Regulation of Dendritic Cell Function by Dectin-1

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I, Emma M. C. Slack, confirm that the work presented in this thesis is my own. Where information has been derived from other sources I confirm that this has been indicated.

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Abstract of Thesis

Innate pattern recognition receptors (PRRs) expressed on dendritic cells (DC) link direct recognition of pathogens to initiation of T cell responses. Here I describe evidence that Dectin-1 is a novel pattern recognition receptor involved the activation of dendritic cells by the yeast cell wall preparation, zymosan.

Zymosan contains ligands for the known PRR Toll-like Receptor-2 (TLR2). Interestingly, recent work in macrophages has implicated the β-glucan receptor Dectin-1 in zymosan recognition. This thesis demonstrates that Dectin-1 can function as a PRR independently of the Toll-like Receptor system to induce DC cytokine production (IL-2, IL-10) and Notch-ligand upregulation (Jagged-1).

My work has helped determine that Dectin-1 can signal via a novel HemITAM motif to the tyrosine kinase Syk. DC stimulated with zymosan upregulate IL-10, IL-2 and Jagged-1 in a Syk-dependent manner. Indeed, IL-10 and Jagged-1 induction is independent of TLR-mediated recognition of zymosan. In addition, zymosan induced ERK activation is entirely dependent on signalling through Syk and is independent of TLR signalling. I demonstrate that this ERK activation is necessary for the induction of IL-2 and IL-10 in response to zymosan. Finally I present preliminary findings on how the unusual cytokine signature of zymosan-stimulated DCs may bias Th1 and Th17 differentiation induced *in vitro*.

Statement of Collaboration

All though all of the data generated here is my own work, it would not have been possible without the collaboration of colleagues in the lab and people around the world. Specific collaborations were as follows:

Neil Rogers, Martijn Nolte and Al Edwards (all currently or previously at the Immunobiology Lab) inspired the work on Syk-dependent cytokine and Notch-ligand upregulation in DC and worked in parallel with me elucidating the nature of interactions between Dectin-1 and Syk. Patrick Costello, Jane Willoughby and Richard Treisman (Cancer Research UK London Research institute) produced bone marrow chimeras with c-fos-deficient, and TCF-deficient foetal liver and provided helpful advice on the ERK project. Edina Schweighoffer from Victor Tybulewicz's laboratory (NIMR, Mill Hill, UK) provided Syk-deficient foetal liver cells. Jagged-1 -/- mice were made by Dr Katsuto Hozumi and kindly donated to us by Soline Estrach (Cancer Research UK London Research institute). Dr Ralf Adams (Cancer Research UK London Research institute) provided ROSA26-YFP mice for testing the GFPCre constructs. Gordon Brown (University of Cape Town, South Africa) and Siamon Gordon (Oxford University, UK) provided antibodies and plasmids used in the dectin-1 project.

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This thesis is dedicated to my incredibly brave sister, Tor Slack.

Table of Abbreviations

APC	Antigen Presenting Cells
BMDC	Bone marrow-derived dendritic cell (GM-CSF-derived unless otherwise stated)
DAMP	Danger Associated Molecular Pattern
DC	Dendritic cell
DLL	Delta-like ligand
FACS	Fluorescence Activated Cell Sorting
GM-CSF	Granulocyte Monocyte – Colony Stimulating Factor
IFN	Interferon
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
LPS	Lipopolysaccharide
LRR	Leucine Rich Repeat
MALP2	Mannosylated lipopeptide 2, a diacylated lipopeptide, originally isolated from mycobacteria
MHC	Major Histocompatibility Complex
MHC II	Major Histocompatibility Complex Class II molecules
MSCV	Murine Stem Cell Virus
Pam3Cys	Tripalmitoylated lipopeptides Pam3CysSerLys4
PAMP	Pathogen Associated Molecular Pattern
PRR	Pattern Recognition Receptor
TCF	Ternary Complex Factor
TGFβ	Tumour Growth Factor β
Th	T-helper
Th1	Type 1 helper T cell
Th17	Type 17 helper T cell
Th2	Type 2 helper T cell

TLR	Toll-like receptor
TNFα	Tumour Necrosis Factor α

1 Chapter 1: Introduction

1.1 Microbial Recognition and Adaptive Immunity

The foundations of this thesis lie in two central paradigms in immunology: 1) The concept of pattern recognition, as proposed by Charlie Janeway Jr in 1989 [1] and 2) the realisation that dendritic cells (DC) are the most efficient cells at translating innate immune activation into priming of adaptive immunity (reviewed in [2, 3]). I hope to explain why the study of pattern recognition is important to our understanding of immunity and to summarise the enormous wealth of data which has made it possible to identify a novel pattern recognition pathway involved in the recognition of yeasts by dendritic cells.

1.1.1 The Mammalian immune system

The innate immune system is evolutionarily ancient. Indeed, it can be argued that parasitism has existed for almost as long as life itself and therefore aspects of "innate immunity" are found in the majority of living organisms [4] [5]. Responses of the innate immune system are rapid and broadly specific, discriminating between classes of pathogens rather than between particular species [6]. We now recognise the striking homology between innate immunity in mammals, flies, and plants both at the level of detection of microbes (e.g. the use of LRR and TIR domains [7]), and at the levels of effectors (e.g. anti-microbial peptides [8]). However, throughout evolution there has been an ongoing 'arms race' between pathogens and hosts such that whilst highly successful strategies are maintained (for example Toll-like receptors), new innate immune proteins are also being selected for (such as NK cell receptors) which give the host a competative advantage in the presence of certain pathogens [9].

Around 412 million years ago something dramatic happened [10]. With the evolution of jawed vertebrates, came the evolution of an adaptive immune system. Unlike the innate immune system, which uses phylogenetically ancient receptors to recognise infection and raise the alarm, the mammalian adaptive immune system uses clonally-expressed receptors that are generated somatically, by the recombination of genome-encoded receptor fragments [11]. The ability of evolution to select for pathogen-specific clones is minimal in this system (beyond biasing the repertoire of possible fragments) [11]. Since the recombination events that generate receptors are error-prone this leads to an enormous range of receptor specificities which will include specificities for self.

In order for the adaptive immune system not to destroy the body it exists to protect it must be tightly regulated by more than cognate antigen-recognition. In the case of T lymphocytes, the antigen recognised is a peptide presented by host MHC molecules [12], [13]. Developing T cells are selected such that cells expressing a T-cell receptor with a high affinity of self-MHC-self-peptide are deleted, as are cells bearing a receptor with very low affinity of self-MHC – a process described as clonal selection [14]. The remaining repertoire is biased towards the recognition of self-MHC presenting particular foreign peptides. However, this is insufficient to explain the observed pattern of T cell activation, for example the adaptive immune system still ignores most of the foreign antigen encountered in food (as discussed in [15] [1]).

1.1.2 Adaptive Immunity requires innate signals

In his seminal opinion of 1989 [1] Janeway proposed the existence of "Pattern Recognition Receptors" (PRRs) that have evolved to recognise components of microbes not present in our own bodies and which are essential for the survival of that microbe – so called pathogen associated molecular patterns (PAMPs). According to this theory, induction of costimulatory factors by signalling through PRRs would lead to the activation of T cells, only where there is evidence of infectious challenge. An alternative hypothesis was proposed by Matzinger in 1994 stating that the real trigger for full-blown immune activation

is "danger" [15]. In this model "danger" is defined as abnormal cell death and inflammatory mediators (see figure 1.1).

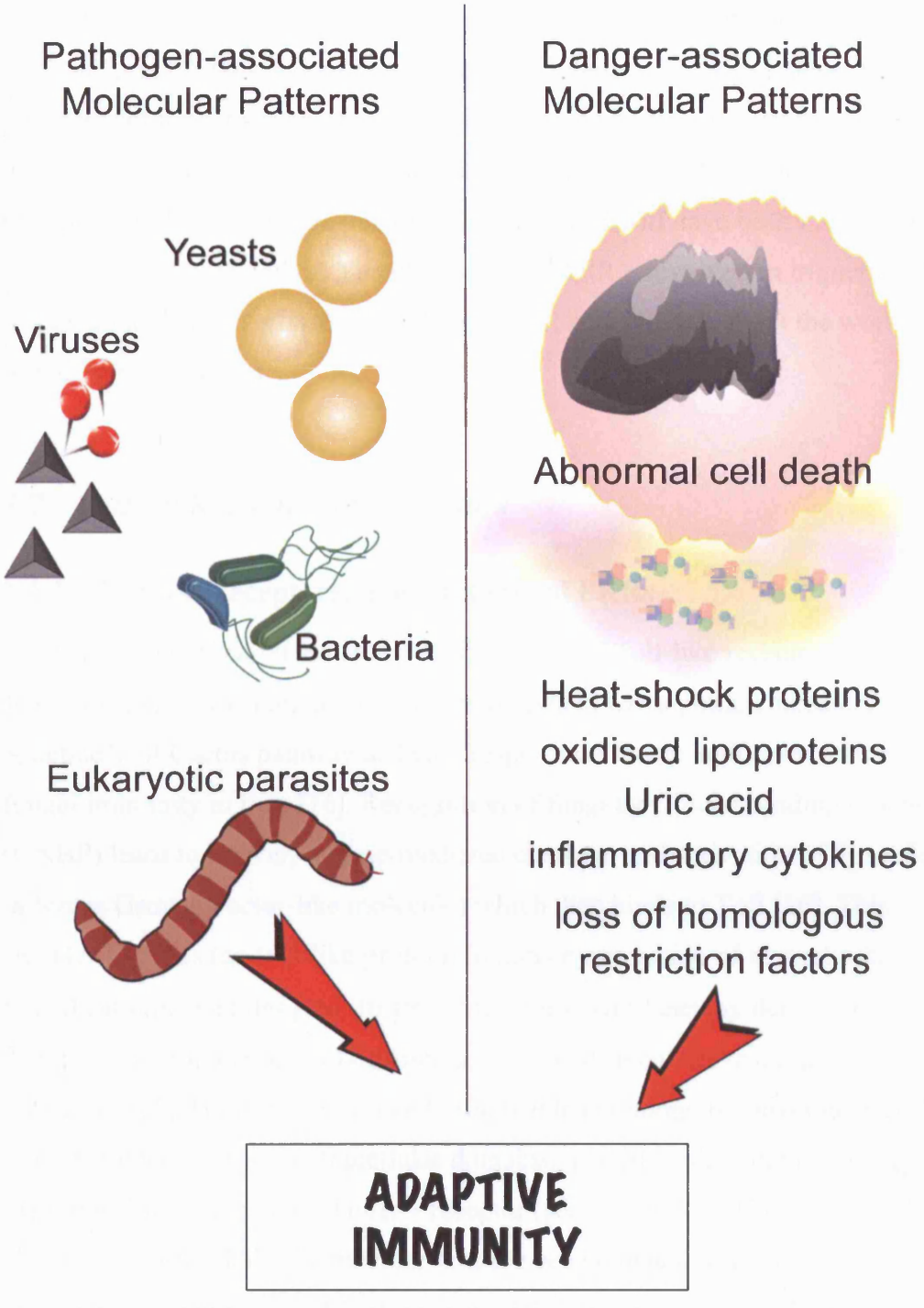


Figure 1-1: PAMPs vs DAMPs in the initiation of adaptive immune responses

Both models explain important observations, such as the requirement for adjuvant in vaccination and the link between tissue necrosis and transplant rejection. A full discussion of their relative merits is not central to this thesis, beyond stating that the innate immune system can clearly respond to both "danger" and PAMPs and in very particular circumstances one or the other may be sufficient for activation of the adaptive immune system. However, in the majority of cases when an adaptive immune response is induced and is beneficial to the host the challenge is infection, and will have both PAMPs and "danger" associated with it. The elucidation of PRR's involved in triggering the responses to different classes of pathogens will reveal much about the workings of the immune system.

1.2 Pattern Recognition Receptors

1.2.1 Toll-like receptors: The archetypal PRRs

Janeway's theoretical PRRs led to the discovery of Toll-like receptors. In 1996 Jules Hoffmann, working on immune responses in Drosophila, characterised the Spaetzle/Toll/Cactus pathway as being responsible for the induction of anti-fungal immunity in flies [16]. Recognition of fungi by Glucan-binding proteins (GNBP) leads to the Persephone-mediated cleavage and activation of Spaetzle (a Nerve Growth Factor-like molecule) which then binds to Toll [16]. This signals to Cactus (an IκB-like protein) to induce expression of several anti-fungal cationic peptides [16]. In 1997 Medzhitov and Janeway demonstrated that a human homologue of Drosophila Toll could signal for macrophage activation [17]. Human Toll showed remarkable homology to Drosophila Toll in both intracellular and extracellular domains, as well as showing homology to the intracellular domain of the IL-1 receptor (see figure 1-2, [17]). Positional cloning identified Toll-like receptor 4 as the gene mutated in LPS-hyporesponsiveness in C3H/HeJ mice and BL/10ScCr mice [18], the same gene identified by Medzhitov and Janeway as stimulating macrophage activation [17]. There are now 11 known mammalian Toll-like Receptors, all implicated as

pattern recognition receptors essential for the activation of adaptive immunity in response to particular microbial adjuvants.

