and Maizels, 2008). DCs fail to upregulate conventional markers of activation in response to stimulation by *S. mansoni* soluble egg antigen (SEA) and yet are potent inducers of Th2 responses both *in vitro* and *in vivo* (Jankovic *et al.*,

2004; MacDonald *et al.*, 2001). By stimulating both DCs and MØs with a range of Th2 driving Ags, we could assess whether the potent, yet muted, phenotype exhibited by Th2 driving DCs was also true of MØs. Additionally we investigated the possible association of *jagged* family ligands with Th2 stimulation as proposed by Amsen et al. (2004) (Amsen *et al.*, 2004). For this purpose, SEA, filarial nematode excretory-secretory product 62 (ES62) (Goodridge *et al.*, 2005) and ProstaglandinE2 (PGE_2) (Kalinski *et al.*, 1997) were all used for their Th2 driving ability. ES62 is a secreted phosphorylcholine-bearing filarial worm glycoprotein that acts as an antagonist to Th1 responses by limiting MØ production of IL-12, and broadly biases towards a Th2 response (Goodridge *et al.*, 2005; Harnett and Harnett, 2006). PGE_2 can act as a Th2 promoting factor by limiting APC production of IL-12 (Kalinski *et al.*, 1997; Kapsenberg, 2003).

AIMS

1) To establish whether DCs and MØs display defined expression profiles of Notch ligands in response to Th1 or Th2 stimuli
2) To assess the comparative ability of DCs and MØs to activate a polarise $CD4^+$ T cells *in vivo*

3.2 How are DCs and M∅s activated in response to distinct pathogen challenges?

Before assessing expression profiles of Notch ligands, we first tested the comparative responses of BM-DCs or BM-MØs to complex pathogens or their products. Cytokine levels were assessed over a time course, with DC or M∅

secretion of IL-10, TNFα, IL-6, IL-12p40 and IL-12p70 measured after exposure to biologically relevant pathogens or their products. Initial dose curves were previously conducted to establish the necessary quantity for each stimuli to illicit a robust cytokine response, whilst maintaining cell viability and the capacity to drive T cell responses *in vivo*. Over the course of multiple experiments a variety of different stimuli were used at several different time points. Following exposure to either Th1 (St, Pa, LPS) or Th2 (SEA) stimuli, supernatants from DC and MØ cultures were collected at 1.5, 12 or 24 and representative data for each cytokine measured is shown in **Figure 3.1**. In keeping with previous reports, DCs exposed to SEA produced similar levels of cytokine to unstimulated controls (MacDonald *et al.*, 2001). Similarly, we found that MØ activation in response to SEA was also muted. In contrast, both DCs and MØs responded to exposure to Th1 pathogens or their products by producing a range of cytokines. On a per cell basis, DCs produced more IL-12p40, as well as the biologically active heterodimer IL-12p70, than MØs, although the kinetics of cytokine expression remain largely the same. DCs and M∅s also shared a similar pattern of expression for IL-6 and TNFα secretion, again with comparably greater levels of this cytokine produced by DCs than MØs while IL-10 expression between the two cell types was more directly comparable. This data is in agreement with the perception that DCs tend to be the foremost cell type involved in the priming of $CD4^+$ T cells during a Th1 immune response, where it is understandable that DCs might benefit from elevated production of cytokines such as IL-12 (Robson *et al.*, 2003).

We then tested whether DCs and MØs displayed similar co-stimulatory molecule expression profiles in response to stimulation with diverse pathogens. DCs

and MØs were identified by flow cytometry using the markers CD11c and F4/80 respectively. CD11c is an integrin involved in cell adhesion (Stacker and Springer, 1991). Although its exact function is unknown, F4/80 may have a role in immunological tolerance (van den Berg and Kraal, 2005), and has widely been used as a murine MØ marker (van den Berg and Kraal, 2005). Measurement of high levels of CD11c expression, low autofluoresence and high levels of MHC II and co-stimulatory molecules is sufficient to characterize cells as DCs (Vakkila *et al.*, 2005). M∅s, in contrast, express F4/80 with low levels of CD11c and MHC II and co-stimulatory molecules (Vermaelen and Pauwels, 2004).

24 hours after stimulation with St, Pa or SEA, cells were analysed by flow cytometry for CD11c, F4/80, MHC II, CD40, CD80 and CD86 expression (**Figure 3.2, Table 3.1**). Consistent with previously published reports, we observed higher basal MHC II expression by DCs, both unstimulated and in response to bacterial Ag, than similarly stimulated MØs (Vermaelen and Pauwels, 2004; Yrlid *et al.*, 2000). DCs also expressed a greater level of CD80 and CD86 than MØs. Notably, consistent with what was seen with DCs, MØ stimulation by SEA had no measurable impact on cell surface phenotype. Unexpectedly, and in contrast to other molecules examined, MØs displayed a comparable level of CD40 upregulation to their DC counterparts. Together this data supports the consensus that DCs generally express greater levels of both co-stimulatory markers and cytokines than MØs, with the exception of CD40. Further, they reveal that MØs, like DCs, fail to be overtly activated by the Th2 stimulus provided by SEA.

3.3 Does the low activation phenotype of MØs relate to their ability to prime T cell responses *in vivo*?

Although it was clear from these experiments that BM-DCs secreted greater quantities of cytokine and expressed generally higher levels of activation markers than MØs, it was important to determine whether this accurately reflected their respective T cell priming capacities. APC priming ability *in vivo* was assessed by adoptive transfer, a method previously developed and optimised in the laboratory for DCs but not previously applied to MØs (MacDonald *et al.*, 2001; MacDonald *et al.*, 2002b). Following overnight stimulation with Ag, $5x10^5$ DCs or MØs were injected intra peritoneally (i.p.) into naïve recipient mice. One week later, splenocytes were removed, stimulated with Ag and then assessed for cytokine production by ELISA. Previous reports have shown that DCs stimulated with SEA prime potent IL-4, IL-5 and IL-13 recall responses in splenocytes from recipient mice, while DCs matured in the presence of Th1 polarising Ag induce elevated IFN-γ production (MacDonald and Pearce, 2002; MacDonald *et al.*, 2001; MacDonald *et al.*, 2002b).

Given the diminished capacity of MØs to secrete T cell polarising cytokines (**Figure 3.1**) or upregulate co-stimulatory molecules (**Figure 3.2**), we predicted that MØs would be significantly less capable than DCs at induction of $CD4^+$ T cell responses. Since both DCs and MØs stimulated with SEA were indistinguishable from unstimulated cells by the parameters measured, it was not clear whether there would be a difference in their ability to prime an SEA specific Th2 response.

Astonishingly, although SEA specific IL-4 elicited by MØs was reduced in comparison to DC primed IL-4 (**Figure 3.3A**), MØs stimulated with St were found to be as competent at inducing an IFN-γ response as similarly activated DCs (**Figure**

3.3B). These results suggest that, when stimulated with SEA, DCs are indeed more capable at priming a Th2 response, as indicated by significantly greater levels of Ag-specific IL-5 and IL-13 induced by DCs in comparison to MØs. However, when stimulated with *S. typhimurium*, both APC types appeared equally proficient at inducing a Th1 response. This was especially surprising given the limited MØ cytokine, co-stimulatory marker and MHC II expression in comparison to similarly treated DCs (**Figure 3.1 and 3.2**). Interestingly, Pa stimulated M∅s did not display a similarly competent ability to generate an IFN-γ response (**Figure 3.3C**).SEA-treated DCs also drove a greater production of Th2 cytokines than SEA-treated MØs following restimulation of spleen cells with plate-bound αCD3. However, IFN-γ production by spleen cells stimulated with αCD3 was comparable following exposure to either DCs or MØs treated with Pa or St. This result indicates that the discrepancy between Th2 cytokines driven by SEA-treated DC or SEA-treated MØs may be due to the greater inherent capacity of DCs to drive T cell Th2 cytokines rather than an inherent ability to process and present SEA antigen, whereas both DCs and MØs share a roughly equivalent capacity to drive Th1 responses. It should be noted that the quantity of IFN-γ produced by spleen cells restimulated with αCD3 following exposure to St-treated DCs or MØs is expected to be much higher. Further experiments are needed to confirm the quantity of IFN-γ secreted by spleen cells following transfer St-treated APC and restimulation by αCD3.

These results highlight the importance of functional assays to address hypotheses generated from *in vitro* studies. Often assumptions about an APC's aptitude for induction of effector T cell responses are derived solely by measurement of cytokine production or co-stimulatory molecule expression *in vitro*. However, the

data presented here (**Figure 3.3**) clearly illustrates that such assumptions may be misleading, and that between *in vitro* culture of APCs, and actual function *in vivo,* there remain several variables either not measured, not accounted for, or simply not yet known.

3.4 Do separate but similarly polarising pathogenic stimuli drive distinct DC cytokine expression profiles?

As DC and MØ comparisons demonstrated that DCs generated the greatest production of cytokine *in vitro*, and superior Th2 inductive capacity *in vivo*, we then focussed on this APC type. DC activation was assessed in greater detail in an additional time course study utilizing a more exhaustive array of pathogen-derived products. DCs were exposed to either Th1 (St, Pa, LPS) or Th2 (SEA, PGE_2, ES62) associated stimuli for 1.5, 6, 12 and 24 hours and cytokine secretions measured by ELISA (**Figure 3.4**).

While optimal IL-12p70 cytokine production took at least 12 hours, IL-12p40, IL-6 and TNFα attained peak production by 6 hours post stimulation with Th1 Ags (**Figure 3.4**). IL-10 meanwhile typically peaked at 12 hours following exposure to St and Pa stimulation and then decreased over the remainder of the time course. Although all Th1 polarising pathogens assessed triggered similar levels of DC IL-12p40, IL-6 and TNFα, there was a substantial difference in the profile of IL-10 and IL-12p70 secretion. Of the Th1 associated Ag preparations, stimulation via Gram-positive *P. acnes* yielded the highest IL-10 production and the lowest IL-12p70, while exposure to Gram-negative *S. typhimurium* triggered elevated IL-12p70 and comparably less IL-10. LPS induced moderate levels of IL-12p70 but no more IL-10

than unstimulated controls. Th2 polarising pathogens or Th2 associated molecules, meanwhile, failed to provoke secretion of any of the cytokines measured above the level produced by unstimulated control DCs.

3.5 Notch: A DC activation marker associated with specific pathogens?

Not only have Notch ligands been suggested to play a role in translating the recognition of microbial products into specific signals instructing T cells responses, (Amsen *et al.*, 2004; Hoyne *et al.*, 2001; Maekawa *et al.*, 2003; Osborne and Minter, 2007; Wong *et al.*, 2003) but distinct Notch ligand families have been associated with induction of either Th1 or Th2 differentiation (Amsen *et al.*, 2004; Maekawa *et al.*, 2003; Tu *et al.*, 2005). We wished to examine whether the association of *delta* ligands with Th1 and *jagged* with Th2 remained consistent when DCs were treated with complex pathogen-derived Ag preparations as opposed to the single TLR agonists that had previously been studied (Amsen *et al.*, 2004). In order to determine if Notch ligand expression by APCs might relate to their ability to provide 'signal 3' to $CD4^+$ T cells, quantitative PCR was used to assess *delta1*, *delta3*, *delta4*, *jagged1* and *jagged2* mRNA levels in APCs exposed up to 24 hours to various Th polarising stimuli.

Measurement of mRNA by quantitative PCR required optimising primers, reagents and temperatures necessary for this technique. For optimisation, reactions were run repeatedly across a temperature gradient and the conditions that produced the most reliable measurements with a satisfactory melting curve were subsequently used. *jagged2* primers were designed in house using Ensembl genome browser (www.ensembl.org), *delta4* and *delta3* primers were the same as used by Amsen et

al. (2004), while *jagged1* and *delta1* primers were designed by Dr P Loke. However, throughout these studies, no evidence of *delta3* expression was found in either DCs or MØs (data not shown).

After stimulation, 10^6 DCs or MØs were stored in Trizol (Invitrogen) at -80° C and cDNA was generated and stored at -20° C. Initially, cDNA copies were produced using Oligo$(dT)_{15}$ and the housekeeping gene β-actin was used as an indicator of total RNA extracted. Oligo(dT) primers are the most widely used method for conversion of mRNA into cDNA as they anneal to the poly(A) sequences present at the 3' end of the majority of mRNA sequences (Hagenbuchle *et al.*, 1979; Nam *et al.*, 2002; Verma, 1978; Weiss *et al.*, 1976). β-actin is a cytoskeletal protein whose mRNA is ubiquitously expressed at moderate levels in most cell types and was one of the RNAs to be used as an internal standard (Bustin, 2000). However, it was later found that β-actin was not a reliable normalization tool as it is differentially expressed upon APC activation (Bustin, 2000). Instead, 18s, a ribosomal RNA component, was used for all subsequent quantitative PCR experiments as it is thought to remain at a constant proportion to the total RNA (Bustin, 2000). As a result of this alteration in protocol, the technique for cDNA synthesis was also changed and Random Hexamers (Promega) were used instead of Oligo(dT), since 18s is a ribosomal component and thus does not have a poly(A) tail. Intriguingly, 18s real time amplification was conducted on cDNA generated with Oligo(dT) primers and an abundant product was constitutively observed despite the supposed absence of a 18s poly(A) tail (data not shown). Although there is very little literature discussing the possibility of polyadenylated ribosomal RNA there have been some studies indicating the existence of 18s rRNA-like mRNAs (Mauro and Edelman,

1997) or polyadenylated rRNA (Slomovic *et al.*, 2006). Nevertheless, as the reliability of 18s measurement using Oligo(dT) was not sufficiently established, further work was conducted with cDNA synthesised using Random Hexamers.

RNA was extracted from 10^6 DCs stimulated for 6, 12 or 24 hours with either medium alone (open bars), SEA (black bars), St (grey cross-hatched bars) or Pa (grey bars), and then quantitative PCR was performed to assess mRNA levels of *jagged1*, *jagged2*, *delta1* and *delta4* (**Figure 3.5**). Across multiple experiments *delta4* expression was significantly increased over unstimulated controls when activated by Pa or St, beginning at 6 hours and maintained throughout the time course, although St induced *delta4* returned to levels comparable to medium controls by 24 hours post Ag exposure. *delta1* showed a trend for elevated expression at 6 hours before returning to control levels by 12 hours in both St and Pa stimulated DCs. It is important to note that *delta1* and *delta4*, although both upregulated in response to Th1 stimuli, did not display the same expression kinetics, indicating that Notch ligand family members are not necessarily regulated in the same manner. Surprisingly, given the suggestion that Jagged ligands are associated with Th2 stimuli (Amsen *et al.*, 2004), *jagged1* also displayed a trend for upregulation at 6 hours in response to Pa and St. *jagged2* was significantly decreased 6 hours following bacterial stimulation across multiple experiments in comparison to controls, but returned to unstimulated levels by 12 hours (**Figure 3.5**).

In contrast to Pa, SEA stimulation showed no significant upregulation of any of the Notch ligands measured over that of unstimulated controls. Although there was a trend for upregulation of *jagged2* 6 hours after SEA stimulation, this was not consistent across multiple experiments. This suggests that, contrary to expectation,

jagged1 and *jagged2* expression are not indicative of DCs stimulated by Th2 pathogens.

In order to generate a better picture of the overall Notch ligand expression profile, the fold change in expression between unstimulated DCs and those exposed to Ag were compiled (**Figure 3.6**). One of the more striking observations from these results was the maintenance of *jagged2* expression following SEA stimulation compared to the distinct downregulation of *jagged2* by Pa stimulated DCs. Additionally, this presentation format highlights the dramatic increase in *delta4* expression by DCs that are bacterially activated.

MØs were also assessed for expression of Notch ligands. However, during DC and MØ comparisons quantitative PCR methods had not yet been optimised and, consequently, results were variable. Across several experiments MØs showed increased expression of *jagged2* in response to St and SEA, but not Pa stimulation (**Figure 3.7**). *delta4* was substantially increased by St stimulation at both 1.5 and 24 hours, although apparently not at 6 hours. Unfortunately, in these experiments data was not generated for MØs exposed to Pa for 24 hours.

Although further study is required for a more comprehensive overview of M∅ Notch ligand expression, this initial comparison of DCs and MØs does suggest that different APCs may utilise Notch ligands in distinct manners. Indeed, it appears that activation with the same stimuli may trigger entirely different patterns of Notch ligand expression between the two cell types, suggesting that Notch ligand expression profiles may not only be subject to timing and stimulation, but also to APC type. Additionally, more work is needed in determining the impact of activation on Notch ligand protein expression. There may yet be substantial regulation of

surface expression of Notch ligands subsequent to mRNA expression, however, at the time of experiments reliable antibodies were unavailable.

3.6 Is Notch ligand expression altered during infection?

As an initial assessment of *jagged2* expression during Th2 immunity we examined samples isolated from *Schistosoma mansoni* infected mice. Mesenteric lymph node, spleen, liver and gut ileal samples were removed from mice infected with 75 *S. mansoni* cercariae 8 weeks post infection, at the peak of the Th2 response, and expression of *jagged2* was then measured by quantitative PCR (**Figure 3.8**). Interestingly, spleens of infected mice displayed abundant expression of *jagged2* whereas expression levels in uninfected mice were undetectable. In mesenteric lymph nodes and ileal samples, there were detectable levels of *jagged2* expression, but no significant difference was observed between infected or non-infected animals. There were no detectable levels of *jagged2* from liver samples (data not shown).

Although this is a very preliminary insight into the affect of Th2 driving pathogen on *jagged2* expression, it appears to indicate that, at least in the spleen, *S. mansoni* infection does have a substantial impact on the overall expression profile of *jagged2*. This suggests an association between *jagged2* expression in some tissues and Th2 infections. Whether or not this upregulation is due to DCs or other cell types is unclear, and future studies in which cells could be isolated *ex vivo* for Notch ligand analysis are required.

3.7 Conclusions

CD4$^+$ T cells orchestrate many of the activities of the adaptive immune system, but in order to exercise their function they must first be provided instruction by cells displaying their specific Ag in the context of MHC II. Unlike MHC I, which is expressed ubiquitously, MHC II has a much more confined distribution and is restricted to APCs such as B cells, MØs and DCs. However, these APCs do not merely serve to provide signalling through the T cell receptor (TCR), but also modify and adapt the T cell response to suit the invading pathogen. Thus APCs behave as the "conductor to the immune orchestra" dictating the tone of the adaptive immune response through a range of secreted and surface bound molecular signals tailored by the APC's innate capacity to recognise pathogens. DCs are considered more effective than either MØs or B cells at activating naïve T cells (Banchereau and Steinman, 1998; Mellman and Steinman, 2001; Mellman *et al.*, 1998; Steinman and Inaba, 1989).

Culturing both DCs and MØs from bone marrow under similar conditions allowed us to directly compare the extent of cytokine and co-stimulatory marker expression in response to a variety of Th1 and Th2 pathogens or their products. Although a further comparison involving B cells would have been of interest, it was not possible to carry out such experiments due to time constraints. Furthermore, work of others has disregarded B cells as potential professional APCs since they concentrate self antigen too readily and, if allowed to activate a virgin T cell to the same extent as other APC, may overload the immune system with autoreactive antigens (Epstein *et al.*, 1995).

One advantage of comparing DCs and MØs in the manner outlined in this chapter is that the culture conditions used to generate the cells were extremely

controlled. DCs grown in the presence of recombinant GM-CSF (Lutz *et al.*, 1999) yielded CD11c$^+$ cells of high purity in a predominantly immature state. MØs were cultured for fewer days in the presence of recombinant M-CSF in order to generate F4/80$^+$ CD11c^{low} cells that were also immature (Fischer *et al.*, 1988). L929 medium is commonly used to grow MØs. However, rM-CSF was chosen instead amidst concerns that components within L929 medium might influence MØ activation. A recent study comparing the two culture methods found slight differences in the activation status of MØs grown either using rM-CSF or L929 medium, which was attributed to soluble factors secreted by the fibroblasts (including IL-1β, IL-2, IL-12, GM-CSF, IFN-γ, VEGF and RANTES) (Gersuk *et al.*, 2008). By limiting the possibility of exogenous activation factors in the growth of these two APC types, the expression of T cell polarising signals should be specifically derived from exposure to pathogen derived Ags.

Assessment of the response of the different APCs over a detailed time course clearly indicated that BM-DCs expressed greater levels of cytokines and co-stimulatory molecules compared to their BM-derived MØ counterparts, on a per cell basis. DCs produced greater levels of IL-12, TNFα and IL-6 relative to MØs, as well as expressing a more pronounced up-regulation of MHC II, CD80 and CD86. One possible explanation is that the manner in which these MØs were grown inherently biased the cells towards an alternatively activated state in which MHC II expression and overall APC function was reduced. Early studies have demonstrated that MØs grown with M-CSF exhibit transient antigen-presentation capability when compared to MØ grown using GM-CSF, or when cultured with IFN-γ (Fischer *et al.*, 1988; Germann *et al.*, 1992). Lee *et al.* (2005) observed that MØs grown from BM with M-

CSF in the culture medium yielded cells that were F4/80$^+$, MHC Class II$^+$, and CD11c$^-$ but were unable to induce proliferation of anti-CD3 mAb-primed T cells. However, proliferation of T cells was significantly enhanced if chemokines such as Lkn-1 are added to the MØ culture media (Lee *et al.*, 2005). A more recent study investigated the impact of colony stimulating factor – 1 (CSF-1) on the MØ expression of the Notch ligand *jagged1* (Goh *et al.*, 2008). Interestingly, it was shown that BM-MØs are able to express more *jagged1* than BM-DCs in response to SEA, however this *jagged1* expression was impaired by the presence of CSF-1 (Goh *et al.*, 2008). This study highlights the fact that DC and MØ activation, including expression of Notch ligands, are susceptible to the presence of cytokine and tissue factors in culture.

Nevertheless, when Ag-activated DCs and MØs were transferred into C57BL/6 mice, not only did the MØs stimulate an immune response, but when MØs were activated with heat-killed *S. typhimurium* prior to transfer, recipient mice produced similar levels of IFN-γ to mice that had received St pulsed DCs. While *in vitro* data would suggest that DCs are far more proficient at initiating primary immune responses, this *in vivo* data argues that, depending on the Ag, MØs can be fully capable of stimulating T cells to the same extent as DCs. This view is supported by recent publications demonstrating that MØs can directly stimulate T cells *in vivo* to proliferate and mature into both effector and memory cells (Pozzi *et al.*, 2005) and that both DCs and MØs can present live *Salmonella enterica* antigen to T cells (Kalupahana *et al.*, 2005). In these studies MØs were either grown using L929 conditioned medium (Pozzi *et al.*, 2005) or were a cell line (Kalupahana *et al.*, 2005) and thus a direct comparison to BM-DC cultured with GM-CSF should be treated

with caution. Pozzi et al. (2005) found that both DCs and MØs were equally proficient at driving Tg T cell proliferation as well as stimulating the generation of effector T cells capable of producing IFN-γ (Pozzi *et al.*, 2005). These findings using transgenic T cells are in accordance with the data presented in this chapter using the heat-killed Gram-negative bacterium St. Since MØs grown using M-CSF do produce significantly less IL-12p70 (**Figure 3.1**) and express very little MHC class II (**Figure 3.2**) compared to DCs, and yet stimulate an St-specific Th1 immune response as efficiently (**Figure 3.3**), then there must be some as yet poorly understood mechanism occurring *in vivo* that allows efficient antigen presentation which has not yet been revealed by *in vitro* phenotyping experiments.

It is possible that IFN-γ, other cytokines, or migratory chemokines produced by the recipient mouse and not recreated *in vitro*, induce further DC cytokine production and MHC II expression, promoting the innate antigen presenting capability of M∅s (Germann *et al.*, 1992). Injection of cells into a recipient mouse causes local inflammation, encouraging the release of IFN-γ by NK cells (Kobayashi *et al.*, 1989) or peripheral T cells (Skeen and Ziegler, 1995). One alternative explanation is that transferred M∅s act as a reservoir of antigen and, although they themselves do not directly activate T cells, other APCs within the recipient mice might internalize Ag released from lysed injected cells for presentation to T cells. However, previous work from our laboratory has shown that DCs deficient in MHC II are not able to induce Ag specific Th responses after *in vivo* injection (MacDonald *et al.*, 2001). This suggests that Ag is being directly presented by the cells injected and not by resident cells that obtained Ag from the injected cells. This experiment has not yet been repeated using MHC II deficient M∅s.

Time courses of DC activation by an array of pathogen derived products revealed two things. Firstly, kinetic patterns of separate cytokine and co-stimulatory molecules were distinct depending upon the stimuli. Secondly, diverse stimuli, even if they are associated with similar $CD4^+$ T cell polarisation, displayed discrete profiles of maturation. Early expression of some cytokines (such as TNFα and IL-12p40) versus the later peak of expression found in IL-12p70 (and to some extent IL-6) (**Figure 3.4**) emphasizes shifting cytokine secretion profiles of DCs over time. Interestingly, stimulation with broadly similar Th polarising pathogens or products yielded variable levels of cytokine secretion. This result exemplifies the notion that DCs are able to specifically tailor their response to the unique pathogen profile they encounter. It should be noted, however, that there remains a great deal of difficulty in directly comparing cellular responses to different whole heat killed bacteria. In this case, the quantity and diversity of TLR agonists provided by each bacterium is difficult to elucidate. Furthermore, the engagement of multiple different PRRs may yield entirely different outcomes than would otherwise be expected from a single PAMP (Napolitani *et al.*, 2005). Of course it is the very complexity endowed by whole pathogen derived Ag that is of interest, since the more closely the APC stimulus resembles what the immune system would naturally encounter, the more relevant the induced response is likely to be.

Although the signalling mechanisms APCs employ to drive Th1 polarisation is relatively well characterised, how APCs promote Th2 differentiation remains unclear (MacDonald and Maizels, 2008; MacDonald *et al.*, 2001). Adoptive transfer of Th2 polarised DCs initiates a Th2 recall response in a manner dependent on MHC II (MacDonald *et al.*, 2001), NF-kB (Artis *et al.*, 2005), CD40 (MacDonald *et al.*,

2002b), and OX40L (Jenkins *et al.*, 2007). However, the precise mechanisms underlying this interaction have proven elusive. Importantly, levels of expression of these molecules remain unchanged following DC stimulation with SEA. So, although the presence of these molecules is critical, none of them require significant upregulation in order to drive Th2 differentiation. Discovery of that would indicate an APC primed for induction of Th2 responses has been an ongoing goal of our laboratory and others for many years. It is in this context that we investigated the potential role for Notch ligands as markers of Th2 activation of DCs, particularly given the recent suggestion that the Jagged family of Notch ligands could potentially be a mechanism of Th2 instruction by APC to naïve T cells (Amsen *et al.*, 2007; Amsen *et al.*, 2004; Tu *et al.*, 2005).

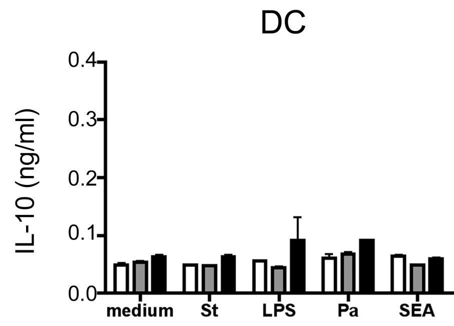
The Notch ligand expression data presented in this chapter demonstrates that DCs can express both the Delta and Jagged family of ligands concurrently in either Th1- or Th2-priming conditions, but their relative expression changes depending on the stimulus encountered. Previous work had demonstrated enhanced expression of the Notch ligands *delta4* and *jagged1* by BM-DC in response to stimulation by LPS, whereas stimulation with the 'Th2 inducers' cholera toxin and PGE_2 promoted *jagged2* expression (Amsen *et al.*, 2004). However, this work only examined expression after a single time point (6 hours post stimulation) and the Th2 nature of these stimuli is open to question. Our results agree with the suggestion that Th1 related Ags induce *delta4* expression and early *delta1* in DCs. However, DCs stimulated with the Th2 Ag SEA only displayed a trend for enhanced *jagged2*, and this was solely evident at 6 hours following exposure and not later time points. Intriguingly, there was a significant decrease in *jagged2* expression by DCs in

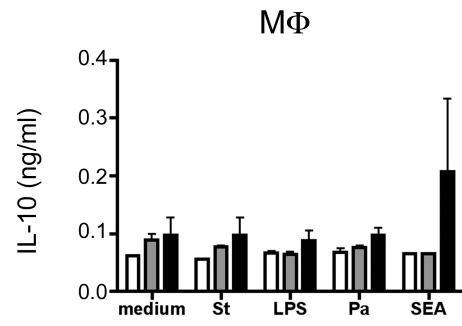
response to stimulation by heat-killed *P. acnes*, a trend that was also consistent in St-activated cells. Conceivably, the ratio of different Notch ligands expressed by APCs may ultimately determine the manner in which Notch signalling affects the differentiation of a naïve T cell. Although these results do not support the simplistic association of Jagged ligand expression by APCs exposed to all Th2 pathogens, it is clear that the relative expression profile of Notch ligands is influenced by the activation status, and may relate to the Th priming capacity, of the DC. Therefore, it remains a distinct possibility that Notch ligands expressed by APC are utilized as an additional mechanism to provide "signal 3" for driving a T helper cell response.

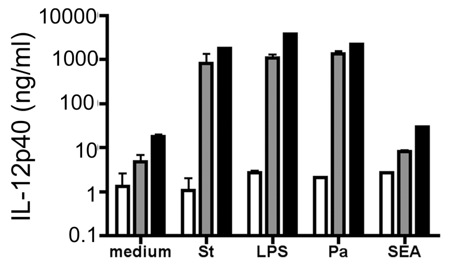
By examining the effect of *S. mansoni* infection on *jagged2* expression in immunologically relevant sites we could begin to assess whether Notch ligands may be relevant during Th2 dominated disease. The liver and gut are the primary sites of the immune response during *S. mansoni* infection while the mesenteric lymph nodes and spleen are secondary lymphoid tissues facilitating the interaction of APCs and lymphocytes. Intriguingly, there did appear to be an increase in overall expression of *jagged2* in the spleen of *S. mansoni* infected animals, although which cell types account for this increase is unclear. If, as this data suggests, there is no significant increase in *jagged2* expression at the site of egg deposition in the gut ileum, or any detectable *jagged2* in liver, then the proposition that *jagged2* expression is directly affected during interaction with pathogen products seems unlikely. Rather, the evidence that *jagged2* is upregulated in lymphoid tissues, such as the spleen, suggests that, if Notch ligands do fulfil an immunological role, it is likely involved in the induction of adaptive immunity. However, given that no significant difference was found between the expression of *jagged2* in mesenteric lymph nodes from

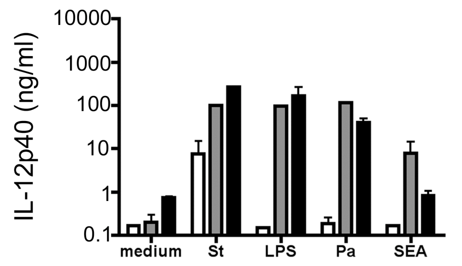
infected or non-infected mice, the particular environment of the spleen may be more conducive to fluctuation in the expression of Notch ligands.

Ideally, the use of Notch ligand specific antibodies and immunohistochemistry could provide a more detailed insight into the cell-specific surface expression of Notch ligands during infection. Unfortunately, due to the highly conserved nature of Notch proteins throughout mammalian species, the generation of antibodies suitable for flow cytometry, western blotting or immunohistochemistry was unreliable at the time of these experiments. Future studies into the cell-specific *in vivo* expression of Notch ligands during immunological challenge would prove invaluable to our understanding of these ligands and their role in immunology.


DC
IL-10 (ng/ml)
0.4
0.3
0.2
0.1
0.0
medium
St
LPS
Pa
SEA


MΦ
IL-10 (ng/ml)
0.4
0.3
0.2
0.1
0.0
medium
St
LPS
Pa
SEA


IL-12p40 (ng/ml)
10000
1000
100
10
1
0.1
medium
St
LPS
Pa
SEA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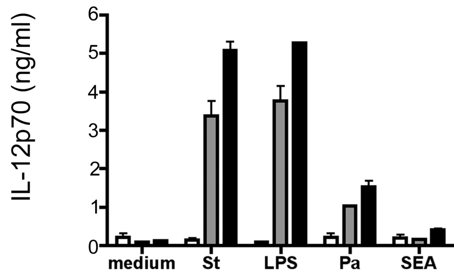
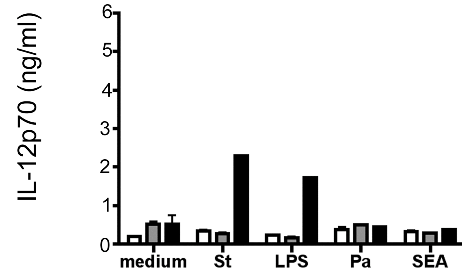
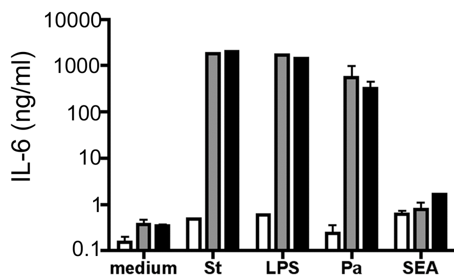
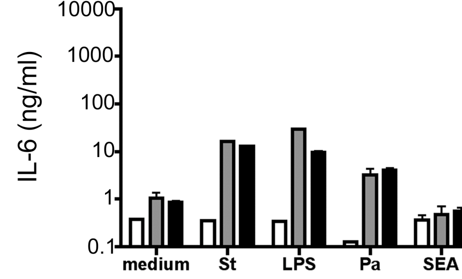
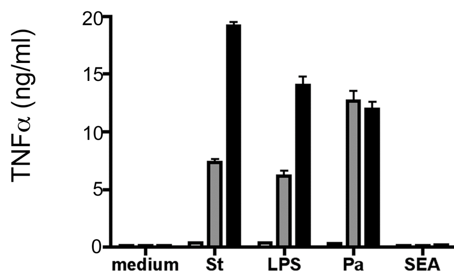
10000
1000
100
10
1
0.1
medium
St
LPS
Pa
SEA


IL-12p70 (ng/ml)
6
5
4
3
2
1
0
medium
St
LPS
Pa
SEA


IL-12p70 (ng/ml)
6
5
4
3
2
1
0
medium
St
LPS
Pa
SEA


IL-6 (ng/ml)
10000
1000
100
10
1
0.1
medium
St
LPS
Pa
SEA


IL-6 (ng/ml)
10000
1000
100
10
1
0.1
medium
St
LPS
Pa
SEA


TNFα (ng/ml)
20
15
10
5
0
medium
St
LPS
Pa
SEA

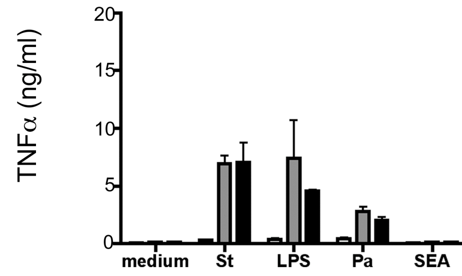

TNFα (ng/ml)
20
15
10
5
0
medium
St
LPS
Pa
SEA

Figure 3.1 Comparison of cytokine production by DCs and M∅s in response to a variety of stimuli. 10^6 DCs or M∅s were stimulated with medium alone (medium), St (2 μg/ml), LPS (50 ng/ml) Pa (10 μg/ml) or SEA (25 μg/ml) and supernatants harvested 1.5 (open bars), 12 (grey bars) or 24 hours later (black bars) for analysis by using ELISA. Bars represent mean + standard error from triplicate culture wells. Data shown are representative of 5 different experiments.

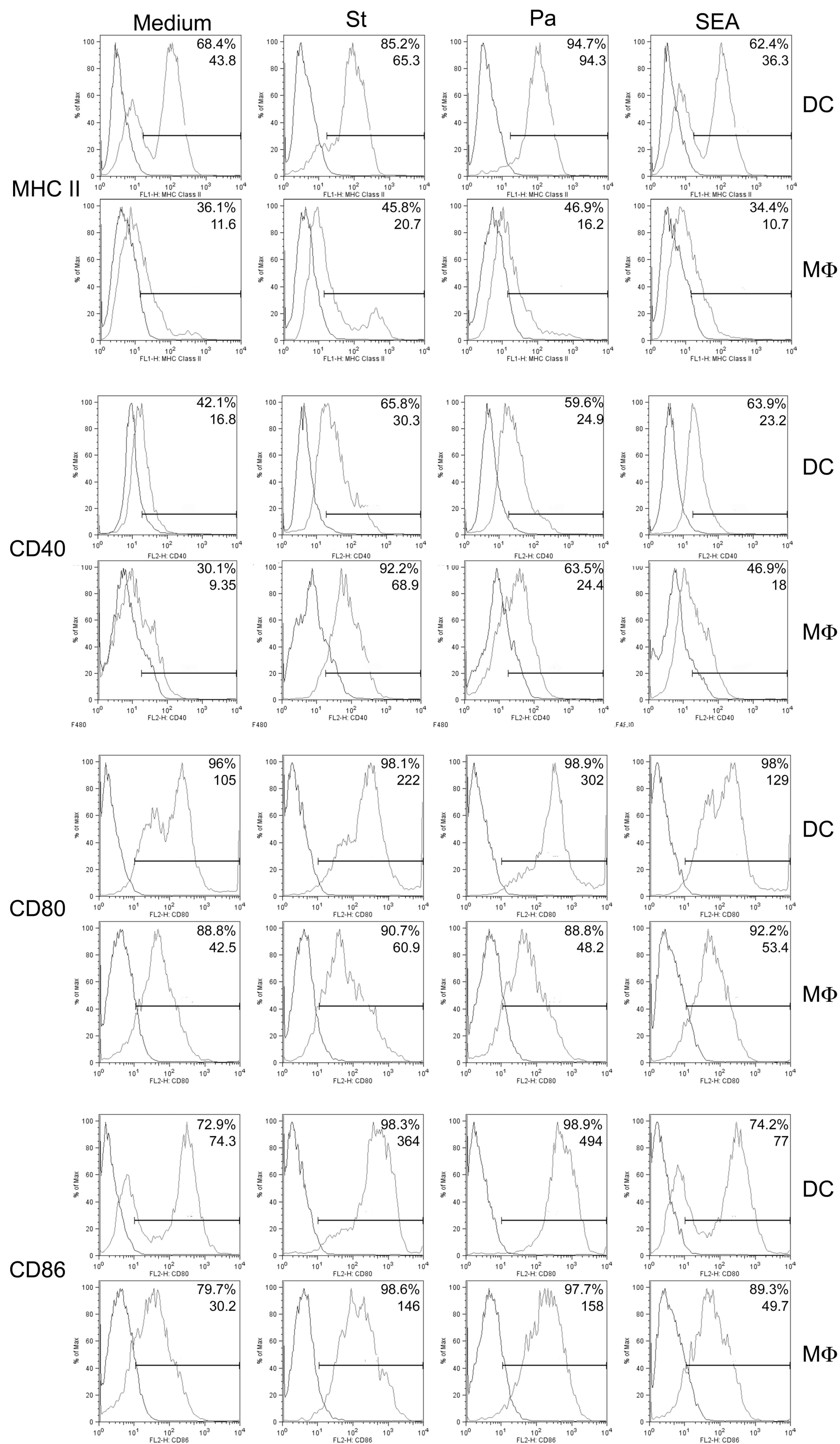

Medium
St
Pa
SEA
MHC II
CD40
CD80
CD86
DC
MΦ
68.4%
43.8
85.2%
65.3
94.7%
94.3
62.4%
36.3
36.1%
11.6
45.8%
20.7
46.9%
16.2
34.4%
10.7
42.1%
16.8
65.8%
30.3
59.6%
24.9
63.9%
23.2
30.1%
9.35
92.2%
68.9
63.5%
24.4
46.9%
18
96%
105
98.1%
222
98.9%
302
98%
129
88.8%
42.5
90.7%
60.9
88.8%
48.2
92.2%
53.4
72.9%
74.3
98.3%
364
98.9%
494
74.2%
77
79.7%
30.2
98.6%
146
97.7%
158
89.3%
49.7

Figure 3.2 Phenotypic activation of DCs and M∅s in response to diverse pathogens. 10^6 DCs or M∅s were cultured with medium alone (medium), St (2 μg/ml), Pa (10 μg/ml) or SEA (25 μg/ml) for 24 hours. Surface expression of CD11c (DCs), F4/80 (M∅s) MHC class II, CD40, CD80 and CD86 were then analysed by flow cytometry. Dark histograms depict isotype control staining while lighter histograms indicate cells stained against the marker indicated. DCs were gated on live $CD11c^+$ cells while M∅ were gated on live cells $F4/80^+$ cells. Figures provided in the upper right hand corner of each graph refers to percentage positive and geometric mean fluorescence intensity for the marker indicated. Data shown are representative of 5 different experiments.