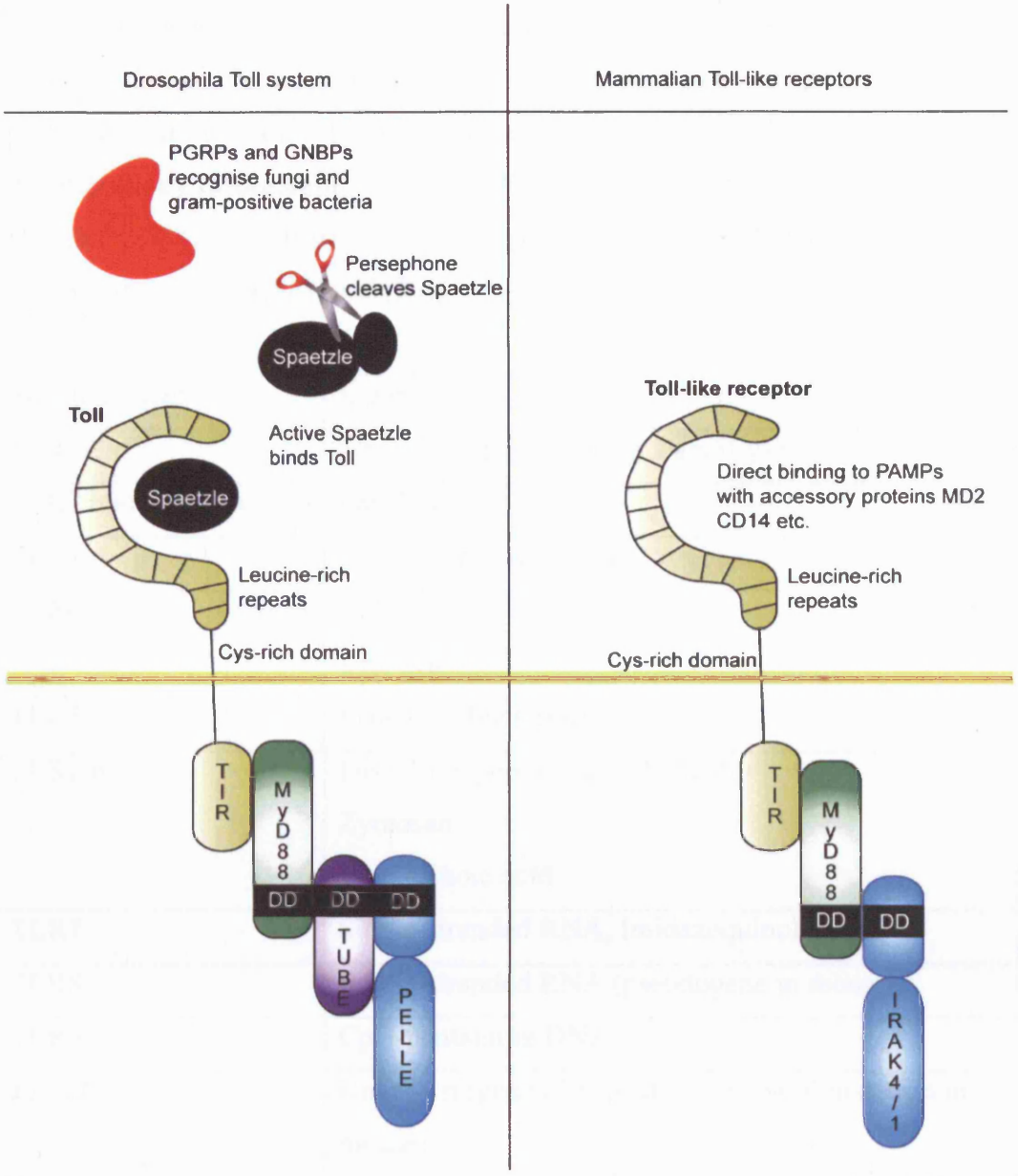


Figure 1-2: Homology between Drosophila Toll and mammalian TLR signalling. Proteins represented in the same colours are direct homologues (drawn with reference to [19])

Physiological ligands have been identified for most of the 11 known Toll-like receptors characterised. In keeping with Janeway's original hypothesis [1] the majority of the ligands are essential components of the microorganisms from which they are derived, and include viral genomes, bacterial cell wall

components or structures involved in bacterial motility and adhesion. There are two clusters within the mammalian TLRs made up of TLRs 1, 2, 6 and 10 [20] which recognise lipopeptides at the cell surface, and TLRs3, 7 and 9 [21] which recognise nucleic acids in endosomes (recently reviewed in [22]). Notably TLR1, TLR6 and TLR10 are very close together on mouse chromosome 5, and on human chromosome 4 (human genome resources; National Centre for Biotechnology Information, ncbi.nlm.nih.gov), and all pair with TLR2 in order to signal [22] [20]. Toll-like receptors and their ligands are shown in the table below (adapted from [23] and [20])

Toll-like receptor	Ligands
TLR1/2	Triacyl lipopeptides (e.g. Pam3Cys)
TLR2 homodimers	Pam2Cys
TLR3	Double-stranded RNA
TLR4	LPS Uric acid
TLR5	Flagellin (Bacterial)
TLR2/6	Diacyl lipopeptides (e.g.MALP2) Zymosan Lipoteichoic acid
TLR7	Single-stranded RNA, Imidazoquinolines
TLR8	Single-stranded RNA (pseudogene in mouse)
TLR9	CpG-containing DNA
TLR10	Unknown (gene disrupted by retroviral insertion in mouse)
TLR11	Profilin-like protein (*T. gondii*) Uropathogenic bacterial

Toll like receptors are type I membrane proteins with an N-terminal (extracellular) leucine-rich repeat (LRR) domain, followed by one or more cysteine-rich domains. The transmembrane domain connects this to the intracellular TIR (Toll-Interleukin-1-Receptor) domain (reviewed in [22]. As with many biological systems, Toll is used multiple times during the life-time of

drosophila, for immunity in adults and larvae and for dorsal-ventral patterning in the embryo [16]. Studies of Toll in Drosophila embryonic development identified mutations indicative of constitutively active dToll [24, 25]. Of these several were found to be deletions or mutations of the cysteine residues within the cysteine-rich domain, or complete deletion of this domain [24, 25]. The original Medzhitov and Janeway paper of 1997 [17] used this information to generate a constitutively active human TLR4 molecule in which the LRR and most of the cysteine-rich domain were replaced with human CD4, suggesting that mammalian TLRs are also activated by ligand-induced conformational changes in the cysteine-rich regions.

A notable difference between TLRs in mammals and Toll in flies is that Toll is an indirect PRR activated after PAMP-induced cleavage and activation of the cytokine-like protein Spaetzle [16]. There does not appear to be a Spaetzle homologue involved in innate immunity in mammals. It seems likely, in fact that TLRs in mammals are direct pattern recognition receptors [26] [27]. In mice, TLR4 signalling can be activated by the presence of LPS or the partial LPS structure tetraacyl LPS [27]. However, in humans complete LPS, but not tetraacyl structures lead to TLR4 signalling [27]. Complementation of murine TLR4-mutant (C3H/HeJ) macrophages with human TLR4 confers the ability only to recognise the complete LPS implying that the ability to recognise different LPS structures lies with TLR4 itself [27]. It is likely that this is achieved by direct binding of LPS by TLR4.

Ligand-activated TLRs form homotypic TIR-TIR interactions with adaptor proteins MyD88, TRIF, TIRAP and TRAM (recently reviewed in [22]). All TLRs excluding TLR3 require the TIR-containing adaptor MyD88 to signal Recently reviewed in [28]. TLR2 and TLR4 require TIRAP [29], a further TIR-containing adaptor molecule, whilst TLRs 3 and 4 require TRIF [30], and TLR4 additionally requires TRAM [31](see figure 1.3), demonstrating that although all the TLRs use the same domain to signal there is some variation in the usage of TIR-containing adaptors.

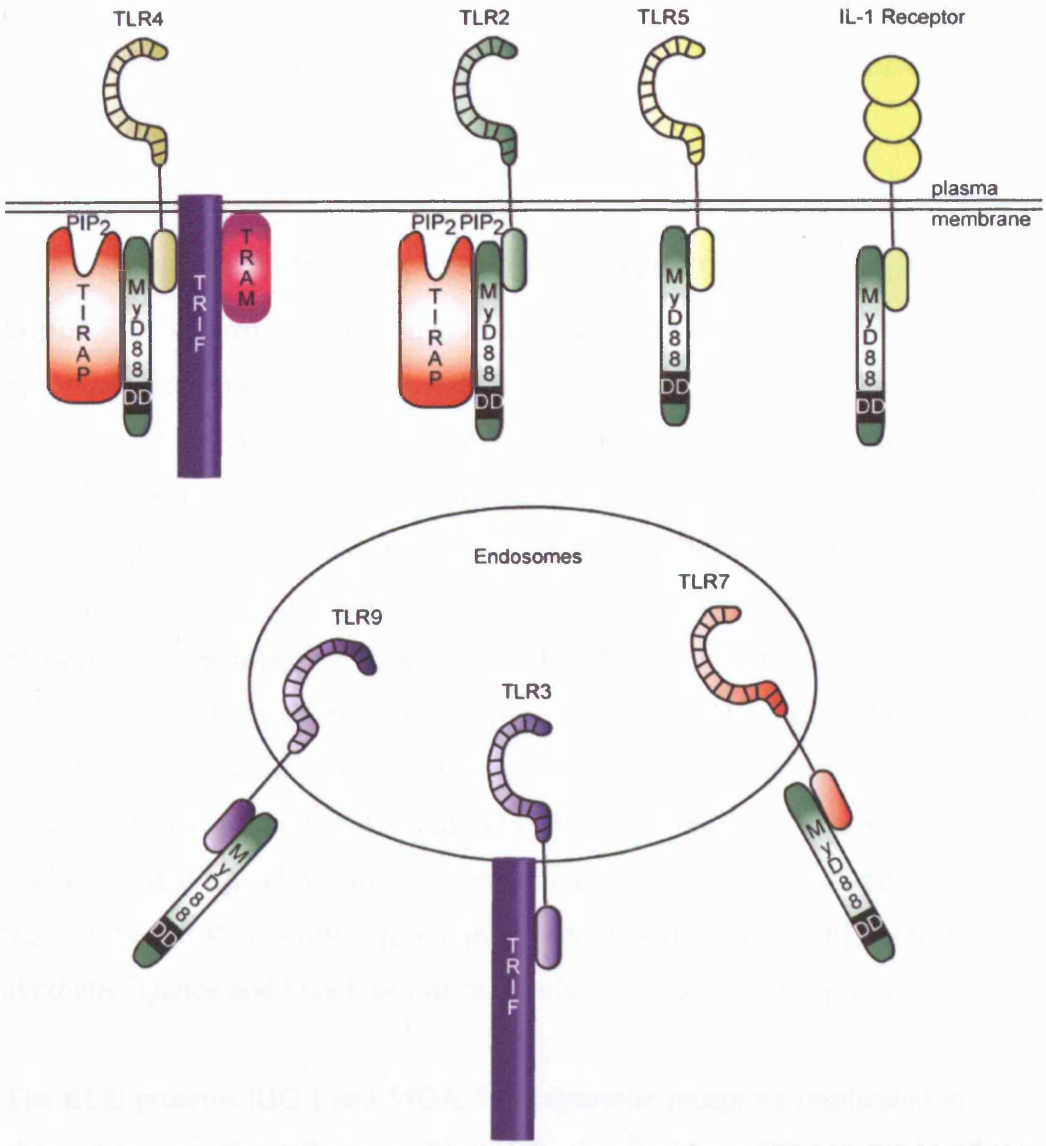


Figure 1-3: Mammalian TLRs associate with TIR-containing adaptors MyD88, TIRAP, TRAM and TRIF (drawn with reference to [28])

Many real infections encountered by the immune system will present ligands for more than one TLR. It has been observed that co-ligation of TLRs linking to different TIR-containing adaptors (such as TLR3 using TRIF, with TLR7 using MyD88) results in a synergistic increase in cytokine production [32] [33]. The same is true when LPS, signalling via TLR4 and all four known TIR-containing adaptors, is combined with R848, signalling via TLR7 and only MyD88. Assuming uniform expression of TLR7 and TLR9 in this system, this would indicate that MyD88 activation via TLR7 is not functionally equivalent to MyD88-dependent TLR4 signalling as there is at least an additive effect of

combining the ligands at saturating doses [32] [33]. The ability of PRRs to synergise may be critical to our understanding of the responses to infection where this is likely to be commonplace.

1.2.2 Non-TLR Pattern Recognition Receptors: NLRs and RLHs

Whilst TLR's remain the paradigm of true pattern recognition receptors, it is becoming clear that other systems exist. A number of PRRs have been defined that recognise PAMPs in the cytosol. These are the NOD-like receptors (NLRs) [34] [35] and RIG-Like helicases (RLHs) (Reviewed in [35]). NLRs are soluble cytosolic proteins with LRR domains, a central NACHT (nucleotide-binding- and oligomerisation) domain and either a CARD (caspase activation and recruitment domain) or Pyrin domain [35]. NOD proteins have CARD domains and are involved in sensing cytosolic peptidoglycan [34], whilst NALP proteins have N-terminal pyrin domains and are required for the response to a wide range of stimuli (recently reviewed in [35]). It is currently unclear whether an exclusive NLR ligand would act as an adjuvant for adaptive immunity or whether the NLRs modify ongoing innate immune responses [36] but use of synthetic ligands and knock-out mice should soon address this question.

The RLH proteins RIG-I and MDA-5 are cytosolic receptors implicated in cytosolic recognition of viruses. Mice defective in either of these proteins show a marked increased susceptibility to particular viral infections (reviewed in [35]).

Recognition of yeasts by the immune system can occur via TLR2 and TLR4 (Reviewed in [37, 38]). However, several studies have revealed certain aspects of innate immune activation in the absence of TLR signalling when animals, or isolated cells, are challenged with yeast extracts such as zymosan, hinting at the existence of non-TLR pattern recognition in anti-fungal responses (reviewed in [38]).

1.2.3 Zymosan is a source of fungal PAMPs

Zymosan is the insoluble residue that remains after Saccharomyces cerevisiae is extracted in hot water, trypsanised and extensively washed. This produces particles of denatured yeast cell wall with a composition of 54.7% glucan, 18.8% mannan, 14.5% protein and 12% lipids, inorganic materials and chitin [39]. Yeast cells walls are well conserved between yeast species. They consist of single or triple helices of 1,3-β-glucans with 1,6-β-glucan chains that cross-link the glucans to extensively O- and N-glycosylated mannoproteins, either via glycosyl phosphatidylinositol linkage or by unknown alkali-sensitive bonds [40-42] (Schematic diagram; Figure 1.4). The wall is built up of minimal units that are non-covalently associated in the basal β-glucan layer, and covalently cross-linked in the outer mannoprotein layer [40]. Both β-glucans and mannoproteins are essential for yeast growth and survival. Chitin forms a further essential component of yeast cell walls although it is less abundant than glucans and mannans [43]. It consists of N-acetlyglucosamine chains which form a crystal-like rigid structure. Chitin is particularly critical at yeast budding where it forms the primary septum between the bud and the mother cell. A secondary septum of β-glucan and mannoprotein is then laid-down before chitinase digests the primary septum to release the daughter cells [41]. Chitin is therefore greatly enriched at bud scars, along with exposed β-glucans, possibly making both of these compounds good markers of actively replicating yeasts. Chitin is also enriched in hyphal walls where β-glucan molecules are well masked.

Studies on Candida have confirmed that this opportunistic pathogen has a cell wall, almost identical in composition to that described for S. cerevisiae (Reviewed in [44]). Although the structure of yeast cell wall has been disrupted in zymosan, the majority of essential components are maintained, and it is reasonable to use zymosan as a source of yeast PAMPs.

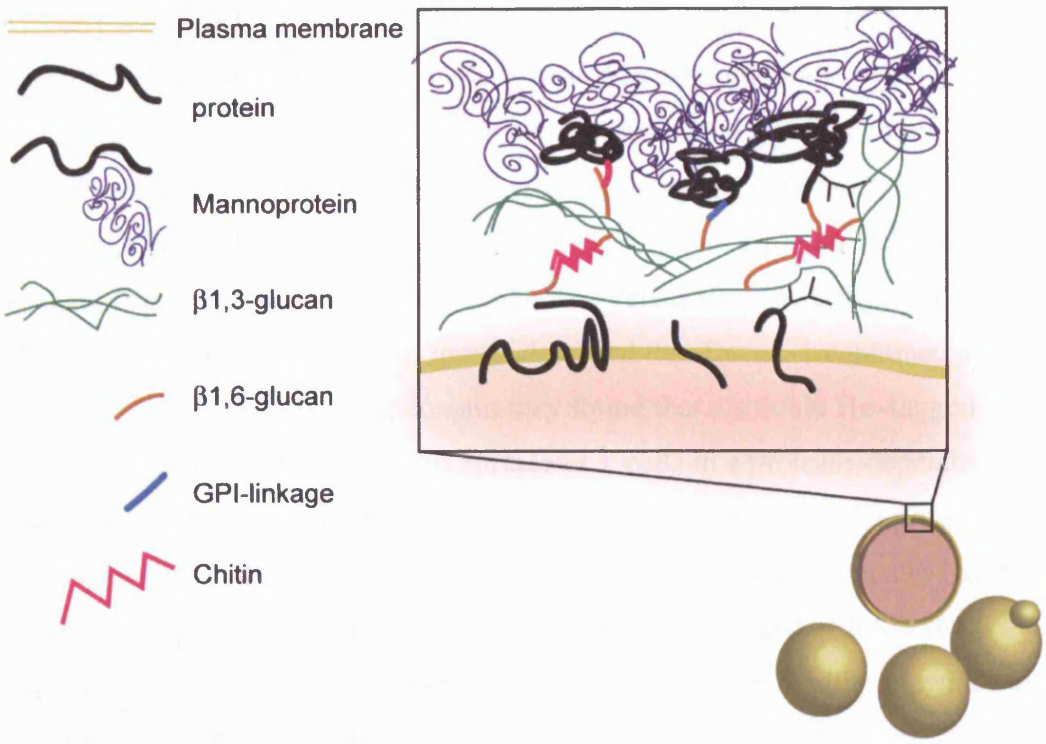


Figure 1-4: A schematic diagram of yeast cell walls adapted from Lipke and Ovale 1998 [40]

1.2.4 Innate recognition of zymosan

Recognition of zymosan by the innate immune system has been proposed to involve complement receptors [45], mannose receptors [46] [47] and β-glucan receptors [48, 49]. Zymosan is a very efficient surface for complement fixation and complement is likely to play an important role in clearing yeast infections *in vivo* [50]. However, complement does not distinguish between different types of infectious organism and therefore will not be considered further. The role of mannose receptors is controversial. The original observation by Sung et al [46] that phosphomannan inhibits binding and phagocytosis of zymosan by murine peritoneal macrophages is brought into question by the observations of Kaddish and Goldman [48, 49] that β-glucan is far more effective at inhibiting yeast phagocytosis and that treatment of mannan preparations with a β-glucanase to remove any traces of contaminating β-glucan abrogated the inhibitory effect of mannan on zymosan phagocytosis by macrophages. It now appears that the major yeast PAMP recognised by macrophages is β-glucan, an essential structural component of yeast cell walls.

Dectin1 is a type II membrane protein with an extracellular C-type lectin domain lacking the residues normally involved in calcium binding [51] [52]. The intracellular tail is just 32αα long and contains an ITAM-like motif [52]. It shows significant homology to LOX-1 (the oxidised LDL-receptor), CD94, CD69 and NKG2D [53]. The receptor was originally identified by subtractive cloning from a murine DC-like cell line XS52 versus the macrophage-like line J774 [52]. However, whilst this group identified that Dectin-1 contained a putative carbohydrate binding domain they found that a soluble His-tagged version of the protein bound to the surface of T cells in a protease-dependent but N-glycosidase independent manner suggesting the presence of a protein ligand for Dectin-1 expressed on T cells. Shortly afterwards Brown and Gordon identified Dectin-1 as a zymosan-binding receptor in a screen of RAW264.7 cDNAs [54]. They, along with Gantner et al, found that the binding of zymosan by Dectin-1 transfectants was efficiently inhibited by β-1,3-linked and β-1,6-linked β-glucans such as Laminarin and glucan phosphate [55, 56]. Using a carbohydrate array it was suggested that it is the presence of β1,3-linked glucans that lends Dectin-1 binding specificity [57]. Site-directed mutagenesis identified a triple αα Trp-Ile-His motif as well as the 4th cysteine residue as critical for binding both to β-glucan and an antagonistic antibody [58]. The Trp-Ile-His motif is not shared by the NKG2 proteins, supporting the possibility that it is important for the specific binding of Dectin-1. Interestingly, the binding of T cells to Dectin-1 transfectants was unaffected by soluble β-glucans suggesting that an alternative ligand-binding site on Dectin-1 exists.

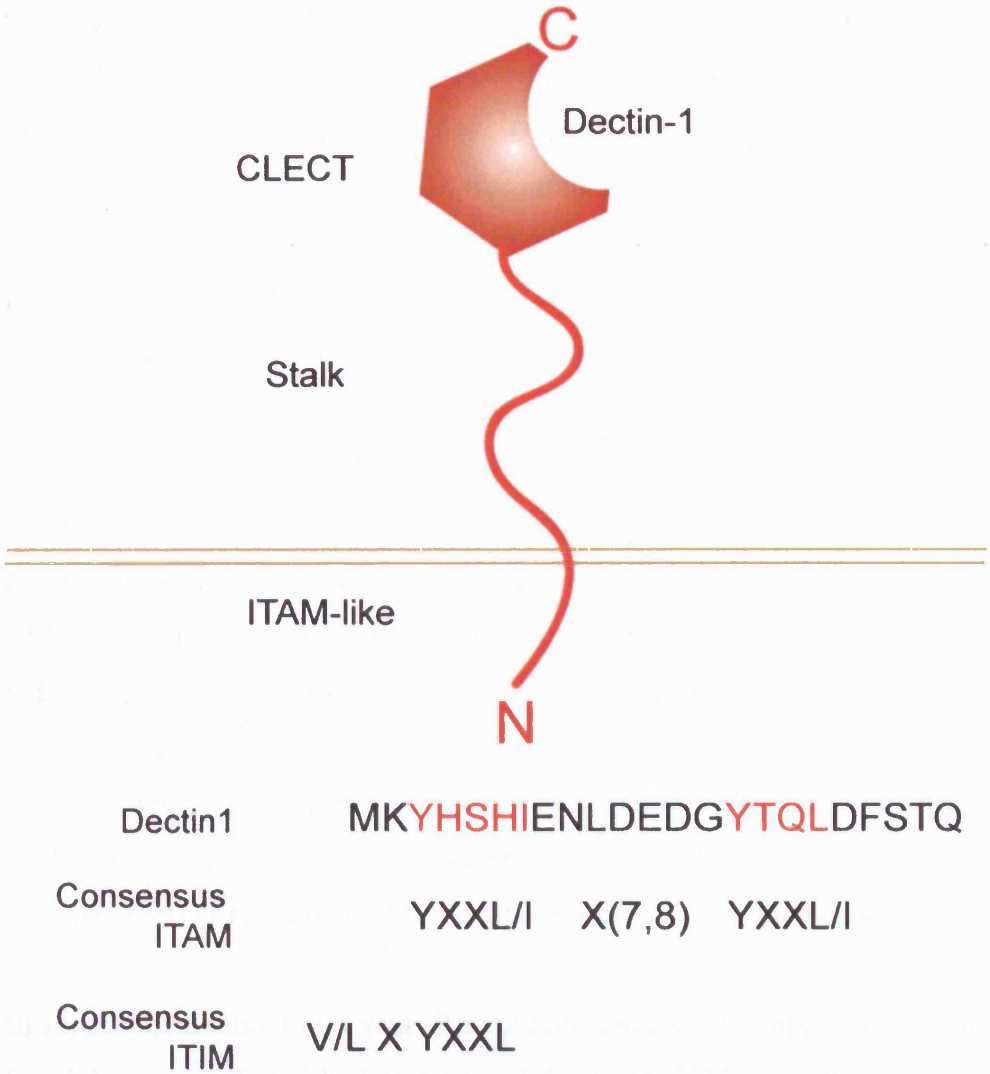


Figure 1-5: Dectin-1 is a C-type lectin with extracellular CLECT domain and intracellular ITAM-like motif, shown compared to the consensus sequences for ITAMs and ITIMs. Drawn with reference to [38].

The PRR for zymosan had previously been identified as TLR2/6 heterodimers [59], although the TLR2/6 ligands within zymosan have not yet been identified. Inhibiting the interaction between Dectin-1 and zymosan with soluble β-glucans, or an anti-Dectin-1 antibody, decreased zymosan binding by macrophages [55, 56, 60]. Inhibiting Dectin-1 binding also caused a decrease in TLR2-dependent production of TNFα in response to zymosan [55, 56]. However, using inhibition alone it is impossible to distinguish between a simple requirement of Dectin-1 to present zymosan to TLR2 for pattern recognition,

and a requirement for Dectin-1 signalling. Over-expression of wild-type Dectin-1 was demonstrated to amplify TNFα production stimulated by zymosan in the macrophage-like cell-line RAW264.7. Dectin-1 mutants which either lack the intracellular tail, or lack the ITAM-like motif are unable to induce this amplification despite efficiently increasing zymosan binding. This indicates that the Dectin-1 intracellular tail may play a role in signalling for TNFα production [55]. A similar system in which wild-type Dectin-1 or mutants lacking intracellular signalling capacity were cotransfected into 293T cells with TLR2 and an NF-κB reporter demonstrated that only wild-type Dectin-1 could amplify NF-κB activation induced by zymosan [56]. Intriguingly, it was demonstrated that β-glucan recognition alone was sufficient to induce an oxidative burst in TLR2-deficient macrophages [56], implying that Dectin-1 may have a TLR2-independent signalling role, beyond binding and uptake of yeasts. In dendritic cells TLR signalling is completely dispensable for zymosan induction of IL-10 suggesting the existence of a TLR-independent pattern-recognition pathway for yeasts, potentially involving β-glucan recognition by Dectin-1. Since β-glucan fills the criteria of a yeast PAMP very well, Dectin-1 is a good candidate PRR.

In support of Dectin-1 as a yeast PAMP receptor, Dectin-1 appears to recognise Candida yeasts effectively, but not Candida hyphae [61]; as well as *Aspergillus fumigatus* conidia, but not hyphae [62, 63](reviewed in [64]). Cytokine production by peritoneal macrophages stimulated with the primary pathogen *Coccidioides posadasii* requires signalling through TLR2, MyD88 and Dectin-1 [65].

Innate recognition of β-glucans is phylogenetically ancient. In drosophila, and the silkworm *Bombyx mori,* yeast β-glucans are recognised by soluble, or GPI-anchored PRR's with homology to the β-1,3-glucanase of *Bacillus circulans* leading to the indirect activation of Toll signalling [66] [67]. In jawed vertebrates this mechanism appears to have been superceded by Dectin-1, providing direct pattern recognition. Dectin-1 is encoded within a cluster of C-type lectins [53], many of which have immune functions, such as the NKG2

proteins. This suggests it has evolved as the result of gene duplications relatively recently in evolutionary history.

1.3 Translating innate into adaptive immunity

1.3.1 PRRs link the type of challenge encountered to recruitment of appropriate effector mechanisms

It has long been recognised that the immune response to an infection must not simply be activated but must also be directed to include effector mechanisms that will best combat that infection [1]. For example successful immune responses to viruses require the production of high levels of type I interferons [68] cytotoxic T cells, NK cells and neutralising antibodies (predominantly Th1 responses). Gut nematode infections such as trichuriasis and ascariasis are instead associated with eosinophilia, high levels of Ig-E and Ig-A, mastocytosis, increased mucous secretion, mucosal permeability and gut motility (predominantly Th2 responses)[69]. Induction of inappropriate (Th2) responses to mycobacteria results in progressive disease, highlighting the importance of controlling the class of immune response induced (Reviewed in [70], [71], [72]).

There is evidence that a similar story exists in Drosophila. Anti-microbial peptides targeted against either fungi (including Metchnikowin,and Drosomycin) or gram-negative bacteria (including Defensin, Cecropin, and Attacin) are induced downstream of receptors which recognise these classes of infection; Toll and 18-wheeler respectively [73]. This suggests that evolutionarily ancient mechanisms not only alert the immune system to the presence of potential infections but also provide some general information on the nature of the challenge.

How does recognition of microbial stimuli lead to the observed priming of appropriate adaptive immune responses? The 'real' answer to this is exceedingly complex since the immune system operates in an intricately

interconnected manner to initiate, propagate, feedback and hone the responses induced. In most cases when a pathogen is encountered an acute inflammatory reaction is triggered [6]. Tissue fluid and inflammatory cells (neutrophils, macrophages, NK cells) accumulate in the region of tissue affected, cascades of tissue factors lead to the production of bradykinin and extracellular matrix remodelling, inducing pain and behavioural changes and favouring containment of infection and migration of leukocytes [6]. The majority of these responses will occur regardless of whether the challenge is viral, bacterial, eukaryotic, or even sterile injury such as myocardial infarction (e.g. complement fixation producing the potent anaphylatoxins C3a and C5a will occur in all of these situations [74, 75]). If the acute inflammatory response was triggered by microbial stimuli acting via PRRs, then immediately a specific set of events will be set in motion. For example we know that production of extremely high systemic levels of type I interferons occurs very rapidly after viral inoculation (reviewed in [76]) when compared to infection with gut nematodes. These initially minor differences become amplified as different cell-types and humoral effectors are recruited to the site of infection.

1.3.2 Dendritic cells translate innate into adaptive immunity

Dendritic cells are a heterogeneous group of leukocytes with superior abilities to capture antigen and integrate signals from the site of acute inflammation [77]. Integrated information on the nature of the challenge is then translated into signals that prime appropriate T cell responses [78]. Concentrating on how DC respond to particular challenges, and how T cells respond to particular DC provides a powerful framework on which we can start to ask how appropriate adaptive immune responses are initiated. It is important to recognise the reductionist nature of this line of enquiry, as necessarily it is only an approximation of the complex behaviour demonstrated during immune priming *in vivo*.

Dendritic cells were first identified by Steinman and Cohn in 1973 as a novel population of splenocytes [79]. Subsequently these cells were found to express

high levels of MHC molecules and to be extremely potent stimulators of MLR responses [80], [81], [82]. DC were found to be the main antigen-bearing cells after intravenous or intraperitoneal injection of antigen in mice [83] hinting at their powerful antigen-capture abilities *in vivo*.

Langerhans cells in skin had been identified some time earlier and suggested to play a role in T cell activation. However, their ability to stimulate T cells was found to be only comparable to that of macrophages [84]. Schuler and Steinman [85] found that *ex vivo* culture of Langerhans cells caused the upregulation of MHCII which correlated with an increase in MLR-stimulating ability and hence defined Langerhans cells as efficient antigen-presenting cells. They also proposed that Langerhans cells are the immature counterparts of immunogenic DC in secondary lymphoid tissues.