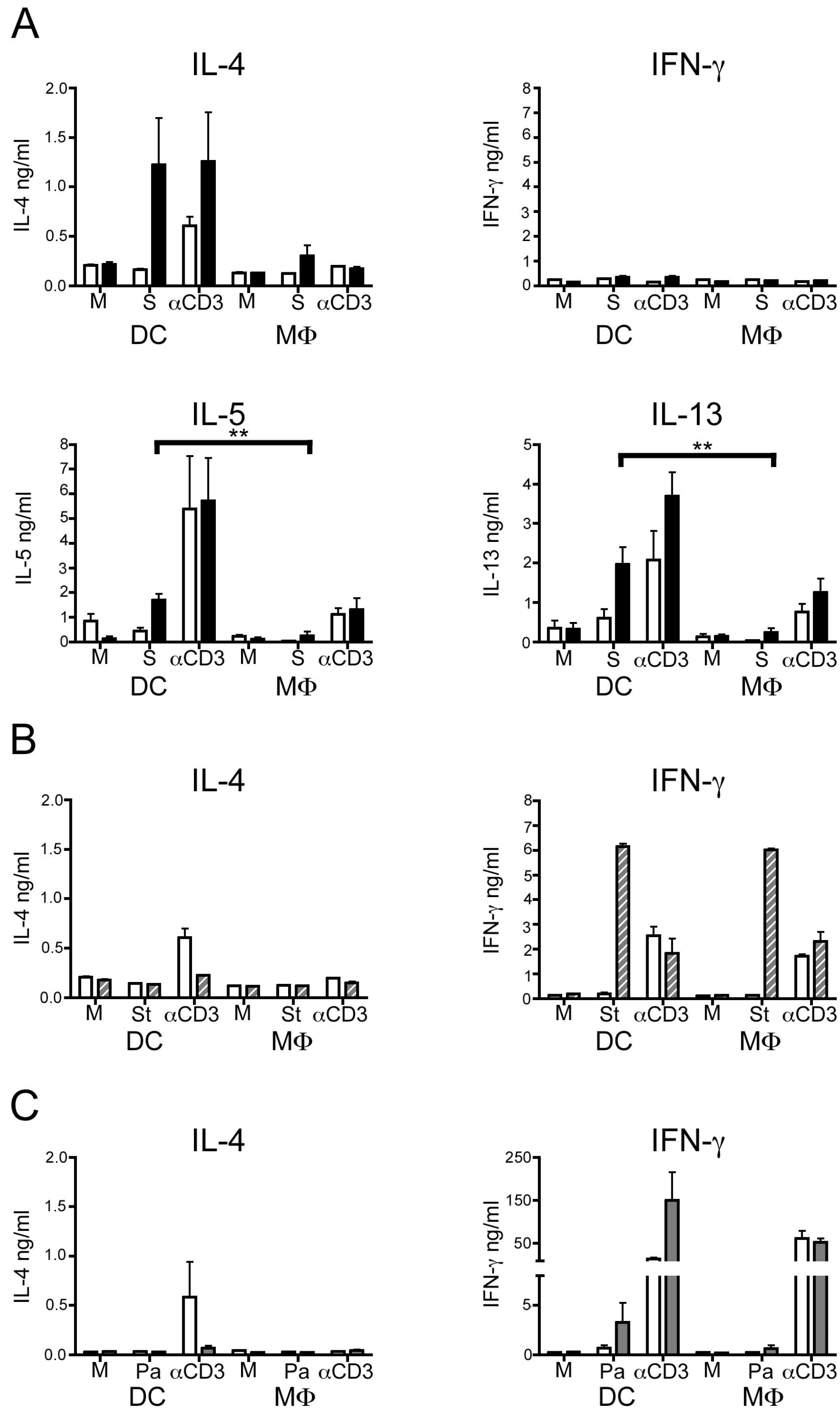


Figure 3.3 T cell polarisation by DCs and M∅s *in vivo*. 5 x 10^5 bone marrow-derived DCs or M∅s stimulated with medium alone (M), **A)** SEA (S, 25 μg/ml), **B)** St (2 μg/ml) or **C)** Pa (P, 10 μg/ml) and injected intraperitoneally (i.p.) into naïve

wild type recipient mice. A week later, splenocytes were cultured for 72 hours with medium (open bars), St (1 μg/ml, grey cross-hatched bars), Pa (5 μg/ml, grey bars), SEA (15 μg/ml, black bars) or plate-bound αCD3 antibodies (16.6 μg/ml, labelled αCD3). Supernatants were then harvested for cytokine measurement by ELISA. Error bars represent mean + SEM from 3 mice per group. Data shown are representative of three experiments total. ** = $p < 0.01$

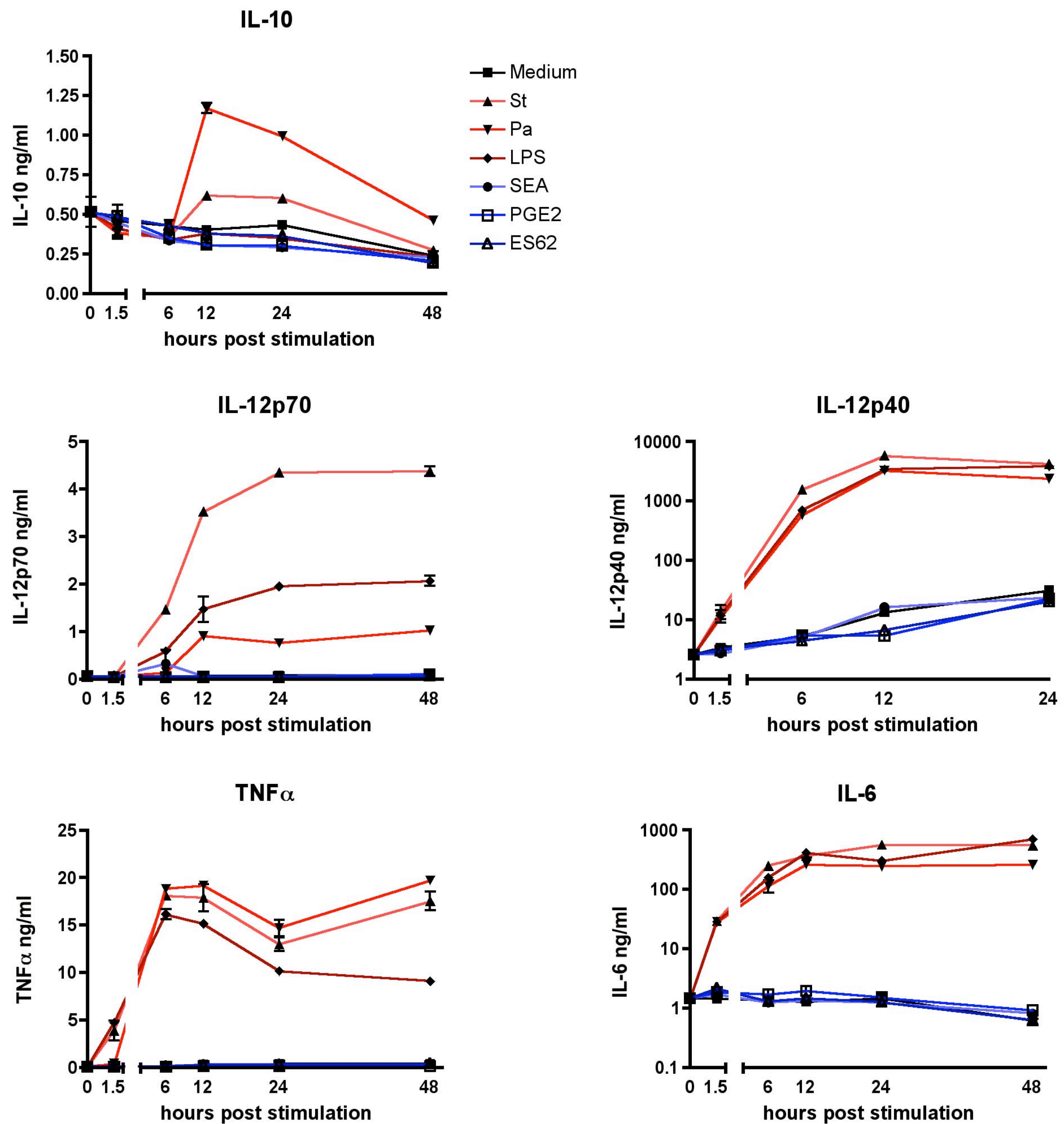


Figure 3.4 Stimulation with diverse pathogen preparations induces distinct cytokine production profiles by DCs. 2x10^{6} DCs were stimulated for either 0, 1.5, 6, 12, 24 or 48 hours with St (2 μg/ml), Pa (10 μg/ml), LPS (50 ng/ml), SEA (25 μg/ml), PGE_2 (10^{-6} Mol) or ES62 (2 μg/ml). Supernatants were harvested and analysed for cytokine production by ELISA. Data represents mean + SEM of duplicate wells and are representative of at least 2 separate experiments per antigen.

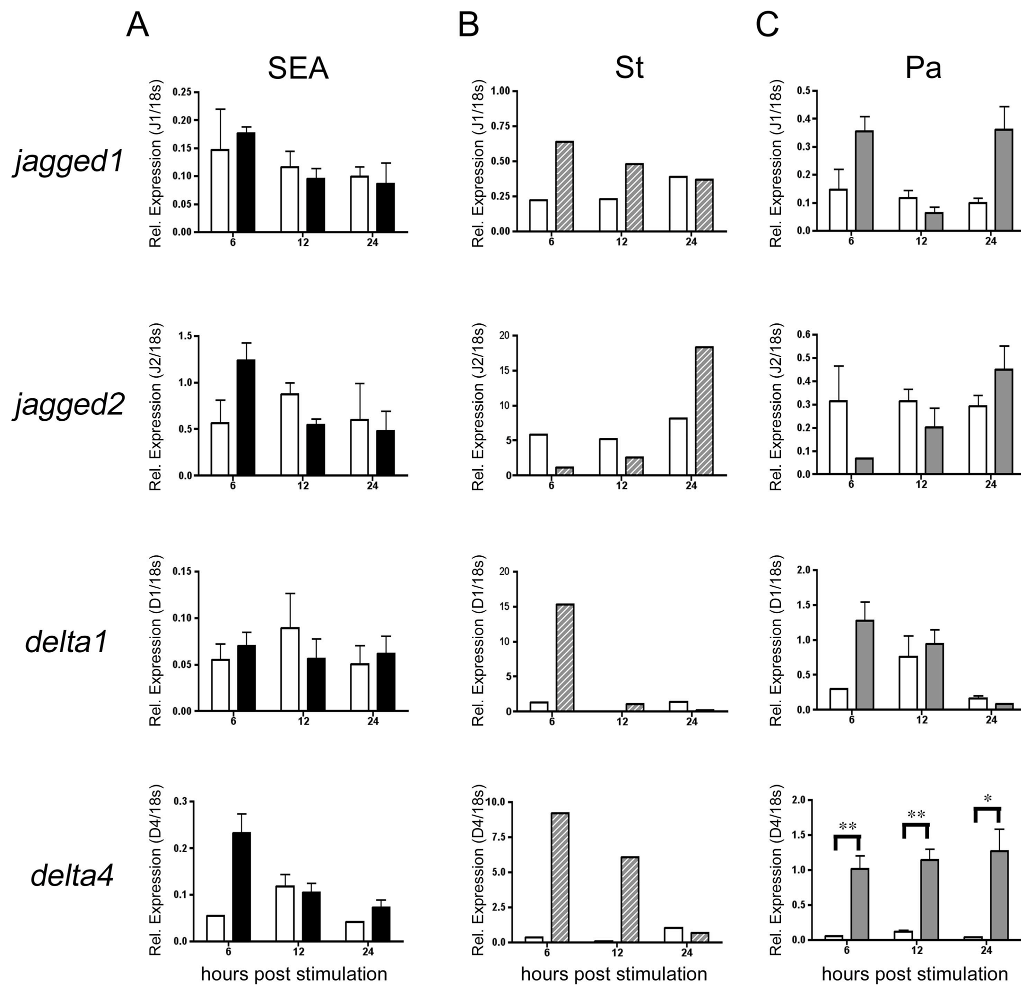


Figure 3.5 DC Notch ligand expression in response to SEA, Pa or St. 2x10^{6} DCs were stimulated for 6, 12 or 24 hours with medium alone (open bars), **A)** SEA (25 μg/ml, black bars), **B)** St (2 μg/ml, grey cross-hatched bars), or **C)** Pa (10 μg/ml, grey bars). *jagged1, jagged2, delta1,* and *delta4* mRNA was assessed by quantitative PCR, normalised against 18s rRNA. **A)** and **C)** Errors bars indicate mean + SEM of triplicate culture wells and are representative of four separate experiments. **B)** data shown are representative of three separate experiments derived from single culture wells. * = $p < 0.05$, ** = $p < 0.01$

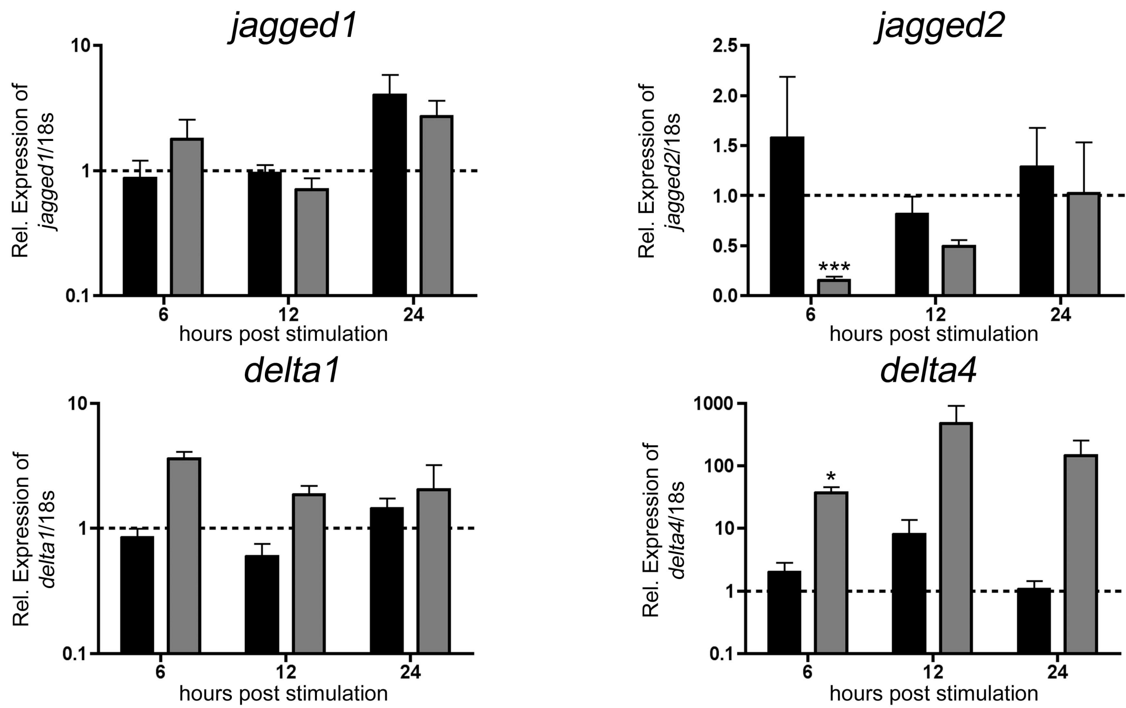


Figure 3.6 Relative expression of Notch ligands by DCs in response to SEA or Pa stimulation. Expression of *jagged1, jagged2, delta1* and *delta4,* as determined by quantitative PCR, were normalised to 18s RNA. Data indicate fold change of expression over unstimulated cells (dotted line) by DCs exposed to SEA (25 μg/ml, black bars) or Pa (10 μg/ml, grey bars) for 6, 12 or 24 hours. Data are mean + SEM of three to six combined experiments. * = $p < 0.05$, *** = $p < 0.001$ comparing expression by SEA- or Pa- stimulated groups relative to medium controls.

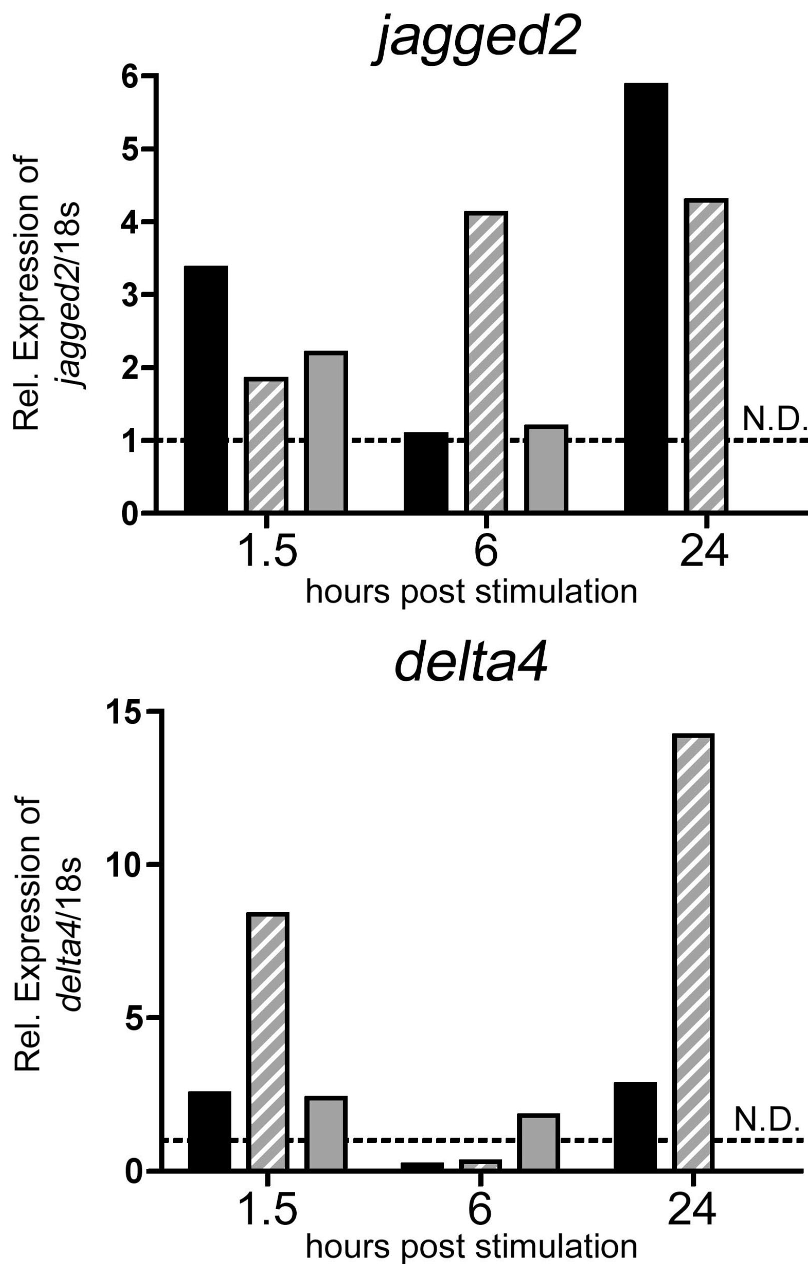


Figure 3.7 M∅ Notch ligand expression in response to SEA, St and Pa. Expression of *jagged2* and *delta4* were measured by quantitative PCR and normalised to 18s RNA. Data indicate fold change of expression over unstimulated cells (dotted lines) by M∅s exposed to SEA (25 μg/ml, black bars), St (2 μg/ml, grey cross-hatched bars), or Pa (10 μg/ml, grey bars) for 6, 12 or 24 hours. Data are representative of three experiments comparing expression of SEA-, St- or Pa-stimulated groups relative to medium controls using triplicate wells. N.D. = not done

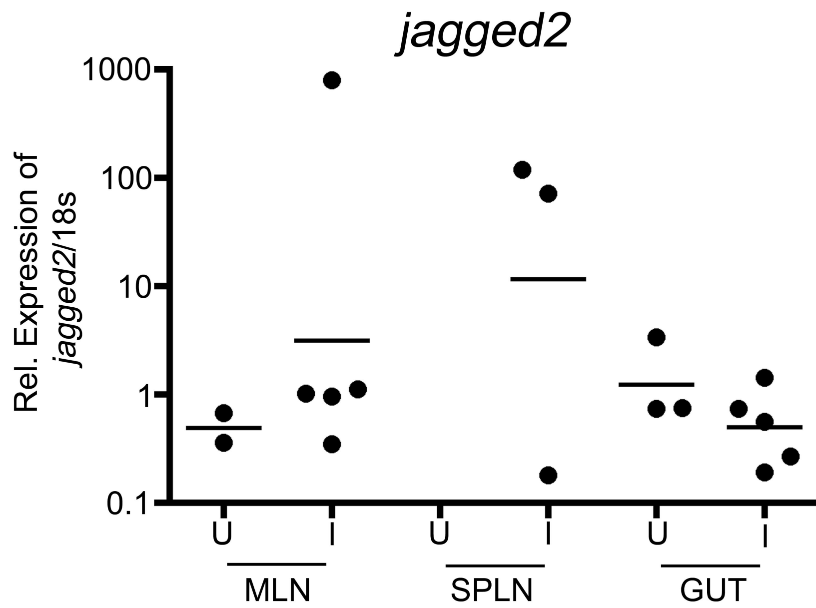


Figure 3.8 ***jagged2*** **expression in** ***Schistosoma mansoni*** **infection.** The expression of *jagged2* was determined in mesenteric lymph nodes (MLN), spleen (SPLN) and gut ileum from samples of uninfected controls (U, 2-3 mice) or *S. mansoni* infected animals (I, 5 mice). Data points represent individual mice.

Dendritic Cells

MHC

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	43.8	73.6	81.2	24.8	36.9	52.06	24.26042044
St	65.3	104	46.2	221	47	96.7	73.33157574
Pa	94.3	75.8		202	43.3	103.85	68.74525438
SEA	36.3	116	77.3	26	29.3	56.98	38.8904487

CD40

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	4.8	16.8	2.38	21.3	7.95	10.646	8.080073019
St	9.35	30.3	25.5	199	14	55.63	80.59044298
Pa	12	24.9		146	9.94	48.21	65.52861258
SEA	6.1	23.2	10.6	27.4	5.51	14.562	10.10780491

CD80

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	105	97.9	21.2	323	44.2	118.26	107.158156
St	222	330	27.6	1273	286	427.72	435.097407
Pa	302	229		775	124	357.5	249.2092494
SEA	129	110	20.2	424	101	156.84	138.6593754

CD86

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	74.3	82	22.5	276	42	99.36	90.91610638
St	364	224	43.6	1207	265	420.72	406.6006316
Pa	494	231		931	173	457.25	299.0722112
SEA	77	139	25.4	357	90.5	137.78	115.4276986

Macrophages

MHC

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	7.28	49.9	4.31	11.6	12.6	17.138	18.6168558
St	4.76	13.5	5.49	20.7	3.47	9.584	7.353796978
Pa	7.55	35.7		16.2	10.2	17.4125	12.71733561
SEA	6.06	22.1	5.05	10.7		10.9775	7.812470267

CD40

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	6.95	37.3	3.22	11.4	23.2	16.414	13.88401887
St	7.47	66.9	17.8	162	91.7	69.174	62.40178187
Pa	8.97	46.6		98.1	52.1	51.4425	36.53636396
SEA	6.33	39.6	6.69	11.6		16.055	15.87968199

CD80

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	12.7	58.3	4.31	42.5	36.9	30.942	22.13356772
St	14.7	57.8	14.2	60.9	62.7	42.06	25.26584651
Pa	15.8	47.6		48.2	50.9	40.625	16.61211907
SEA	15.4	61.4	13.3	53.4		35.875	25.08324474

CD86

	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Average	STD
Medium	11.1	54.7	4.91	30.2	51.6	30.502	22.70544032
St	6.09	55.7	19	146	43.6	54.078	54.98951646
Pa	10	62.4		158	39.4	67.45	64.06275569
SEA	9.7	52.7	18.7	49.7		32.7	21.71021265

Table 3.1 Combined Data from DC and M∅ flow staining. 10^6 DCs or M∅s were cultured with medium alone (medium), St (2 μg/ml), Pa (10 μg/ml) or SEA (25 μg/ml) for 24 hours. Surface expression of CD11c (DCs), F4/80 (M∅s) MHC class II, CD40, CD80 and CD86 were then analysed by flow cytometry. Mean fluorescence Intensity is shown as gated on live $CD11c^+$ DCs or F4/80+ M∅ from each of five independent experiments. The average of these values and the standard deviation is show n at right. Experiments 3 did not include Pa stimulation, and Experiment 5 did not have an SEA stimulus for M∅s.

Chapter 4

Does the expression of Notch Ligands by Dendritic Cells affect their ability to prime a CD4$^+$ T cell response?

4.1 Introduction

Given its ubiquity in expression, and diversity of function, identifying the relationship between Notch signalling and the establishment of an immune response presents a complex and daunting challenge. Interest in the role of Notch signalling during peripheral immunity has been present since the first human *notch1* was isolated from a T cell-leukaemia (Ellisen *et al.*, 1991; Hoyne *et al.*, 2000a; Milner and Bigas, 1999; Robey, 1999). In addition to the considerable research examining the function of the Notch pathway during haematopoiesis it was Hoyne et al. (2000) who first postulated that Notch ligand expression by APC may have a direct impact on the differentiation of naive T cells in the periphery (Hoyne *et al.*, 2001; Hoyne *et al.*, 2000a; Hoyne *et al.*, 2000b). With the observation that both Notch receptors and ligands are differentially expressed by CD4$^+$ T cells and DCs in response to tolerance induction, Hoyne *et al*. (2000) suggested that by expression of *serrate1* (now called *jagged1* in mammalian cells) DCs may promote tolerance (Hoyne *et al.*, 2000b). As further studies examined the distinctive expression patterns of Notch ligands on APCs (Yamaguchi *et al.*, 2002) and the impact of Notch receptor signalling on peripheral T cells (Maekawa *et al.*, 2003; Vigouroux *et al.*, 2003; Wong *et al.*, 2003; Yvon *et al.*, 2003) the possibility that APCs may directly regulate their Notch ligand expression profile to influence T cell polarisation became evident. Several studies

now suggest that signalling through Notch receptors has an effect on both T cell proliferation and mature T cell commitment (Amsen *et al.*, 2004; Hoyne *et al.*, 2001; Hoyne *et al.*, 2000b; Maekawa *et al.*, 2003; Vigouroux *et al.*, 2003). In the context of $CD4^+$ T cell polarisation, a recent *in vivo* study showed that blocking the ability of T cells to respond to signalling in Notch receptors 1-4 resulted in impaired Th2, but not Th1, responses (Tu *et al.*, 2005). In addition, studies *in vitro* have suggested that both Delta and Jagged ligand families may be associated with T cell differentiation but that Delta ligands promote Th1 whereas Jagged ligands promote Th2 polarisation (Amsen *et al.*, 2004; Maekawa *et al.*, 2003; Napolitani *et al.*, 2005). However, this contention remains controversial, with other reports suggesting that no such association exists, that Delta can inhibit T cell cytokine production rather than promote Th1 differentiation (Stallwood *et al.*, 2006; Wong *et al.*, 2003), or that Jagged may induce Treg, rather than Th2, differentiation (Dallman *et al.*, 2003; Hoyne *et al.*, 2000b; Minter *et al.*, 2005; Vigouroux *et al.*, 2003; Wong *et al.*, 2003; Yvon *et al.*, 2003), or even blockade Th1 (Sun *et al.*, 2008). The contrasting nature of these separate studies indicate that proper analysis of a pathway as complicated as Notch in a system as sophisticated as peripheral immunity will require insights from both multi-variant *in vivo* approaches as well as reductionist *in vitro* experiments.

Following the apparent association between relative expression of Notch ligands and Th1 or Th2 inducing stimuli (**Figure 3.6**), we went on to test their relevance in these systems by manipulating the expression pattern of Notch ligands on DCs. DCs were used primarily for these experiments as they are considered the most proficient APC at activating naïve T cells (Steinman, 2007), a reflection supported by our own experiments comparing DCs and M∅s in (**Chapter 3**). Their

ability to dictate the tone of the developing $CD4^+$ T cell response is thought to be largely determined by the nature of the stimulus they encounter (Banchereau and Steinman, 1998). However, particularly in the case of Th2 development, the molecular mechanisms utilized by DCs to influence and instruct T cell polarisation are not completely understood (MacDonald and Maizels, 2008; Reis e Sousa, 2006).

Initially, RNA interference (RNAi) was used in an attempt to manipulate expression of Notch ligands by DCs. RNAi is an evolutionarily conserved gene silencing mechanism present in a vast array of eukaryotic organisms that serves as a safeguard against the threat of exogenous DNA either from viral infection or mobile genetic elements. First reported in 1990 as an inducible process for suppression of gene expression (Napoli *et al.*, 1990; van der Krol *et al.*, 1990), the mechanism of RNAi was finally unravelled in 1998 (Fire *et al.*, 1998) as short double stranded RNA was used to inhibit expression of complementary RNA sequences. Since then, RNAi has become an invaluable technique for the targeted silencing of specific genetic expression in a variety of tissues and organisms. Once introduced, long double stranded RNAs are processed by an RNase-III like enzyme known as Dicer into smaller 20-25 nucleotide segments known as small interfering RNAs (siRNA). These siRNAs then activate the RNA-induced silencing complexes (RISC) pathways which cleave and degrade complementary RNA strands (Hammond *et al.*, 2000; Hannon, 2002). Unfortunately, in mammals, the addition of long double stranded RNA activates innate anti-viral mechanisms resulting in nonspecific inhibition of protein synthesis and RNA degradation (Gao and Zhang, 2007). However, the direct addition of siRNA to the cell can bypass this potent anti-viral response (Stallwood *et al.*, 2006). There are several possible techniques for taking advantage of the RNAi

mechanism in order to achieve targeted gene knockdown including direct application of siRNA or through the expression of short hairpin RNA (shRNA) using vectors which are then cleaved into the necessary siRNA sequence (Gao and Zhang, 2007).

Recent reviews suggest that more effective, and perhaps more consistent, gene expression knockdown can be achieved by transfecting shRNA using viral vectors (Gao and Zhang, 2007). However, this method entails the cultured generation of cell lines with incubation of the vector-mediated shRNA transfection. In contrast, siRNA would allow for the inhibition of any combination of Notch ligands at any point during the experiment once the appropriate complementary RNA strands are constructed and optimal transfection method ascertained (Stallwood *et al.*, 2006). Furthermore, siRNA has been successfully demonstrated in primary immune cells such as T cells and DCs (Hill *et al.*, 2003; Laderach *et al.*, 2003; Liu *et al.*, 2004; McManus *et al.*, 2002; Stallwood *et al.*, 2006).

Transgenic mice provide an alternative approach for investigation into the function of Notch ligand expression by DCs. Unfortunately, induced deficiency in any of the Notch pathway genes is almost universally embryonic lethal (Maillard *et al.*, 2005). However, this technical difficulty can be bypassed through the use of foetal liver chimeras. Briefly, foetal livers are taken from embryonic day 15 in mice and then injected into irradiated recipient mice, resulting in notch gene deficient haematopoietic cells (Jiang *et al.*, 1998; Washburn *et al.*, 1997). The caveat of this system is that only cells whose progenitors arise from a haematopoietic origin will be deficient in gene expression.

Additional techniques used for interfering with the Notch pathway during the process of antigen presentation include the addition of a pharmacological blockade to

disrupt signalling. Several studies have investigated the role of Notch signalling in immune cells through the use of γ secretase inhibitors which block Notch receptor cleavage and the release of the Notch intracellular domain (N-IC), disabling the classical CSL-dependent pathway (Adler *et al.*, 2003; Eagar *et al.*, 2004; Stallwood *et al.*, 2006). Importantly, the use of γ secretase inhibitors, such as N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT), do not discriminate between the effects of different Notch ligands in their signalling inhibition (Geling *et al.*, 2002). This pharmacological blockade serves as a whole-scale inhibition of the entire Notch pathway. It is possible that there are cleavage independent forms of Notch signalling, in which case γ secretase inhibitors may only disrupt a subset of Notch functions (Stallwood *et al.*, 2006).

Although many fascinating avenues of research present themselves in the investigation of Notch signalling in the immune system, we focussed first on the specific role, if any, Notch signalling has when Notch ligands are presented by DCs to Notch receptors on naïve T cells. A major goal of this thesis was to attempt to understand whether DCs utilize these molecular tools during antigen presentation. Specifically, do the changes in Notch ligand expression by stimulated DCs play an active role in the process of conferring specific Th differentiation?

AIMS

1) To devise a successful method for the manipulation of Notch ligand expression by DCs
2) To assess whether the manipulation of Notch ligand expression by DCs influences their development or activation

3) To determine whether Notch ligand expression by DCs is required, redundant or irrelevant in regard to their ability to prime a Th response

4.2 Can RNA interference be used to inhibit Dendritic cell expression of Notch ligands?

Successful post-transcriptional inhibition of gene expression by RNAi has only recently been demonstrated in mammalian DCs (Hill *et al.*, 2003; Laderach *et al.*, 2003; Liu *et al.*, 2004; Stallwood *et al.*, 2006). Employing RNAi in DCs is particularly difficult since not only can double stranded RNA elicit a type-1 IFN response, inducing non-specific inhibition of transcription and cell death, but it can also activate PRRs, such as TLR3 (Kadowaki *et al.*, 2001). For this reason we chose to use Stealth™ RNAi technology (Invitrogen), which utilizes proprietary chemical modifications specifically designed by the manufacturer to enhance knockdown of expression while avoiding innate activation of the recipient cell. To assess the precise contribution of the Notch ligand targeted for knockdown, it was first essential to exclude the possibility that the process of RNA interference influences the activation status of the DC. DCs receiving siRNA treatment were checked by ELISA and flow cytometry for hallmarks of activation (MHC II, CD86, IL-12, IL-10 and IL-6) to monitor whether the addition of the siRNA oligos themselves induced DC maturation. To determine whether successful knockdown was achieved, initial experiments used siRNA complementary to the gene encoding CD86 using the companies own algorithm software. Since DC surface expression of CD86 can be easily measured by flow cytometry, it was chosen as a useful target for determining the efficiency of the RNA inhibition, as opposed to Notch ligands, for which there

were no specific antibodies available for flow cytometry at the time of the experiments.

Several parameters required optimisation before RNA interference could be used for determining the specific effects of reduced DC Notch ligand expression. For maximum transfection efficiency and efficacy, the concentration of siRNA oligos, transfection reagent and timing of siRNA addition had to be calibrated. Furthermore, RNAi techniques had to be optimised to reduce additional activation of DCs. Assessment of RNAi effectiveness by quantitative PCR demonstrates the overall reduction of mRNA expression across a population of cultured DCs. However, it does not demonstrate either the efficacy of siRNA on a per cell basis, or whether knockdown of gene transcription correlates with knockdown of protein expression. To address these details, DCs were exposed to fluorescent RNA oligonucleotides under the same conditions as standard siRNA in order to determine the transfection efficiency of the system (**Figure 4.1A**). Back-gating from fluorscent-oligo positive cells indicates that the majority of successfully transfected cells were a discrete population of live cells. However, by 24 hours the proportion of living fluorescent-oligo containing cells had decreased, potentially indicating that the transfection process may negatively impact long-term cell viability (**Figure 4.1B)**. Additionally, DCs treated with siRNA were assessed by flow cytometry to measure MHC II as a marker of activation (**Figure 4.1C**). There were no significant differences in MHC II expression under all treatment conditions used indicating little change in DC activation status due to siRNA treatment. However, treatment did appear to have differing effects on fluorescent oligonucleotide transfection rate depending on whether cells were stimulated, and the time point at which they were assessed. *S.*

typhimurium was used as a control to test for the impact of DC activation on transfection efficiency. At each time point, a greater percentage of DCs incorporated fluorescent oligonucleotides when siRNA was given prior to bacterial stimulation. Cytokine production was also measured to observe whether siRNA treatment activated DCs, but no significant difference in cytokine secretion between control siRNA oligonucleotides, functional siRNA oligonucleotides or the absence of oligonucleotides was found (**Figure 4.2**).

To determine both the effectiveness and duration of expression knockdown, siRNA specific for CD86 was added to DCs either concurrently or previous to treatment with either medium alone or *S. typhimurium*. Then, CD86 mRNA expression was measured by quantitative PCR after 6 or 24 hours (**Figure 4.3**). As introduced in the previous chapter, *S. typhimurium* strongly activates DCs and thus markedly up-regulates CD86 expression. siRNA appeared to successfully inhibit CD86 mRNA expression by 24 hours in culture (**Figure 4.3A and 4.3B**). Addition of siRNA prior to stimulation with *S. typhimurium* appeared to improve the effectiveness of expression knockdown (**Figure 4.3A and 4.3B**). However, as samples were derived from single culture wells, statistical analysis was not possible. Although *S. typhimurium* stimulated DCs expressed greater levels of CD86 mRNA than media controls at 6 hours, 24 hours after stimulation these results were reversed. This may be due to a feedback mechanism whereby after an initial increase in CD86 mRNA expression following stimulation, this mRNA expression is then reduced. It is unclear what exactly influences this change in expression at a later time point, but the result exemplifies the importance of timing when assessing mRNA expression. Unfortunately, elevated CD86 expression by DCs exposed to scrambled siRNA

controls indicated that addition of RNA alone was encouraging DC activation (data not shown).

Since the extent of expected gene knockdown was unknown, it was thought that inhibiting CD86 expression during its peak production would more easily reveal the success of RNAi as opposed to homeostatic conditions. However, it is unclear whether addition of siRNA simultaneously with antigen disrupts the RNAi pathway during such a transcriptionally active and dynamic period as DC maturation.

To complement the quantitative PCR approach, flow cytometric analysis was used to address whether RNAi affected the cell surface expression of CD86 protein (**Figure 4.4**). Although by 24 hours CD86 mRNA expression was reduced (**Figure 4.3**), the cell surface expression of CD86 by DCs treated with siRNA oligos was equivalent to DCs with no siRNA treatment as measured by flow cytometry (**Figure 4.4**). One possible reason for the discrepancy between mRNA expression and cell surface protein expression may be retention of CD86. Even in the presence of siRNA complementary to CD86 mRNA, there may be a reservoir of protein, which has already transcribed and is awaiting deployment to the cell surface. If so, cell surface expression would potentially be unaffected by recent mRNA inhibition.

We also tested whether the siRNA approach might still be valid for inhibition of expression of Notch ligands by DCs. Once the quantity of lipofectamine and siRNA concentration had been optimised for transfection efficiency, siRNA specific for both *jagged2* and *delta4* were transfected into DCs and knockdown assessed by quantitative PCR. These two genes were chosen since these displayed the most dramatic change in expression in differentially activated DC (**Figure 3.6**) and have been implicated in other studies as influencing naïve $CD4^+$ T cell differentiation

(Amsen *et al.*, 2004). When effective knockdown was not achieved (data not shown), additional transfection reagents were tested including Hipofectamine 2000 (Invitrogen), as well as alternative siRNAs specific for the same Notch ligands. All together 16 separate experiments were conducted in order to both optimise conditions and attempt to attain successful and consistent knockdown of expression, however no significant knockdown of expression of any targeted gene was achieved.

There are several possibilities for why Notch ligand expression by DCs was not impaired by siRNA, but most likely a combination of factors was the cause of such inconsistent results. As measured by fluorescent siRNA, transfection efficiency typically varied between 70-85%. However, according to manufacturer's instruction, such quality of transfection is on the borderline of effectiveness. If the siRNA oligos used to inhibit mRNA expression of targeted genes were also sub-optimal, then this, in combination with mediocre transfection, may explain the marked variation in effectiveness of attempted gene knockdown between experiments. Potentially, the use of a broader range of siRNA oligonucleotides would have yielded more successful results.