In fact Langerhans cells are not precursors of splenic DC but are an epidermal dendritic cell subset, the mature counterparts of which are found in skin-draining lymph nodes [86]. Several subsets of DC have now been described in spleen, lymph nodes and tissues under steady state conditions [86] with additional populations found during inflammatory responses [87]. To take splenic DC as an example, these are now classified into at least 4 subsets that do not interconvert. Three populations are defined as 'conventional' DC and are defined by high expression of CD11c and MHC II combined with expression of CD4, CD8 or neither. Functional differences have been described between the subsets, for example CD8-positive DC are extremely good at cross-presenting cell-associated antigen (reviewed in [88]) and produce high levels of IL-12 p70, whereas CD4-positive cDC are relatively poor producers of IL-12 p70 (for example TLR7 is expressed in CD8-negative, but not CD8-positive DC [89]). In addition there are differences in the expression pattern of PRR's. The significance of this is not entirely clear, as at least *in vitro*, all subsets are capable of flexibly inducing different patterns of T cell differentiation depending on the PRR triggered [90] [78]. The fourth splenic DC subset are plasmacytoid DC. These have a markedly different morphology to cDC and are

notable in their ability to produce very high levels of type I interferons in response to extracellular viral PAMPs [91].

The majority of work in this thesis has employed dendritic cells differentiated from bone marrow in the presence of GM-CSF [92]. These have recently been reported to bear similarity to a DC subset that differentiates from monocytes during inflammatory conditions *in vivo* [87].

A further implication of the observations of Schuler and Steinman was that DC are not constitutively immunogenic. Koide et al [93]demonstrated that the proposed role of IL-1 as a costimulator of T cell responses was actually due to its effect on dendritic cells, rather than direct costimulation of T cells [93] further supporting this notion. It is now well accepted that the majority of DC, regardless of subset, are in an "immature" state in a healthy animal and acquire the ability to instruct T cells only after triggering of maturation.

1.3.3 Dendritic cell maturation is induced by innate signals

Immature DC express low levels of surface costimulatory molecules and MHC and have high pinocytic and endocytic activity [77]. On stimulation, a maturation programme is initiated in which captured antigen is loaded onto MHC and delivered to the cell surface, costimulatory molecules such as B7.1, B7.2 and CD40 are displayed, cytokines are produced and DC migrate to the T cell areas of secondary lymphoid tissues [77]. There is considerable flexibility in phenotype of mature DC produced depending the stimulus [94], [95], [96]. Quantitative differences in MHC upregulation (Recently reviewed in [97, 98]); differential display of a wide range of costimulatory molecules with both positive and negative effects on T cell differentiation (B7-related molecules B7.1, B7.2, B7-H3, PD-L1, B7-H4 and TNF-TNFR relations OX40L, TRAIL/LIGHT, CD70, GITRL, HVEM, 4BBL amongst others, (recently reviewed in [99], [100])) and production of a variable cocktail of cytokines define these phenotypes. The particular combination of costimulatory molecules, cytokines etc produced by a dendritic cell (and possibly the timing of production of such factors) can profoundly influence the differentiation of a T cell clone encountering its cognate antigen on that particular dendritic cell [101], [102], [103], [104].

A good, if extreme, example of this is in mixed bone marrow chimeras in which half of the DC population cannot respond to the TLR9 ligand CpG [101]. Injection of CpG into the mixed chimeric mouse will directly stimulate only the TLR-9 sufficient DC. Inflammatory mediators produced by the TLR9-sufficient cells are sufficient to result in upregulation of surface MHC and surface costimulatory molecules on the TLR9-deficient DC. However, despite almost equivalent maturation in terms of surface markers only the TLR9-sufficient DC can produce IL-12 [101]. If an MHC mismatch is introduced such that TLR9-expressing DC cannot present to antigen-specific T cells but TLR9-deficient DC can, it is found that T cells primed by this route proliferate almost as well as T cells primed in control chimeras in which TLR9-sufficient DC can also present

cognate antigen. However T cells primed on indirectly activated DC do not differentiate into IFNγ-producing effector cells whilst those primed in TLR9-sufficient DC do [101]. This indicates that DC stimulation by inflammatory mediators is sufficient for costimulatory molecule upregulation and instruction of T cell proliferation by DC. However, inflammatory mediators alone cannot induce DC cytokine production and fail to instruct effector T cell differentiation *in vivo*. There are also numerous reports of cytokines such as TNFα, IFNα, and IL-15 [105-107] leading to upregulation of surface markers on DC without inducing DC cytokine production.

1.3.4 Intracellular Signalling linking PRRs and DC activation

A well characterised trigger of DC maturation is TLR ligation, therefore a good starting point for study of signalling in DC maturation is to assess the signalling requirements in this specific example. As already mentioned, ligand-induced dimerisation leads to the recruitment of TIR-containing adaptor proteins (MyD88 and TRIF) to the TIR domains of TLRs through TIR-TIR interactions. It has recently been demonstrated that in the case of TLR4 (and by analogy TLR2) a second TIR-containing adaptor, TIRAP, is crucial to deliver MyD88 to PIP2-enriched membrane domains where these TLRs are localised, implying that TIRAP is a "sorting adaptor" [108]. By analogy it seems likely that TRAM plays a similar role in bringing TRIF into the correct location for TLR4 signalling [31] [108]. TRIF is required for TLR3 signalling but TRAM is not, fitting with the observation that TLR3 is present in a different endomembrane compartment (endosomes) and would hence require a different sorting adaptor. IL-1-Receptor, TLR5, TLR7 and TLR9 signal via MyD88 with no requirement for TIRAP possibly also due to alternative membrane localisation. (Reviewed in [23])

MyD88 interacts with the serine/threonine kinases IRAK1 and IRAK4 via its death domain. This leads to the recruitment and activation of the E3 ubiquitin ligase TRAF6 and the MAP3K TAK1. K63-polyubiquitination of TAK1 leads to the activation of MKK3/6 and the p38 MAPK cascade and to K63-

polyubiquitination of NEMO/IKKγ and NK-κB activation. (reviewed in [22], depicted in figure 1.6). IRAK4-deficient mice show massively decreased sensitivity to Interleukin-1, LPS (TLR4), CpG (TLR9) and slight decreases in response to Peptidoglycan (TLR2/NOD2), and polyI:C (TLR3) [109] reflecting the TRIF-dependence of TLR3 signalling and the divergent signalling downstream of NOD-protein recognition [109]. TRAF6-/- mice show no MAP kinase phosphorylation and no IκB degradation in response to CpG or Pam3Cys (TLR2/1) but partial responses to LPS and wild-type responses to polyI:C [110] indicating an essential role in MyD88 signalling, but not TRIF signalling (See figure 1.6).

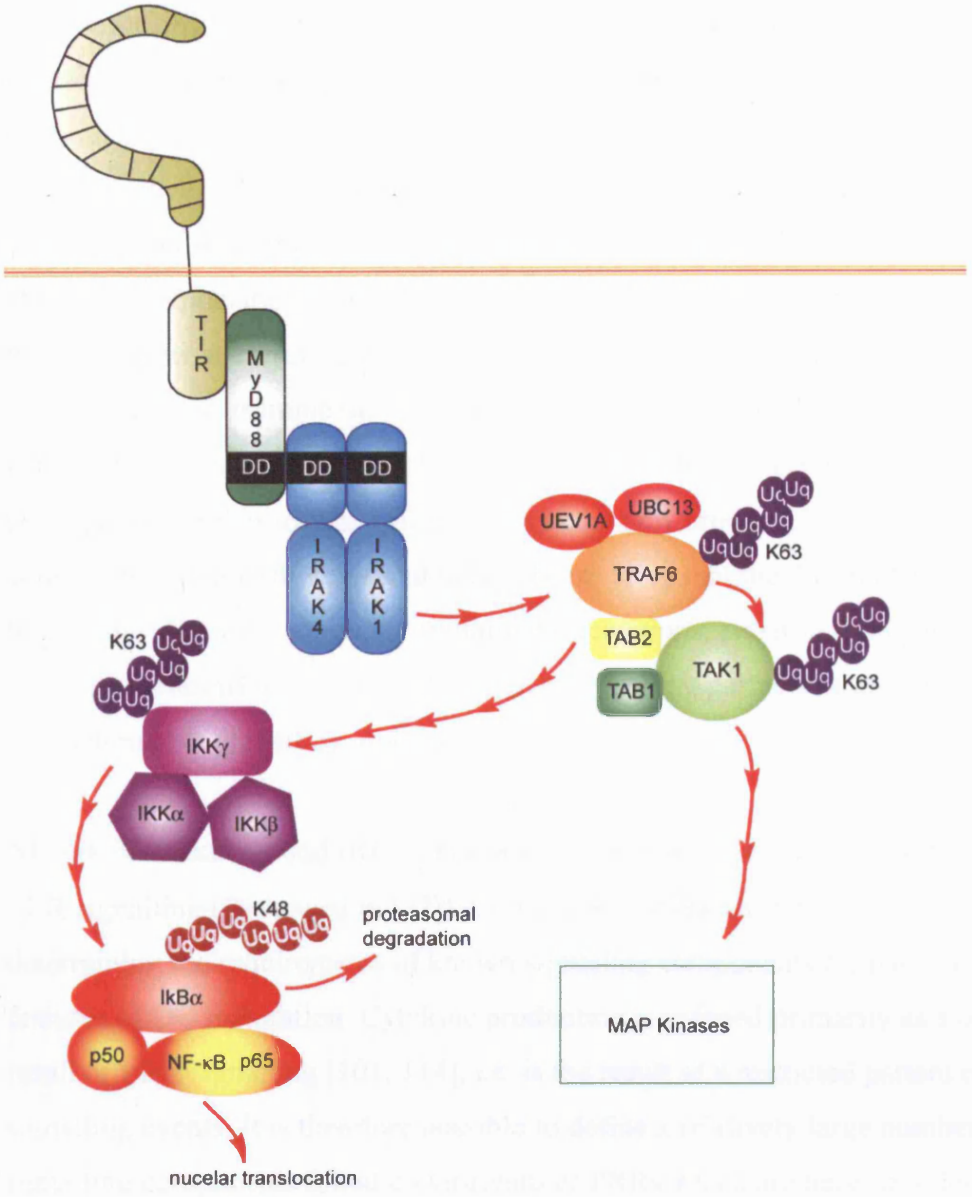


Figure 1-6: MyD88-dependent TLR signalling. (drawn with reference to [22])

TRIF, like MyD88, associates with particular TLRs through a TIR domain, but it bears little homology to MyD88 over the rest of the molecule and signals in a correspondingly divergent manner (as reviewed in [111]). Signalling downstream of TRIF appears to require TRAF3 [110]and TBK1 leading to the activation of IRF3/7 and production of type I interferons [112].

It has been proposed that the use of different signalling adaptors is sufficient to explain the differences in cytokine production observed downstream of TLRs

[28]. This is an attractive idea but implies the existence of further adaptors yet to be described that explain the subtleties of TLR5, TLR7, TLR9 and Interleukin-1 responses which all use the MyD88 adaptor exclusively but elicit dramatically different responses from a single purified subset of dendritic cells (for example in GM-CSF BMDC, TLR9 ligands induce high levels of IL-12 p40, IL-6 and IL-2 [96] whereas IL-1 does not induce cytokine secretion). An alternative explanation is that the localisation of TLRs to different membrane microdomains or compartments gives access to slightly different signalling components, for example the delivery of CpG to different endosomal compartments can alter the ability to induce production of IFNα from PDC [113] [113]. Further to this, TIR domains show only around 20% sequence conservation (Recently reviewed in [111]) implying that the different TLRs have evolved to form divergent signalling interactions, either in terms of affinity/kinetics of interactions with known signalling partners or association with alternative signalling molecules.

NF-κB, MAP kinase and IRF3/7 pathways are known to be activated directly by TLR signalling (Reviewed in [22]). Considerable work has been devoted to determining the requirements of known signalling components for particular features of DC maturation. Cytokine production is induced primarily as a direct result of PRR signalling [101, 114], i.e. is the result of a restricted pattern of signalling events. It is therefore possible to define a relatively large number of signalling components found downstream of PRRs which are necessary for cytokine production.

IL-12 p40 is produced by DC in response to TLR ligation (with the possible exception of TLR5) and in response to zymosan ([96], Chapter 4). Induction of IL-12 p40 is inhibited by disruption of the p38 MAPK [115] pathway or the NF-κB pathway [116]. In addition, IκBζ, an immediate-early gene induced downstream of TLR signalling is essential for IL-12 p40 production [117]. ICSBP (IRF8), IRF1 and NFAT have all been proposed to play a role in the induction of IL-12 by various stimuli in macrophages and DC [118-122]. However it is unclear how these transcription factors become active after TLR

ligation. Conversely, inhibition of ERK MAPK increases IL-12 production in response to a number of stimuli [123] [124]. This may be partially due to the role of ERK in the induction of IL-10, which can negatively feedback to inhibit IL-12 production .

Several reports suggest similarity in the requirements for IL-6 and IL-12 production by DC. Production of IL-6 also requires IκBζ [117]. In addition an elegant paper by Saccani et al suggests that robust p38 MAPK activation is essential to phosphorylate Histone H3 at the promoters of IL-6, IL-8, MCP-1 and IL-12 p40 to allow recruitment of NF-κB[125]. The authors demonstrated their hypothesis directly for the MCP-1 and IL-8 promoters, although poor quality of c-Rel antibodies made direct observations of IL-12 p40 and IL-6 NF-κB recruitment impossible. The authors further go on to show that stimulation of DCs with TNFα, CD40L or IL-1β resulted in similar NF-κB activation but much weaker p38 MAPK activation when compared to LPS. This correlated with decreased H3 phosphorylation suggesting that the ability of a stimulus to induce cytokine transcription may dependent on its ability to induce a strong p38 MAPK signal.

The 3' UTR of mRNAs for IL-6, TNFα and IL-2 contain AU-rich elements. Tristetraprolin binds ARE-containing mRNAs by sequestering mRNA into stress-granules or related protein complexes that inhibit translation and promote mRNA degradation [126]. MAPKAP Kinase 2 (MK2) the MAP kinase target, phosphorylates Tristetraprolin leading to association with 14-3-3 proteins, relocalisation and therefore mRNA stabilisation [126]. Macrophages deficient in MK2 show a dramatic decrease in IL-6 and TNFα production in response to LPS and TNFα production becomes MK2-independent if the AU-rich element is deleted from the TNFα mRNA 3'UTR [127]. MK2-deficient mice show a defect in the induction of IL-12, IL-10, IL-6, IL-1β and TNF from splenocytes after LPS injection [128] although it is not clear whether all of these cytokines are regulated at the translational level or whether MK2 has additional functions. It has been suggested that ERK signalling regulates nuclear export of TNFα mRNA [129]. There are further reports that the ERK pathway can influence

cap-dependent translation initiation through the phosphorylation of eIF4E and eIF2α by promoting Protein Phosphatase 1 activity, or via Mnk1 and 2 respectively [130, 131]. Clearly both transcriptional and post-transciptional events control cytokine production downstream of TLR ligation.