4.3 Does the absence of jagged2 have an impact on the development and activation of DCs?

Given the lack of success of the siRNA approach, we attempted an alternative method for generating DCs deficient in expression of specific Notch ligands. *jagged2* deficiency is embryonic lethal (Jiang *et al*., 1998) and, Notch signalling is required for the development of many haematopoietic cell types (Cheng *et al*., 2003; Olivier *et al*., 2005). *jagged2* was targeted for gene knockout both due to its potential

association with distinct Th responses (Amsen *et al.*, 2004) and the fact that, unlike *jagged1* or *delta1*, it is not thought to be essential for DC differentiation (Ohishi *et al.*, 2001; Olivier *et al.*, 2005; Schroeder and Just, 2000b). In order to circumvent the difficulties of generating viable Notch deficient mice, *jagged2* deficient bone marrow was generated from foetal liver chimeras, and kindly supplied by Caetano Reis e Sousa. Foetal livers from Ly5.2^{+} *jagged2*$^{+/-}$ x *jagged2*$^{+/-}$ matings were removed from d14.5 embryos, and *jagged2*$^{-/-}$ tissues were identified by PCR (Jiang *et al.*, 1998; Washburn *et al.*, 1997). Irradiated Ly5.1^{+} recipients were reconstituted with cells from *jagged2*$^{-/-}$ or *jagged2*$^{+/+}$ womb mates. DCs were grown from BM isolated from chimeras 8-20 weeks later. Donor origin of DCs was verified by flow cytometry (**Figure 4.5**) to ensure that DCs grown from *jagged2* deficient bone marrow were Ly5.2^{+} but not Ly5.1^{+}. After standard ten day culture, cell yields of *jagged2*$^{-/-}$ BM-DC were equivalent to *jagged2*$^{+/+}$ controls, suggesting that the growth and development of DCs from this bone-marrow was not impaired by *jagged2* deficiency (data not shown).

To ensure that DCs were *jagged2* deficient, quantitative PCR was used to assess the level of *jagged2* mRNA (**Figure 4.6**). Analysis of Notch ligand expression by these DCs revealed that they neither expressed *jagged2* mRNA, nor was expression of other Notch ligands upregulated as a compensatory mechanism. The lack of *jagged1* up-regulation, for example, suggests that any effect upon development, activation or function of these DCs would solely be the result of *jagged2* deficiency.

In addition to possible effects on the expression of other Notch ligands, the issue of whether the absence of Jagged2 made an impact on more classical indicators

of DC activation was examined. Both *jagged2*$^{+/+}$ and *jagged2*$^{-/-}$ DC were analysed for secretion of cytokines and expression of co-stimulatory molecules (**Figures 4.7 and 4.8**) in response to stimulation with a heat-killed preparation of *P. acnes*, or SEA. For all parameters measured, the markers of activation were unaffected by the absence of *jagged2*. Thus, *jagged2*$^{-/-}$ DC appeared similar to their wildtype counterparts other than lacking *jagged2* expression.

4.4 Are *jagged2*-deficient DCs impaired in their ability to influence T cell activation and proliferation?

Once it had been established that the absence of *jagged2* had no major effect on DC development or activation, we set out to determine whether the absence of *jagged2* alone impacts the DC's ability to prime a T cell response. In order to dissect whether expression of *jagged2* specifically impacts proliferation and polarisation of CD4^{+} T cells, we first used an *in vitro* co-culture system of DCs and transgenic OVA specific OTII T cells.

DCs exposed to SEA or Pa were pulsed with either whole OVA protein or OVA peptide$_{323\text{-}329}$, then co-cultured with purified CD4^{+} T cells from OTII mice, which express a T cell receptor (TCR) specific for ovalbumin peptide. Co-culturing DCs with OTII Tg T cells provides a reductionist experimental system, removing the possible influence of other cell types such as B cells, M∅s or NK cells. Thus, any differences observed using *jagged2* deficient DCs are attributable solely to the expression of Notch ligand by the APC. At first, a similar protocol was used to that reported by Jankovic *et al.* in 2004 (Jankovic *et al.*, 2004) wherein BM-DC where stimulated overnight with SEA (50μg/ml). DCs were then added at 2.5x10^{5} cells per

well to $2x10^5$ purified $CD4^+$ T cells from OTII mice in a volume of 0.2 ml with $OVA_{323-329}$ and co-cultured for 5 days, with the addition of rIL-2 for the last 2 days. However, using this method Th2 specific induction of IL-4 as assessed by intracellular staining or supernatant ELISA was not achieved.

Over the course of subsequent experiments, several parameters of the protocol were altered, including timing of DC stimulation, comparison of OVA protein to peptide, DC:T cell ratio, and variation of the period of time DCs were incubated with T cells in co-culture, in order to optimise the system. Throughout this process of optimisation, only DCs derived from wild type C57BL/6 mice were used, as the supply of *jagged2* deficient bone marrow was limited. The use of either $OVA_{323-329}$ peptide (50 ng/ml) or whole OVA protein (200 μg/ml) was compared in order to determine whether optimal T cell polarisation by DCs required the processing of entire protein or, should protein degradation and MHC loading interfere with the maturation process, the use of $OVA_{323-329}$. In addition, fewer DCs were cultured with $CD4^+$ T cells in accordance with Artis et al. (2005) (Artis *et al.*, 2005). Initial experiments showed enhanced T cell proliferation in response to whole protein (**Figure 4.9A**). However, significant T cell expansion occurred even in the absence of DC maturation. Based on this, $OVA_{323-329}$ was used for subsequent work, as the resulting proliferation profile was the clearest for all stimulation conditions carried out, including the extension of co-culture to 5 days (**Figure 9B**).

Once the *in vitro* co-culture csystem had been optimised with wild type C57BL/6 DCs, the capacity of *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DC to activate and polarise OTII TCR Tg T cells was assessed. Although both were equally proficient in stimulating T cell proliferation as measured by CFSE staining (**Figure 4.10**),

jagged2$^{-/-}$ DC displayed a dramatic impairment in their ability to induce SEA specific IL-4 production in comparison to their jagged2$^{+/+}$ counterparts (**Figure 4.11**). The absence of *jagged2* expression by DCs reduced the proportion of IL-4 producing CD4^{+} T cells from 11.8% to 4.5%, or from 10.8% to 5.2%. Interestingly, the proportion of IFN-γ producing CD4^{+} OTII cells was marginally increased by both *P. acnes* and SEA treated *jagged2* deficient DC in comparison to *jagged2* sufficient counterparts (**Figure 4.11**).

4.5 How is T cell polarisation and proliferation affected by the inhibition of Notch receptor signalling?

As an additional approach to manipulate Notch signalling *in vitro*, the γ-secretase inhibitor N-[N-(3,5-difluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester (DAPT) was used (Dovey *et al.*, 2001). DAPT inhibits the protein complex involved in one of the key cleavage events required for Notch receptor signalling (Geling *et al.*, 2002; Sastre *et al.*, 2001). Addition of DAPT thus prevents all signalling through the Notch receptor pathway, and addresses the question of how Notch signalling influences T cell activation independent of specific Notch ligand expression. When added to co-culture wells (5 μM) containing DCs ($2x10^4$/well), OTII T cells ($2x10^5$/well), $OVA_{323-329}$ (50 ng/ml) and SEA (50 μg/ml), there was a marked impairment of SEA-specific IL-4 production compared to culture wells in the absence of DAPT (**Figure 4.12**). It is interesting to note that the blockade of Notch receptor signalling was comparable to the absence of DC *jagged2* expression as measured by this *in vitro* co-culture system (**Figure 4.11 and 4.12**).

These combined results indicated that the process by which Notch signalling affects Th2 differentiation *in vitro* is critically dependant on *jagged2* expression by DCs despite it's lack of significant upregulation by Th2 stimuli. It is unclear, however, whether the Notch ligand *jagged2* alone is required for Th2 induction, or if the absence of any individual Notch ligand would impair Th2 induction in this setting.

4.6 Does the absence of jagged2 expression by DCs affect their ability to prime an immune response in vivo?

We then asked whether expression of *jagged2* was also important for polarisation of Th cells by DCs in a more complex, and arguably more relevant, *in vivo* setting. *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DCs were stimulated with Pa or SEA and then injected into the footpads of naïve C57BL/6 mice. Four days later, draining LN were removed and examined for cytokine secretion following restimulation with Ag in vitro. Pa activated DC induced a marked Th1 response with Pa-specific IFN-γ detected in culture supernatants irrespective of whether transferred DC were *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ (**Figure 4.13**). Contrary to expectation, SEA stimulated *jagged2*$^{-/-}$ DCs induced an equivalent or higher IL-4 response after transfer into naïve wild type animals. This was irrespective of the route of immunization as *jagged2*$^{+/+}$ and *jagged2*$^{-/-}$ DCs given i.p. also showed equivalent ability to induce a Th2 response whether measured by IL-5, IL-13 or IL-10 (**Figure 4.13**). Thus, despite the fact that DCs maintain expression of *jagged2* after exposure to SEA *in vitro* (**Figure 3.5**), and *jagged2*$^{-/-}$ DCs are severely impaired in their ability to instruct Th2 polarization *in*

vitro (**Figure 4.11**), these data suggest that DC expression of this Notch ligand is not essential for the establishment of a Th2 response *in vivo*.

4.7 Discussion

Current literature strongly suggests that Notch signalling plays a role in the development of an adaptive immune response (Amsen *et al.*, 2007; Amsen *et al.*, 2004; Dallman *et al.*, 2003; Maekawa *et al.*, 2003; Maillard *et al.*, 2003; Rutz *et al.*, 2008; Tu *et al.*, 2005). Our intention was to first use RNA interference to systematically deplete DC expression of individual Notch ligands, or sets of ligands, in order to determine the importance of each one alone or in combination. Unfortunately, effective RNA inhibition was found to be difficult to achieve, perhaps because the DCs undergoing transfection were primary cells specialised in recognition and response to foreign RNA. In this project, achieving consistent measurable knockdown of DC protein expression using siRNA, while refraining from unnecessary DC activation, proved elusive.

There are several main considerations when attempting to inhibit gene expression via RNA inhibition. First, the sequence of the interfering RNA must be sufficient to engage the RNA inhibitory pathway (Hannon, 2002). Secondly, the RNA interfering sequence must be effectively transported into the cell and allowed to engage with the RISC pathway (Tijsterman and Plasterk, 2004). Finally, effective gene knockdown must be achieved without activating the cell's own anti-viral response (Gao and Zhang, 2007; Tijsterman and Plasterk, 2004). Although a great deal of time and effort was spent trying to optimise this system, reliable results were not obtained.

Inhibition of Notch ligand expression has now been carried out in human cells (Stallwood *et al.*, 2006), where matured human monocyte derived DC were exposed to siRNA specific for *delta1*, *jagged1* or *jagged2* and then co-cultured with allogeneic T cells in a MLR. This study found that a reduction in DC expression of any of the three Notch ligands resulted in enhanced T cell IFN-γ production and proliferation (Stallwood *et al.*, 2006). However, it is important to note that in this study the DCs being transfected with siRNA were in no way primed to drive either a Th1 or Th2 response, but instead were matured by the addition of 20 ng/ml of TNFα. As shown in the previous chapter, DCs exposed to biologically relevant pathogens display distinct expression patterns of Notch ligands corresponding with their T cell polarising nature. Furthermore, no IL-4 was detectable in this report (Stallwood *et al.*, 2006), so it remains to be seen whether the use of RNA inhibition of Notch ligand expression by DCs which are subsequently exposed to pathogens or their products influences their capacity to drive a T helper cell response, particularly Th2 responses.

The advantage of a successful siRNA approach to reduce Notch ligand expression by DC would be that, with the appropriate specificity, any Notch ligand (or combination of ligands) could be targeted for study at the time of the investigator's choosing. The disadvantages are that the effects may be transient and, furthermore, RNAi cannot guarantee complete knockout, only decreased expression (Gao and Zhang, 2007). Therefore, interpretation of any RNAi results would have to take into consideration that small amounts of protein expression may remain, or normal expression may resume later on during the course of the experiment. In the case of Notch signalling, a pathway that is particularly sensitive to small changes in

expression (Artavanis-Tsakonas *et al.*, 1999), a reduction may yield a very different result from complete absence. Additionally, at the time these experiments were being conducted, reliable Notch ligand specific antibodies were unavailable, making confirmation of reduced Notch ligand expression at the protein level technically difficult. Reduction in Notch ligand mRNA may not necessarily correlate with reduction of Notch protein expression on the cell surface.

Having *jagged2* deficient bone marrow available proved a key resource and addressed some of the questions RNAi would not be able to, even if it had worked. Importantly, *jagged2*$^{-/-}$ DC were phenotypically similar to *jagged2*$^{+/+}$ for all the conventional markers of activation measured. Further, *jagged2*$^{-/-}$ DCs did not compensate by up-regulating expression of *jagged1*, *delta1*, or *delta4* in response to stimulation with either *P. acnes* or SEA. Therefore, *jagged2* signalling does not intrinsically affect a DC's ability to grow and develop, or interfere with the expression of cytokines or co-stimulatory markers following *P. acnes* or SEA stimulation. Additionally, any differences in the T cell polarising capacity of *jagged2*$^{-/-}$ and *jagged*$^{+/+}$ DCs are also more likely to be directly due to the presence or absence of *jagged2* and not through a third party pathway.

Our results clearly show that DC expression of *jagged2* alone can be critical for Th2 polarization of naïve CD4^{+} T cells *in vitro*. The presence of the other Notch ligands could not compensate for the absence of *jagged2* in this setting. This indicates that *jagged1*, *delta1*, and *delta4* are all unable to fulfil the signalling requirement by Notch receptors on CD4^{+} T cells in the establishment a Th2 response *in vitro*. Interestingly, inhibition of Notch receptor signalling using *in vitro* DAPT had a similar effect on T cell activation to using *jagged2* deficient DCs (**Figure**

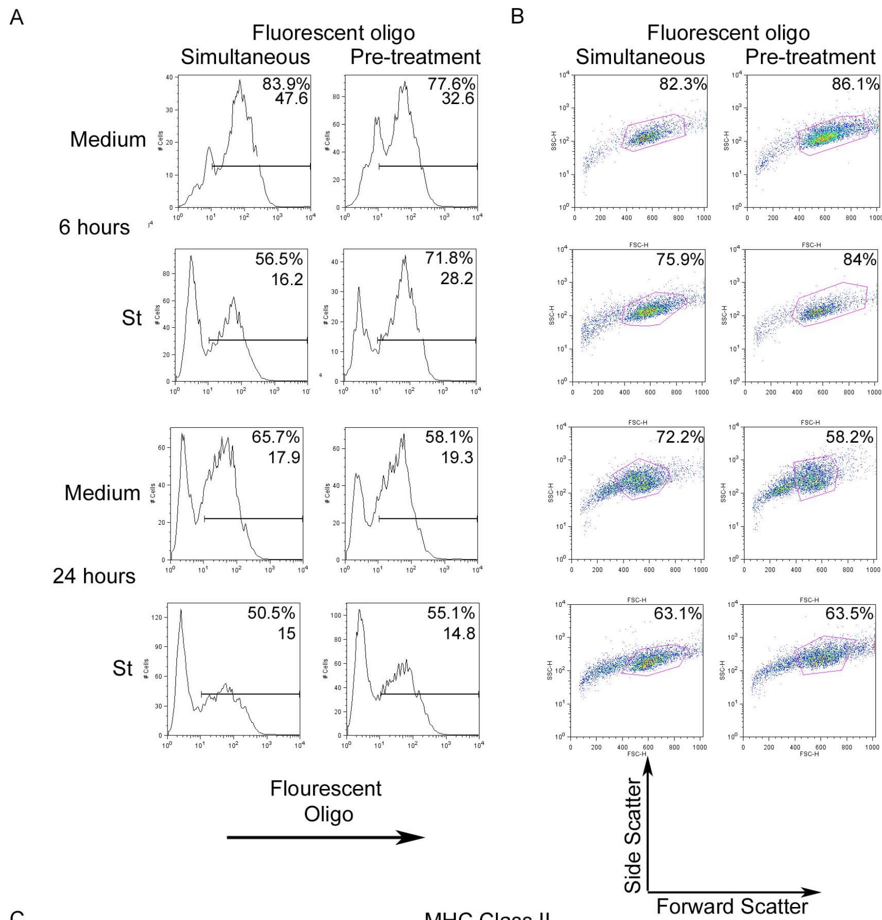
4.12). This suggests that in a reductionist Th2 setting limited to DC and Tg T cells, *jagged2* is the primary Notch ligand utilized by the DC in order to promote a Th2 response. Since this DAPT experiment was only carried out once, additional repeats are needed to confirm and expand this surprising finding.

In the more complex *in vivo* setting, by contrast, DC expression of *jagged2* was found to be dispensable for Th2 induction (**Figure 4.13**). Taken together, our *in vitro* and *in vivo* results suggest that jagged2 signalling is important for Th2 polarisation, yet *jagged2* expression need not be limited to the APC driving the response. For example, the important interaction in vivo may not actually be between a Notch ligand bearing APC and a naïve $CD4^+$ T cell, but rather between APC and NKT cells, or a subset of memory $CD4^+$ T cells capable of producing IL-4 rapidly and independently of STAT6 (Tanaka *et al.*, 2006; van der Vliet *et al.*, 2001). It is therefore possible that, *in vivo*, the multivariant expression of Notch ligands by APCs involves interactions with multiple cell types, since both Notch receptors and ligands can be found in diverse cell types in addition to T cells and DC (Amsen *et al.*, 2004; Baron, 2003).

In future experiments, the importance of provision of *jagged2* by haematopoietic cells could be addressed by adoptive transfer of WT or *jagged2*$^{-/-}$ DCs into chimeric recipient mice grafted with *jagged2* deficient bone marrow, or by infecting such mice with a Th2 dominated infection such as *S. mansoni* or *Trichuris muris*. Another avenue of investigation *in vitro* could be to attempt to rescue the absence of SEA-specific IL-4 by $CD4^+$ T cells stimulated by *jagged2*$^{-/-}$ DC by providing soluble Jagged ligand or co-culturing with non-Ag loaded *jagged2*$^{+/+}$ DCs, M∅s or even epithelial cells. Successful induction of IL-4 by this method would

indicate whether *jagged2* provision is required by the primary APC or if *jagged2* expression by adjacent cell types is sufficient.

Our *in vivo* experiments, in which all such cells were present, revealed that DC expression of the Notch ligand *jagged2* does not play a dominant role during SEA-specific Th2 induction. Whether this finding is indicative of the provision of jagged2 by additional cell types, a redundant pathway, or compensation via other Notch ligands remains to be determined. Even so, these results challenge the model that selective expression of Jagged ligands by DCs is responsible for determining Th2 differentiation, and suggest that reliance on reductionist *in vitro* systems to investigate the consequence of immune interactions can be misleading.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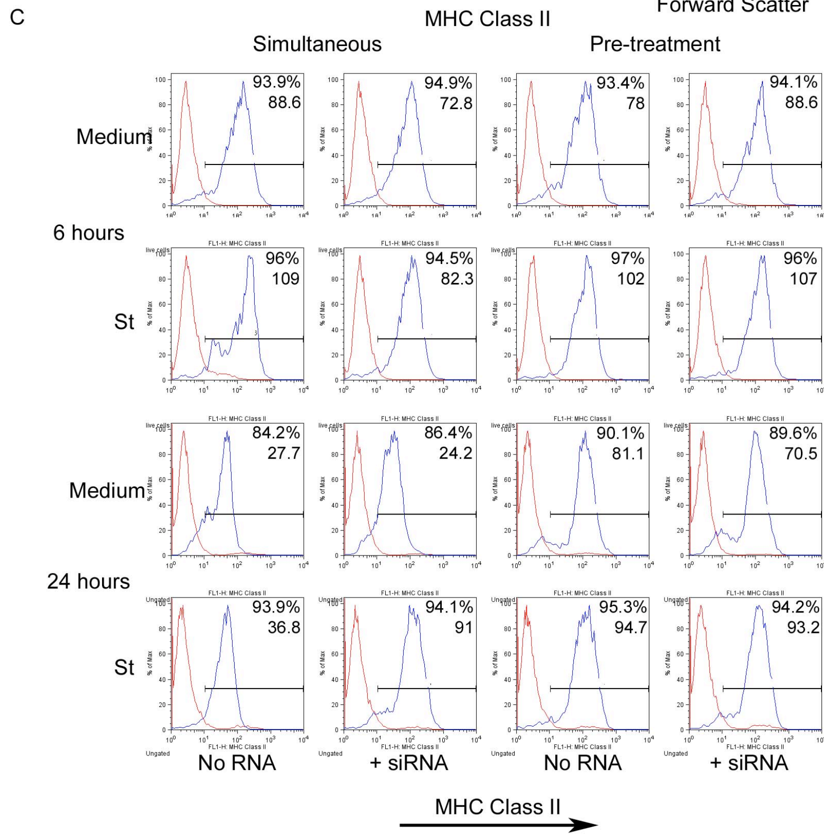
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27.7
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36.8
94.1%
91
95.3%
94.7
94.2%
93.2
No RNA
+ siRNA
No RNA
+ siRNA
MHC Class II

Figure 4.1 Optimal transfection efficiency occurs when DCs are exposed to siRNA prior to stimulation. 10^6 DCs per well received siRNA complementary to CD86 either concurrently (Simultaneous) or 2 hours prior (Pre-treatment) to stimulation with either medium alone (Medium) or *Salmonella typhimurium* (St, 2 μg/ml). **A)** Transfection efficiency was determined by DCs treated with fluorescent RNA oligonucleotides for 6 or 24 hours, and **B)** Forward and side scatter plots were back-gated for cells positive for fluorescent oligo expression. **C)** Flow cytometry was used to measure activation status via MHC II. Histograms are gated on live $CD11c^+$ DCs. Figures in upper right of graphs depict percentage positive and geometric mean fluorescence. Data shown is representative of three separate experiments.

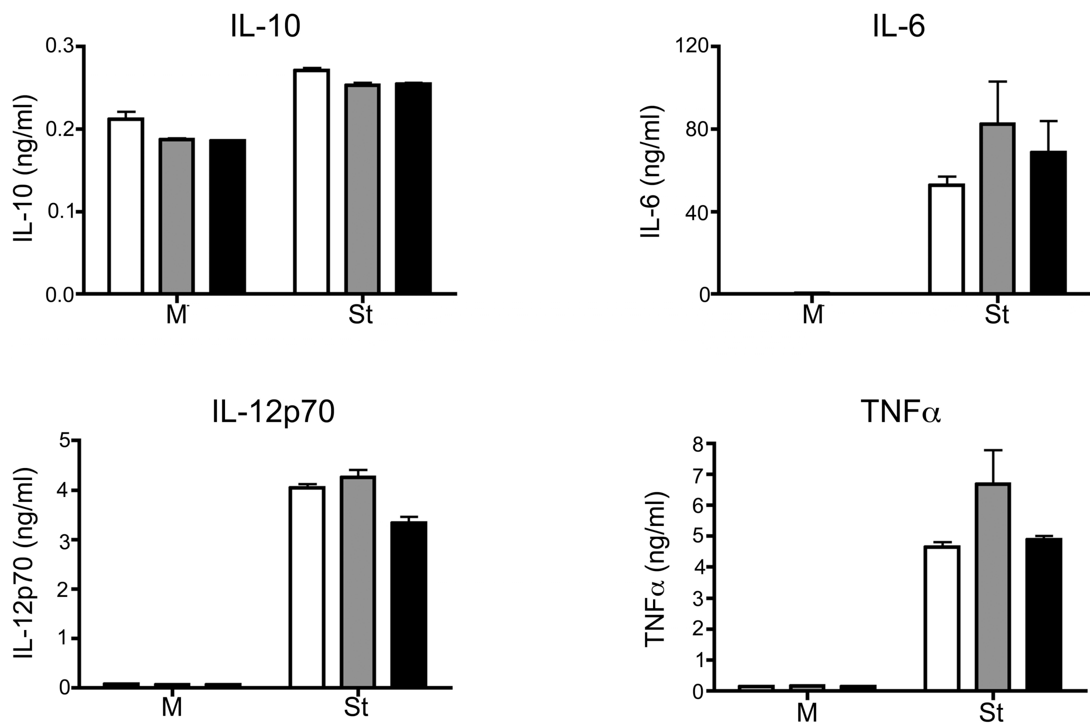

Figure 4.2 Cytokine secretion by DCs is not affected by siRNA treatment. 1.5 x10^6 DCs per well received either no RNA (open bars) scrambled control siRNA (gray bars, 150 nMol) or siRNA complementary to CD86 (black bars, 150 nMol) in addition to stimulation with either medium alone (M) or *S. typhimurium* (St, 1 μg/ml) for 24 hours. Data shown is mean + SEM of cytokine measured by ELISA of duplicate wells and are representative of four separate experiments.

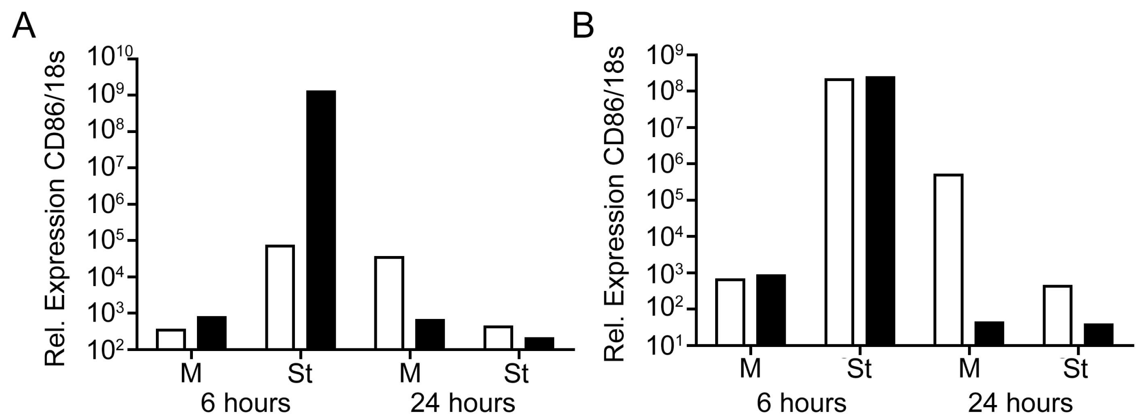

Figure 4.3 Impact of siRNA on DC expression of CD86. DCs were stimulated with medium alone (M) or *S. typhimurium* (St, 2 μg/ml) for either 6 hours or 24 hours with either no RNA (open bars) or CD86 siRNA (black bars) added **A)** simultaneously or **B)** 2 hours previous to antigenic stimulation. siRNA against CD86 was added at a concentration of 150 nM with 1 μg/mL lipofectamine (Invitrogen). CD86 mRNA levels were measured by quantitative PCR and normalised to 18s. Bars represent single culture wells and thus no error bars are applicable. Data representative of three experiments.

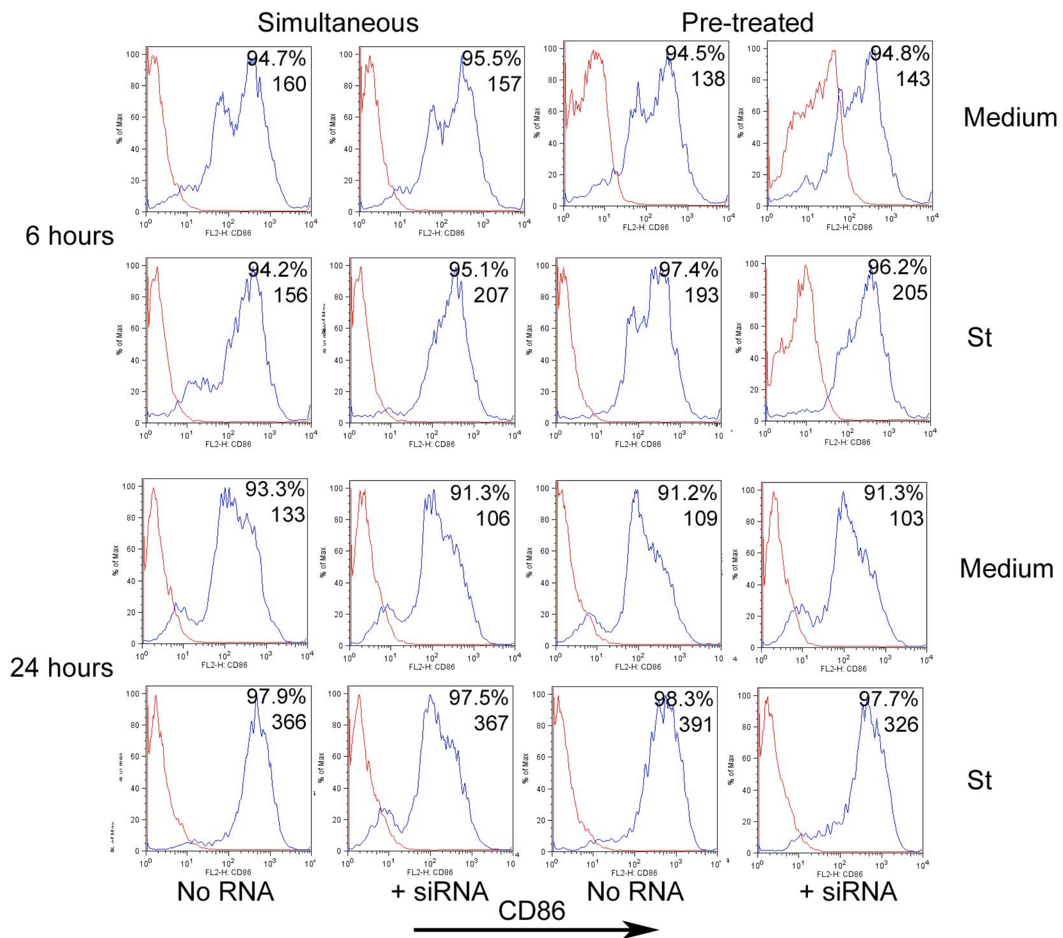

Figure 4.4 Impact of siRNA on cell surface expression of CD86 by DCs. 10^6 DCs per well received siRNA complementary to CD86 either concurrently (Simultaneous) or 2 hours prior (Pre-treated) to stimulation with either medium alone (Medium) or *S. typhimurium* (St, 2 μg/ml) for 6 or 24 hours. Histograms are gated on live CD11c$^+$ DCs. Figures in the upper right of graphs depict percentage positive and geometric mean fluorescence. Data are representative of three separate experiments.

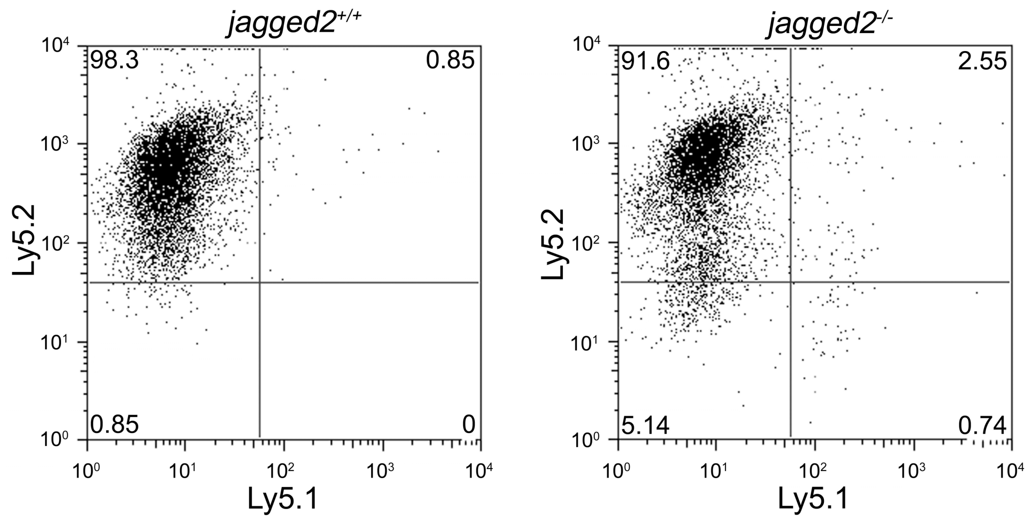

Figure 4.5 *jagged2*$^{-/-}$ bone marrow is of donor origin. DC derived from BM from chimeric Ly5.2^{+} *jagged2*$^{+/-}$ x *jagged2*$^{+/-}$ matings were grown for 11 days in the presence of GM-CSF and then analysed by flow cytometry for expression of Ly5.1 and Ly5.2. Dot plots are gated on live CD11c^{+} cells and are representative of two experiments.

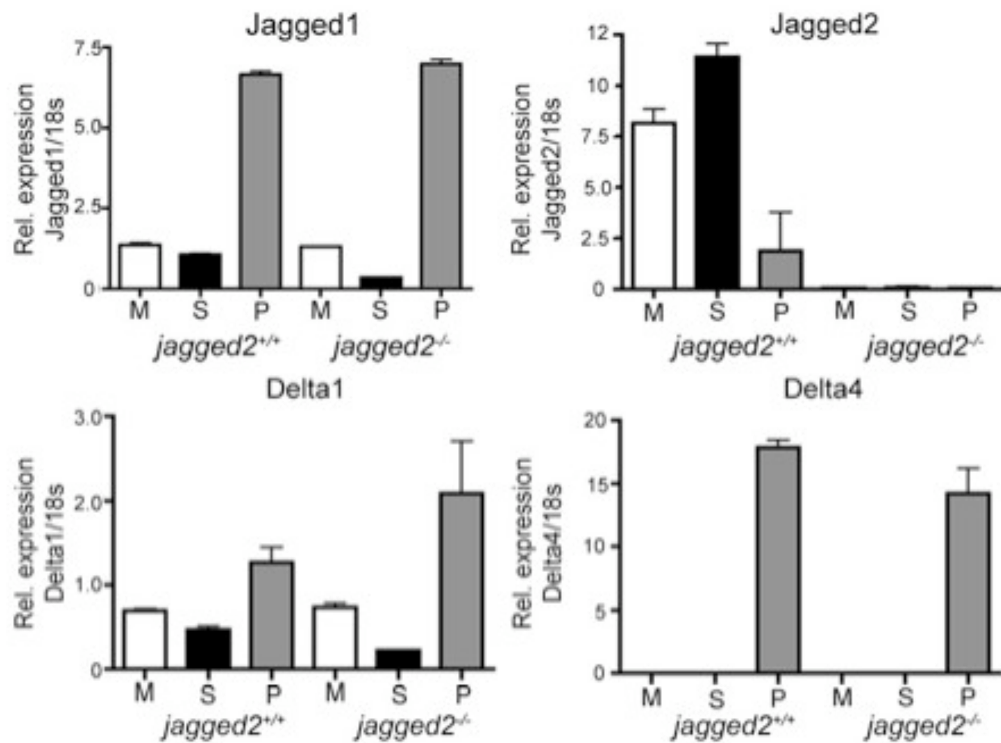

Figure 4.6 Notch ligand expression by *jagged2*$^{-/-}$ DCs. Expression of *jagged1*, *jagged2*, *delta1* and *delta4* by DCs exposed to medium (open bars), Pa (gray bars) or SEA (black bars) was assessed by quantitative PCR. Data shown are mean + SEM of mRNA expression levels measured in duplicate relative to 18s, and are representative of five separate experiments.

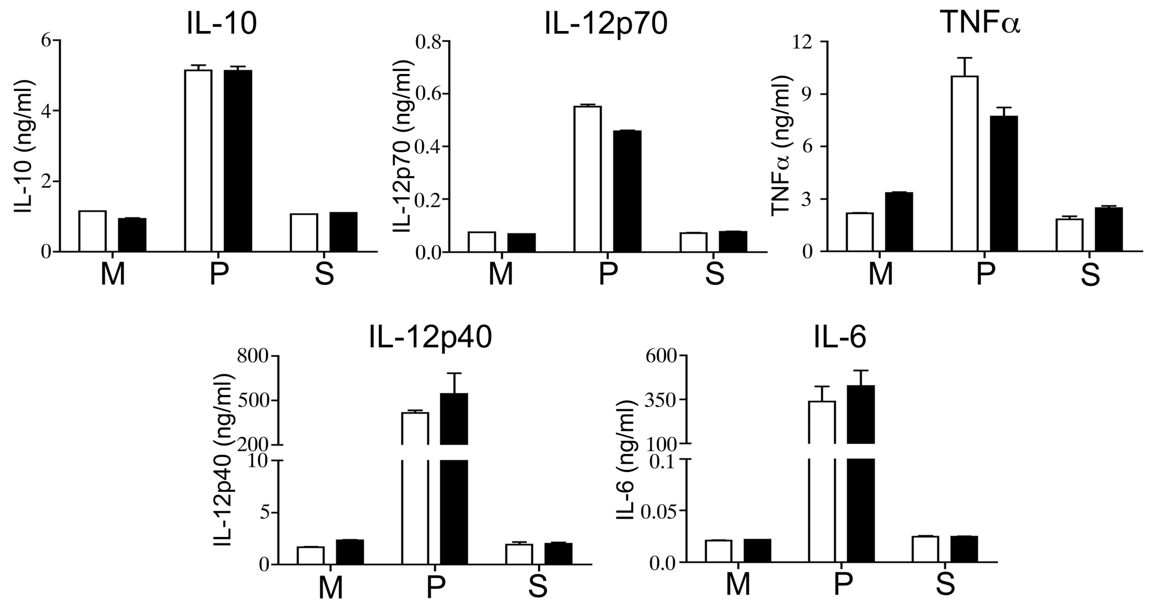

Figure 4.7 Cytokine production by *jagged2*$^{-/-}$ DCs. DCs were grown from BM derived from *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ chimeras and exposed to either medium alone (M), Pa (P, 10 μg/mL) or SEA (S, 25 µg/ml) for 24h. Cytokine production by *jagged2*$^{+/+}$ (open bars) or *jagged2*$^{-/-}$ (black bars) DCs was measured by ELISA and data shown are mean + SEM of duplicate wells, and are representative of two separate experiments.

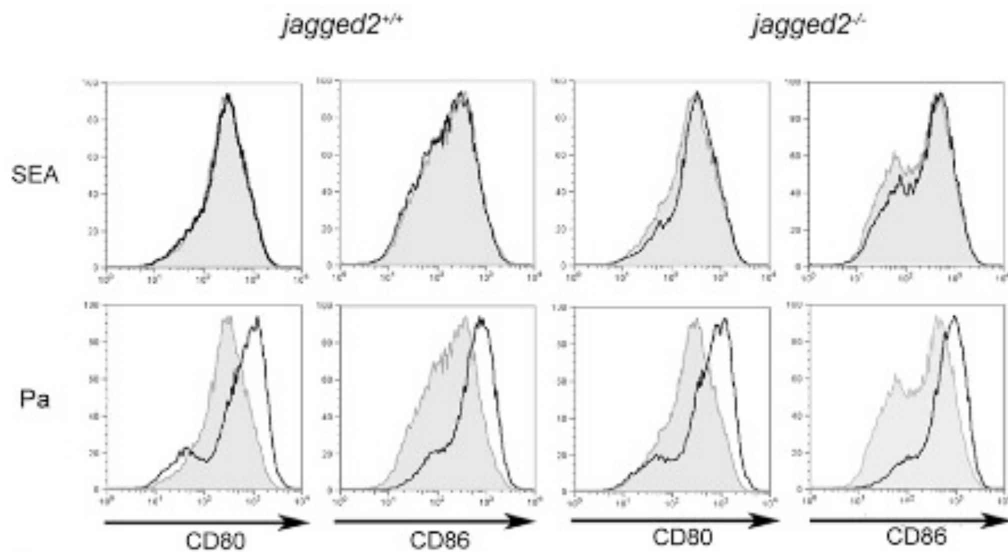

Figure 4.8 Phenotypic activation in *jagged2$^{-/-}$* DCs. Surface expression of CD80 and CD86 on *jagged2$^{+/+}$* or *jagged2$^{-/-}$* DCs. Light grey filled histograms indicate unstimulated DC; black unfilled histograms, DCs stimulated with SEA or Pa. Graphs are representative of two separate experiments. This specific experiment was done in conjunction with Salome LiebundGut-Landmann.

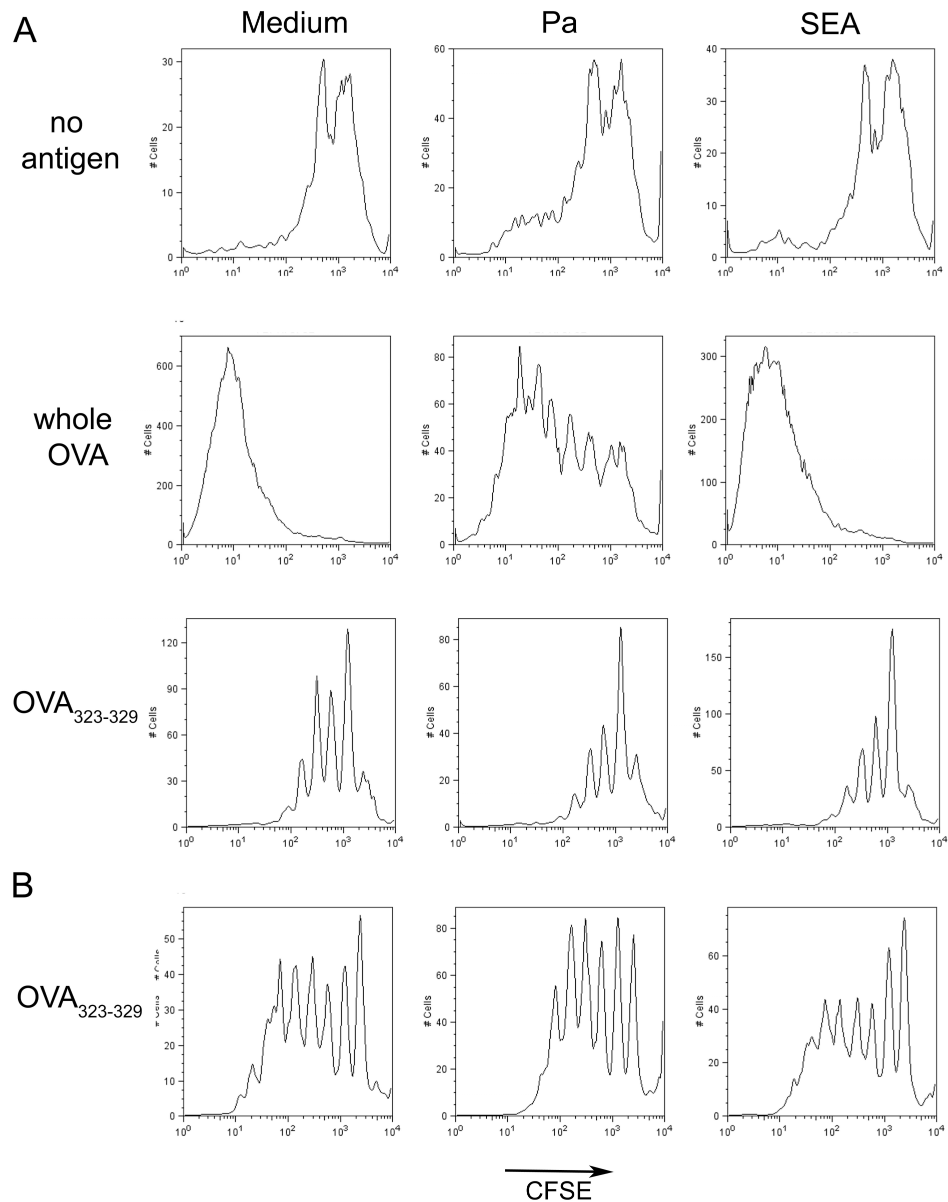

Figure 4.9 Comparison of whole OVA protein and $OVA_{323\text{-}339}$ in the proliferation of OTII cells. $2x10^5$ CD4 OTII cells were co-cultured with $2x10^4$ DCs stimulated with either medium alone (Medium), *P. acnes* (Pa, 10 μg/ml) or SEA (50 μg/ml). Either whole OVA protein (200 μg/ml) or $OVA_{323\text{-}339}$ peptide (50 ng/mL) were also added to co-cultures for **A)** 3 days. **B)** An additional culture using $OVA_{323\text{-}339}$ peptide (50 ng/mL) was maintained for 5 days to assess the impact on proliferation. OTII cells were labelled with CD4 and CFSE. Data are representative of 5 separate experiments

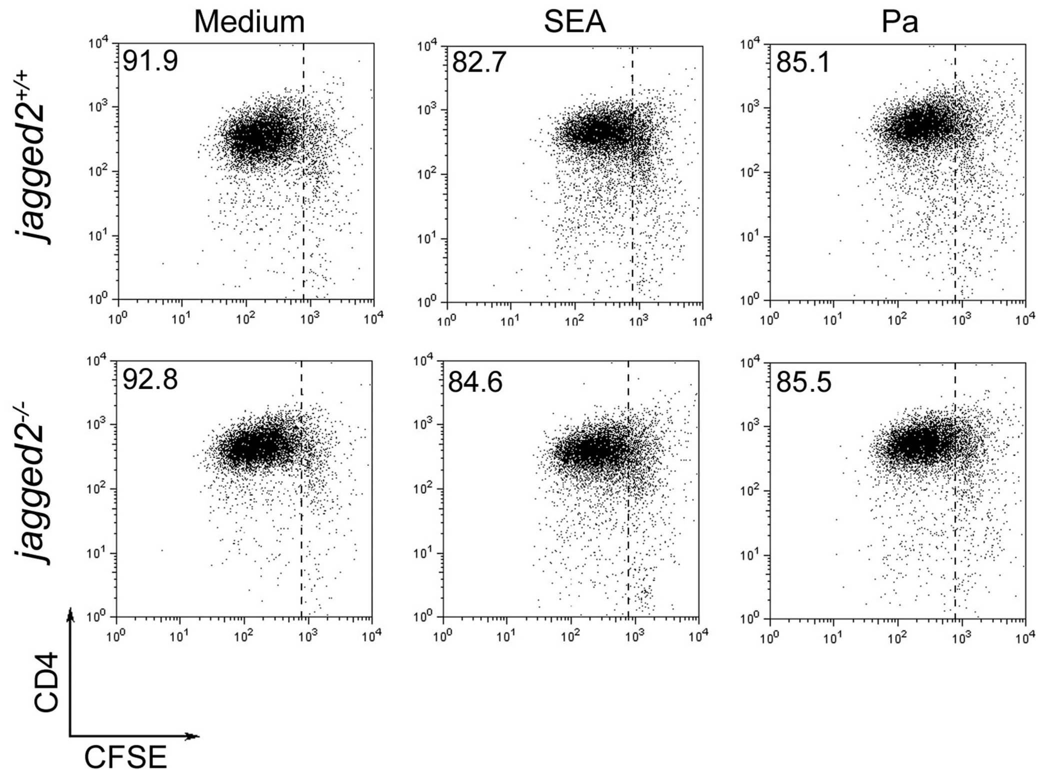

Figure 4.10 *jagged2$^{-/-}$* DCs are not impaired in their capacity to drive T cell proliferation. CD4^{+} OTII cells were co-cultured with *jagged2$^{+/+}$* or *jagged2$^{-/-}$* DCs in the presence of $OVA_{323\text{-}339}$ peptide (50 ng/mL) alone (Medium) or in conjunction with SEA (50 μg/mL) or Pa (10μg/mL). OTII cells were labelled with CD4 and CFSE to assess proliferation. Dotted lines represent CFSE levels on cells cultured in the absence of peptide and figures refer to percentage of dividing cells. Results are representative of three independent experiments.

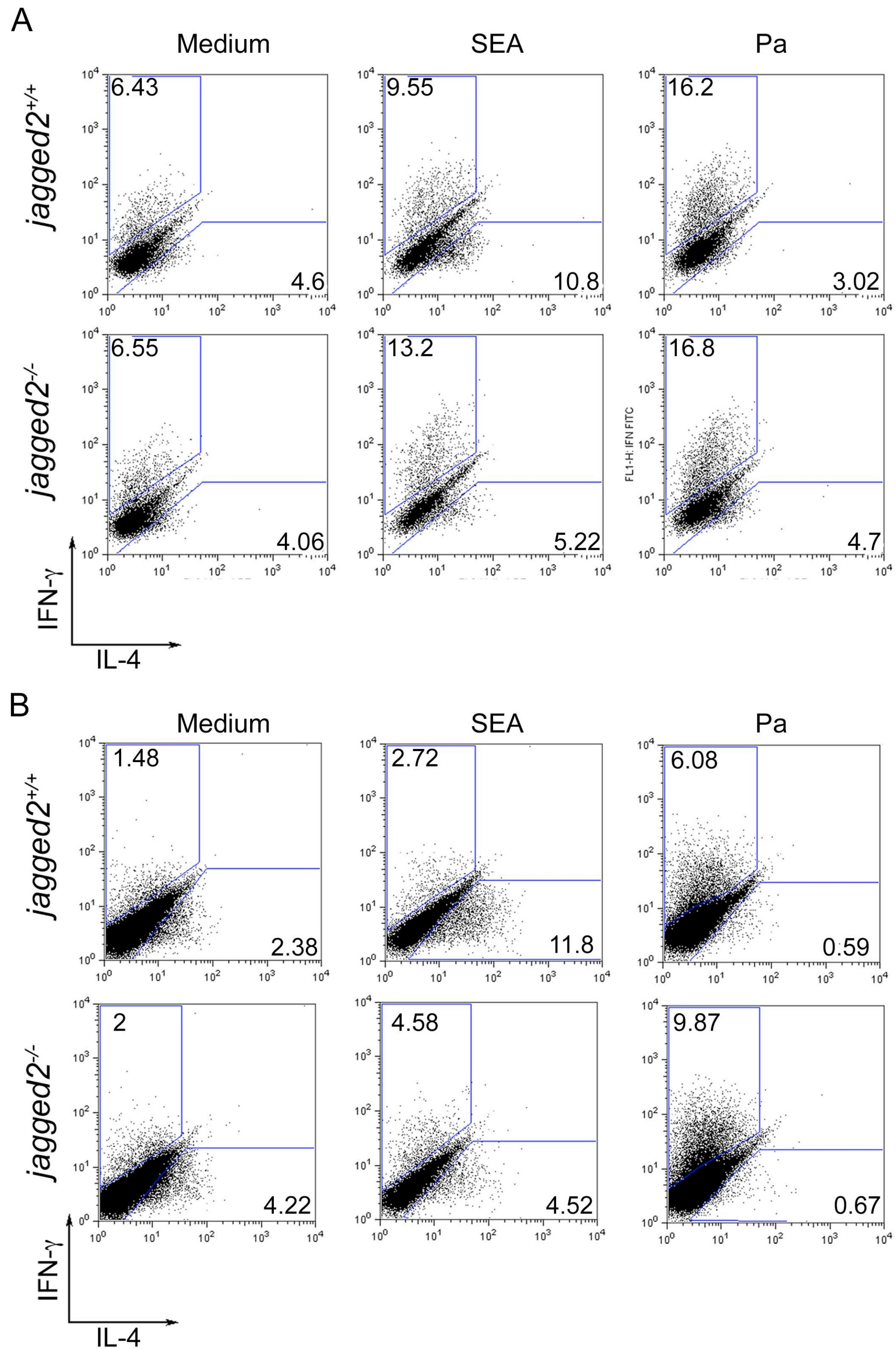

Figure 4.11 Impaired Th2 induction by *jagged2*$^{-/-}$ DCs *in vitro*. $CD4^+$ OTII cells were co-cultured with *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DCs in the presence of $OVA_{323\text{-}339}$ peptide (50 ng/mL) alone (Medium) or in conjunction with SEA (50 μg/mL) or Pa (10μg/mL). OTII cells were

examined for intracellular staining Plots depicting intracellular staining in are gated on live CD4$^+$ cells and figures refer to cytokine producing cells. **A)** and **B)** represent two independent experiments.

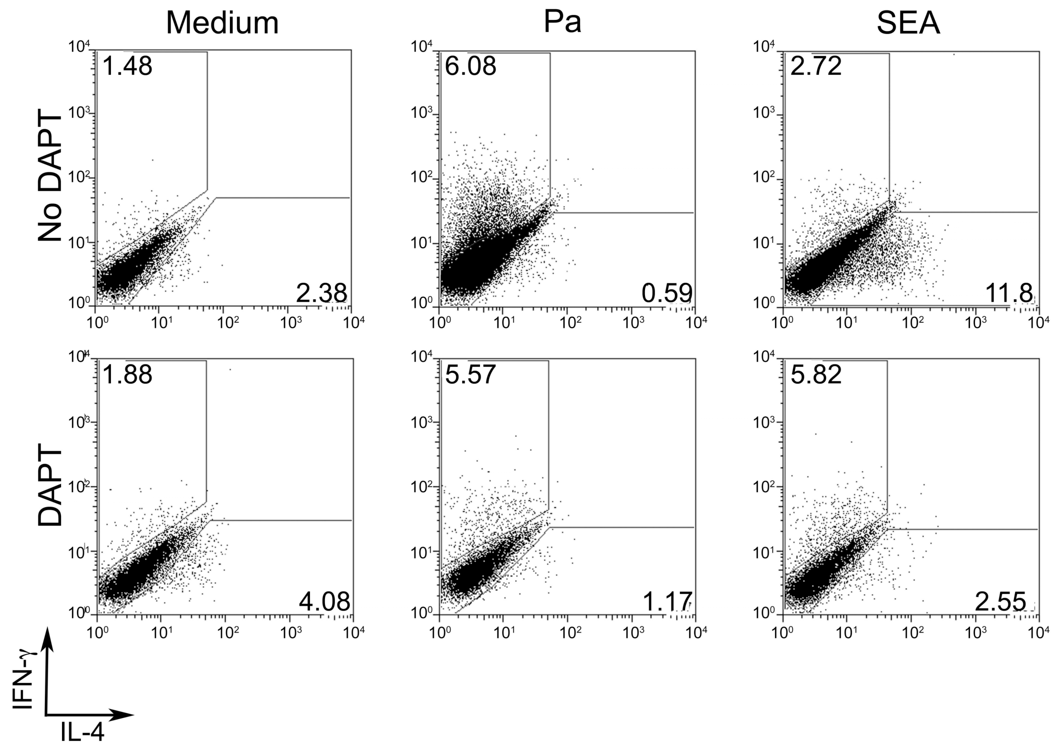

Figure 4.12 Blockade of Notch signaling using DAPT impairs Th2 differentiation. CD4 purified OTII cells were co-cultured with DC in the presence of OVA323-339 peptide (50 ng/mL) alone (Medium) or in conjunction with SEA (50 μg/mL) or Pa (10 μg/mL) with or without DAPT (5μM). OTII cells were labelled with CD4, IL-4 and IFN-γ and were examined for intracellular staining following 3 days of co-culture.

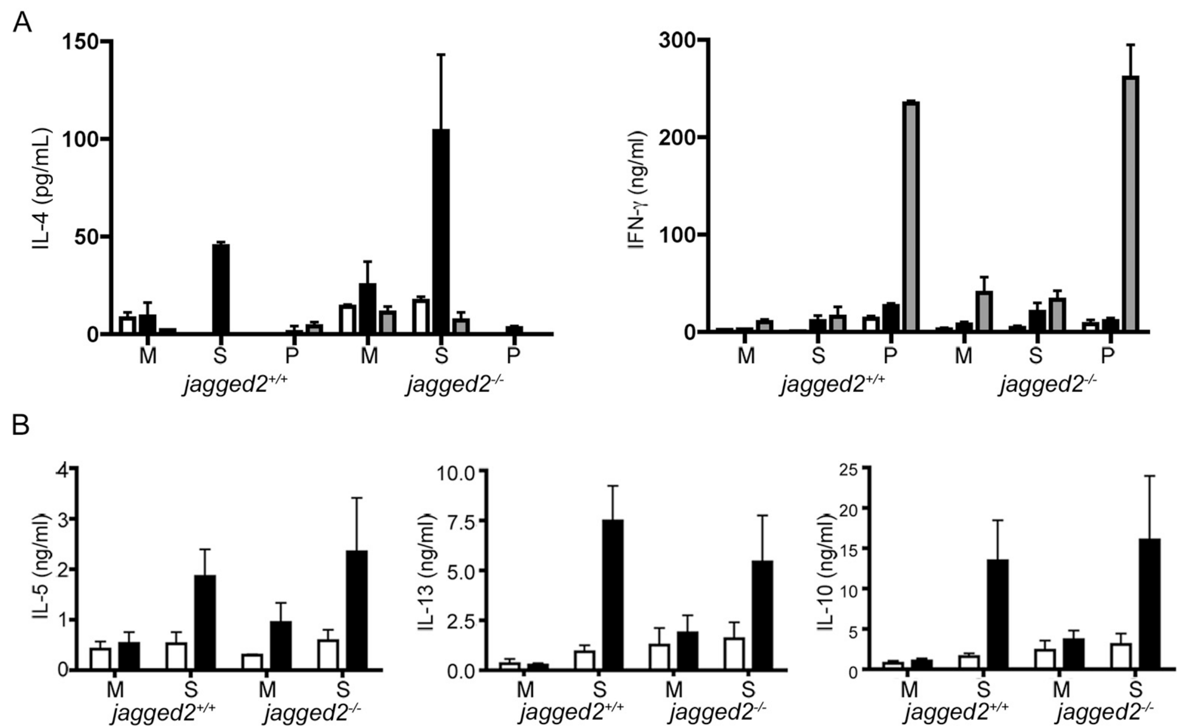

Figure 4.13 DC expression of *jagged2* is dispensable for Th2 or Th1 induction *in vivo*. *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DCs were exposed to medium alone (M), Pa (P, 10 μg/mL) or SEA (S, 25 μg/mL), then injected into the footpad **A)**, or i.p. **B)** of naïve C57BL/6 recipient mice. Popliteal LN cells **A)** or splenocytes **B)** were removed 4d **A)** or 7d **B)** later and then stimulated *in vitro* with medium (open bars), SEA (black bars) or Pa (grey bars). Data shown are mean + SD of cytokine measured by ELISA of triplicate wells of combined LN cells **A),** or mean + SEM of cytokine measured by ELISA of three to five mice per group **B)**. Data shown are representative of three **A)**, or two **B)**, separate experiments. This experiment was conducted with Salome LeibundGut-Landmann.

Chapter 5

Alternative Strategies for identification of molecular requirements for DC polarisation of T cells *in vivo*

5.1 Introduction

There is an extensive difference between the environment in which DCs are stimulated *in vitro* and the signalling molecules, tissue factors, structural relationships and physiological conditions under which a DC would encounter the same stimulus *in vivo*. This is illustrated by the often-conflicting results of our *in vitro* and *in vivo* comparisons, as previously detailed (**Figure 3.3 and Figure 4.13**). These observations prompted assessment of the extent to which DC activation and function is affected by the presence of infection-associated tissue factors during exposure to Ag *in vitro*. However, determining which *in vivo* components play a critical role in the maturation process of DCs and how they may exert their influence is a daunting task, particularly in a Th2 setting. No clear molecular indicator of a Th2 primed DC has yet been identified and Th2 conditioned DCs display no significant upregulation of any hallmarks of conventional maturation, and only minor increases in MHC II expression (MacDonald *et al.*, 2001). Thus, determining whether external factors impact the Th2 maturation status of a DC at all is challenging. We hypothesized that candidates should be present *in vivo* in the context of Th2 pathogens, and have the potential to influence DC maturation status. Since SEA has been the most consistently used Th2 stimulus throughout this work, we reasoned that

the *in vitro* addition of molecular factors commonly associated with S. *mansoni* infection would be most relevant.

The development of an immune response varies considerably over the course of *S. mansoni* infection. However, we chose to focus on the host-parasite environment during the Th2 dominated stage of infection induced primarily by egg antigens (Pearce and MacDonald, 2002; Perona-Wright *et al.*, 2006). Obvious molecular candidates present during a Th2 response, and whose absence *in vitro* may mask the ability to define a Th2 primed DC, include IL-4 and IL-13. Both cytokines predominate during the egg-laying stage of infection and are primarily responsible for the severe hepatic fibrosis that can occur during infection (MacDonald *et al.*, 2002a; Wynn *et al.*, 2004).

Although an important Th2 cytokine, IL-4 has been previously shown to play a more nuanced role in the maturation of APCs. Recent studies have shown that IL-4 treatment of DC may upregulate IL-12 in response to LPS, which seems in contradiction with its established Th2 role (Kalinski *et al.*, 2000; Yao *et al.*, 2005). Importantly, however, these studies did not investigate whether IL-4 treatment of DCs affected their capacity to polarise T cell responses *in vivo*. Another study has demonstrated that DCs purified from lymph nodes from mice infected with *Leishmania major* and injected with IL-4 i.p. 8 hours later expressed greater levels of IL-12 mRNA, and that this IL-12 was responsible for an enhanced Th1 response (Biedermann *et al.*, 2001). However, the prevailing immune response from *L. major* infected mice given IL-4 for 64 hours became strongly Th2 (Biedermann *et al.*, 2001). This highlights the pluripotent effect of these infection related cytokines, and emphasizes the need to ascertain the impact of environment on the maturation of

DCs. In this particular study the only readout for DC maturation was IL-12 production (Biedermann *et al.*, 2001). Assessment of a broader array of activation markers, including Notch ligands, may provide greater insight into the mechanisms by which Th2 associated tissue factors can influence DC activation and function.

More recently identified Th2 tissue factors include cytokines such as TSLP and IL-25. TSLP is an epidermal derived IL-7 related cytokine, originally identified as a growth factor in the generation of immature B cells which is now implicated in the pathogenesis of dermatitis, asthma and may be associated with certain helminth infections (Astrakhan *et al.*, 2007; Demehri *et al.*, 2008; Huston and Liu, 2006; Zaph *et al.*, 2007; Zhou *et al.*, 2005). Predominantly secreted by barrier epithelial cells, TSLP is also thought to be a potent activator of myeloid cell types (including DCs), and thus may play a significant role in the initial milieu in which DCs encounter Th2 Ags. IL-25 (IL-17E) was identified as being structurally related to IL-17 (IL-17A) but with markedly different biological activities both *in vitro* and *in vivo* (Fort *et al.*, 2001). IL-17 typically induces production of pro-inflammatory cytokines such as IL-1β, IL-6 and TNFα in M∅s (Jovanovic *et al.*, 1998), whereas injection of mice with IL-25 induces IL-4, IL-5 and IL-13 expression and is predominantly expressed in Th2 polarised $CD4^+$ T cells (Fort *et al.*, 2001; Hurst *et al.*, 2002). Although it has been suggested that IL-25 may directly regulate the Th2 differentiation of $CD4^+$ T cells (Angkasekwinai *et al.*, 2007; Wang *et al.*, 2007) the extent of cell types receptive to IL-25 signalling remains unclear, and the impact of IL-25 on the Th2 inductive potential of DCs is as yet unknown.

The addition of exogenous cytokines to DCs *in vitro* serves two aims. First, the co-administration of antigen with Th2 associated cytokines may reveal which

components of the host immune response are responsible for the discrepancies observed between co-culture experiments *in vitro* and DC transfer *in vivo*. Secondly, the presence of Th2 associated cytokines may be required to reveal changes in the expression of molecules indicating a Th2 primed DC, such as Notch ligands. For example, it is possible that upregulation of *jagged2* in response to SEA may only be readily apparent in the presence of Th2 cytokines, such as IL-4.

As an alternative strategy for investigating the *in vivo* requirements for DC function, we also assessed the antigen presenting capacity of DCs deficient in pathogen recognition. We reasoned that use of DCs derived from transgenic mice deficient in their ability to receive pathogen recognition signals may demonstrate what distinguishes the requirements for activation of Th1 driving DCs from Th2. TLRs are possibly the best studied of receptors responsive to such stimuli. The majority of TLRs, with the exception of TLR3, make use of the universal adaptor protein MyD88 to activate transcription factor NFκB (Medzhitov *et al.*, 1998) and MyD88 deficient mice provide a useful gene target for impairing majority of TLR signalling. Given that MyD88 deficient DCs severely reduced responses to Th1 stimuli (Kaisho *et al.*, 2001), our expectation was that they would have a diminished capacity to drive Th1 responses *in vivo*. It has previously been shown in an *in vitro* system that when DCs stimulated with soluble *T. gondii* tachyzoite extract (STAg) are co-cultured with naïve $CD4^+$ T cells from TCR Tg DO.11.10 X RAG2 knockout mice together with $OVA_{323-329}$ peptide, the resulting induction of IFN-γ producing $CD4^+$ T cells was entirely MyD88 dependent (Jankovic *et al.*, 2004). By contrast, SEA-DCs cocultured in a similar fashion induced IL-4 production by $CD4^+$ T cells in a MyD88 independent fashion (Jankovic *et al.*, 2004). However, whether this might

also be the case *in vivo* has yet to be shown. Thus, we investigated the requirements for DC function *in vivo* using multiple approaches, including cumulative addition of cytokines and reductionist gene knockout experiments, with a particular focus on mechanisms utilised by DCs in Th2 induction.

A primary motivation for investigating the role of Notch proteins in DC function was to observe whether the upregulation of specific Notch ligands could act as a marker for Th2 maturation. Although SEA stimulated DCs did not enhance expression of any Notch ligands measured in either a significant or consistent manner (**Figure 3.6**), other molecules have recently been shown to be upregulated by M∅s in a Th2 setting, including Ym1 and RELMα (Chang *et al.*, 2001; Nair *et al.*, 2003; Nair *et al.*, 2005). Ym1 and RELMα gained notoriety in the field of Th2 immunology after M∅s were found to secrete these two proteins in abundance during nematode infection (Chang *et al.*, 2001; Falcone *et al.*, 2001). Ym1 is a member of a family of mammalian proteins that share a homology with chitinases in other species which have recently been associated with development of allergic airways disease (Zhu *et al.*, 2004). Chitin is the second most abundant polysaccharide in nature and typically acts as a protective layer separating the harsh environment of a host from a pathogen such as a parasitic nematode (Shahabuddin and Vinetz, 1999; Zhu *et al.*, 2004) Since chitin itself is not utilized by mammals, it was assumed until recently that chitinases were also lacking. However the recent discovery of chitinases such as Acidic mammalian chitinase (AMCase) in humans or YM-1 (YM-2 in mice) and, particularly, their expression in Th2 inflammation, implies that these proteins may be directly involved the defence against chitin-containing pathogens. Furthermore, BM-DC have been shown to upregulate Ym1

and RELMα in the presence of IL-4 (Nair *et al.*, 2005). However, it is as yet unknown how pathogen stimulated DCs respond in terms of their expression of either Ym1 or RELMα and whether these molecules could act as an indicator demarking a Th2 primed DC.

Thus, by focussing on Th2 associated cytokines, MyD88 and Notch ligands, we expanded our investigation of what might be the critical components for DC function *in vivo*, while increasing our repertoire of potential markers of Th2 activation.

AIMS

1) To test Th2 related tissue factors for their ability to alter DC activation and function.
2) To determine whether activation and function of Th2 conditioned DCs is dependent upon MyD88 signaling.
3) To ascertain if hallmarks of alternatively activated M∅s, such as Ym1 or RELMα, may represent markers of Th2 primed DCs.

5.2 Can exogenous cytokines measurably alter DC maturation status *in vitro*?

Predominantly, we have conducted DC experiments by stimulating cells *in vitro* and then either directly observing their antigen presenting capacity in culture, or transferring matured DCs into recipient mice. In either circumstance, the *in vitro* process of DC activation is carried out in the absence of the environment in which an APC naturally encounters pathogens or their Ags. To better represent the tissue

environment in which DCs might be activated *in vivo*, IL-4 and αCD40 were added to DCs during Ag exposure *in vitro*. IL-4 is produced within tissues and detectable in sera during Th2 infection settings (Jankovic *et al.*, 2001; Pearce and MacDonald, 2002). Agonistic αCD40 antibody was also added to DCs stimulated by Ag to mimic CD154 provision by activated T cells or other tissue sources (Jenkins *et al.*, 2008).

In keeping with previous reports (Biedermann *et al.*, 2001; Hochrein *et al.*, 2000; Kalinski *et al.*, 2000; Koch *et al.*, 1996; Yao *et al.*, 2005), the addition of IL-4 cytokine in conjunction with the strong Th1 stimulus of heat-killed *P. acnes* significantly enhanced DC secretion of IL-12p70 (**Figure 5.1**). A similar increase in IL-12p70 production occurred when Pa-DCs were treated with αCD40 antibody 4 hours following Ag stimulation, again consistent with other studies (Schulz *et al.*, 2000). However, IL-4 and αCD40 antibody together shared strong synergy in this capacity dramatically increasing DC IL-12p70 secretion (**Figure 5.1**). Elevated cytokines production was not observed in all cases, IL-6 secretion was unaffected by the presence of either IL-4 or αCD40 antibody alone or in conjunction. However, IL-10 secretion by Pa-DCs appeared to be impaired in the presence of IL-4, but not in the presence of agonistic αCD40 antibody. This trend was consistent across multiple experiments, but not significant when IL-4 was present at a concentration of 1 ng/ml.

Although displaying a clear effect on the secretion of IL-12p70 by bacterially stimulated DCs, neither IL-4 nor αCD40 antibody exerted measurable effects on cytokine production by SEA treated DCs (**Figure 5.1**). There were, however, some observable differences in DC expression of Notch ligands (**Figure 5.2**). Intriguingly, the addition of IL-4 impaired *delta4* expression by Pa-DCs, although there was no corresponding increase in *jagged2* expression. SEA-DCs behaved somewhat

differently. Unlike Pa-DCs, SEA-DCs substantially increased expression of *jagged2* in response to IL-4 in conjunction with a slight decrease in *delta4* expression compared to unstimulated controls. Stimulation of CD40 through the use of agonistic αCD40 antibody following either Pa or SEA stimulation dramatically increased expression of both *delta4* and *jagged2*. However, it is difficult to draw any firm conclusions due to the preliminary nature of the data.

The presence of IL-4 during DC stimulation has been shown to influence the secretion of IL-12 (Hochrein *et al.*, 2000; Kalinski *et al.*, 2000; Yao *et al.*, 2005) as well as the expression of Notch ligands (**Figure 5.2)**. However, the impact of Th2 associated cytokines on the ability of DCs to generate immune responses *in vivo* remains unclear. We went on to address whether IL-4 and additional Th2-related tissue factors may affect the T cell polarising capacity of DCs *in vivo*. Recent work had suggested a role for the cytokines IL-25 and TSLP in the generation of a Th2 response (Huston and Liu, 2006; Perrigoue *et al.*, 2008; Wang *et al.*, 2007; Zhou *et al.*, 2005; Ziegler and Liu, 2006). Thus, DCs were treated with IL-4, IL-25 or TSLP over a range of doses and in conjunction with *P. acnes* stimulation and their cytokine production measured (**Figure 5.3**). As shown previously (**Figure 5.1**), IL-4 decreased IL-10, and increased IL-12p70 production, by Pa-DCs, whereas IL-6 and IL-12p40 remained unchanged. At higher doses of TSLP, there was a trend for enhanced DC IL-12p70 production, similar to that seen with IL-4, but this was not statistically significant. The intention was to go on to identify how IL-25 and TSLP impacted Notch ligand expression. Unfortunately, due to time constraints the data generated to date remain preliminary.

5.3 Do Th2 associated cytokines influence DC priming of T cell responses *in vivo*?

To investigate whether DC treatment with Th2 related cytokines *in vitro* had influenced their *in vivo* function, DCs activated with Pa in the presence or absence of IL-4, IL-25 or TSLP were then transferred into naïve recipient mice. We reasoned that, since stimulation of DCs with IL-4 resulted in enhanced IL-12p70 production, we would expect to see elevated IFN-γ in recall responses from mice that had received DCs stimulated with Pa and IL-4. Contrary to expectation, mice that had received DCs stimulated with Pa in conjunction with IL-4, IL-25 or TSLP all produced significantly lower levels of IFN-γ, in comparison to recipients of DCs stimulated with Pa alone (**Figure 5.4**). Furthermore, there was a significant decrease in the production of IL-17 by recipients of Pa-DCs treated with IL-25 or TSLP. This result demonstrates that, although the addition of IL-4 enhanced DC secretion of Th1-driving cytokines such as IL-12 *in vitro* (**Figure 5.3**), this was not translated into increased Th1 driving capacity by these same cells *in vivo*. Further, DC exposure to IL-25 and TSLP had a significant impact on *in vivo* Th1 responses despite having little impact on *in vitro* cytokine secretion. This data suggests that DCs stimulated in the presence of Th2 related cytokines have an impaired ability to drive a Th1 response by a mechanism that is yet to be determined. In addition, this data again challenges the wisdom of speculating T cell polarising ability of DCs based on *in vitro* cytokine production alone.

5.4 What is the impact of MyD88 deficiency on DC function *in vitro* and *in vivo*?

In addition to determining the impact of exogenous cytokines on DC function, we assessed the effect of curtailing DC cytokine production on their T cell priming ability through the use of MyD88 deficient DC. MyD88 is a crucial adaptor protein for the majority of TLR signalling pathways, excluding TLR3, and plays an important role in DC function. (Eisenbarth *et al.*, 2002; Kaisho and Akira, 2001; Kawai and Akira, 2007) When stimulated with Pa, MyD88$^{-/-}$ DCs displayed a similar ability to upregulate MHC II, CD40, CD80 and CD86 to WT DC (**Figure 5.5**). In fact, MyD88 deficient DCs appeared to have a slightly greater basal activation status than WT DCs. However, cytokine secretion by MyD88$^{-/-}$ DCs was strikingly impaired, as MyD88 deficient Pa-DCs failed to secrete any measurable IL-12p70, while 12p40, IL-6 and TNFα were all dramatically reduced (**Figure 5.6**). These results agree with previous studies showing that the impairment of MyD88 signalling in DCs either by small interfering RNAs (Zhu *et al.*, 2008) or by transgenic deficiency (Kaisho *et al.*, 2002; Kaisho *et al.*, 2001; Tesar *et al.*, 2004) dramatically reduces (although does not eliminate) Th1-associated cytokine production yet maintains co-stimulatory molecule expression.

We then examined Notch ligand expression in MyD88 deficient DCs (**Figure 5.7**). Interestingly, *delta4* expression by Pa-DCs was completely abrogated in the absence of MyD88. It has been previously reported that LPS stimulation induces a MyD88 dependent enhancement of *delta4* expression in addition to a MyD88 independent *jagged1* upregulation (Amsen *et al.*, 2004). Down-regulation of *jagged2* by Pa stimulation, as described previously (**Figure 3.6**), was maintained in the absence of MyD88, indicating that the inhibition of *jagged2* expression in response to Pa is not dependent upon MyD88 signalling. However, at 6 hours MyD88

deficient Pa-DCs and unstimulated DCs did express higher levels of *jagged2* than their WT counterparts. Future assessment of *jagged1* and *delta1* expression by MyD88 deficient DCs would also reveal the extent to which other Notch ligands are responsive to stimulation and reliant on MyD88 signalling.

The ability of WT and MyD88 deficient DCs to induce Th1 and Th2 responses was then compared *in vivo*. As described in previous chapters, $5x10^5$ DCs were injected i.p. into naïve recipient mice, and splenic recall responses assessed one week later. Given that the absence of MyD88 mediated TLR signalling severely disrupted the bacterially triggered secretion of T cell polarising cytokines such as IL-12 (**Figure 5.6**), in addition to the loss of Pa-specific *delta4* expression, (**Figure 5.7)** our expectation was to observe dramatically diminished capacity for Pa-stimulated $MyD88^{-/-}$ DCs to drive a Pa-specific IFN-γ response, in comparison to WT DCs. In striking contrast to this expectation, Pa-DCs deficient in MyD88 signalling induced comparable IFN-γ responses to Pa stimulated MyD88 sufficient DCs (**Figure 5.8A**). Additionally, in four separate experiments there was also a trend for increased IL-13 induction by $MyD88^{-/-}$ Pa-DCs, in comparison to WT controls. These results were surprising since the impaired pro-inflammatory cytokine secretion by Pa-DCs in the absence of MyD88 (**Figure 5.6**) appears to have had no measurable effect on their ability to induce an IFN-γ response. This suggests that additional MyD88-independent mechanisms must be responsible for Th1 induction by Pa-DCs.

Importantly, in addition to displaying impaired IL-12p70 production, MyD88 deficient DCs were also limited in their capacity to upregulate *delta4* in response to Pa stimulation (**Figure 5.7**). As *delta4* is the Notch ligand most closely associated with Th1 stimuli (**Figure 3.5**), it was of great interest to determine the role it may

play in the establishment of a Th1 response. Since the transfer of Pa stimulated MyD88$^{-/-}$ DC yielded no discernible impairment of Th1 response induction, it can be argued by proxy that *delta4* is also not likely a requirement for the induction of a Th1 response by Pa-DC.

Similarly to Pa-DCs, MyD88 signalling was not required for Th2 induction by SEA-DCs *in vivo* (**Figure 5.8B**), in agreement with previously published work using TCR Tg T cells *in vitro* (Jankovic et al., 2004). Thus, whichever mechanisms are utilised by SEA-DCs for the induction of SEA-specific Th2 responses must be invoked in a MyD88 independent fashion. This result argues against the requirement of TLR involvement in the activation of an DCs by SEA (Thomas *et al.*, 2003; van der Kleij *et al.*, 2002).

5.5 What alternative candidates might there be for identification of Th2 driving DCs?

One of the aims of the work articulated in this thesis was to ascertain whether or not Notch Ligands provide a mechanism for Th2 induction by DCs. Unfortunately, our data did not support a role for the specific Notch ligand we investigated (*jagged2*) in the determination of a Th2 response (**Figure 4.13**). However, Ym1 and RELMα are molecules that have been found to be highly expressed by alternatively activated M∅s or M∅s elicited from nematode infected mice (NeM∅) (Falcone *et al.*, 2001; Loke *et al.*, 2002; Nair *et al.*, 2003; Raes *et al.*, 2002b; Welch *et al.*, 2002). Nair et al. (2005) demonstrated that DCs treated with IL-4 have enhanced expression of Ym1 and RELMα (Nair *et al.*, 2005). However, this

was only carried out with IL-4 alone and not in the context of stimulation with pathogens or their products.

To address the relevance of these markers during stimulation with pathogens, DCs were first exposed to either Pa or SEA for 6 hours or 24 hours. Additionally, WT or MyD88$^{-/-}$ mice were compared in order to assess whether any observed alteration in expression following Ag stimulation might be MyD88 dependent. The expression of Ym1 and RELMα were then measured by quantitative RT-PCR (**Figure 5.9**). Similar to what was observed for cytokines, co-stimulatory molecules and Notch ligands, neither Ym1 nor RELMα were measurably upregulated in a Th2 Ag-dependant manner. Moreover, SEA alone did not significantly increase either Ym1 or RELMα expression over the level of unstimulated DC. However, Pa significantly decreased expression of both Ym1 and RELMα at 6 and 24-hour time points. Strikingly, this reduction in Ym1 and RELMα expression by DCs following Pa stimulation remained significant despite the absence of MyD88 signalling.

To test whether stimulation of DCs with SEA in conjunction with Th2 tissue factors or a T cell mimic might enhance expression of either Ym1 or RELMα, IL-4 and/or αCD40 agonistic antibody were added to unstimulated, Pa or SEA activated DCs (**Figure 5.10**). As demonstrated by others (Nair *et al.*, 2005), the addition of IL-4 alone was sufficient to upregulate DC expression of Ym1. However, SEA stimulation yielded no greater expression of either Ym1 or RELMα. In contrast to previous reports (Nair *et al.*, 2005), IL-4 alone did not significantly increase the expression of RELMα by DCs. Addition of agonistic αCD40 antibody into culture had no discernible effect on the DC expression of either Ym1 or RELMα.

Whenever molecular expression profiles are inferred by mRNA readout alone there is always the risk that much of the regulation actually occurs post-transcriptionally. Preliminary work was initiated to assess surface expression of Ym1 and RELMα using Immunofluorescent antibody (IFA) staining, however time constraints prevented the derivation of firm conclusions.

5.6 Discussion

The work detailed in this thesis has shown that DC expression of defined Notch ligands is not necessarily critical to the establishment of an immune response *in vivo* (**Chapter 4**), and the expression pattern of Notch ligands are decidedly more nuanced than a simple delta/Th1 jagged/Th2 paradigm (**Chapter 3**). We then selectively exposed DCs *in vitro* to factors commonly present during Th2 infection *in vivo*, reasoning that their presence may provide greater insight into the behaviour of DCs in a more physiologically relevant setting. The curious result that addition of IL-4 to DCs stimulated with a Th1 antigen enhances IL-12 production has been previously described (Biedermann *et al.*, 2001; Yao *et al.*, 2005) with those reports suggesting that IL-4 may actually assist in the establishment of a Th1 response under certain conditions; perhaps providing a feedback mechanism for endogenous regulation of Th2 responses. However, our results indicate that these IL-4 treated DCs, despite displaying elevated levels of IL-12 secretion, in fact exhibit impaired Th1 inductive ability *in vivo*. Although this result is in contradiction to a previous report suggesting that addition of IL-4 can promote DC Th1 induction (Biedermann *et al.*, 2001), it should be noted that in this case mice were infected with *L. major* and then subsequently injected with IL-4 cytokine i.p. Thus, the addition of IL-4 in this study might influence the behaviour of many cell types in addition to DCs, such as

resident mast cells or basophils. In contrast, our results focus on the impact of IL-4 specifically on DCs prior to their injection into recipient mice. Thus, the impaired Th1 response we have observed can be solely attributed to the influence of IL-4 on the DCs that were transferred. These results caution against making inferences on *in vivo* DC function based on restricted molecular readouts *in vitro*.

In contrast to IL-4, TSLP and IL-25 did not significantly influence the cytokine profile of Pa-DCs, although there was a trend for increased IL-12 production by DCs exposed to TSLP. Surprisingly however, both IL-25 and TSLP exposed Pa-DCs displayed impaired ability to induce IFN-γ and IL-17 production in recipient mice, suggesting a possible role for both these cytokines in the inhibition of either Th1 or Th17 induction. These preliminary data have formed the platform for further work in the lab to define the role of these two cytokines in the establishment of a Th2 response.