Control of IL-2 production in T cells is also mediated by Tristetraprolin-binding to the AU-rich element within the 3' UTR of IL-2 mRNA [132]. Although this is yet to be characterised in DC, it is a reasonable hypothesis that MK2 phosphorylation of TTP is also an important step in IL-2 production in DC. Other known requirements for IL-2 production by dendritic cells include a requirement for paracrine or autocrine signalling by IL-15[133].

IL-10 is associated with the suppression of inflammatory responses and inhibition of Th1 polarisation (reviewed in [134]). IL-10 can decrease the production of IL-12 p70 by DC, and blocking of IL-10 will increase IL-12 p70 production in response to ligands for TLR2, TLR4 or TLR9 [132]. The induction of IL-10 by TLR ligands requires ERK activation via TPL2/NF-κB1. TPL2 is a MEK kinase that becomes active on NF-κB p105 degradation[135]. Downstream of ERK, c-fos is required for transcription of the IL-10 gene [136].

Although attempts have been made to study signalling required for surface marker upregulation, results are highly variable and can be contradictory. A case in point is the upregulation of B7 costimulatory molecules during DC maturation. Multiple interconnected signalling pathways, including the production of autocrine and paracrine intercellular signals such as IFNα[106] and IL-15[107], play a role in the upregulation of costimulatory molecules by DC. Inhibition of NF-κB by overexpression of IκB or use of inhibitory peptides limits B7 upregulation in response to LPS (signalling through TLR4) and CD40L [116, 137, 138]. However, TREM2 signalling through DAP12 appears not to activate the NF-κB pathway in DC but is still capable of producing robust B7 upregulation [139]. ERK and p38 MAPK inhibitors appear to have either neutral [139, 140], positive [140]or negative effects on B7 levels [141], possibly

depending on whether the stimulus applied elicits a MAPK-dependent inhibitor or promoter of DC maturation.

DC must respond specifically to an enormous number of external cues using a comparatively limited number of signalling pathways. There is unlikely to be a specific pathway for each PRR. A plausible alternative is that each PRR initiates a specific sequence of intricately networked events, the identity, but also the timing and localisation of which encode the signal received.

The ERK MAP Kinase pathway provides a good example of how localisation of signalling is controlled. ERK MAP kinase signalling could propagate as a series of phospho-protein waves from the cell surface to the nucleus, but this process would be rapidly attenuated by phosphatases that limit the half-life of active kinases and therefore the distance over which the signal can be propagated by diffusion (as discussed in [142]). It now appears that scaffolds promote the physical association of kinases in a cascade (for example KSR can bind to Raf1, MEK and ERK – reviewed in [143]). This induced proximity increases processivity of the cascade. In addition KSR can control the localisation of ERK activity. In response to growth factor signalling KSR translocates to the plasma membrane from lipid rafts or the cytosol (reviewed in [143]). Evidence suggests that scaffolding proteins can associate with molecular motors or trafficking endosomes to propagate signals between two or more compartments. Sef, an alternative ERK scaffold appears to retain active ERK on the Golgi apparatus preventing phosphorylation of nuclear targets and promoting the phosphorylation of cytoplasmic proetins (reviewed in [142], [143]). These observations, combined with the classic PC12 cell experiment in which prolonged ERK activation promotes differentiation whilst transient ERK activation promotes proliferation (reviewed in [144]) suggest that even single components in signalling networks can instruct very different outcomes. Whilst it is possible to identify important players in the signalling networks that are absolutely required for signal propagation the actual outcome of signalling may often depend on much more subtle interactions that are not revealed by deletion of specific components. It is relatively simple therefore to show that in response

to a defined signal a signalling component links the network to a particular facet of the response. To demonstrate that signalling via a particular pathway or component is sufficient for a given response is almost impossible due to the high levels of interconnectivity.

The production of cytokines by murine DC, as discussed earlier, appears to reflect bona fide direct pattern recognition [101], suggesting an absolute requirement for particular patterns of signalling occurring exclusively downstream of PRRs. This correlates well with the greater number of genetic and chemical disruptions found to inhibit the production of particular dendritic cell cytokines. For these reasons cytokine production is used in this thesis as a mark of direct pattern recognition by DC, and as a useful phenomenon to elucidate the signalling downstream of zymosan recognition.

1.3.5 Intracellular Signalling linking Zymosan recognition and DC maturation

Innate recognition of zymosan by dendritic cells induces upregulation of MHC class II, B7.1, B7.2 and CD40 and production of large amounts of IL-12 p40, IL-10 [96] and IL-2 [145]. In chapter 4 of this thesis I demonstrate that BMDC also produce large amounts of TNFα, IL-6 and IL-12p19 (IL-23) and upregulate the Notch-ligands DLL1, DLL4, Jagged-1 and Jagged-2. Both TLR2/6 heterodimers and β-glucan recognition, putatively via Dectin-1 are implicated in these responses. Whilst the production of IL-2, IL-6, IL-12 and DLL4 has a strong TLR2/MyD88-dependent component, IL-10 and Jagged-1 are upregulated in a TLR-independent fashion. It has previously been demonstrated that upregulation of surface markers is also completely TLR-independent [96].

TLR2/6 signalling via MyD88 stimulates the ERK pathway strongly [136]in a TPL2-dependent fashion (Dr MJ Robinson, Cancer Research UK, London. personal communication). This leads to the induction of IL-10 in response to well-defined TLR2 ligands such as synthetic lipopeptides. However, I have found that the amount of IL-10 produced in response to lipopeptides is orders of

magnitude lower than that induced in response to zymosan (see chapter 4). MyD88-deficient DC show no defect in IL-10 induction suggesting that the Dectin-1 pathway alone provides a potent IL-10 –inducing stimulus.

Dectin-1 signalling is implied by the presence of an ITAM-like motif in the cytoplasmic tail [52]. We demonstrate that this ITAM-like motif couples to Syk to mediate downstream signalling. Syk-deficient DC produce almost no IL-10 or IL-2 in response to zymosan although production of IL-12 p40 is unaltered (see chapter 4, [146]). Also, ERK activation is completely dependent on Syk in response to zymosan and appears to require different downstream targets to induce IL-10 transcription from those implicated downstream of TLRs (see chapter 5).

Dectin-1 signalling through Syk may deviate slightly from classical ITAM signalling such as that occurring downstream of the B cell receptor. A very recent example of this is the report that Dectin-1 mediated NF-κB activation is induced through Bcl10 and MALT1 [147], both of which are used in antigen-receptor signalling. However the upstream activator of Bcl10 in Dectin-1 signalling is CARD9 and not CARMA1, which is normally used in ITAM signalling [147].

Dectin-1 is the first example of a bona-fide cell surface PRR which is evolutionarily distinct from the TLRs and uses distinct signalling pathways to mediate DC activation and cytokine production.

1.4 Instruction of T cell differentiation into appropriate effectors

The process of DC maturation leads to the instruction of optimal T cell responses. As discussed previously dendritic cells are extremely efficient at inducing effector T cells *in vitro* and *in vivo* and may be the only cell types capable of priming naïve T cells. Upregulation of antigen-presentation and costimulatory molecules on DC by inflammatory stimuli is sufficient to induce T cells proliferation *in vivo* [101]. However most evidence suggests that these

signals alone are not sufficient for T cells to acquire effector functions. DC that have been stimulated via bona fide PRRs induce the differentiation of effector T cells [78], [90].

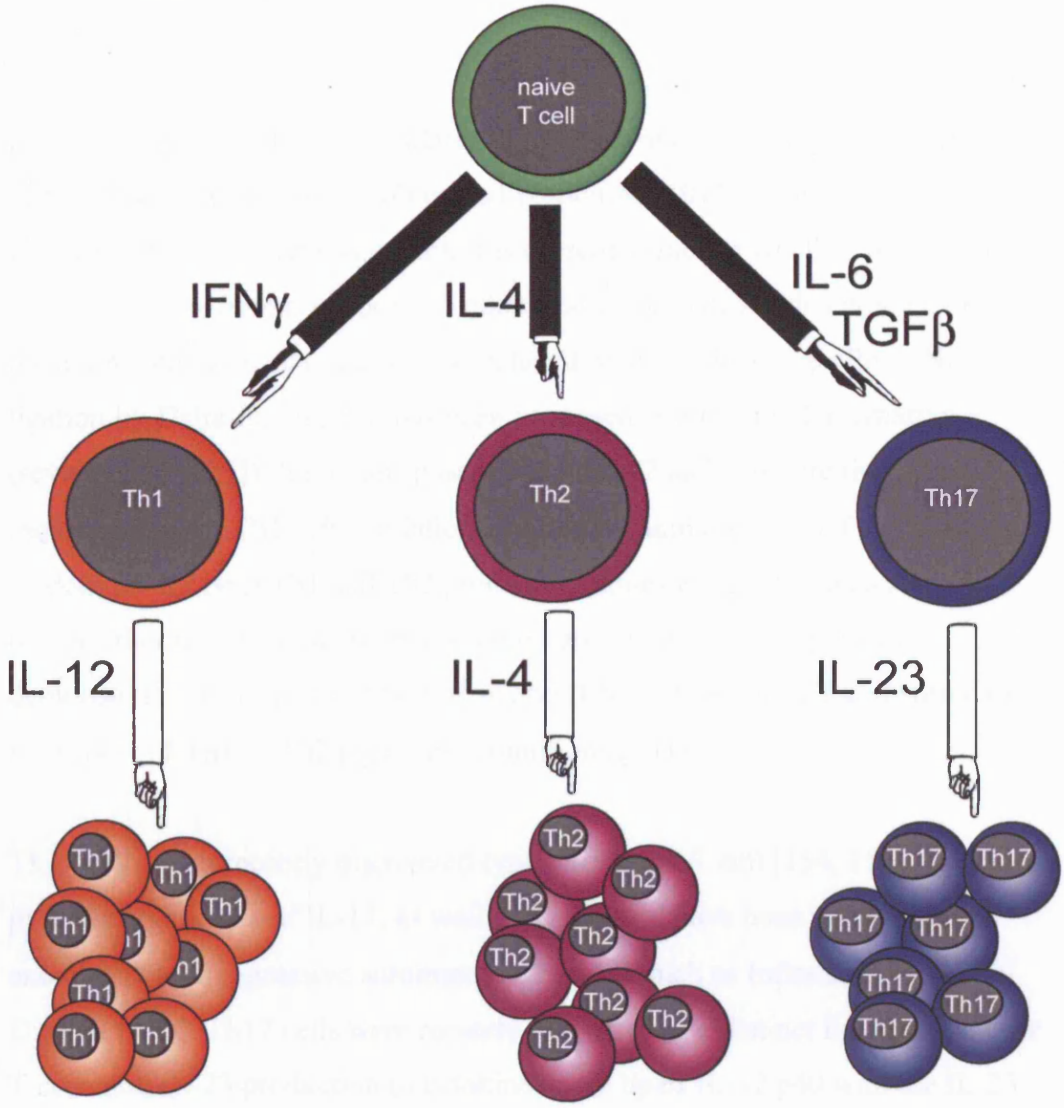


Figure 1-7: T cell differentiation into Th1, Th2 or Th17 effector T cells can be controlled by cytokines from innate immune cells.

Th1 effector T cells produce IFNγ, TNFα, IL-2 and occasionally IL-10 [148]. IL-12 produced by TLR-stimulated DC correlates well with Th1 induction [103] and mice deficient in any of the components of IL-12 production or signalling show severely impaired Th1 responses [149, 150].

Expression of the T-bet transcription factor is both necessary and sufficient for Th1 polarisation. It has been suggested that IFNγ can directly turn on T-bet

expression and although production of IFNγ by murine DC has not been convincingly demonstrated, this agrees well with data suggesting that activated DC can recruit IFNγ-producing NK cells into lymph nodes to promote Th1 priming [151].

It is clear *in vivo* that DC stimulated by PAMPs and delivering IL-12 in cis can instruct the development of robust Th1 responses [101] whilst indirectly activated DC are much less potent. It is currently unclear whether IL-12 is the decisive cis-restricting factor or whether IL-12 correlates with a novel Th1-polarising stimulus that can only be induced by direct PRR signalling. Notch ligation by Delta-like ligands has been proposed as a potential alternative (reviewed in [152]). It remains possible that IL-12 and IFNγ are the critical requirements for Th1 differentiation and that the initial stage of T cell priming is identical for both Th1 and Th2 producing clones which stochastically adopt one or other fate. In support of this notion an elegant set of experiments demonstrate transcription of both IL-4 and IFNγ within 1hr of T cell stimulation regardless of Th1 or Th2 polarising conditions [153].

Th17 cells are a recently discovered type of effector T cell [154, 155]. These produce high levels of IL-17, as well as TNFα and have been predominantly associated with aggressive autoimmune diseases such as Inflammatory Bowel Disease [156]. Th17 cells were recently identified as a distinct lineage of helper T cells and IL-23 production (a cytokine made up of IL-12 p40 with the IL-23 p19 homologue of IL-12 p35) was implicated in promoting Th17 responses [154, 155]. It now appears that TGFβ plays an instructive role in Th17 differentiation [102] in combination with IL-6, whilst IL-23 is critical for the survival and expansion of Th17 clones.

Th2 cells produce IL-4, IL-5 and IL-13. Th2 responses are critical for the clearance of large eukaryotic parasites such as the gut nematodes ascaris and trichuris [69]. The link between DC maturation and Th2 induction has been relatively elusive. Th2 inducing stimuli such as Schistosome Egg Antigen (SEAg) are poor inducers of DC maturation both at the level of costimulatory

molecules and in cytokine production [157]. The presence of IL-4 is suggested to play a critical role as IL-4 deficient, and STAT6 deficient mice have a severe defect in Th2 development (reviewed in [158]) and in clearance of gut nematode infections [159]. C-Maf and Mel18 interact with GATA3 and mice deficient in any of these proteins have defective Th2 responses [158]. Recently there has been significant interest in the role of Notch signalling in Th2 specification (reviewed in [152]).

1.4.1 Notch-ligands and Signalling

Interest in the role of Notch-ligands in T cell differentiation has been ignited by a recent claim that Jagged-like ligands may be the elusive dendritic cell factors that drive Th2 differentiation [160]. However, this is still some way from being conclusively proven.