Crucially, we assessed the impact of Th2 associated cytokines on DC activation by measuring the production of an array of cytokines and Notch ligands as well as Ym1 and RELMα, as opposed to limiting readout of DC activation to a single cytokine. It was most unfortunate that time did not permit a full assessment by quantitative PCR of DC Notch ligand expression following exogenous cytokine-mediated stimulation. However, the evidence gathered to date suggests cytokines such as IL-4 present during DC stimulation can have a direct impact on the expression of Notch ligands as well as Ym1, RELMα and cytokines.

Experiments involving transfer of MyD88 deficient DCs were remarkably revealing in which aspects of measured maturation are actually relevant for T cell polarisation by DCs *in vivo*. Despite the impaired capacity of MyD88 deficient DCs

to produce IL-12, IL-6, TNFα, or *delta4 in vitro*, Pa stimulated MyD88$^{-/-}$ DC were just as able as WT DCs at inducing Pa-specific IFN-γ following *in vivo* transfer. It is also interesting that these experiments revealed a trend for increased IL-13 following *in vivo* transfer of MyD88 deficient Pa-DCs. Earlier evidence has indicated MyD88 deficiency can be linked to enhanced IL-13 responses (Schnare *et al.*, 2001). However, this was concluded to be a result of decreased IFN-γ detected when stimulated with *Mycobacterium tuberculosis*. In our hands this could not be the explanation, since we saw no IFN-γ deficiency when priming was carried out by MyD88 deficient Pa-DCs. In another study MyD88 deficient DCs stimulated with soluble antigen extract of *Toxoplasma gondii* (STAg) were impaired in their capacity to drive IFN-γ production by OTII Tg T cells in an *in vitro* co-culture system (Jankovic *et al.*, 2004). It has already been shown that DC IL-12 production is not necessarily required for the generation of a Pa-specific Th1 response (MacDonald and Pearce, 2002) and it is possible that the MyD88 independent maintenance of IFN-γ production is unique to stimulation with Pa, or other Gram positive bacteria.

We also found that MyD88 deficiency did not impair DC capacity to drive SEA-specific Th2 responses *in vivo*, in agreement with previous studies (Jankovic *et al.*, 2004; Kane *et al.*, 2008). Prior studies have shown that a lipid fraction from *S. mansoni* eggs promote DCs to induce Th2 differentiation through a TLR2-dependent mechanism (van der Kleij *et al.*, 2002), or that a synthetic *S. mansoni* egg glycan promoted Th2 induction via TLR4 (Thomas *et al.*, 2003). Potentially SEA binds to an as yet unknown PRR in order to promote Th2 differentiation. The effect of MyD88 signalling on DC Th2 induction, if involved at all, remains unclear as we still lack a defining indicator of a Th2 conditioned DC.

Finally, we measured mRNA expression of YM1 and RELMα, reasoning that it was possible that these molecules could provide an alternative readout of DC Th2 activation. However, SEA exposure did not yield any more Ym1 or RELMα expression than was measured in unstimulated DCs. Thus, although the experiments detailed in this chapter have shown that Th2 associated cytokines can have a substantial impact on DC activation and function, they have not revealed a clear measure to distinguish SEA-primed DCs from unstimulated DCs in any of the conditions measured.

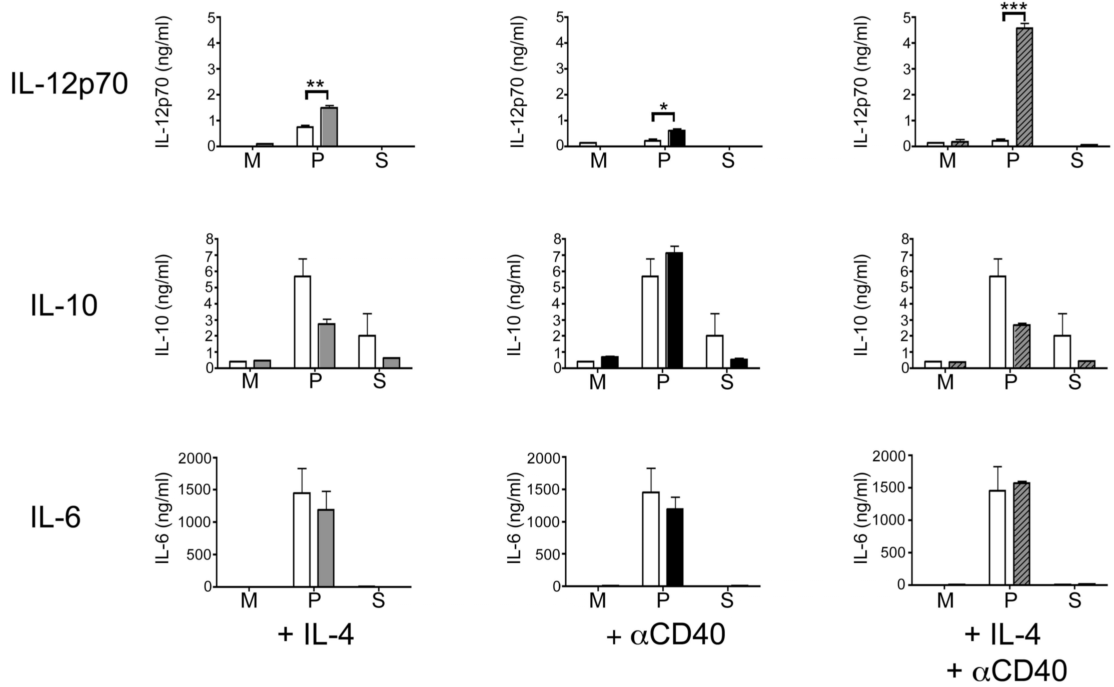

Figure 5.1. Impact of cytokines or antibodies mimicking T cell interaction on DC cytokine production. 10^6 DCs were stimulated with medium alone (M), *P. acnes* (P, 10 μg/ml) or SEA (S, 25 μg/ml) and then 4 hours later with medium alone (open bars) or medium and IL-4 (1 ng/ml, grey), αCD40 (30 μg/ml, black bars) or IL-4 and αCD40 (grey with black stripes). Cells were left for 24 hours and then cytokine measured by ELISA. Data shown are mean + SEM of cytokine measured in supernatants from duplicate wells, and are representative of four separate experiments. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

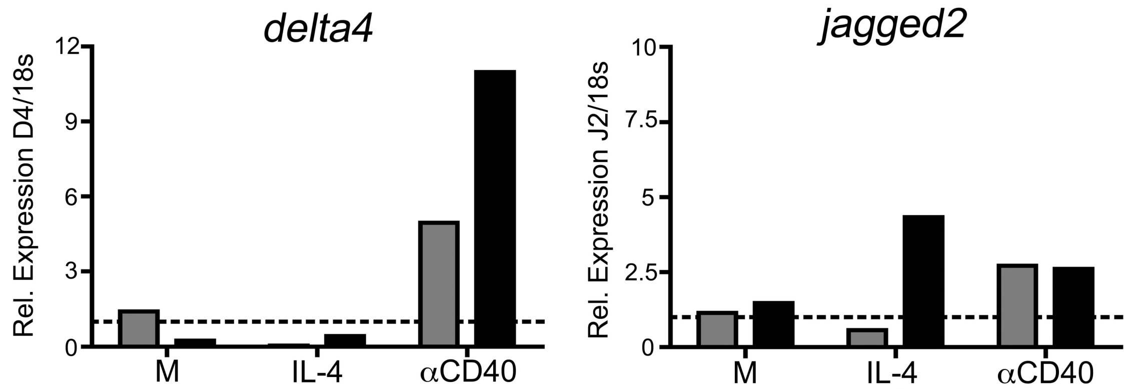

Figure 5.2 Notch ligand expression in response to Ag stimulation is affected by other cytokines or antibodies mimicking T cell interaction. 10^6 DCs stimulated with medium alone (M), *P. acnes* (10 μg/ml, grey bars) or SEA (25 μg/ml, black bars) and then 4 hours later medium alone (M), medium and IL-4 (1 ng/ml), or αCD40 (30 μg/ml) were added to culture. Cells were left for either 24 (*jagged2*) or 48 hours (*delta4*) and *delta4* or *jagged2* mRNA were assessed by quantitative PCR normalised against 18s rRNA. Bars represent average of duplicate runs as a fold change of expression over unstimulated cells (dotted line).

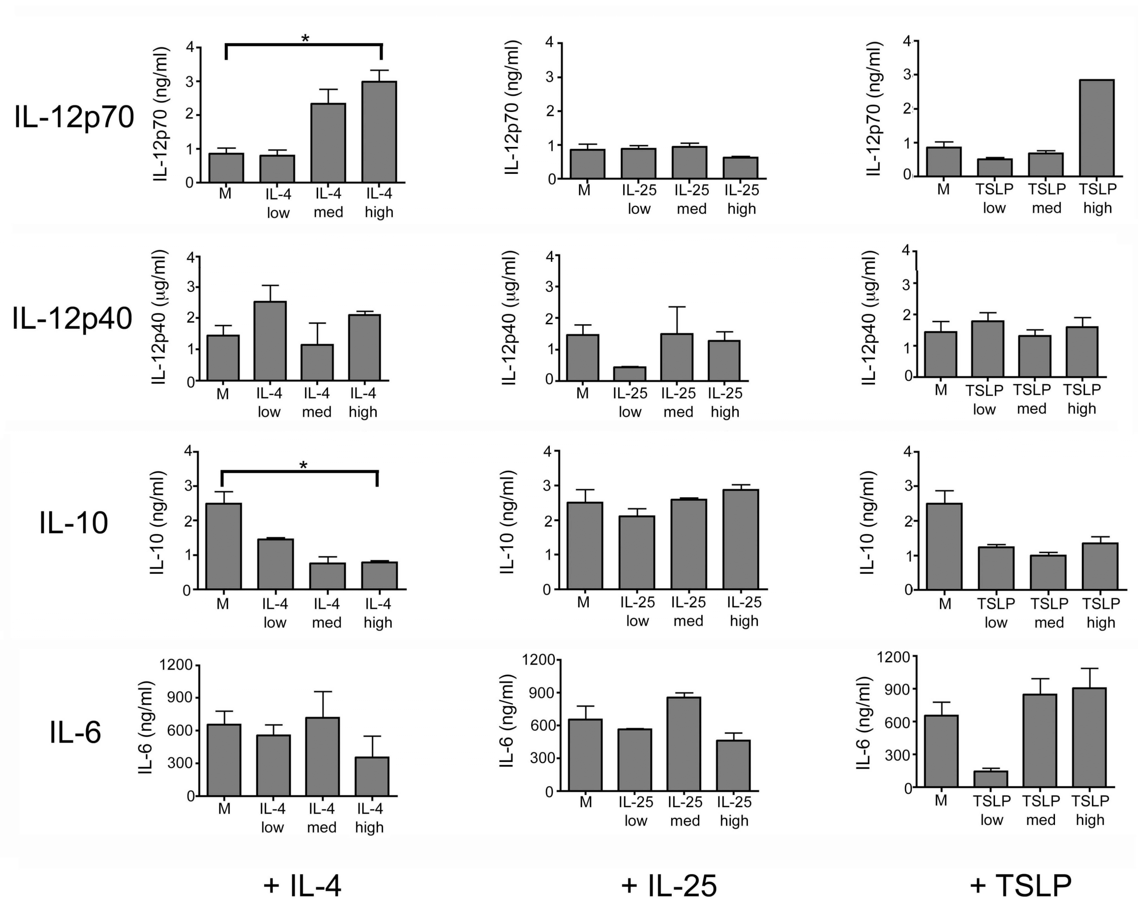

Figure 5.3 Th2 tissue factors can influence DC cytokine secretion. 10^6 DCs were stimulated with *P. acnes* (10 μg/ml) in the presence of medium (M) or 3 separate doses of IL-4 (0.1 ng/ml low, 1 ng/ml med, 10 ng/ml high), IL-25 (2 ng/ml low, 20 ng/ml med, 100 ng/ml high) or TSLP (1 ng/ml low, 10 ng/ml med, 50 ng/ml high). Cells were left for 24 hours and then cytokine in supernatant measured by ELISA. Data shown are mean + SEM of cytokine measured by ELISA and are representative of two separate experiments. *, $P < 0.05$

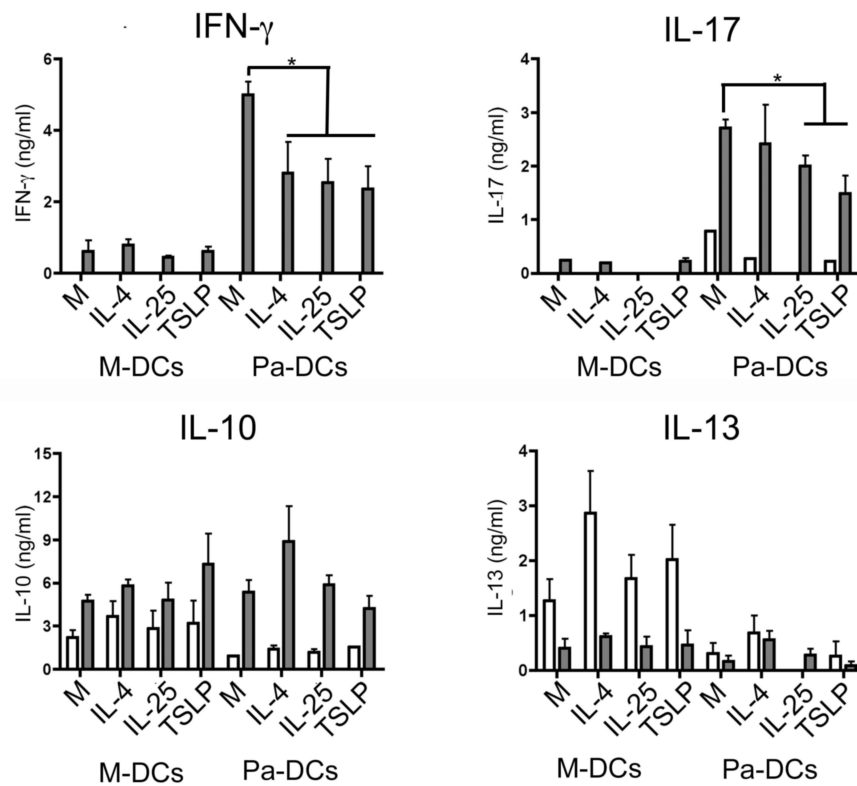

Figure 5.4 Th2 tissue factors impair Th1/Th17 priming by bacterially stimulated DCs. 10^6 DCs were stimulated with medium alone (M-DCs) or *P. acnes* (Pa-DCs, 10 μg/ml) in addition to IL-4 (1 ng/ml), IL-25 (20 ng/ml) or TSLP (10 ng/ml). 24 hours later 5×10^5 cells were injected i.p. into naïve recipient mice and 7 days later spleens were removed and restimulated *in vitro* with either medium alone (open bars) or Pa (gray bars). Cells were cultured for 72 hours and then cytokine measured in supernatants by ELISA. Data shown are mean + SEM of cytokine measured by ELISA of five mice per group. *, P < 0.05. This experiment was carried out with the assistance of Lowri Griffiths.

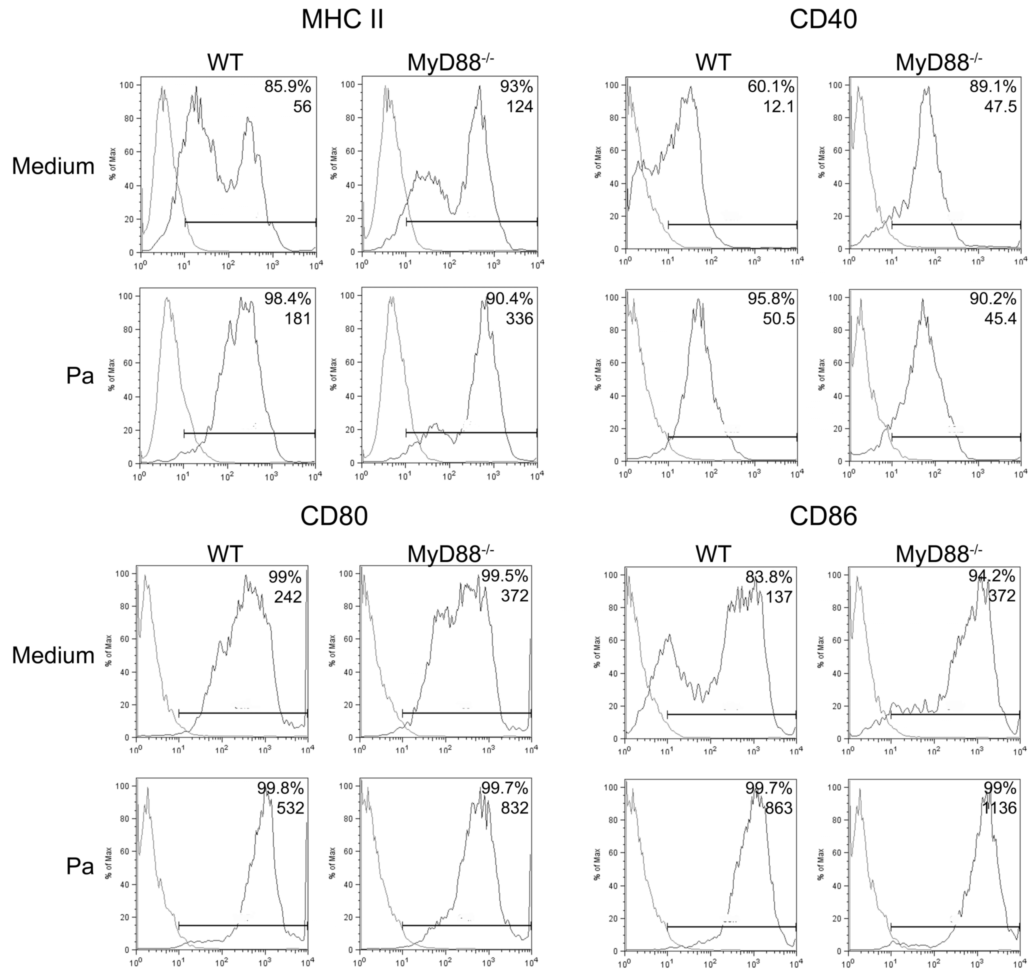

Figure 5.5 MyD88 deficient DCs retain responsiveness to phenotypic activation by bacterial stimulation. 10^6 DCs were stimulated for 24 hours with medium alone (Medium) or Pa (10 μg/ml). Surface expression of MHC II, CD40, CD80 and CD86 was then analysed by flow cytometry. Light histograms depict isotype control staining while darker histograms indicate cells stained with the marker indicated. Plots shown are gated on live $CD11c^+$ cells and percentage positive and geometric mean fluorescence is shown in each graph. Data shown are representative of 5 individual experiments.

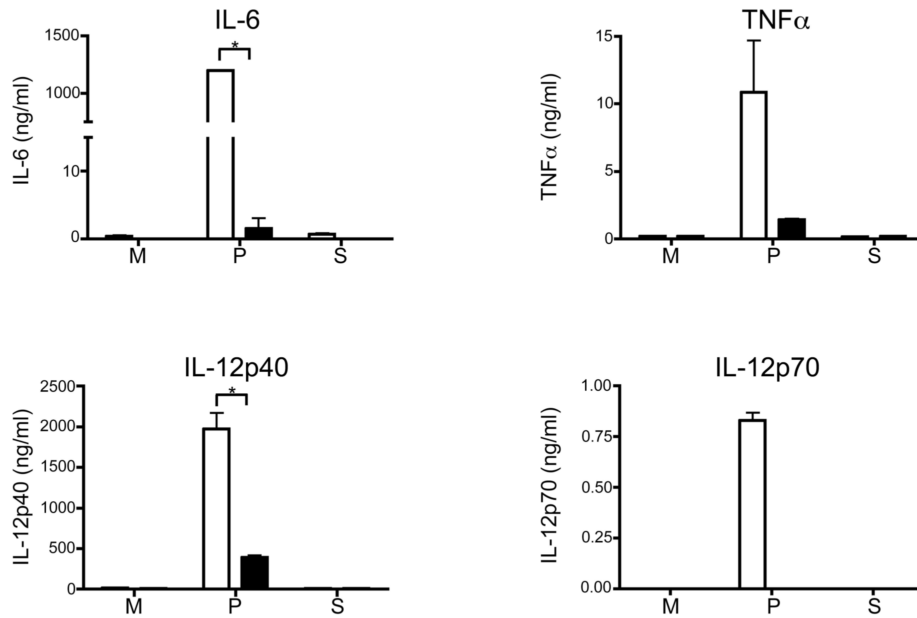

Figure 5.6 MyD88 deficient DCs display impaired cytokine production in response to bacterial stimulation. 10^6 WT (open bars) or MyD88$^{-/-}$ DCs (black bars) were stimulated for 24 hours with medium alone (Medium), SEA (50 µg/mL) or Pa (10µg/mL). Data shown are mean + SEM of cytokine levels in supernatants measured by ELISA and are representative of five separate experiments. *, $P < 0.05$

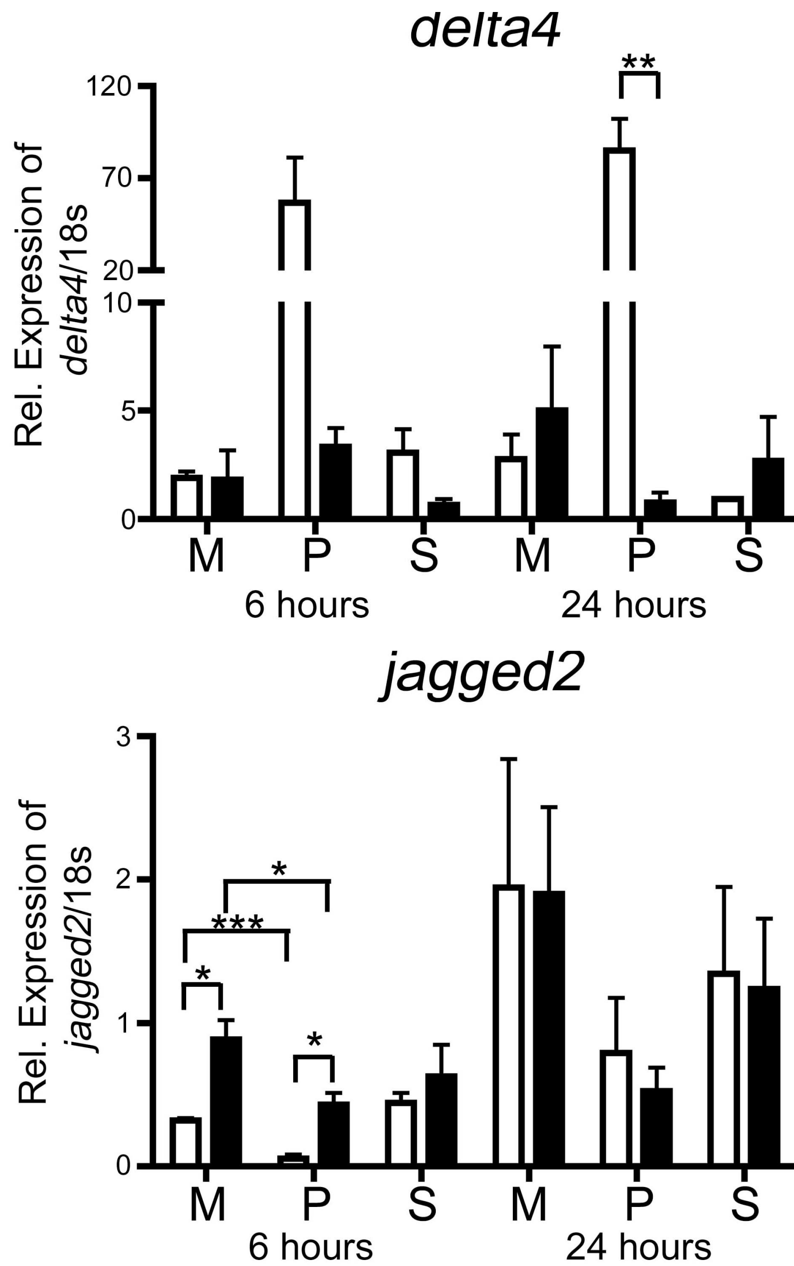

Figure 5.7 Pa specific *delta4* upregulation by DCs is MyD88 dependent. 10^6 WT (open bars) or MyD88$^{-/-}$ (black bars) DCs were stimulated for either 6 or 24 hours with medium alone (M, open bars) Pa (P, 10 μg/ml) or SEA (S, 25 μg/ml). *jagged2* or *delta4* mRNA were assessed by quantitative PCR and normalised against 18s rRNA. Error bars indicate mean + SEM of triplicate culture wells. Data shown are representative of three separate experiments. * = P < 0.05, ** = P < 0.01, *** = p < 0.001

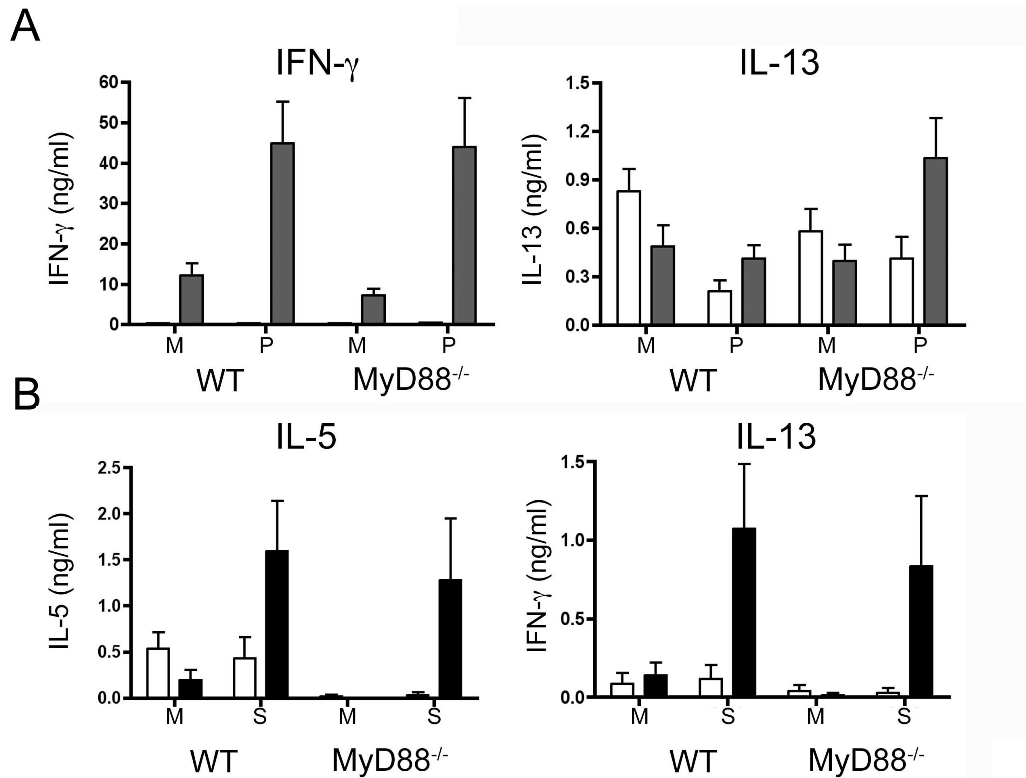

Figure 5.8 T cell polarisation by MyD88$^{-/-}$ DCs *in vivo*. WT or MyD88 deficient DCs were exposed to medium alone (M), **A)** Pa (P, 10 µg/mL) or **B)** SEA (S, 25 µg/mL). Cells were then injected i.p. into naïve C57BL/6 recipient mice. Splenocytes were removed 7d later and then stimulated *in vitro* with medium (open bars), SEA (black bars) or Pa (grey bars). Data shown are mean + SEM of cytokine measured by ELISA five mice per group and representative of five separate experiments.

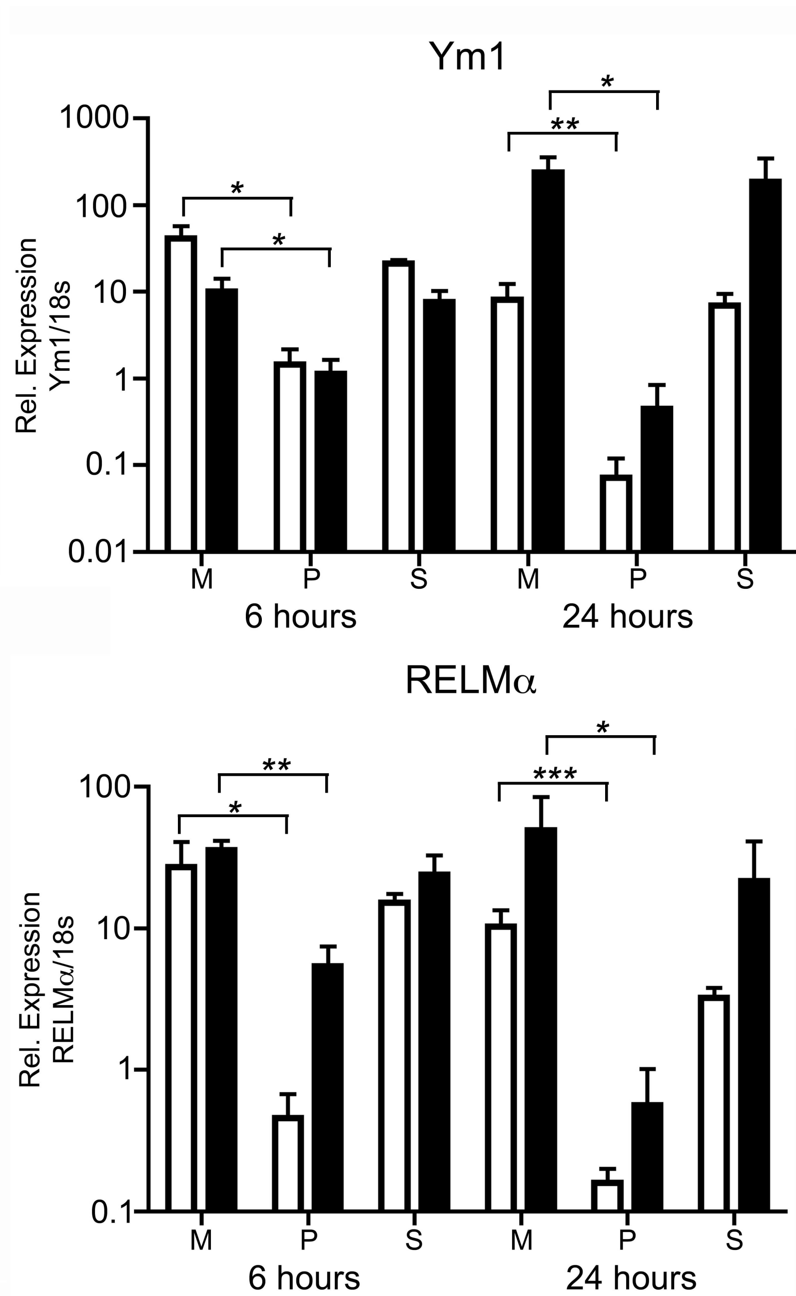

Figure 5.9 Pa stimulation downregulates RELMα in a partially MyD88 dependent manner. 10^6 WT (open bars) or MyD88$^{-/-}$ (black bars) DCs were stimulated for either 6 or 24 hours with medium alone (Medium), Pa (10 μg/ml) or SEA (25 μg/ml) and then Ym1 or RELMα mRNA expression measured by quantitative PCR and normalised to 18s RNA. Error bars indicate mean + SEM of triplicate wells of DCs. Data shown are representative of two independent experiments. * = $P < 0.05$, ** = $P < 0.01$, *** = $p < 0.001$

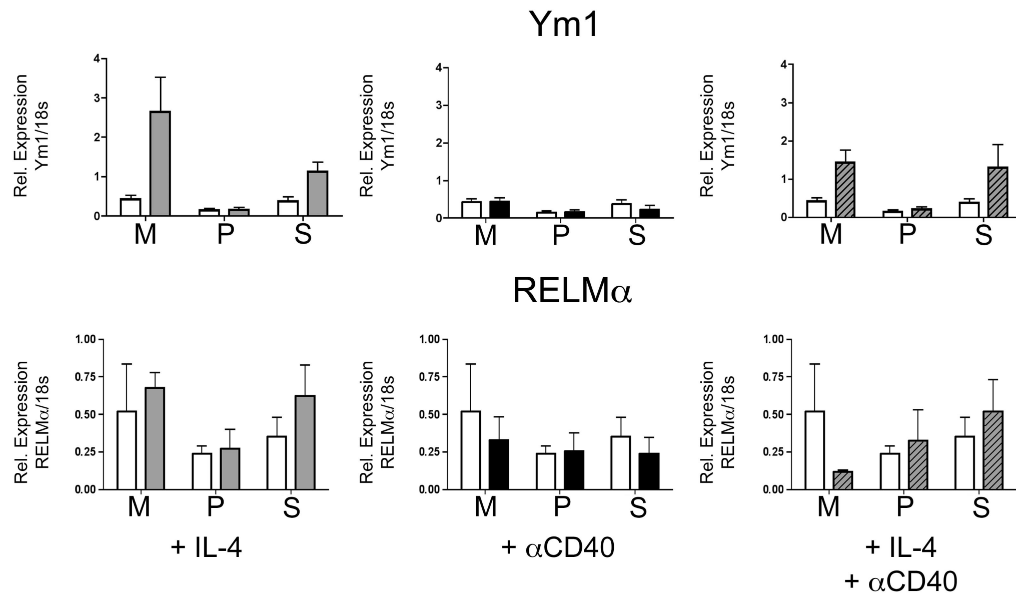

Figure 5.10 Pa prevents IL-4 mediated upregulation of Ym1 and RELMα by DCs. 10^6 DCs were stimulated for 24 hours with medium alone (M), Pa (P, 10 μg/ml) or SEA (S, 25 μg/ml) and then 4 hours later were treated with medium alone (open bars) or IL-4 (1 ng/ml, grey bars), αCD40 (30 μg/ml, black bars), or both IL-4 and αCD40 (grey bars with black cross-hatched). Ym1 or RELMα mRNA expression was measured by quantitative PCR and normalised to 18s rRNA. Error bars indicate mean + SEM of triplicate wells of DCs. Data shown are representative of three independent experiments.

Chapter 6

Final Discussion

6.1 General Discussion

The processes by which APCs instruct T cell polarisation, particularly Th2 differentiation, remain poorly understood. By most standard measures of maturation, DCs stimulated by Th2 pathogens such as *S. mansoni* are phenotypically indistinguishable from unstimulated DCs. The central hypothesis of this thesis was that Notch ligands may play a role in the "third signal" (Kalinski *et al.*, 1999) provided by APCs to polarise naïve $CD4^+$ T cells.

That SEA-treated DCs display few of the conventional markers of activation, and yet are able to potently drive SEA specific Th2 immune responses, is well established (Perona-Wright *et al.*, 2006). Although DC expression of MHC II (MacDonald *et al.*, 2001), NF-κB (Artis *et al.*, 2005), CD40 (MacDonald *et al.*, 2002b) and OX40L (Jenkins *et al.*, 2007) have been shown to be critical to the generation of a Th2 response, none of these molecules are significantly upregulated by SEA stimulation. However, Th2 priming by SEA stimulated DCs is not considered a "default" process, but rather an active induction, as evidenced by the fact that DCs co-stimulated by SEA and *P. acnes* drive separate, yet concurrent, Th1 and Th2 responses (Cervi *et al.*, 2004). Thus, the question remains: by which mechanisms are DCs able to actively drive Th2 differentiation when they appear to display no clear signs of activation when compared to immature DCs? This question is pertinent, as it not only addresses our fundamental understanding of DC biology and the invocation of adaptive immunity, but could also provide therapeutic targets for protection against helminth infection and other Th2 dominated disease settings.

6.2 Is DC Notch ligand expression associated with specific stimuli?

The focus of the work in this thesis was to determine whether an association exists between Notch ligand expression and DC ability to polarise $CD4^+$ T cells. Notch ligand expression by DCs was investigated for providing a novel 'signal 3' component for T cell polarisation. Although there is gathering evidence supporting a role for Notch receptor signalling in T cells (Amsen *et al.*, 2007; Tu *et al.*, 2005), it has not yet been determined if APCs provide the source of Notch ligand necessary to activate that signal. Enticingly, previous studies had proposed that not only might Notch receptor signalling be important for naïve $CD4^+$ T cell differentiation (Adler *et al.*, 2003; Eagar *et al.*, 2004; Izon *et al.*, 2001; Palaga *et al.*, 2003; Vigouroux *et al.*, 2003; Wong *et al.*, 2003; Yvon *et al.*, 2003), but also that the precise Notch ligand profile displayed to T cells may instruct a specific polarisation fate (Amsen *et al.*, 2004; Maekawa *et al.*, 2003; Tanigaki *et al.*, 2004) with *delta* ligands directing Th1 differentiation and *jagged* ligands Th2.

Importantly, my work has demonstrated, in the context of biologically relevant pathogens, that the nature of the stimulus to which DCs are exposed does have an impact on the expression of Notch ligands (**Figure 3.6**). Although we did not find significant upregulation of *jagged2* expression on SEA stimulated DCs, there was an association between elevated *delta1* and *delta4* expression, and decreased *jagged2* expression, in DCs responding to Pa (**Figure 3.6**). Furthermore, these observed expression patterns were MyD88 dependent (**Figure 5.7**) supporting the notion that such changes in Notch ligand expression are a direct result of DC TLR signalling and thus influenced by pathogen recognition. Our results are similar to

those reported by Sun et al (2008), which suggest that the Notch ligands *delta1* and *delta4* may be used to actively suppress Th2 development in a MyD88 dependent manner (Sun *et al.*, 2008).

Our data demonstrating that complex pathogen preparations induce DC expression of a specific cohort of Notch ligands, and that the expression of distinct Notch ligands are not consistent within their family, emphasize the potential of these markers as precise indicators of DC stimulation. Further characterisation of Notch ligand expression in all APCs in both mice and humans may provide us with an invaluable tool for carefully defining the nature of an antigenic stimulus. Ideally, profiling Notch ligand expression could be used as a diagnostic tool for determining the exact maturation status of an APC in a more specific manner than cytokine secretion alone.

6.3 Is DC *jagged2* expression essential for Th2 differentiation *in vitro* or *in vivo*?

Few studies have directly explored a role for Notch-Notch ligand interactions *in vivo*. In one case, where $CD4^+$ cell specific *Notch1* deficient mice were infected with *Leishmania major*, the authors found that the absence of *Notch1* receptor on $CD4^+$ T cells did not impair the host's ability to mount a protective Th1 response (Tacchini-Cottier *et al.*, 2004). More recently, a study utilized $CD4^+$ specific expression of a dominant negative mutant of the MAML protein (essential for the recruitment of RBP-J co-activators) thus disrupting RBP-J dependent Notch signalling via all Notch receptors, in $CD4^+$ T cells (Tu *et al.*, 2005). When these mice were infected with either *T. muris* or *L. major* they were able to mount a successful

Th1 response to *L. major* infection, but were susceptible to *T. muris* infection (Tu *et al.*, 2005). Although this work did not specifically investigate the roles for specific Notch ligands in the promotion of a Th1 or Th2 response, it provided strong evidence that the Notch signalling pathway may be more crucial in the establishment of Th2 than Th1 adaptive immunity.

Based on the proposed Jagged/Th2, Delta/Th1 paradigm (Amsen *et al.*, 2004), and within the limits of current technical ability, we directly assessed the importance of *jagged2* expression by DCs in the process of Th2 induction by DCs *in vitro* and *in vivo*. Despite the absence of a clear enhancement of *jagged2* expression by DCs in response to SEA stimulation, the inability of *jagged2* deficient DCs to instruct SEA specific IL-4 production by $CD4^+$ T cells *in vitro* suggests that provision of this Notch ligand is required for Th2 differentiation (**Figure 4.11**). However, adoptive transfer experiments revealed that DC expression of *jagged2* was not required for an SEA specific Th2 response *in vivo* (**Figure 4.13**). These contradictory results suggest a more nuanced system of Notch provision in the activation and polarisation of $CD4^+$ T cells than we might have expected.

Potentially, Notch signal transduction maybe a redundant mechanism that reinforces the polarisation outcome predominantly determined by an alternate 'signal 3' provision by APCs. Recent work by Ong et al. (2008) has shown that Chinese hamster ovary (CHO) cells transfected with either *delta1* or *jagged1* ligands were not able to redirect T cells towards a different cell fate than that which cytokine conditions would specify (Ong *et al.*, 2008). For example, DO11.10 $CD4^+$ T cells could not overcome Th2 polarising conditions, and still produced high levels of IL-4 even when stimulated by CHO cells transfected with MHC II, CD80 and *delta1*

ligand (Ong *et al.*, 2008). Although this study focused on artificial APCs in the absence of pathogen specific T cell polarising cytokines normally produced during APC maturation, it demonstrates that Notch signalling may be overruled by polarising cytokines.

Another possibility is that Notch signalling is involved as a survival signal administered to previously polarised T cells rather than in the initial priming of T cell polarisation. Although our results show a requirement for DC *jagged2* expression in the induction of SEA-specific IL-4 production by $CD4^+$ T cells *in vitro*, the absence of *jagged2*, or presence of the presenilin inhibitor DAPT, did not completely abolish IL-4 production (**Figure 4.11 and Figure 4.12**). This implies that initial generation of IL-4 is still possible in the absence of Notch signalling, but the sustained production of this Th2 cytokine may require Notch signalling. A link between Notch receptor signalling and the transcription of GATA3, as well as the IL-4 gene locus has been established in other studies (Amsen *et al.*, 2007; Amsen *et al.*, 2004). *notch1* and *notch2* deficiency has been shown to dramatically reduce the expression of GATA3 in Th2 priming conditions, and RBP-Jκ binding sites were present in the upstream promoter region of GATA3 (Amsen *et al.*, 2007). However, in the absence of GATA3 the addition of NICD enhanced Th1 responses (Amsen *et al.*, 2007). An interpretation of these studies is that transcription factors such as GATA3 or T-bet may exert a greater influence on Notch signalling than Notch signalling has on transcription factor expression. With more time for additional experiments, assessment of Notch signal transduction in OTII $CD4^+$ T cells by Q-PCR, such as measurement of HES and GATA3 transcription during stimulation with *jagged2*$^{-/-}$ SEA-DC, could help reveal whether it is Th2 initiation or maintenance that requires

jagged2 provision. Furthermore, examination of potential binding sites for transcription factors such as GATA3 or STAT6 (the downstream transcription factor from IL-4 signalling) within Notch ligand promoter regions would shed light on whether Notch ligands are receptive to Th1 or Th2 differentiation. STAT binding motifs can be quite variable, but typically utilise the 5'-TTC…GAA-3' core palindrome (Kraus *et al.*, 2003). The use of radioactive probes or RNAse protection assays could be used to confirm the activity of these transcription factors in Notch ligand promoter regions.

Finally, given that Notch ligands and receptors are expressed on a wide variety of cell types (Yamaguchi *et al.*, 2002), it is also possible that other cells could provide the Notch ligands necessary to induce $CD4^+$ T cell differentiation. In our *in vitro* co-culture system, only DCs or other $CD4^+$ T cells were available for Notch ligand provision. It would be interesting in future experiments to discern whether additional cell types, which are *jagged2* sufficient and not involved in antigen presentation, are capable of restoring Th2 differentiation. It is also important to note that during *in vitro* co-culture, $CD4^+$ T cells are *jagged2* sufficient, and thus provision of *jagged2* either through *cis*-interactions or via other T cells are not capable of inducing SEA-specific IL-4 production. In addition to investigating Notch ligands as a mechanism by which APCs induce $CD4^+$ T cell polarisation, future work should also examine the extent to which Notch signalling is utilized by many other cell types within peripheral immunity. Recent work has suggested that Notch signalling may be important for NK cell activation (Kijima *et al.*, 2008), NKT cells interaction (Wiethe *et al.*, 2008) and T cell cytotoxicity (Maekawa *et al.*, 2008). It is

important to remember that we have merely scratched the surface of the extent to which this dynamic signalling mechanism may be involved in the immune system.

6.4 Is DC maturation impacted by infection related cytokines?

Although Notch ligand expression by DCs may assist in the determination of a $CD4^+$ T cell polarisation, ligand expression *in vivo* may be subject to influence by additional factors provided by the tissue microenvironment, such as TSLP, not present *in vitro*. To begin to address this possibility we exposed DCs to a panel of infection related cytokines, such as IL-4, IL-25, TSLP during their *in vitro* stimulation, or mimicked T cell interactions using an agnostic αCD40 antibody (**Figures 5.1, 5.2 and 5.3**). Overall, several tissue factors did have an impact on DC responses to stimuli, supporting the idea that the context of Ag exposure may be a key factor in subsequent activation. Addition of IL-4 was found to decrease IL-10 production and increase IL-12p70 production by DCs. TSLP exposure showed a similar trend although these results were not significant. However, despite enhanced IL-12 production *in vitro*, transferred Pa-DCs stimulated in conjunction with IL-4, IL-25 or TSLP induced significantly less IFN-γ than DCs stimulated by Pa alone (**Figure 5.4)**. This fascinating outcome demonstrates that infection-related cytokines impact both the measurable activation status of DCs as well as their ability to polarise $CD4^+$ T cell responses *in vivo*. The implications of these results impact all *in vitro* DC work, as it highlights the fact that cytokine micro-environment plays a substantial role in DC activation. Additionally, given the association of DC IL-12 with Th1 induction (Gately *et al.*, 1998), these results once again underline that conclusions drawn purely from *in vitro* assessment are not necessarily relevant *in*

vivo. More broadly, DC maturation and polarising behaviour may be profoundly altered within an infection context and all inferences from strictly *in vitro* analyses should take this into consideration.

6.5 Are there any markers to distinguish SEA treated DC from unstimulated DC?

The search for any measurable response by DCs to SEA treatment remains ongoing. Importantly, the work in this thesis has shown clearly that individual Notch ligands may not be useful as markers of Th2 stimulated DCs. We investigated other potential indicators of Th2 maturation by assessing DC expression of Ym1 and RELMα, (**Figure 5.9 and 5.10**). However DCs exposed to SEA were no different from unstimulated DCs in the expression of these molecules. Doubtless, additional potential markers of Th2 activation will present themselves in the years to come and the discovery of such would perhaps be aided by a more broad screening approach to identifying new candidates.

6.6 What defines the differences in antigen presenting capacity between DCs and M∅s?

In the initial work of this thesis, DCs were compared to another major APC cell type, M∅s. By contrasting their relative capacities to induce *in vivo* immune responses in relation to their production of cytokine and expression of co-stimulatory markers *in vitro* the intention was to discern which mechanisms might be essential for the "professionalism" of DCs in terms of T cell polarising ability. That DCs in general secreted or expressed greater levels of most conventional markers of

activation (**Figures 3.2 and 3.3**) was not unexpected (Mellman *et al.*, 1998). However, given their reduced activation state in comparison to DCs, the similar ability of both DCs and M∅s to induce an St-specific IFN-γ response following transfer into naïve recipient mice was surprising (**Figure 3.3**). Interestingly, this APC ability equivalency was not seen when DCs or M∅s were instead stimulated with the gram-positive bacterium *P. acnes*. Irrespective of this, several possibilities arise from these data. Either the threshold for cytokine secretion required by APCs for the establishment of an St-specific immune response is less than for *P. acnes*, or an exogenous factor encountered following M∅ transfer *in vivo* may heighten APC function by M∅, or an additional mechanism stimulated by *S. typhimurium* (not measured in these studies) might be responsible for the unexpectedly effective M∅ induced inflammatory response.

6.7 Concluding Remarks

The work presented in this thesis illustrates an association of DC Notch ligand expression profiles with Th1 versus Th2 driving pathogens or pathogen products, and that the expression of specific Notch ligands by DCs may be critical *in vitro* but redundant *in vivo*. While this thesis supports the hypothesis that Notch ligands should be included in the array of signals considered to be involved in instruction of T cell polarisation, it is clear that other mechanisms for directing the immune ‘orchestra’ remain to be identified.

References

Abbas AK, Murphy KM, Sher A (1996) Functional diversity of helper T lymphocytes. *Nature* 383:787-93.

Aderem A, Underhill DM (1999) Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 17:593-623.

Adler SH, Chiffoleau E, Xu L, Dalton NM, Burg JM, Wells AD*, et al.* (2003) Notch signaling augments T cell responsiveness by enhancing CD25 expression. *J Immunol* 171:2896-903.

Agnello D, Lankford CS, Bream J, Morinobu A, Gadina M, O'Shea JJ*, et al.* (2003) Cytokines and transcription factors that regulate T helper cell differentiation: new players and new insights. *J Clin Immunol* 23:147-61.

Ahmed R, Gray D (1996) Immunological memory and protective immunity: understanding their relation. *Science* 272:54-60.

Akira S, Uematsu S, Takeuchi O (2006) Pathogen recognition and innate immunity. *Cell* 124:783-801.

Alegre ML, Frauwirth KA, Thompson CB (2001) T-cell regulation by CD28 and CTLA-4. *Nat Rev Immunol* 1:220-8.

Allman D, Punt JA, Izon DJ, Aster JC, Pear WS (2002) An invitation to T and more: notch signaling in lymphopoiesis. *Cell* 109 Suppl:S1-11.

Amsen D, Antov A, Jankovic D, Sher A, Radtke F, Souabni A*, et al.* (2007) Direct regulation of Gata3 expression determines the T helper differentiation potential of Notch. *Immunity* 27:89-99.

Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA (2004) Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 117:515-26.

Angkasekwinai P, Park H, Wang YH, Wang YH, Chang SH, Corry DB*, et al.* (2007) Interleukin 25 promotes the initiation of proallergic type 2 responses. *J Exp Med* 204:1509-17.

Anthony RM, Rutitzky LI, Urban JF, Jr., Stadecker MJ, Gause WC (2007) Protective immune mechanisms in helminth infection. *Nat Rev Immunol* 7:975-87.

Artavanis-Tsakonas S, Rand MD, Lake RJ (1999) Notch signaling: cell fate control and signal integration in development. *Science* 284:770-6.

Artis D, Kane CM, Fiore J, Zaph C, Shapira S, Joyce K*, et al.* (2005) Dendritic cell-intrinsic expression of NF-kappa B1 is required to promote optimal Th2 cell differentiation. *J Immunol* 174:7154-9.

Askenasy N, Kaminitz A, Yarkoni S (2008) Mechanisms of T regulatory cell function. *Autoimmun Rev* 7:370-5.

Askonas BA, Auzins I, Unanue ER (1968) Role of macrophages in the immune response. *Bull Soc Chim Biol (Paris)* 50:1113-28.

Aster JC, Pear WS, Blacklow SC (2008) Notch signaling in leukemia. *Annu Rev Pathol* 3:587-613.

Astrakhan A, Omori M, Nguyen T, Becker-Herman S, Iseki M, Aye T*, et al.* (2007) Local increase in thymic stromal lymphopoietin induces systemic alterations in B cell development. *Nat Immunol* 8:522-31.

Axelrod JD, Matsuno K, Artavanis-Tsakonas S, Perrimon N (1996) Interaction between Wingless and Notch signaling pathways mediated by dishevelled. *Science* 271:1826-32.

Balaram P, Kien PK, Ismail A (2008) Toll-like receptors and cytokines in immune responses to persistent mycobacterial and Salmonella infections. *Int J Med Microbiol*.

Balfour BM, Buttifant L, O'Brien J, Clarke J, Knight SC (1985) Veiled cell lymphocyte interaction. *Adv Exp Med Biol* 186:395-9.

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ*, et al.* (2000) Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767-811.

Banchereau J, Palucka AK (2005) Dendritic cells as therapeutic vaccines against cancer. *Nat Rev Immunol* 5:296-306.

Banchereau J, Steinman RM (1998) Dendritic cells and the control of immunity. *Nature* 392:245-52.

Banz A, Pontoux C, Papiernik M (2002) Modulation of Fas-dependent apoptosis: a dynamic process controlling both the persistence and death of CD4 regulatory T cells and effector T cells. *J Immunol* 169:750-7.

Baron M (2003) An overview of the Notch signalling pathway. *Semin Cell Dev Biol* 14:113-9.

Bassing CH, Swat W, Alt FW (2002) The mechanism and regulation of chromosomal V(D)J recombination. *Cell* 109 Suppl:S45-55.

Belvin MP, Anderson KV (1996) A conserved signaling pathway: the Drosophila toll-dorsal pathway. *Annu Rev Cell Dev Biol* 12:393-416.

Beninga J, Rock KL, Goldberg AL (1998) Interferon-gamma can stimulate post-proteasomal trimming of the N terminus of an antigenic peptide by inducing leucine aminopeptidase. *J Biol Chem* 273:18734-42.

Benoit M, Desnues B, Mege JL (2008) Macrophage polarization in bacterial infections. *J Immunol* 181:3733-9.

Benson RA, Lowrey JA, Lamb JR, Howie SE (2004) The Notch and Sonic hedgehog signalling pathways in immunity. *Mol Immunol* 41:715-25.

Berdnik D, Torok T, Gonzalez-Gaitan M, Knoblich JA (2002) The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. *Dev Cell* 3:221-31.

Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M*, et al.* (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235-8.

Biedermann T, Zimmermann S, Himmelrich H, Gumy A, Egeter O, Sakrauski AK*, et al.* (2001) IL-4 instructs TH1 responses and resistance to Leishmania major in susceptible BALB/c mice. *Nat Immunol* 2:1054-60.

Bigas A, Martin DI, Milner LA (1998) Notch1 and Notch2 inhibit myeloid differentiation in response to different cytokines. *Mol Cell Biol* 18:2324-33.

Blackwell JM, Jamieson SE, Burgner D (2009) HLA and infectious diseases. *Clin Microbiol Rev* 22:370-85, Table of Contents.

Boes M, Bertho N, Cerny J, Op den Brouw M, Kirchhausen T, Ploegh H (2003) T cells induce extended class II MHC compartments in dendritic cells in a Toll-like receptor-dependent manner. *J Immunol* 171:4081-8.

Brunet LR, Finkelman FD, Cheever AW, Kopf MA, Pearce EJ (1997) IL-4 protects against TNF-alpha-mediated cachexia and death during acute schistosomiasis. *J Immunol* 159:777-85.

Bryant P, Ploegh H (2004) Class II MHC peptide loading by the professionals. *Curr Opin Immunol* 16:96-102.

Bueno SM, Gonzalez PA, Schwebach JR, Kalergis AM (2007) T cell immunity evasion by virulent Salmonella enterica. *Immunol Lett* 111:14-20.

Bush G, diSibio G, Miyamoto A, Denault JB, Leduc R, Weinmaster G (2001) Ligand-induced signaling in the absence of furin processing of Notch1. *Dev Biol* 229:494-502.

Bustin SA (2000) Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. *J Mol Endocrinol* 25:169-93.

Carreno BM, Collins M (2002) The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. *Annu Rev Immunol* 20:29-53.

Cervi L, MacDonald AS, Kane C, Dzierszinski F, Pearce EJ (2004) Cutting edge: dendritic cells copulsed with microbial and helminth antigens undergo modified maturation, segregate the antigens to distinct intracellular compartments, and concurrently induce microbe-specific Th1 and helminth-specific Th2 responses. *J Immunol* 172:2016-20.

Chabriat H, Vahedi K, Iba-Zizen MT, Joutel A, Nibbio A, Nagy TG*, et al.* (1995) Clinical spectrum of CADASIL: a study of 7 families. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy. *Lancet* 346:934-9.

Chang NC, Hung SI, Hwa KY, Kato I, Chen JE, Liu CH*, et al.* (2001) A macrophage protein, Ym1, transiently expressed during inflammation is a novel mammalian lectin. *J Biol Chem* 276:17497-506.

Chen AI, McAdam AJ, Buhlmann JE, Scott S, Lupher ML, Jr., Greenfield EA*, et al.* (1999) Ox40-ligand has a critical costimulatory role in dendritic cell:T cell interactions. *Immunity* 11:689-98.

Chen X, Jensen PE (2008) The role of B lymphocytes as antigen-presenting cells. *Arch Immunol Ther Exp (Warsz)* 56:77-83.

Chen Y, Kuchroo VK, Inobe J, Hafler DA, Weiner HL (1994) Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis. *Science* 265:1237-40.

Cheng P, Nefedova Y, Corzo CA, Gabrilovich DI (2006) Regulation of dendritic cell differentiation by bone marrow stroma via different Notch ligands. *Blood*.

Cheng P, Nefedova Y, Corzo CA, Gabrilovich DI (2007) Regulation of dendritic-cell differentiation by bone marrow stroma via different Notch ligands. *Blood* 109:507-15.

Cheng P, Nefedova Y, Miele L, Osborne BA, Gabrilovich D (2003) Notch signaling is necessary but not sufficient for differentiation of dendritic cells. *Blood* 102:3980-8.

Chiaramonte R, Basile A, Tassi E, Calzavara E, Cecchinato V, Rossi V*, et al.* (2005) A wide role for NOTCH1 signaling in acute leukemia. *Cancer Lett* 219:113-20.

Chitsulo L, Engels D, Montresor A, Savioli L (2000) The global status of schistosomiasis and its control. *Acta Trop* 77:41-51.

Chitsulo L, Loverde P, Engels D (2004) Schistosomiasis. *Nat Rev Microbiol* 2:12-3.

Coffman RL, Mosmann TR (1991) CD4+ T-cell subsets: regulation of differentiation and function. *Res Immunol* 142:7-9.

Coffman RL, Seymour BW, Hudak S, Jackson J, Rennick D (1989) Antibody to interleukin-5 inhibits helminth-induced eosinophilia in mice. *Science* 245:308-10.

Collins M, Ling V, Carreno BM (2005) The B7 family of immune-regulatory ligands. *Genome Biol* 6:223.

Conlon RA, Reaume AG, Rossant J (1995) Notch1 is required for the coordinate segmentation of somites. *Development* 121:1533-45.

Cottrez F, Groux H (2001) Regulation of TGF-beta response during T cell activation is modulated by IL-10. *J Immunol* 167:773-8.

Creagh EM, O'Neill LA (2006) TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity. *Trends Immunol* 27:352-7.

Croft M (2003) Costimulation of T cells by OX40, 4-1BB, and CD27. *Cytokine Growth Factor Rev* 14:265-73.

Crow MK (2006) Modification of accessory molecule signaling. *Springer Semin Immunopathol* 27:409-24.

D'Souza B, Miyamoto A, Weinmaster G (2008) The many facets of Notch ligands. *Oncogene* 27:5148-67.

Dale DC, Boxer L, Liles WC (2008) The phagocytes: neutrophils and monocytes. *Blood* 112:935-45.

Dallman MJ, Champion B, Lamb JR (2003) Notch signalling in the peripheral immune system. *Novartis Found Symp* 252:268-76; discussion 76-8.

Darrah PA, Patel DT, De Luca PM, Lindsay RW, Davey DF, Flynn BJ*, et al.* (2007) Multifunctional TH1 cells define a correlate of vaccine-mediated protection against Leishmania major. *Nat Med* 13:843-50.

Davies SJ, Grogan JL, Blank RB, Lim KC, Locksley RM, McKerrow JH (2001) Modulation of blood fluke development in the liver by hepatic CD4+ lymphocytes. *Science* 294:1358-61.

Davies SJ, McKerrow JH (2003) Developmental plasticity in schistosomes and other helminths. *Int J Parasitol* 33:1277-84.

de Jong EC, Vieira PL, Kalinski P, Schuitemaker JH, Tanaka Y, Wierenga EA*, et al.* (2002) Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. *J Immunol* 168:1704-9.

de La Coste A, Freitas AA (2006) Notch signaling: distinct ligands induce specific signals during lymphocyte development and maturation. *Immunol Lett* 102:1-9.

Del Prete G, De Carli M, Almerigogna F, Giudizi MG, Biagiotti R, Romagnani S (1993) Human IL-10 is produced by both type 1 helper (Th1) and type 2 helper (Th2) T cell clones and inhibits their antigen-specific proliferation and cytokine production. *J Immunol* 150:353-60.

Demehri S, Liu Z, Lee J, Lin MH, Crosby SD, Roberts CJ*, et al.* (2008) Notch-deficient skin induces a lethal systemic B-lymphoproliferative disorder by secreting TSLP, a sentinel for epidermal integrity. *PLoS Biol* 6:e123.

Dennehy KM, Ferwerda G, Faro-Trindade I, Pyz E, Willment JA, Taylor PR*, et al.* (2008) Syk kinase is required for collaborative cytokine production induced through Dectin-1 and Toll-like receptors. *Eur J Immunol* 38:500-6.

Dichgans M, Ludwig H, Muller-Hocker J, Messerschmidt A, Gasser T (2000) Small in-frame deletions and missense mutations in CADASIL: 3D models predict misfolding of Notch3 EGF-like repeat domains. *Eur J Hum Genet* 8:280-5.

Dichgans M, Mayer M, Uttner I, Bruning R, Muller-Hocker J, Rungger G*, et al.* (1998) The phenotypic spectrum of CADASIL: clinical findings in 102 cases. *Ann Neurol* 44:731-9.

Donelson JE, Hill KL, El-Sayed NM (1998) Multiple mechanisms of immune evasion by African trypanosomes. *Mol Biochem Parasitol* 91:51-66.

Dovey HF, John V, Anderson JP, Chen LZ, de Saint Andrieu P, Fang LY*, et al.* (2001) Functional gamma-secretase inhibitors reduce beta-amyloid peptide levels in brain. *J Neurochem* 76:173-81.

Dragon S, Saffar AS, Shan L, Gounni AS (2008) IL-17 attenuates the anti-apoptotic effects of GM-CSF in human neutrophils. *Mol Immunol* 45:160-8.

Dunwoodie SL, Clements M, Sparrow DB, Sa X, Conlon RA, Beddington RS (2002) Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene Dll3 are associated with disruption of the segmentation clock within the presomitic mesoderm. *Development* 129:1795-806.

Dutton RW, Bradley LM, Swain SL (1998) T cell memory. *Annu Rev Immunol* 16:201-23.

Eagar TN, Tang Q, Wolfe M, He Y, Pear WS, Bluestone JA (2004) Notch 1 signaling regulates peripheral T cell activation. *Immunity* 20:407-15.

Eisenbarth SC, Piggott DA, Huleatt JW, Visintin I, Herrick CA, Bottomly K (2002) Lipopolysaccharide-enhanced, toll-like receptor 4-dependent T helper cell type 2 responses to inhaled antigen. *J Exp Med* 196:1645-51.

Ellisen LW, Bird J, West DC, Soreng AL, Reynolds TC, Smith SD*, et al.* (1991) TAN-1, the human homolog of the Drosophila notch gene, is broken by chromosomal translocations in T lymphoblastic neoplasms. *Cell* 66:649-61.

Epstein MM, Di Rosa F, Jankovic D, Sher A, Matzinger P (1995) Successful T cell priming in B cell-deficient mice. *J Exp Med* 182:915-22.

Falcone FH, Loke P, Zang X, MacDonald AS, Maizels RM, Allen JE (2001) A Brugia malayi homolog of macrophage migration inhibitory factor reveals an important link between macrophages and eosinophil recruitment during nematode infection. *J Immunol* 167:5348-54.

Fallon PG, Richardson EJ, McKenzie GJ, McKenzie AN (2000) Schistosome infection of transgenic mice defines distinct and contrasting pathogenic roles for IL-4 and IL-13: IL-13 is a profibrotic agent. *J Immunol* 164:2585-91.

Fickenscher H, Hor S, Kupers H, Knappe A, Wittmann S, Sticht H (2002) The interleukin-10 family of cytokines. *Trends Immunol* 23:89-96.

Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* 391:806-11.

Fischer HG, Opel B, Reske K, Reske-Kunz AB (1988) Granulocyte-macrophage colony-stimulating factor-cultured bone marrow-derived macrophages reveal accessory cell function and synthesis of MHC class II determinants in the absence of external stimuli. *Eur J Immunol* 18:1151-8.

Fleer A, Krediet TG (2007) Innate immunity: toll-like receptors and some more. A brief history, basic organization and relevance for the human newborn. *Neonatology* 92:145-57.

Fleming RJ, Gu Y, Hukriede NA (1997) Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. *Development* 124:2973-81.

Fort MM, Cheung J, Yen D, Li J, Zurawski SM, Lo S*, et al.* (2001) IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity* 15:985-95.

Fortini ME (2002) Gamma-secretase-mediated proteolysis in cell-surface-receptor signalling. *Nat Rev Mol Cell Biol* 3:673-84.

Fujiwara N, Kobayashi K (2005) Macrophages in inflammation. *Curr Drug Targets Inflamm Allergy* 4:281-6.

Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, Valenzuela DM*, et al.* (2004) Haploinsufficiency of delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc Natl Acad Sci U S A* 101:15949-54.

Gallegos AM, Bevan MJ (2006) Central tolerance: good but imperfect. *Immunol Rev* 209:290-6.

Gao X, Zhang P (2007) Transgenic RNA interference in mice. *Physiology (Bethesda)* 22:161-6.

Gately MK, Renzetti LM, Magram J, Stern AS, Adorini L, Gubler U*, et al.* (1998) The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* 16:495-521.

Geling A, Steiner H, Willem M, Bally-Cuif L, Haass C (2002) A gamma-secretase inhibitor blocks Notch signaling in vivo and causes a severe neurogenic phenotype in zebrafish. *EMBO Rep* 3:688-94.

Genoud S, Lappe-Siefke C, Goebbels S, Radtke F, Aguet M, Scherer SS*, et al.* (2002) Notch1 control of oligodendrocyte differentiation in the spinal cord. *J Cell Biol* 158:709-18.

Germain RN (2004) An innately interesting decade of research in immunology. *Nat Med* 10:1307-20.

Germann T, Mattner F, Partenheimer A, Schmitt E, Reske-Kunz AB, Fischer HG*, et al.* (1992) Different accessory function for TH1 cells of bone marrow derived macrophages cultured in granulocyte macrophage colony stimulating factor or macrophage colony stimulating factor. *Int Immunol* 4:755-64.

Gersuk GM, Razai LW, Marr KA (2008) Methods of in vitro macrophage maturation confer variable inflammatory responses in association with altered expression of cell surface dectin-1. *J Immunol Methods* 329:157-66.

Glittenberg M, Pitsouli C, Garvey C, Delidakis C, Bray S (2006) Role of conserved intracellular motifs in Serrate signalling, cis-inhibition and endocytosis. *Embo J* 25:4697-706.

Gluckman JC, Canque B, Chapuis F, Rosenzwajg M (1997) In vitro generation of human dendritic cells and cell therapy. *Cytokines Cell Mol Ther* 3:187-96.

Goh F, Irvine KM, Lovelace E, Donnelly S, Jones MK, Brion K, *et al.* (2008) Selective induction of the Notch ligand Jagged-1 in macrophages by soluble egg antigen from Schistosoma mansoni involves ERK signalling. *Immunology*.

Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ (2005) Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174:1783-6.

Goodridge HS, Stepek G, Harnett W, Harnett MM (2005) Signalling mechanisms underlying subversion of the immune response by the filarial nematode secreted product ES-62. *Immunology* 115:296-304.

Gordon S (2003) Alternative activation of macrophages. *Nat Rev Immunol* 3:23-35.

Gramaglia I, Weinberg AD, Lemon M, Croft M (1998) Ox-40 ligand: a potent costimulatory molecule for sustaining primary CD4 T cell responses. *J Immunol* 161:6510-7.

Gray D, Gray M, Barr T (2007) Innate responses of B cells. *Eur J Immunol* 37:3304-10.

Greenwald RJ, Freeman GJ, Sharpe AH (2005) The B7 family revisited. *Annu Rev Immunol* 23:515-48.

Grewal IS, Flavell RA (1998) CD40 and CD154 in cell-mediated immunity. *Annu Rev Immunol* 16:111-35.

Groux H, Powrie F (1999) Regulatory T cells and inflammatory bowel disease. *Immunol Today* 20:442-5.

Gryseels B, Polman K, Clerinx J, Kestens L (2006) Human schistosomiasis. *Lancet* 368:1106-18.

Grzych JM, Pearce E, Cheever A, Caulada ZA, Caspar P, Heiny S, *et al.* (1991) Egg deposition is the major stimulus for the production of Th2 cytokines in murine schistosomiasis mansoni. *J Immunol* 146:1322-7.

Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, *et al.* (1999) Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *J Exp Med* 189:451-60.

Hagenbuchle O, Krikeles MS, Sprague KU (1979) The nucleotide sequence adjacent to poly(A) in silk fibroin messenger RNA. *J Biol Chem* 254:7157-62.

Haines N, Irvine KD (2003) Glycosylation regulates Notch signalling. *Nat Rev Mol Cell Biol* 4:786-97.

Hamada Y, Kadokawa Y, Okabe M, Ikawa M, Coleman JR, Tsujimoto Y (1999) Mutation in ankyrin repeats of the mouse Notch2 gene induces early embryonic lethality. *Development* 126:3415-24.

Hammond SM, Bernstein E, Beach D, Hannon GJ (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 404:293-6.

Han H, Tanigaki K, Yamamoto N, Kuroda K, Yoshimoto M, Nakahata T*, et al.* (2002) Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* 14:637-45.

Hannon GJ (2002) RNA interference. *Nature* 418:244-51.

Harnett W, Harnett MM (2006) Filarial nematode secreted product ES-62 is an anti-inflammatory agent: therapeutic potential of small molecule derivatives and ES-62 peptide mimetics. *Clin Exp Pharmacol Physiol* 33:511-8.

Heath WR, Belz GT, Behrens GM, Smith CM, Forehan SP, Parish IA*, et al.* (2004) Cross-presentation, dendritic cell subsets, and the generation of immunity to cellular antigens. *Immunol Rev* 199:9-26.

Herbert DR, Holscher C, Mohrs M, Arendse B, Schwegmann A, Radwanska M*, et al.* (2004) Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology. *Immunity* 20:623-35.

Hesse M, Modolell M, La Flamme AC, Schito M, Fuentes JM, Cheever AW*, et al.* (2001) Differential regulation of nitric oxide synthase-2 and arginase-1 by type 1/type 2 cytokines in vivo: granulomatous pathology is shaped by the pattern of L-arginine metabolism. *J Immunol* 167:6533-44.

Hilkens CM, Kalinski P, de Boer M, Kapsenberg ML (1997) Human dendritic cells require exogenous interleukin-12-inducing factors to direct the development of naive T-helper cells toward the Th1 phenotype. *Blood* 90:1920-6.

Hill JA, Ichim TE, Kusznieruk KP, Li M, Huang X, Yan X*, et al.* (2003) Immune modulation by silencing IL-12 production in dendritic cells using small interfering RNA. *J Immunol* 171:691-6.

Hochrein H, O'Keeffe M, Luft T, Vandenabeele S, Grumont RJ, Maraskovsky E*, et al.* (2000) Interleukin (IL)-4 is a major regulatory cytokine governing bioactive IL-12 production by mouse and human dendritic cells. *J Exp Med* 192:823-33.

Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO*, et al.* (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* 424:743-8.

Hope JC, Thom ML, McCormick PA, Howard CJ (2004) Interaction of antigen presenting cells with mycobacteria. *Vet Immunol Immunopathol* 100:187-95.

Hoyne GF, Dallman MJ, Champion BR, Lamb JR (2001) Notch signalling in the regulation of peripheral immunity. *Immunol Rev* 182:215-27.

Hoyne GF, Dallman MJ, Lamb JR (2000a) T-cell regulation of peripheral tolerance and immunity: the potential role for Notch signalling. *Immunology* 100:281-8.

Hoyne GF, Le Roux I, Corsin-Jimenez M, Tan K, Dunne J, Forsyth LM, *et al.* (2000b) Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells. *Int Immunol* 12:177-85.

Hozumi K, Negishi N, Suzuki D, Abe N, Sotomaru Y, Tamaoki N, *et al.* (2004) Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat Immunol* 5:638-44.

Hrabe de Angelis M, McIntyre J, 2nd, Gossler A (1997) Maintenance of somite borders in mice requires the Delta homologue DII1. *Nature* 386:717-21.

Hsieh CS, Macatonia SE, O'Garra A, Murphy KM (1993a) Pathogen-induced Th1 phenotype development in CD4+ alpha beta-TCR transgenic T cells is macrophage dependent. *Int Immunol* 5:371-82.

Hsieh CS, Macatonia SE, Tripp CS, Wolf SF, O'Garra A, Murphy KM (1993b) Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages. *Science* 260:547-9.

Huppert SS, Jacobsen TL, Muskavitch MA (1997) Feedback regulation is central to Delta-Notch signalling required for Drosophila wing vein morphogenesis. *Development* 124:3283-91.

Huppert SS, Le A, Schroeter EH, Mumm JS, Saxena MT, Milner LA, *et al.* (2000) Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. *Nature* 405:966-70.

Hurst SD, Muchamuel T, Gorman DM, Gilbert JM, Clifford T, Kwan S, *et al.* (2002) New IL-17 family members promote Th1 or Th2 responses in the lung: in vivo function of the novel cytokine IL-25. *J Immunol* 169:443-53.

Huston DP, Liu YJ (2006) Thymic stromal lymphopoietin:a potential therapeutic target for allergy and asthma. *Curr Allergy Asthma Rep* 6:372-6.

Inaba K, Steinman RM (1984) Resting and sensitized T lymphocytes exhibit distinct stimulatory (antigen-presenting cell) requirements for growth and lymphokine release. *J Exp Med* 160:1717-35.

Ito T, Wang YH, Duramad O, Hori T, Delespesse GJ, Watanabe N*, et al.* (2005) TSLP-activated dendritic cells induce an inflammatory T helper type 2 cell response through OX40 ligand. *J Exp Med* 202:1213-23.

Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ*, et al.* (2006) The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 126:1121-33.

Izon DJ, Punt JA, Pear WS (2002) Deciphering the role of Notch signaling in lymphopoiesis. *Curr Opin Immunol* 14:192-9.

Izon DJ, Punt JA, Xu L, Karnell FG, Allman D, Myung PS*, et al.* (2001) Notch1 regulates maturation of CD4+ and CD8+ thymocytes by modulating TCR signal strength. *Immunity* 14:253-64.

Jacobsen TL, Brennan K, Arias AM, Muskavitch MA (1998) Cis-interactions between Delta and Notch modulate neurogenic signalling in Drosophila. *Development* 125:4531-40.

Jaleco AC, Neves H, Hooijberg E, Gameiro P, Clode N, Haury M*, et al.* (2001) Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J Exp Med* 194:991-1002.

Janeway CA, Jr. (1989) Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1:1-13.

Janeway CA, Jr., Medzhitov R (2002) Innate immune recognition. *Annu Rev Immunol* 20:197-216.

Jankovic D, Kullberg MC, Caspar P, Sher A (2004) Parasite-induced Th2 polarization is associated with down-regulated dendritic cell responsiveness to Th1 stimuli and a transient delay in T lymphocyte cycling. *J Immunol* 173:2419-27.

Jankovic D, Kullberg MC, Feng CG, Goldszmid RS, Collazo CM, Wilson M*, et al.* (2007) Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J Exp Med* 204:273-83.

Jankovic D, Liu Z, Gause WC (2001) Th1- and Th2-cell commitment during infectious disease: asymmetry in divergent pathways. *Trends Immunol* 22:450-7.

Jenkins SJ, Perona-Wright G, MacDonald AS (2008) Full development of Th2 immunity requires both innate and adaptive sources of CD154. *J Immunol* 180:8083-92.

Jenkins SJ, Perona-Wright G, Worsley AG, Ishii N, MacDonald AS (2007) Dendritic cell expression of OX40 ligand acts as a costimulatory, not polarizing, signal for optimal Th2 priming and memory induction in vivo. *J Immunol* 179:3515-23.

Jensen PE (2007) Recent advances in antigen processing and presentation. *Nat Immunol* 8:1041-8.

Jiang R, Lan Y, Chapman HD, Shawber C, Norton CR, Serreze DV*, et al.* (1998) Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* 12:1046-57.

Joutel A, Dodick DD, Parisi JE, Cecillon M, Tournier-Lasserve E, Bousser MG (2000) De novo mutation in the Notch3 gene causing CADASIL. *Ann Neurol* 47:388-91.

Jovanovic DV, Di Battista JA, Martel-Pelletier J, Jolicoeur FC, He Y, Zhang M*, et al.* (1998) IL-17 stimulates the production and expression of proinflammatory cytokines, IL-beta and TNF-alpha, by human macrophages. *J Immunol* 160:3513-21.

Jundt F, Schwarzer R, Dorken B (2008) Notch signaling in leukemias and lymphomas. *Curr Mol Med* 8:51-9.

Justice NJ, Jan YN (2002) Variations on the Notch pathway in neural development. *Curr Opin Neurobiol* 12:64-70.

Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F*, et al.* (2001) Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* 194:863-9.

Kaisho T, Akira S (2001) Dendritic-cell function in Toll-like receptor- and MyD88-knockout mice. *Trends Immunol* 22:78-83.

Kaisho T, Hoshino K, Iwabe T, Takeuchi O, Yasui T, Akira S (2002) Endotoxin can induce MyD88-deficient dendritic cells to support T(h)2 cell differentiation. *Int Immunol* 14:695-700.

Kaisho T, Takeuchi O, Kawai T, Hoshino K, Akira S (2001) Endotoxin-induced maturation of MyD88-deficient dendritic cells. *J Immunol* 166:5688-94.

Kaisho T, Tanaka T (2008) Turning NF-kappaB and IRFs on and off in DC. *Trends Immunol* 29:329-36.

Kalinski P, Hilkens CM, Snijders A, Snijdewint FG, Kapsenberg ML (1997) IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells. *J Immunol* 159:28-35.

Kalinski P, Hilkens CM, Wierenga EA, Kapsenberg ML (1999) T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today* 20:561-7.

Kalinski P, Smits HH, Schuitemaker JH, Vieira PL, van Eijk M, de Jong EC, *et al.* (2000) IL-4 is a mediator of IL-12p70 induction by human Th2 cells: reversal of polarized Th2 phenotype by dendritic cells. *J Immunol* 165:1877-81.

Kallies A (2008) Distinct regulation of effector and memory T-cell differentiation. *Immunol Cell Biol* 86:325-32.

Kalupahana RS, Mastroeni P, Maskell D, Blacklaws BA (2005) Activation of murine dendritic cells and macrophages induced by Salmonella enterica serovar Typhimurium. *Immunology* 115:462-72.

Kane CM, Jung E, Pearce EJ (2008) Schistosoma mansoni egg antigen-mediated modulation of Toll-like receptor (TLR)-induced activation occurs independently of TLR2, TLR4, and MyD88. *Infect Immun* 76:5754-9.

Kapsenberg ML (2003) Dendritic-cell control of pathogen-driven T-cell polarization. *Nat Rev Immunol* 3:984-93.

Kawai T, Akira S (2007) TLR signaling. *Semin Immunol* 19:24-32.

Kellermann SA, Hudak S, Oldham ER, Liu YJ, McEvoy LM (1999) The CC chemokine receptor-7 ligands 6Ckine and macrophage inflammatory protein-3 beta are potent chemoattractants for in vitro- and in vivo-derived dendritic cells. *J Immunol* 162:3859-64.

Khan N, Ghousunnissa S, Jegadeeswaran SM, Thiagarajan D, Hasnain SE, Mukhopadhyay S (2007) Anti-B7-1/B7-2 antibody elicits innate-effector responses in macrophages through NF-kappaB-dependent pathway. *Int Immunol* 19:477-86.

Kijima M, Yamaguchi T, Ishifune C, Maekawa Y, Koyanagi A, Yagita H, *et al.* (2008) Dendritic cell-mediated NK cell activation is controlled by Jagged2-Notch interaction. *Proc Natl Acad Sci U S A* 105:7010-5.

Kissenpfennig A, Henri S, Dubois B, Laplace-Builhe C, Perrin P, Romani N, *et al.* (2005) Dynamics and function of Langerhans cells in vivo: dermal dendritic cells colonize lymph node areas distinct from slower migrating Langerhans cells. *Immunity* 22:643-54.

Kloetzel PM (2004) Generation of major histocompatibility complex class I antigens: functional interplay between proteasomes and TPPII. *Nat Immunol* 5:661-9.

Knight SC, Balfour BM, O'Brien J, Buttifant L, Sumerska T, Clarke J (1982) Role of veiled cells in lymphocyte activation. *Eur J Immunol* 12:1057-60.

Kobayashi M, Fitz L, Ryan M, Hewick RM, Clark SC, Chan S, *et al.* (1989) Identification and purification of natural killer cell stimulatory factor (NKSF), a cytokine with multiple biologic effects on human lymphocytes. *J Exp Med* 170:827-45.

Koch F, Stanzl U, Jennewein P, Janke K, Heufler C, Kampgen E*, et al.* (1996) High level IL-12 production by murine dendritic cells: upregulation via MHC class II and CD40 molecules and downregulation by IL-4 and IL-10. *J Exp Med* 184:741-6.

Kopf M, Le Gros G, Bachmann M, Lamers MC, Bluethmann H, Kohler G (1993) Disruption of the murine IL-4 gene blocks Th2 cytokine responses. *Nature* 362:245-8.

Kraus J, Borner C, Hollt V (2003) Distinct palindromic extensions of the 5'-TTC...GAA-3' motif allow STAT6 binding in vivo. *Faseb J* 17:304-6.

Krebs LT, Xue Y, Norton CR, Shutter JR, Maguire M, Sundberg JP*, et al.* (2000) Notch signaling is essential for vascular morphogenesis in mice. *Genes Dev* 14:1343-52.

Krebs LT, Xue Y, Norton CR, Sundberg JP, Beatus P, Lendahl U*, et al.* (2003) Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis* 37:139-43.