There are 4 mammalian Notch receptors (Notch1-4) and 5 ligands (Jagged-1, Jagged-2, DLL-1, DLL-2 and DLL-4) (reviewed in [161]). Variable levels of ligands and receptors are expressed by both T cells and antigen-presenting cells (reviewed in [161] [152]). Notch activation occurs when the receptor binds to a ligand presented on an adjacent cell. Proteases of the ADAM/TACE family then mediate cleavage of Notch at the extracellular S2 site. S2-cleaved Notch is endocytosed by the T cell and then further cleaved by the γ-secretase complex (S3 cleavage) releasing the intracellular domain of Notch (NICD) into the cytosol (reviewed in [162] [161]). NICD translocates into the nucleus where it associates with RPBJκ/CSL and forms protein complexes that activate transcription from particular promoters (reviewed in [161]).

There is now strong evidence for the modulation of mRNA levels of Notch-ligands in APC on microbial recognition [32] [160] as well as some evidence for modulation at the protein level (chapter 4). The different Notch-ligands differ significantly in their extracellular and intracellular domains but all contain the conserved DSL motif, required for Notch-binding, and a number of EGF repeats (reviewed in [161]). These differences in structure are at least partially responsible for observed differences in binding of Notch-ligand-Fc-fusion-

proteins to naïve T cells. DLL4-Fc strongly stains murine naïve T cells, whilst DLL1-Fc gives intermediate staining and Jagged-1-Fc mediates only very weak staining as compared to isotype controls [163]. The ability of the immobilised recombinant ligands to bind T cells correlates well with the induction of classical notch signalling (reviewed in [152]).

The Notch receptors and ligands are heavily glycosylated and regulation of this glycosylation can affect the affinity of Notch/Notch-ligand interactions [164]. Fringe (O-fucose-β1,3-N-acetylglucosaminyltransferases) proteins add β1,3-N-acetylglucosamine residues onto extensively O-fucosylated EGF repeats of Notch receptors, altering interactions with Delta-like ligands and Jagged-ligands [164]. Effects of Fringe expression are however difficult to predict as even when considering Notch-1 in isolation the three mammalian Fringes can either increase or decrease Notch-1 signalling in response to Jagged-1, although all three Fringes increase the sensitivity of Notch-1 to Delta [164]. The effect of Fringe on Notch-signalling appears to act at the level of induction of S2 cleavage rather than inhibition of binding suggesting that Fringe expression is not responsible for the variable binding of purified Notch-ligands to naïve T cells [163]. At the time of writing, published data on the regulation of Fringe expression in T cells was not available.

Simply ligating Notch is insufficient for activation. Studies on Drosophila mutants suggest that dynamin-dependent endocytosis of the ligand is required to activate signalling through the Notch receptor [162]. Mice have been generated which carry a deletion of the E3 ubiquitin-ligase Mind bomb 1 which is implicated in ligand endocytosis [165]. Homozygous embryos die at E11.5 due to multiple defects attributable to deficient Notch signalling. Cells from mutant embryos show an accumulation of DLL1 at the cell surface, compared to predominant perinuclear distribution in wild-type cells, demonstrating a defect in ligand endocytosis that phenocopies the defect in drosophila lacking Mind bomb [165]. It is unclear whether the requirement for ligand endocytosis is a physical requirement for S2 cleavage of Notch or whether recycling through endosomes is necessary for the generation of active Notch-ligands [162]. Free,

soluble ligands can antagonise Notch activity induced by both membrane-bound Notch-ligands and plastic-bound Notch-ligands, apparently by allowing S2, but not S3 cleavage to take place [162] [166]. Intriguingly, S3 cleavage requires endocytosis of S2-cleaved Notch, possibly due to the localisation of γ-sectretase complexes within endosomes (Reviewed in [166] [162]).

1.4.2 Notch-ligands and T cell differentiation

A high profile paper recently implicated Jagged-1 and Jagged-2 as Th2-inducing ligands induced on DC by typical Th2 stimuli whilst DLL1 and DLL4 acted as Th1-inducing ligands [160]. This was concluded as a results of overexpressing either human Jagged-1 or DLL1 on a mouse fibroblast cell line previously engineered to express murine MHC-class II and B7 molecules (DCEK cells). Ectopic expression of human Jagged-1 induced Th2 differentiation of naïve T cells, DLL-1 expression induced Th1 differentiation [160]. In addition, Jagged-2 upregulation correlates with DC recognition of classic Th2 stimuli such as Cholera toxin and Prostaglandin E2, whilst DLL4 is upregulated by strong Th1-inducing stimuli [160]. The authors' statement that Jagged-1 and Jagged-2 are functionally equivalent is slightly dangerous generalisation in this context as the primary structure of these ligands is relatively divergent (reviewed in [161]). More compelling is the observation that Th2 differentiation in the presence of IL-4, a strong Th2 polarising stimulus, is impaired in RBPJκ-/- T cells and that expression of the Notch1 ICD in T cells can upregulate IL-4 and GATA3 expression and down-regulate T-bet [160].

A role of DLL-1 in Th1 polarisation has been suggested previously using DLL1-Fc fusion proteins to promote Th1 responses *in vitro* [167]. In addition, treatment of BALB/c mice with DLL1-Fc at the site of infection with *Leishmania major* resulted in increased Th1 responses and improved pathogen clearance. The authors demonstrate that Fc-receptor binding by the DLL1-Fc is critical for the enhancement of Th1 differentiation, presumably allowing DLL1-Fc endocytosis and therefore full Notch-cleavage [167].

Notch-1 is recruited into the immunological synapse where it can participate in signalling for T cell activation [167]. Further support for a role of Notch in Th2 priming was obtained using T-cell specific expression of dominant-negative MAML to block classical Notch-signalling [168]. This was found to impair Th2 differentiation *in vitro* and *in vivo*. In addition, mice expressing DN-MAML showed significantly delayed clearance of a gut nematode infection due to impaired Th2 responses [168] suggesting that this is physiologically relevant to Th2 differentiation.

This picture of the literature is slightly selective. Several papers propose that Jagged-1 expression by APC leads to the induction of antigen-specific regulatory T cells [169] [170]. It has also been suggested that Notch-ligands modulate CD25 expression and therefore IL-2 sensitivity of T cells [171], and that Notch-1 signalling increases the production of IFNγ, IL-4, IL-5, IL-10, TNFα and IL-2 [167]. Contrary to many of these observations, T cell-specific deletion of Notch-1 has no effect on T cell differentiation [172], although this is easily explained by invoking redundancy between the 4 Notch-receptors.

Confusion in the literature arises from the widely different models employed by different groups, for example the use of dendritic cells or B cells as antigen presenting cells, and use of total splenocytes versus purified T cells as the responders. In addition, use of γ-secretase inhibitors to block Notch signalling yields variable results depending on the particular inhibitor used [167] which suggests variable selectivity of these inhibitors. Careful genetic dissection of the Notch receptors and ligands required in T cell differentiation induced by different microbial stimuli will be important in establishing the actual effects of Notch signalling in these processes.

1.4.3 Is a "third cell" required for T cell differentiation?

The combination of regulatory T cells with dendritic cells making inflammatory cytokines biases the development of Th17 cells in a TGFβ-dependent manner

[102]. It is interesting to note a slightly earlier observation that TLR-mediated activation of DC induced resistance to T_{reg}-mediated suppression in a process partially dependent on IL-6 [173]. In both Th1 differentiation where NK cells are proposed to be an initial source of IFNγ for T cell priming [151]and in Th17 where regulatory T cells induce TGFβ production for Th17 priming [102], there is evidence that at least 3 cell-types contribute to the instruction of T cell fate. As of yet the 3rd cell has not been positively identified in Th2 differentiation but several innate immune cells are reported to produce IL-4 directly in response to SEAg, most notably basophils (reviewed in [174]). This hints that the robust induction of appropriate effectors *in vivo*, when compared to highly variable results *in vitro*, may reflect the absence of reinforcing cells that respond to mature DC by helping to polarise differentiating T cells.

1.4.4 T cell differentiation induced by Zymosan

Dendritic cell recognition of zymosan leads to the upregulation of MHC class II and costimulatory molecules B7.1 and B7.2 thus making DC competent to induce the proliferation of antigen-specific T cells [96]. In addition a broad spectrum of factors that could contribute to T cell differentiation are produced such as IL-10, IL-23, IL-2 and IL-6 (data presented in chapter 4). The effector function of these T cells has typically been reported as mixed Th1 and Th2 [90], with further reports that use of zymosan as an adjuvant *in vivo* actually induces dominant tolerance to co-injected antigen [175]. *In vivo* experiments with zymosan are complicated by the particulate nature of zymosan. Particles injected intravenously are almost exclusively taken up by granulocytes (Dr MN Nolte, unpublished observations 2004) suggesting that DC activated in this situation may be indirectly activated, correlating with the increased tolerance to secondary challenge observed [175]. *In vitro* the situation is clearer as purified cell populations are used and heat-inactivated serum minimises any effects of complement fixation that will occur on the surface of zymosan *in vivo*. In experiments by Manickasingham et al [90], zymosan stimulation of any of the splenic DC subsets did not particularly bias the development of Th1 or Th2 cells.

Of the DC factors induced by zymosan, I am intrigued by IL-10, IL-2 and the Notch-ligand Jagged-1. As will be described in chapter 4 of this thesis, the levels of IL-10, IL-2 and Jagged-1 are much higher in zymosan-stimulated DCs that in DCs sitmulated with conventional TLR ligands.

IL-2 has controversial roles in T cell differentiation. It can be produced by Th1 cells but can also promote Th2 polarisation. IL-2 is essential for the maintenance of regulatory T cells in the periphery suggesting DC-derived IL-2 may play a role in the crosstalk between naïve T cells, DC and T_{regs} [176],[177]. IL-2 can also promote NK cell recruitment and activation, potentially modulating the "3rd cells" to promote Th1 differentiation [178]. IL-2 is also necessary for the induction of Th2 responses to *Nippostrongylus brasiliensis* as determined by antibody production [179]. Administration of IL-2 with anti-CD40 to mice infected with the pathogenic fungus *Cryptococcus neoformans* increased survival times in an IFNγ-dependent but CD4-T cell independent manner, further hinting at a role for NK cell activation by IL-2 in anti-fungal responses. Despite all of these reported functions, it has also been found that DC-derived IL-2 is apparently completely dispensable for T cell polarisation when LPS is used as the adjuvant *in vivo* [180].

IL-10 is typically associated with suppression of inflammatory responses and inhibition of Th1 induction (reviewed in [134]). IL-10 deficient animals develop enterocolitis [181], suggesting that IL-10 may play a role in control of microflora and/or regulation of inappropriate responses to microflora in these locations. IL-10/ IL-23 p19 doubly deficient mice, but not IL-10/ IL-12 p35 doubly deficient mice, have a milder disease phenotype than IL-10-deficient mice, attributable to defective Th17 differentiation [182]. IL-17-producing T cells are over-represented in ulcerative colitis or Irritable Bowel Disease patient samples [156]. This suggests that excessive Th17 differentiation contributes to pathology in IL-10-/- animals. As with many models of gut inflammation, it is yet to be determined whether the pathology is due to failure to control the expansion and gut lumen restriction of commensals or to inappropriate inflammatory responses to normally abundant and normally localised

commensals. IL-10 may act to limit the aggressive immunopathology that can arise from excessive Th17 induction. IL-10 induction via TLR2 in response to *Candida* appears to delay clearance of the infection, in keeping with its ability to limit inflammatory immune responses [183]. It is paradoxical that pattern recognition leads to worse clearance of a pathogen. A possible reconciliation of these data is suggested by Montagnoli et al [184] who propose that IL-10 producing DC turn on CD4+,CD25+ T_{regs} which are necessary for acquired resistance to reinfection.

The role of Jagged-1 in T cell differentiation has been suggested in a number of T cell differentiation assays *in vitro*. When overexpressed on DC-EK cells, Jagged-1 led to Th2 differentiation of AND T cells specific for pigeon cytochrome C [185]. When used as a soluble ligand, ligand immobilised on plastic, or when over-expressed on B cells Jagged-1 can induce the differentiation of regulatory T cells [152, 170, 186].

The majority of the clues suggest that zymosan has the ability to induce patterns of T cell differentiation that differ from those induced by pure TLR-ligands due to the induction of particular factors by TLR-independent pattern recognition receptors. The exact nature of this response is being worked on currently, and the initial results are presented in chapter 6.

1.4.5 Immunology of Yeast Infections

Yeasts infections can be divided into those that infect healthy individuals, i.e. are primary pathogens; and those which only infect immunocompromised individuals, i.e. are opportunistic pathogens. Primary pathogens include *Coccidoides immitis, Histoplasma capsulatum*, Blastomyces and Paracoccoides and are extremely rare [187]. The opportunistic pathogens, *Candida albicans, Aspergillus fumigatus*, Trichosporon, and Fusarium, are increasingly common infections at least partly due to the increased numbers of immunocompromised individuals [187]. Susceptibiltiy to systemic opportunistic infection is associated with prolonged corticosteroid use or generalised leukopaenia,

particularly neutropaenia, (mycoses are commonly lethal in these patient groups) with lymphopaenia alone providing only a small increased risk above that of the general population. This suggests that the innate immune system normally prevents the invasion of yeasts through mucosal barriers. However, superficial yeast infections show a high incidence in lymphopaenic patients, to the extent that oral Candidiasis is now almost diagnostic for HIV. It seems likely that a functional T cell response is essential to control superficial yeast infections (reviewed in [188] [187]). In addition to systemic and opportunistic mycoses, fungi are associated with a number of severe allergic disorders in humans including allergic broncho-pulmonary aspergillosis and A. fumigatus-related allergic asthma [187]. Additionally, anti-*Saccharomyces cerevisiae* antibodies are considered diagnostic for Crohn's disease but do not fluctuate with disease severity leading to the hypothesis that a propensity to develop inappropriate responses to yeasts is linked to the genetic susceptibility for Crohn's disease [189].

Predominant Th1 responses are associated with clearance of fungal infections. It has been observed that IL-12 treatment of BALB/c mice during Coccidoidies infection aids recovery [190] putatively by favouring Th1 responses. More recently it has been reported that IL-17-deficient mice are very susceptible to Candida infection implying a role for Th17 cells [191]. Before work on Th17, it was observed that TGFβ production by splenocytes after *Candida albicans* infection correlated with clearance of the infection [192]. Blockade of TGFβ signalling during infection with a live-vaccinating strain severely impaired acquired resistance of infection with virulent Candida [192]. We now know that TGFβ can play an instructive role in Th17 differentiation [102]so it seems possible that the observed effect of TGFβ in this system was on induction of Th17 differentiation. It is therefore of considerable interest whether zymosan-stimulated DC are particularly good at inducing Th17 differentiation.