Kumagai Y, Takeuchi O, Akira S (2008) Pathogen recognition by innate receptors. *J Infect Chemother* 14:86-92.

Kumano K, Chiba S, Kunisato A, Sata M, Saito T, Nakagami-Yamaguchi E*, et al.* (2003) Notch1 but not Notch2 is essential for generating hematopoietic stem cells from endothelial cells. *Immunity* 18:699-711.

Kusumi K, Sun ES, Kerrebrock AW, Bronson RT, Chi DC, Bulotsky MS*, et al.* (1998) The mouse pudgy mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. *Nat Genet* 19:274-8.

Laderach D, Compagno D, Danos O, Vainchenker W, Galy A (2003) RNA interference shows critical requirement for NF-kappa B p50 in the production of IL-12 by human dendritic cells. *J Immunol* 171:1750-7.

Ladi E, Nichols JT, Ge W, Miyamoto A, Yao C, Yang LT*, et al.* (2005) The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. *J Cell Biol* 170:983-92.

Lai EC (2004) Notch signaling: control of cell communication and cell fate. *Development* 131:965-73.

Lamb CA, Cresswell P (1992) Assembly and transport properties of invariant chain trimers and HLA-DR-invariant chain complexes. *J Immunol* 148:3478-82.

Langer JA, Cutrone EC, Kotenko S (2004) The Class II cytokine receptor (CRF2) family: overview and patterns of receptor-ligand interactions. *Cytokine Growth Factor Rev* 15:33-48.

Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD*, et al.* (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233-40.

Le Gros G, Ben-Sasson SZ, Seder R, Finkelman FD, Paul WE (1990) Generation of interleukin 4 (IL-4)-producing cells in vivo and in vitro: IL-2 and IL-4 are required for in vitro generation of IL-4-producing cells. *J Exp Med* 172:921-9.

Lee JK, Kim JK, Lee YR, Kim HS, Im SA, Kim K*, et al.* (2005) Exposure to chemokines during maturation modulates antigen presenting cell function of mature macrophages. *Cell Immunol* 234:1-8.

Lehman R, Jimenez F, Dietrich U, Campos-Ortega JA (1983) On the phenotype and development of mutants of early neurogenesis in Drosophila melanogaster. *Roux's Arch Dev Biol* 192:62-74.

Lemaitre B, Nicolas E, Michaut L, Reichhart JM, Hoffmann JA (1996) The dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* 86:973-83.

Li L, Krantz ID, Deng Y, Genin A, Banta AB, Collins CC*, et al.* (1997) Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. *Nat Genet* 16:243-51.

Li L, Milner LA, Deng Y, Iwata M, Banta A, Graf L*, et al.* (1998) The human homolog of rat Jagged1 expressed by marrow stroma inhibits differentiation of 32D cells through interaction with Notch1. *Immunity* 8:43-55.

Lighvani AA, Frucht DM, Jankovic D, Yamane H, Aliberti J, Hissong BD*, et al.* (2001) T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells. *Proc Natl Acad Sci U S A* 98:15137-42.

Lindsell CE, Boulter J, diSibio G, Gossler A, Weinmaster G (1996) Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol Cell Neurosci* 8:14-27.

Liu G, Ng H, Akasaki Y, Yuan X, Ehtesham M, Yin D*, et al.* (2004) Small interference RNA modulation of IL-10 in human monocyte-derived dendritic cells enhances the Th1 response. *Eur J Immunol* 34:1680-7.

Liu YJ (2001) Dendritic cell subsets and lineages, and their functions in innate and adaptive immunity. *Cell* 106:259-62.

Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, Malefyt Rde W*, et al.* (2007) TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. *Annu Rev Immunol* 25:193-219.

Loke P, Gallagher I, Nair MG, Zang X, Brombacher F, Mohrs M*, et al.* (2007) Alternative activation is an innate response to injury that requires CD4+ T cells to be sustained during chronic infection. *J Immunol* 179:3926-36.

Loke P, Nair MG, Parkinson J, Guiliano D, Blaxter M, Allen JE (2002) IL-4 dependent alternatively-activated macrophages have a distinctive in vivo gene expression phenotype. *BMC Immunol* 3:7.

Lubetzki C, Stankoff B (2000) [Role of axonal signals in myelination of the central nervous system]. *Pathol Biol (Paris)* 48:63-9.

Lutz MB, Kukutsch N, Ogilvie AL, Rossner S, Koch F, Romani N*, et al.* (1999) An advanced culture method for generating large quantities of highly pure dendritic cells from mouse bone marrow. *J Immunol Methods* 223:77-92.

Lyons AB, Parish CR (1994) Determination of lymphocyte division by flow cytometry. *J Immunol Methods* 171:131-7.

MacDonald AS, Araujo MI, Pearce EJ (2002a) Immunology of parasitic helminth infections. *Infect Immun* 70:427-33.

MacDonald AS, Maizels RM (2008) Alarming dendritic cells for Th2 induction. *J Exp Med* 205:13-7.

MacDonald AS, Pearce EJ (2002) Cutting edge: polarized Th cell response induction by transferred antigen-pulsed dendritic cells is dependent on IL-4 or IL-12 production by recipient cells. *J Immunol* 168:3127-30.

MacDonald AS, Straw AD, Bauman B, Pearce EJ (2001) CD8- dendritic cell activation status plays an integral role in influencing Th2 response development. *J Immunol* 167:1982-8.

MacDonald AS, Straw AD, Dalton NM, Pearce EJ (2002b) Cutting edge: Th2 response induction by dendritic cells: a role for CD40. *J Immunol* 168:537-40.

Maekawa Y, Minato Y, Ishifune C, Kurihara T, Kitamura A, Kojima H*, et al.* (2008) Notch2 integrates signaling by the transcription factors RBP-J and CREB1 to promote T cell cytotoxicity. *Nat Immunol*.

Maekawa Y, Tsukumo S, Chiba S, Hirai H, Hayashi Y, Okada H*, et al.* (2003) Delta1-Notch3 interactions bias the functional differentiation of activated CD4+ T cells. *Immunity* 19:549-59.

Maillard I, Adler SH, Pear WS (2003) Notch and the immune system. *Immunity* 19:781-91.

Maillard I, Fang T, Pear WS (2005) Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu Rev Immunol* 23:945-74.

Maillard I, Weng AP, Carpenter AC, Rodriguez CG, Sai H, Xu L*, et al.* (2004) Mastermind critically regulates Notch-mediated lymphoid cell fate decisions. *Blood* 104:1696-702.

Maldonado-Lopez R, De Smedt T, Pajak B, Heirman C, Thielemans K, Leo O*, et al.* (1999) Role of CD8alpha+ and CD8alpha- dendritic cells in the induction of primary immune responses in vivo. *J Leukoc Biol* 66:242-6.

Manz MG, Traver D, Miyamoto T, Weissman IL, Akashi K (2001) Dendritic cell potentials of early lymphoid and myeloid progenitors. *Blood* 97:3333-41.

Marks MS, Blum JS, Cresswell P (1990) Invariant chain trimers are sequestered in the rough endoplasmic reticulum in the absence of association with HLA class II antigens. *J Cell Biol* 111:839-55.

Martinez-Pomares L, Gordon S (2007) Antigen presentation the macrophage way. *Cell* 131:641-3.

Mastronardi FG, Moscarello MA (2005) Molecules affecting myelin stability: a novel hypothesis regarding the pathogenesis of multiple sclerosis. *J Neurosci Res* 80:301-8.

Matsui K, Yoshimoto T, Tsutsui H, Hyodo Y, Hayashi N, Hiroishi K*, et al.* (1997) Propionibacterium acnes treatment diminishes CD4+ NK1.1+ T cells but induces type I T cells in the liver by induction of IL-12 and IL-18 production from Kupffer cells. *J Immunol* 159:97-106.

Matsuno K, Diederich RJ, Go MJ, Blaumueller CM, Artavanis-Tsakonas S (1995) Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* 121:2633-44.

Matsuno K, Eastman D, Mitsiades T, Quinn AM, Carcanciu ML, Ordentlich P*, et al.* (1998) Human deltex is a conserved regulator of Notch signalling. *Nat Genet* 19:74-8.

Matzinger P (1998) An innate sense of danger. *Semin Immunol* 10:399-415.

Mauro VP, Edelman GM (1997) rRNA-like sequences occur in diverse primary transcripts: implications for the control of gene expression. *Proc Natl Acad Sci U S A* 94:422-7.

McManus MT, Haines BB, Dillon CP, Whitehurst CE, van Parijs L, Chen J*, et al.* (2002) Small interfering RNA-mediated gene silencing in T lymphocytes. *J Immunol* 169:5754-60.

Medzhitov R (2001) Toll-like receptors and innate immunity. *Nat Rev Immunol* 1:135-45.

Medzhitov R, Janeway CA, Jr. (1997) Innate immunity: impact on the adaptive immune response. *Curr Opin Immunol* 9:4-9.

Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. (1997) A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388:394-7.

Medzhitov R, Preston-Hurlburt P, Kopp E, Stadlen A, Chen C, Ghosh S*, et al.* (1998) MyD88 is an adaptor protein in the hToll/IL-1 receptor family signaling pathways. *Mol Cell* 2:253-8.

Mekori YA, Metcalfe DD (1999) Mast cell-T cell interactions. *J Allergy Clin Immunol* 104:517-23.

Mellman I, Steinman RM (2001) Dendritic cells: specialized and regulated antigen processing machines. *Cell* 106:255-8.

Mellman I, Turley SJ, Steinman RM (1998) Antigen processing for amateurs and professionals. *Trends Cell Biol* 8:231-7.

Meyer S, van Liempt E, Imberty A, van Kooyk Y, Geyer H, Geyer R*, et al.* (2005) DC-SIGN mediates binding of dendritic cells to authentic pseudo-LewisY glycolipids of Schistosoma mansoni cercariae, the first parasite-specific ligand of DC-SIGN. *J Biol Chem* 280:37349-59.

Micchelli CA, Rulifson EJ, Blair SS (1997) The function and regulation of cut expression on the wing margin of Drosophila: Notch, Wingless and a dominant negative role for Delta and Serrate. *Development* 124:1485-95.

Miele L (2006) Notch signaling. *Clin Cancer Res* 12:1074-9.

Miele L, Osborne B (1999) Arbiter of differentiation and death: Notch signaling meets apoptosis. *J Cell Physiol* 181:393-409.

Mills KH (2008) Induction, function and regulation of IL-17-producing T cells. *Eur J Immunol* 38:2636-49.

Milner LA, Bigas A (1999) Notch as a mediator of cell fate determination in hematopoiesis: evidence and speculation. *Blood* 93:2431-48.

Milner LA, Bigas A, Kopan R, Brashem-Stein C, Bernstein ID, Martin DI (1996) Inhibition of granulocytic differentiation by mNotch1. *Proc Natl Acad Sci U S A* 93:13014-9.

Minter LM, Turley DM, Das P, Shin HM, Joshi I, Lawlor RG*, et al.* (2005) Inhibitors of gamma-secretase block in vivo and in vitro T helper type 1 polarization by preventing Notch upregulation of Tbx21. *Nat Immunol* 6:680-8.

Mohr OL (1919) Character Changes Caused by Mutation of an Entire Region of a Chromosome in Drosophila. *Genetics* 4:275-82.

Moloney DJ, Panin VM, Johnston SH, Chen J, Shao L, Wilson R*, et al.* (2000) Fringe is a glycosyltransferase that modifies Notch. *Nature* 406:369-75.

Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19:683-765.

Morgan TH (1917) The Theory of the Gene. *Am Nat* 51:513.

Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL (1986) Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol* 136:2348-57.

Murata K, Ishii N, Takano H, Miura S, Ndhlovu LC, Nose M*, et al.* (2000) Impairment of antigen-presenting cell function in mice lacking expression of OX40 ligand. *J Exp Med* 191:365-74.

Nair MG, Cochrane DW, Allen JE (2003) Macrophages in chronic type 2 inflammation have a novel phenotype characterized by the abundant expression of Ym1 and Fizz1 that can be partly replicated in vitro. *Immunol Lett* 85:173-80.

Nair MG, Gallagher IJ, Taylor MD, Loke P, Coulson PS, Wilson RA*, et al.* (2005) Chitinase and Fizz family members are a generalized feature of nematode infection with selective upregulation of Ym1 and Fizz1 by antigen-presenting cells. *Infect Immun* 73:385-94.

Nam DK, Lee S, Zhou G, Cao X, Wang C, Clark T*, et al.* (2002) Oligo(dT) primer generates a high frequency of truncated cDNAs through internal poly(A) priming during reverse transcription. *Proc Natl Acad Sci U S A* 99:6152-6.

Napoli C, Lemieux C, Jorgensen R (1990) Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* 2:279-89.

Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A (2005) Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 6:769-76.

Nussenzweig MC, Steinman RM, Gutchinov B, Cohn ZA (1980) Dendritic cells are accessory cells for the development of anti-trinitrophenyl cytotoxic T lymphocytes. *J Exp Med* 152:1070-84.

O'Garra A, Trinchieri G (2004) Are dendritic cells afraid of commitment? *Nat Immunol* 5:1206-8.

O'Sullivan BJ, Thomas R (2002) CD40 ligation conditions dendritic cell antigen-presenting function through sustained activation of NF-kappaB. *J Immunol* 168:5491-8.

Oda T, Elkahloun AG, Pike BL, Okajima K, Krantz ID, Genin A*, et al.* (1997) Mutations in the human Jagged1 gene are responsible for Alagille syndrome. *Nat Genet* 16:235-42.

Ohishi K, Varnum-Finney B, Flowers D, Anasetti C, Myerson D, Bernstein ID (2000) Monocytes express high amounts of Notch and undergo cytokine specific apoptosis following interaction with the Notch ligand, Delta-1. *Blood* 95:2847-54.

Ohishi K, Varnum-Finney B, Serda RE, Anasetti C, Bernstein ID (2001) The Notch ligand, Delta-1, inhibits the differentiation of monocytes into macrophages but permits their differentiation into dendritic cells. *Blood* 98:1402-7.

Okano M, Satoskar AR, Nishizaki K, Abe M, Harn DA, Jr. (1999) Induction of Th2 responses and IgE is largely due to carbohydrates functioning as adjuvants on Schistosoma mansoni egg antigens. *J Immunol* 163:6712-7.

Okano M, Satoskar AR, Nishizaki K, Harn DA, Jr. (2001) Lacto-N-fucopentaose III found on Schistosoma mansoni egg antigens functions as adjuvant for proteins by inducing Th2-type response. *J Immunol* 167:442-50.

Olivier A, Lauret E, Gonin P, Galy A (2005) The Notch ligand delta-1 is a hematopoietic development co- factor for plasmacytoid dendritic cells. *Blood*.

Ong CT, Sedy JR, Murphy KM, Kopan R (2008) Notch and presenilin regulate cellular expansion and cytokine secretion but cannot instruct Th1/Th2 fate acquisition. *PLoS ONE* 3:e2823.

Opherk C, During M, Peters N, Karpinska A, Rosner S, Schneider E*, et al.* (2009) CADASIL mutations enhance spontaneous multimerization of NOTCH3. *Hum Mol Genet*.

Opherk C, Peters N, Herzog J, Luedtke R, Dichgans M (2004) Long-term prognosis and causes of death in CADASIL: a retrospective study in 411 patients. *Brain* 127:2533-9.

Ordentlich P, Lin A, Shen CP, Blaumueller C, Matsuno K, Artavanis-Tsakonas S*, et al.* (1998) Notch inhibition of E47 supports the existence of a novel signaling pathway. *Mol Cell Biol* 18:2230-9.

Osborne BA, Minter LM (2007) Notch signalling during peripheral T-cell activation and differentiation. *Nat Rev Immunol* 7:64-75.

Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T (2003) TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat Immunol* 4:161-7.

Ouyang W, Kolls JK, Zheng Y (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* 28:454-67.

Palaga T, Miele L, Golde TE, Osborne BA (2003) TCR-mediated Notch signaling regulates proliferation and IFN-gamma production in peripheral T cells. *J Immunol* 171:3019-24.

Parker DC (1993) T cell-dependent B cell activation. *Annu Rev Immunol* 11:331-60.

Pasare C, Medzhitov R (2003) Toll-like receptors: balancing host resistance with immune tolerance. *Curr Opin Immunol* 15:677-82.

Pasare C, Medzhitov R (2004a) Toll-like receptors and acquired immunity. *Semin Immunol* 16:23-6.

Pasare C, Medzhitov R (2004b) Toll-like receptors: linking innate and adaptive immunity. *Microbes Infect* 6:1382-7.

Peaper DR, Cresswell P (2008) Regulation of MHC class I assembly and peptide binding. *Annu Rev Cell Dev Biol* 24:343-68.

Pearce EJ (2005) Priming of the immune response by schistosome eggs. *Parasite Immunol* 27:265-70.

Pearce EJ, Caspar P, Grzych JM, Lewis FA, Sher A (1991) Downregulation of Th1 cytokine production accompanies induction of Th2 responses by a parasitic helminth, Schistosoma mansoni. *J Exp Med* 173:159-66.

Pearce EJ, Freitas TC (2008) Reverse genetics and the study of the immune response to schistosomes. *Parasite Immunol* 30:215-21.

Pearce EJ, MacDonald AS (2002) The immunobiology of schistosomiasis. *Nat Rev Immunol* 2:499-511.

Perona-Wright G, Jenkins SJ, MacDonald AS (2006) Dendritic cell activation and function in response to Schistosoma mansoni. *Int J Parasitol* 36:711-21.

Perrigoue JG, Marshall F, Artis D (2008) On the hunt for helminths: Innate immune cells in the recognition and response to helminth parasites. *Cell Microbiol*.

Piccirillo CA, Letterio JJ, Thornton AM, McHugh RS, Mamura M, Mizuhara H*, et al.* (2002) CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. *J Exp Med* 196:237-46.

Piccirillo CA, Shevach EM (2001) Cutting edge: control of CD8+ T cell activation by CD4+CD25+ immunoregulatory cells. *J Immunol* 167:1137-40.

Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X*, et al.* (1998) Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282:2085-8.

Poulson DF (1937) Chromosomal Deficiencies and the Embryonic Development of Drosophila Melanogaster. *Proc Natl Acad Sci U S A* 23:133-7.

Pozzi LA, Maciaszek JW, Rock KL (2005) Both dendritic cells and macrophages can stimulate naive CD8 T cells in vivo to proliferate, develop effector function, and differentiate into memory cells. *J Immunol* 175:2071-81.

Pui JC, Allman D, Xu L, DeRocco S, Karnell FG, Bakkour S*, et al.* (1999) Notch1 expression in early lymphopoiesis influences B versus T lineage determination. *Immunity* 11:299-308.

Pulendran B, Lingappa J, Kennedy MK, Smith J, Teepe M, Rudensky A*, et al.* (1997) Developmental pathways of dendritic cells in vivo: distinct function, phenotype, and localization of dendritic cell subsets in FLT3 ligand-treated mice. *J Immunol* 159:2222-31.

Pulendran B, Smith JL, Caspary G, Brasel K, Pettit D, Maraskovsky E*, et al.* (1999) Distinct dendritic cell subsets differentially regulate the class of immune response in vivo. *Proc Natl Acad Sci U S A* 96:1036-41.

Radtke F, Ferrero I, Wilson A, Lees R, Aguet M, MacDonald HR (2000) Notch1 deficiency dissociates the intrathymic development of dendritic cells and T cells. *J Exp Med* 191:1085-94.

Radtke F, Wilson A, Stark G, Bauer M, van Meerwijk J, MacDonald HR*, et al.* (1999) Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* 10:547-58.

Raes G, De Baetselier P, Noel W, Beschin A, Brombacher F, Hassanzadeh Gh G (2002a) Differential expression of FIZZ1 and Ym1 in alternatively versus classically activated macrophages. *J Leukoc Biol* 71:597-602.

Raes G, Noel W, Beschin A, Brys L, de Baetselier P, Hassanzadeh GH (2002b) FIZZ1 and Ym as tools to discriminate between differentially activated macrophages. *Dev Immunol* 9:151-9.

Ramain P, Khechumian K, Seugnet L, Arbogast N, Ackermann C, Heitzler P (2001) Novel Notch alleles reveal a Deltex-dependent pathway repressing neural fate. *Curr Biol* 11:1729-38.

Randolph GJ, Ochando J, Partida-Sanchez S (2008) Migration of dendritic cell subsets and their precursors. *Annu Rev Immunol* 26:293-316.

Reis e Sousa C (2006) Dendritic cells in a mature age. *Nat Rev Immunol*.

Reis ESC (2006) Dendritic cells in a mature age. *Nat Rev Immunol*.

Robey E (1999) Regulation of T cell fate by Notch. *Annu Rev Immunol* 17:283-95.

Robey E, Chang D, Itano A, Cado D, Alexander H, Lans D*, et al.* (1996) An activated form of Notch influences the choice between CD4 and CD8 T cell lineages. *Cell* 87:483-92.

Robey EA, Bluestone JA (2004) Notch signaling in lymphocyte development and function. *Curr Opin Immunol* 16:360-6.

Robson NC, Beacock-Sharp H, Donachie AM, Mowat AM (2003) The role of antigen-presenting cells and interleukin-12 in the priming of antigen-specific CD4+ T cells by immune stimulating complexes. *Immunology* 110:95-104.

Roca C, Adams RH (2007) Regulation of vascular morphogenesis by Notch signaling. *Genes Dev* 21:2511-24.

Rock KL, York IA, Goldberg AL (2004) Post-proteasomal antigen processing for major histocompatibility complex class I presentation. *Nat Immunol* 5:670-7.

Romagnani S (2000) T-cell subsets (Th1 versus Th2). *Ann Allergy Asthma Immunol* 85:9-18; quiz , 21.

Ropke A, Kujat A, Graber M, Giannakudis J, Hansmann I (2003) Identification of 36 novel Jagged1 (JAG1) mutations in patients with Alagille syndrome. *Hum Mutat* 21:100.

Rothenberg EV, Moore JE, Yui MA (2008) Launching the T-cell-lineage developmental programme. *Nat Rev Immunol* 8:9-21.

Rutz S, Janke M, Kassner N, Hohnstein T, Krueger M, Scheffold A (2008) Notch regulates IL-10 production by T helper 1 cells. *Proc Natl Acad Sci U S A* 105:3497-502.

Saito T, Chiba S, Ichikawa M, Kunisato A, Asai T, Shimizu K*, et al.* (2003) Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* 18:675-85.

Saito T, Gale M, Jr. (2008) Differential recognition of double-stranded RNA by RIG-I-like receptors in antiviral immunity. *J Exp Med* 205:1523-7.

Sakamoto K, Yamaguchi S, Ando R, Miyawaki A, Kabasawa Y, Takagi M*, et al.* (2002) The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. *J Biol Chem* 277:29399-405.

Sallusto F, Lanzavecchia A (1994) Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. *J Exp Med* 179:1109-18.

Sallusto F, Palermo B, Lenig D, Miettinen M, Matikainen S, Julkunen I*, et al.* (1999) Distinct patterns and kinetics of chemokine production regulate dendritic cell function. *Eur J Immunol* 29:1617-25.

Sandler NG, Mentink-Kane MM, Cheever AW, Wynn TA (2003) Global gene expression profiles during acute pathogen-induced pulmonary inflammation reveal divergent roles for Th1 and Th2 responses in tissue repair. *J Immunol* 171:3655-67.

Sastre M, Steiner H, Fuchs K, Capell A, Multhaup G, Condron MM*, et al.* (2001) Presenilin-dependent gamma-secretase processing of beta-amyloid precursor protein at a site corresponding to the S3 cleavage of Notch. *EMBO Rep* 2:835-41.

Sato K, Fujita S (2007) Dendritic cells: nature and classification. *Allergol Int* 56:183-91.

Saunders SS, Walsh CM, Barlow JL, Mangan NE, Taylor PR, McKenzie AN*, et al.* (2008) The C-type lectin SIGNR1 binds Schistosoma mansoni antigens in vitro but SIGNR1-deficient mice have normal responses during schistosome infection. *Infect Immun*.

Schmitt TM, Zuniga-Pflucker JC (2002) Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* 17:749-56.

Schmittgen TD, Zakrajsek BA (2000) Effect of experimental treatment on housekeeping gene expression: validation by real-time, quantitative RT-PCR. *J Biochem Biophys Methods* 46:69-81.

Schnare M, Barton GM, Holt AC, Takeda K, Akira S, Medzhitov R (2001) Toll-like receptors control activation of adaptive immune responses. *Nat Immunol* 2:947-50.

Schroeder T, Just U (2000a) mNotch1 signaling reduces proliferation of myeloid progenitor cells by altering cell-cycle kinetics. *Exp Hematol* 28:1206-13.

Schroeder T, Just U (2000b) Notch signalling via RBP-J promotes myeloid differentiation. *Embo J* 19:2558-68.

Schulz O, Edwards AD, Schito M, Aliberti J, Manickasingham S, Sher A*, et al.* (2000) CD40 triggering of heterodimeric IL-12 p70 production by dendritic cells in vivo requires a microbial priming signal. *Immunity* 13:453-62.

Scotet E, Nedellec S, Devilder MC, Allain S, Bonneville M (2008) Bridging innate and adaptive immunity through gammadelta T-dendritic cell crosstalk. *Front Biosci* 13:6872-85.

Segura JJ, Calvo JR, Guerrero JM, Jimenez-Planas A, Sampedro C, Llamas R (1997) EDTA inhibits in vitro substrate adherence capacity of macrophages: endodontic implications. *J Endod* 23:205-8.

Seifert U, Maranon C, Shmueli A, Desoutter JF, Wesoloski L, Janek K*, et al.* (2003) An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. *Nat Immunol* 4:375-9.

Seljelid R, Eskeland T (1993) The biology of macrophages: I. General principles and properties. *Eur J Haematol* 51:267-75.

Shahabuddin M, Vinetz JM (1999) Chitinases of human parasites and their implications as antiparasitic targets. *Exs* 87:223-34.

Shi HZ (2004) Eosinophils function as antigen-presenting cells. *J Leukoc Biol* 76:520-7.

Shortman K, Liu YJ (2002) Mouse and human dendritic cell subtypes. *Nat Rev Immunol* 2:151-61.

Skeen MJ, Ziegler HK (1995) Activation of gamma delta T cells for production of IFN-gamma is mediated by bacteria via macrophage-derived cytokines IL-1 and IL-12. *J Immunol* 154:5832-41.

Slomovic S, Laufer D, Geiger D, Schuster G (2006) Polyadenylation of ribosomal RNA in human cells. *Nucleic Acids Res* 34:2966-75.

Snijders A, Kalinski P, Hilkens CM, Kapsenberg ML (1998) High-level IL-12 production by human dendritic cells requires two signals. *Int Immunol* 10:1593-8.

Soares H, Waechter H, Glaichenhaus N, Mougneau E, Yagita H, Mizenina O*, et al.* (2007) A subset of dendritic cells induces CD4+ T cells to produce IFN-gamma by an IL-12-independent but CD70-dependent mechanism in vivo. *J Exp Med* 204:1095-106.

Stacker SA, Springer TA (1991) Leukocyte integrin P150,95 (CD11c/CD18) functions as an adhesion molecule binding to a counter-receptor on stimulated endothelium. *J Immunol* 146:648-55.

Stallwood Y, Briend E, Ray KM, Ward GA, Smith BJ, Nye E*, et al.* (2006) Small interfering RNA-mediated knockdown of notch ligands in primary CD4+ T cells and dendritic cells enhances cytokine production. *J Immunol* 177:885-95.

Steinbrink K, Wolfl M, Jonuleit H, Knop J, Enk AH (1997) Induction of tolerance by IL-10-treated dendritic cells. *J Immunol* 159:4772-80.

Steinman R, Inaba K (1989) Immunogenicity: role of dendritic cells. *Bioessays* 10:145-52.

Steinman RM (2007) Dendritic cells: understanding immunogenicity. *Eur J Immunol* 37 Suppl 1:S53-60.

Steinman RM, Cohn ZA (1973) Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137:1142-62.

Steinman RM, Hemmi H (2006) Dendritic cells: translating innate to adaptive immunity. *Curr Top Microbiol Immunol* 311:17-58.

Steinman RM, Witmer MD (1978) Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* 75:5132-6.

Stelekati E, Orinska Z, Bulfone-Paus S (2007) Mast cells in allergy: innate instructors of adaptive responses. *Immunobiology* 212:505-19.

Stockinger B, Veldhoen M, Martin B (2007) Th17 T cells: linking innate and adaptive immunity. *Semin Immunol* 19:353-61.

Stoltze L, Schirle M, Schwarz G, Schroter C, Thompson MW, Hersh LB*, et al.* (2000) Two new proteases in the MHC class I processing pathway. *Nat Immunol* 1:413-8.

Strehl B, Seifert U, Kruger E, Heink S, Kuckelkorn U, Kloetzel PM (2005) Interferon-gamma, the functional plasticity of the ubiquitin-proteasome system, and MHC class I antigen processing. *Immunol Rev* 207:19-30.

Stuber E, Neurath M, Calderhead D, Fell HP, Strober W (1995) Cross-linking of OX40 ligand, a member of the TNF/NGF cytokine family, induces proliferation and differentiation in murine splenic B cells. *Immunity* 2:507-21.

Sun J, Krawczyk CJ, Pearce EJ (2008) Suppression of th2 cell development by notch ligands delta1 and delta4. *J Immunol* 180:1655-61.

Sun X, Artavanis-Tsakonas S (1997) Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in Drosophila. *Development* 124:3439-48.

Sundquist M, Johansson C, Wick MJ (2003) Dendritic cells as inducers of antimicrobial immunity in vivo. *Apmis* 111:715-24.

Suzuki Y, Orellana MA, Schreiber RD, Remington JS (1988) Interferon-gamma: the major mediator of resistance against Toxoplasma gondii. *Science* 240:516-8.

Swain SL, Croft M, Dubey C, Haynes L, Rogers P, Zhang X*, et al.* (1996) From naive to memory T cells. *Immunol Rev* 150:143-67.

Swain SL, Weinberg AD, English M, Huston G (1990) IL-4 directs the development of Th2-like helper effectors. *J Immunol* 145:3796-806.

Swiatek PJ, Lindsell CE, del Amo FF, Weinmaster G, Gridley T (1994) Notch1 is essential for postimplantation development in mice. *Genes Dev* 8:707-19.

Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH (2000) A novel transcription factor, T-bet, directs Th1 lineage commitment. *Cell* 100:655-69.

Tacchini-Cottier F, Allenbach C, Otten LA, Radtke F (2004) Notch1 expression on T cells is not required for CD4+ T helper differentiation. *Eur J Immunol* 34:1588-96.

Tanaka S, Tsukada J, Suzuki W, Hayashi K, Tanigaki K, Tsuji M*, et al.* (2006) The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. *Immunity* 24:689-701.

Tanigaki K, Tsuji M, Yamamoto N, Han H, Tsukada J, Inoue H*, et al.* (2004) Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity* 20:611-22.

Tanioka T, Hattori A, Masuda S, Nomura Y, Nakayama H, Mizutani S*, et al.* (2003) Human leukocyte-derived arginine aminopeptidase. The third member of the oxytocinase subfamily of aminopeptidases. *J Biol Chem* 278:32275-83.

Tesar BM, Zhang J, Li Q, Goldstein DR (2004) TH1 immune responses to fully MHC mismatched allografts are diminished in the absence of MyD88, a toll-like receptor signal adaptor protein. *Am J Transplant* 4:1429-39.

Thomas PG, Carter MR, Atochina O, Da'Dara AA, Piskorska D, McGuire E*, et al.* (2003) Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. *J Immunol* 171:5837-41.

Thompson AJ, Locarnini SA (2007) Toll-like receptors, RIG-I-like RNA helicases and the antiviral innate immune response. *Immunol Cell Biol* 85:435-45.

Thornton AM, Shevach EM (2000) Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. *J Immunol* 164:183-90.

Tian L, Wu X, Chi C, Han M, Xu T, Zhuang Y (2008) ADAM10 is essential for proteolytic activation of Notch during thymocyte development. *Int Immunol* 20:1181-7.

Tijsterman M, Plasterk RH (2004) Dicers at RISC; the mechanism of RNAi. *Cell* 117:1-3.

Tu L, Fang TC, Artis D, Shestova O, Pross SE, Maillard I*, et al.* (2005) Notch signaling is an important regulator of type 2 immunity. *J Exp Med* 202:1037-42.

Underhill DM, Ozinsky A (2002) Phagocytosis of microbes: complexity in action. *Annu Rev Immunol* 20:825-52.

Vakkila J, Lotze MT, Riga C, Jaffe R (2005) A basis for distinguishing cultured dendritic cells and macrophages in cytospins and fixed sections. *Pediatr Dev Pathol* 8:43-51.

Valenzuela J, Schmidt C, Mescher M (2002) The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells. *J Immunol* 169:6842-9.

Valladeau J, Duvert-Frances V, Pin JJ, Dezutter-Dambuyant C, Vincent C, Massacrier C*, et al.* (1999) The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface. *Eur J Immunol* 29:2695-704.

van den Berg TK, Kraal G (2005) A function for the macrophage F4/80 molecule in tolerance induction. *Trends Immunol* 26:506-9.

van der Kleij D, Latz E, Brouwers JF, Kruize YC, Schmitz M, Kurt-Jones EA*, et al.* (2002) A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine activates toll-like receptor 2 and affects immune polarization. *J Biol Chem* 277:48122-9.

van der Krol AR, Mur LA, Beld M, Mol JN, Stuitje AR (1990) Flavonoid genes in petunia: addition of a limited number of gene copies may lead to a suppression of gene expression. *Plant Cell* 2:291-9.

van der Vliet HJ, Nishi N, Koezuka Y, von Blomberg BM, van den Eertwegh AJ, Porcelli SA*, et al.* (2001) Potent expansion of human natural killer T cells using alpha-galactosylceramide (KRN7000)-loaded monocyte-derived dendritic cells, cultured in the presence of IL-7 and IL-15. *J Immunol Methods* 247:61-72.

van Kooten C, Banchereau J (2000) CD40-CD40 ligand. *J Leukoc Biol* 67:2-17.

van Liempt E, van Vliet SJ, Engering A, Garcia Vallejo JJ, Bank CM, Sanchez-Hernandez M*, et al.* (2007) Schistosoma mansoni soluble egg antigens are internalized by human dendritic cells through multiple C-type lectins and suppress TLR-induced dendritic cell activation. *Mol Immunol* 44:2605-15.

van Niel G, Wubbolts R, Stoorvogel W (2008) Endosomal sorting of MHC class II determines antigen presentation by dendritic cells. *Curr Opin Cell Biol* 20:437-44.

van Vliet SJ, den Dunnen J, Gringhuis SI, Geijtenbeek TB, van Kooyk Y (2007) Innate signaling and regulation of Dendritic cell immunity. *Curr Opin Immunol* 19:435-40.

Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B (2006) TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 24:179-89.

Veldhoen M, Uyttenhove C, van Snick J, Helmby H, Westendorf A, Buer J*, et al.* (2008) Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol* 9:1341-6.

Vella AT, Scherer MT, Schultz L, Kappler JW, Marrack P (1996) B cells are not essential for peripheral T-cell tolerance. *Proc Natl Acad Sci U S A* 93:951-5.

Verma IM (1978) Genome organization of RNA tumor viruses. I. In vitro synthesis of full-genome-length single-stranded and double-stranded viral DNA transcripts. *J Virol* 26:615-29.

Vermaelen K, Pauwels R (2004) Accurate and simple discrimination of mouse pulmonary dendritic cell and macrophage populations by flow cytometry: methodology and new insights. *Cytometry A* 61:170-77.

Vignali DA, Collison LW, Workman CJ (2008) How regulatory T cells work. *Nat Rev Immunol* 8:523-32.

Vigouroux S, Yvon E, Wagner HJ, Biagi E, Dotti G, Sili U*, et al.* (2003) Induction of antigen-specific regulatory T cells following overexpression of a Notch ligand by human B lymphocytes. *J Virol* 77:10872-80.

Visan I, Tan JB, Yuan JS, Harper JA, Koch U, Guidos CJ (2006) Regulation of T lymphopoiesis by Notch1 and Lunatic fringe-mediated competition for intrathymic niches. *Nat Immunol* 7:634-43.

Vyas JM, Kim YM, Artavanis-Tsakonas K, Love JC, Van der Veen AG, Ploegh HL (2007) Tubulation of class II MHC compartments is microtubule dependent and involves multiple endolysosomal membrane proteins in primary dendritic cells. *J Immunol* 178:7199-210.

Vyas JM, Van der Veen AG, Ploegh HL (2008) The known unknowns of antigen processing and presentation. *Nat Rev Immunol* 8:607-18.

Wajant H, Pfizenmaier K, Scheurich P (2003) Tumor necrosis factor signaling. *Cell Death Differ* 10:45-65.

Wang S, Chen L (2004) T lymphocyte co-signaling pathways of the B7-CD28 family. *Cell Mol Immunol* 1:37-42.

Wang YH, Angkasekwinai P, Lu N, Voo KS, Arima K, Hanabuchi S*, et al.* (2007) IL-25 augments type 2 immune responses by enhancing the expansion and functions of TSLP-DC-activated Th2 memory cells. *J Exp Med* 204:1837-47.

Washburn T, Schweighoffer E, Gridley T, Chang D, Fowlkes BJ, Cado D*, et al.* (1997) Notch activity influences the alphabeta versus gammadelta T cell lineage decision. *Cell* 88:833-43.

Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM (2006) Th17: an effector CD4 T cell lineage with regulatory T cell ties. *Immunity* 24:677-88.

Weber SE, Harbertson J, Godebu E, Mros GA, Padrick RC, Carson BD*, et al.* (2006) Adaptive islet-specific regulatory CD4 T cells control autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo. *J Immunol* 176:4730-9.

Weijzen S, Velders MP, Elmishad AG, Bacon PE, Panella JR, Nickoloff BJ*, et al.* (2002) The Notch ligand Jagged-1 is able to induce maturation of monocyte-derived human dendritic cells. *J Immunol* 169:4273-8.

Weinmaster G, Roberts VJ, Lemke G (1991) A homolog of Drosophila Notch expressed during mammalian development. *Development* 113:199-205.

Weinmaster G, Roberts VJ, Lemke G (1992) Notch2: a second mammalian Notch gene. *Development* 116:931-41.

Weiss GB, Wilson GN, Steggles AW, Anderson WF (1976) Importance of full size complementary DNA in nucleic acid hybridization. *J Biol Chem* 251:3425-31.

Welch JS, Escoubet-Lozach L, Sykes DB, Liddiard K, Greaves DR, Glass CK (2002) TH2 cytokines and allergic challenge induce Ym1 expression in macrophages by a STAT6-dependent mechanism. *J Biol Chem* 277:42821-9.

Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C*, et al.* (2004) Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science* 306:269-71.

Weng AP, Nam Y, Wolfe MS, Pear WS, Griffin JD, Blacklow SC*, et al.* (2003) Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol Cell Biol* 23:655-64.

Wiethe C, Debus A, Mohrs M, Steinkasserer A, Lutz M, Gessner A (2008) Dendritic cell differentiation state and their interaction with NKT cells determine Th1/Th2 differentiation in the murine model of Leishmania major infection. *J Immunol* 180:4371-81.

Wilson HL, O'Neill HC (2003) Murine dendritic cell development: difficulties associated with subset analysis. *Immunol Cell Biol* 81:239-46.

Wolfe MS (2001) gamma-Secretase inhibitors as molecular probes of presenilin function. *J Mol Neurosci* 17:199-204.

Wolk K, Kunz S, Asadullah K, Sabat R (2002) Cutting edge: immune cells as sources and targets of the IL-10 family members? *J Immunol* 168:5397-402.

Wolowczuk I, Roye O, Nutten S, Delacre M, Trottein F, Auriault C (1999) Role of interleukin-7 in the relation between Schistosoma mansoni and its definitive vertebrate host. *Microbes Infect* 1:545-51.

Wong KK, Carpenter MJ, Young LL, Walker SJ, McKenzie G, Rust AJ*, et al.* (2003) Notch ligation by Delta1 inhibits peripheral immune responses to transplantation antigens by a CD8+ cell-dependent mechanism. *J Clin Invest* 112:1741-50.

Wu G, Lyapina S, Das I, Li J, Gurney M, Pauley A*, et al.* (2001a) SEL-10 is an inhibitor of notch signaling that targets notch for ubiquitin-mediated protein degradation. *Mol Cell Biol* 21:7403-15.

Wu L, D'Amico A, Hochrein H, O'Keeffe M, Shortman K, Lucas K (2001b) Development of thymic and splenic dendritic cell populations from different hemopoietic precursors. *Blood* 98:3376-82.

Wynn TA (2003) IL-13 effector functions. *Annu Rev Immunol* 21:425-56.

Wynn TA, Thompson RW, Cheever AW, Mentink-Kane MM (2004) Immunopathogenesis of schistosomiasis. *Immunol Rev* 201:156-67.

Xue Y, Gao X, Lindsell CE, Norton CR, Chang B, Hicks C*, et al.* (1999) Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* 8:723-30.

Yamaguchi E, Chiba S, Kumano K, Kunisato A, Takahashi T, Hirai H (2002) Expression of Notch ligands, Jagged1, 2 and Delta1 in antigen presenting cells in mice. *Immunol Lett* 81:59-64.

Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H*, et al.* (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301:640-3.

Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T*, et al.* (2002) Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. *Nature* 420:324-9.

Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS*, et al.* (2007) STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem* 282:9358-63.

Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y*, et al.* (2008) T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR alpha and ROR gamma. *Immunity* 28:29-39.

Yao Y, Li W, Kaplan MH, Chang CH (2005) Interleukin (IL)-4 inhibits IL-10 to promote IL-12 production by dendritic cells. *J Exp Med* 201:1899-903.

Ye P, Garvey PB, Zhang P, Nelson S, Bagby G, Summer WR*, et al.* (2001a) Interleukin-17 and lung host defense against Klebsiella pneumoniae infection. *Am J Respir Cell Mol Biol* 25:335-40.

Ye P, Rodriguez FH, Kanaly S, Stocking KL, Schurr J, Schwarzenberger P*, et al.* (2001b) Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. *J Exp Med* 194:519-27.

York IA, Goldberg AL, Mo XY, Rock KL (1999) Proteolysis and class I major histocompatibility complex antigen presentation. *Immunol Rev* 172:49-66.

Yrlid U, Svensson M, Johansson C, Wick MJ (2000) Salmonella infection of bone marrow-derived macrophages and dendritic cells: influence on antigen presentation and initiating an immune response. *FEMS Immunol Med Microbiol* 27:313-20.

Yuan ZR, Kobayashi N, Kohsaka T (2006) Human Jagged 1 mutants cause liver defect in Alagille syndrome by overexpression of hepatocyte growth factor. *J Mol Biol* 356:559-68.

Yvon ES, Vigouroux S, Rousseau RF, Biagi E, Amrolia P, Dotti G*, et al.* (2003) Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells. *Blood* 102:3815-21.

Zaph C, Troy AE, Taylor BC, Berman-Booty LD, Guild KJ, Du Y*, et al.* (2007) Epithelial-cell-intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* 446:552-6.

Zheng SG, Wang JH, Gray JD, Soucier H, Horwitz DA (2004) Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. *J Immunol* 172:5213-21.

Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB*, et al.* (2005) Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol* 6:1047-53.

Zhu C, Xu H, Zhang G, Lu C, Ji M, Wu W (2008) Myeloid differentiation factor 88-silenced bone marrow-derived dendritic cells exhibit enhanced tolerogenicity in intestinal transplantation in rats. *Transplant Proc* 40:1625-8.

Zhu J, Paul WE (2008) CD4 T cells: fates, functions, and faults. *Blood* 112:1557-69.

Zhu Z, Zheng T, Homer RJ, Kim YK, Chen NY, Cohn L*, et al.* (2004) Acidic mammalian chitinase in asthmatic Th2 inflammation and IL-13 pathway activation. *Science* 304:1678-82.

Ziegler SF, Liu YJ (2006) Thymic stromal lymphopoietin in normal and pathogenic T cell development and function. *Nat Immunol* 7:709-14.

Appendix 1: Dendritic cell expression of the Notch ligand jagged2 is not essential for Th2 response induction *in vivo*

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Manuscript published in *European Journal of Immunology* (2008)

European Journal of Immunology

SHORT COMMUNICATION

Dendritic cell expression of the Notch ligand *jagged2* is not essential for Th2 response induction *in vivo*

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We have addressed the hypothesis that Notch ligands play a decisive role in determining the ability of antigen-presenting cells to influence T cell polarization. Dendritic cells displayed distinct expression profiles of Delta and Jagged ligands for Notch when exposed to biologically relevant pathogen preparations associated with Th1 or Th2 responses. Expression of *delta4* was increased, and *jagged2* decreased, after dendritic cell exposure to the Th1-promoting bacterium *Propionibacterium acnes*. In contrast, soluble egg antigen (SEA) from the parasitic helminth *Schistosoma mansoni*, a potent Th2 inducer, failed to significantly alter dendritic cell expression of any of the Notch ligands measured. Irrespective of this, *jagged2*-deficient dendritic cells were severely impaired in their ability to instruct Th2 polarization of naive T cells *in vitro*. However, the ability of SEA-pulsed *jagged2*-deficient dendritic cells to induce a Th2 response *in vivo* was unimpaired relative to *jagged2*-sufficient dendritic cells. Further, *jagged2*-deficient dendritic cells activated by *P. acnes* exhibited no evidence of enhanced (or impaired) Th1 induction *in vivo*. These data suggest that, although involved in Th2 direction *in vitro*, *jagged2* is not fundamentally required for Th2 induction by SEA-activated dendritic cells *in vivo*.

Key words: Cell differentiation · Dendritic cells · Infectious diseases · Notch signalling · T helper cells

Introduction

Conserved throughout the metazoan kingdom, the Notch signalling pathway is remarkable for its extensive versatility, being utilized for exchanging amplification signals, determining cell lineages, and even inducing apoptosis [1, 2]. It has recently been suggested that a possible role for Notch signalling may be to provide a mechanism by which APC can influence T cell polarization [3–7], a situation that is complicated by the existence of four Notch receptors (Notch receptors 1–4) and five Notch ligands (Delta-like1, Delta-like3 and Delta-like4, and Jagged1 and Jagged2) in mammals [1].

Several lines of evidence suggest that signalling through Notch receptors has an effect on both T cell proliferation and mature T cell commitment [3, 4, 6, 8, 9]. In the context of $CD4^+$ T cell polarization, a recent *in vivo* study showed that blocking the signalling ability of Notch receptors 1–4 resulted in impaired Th2, but not Th1, responses [10]. In addition, studies *in vitro* have suggested that both Delta and Jagged ligand families may be associated with T cell differentiation but that Delta ligands promote Th1 whereas Jagged ligands promote Th2 polarization [4, 6, 11]. However, this contention remains controversial, with other reports suggesting that no such association exists, that Delta

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can inhibit T cell cytokine production rather than promote Th1 differentiation [5, 12], or that Jagged may induce Treg, rather than Th2, differentiation [5, 8, 9, 13–15]. Thus, although evidence is emerging that Notch has a function in immunity beyond its developmental role, the exact nature of Notch signalling and the relative contribution of the two ligand families in the adaptive immune response is far from clear [6, 16–18].

Dendritic cells (DC) are the most proficient APC at activating naive T cells. Their ability to dictate the tone of the developing $CD4^+$ T cell response is thought to be largely determined by the nature of the stimulus they encounter. However, the molecular mechanisms that are employed by DC to influence and instruct T cell polarization are not completely understood, particularly in the case of Th2 development [19].

In this study we first assessed whether the profile of expression of Notch ligands by DC was altered in response to Th1- or Th2-polarizing pathogens. We then used both *in vitro* and *in vivo* approaches to examine the direct effect of a specific Notch ligand deficiency in DC on polarization of transgenic and non-transgenic, naturally occurring polyclonal populations of T cells. We found that DC expression of the Notch ligand *jagged2* was unchanged in response to the Th2-associated pathogen *Schistosoma mansoni* and down-regulated in response to the Th1-associated pathogen *Propionibacterium acnes*, and that *jagged2*$^{-/-}$ DC were severely impaired in their ability to direct Th2 polarization of OVA-specific TCR-transgenic T cells *in vitro*. However, the ability of *jagged2*$^{-/-}$ DC to induce either Th2 or Th1 responses *in vivo* was unaffected. Thus, our results support an association of defined patterns of Notch ligand expression by DC responding to different T cell-polarizing pathogens, but suggest a redundant role for *jagged2* in T cell polarization by DC *in vivo*.

Results and discussion

DC express a defined pattern of expression of Notch ligands in response to diverse pathogens

Previous work has shown that stimulation with the Th1-associated bacterial product LPS resulted in substantial up-regulation of Delta by DC, while Th2-associated molecules such as prostaglandin E_2 and cholera toxin promoted Jagged expression [6]. We used two pathogen preparations that are well-characterized as being able to drive either Th1 (heat-killed *P. acnes*, Pa) or Th2 (soluble egg antigen from *S. mansoni*, SEA) induction *via* DC [20, 21] to determine whether we could see any clear pattern of Notch ligand expression associated with either type of stimulus. Over a 12-h time course, DC were activated by these stimuli in a manner consistent with previously published reports [20, 21]. Pa induced DC maturation, provoking up-regulation of MHC class II and co-stimulatory molecules (data not shown), as well as secretion of a range of cytokines including IL-12, IL-6, TNF and IL-10 (Fig. 1A). In contrast, and in keeping with previously published reports [22], SEA-activated DC showed little evidence of phenotypic maturation (data not shown) or cytokine secretion (Fig. 1A) when compared to unstimulated cells.

The influence of the same stimuli on Notch ligand expression by DC was determined using quantitative PCR to measure mRNA levels 6 and 12 h after stimulation (Fig. 1B–D). *delta4* expression was significantly increased over unstimulated control levels in DC activated with Pa ($p<0.03$; Fig. 1B), and this was evident from 6 h post-stimulation. In contrast to this, although showing a trend for elevated expression by 6 h that returned to control levels by 12 h post-stimulation, *delta1* was not significantly increased in response to Pa. Although expression of *jagged1* was unchanged in DC responding to Pa, *jagged2* expression showed a different profile, being strikingly decreased by 6 h post-stimulation ($p<0.001$; Fig. 1B), and returning to a similar level to unstimulated cells by 12 h.

Contrary to the dramatic up-regulation that was evident in response to Pa, expression of *delta4* was not significantly altered in DC exposed to SEA (Fig. 1B). Further, *delta1*, *jagged1*, and *jagged2* were maintained at equivalent levels to unstimulated controls in SEA-activated DC at both 6 and 12 h time points. *delta3* expression in response to either Pa or SEA failed to show a consistent pattern in any of the experiments carried out (data not shown). Although SEA failed to have a marked impact on Notch ligand expression, exposure to SEA resulted in DC that express higher levels of *jagged2* relative to *delta4*, whereas exposure to Pa resulted in DC with the converse phenotype, expressing higher levels of *delta4* relative to *jagged2* (Fig. 1B).

These data suggest that DC expression of a restricted cohort of Notch ligands can be associated with pathogens that induce distinct Th responses. However, they also reveal that the initial description of Jagged and Delta ligands as being Th2- or Th1-associated, respectively, is an oversimplification, and that expression of related members within the same ligand family does not appear to be regulated identically.

Th2 and Th1 responses are capably induced *in vivo* by *jagged2*$^{-/-}$ DC

jagged2 deficiency is embryonic lethal [23]. In order to address the role of expression of *jagged2* in BMDC development and Th induction, *jagged2*$^{-/-}$ chimeras were generated by reconstituting irradiated congenic $Ly5.1^+$ C57BL/6 mice with fetal liver from $Ly5.2^+$ *jagged2*$^{-/-}$ (or *jagged2*$^{+/+}$ control) embryos. DC were then grown from BM isolated from *jagged2*$^{-/-}$ or control chimeras, pulsed overnight with either Pa or SEA, and their activation status compared. The absence of *jagged2* did not significantly affect growth, development or activation of the DC *in vitro* (Fig. 2). Secretion of cytokines (Fig. 2A) and expression of co-stimulatory molecules (Fig. 2B) was similar for both control and *jagged2*$^{-/-}$ DC in response to SEA or Pa. Further, *jagged2*$^{-/-}$ DC did not compensate for *jagged2* deficiency by up-regulating expression of *jagged1*, *delta1*, or *delta4* in response to either stimulus (Fig. 2C). Thus, *jagged2*$^{-/-}$ DC appeared similar to their wild-type counterparts other than lacking *jagged2* expression.

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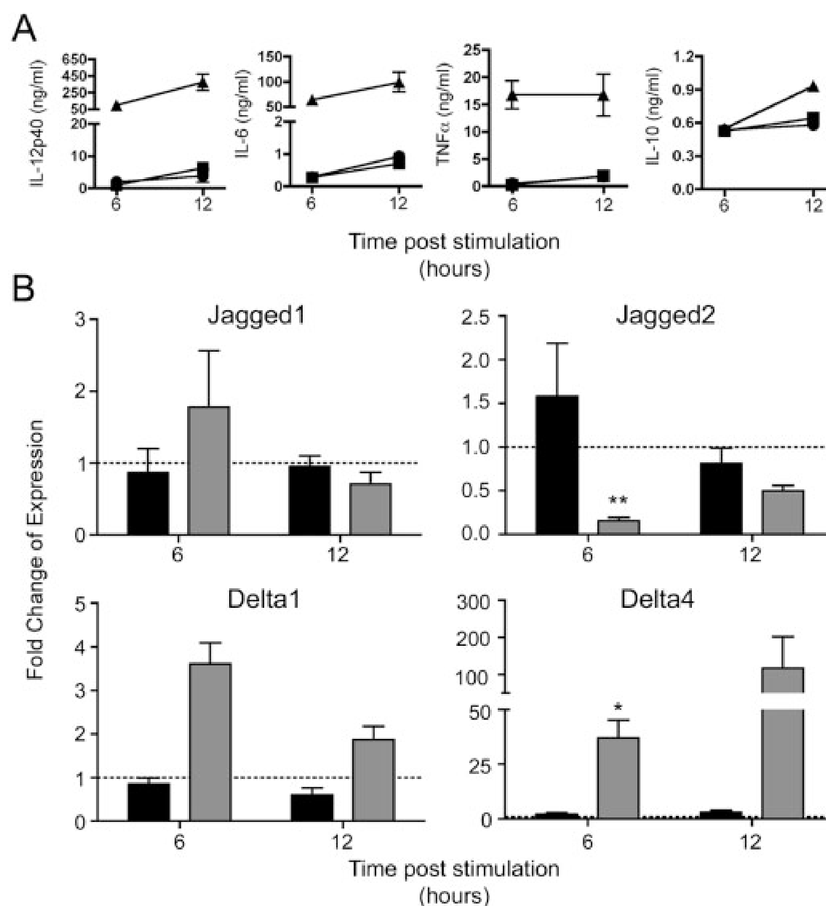

Figure 1. DC response to SEA or Pa. (A) DC were treated for up to 12 h with either medium alone (squares), 25 μg/mL SEA (circles), or 10 μg/mL Pa (triangles) in 24-well plates. Cytokines were measured in culture supernatants. Data shown indicate mean ± SEM of cytokine measured by ELISA of duplicate wells, and are representative of five separate experiments. (B) Notch ligand expression by DC in response to Pa or SEA as detailed in (A) was measured by quantitative PCR. Expression of *delta* and *jagged* mRNA was normalised to 18S RNA. Fold change of Notch ligand expression relative to unstimulated cells (dotted lines) by DC exposed to SEA (black bars) or Pa (grey bars) is shown. Data are mean + SEM of three to six combined experiments; *$p<0.03$, **$p<0.001$, comparing expression by SEA- or Pa-stimulated groups relative to medium controls.

We have previously shown that although DC activated with SEA display a muted activation phenotype, they remain potent inducers of a Th2 response both *in vivo* and *in vitro* [22]. While the exact mechanism by which this occurs remains unclear, Th2 induction by *in vivo* transfer of SEA-stimulated DC is an active process requiring DC expression of MHC class II, CD40, and NF-κB1 [22]. We first assessed the ability of *jagged2*-deficient DC to activate and polarize OVA-specific OTII TCR-transgenic T cells *in vitro*. Although equally proficient at stimulating T cell proliferation (Fig. 3A), *jagged2*$^{-/-}$ DC displayed a striking impairment in their ability to provoke Th2 polarization *in vitro*, in comparison to their *jagged2*$^{+/+}$ counterparts (Fig. 3B).

We then asked whether expression of *jagged2* was also important for polarization of Th cells by DC in a more complex *in vivo* setting. *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DC were activated with Pa or SEA and then injected into the footpads of naive C57BL/6 mice. Four days later draining LN were removed and examined for cytokine secretion following restimulation with antigen *in vitro* (Fig. 3C).

Pa-activated DC induced a marked Th1 response with high Pa-specific IFN-γ levels detected in culture supernatants irrespective of whether transferred DC were *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ (Fig. 3C). Contrary to expectation, SEA-stimulated *jagged2*$^{-/-}$ DC induced an equivalent or higher IL-4 response after transfer into naive wild-type animals (Fig. 3C). This was irrespective of the route of immunization as *jagged2*$^{+/+}$ and *jagged2*$^{-/-}$ DC given i.p. also showed equivalent ability to induce a Th2 response whether measured by IL-4 (data not shown) or IL-5, IL-13, and IL-10 (Fig. 3D). Thus, despite the fact that DC maintain expression of *jagged2* after exposure to SEA *in vitro* (Fig. 1B), and *jagged2*$^{-/-}$ DC are severely impaired in their ability to instruct Th2 polarization *in vitro* (Fig. 3B), our data suggest that this Notch ligand is not essential for the establishment of a Th2 response *in vivo*.

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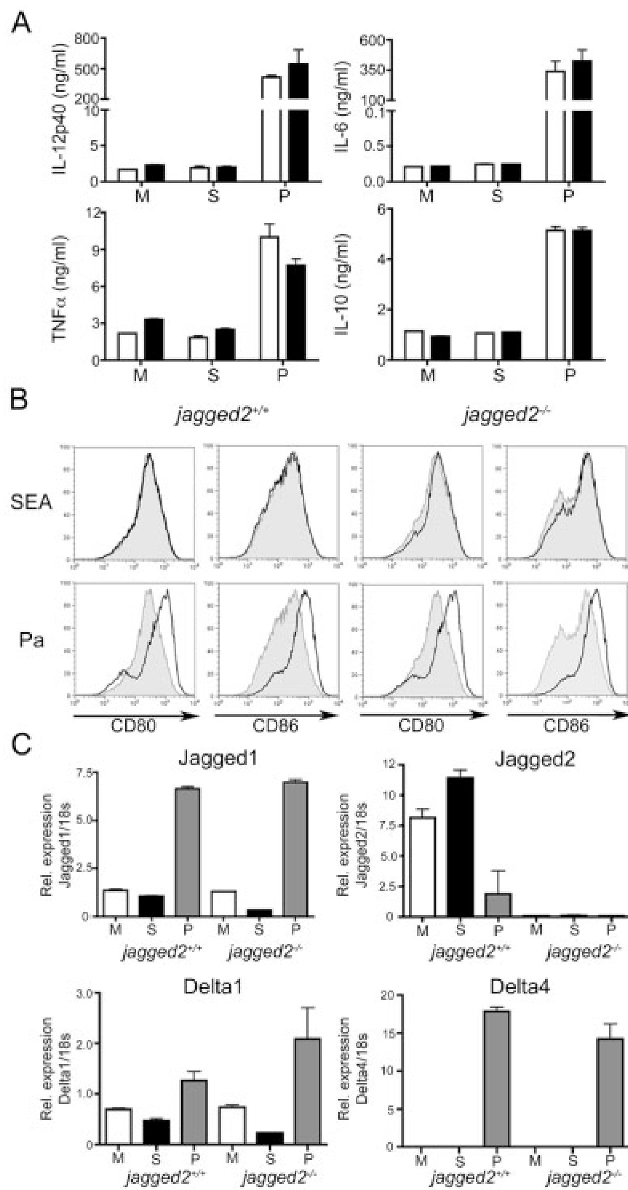

Figure 2. Deficiency in *jagged2* does not interfere with BMDC generation or activation. BMDC were grown from BM derived from *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ chimeras and exposed to either medium alone (M), SEA (S, 25 μg/mL) or Pa (P, 10 μg/mL) for 24 h. (A) Cytokine production by *jagged2*$^{+/+}$ (open bars) or *jagged2*$^{-/-}$ (black bars) DC. Data shown are mean + SEM of cytokine measured by ELISA of duplicate wells and are representative of two separate experiments. (B) Surface expression of CD80 and CD86 on *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DC. Light grey filled histograms indicate unstimulated DC, black unfilled histograms indicate DC stimulated with SEA or Pa. Graphs are representative of two separate experiments. (C) Expression of *jagged1*, *jagged2*, *delta1*, and *delta4* by DC exposed to medium (open bars), Pa (grey bars), or SEA (black bars) was assessed by quantitative PCR. Data shown are mean + SEM of expression levels measured in duplicate relative to 18S, and are representative of five separate experiments.

Concluding remarks

Studies examining the effect of wholesale inhibition of Notch signalling to T cells [6, 10, 15] have demonstrated that signalling through Notch receptors is required for the establishment of an effective T cell response. However, the mechanism by which Notch signalling translates into polarized CD4$^+$ T cell differentiation remains unclear. Tu *et al.* [10] described how inhibition of Notch signalling using dominant-negative MAML-transgenic mice during live infections of either *Leishmania major* or *Trichuris muris* resulted in a fully capable *L. major* Th1 response but an impaired Th2 response and ineffective clearance of *T. muris*. This suggests that in complete biological systems Notch signalling is required for Th2 establishment, but does not reveal which ligands are required to initiate this immunological response *via* Notch, or indeed whether APC expression of Notch ligands is required.

As shown by our expression data, Delta and Jagged ligands are expressed concurrently by DC in either Th1- or Th2-priming conditions, but their relative expression changes dramatically depending upon the stimulus encountered. Conceivably the ratio of different Notch ligands expressed by APC may ultimately determine the manner in which Notch signalling affects the differentiation of a naive T cell. Our results clearly show that DC expression of *jagged2* alone can be critical for Th2 polarization of naive CD4$^+$ T cells *in vitro* (Fig. 3B). The presence of the other Notch ligands (Fig. 2C) could not compensate for the absence of *jagged2* to enable Th2 polarization *in vitro* (Fig. 3B), indicating that *jagged1*, *delta1*, and *delta4* cannot fulfil the signalling requirement by Notch receptors on CD4$^+$ T cells to establish a Th2 response *in vitro*.

In stark contrast to this, in the *in vivo* setting, DC expression of *jagged2* was dispensable for Th2 induction (Fig. 3C, D). Taken together, our *in vitro* and *in vivo* results suggest that *jagged2* signalling is important for Th2 polarization, yet Jagged2 expression need not be limited to the APC driving the response. For example, the important interaction *in vivo* may not actually be between a Notch ligand-bearing APC and a naive CD4$^+$ T cell, but rather between APC and NKT cells, or a subset of memory CD4$^+$ T cells capable of producing IL-4 rapidly and independently of STAT6 [24, 25]. It is therefore possible that *in vivo* the multivariant expression of Notch ligands by APC involves interactions with multiple cell types since both Notch receptors and ligands can be found in diverse cell types in addition to T cells and DC [6, 26].

Our *in vivo* experiments, in which all such cells are present, reveal that DC expression of the Notch ligand *jagged2* plays no major role during SEA-specific Th2 response induction. *jagged2* deficiency impaired neither DC generation nor activation, with *jagged2*$^{-/-}$ DC producing equivalent levels of cytokine and expressing the same levels of surface markers as similarly stimulated *jagged2*$^{+/+}$ controls. Furthermore, transfer of either *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ SEA-activated DC resulted in production of similar quantities of SEA-specific IL-4, IL-5, IL-10, and IL-13 in recipient mice. Whether this finding is indicative of the provision of *jagged2* by additional cell types, a redundant pathway, or compensation *via* other Notch ligands remains to be determined.

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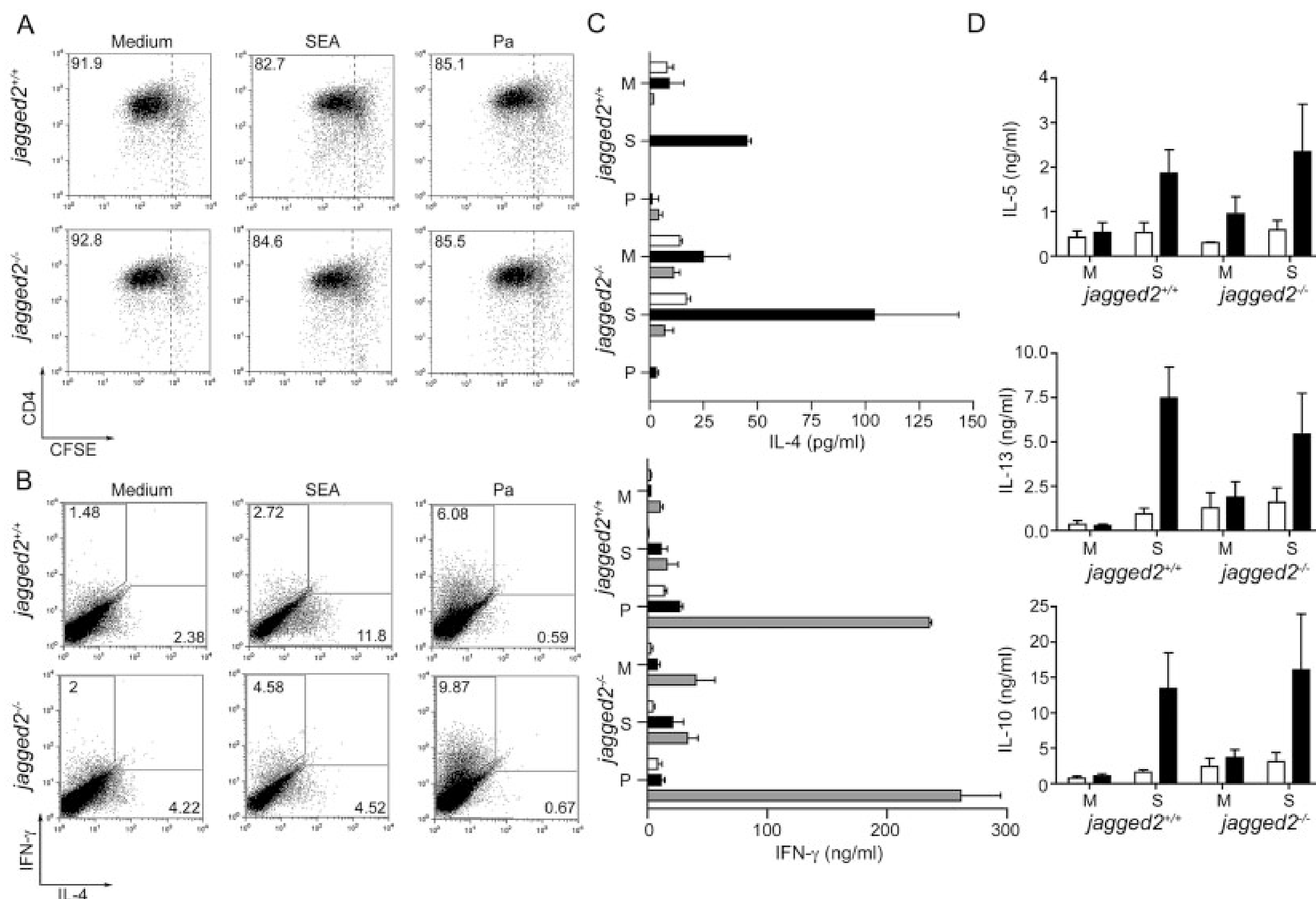

Figure 3. DC expression of *jagged2* is dispensable for Th2 or Th1 induction *in vivo*. (A, B) CD4-purified OTII cells were co-cultured with *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DC in the presence of $OVA_{323-339}$ peptide (50 ng/mL) alone (medium) or in conjunction with SEA (50 μg/mL) or Pa (10 μg/mL). OTII cells were labelled with CD4 and CFSE to assess proliferation (A) or were examined for intracellular staining (B). Dotted lines in (A) represent CFSE levels on cells cultured in the absence of peptide. Plots depicting intracellular staining in (B) are gated on live CD4 cells. Figures refer to percentage of dividing cells (A) or cytokine-producing cells (B) and results are representative of three independent experiments. (C, D) *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DC were exposed to medium alone (M), Pa (P, 10 μg/mL), or SEA (S, 25 μg/mL), then injected into the footpad (C) or i.p. (D) of naive C57BL/6 recipient mice. Popliteal LN cells (C) or splenocytes (D) were removed 4 days (C) or 7 days (D) later and then stimulated *in vitro* with medium (open bars), SEA (black bars), or Pa (grey bars). Data shown are mean + SD of cytokine measured by ELISA of triplicate wells of combined LN cells (C) or mean + SEM of cytokine measured by ELISA of three to five mice per group (D), and are representative of three (C) or two (D) separate experiments.

Even so, these results challenge the model that selective expression of Jagged ligands by DC is responsible for determining Th2 differentiation.

Materials and methods

Animals and reagents

C57BL/6 mice were bred and maintained in the animal facilities at the University of Edinburgh or at Cancer Research UK. A heat-killed preparation of the Gram^{+} bacterium *P. acnes* (ATCC No. 6919) was used as a Th1 stimulus, while SEA (prepared in-house [20]) was used for its Th2-driving capacity. Animal work was carried out under UK Home Office Project license, and was approved locally by Ethical Review Committee.

Dendritic cell culture

Murine BMDC were generated in the presence of rGM-CSF (Peprotech, London, UK) as previously described [20]. DC were stimulated with 25 μg/mL SEA or 10 μg/mL Pa (measured by Bradford assay) over a time course, supernatants assessed for cytokine levels by ELISA, and cells harvested for RNA extraction.

Generation of chimeric bone marrow

Fetal livers from Ly5.2^{+} *jagged2*$^{+/-}$ × *jagged2*$^{+/-}$ matings were removed from day-14.5 embryos, and *jagged2*$^{-/-}$ fetuses were identified by PCR [23]. Irradiated Ly5.1^{+} recipients were reconstituted with cells from *jagged2*$^{-/-}$ or *jagged2*$^{+/+}$ womb mates. DC were grown from BM 8–20 wk later. Donor origin of DC was verified by flow cytometry (data not shown).

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Determination of DC activation status

Cytokine levels were measured in DC supernatants by ELISA using commercial mAb (BD Pharmingen or R&D Systems). Phenotype was assessed by flow cytometry using mAb for CD11c, CD80 and CD86 (Pharmingen). Samples were acquired by FACSCalibur using CellQuest software and analysed using FlowJo software (TreeStar, Ashland, OR).

Assessment of DC priming ability *in vitro*

$CD4^+$ T cells were purified from the spleen and LN of OTII mice by positive selection using magnetic sorting (Miltenyi). For studies measuring proliferation, OTII $CD4^+$ cells were stained with carboxyfluoroscein succinimidyl ester (CFSE, 5 μM). $CD4^+$ cells (2×10^5) were co-cultured for 3 days with 2×10^4 *jagged2*$^{+/+}$ or *jagged2*$^{-/-}$ DC in the presence of $OVA_{323–339}$ peptide (50 ng/mL) alone or in conjunction with SEA (50 μg/mL) or Pa (10 μg/mL). On day 3, CFSE-labelled $CD4^+$ cells were washed, stained with anti-CD4-allophycocyanin (Pharmingen), then assessed by flow cytometry as described above. For measurement of intracellular cytokine, non-CFSE-labelled $CD4^+$ cells were stimulated for 4 h with PMA (10 ng/mL), ionomycin (1 μg/mL), and Brefeldin A (10 μg/mL). Cells were then washed and stained with anti-CD4-allophycocyanin before being fixed using Cytofix/Cytoperm (BD Pharmingen), according to manufacturer's protocol. Intracellular cytokines were labelled with anti-IFN-γ-FITC and anti-IL-4-PE (both from Pharmingen).

Assessment of DC priming ability *in vivo*

DC activated overnight with either SEA or Pa were injected into mice (2×10^5 per footpad or 3×10^5 i.p.). In some experiments, DC were also pulsed with OVA peptide (data not shown). Cell suspensions were prepared from spleens removed 7 days after i.p. DC transfer, or popliteal LN cells 4 days after footpad injection. In some experiments, mice received 4×10^6 OTII cells 1 day previously. Spleen and LN cells were cultured in X-Vivo 15™ serum free medium (Cambrex Bio Science, Wokingham, UK) with 2 mM L-glutamine (Gibco, Paisley, UK) and 50 μM 2-ME (Sigma, Poole, UK) without or with SEA at a final concentration of 15 μg/mL, Pa at 1 μg/mL, or OVA peptide at 1 μM. Supernatants were harvested after 72 h (splenocytes) or 48 h (LN cells) for cytokine analysis by ELISA. Results did not differ significantly when transferred DC had been activated with SEA or Pa for 6 h rather than overnight (data not shown). In OTII co-transfer experiments, SEA was not found to consistently act as an adjuvant for OVA peptide-specific IL-4 production (data not shown).

Determination of Notch ligand expression

Total RNA was extracted from 1×10^6 DC using Trizol (Invitrogen) and cDNA generated using Reverse Transcriptase System with random hexamers (Promega, UK). Notch ligand expression was assessed by quantitative PCR using SYBR green (Invitrogen), a Chromo4 detector and Opticon Monitor software (MJ Research). Relative expression values were calculated by dividing the acquired expression quantity for the gene of interest using SYBR by the expression quantity of 18S rRNA, and using a serially diluted standard of pooled cDNA or using the $2^{-\Delta\Delta C(t)}$ method. Primers used were (5′–3′): murine *jagged2* forward GTCGTCATTCCCTTTCAGTTCG, reverse AGTTCTCATCACAGCG-TACTCG; murine *jagged1* forward GCAACGACCGTAATCGCATC, reverse TGCCTGAGTGAGAAGCCTTTTC; murine *delta4* forward AGGTGCCACTTCGGTTACACAG, reverse CAATCACA-CACTCGTTCCTCTCTTC; *delta1* forward GCACTACTACGGA-GAAGGTTGCTC, reverse TCACACCCTGGAGACAGATTG; 18S forward GTAACCCGTTGAACCCCATT, reverse CCATCCAATCGG-TAGTAGCG.

Statistical analysis

The one-sample *t*-test was used to determine whether means significantly differed in comparison to a standardized control value.

Acknowledgements: This work was funded by the Wellcome Trust, the Medical Research Council UK, and Cancer Research UK. S. LG-L was funded by an EMBO long-term fellowship. Schistosome life cycle stages used to generate SEA for this research were supplied by the NIAID Schistosomiasis Resource Center at the Biomedical Research Institute (Rockville, MD), through contract N01-AI-30026. We thank Tom Gridley (Jackson Laboratory) for originally providing mice deficient in *jagged2* and Malcolm Baldock and Prof. I. Poxton for preparation of Pa.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References

1 **Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J.,** Notch signaling: Cell fate control and signal integration in development. *Science* 1999. **284:** 770–776.

2 **Osborne, B. and Miele, L.,** Notch and the immune system. *Immunity* 1999. **11:** 653–663.

3 **Hoyne, G. F., Dallman, M. J., Champion, B. R. and Lamb, J. R.,** Notch signalling in the regulation of peripheral immunity. *Immunol. Rev.* 2001. **182:** 215–227.

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4 **Maekawa, Y., Tsukumo, S., Chiba, S., Hirai, H., Hayashi, Y., Okada, H., Kishihara, K. and Yasutomo, K.,** Delta1-Notch3 interactions bias the functional differentiation of activated $CD4^+$ T cells. *Immunity* 2003. **19:** 549–559.

5 **Wong, K. K., Carpenter, M. J., Young, L. L., Walker, S. J., McKenzie, G., Rust, A. J., Ward, G.** ***et al.*,** Notch ligation by Delta1 inhibits peripheral immune responses to transplantation antigens by a $CD8^+$ cell-dependent mechanism. *J. Clin. Invest.* 2003. **112:** 1741–1750.

6 **Amsen, D., Blander, J. M., Lee, G. R., Tanigaki, K., Honjo, T. and Flavell, R. A.,** Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. *Cell* 2004. **117:** 515–526.

7 **Osborne, B. A. and Minter, L. M.,** Notch signalling during peripheral T-cell activation and differentiation. *Nat. Rev. Immunol.* 2007. **7:** 64–75.

8 **Hoyne, G. F., Le Roux, I., Corsin-Jimenez, M., Tan, K., Dunne, J., Forsyth, L. M., Dallman, M. J.** ***et al.*,** Serrate1-induced notch signalling regulates the decision between immunity and tolerance made by peripheral CD4(+) T cells. *Int. Immunol.* 2000. **12:** 177–185.

9 **Vigouroux, S., Yvon, E., Wagner, H. J., Biagi, E., Dotti, G., Sili, U., Lira, C.** ***et al.*,** Induction of antigen-specific regulatory T cells following over-expression of a Notch ligand by human B lymphocytes. *J. Virol.* 2003. **77:** 10872–10880.

10 **Tu, L., Fang, T. C., Artis, D., Shestova, O., Pross, S. E., Maillard, I. and Pear, W. S.,** Notch signaling is an important regulator of type 2 immunity. *J. Exp. Med.* 2005. **202:** 1037–1042.

11 **Napolitani, G., Rinaldi, A., Bertoni, F., Sallusto, F. and Lanzavecchia, A.,** Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat. Immunol.* 2005. **6:** 769–776.

12 **Stallwood, Y., Briend, E., Ray, K. M., Ward, G. A., Smith, B. J., Nye, E., Champion, B. R. and McKenzie, G. J.,** Small interfering RNA-mediated knockdown of notch ligands in primary $CD4^+$ T cells and dendritic cells enhances cytokine production. *J. Immunol.* 2006. **177:** 885–895.

13 **Yvon, E. S., Vigouroux, S., Rousseau, R. F., Biagi, E., Amrolia, P., Dotti, G., Wagner, H. J. and Brenner, M. K.,** Overexpression of the Notch ligand, Jagged-1, induces alloantigen-specific human regulatory T cells. *Blood* 2003. **102:** 3815–3821.

14 **Dallman, M. J., Champion, B. and Lamb, J. R.,** Notch signalling in the peripheral immune system. *Novartis Found Symp.* 2003. **252:** 268–276.

15 **Minter, L. M., Turley, D. M., Das, P., Shin, H. M., Joshi, I., Lawlor, R. G., Cho, O. H.** ***et al.*,** Inhibitors of gamma-secretase block *in vivo* and *in vitro* T helper type 1 polarization by preventing Notch upregulation of Tbx21. *Nat. Immunol.* 2005. **6:** 680–688.

16 **Eagar, T. N., Tang, Q., Wolfe, M., He, Y., Pear, W. S. and Bluestone, J. A.,** Notch 1 signaling regulates peripheral T cell activation. *Immunity* 2004. **20:** 407–415.

17 **Hoyne, G. F., Dallman, M. J. and Lamb, J. R.,** T-cell regulation of peripheral tolerance and immunity: The potential role for Notch signalling. *Immunology* 2000. **100:** 281–288.

18 **Tsukumo, S. and Yasutomo, K.,** Notch governing mature T cell differentiation. *J. Immunol.* 2004. **173:** 7109–7113.

19 **Reis e Sousa, C.,** Dendritic cells in a mature age. *Nat. Rev. Immunol.* 2006. **6:** 476–483.

20 **MacDonald, A. S., Straw, A. D., Bauman, B. and Pearce, E. J.,** $CD8^-$ dendritic cell activation status plays an integral role in influencing Th2 response development. *J. Immunol.* 2001. **167:** 1982–1988.

21 **Cervi, L., MacDonald, A. S., Kane, C., Dzierszinski, F. and Pearce, E. J.,** Cutting edge: Dendritic cells copulsed with microbial and helminth antigens undergo modified maturation, segregate the antigens to distinct intracellular compartments, and concurrently induce microbe-specific Th1 and helminth-specific Th2 responses. *J. Immunol.* 2004. **172:** 2016–2020.

22 **Perona-Wright, G., Jenkins, S. J. and MacDonald, A. S.,** Dendritic cell activation and function in response to *Schistosoma mansoni*. *Int. J. Parasitol.* 2006. **36:** 711–721.

23 **Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G. and Gridley, T.,** Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev.* 1998. **12:** 1046–1057.

24 **Tanaka, S., Tsukada, J., Suzuki, W., Hayashi, K., Tanigaki, K., Tsuji, M., Inoue, H.** ***et al.*,** The interleukin-4 enhancer CNS-2 is regulated by Notch signals and controls initial expression in NKT cells and memory-type CD4 T cells. *Immunity* 2006. **24:** 689–701.

25 **van der Vliet, H. J., Nishi, N., Koezuka, Y., von Blomberg, B. M., van den Eertwegh, A. J., Porcelli, S. A., Pinedo, H. M.** ***et al.*,** Potent expansion of human natural killer T cells using alpha-galactosylceramide (KRN7000)-loaded monocyte-derived dendritic cells, cultured in the presence of IL-7 and IL-15. *J. Immunol. Methods* 2001. **247:** 61–72.

26 **Baron, M.,** An overview of the Notch signalling pathway. *Semin. Cell Dev. Biol.* 2003. **14:** 113–119.

Abbreviations: **Pa:** heat-killed *Propionibacterium acnes* · **SEA:** soluble egg antigen from *Schistosoma mansoni*

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Received: 29/3/07
Revised: 13/12/07
Accepted: 22/1/08

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