Fungal β-glucans can induce anaphylactic responses if released into the blood stream and cardiac arteritis is common in patients with disseminated mycoses [42], [187]. Whether this is a due to direct recognition of β-glucan or to cross-

linking of previously induced anti-β-glucan antibody has not been investigated. However, combined with the observation of allergic responses (pathological Th2 responses) to yeasts it appears that yeast-recognition is capable of inducing strong antibody and/or Th2 responses in certain circumstances.

Anti-β-glucan antibodies induced by immunising mice with laminarin (a soluble β-glucan) are protective against systemic and vaginal candidiasis [193] implying that β-glucan binding antibody allows successful clearance of yeast cells. The same antibody provides powerful protection from reinfection. Most protective function was associated with the IgG fraction, rather than IgM fraction of anti-β-glucan antibody, although detailed isotyping was not carried out.

In summary, successful control of yeast infections that cannot be contained by innate immune mechanisms apparently requires either a Th1 or Th17 response, and whilst Th2 responses are found these are generally detrimental to the host.

1.4.6 Questions Addressed in this Thesis

My work was started in order to define the precise mechanisms by which recognition of yeast PAMPs leads to a particular mature dendritic cell phenotype. Experiments are described that aim to link the activation of DC by zymosan to the induction of T cell differentiation. The questions addressed can be summarised as:

1. What T cell polarising factors (cytokines and Notch-ligands) do zymosan-stimulated dendritic cells produce?

2. What are the respective contributions of TLR-dependent and TLR-independent pattern recognition in this response?

3. What signalling components are necessary for the TLR-independent induction of IL-10 and IL-2 in response to zymosan?

4. How might this relate to T cell priming?

2 Chapter 2: Materials and Methods

2.1 : Reagents

2.1.1 : Common Buffers, Solutions and Media

PBS-Dulbeccos: (GIBCO-BRL, Gaithersburg, MD)

PBS-EDTA: PBS-Dulbeccos containing 2mM EDTA (Sigma, Poole, UK)

MACS-Buffer: PBS-Dulbeccos containing 2mM EDTA (Sigma) and 1% FCS (Batch 154-161457: Autogen Bioclear, Mile Elm Calne, UK)

RPMI 1640 medium: (GIBCO-BRL)

R10: RPMI 1640 medium supplemented with 10% FCS (Batch 154-161457: Autogen Bioclear), Penacillin 100U/ml, Streptomycin 100U/ml, L-Glutamine 0.3μg/ml (GIBCO-BRL), 50μM β-mercaptoethanol (GIBCO-BRL)

Dulbeccos Modified Eagle Medium – DMEM: (GIBCO-BRL)

D10: DMEM (GIBCO-BRL) supplemented with 10% FCS (Batch 154-161457: Autogen Bioclear), Penacillin 100U/ml, Streptomycin 100U/ml, L-Glutamine 0.3μg/ml (GIBCO-BRL), 50μM β-mercaptoethanol (GIBCO-BRL)

RBC lysis buffer: 155mM NH_4Cl (Sigma), 10mM $KHCO_3$ (Sigma), 0.1mM EDTA (Sigma), MilliQ H_20, pH 7.0-7.2, sterile filtered.

PBS (For analytical assays only): 8g NaCl, 0.25g KCL, 1.43g Na_2HPO_4, 0.25g KH_2PO_4, (all from Sigma) dissolved in 1l dH_2O, pH 7.2. Solution is autoclaved before use.

FACS buffer: PBS, 5mM EDTA (Sigma), 1% FCS (Autogen Bioclear), 0.02% NaN_3 (w/v) (Sigma)

ELISA block: PBS, 2.5% FCS (Autogen Bioclear), 0.02% NaN_3 (w/v) (Sigma)

ELISA coating buffer: 0.1M NaHCO3 (Sigma) in dH2O, pH 8.2

ELISA wash: 0.05% Tween-20 (Sigma) in PBS

TAE buffer: 0.04M Tris-acetate (Sigma), 0.002M EDTA (Sigma)

2.1.2 : Microbial Stimuli

MALP2, Pam3Cys, and Zymosan were purchased from InvivoGen (San Diego, CA) and resuspended as per the manufacturer's instructions. Curdlan, purified from *Alcaligenes faecalis* was purchased from Wako Pure Chemicals Industries Ltd (Osaka, Japan). Highly purified Salmonella LPS was a kind gift from Dr S. Vogel (University of Maryland, Baltimore, MD). Flagellin was purchased from Alexis Biochemicals (San Diego,CA). CpG 1668 was synthesised by the Cancer Research UK in-house oligonucleotide synthesis service as: TCCATGACGTTCCTGATGCT – all phosphorothioate linked and HPLC-purified.

2.2 : Cells and Mice

2.2.1 : Mice

Wild type C57BL/6 (H-2b) and congenic B6.SJL CD45.1 mice were from the Cancer Research UK animal facility (Clare Hall, South Mimms, UK). OT-II mice bearing a transgenic TCR specific for OVA323-339 presented on I-Ab were a gift from Dr. A. Pirel (Transgenic Alliance, L'Arbresle, France) and are bred at the Cancer Research UK animal facility (Clare Hall, South Mimms, UK) [194]. Mice genetically deficient in MyD88 were a king gift from Dr S Akira [195] and are are bred at the Cancer Research UK animal facility (Clare Hall, South Mimms, UK).

ROSA-YFP mice [196] were a kind gift from Dr R Adams (Cancer Research UK London Research Institute, London, UK).

Sap1-/- [197] and Elk1-/- [198] mice were analysed in collaboration with Dr R Treisman and Dr P Costello (Cancer Research UK London Research Institute, London, UK). Mice heterozygous for deletion of Net [199]were a gift from Dr B Wasylyk and used in breeding double and trible knock-out animals. Net-/- mice die in utero. c-Fos-/- ([200] a kind gift of Dr A. E. Grigoriadis); Sap1,Elk1 double knock-out; and Sap1, Elk1, Net triple knock-out foetal livers were used to reconstitute lethally irradiated B6.SJL hosts as described in 2.2 to generate chimeras as part of the same collaboration.

Syk-/- [201] and litter-mate control foetal liver was a kind gift from Dr V. L. Tybulewicz (National Institute for Medical Research, Mill Hill, UK).

2.2.2 : Bone marrow chimeras

Host mice were kept on acidified drinking water (0.1ml conc.HCL in 840ml water, sterile filtered) for at least one week before irradiation and then for at least 4 weeks after reconstituition. Mice were γ-irradiated using a ^{137}Cs source (IBL 637; CISbio International, Gif-sur-Yvette, France) with two doses of 5.5 Gy (1.24 Gy/min) separated by 3hrs. Mice were reconstituted within 24 hrs with $> 10^6$ bone marrow or foetal liver cells in PBS. The health status of the mice was closely monitored after irradiation and reconstituition. Six weeks after reconstituition blood samples were taken for FACS analysis of congenic marker expression on granulocytes and T cells. Typically close to 100% of the granulocytes were of donor origin at this time-point.

2.2.3 : Isolation of conventional DC from spleen

Freshly isolated spleens were injected with serum-free medium containing Liberase C1 (1.7 Wunsch-U/ml, Roche Diagnostics, Lewes, UK) and DNase I (0.2 mg/ml, Roche Diagnotics) and incubated at 37°C for 30mins to digest extracellular matrix. Digested spleens were mashed through a 50 μm cell sieve

and washed twice with cold MACS buffer (PBS-Dulbeccos (GIBCO), 2mM EDTA (Sigma), 1% FCS (Autogen Bioclear)). Splenocytes were labelled with CD11c-MACS beads (clone N418, Miltenyi Biotec, Bisley, UK) for 10mins at 4°C. After washing in cold MACS buffer CD11c cells were positively selected using either the autoMACS or varioMACS. (Miltenyi Biotec). LS columns were used for varioMACS selection according to the manufacturer's instructions. A "possel" program was used on the autoMACS as per the manufacturer's instructions.

2.2.4 : Isolation of CD4 T cells

T cells were isolated from pooled lymph node and spleen of either OT-II or C57BL/6 mice. Spleen and lymph nodes were digested with Liberase C1 (1.7 Wunsch-U/ml, Roche Diagnostics, Lewes, UK) /DNaseI (0.2 mg/ml, Roche Diagnotics) in serum-free medium for 15mins at 37°C. A cell suspension was produced by mashing lymph nodes and spleen through a 50μM cell strainer. Recovered cells were washed twice in ice-cold MACS buffer. A lineage-depletion cocktail was used to stain the cells. All antibodies were used at 1:100 and were purchased from PharMingen, (Becton-Dickinson, Oxford, UK)

Antibody	Clone
FITC-anti-CD8α	53-6.7
FITC-anti-TCRγδ	GL3
FITC-anti-B220	RA3-6B2
FITC-anti-CD16/CD32	2.4G2
FITC-anti-I-Ab	25-9-17
FITC-anti-CD11c	HL3
FITC-anti-CD11b	M1/70
FITC-anti-Gr-1	RB6-8C5

Aftger 10mins staining at 4°C cells were washed in ice-cold MACS buffer and resuspended in anti-FITC multisort MACS beads (Miltenyi Biotec) for 10mins at 4°C. Cells were then washed and a "depletes" autoMACS program was used to remove FITC-positive cells. The depleted fraction was incubated with

"release reagent" (part of the FITC-multisort kit, Miltenyi Biotec) for at least 30mins at 4°C. After washing, the remaining cells were incubated with Bt-anti-CD4 (PharMingen) for 10mins at 4°C, followed by washing and incubation with Streptavatin-MACS beads (Miltenyi Biotec). CD4+ cells were enriched using the "possel" autoMACS program. Typically the final enriched fraction comprised >95% CD4+ T cells.

Where used, CFSE labelling was carried out directly after isolation. Enriched cells were washed and resuspended in PBS containing 2μM CFSE (Molecular Probes, Invitrogen, Paisley, UK). After 15mins incubation at 37°C, FCS was added to 20% to quench extracellular CFSE. Cells were then washed twice in 10% FCS and resuspended in complete medium.

2.2.5 : Production of GM-CSF BMDC

Mice femurs and tibias were removed under sterile conditions and bone marrow was flushed out with R10 and a 23G needle syringe. The resulting cell suspension was strained through a 70μm cell sieve and resuspended in 10ml R10 per mouse and plated at 10ml per 10cm TC dish (Falcon, Becton-Dickinson, Oxford, UK). After 30mins at 37°C non-adherent cells were recovered into 24ml R10 supplemented with GM-CSF (GM-CSF was made by the Cancer Research UK protein purification service, and batches were titrated to give optimal growth conditions for BM-DCs) and plated in a 6 well plate (Falcon). After 48hrs incubation at 37°C, 5% CO_2 2.5ml of medium is removed from each well and replaced with 3ml R10 supplemented with GM-CSF and cells are incubated overnight at 37°C, 5% CO2. The next day nonadherent and loosely-adherent cells are removed by gentle washing with culture medium. The remaining cells consist of an adherent macrophage-like population and small round DC progenitors. 4ml of complete R10 supplemented with GM-CSF is added to each well. DC can be used up to 2 days later. DC harvested at 120hrs will give a yield of approximately 10^7 cells per mouse.

BMDC are routinely MACS enriched for CD11c before use. Breifly, BMDC are harvested by gently scraping the cells with the plunger from a sterile 1ml syringe. Cells are labelled with CD11c MACS beads (30µl beads per 10^7 cells Miltenyi Biotec) and enriched using MACS LS columns according to the manufacturer's instructions.

2.2.6 : Production of Flt3L BMDC

Mice femurs and tibias were removed under sterile conditions and bone marrow was flushed out with R10 and a 23G needle syringe. The resulting cell suspension was strained through a 70µm cell sieve and all cells except red blood cells were counted. Bone marrow cells are resuspended at 1.5 x 106 cells per ml in R10 supplemented with 50ng/ml FLT3L (R&D systems, Abingdon, UK) and seeded at 5ml per well of a 6 well plate (Falcon).

5 days later 4.5ml of supernatant is removed without disturbing the cell layer and 5ml of fresh R10 containing 50ng/ml FLT3L was replaced. On day 8 the partial medium exchange is repeated as on day 5. Day 10 FLT3L BMDC can be harvested and used, or a partial medium exchange can be performed as on day 5, and cells used on day 11 (with lower yields of B220+ PDC-like cells)

2.2.7 : Cell lines

NIH 3T3 fibroblasts were maintained in R10, 37°C, 5% CO_2 and split regularly. CD40L-fibroblasts were a kind gift from Drs R. Lapointe and P. Hwu (NCI, Bethesda, MD) They were derived by transducing NIH-3T3 with a retroviral vector expressing CD40L from the CMV promoter. SAMEN-3T3 were produced in parallel by transduction with the empty vector.

Two cell lines were used for retroviral production. GP293 cells (Clontech, Becton-Dickinson, Oxford, UK) are 293 cells stably transfected with a gag-pol expression vector and are maintained in D10. Phoenix-ecotropic cells were obtained from Dr. G Nolan and express the gag, pol and env genes from murine

Maloney Leukaemia virus. Phoenix cells were grown in D10 or R10 as indicated.

LK35.2 cells [202] (LK cells, American Type Culture Collection HB-98) were maintained in D10. Full-length, truncated, and point mutant Dectin-1 in pFB-Neo [55] or subcloned into pMSCV-EGFP ([203]; kind gift from Hideki Tsujimura, National Institutes of Health) were used for transfection of LK cells or for retroviral production in an ecotropic packaging cell line [204], followed by LK cell transduction. LK cells expressing Dectin-1 were selected on the basis of EGFP expression (pMSCV-EGFP vector) or neomycin resistance (pFB-Neo vector).

2.3 : Retrovirus production

2.3.1 : "Ping-pong" retrovirus production

GP2-293 cells (Clontech) are seeded at 2.6 x 10^6 per 10cm dish. The following day cells are transfected with retroviral vector and pVSV-G (23μl of Fugene is added to 557μl serum-free RPMI and incubated for 5mins. 7.5μg retroviral vector and 6μg of pVSV-G is added to a second tube and Fugene mixture is added drop-wise. After 15mins incubation at room temperature the transfection mix is gently added to the GP2-293 cells). The following day 2.5 x 10^5 Pheonix-ecotropic cells are plated in a well of a 6 well plate. 24 hrs later, supernatants are harvested and fresh RPMI is added back to the cells. The harvested supernatants are filtered through a 0.45micron Millex-HV PVDF filter (Millipore, Cork, Ireland) and polybrene (Sigma) is added to a final concentration of 5μg/ml. This virus-containing supernatant is used to replace the Phoenix cell medium. Phoenix cells are then centrifuged at 2600rpm at 26°C for 90 minutes. After centrifugation, supernatant is removed and cells are cultured overnight. Infection with GP2-293 virus-containing supernatant is repeated the following day. The resulting Phoenix-ecotropic cells stably produce retrovirus containing the introduced retroviral vector segment. If the viral vector contained a FACS-selectable marker such as GFP, stably transduced Phoenix cells can be FACS sorted to increase the frequency of viral producers.

2.3.2 : Transient transfection of GP2-293 cells

GP2-293 cells (Clontech) are seeded at 2.6 x 10^6 per 10cm dish (Falcon). The following day cells are transfected with retroviral vector and pVSV-G (encoding the envelope protein of Vesicular Stomatosis Virus).

23µl of Fugene6 (Roche Diagnostics, Mannheim, Germany) is added to 557µl serum-free RPMI and incubated for 5mins. 7.5µg retroviral vector and 6µg of pVSV-G is added to a second tube and Fugene mixture is added drop-wise. After 15mins incubation at room temperature the transfection mix is gently added to the GP2-293 cells. At 48hrs and 72hrs post-transfection supernatants containing VSV-G pseudotyped virus are harvested, sterile filtered using 0.45micron syringe-filters (Millipore) and snap-frozen on dry ice.

Retroviruses that include GFP-expression can be easily titred on NIH-3T3 fibroblasts. 10^5 fibroblasts are plated per well of a 6 well plate and rested for 24hrs at 37°C, 5% CO2. Polybrene is added to the wells to a final concentration of 5µg/ml and 10 or 100µl of virus-containing supernatant is added. After 24hrs the medium is exchanged and % transduction is determined by FACS for GFP expression.

2.4 : Retroviral transduction of bone marrow

2.4.1 : Retroviral transduction of GM-CSF BMDC

4 x 106 stably-transduced Phoenix cells are plated in a 15cm tissue culture dish (Falcon) in 18ml R10 and incubated at 37°C, 5% CO_2. A 50µg/ml solution of Retronectin (Takara Mirus Bio, Madison, WI) is made in endotoxin-free water (GIBCO-BRL) and 100µl is added per well of a 24 well plate, the plate is sealed using Parafilm (Peichiney Plastic Packaging, Menasha, WI) and incubated overnight at 4°C.

After 18-24hrs (day 1) supernatant is harvested from Phoenix cells and fresh medium is replaced. Bone marrow is harvested as for the normal BMDC

protocol (see 2.2.5), then red blood cells are lysed in hypertonic buffer and cells are resuspended in virus supernatant (48ml per mouse) plus polybrene. 2ml of cell suspension is plated per well of the prepared retronectin-coated 24well plate. Plates of bone marrow are centrifuged ("spinfected") at 2600rpm (1573g), 26°C, 90mins (Allegra X-15R, Beckman-Coulter, Paulo Alto, CA). Following spinfection, supernatants are removed and spun to recover non-adherent cells. These are returned to the 24 well plate with fresh R10 supplemented with GM-CSF and bone marrow cells are incubated at 37°C, 5% CO_2 overnight. On day 2 Phoenix cell supernatant is harvested and fresh medium is added back to the phoenix cells as on day 1. Supernatants are removed from the BMDC to a 50ml Falcon tube and retained. 2ml of virus supernatant supplemented with 5μg/ml polybrene is added to each well and the plate is centrifuged at 2600rpm, 26°C, for 90mins. After centrifugation the viral supernatants are removed and non-adherent cells are recovered as on day 1 into the retained BMDC supernatant. On day 3 BMDC supernatant is removed and discarded. Phoenix cell supernatant is harvested as on day 1 and phoenix cells can either be split for further passaging or discarded. 2ml of viral supernatant containing 5μg/ml polybrene is added per well and the centrifugation step is carried out as on day 1. After 90 minutes virus supernatant is removed, non-adherent cells are recovered and fresh R10 supplemented with GM-CSF is replaced. On day 4 non-adherent and loosely-adherent cells are removed by gentle washing with culture medium and fresh R10 supplemented with GM-CSF is replaced. BMDC are used on day 5 or day 6. Transduction efficiency with MSCV-EGFP is typically greater than 80%. (For a detailed discussion of this procedure see chapter 3)

2.4.2 : Retroviral transduction of FLT3L BMDC

The protocol for transduction of Flt3L BMDC is an adaptation of the protocol for transduction of GM-CSF BMDC. The specific adaptations are that 50ng/ml FLT3L (R&D systems, Oxford, UK) is used in place of GM-CSF and the BMDC supernatants are retained and replaced after each spinfection, rather than adding fresh supernatant on day 3. After day 3 the protocols diverge. FLT3L DC are left undisturbed at 37°C 5% CO2 on day 4 and day 5. On day 6, 4.5ml of

supernatant is removed without disturbing the cell layer and 5ml of fresh R10 containing 50ng/ml FLT3L was replaced. On day 9 the partial medium exchanges is repeated as on day 6. Day 11 FLT3L BMDC can be harvested and used, or a partial medium exchange can be performed as on day 5, and cells used on day 12. (For a more detailed discussion of this procedure see chapter 3)

2.5 : In vitro assays for DC activation

2.5.1 : *In vitro* stimulation for DC cytokine production

4–20 × 10^4 CD11c-enriched BM-DCs per well (numbers indicated in figure legends) are cultured overnight in 100–200 µl culture medium with or without GM-CSF in 96-well flat-bottomed plates in the presence of stimuli. In some experiments CD40L-expressing fibroblasts (plated on the eve of the experiment at 10^4 cells per well) are included in order to amplify IL-10 levels via CD40 triggering [96]. Supernatants are removed, and cytokine levels measured by sandwich ELISA. For inhibition studies, cells are preincubated for 30 min with glucan phosphate (a gift from Dr D. Williams), latrunculin B (Sigma), UO126 (Calbiochem, Merck Biosceinces, Nottingham, UK), PD98059 (Calbiochem) or SB203580 (Calbiochem) before addition of zymosan (InvivoGen, San Diego, CA). Cells are subsequently are cultured in the continued presence of the inhibitors. An identical protocol is used to identify cytokine production from LK cells stimulated with zymosan.

For intracellular staining of cytokines, DC are stimulated as for measurements of secreted cytokine. After an indicated time-period in the presence of microbial stimuli Brefeldin A is added to a final concentration of 5µg/ml and cells are incubated for a further time period. For analysis DC are isolated and stained for surface markers (APC-CD11c) followed by fixation using "Fix and Perm" reagent A (Caltag). See section 2.8.2 for details of the staining procedure.

2.5.2 : *In vitro* stimulation for mRNA analysis of cytokines and Notch ligands

5 x 10^5 CD11c-enriched BMDC are plated per well of a 24 well plate (Falcon). Microbial stimuli are added and cells incubated at 37°C, 5% CO2 for the indicated times. At the end of the incubation period cells are harvested into a 1.5ml tube (Eppendorf, Fisher Scientific, Loughborough, UK). Cells are pelletted by centrifugation and the pellet is resuspended in 1ml TRIzol (Invitrogen) for RNA isolation.

2.5.3 : *In vitro* stimulation for analysis of MAP kinase activation

5 x 10^5 CD11c-enriched BMDC are plated per well of a 24 well plate. Microbial stimuli are added and cells incubated at 37°C, 5% CO_2 for the indicated times. At the end of the incubation period paraformaldehyde (Sigma) is added to the cultures to a final concentration of 2%. Cells are harvested, pelletted and resuspended in 2% FCS. These cells are then stained for FACS (For a detailed discussion of this procedure see chapter 3)

2.6 : In vitro T cell differentiation assays

CD4+ T cells are isolated as described in 2.2.4. 104 BMDC and 2-5 x 10^4 T cells are added per well of a 96 well U-bottom plate. Where OT-II T cells are used, $OVA_{323-339}$ peptide (ISQAVHAAHAEINEAGR synthesised and HPLC-purified by the Cancer Research UK peptide synthesis service) or OVA protein (Calbiochem) is added at the indicated concentrations along with microbial stimuli. In experiments with C57BL/6 T cells anti-CD3 (2C11, purified from hybridoma supernatant by the Cancer Research UK monoclonal antibody service) is added at the indicated dose along with microbial stimuli. On day 3 each well of T cell-DC co-cultures is split into a fresh well and 100µl of fresh R10 is added. On day 5 T cells are counted and total wells are restimulated for analysis of cytokine production either by plating on immobilised anti-CD3 for 48 hours or by stimulating with PMA and Ionomycin in the presence of monensin for intracellular FACS staining.

2.7 : Molecular Biology and Cloning

2.7.1 : Transformation of Competent E. Coli Strain XL-1 cells

10µl of ice-cold plasmid DNA solution was added to 100µl of DH5α competent cells (Invitrogen) in a 1.5ml tube (Eppendorf) and incubated on ice for 30mins. The tube was then transferred to a 42°C water bath for 30s before immediately returning it to the ice for 10minutes. the suspension was then transferred into a 14ml snap cap (Falcon) and diluted in 2ml of SOC-medium (Invitrogen) and incubated for 90mins on a shaker. 1.5ml of the innoculum was transferred to a fresh 1.5 tube and centrifuged to pellet the cells. The pellet was resuspended in 0.2ml LB (Cancer Research UK media services) and plated on selective agar plates. All plasmids used in this thesis contained Ampicillin resistance genes therefore L-agar (Cancer Research UK media services) plates containing 50µg/ml ampicillin were used. Plates were incubated overnight at 37°C.

2.7.2 : Plasmid isolation

Single resistant clones were picked using sterile pipette-tips and expanded in 5ml LB containing 50µg/ml ampicillin overnight at 37°C on a shaker. The cells were spun down and the pellet processed using the QIAGEN plasmid mini-kit (QIAGEN, Crawley, UK). For large-scale plasmid isolation 2ml of the overnight culture was added to 150ml of LB containing 50µg/ml ampicillin and incubated overnight at 37°C in a shaker. Overnight culture was centrifuged at 3500rpm 30mins (Allegra 6R centriguge, Beckman-Coulter), supernatants were discarded and pellets were processed using the QIAGEN plasmid maxi-kit.

2.7.3 : DNA purification using Phenol/Chloroform

The DNA sample was transferred into at least 100µl of ddH_2O in a clean 1.5ml tube (Eppendorf). An equal volume of phenol was added and the tube was vortexed for 1 minute. Then an equal volume of chloroform:isoamylalcohol 49:1 was added and the tube was vortexed for 1 minute. The rube was then spun at 13000rpm (15000g) and the upper, aqueous phase was transferred into a new Eppendorf tube. 1.5 volumes of chloroform was added and the tube was vortexed for 1 minute and centrifuged again. The aqueous phase was transferred

again into a fresh 1.5ml tube and 0.1 volumes of 3M NaAc plus 2.5 volumes of ethanol (purum, Fulka) were added. The tube was incubated at -80°C for 1hr or -20°C overnight before centrifugation for 5 minutes at 15000g. The pellet was washed once in cold 70% ethanol. The pellet was then air-dried and dissolved in ddH_2O.

2.7.4 : Gel Electrophoresis

DNA samples were prepared in TAE buffer and Sigma DNA loading buffer was added 1:10. 1 or 2% Agarose was suspended in TAE buffer and melted in a microwave oven. 1μg/ml Ethidium Bromide (Sigma) was added and the solution was poured into a gel tray with an appropriate comb. The tray was then placed in a gel chamber filled with TAE buffer and a voltage of 30-120V applied for 0.5 to 2hrs.

2.7.5 : Restriction Enzyme digests

Restriction Enzyme digests were carried out to re-clone GFPCre downstream of the pgk promoter in pMSCV and to check the identity of plasmids obtained during the cloning and of plasmids obtained from outside sources. Restriction Enzymes, Buffers and BSA were purchased from New England Biolabs (Ipswich, UK) and used according to the manufacturer's instructions. Briefly 5μl of plasmid solution (containing up to 2μg plasmid) was added to 2μl 10x buffer, 0.2μl BSA, appropriate amounts of enzyme to ensure complete digestion in 1-3hrs and ddH_2O to make the volume up to 20μl.

2.7.6 : Gel purification of Restriction Enzyme digest fragments

After separating the fragments by gel electrophoresis the desired band was excised using a sterile scalpel, minimising UV exposure of the DNA by working quickly. DNA was purifed from the excised gel fragment using the QIAGEN gel purification kit according to the manufacturer's instruction. 0.1ml NaAc pH 5.2 was routinely added to the dissolved gel fragment as this was found to improve yields.

2.7.7 : Purification of DNA fragment from solution

Between Restriction Enzyme digests and other situations in which DNA needed to be purified away from enzymes or resuspended in fresh buffer DNA was purified using the QIAquick PCR purification mini kit (QIAGEN) according to the manufacturer's instructions. DNA was eluted in 30µl ddH_2O.

2.7.8 : Cloning of pMSCV-GFPCre8

pMSCV-GFP (a kind gift or Dr Hideki Tsujimura, National Institutes of Health) was cut with HindIII (New England Biolabs) (just downstream of the pgk promoter) and ClaI (New England Biolabs) (downstream of GFP) to remove the GFP sequence, leading a 5kb backbone fragment including both LTRs, the pgk promoter, the multiple cloning site and an Ampicillin resistance gene. This 5kb fragment was gel purified and then treated with mung bean nuclease (New England Biolabs) according to the manufacturer's instructions to remove sticky-ends, followed by treatment with Shrimp Alkaline Phosphatase (Roche Diagnostics) used according to the manufacturer's instructions to remove 5' phosphates and therefore prevent vector self-ligation.

pMSCV-GFPCre-neo was cut with EcoRI (New England Biolabs) and BglII (New England Biolabs)to excise a 2kb fragment containing the GFPCre sequence. The 2kb fragment was gel purified as described and treated with mung bean nuclease to remove sticky ends.

Between each set of the cloning protocol when different buffers were required DNA was purified using the QIAquick PCR purification mini kit (QIAGEN) as described.

Ligations were set up using an 8:1 molar ratio of insert to vector and the T4 ligase () and incubated overnight at 25°C. After overnight ligation, the resulting DNA was transformed into DH5α competent cells (Invitrogen) and plated out on ampicillin-selective plates.

26 colonies were obtained and expanded for mini-preps as described. XhoI (NEB) digestions which cuts at the 3' end of the GFPCre sequence and in the multiple cloning site 5' of the pgk promoter to produce 2 bands of 3kb and 4kb from the correctly assembled plasmids. One clone was obtained with the correct orientation and was used for large scale plasmid production, "ping-pong" production of stable Pheonix cell lines and retroviral transduction of ROSA26-YFP bone marrow to demonstrate activity of the Cre protein.

2.7.9 : Sequencing of pMSCV-GFPCre8

DNA sequencing was carried out by the Cancer Research UK sequencing facility. Breifly, a reaction mix was made using 8µl of BigDye Terminator mix v3.1 (Cancer Research UK in-house), 2µl of sequencing primer (equivalent of 3.2pmol) and 10µl of ddH2O containing 150-200ng of plasmid (parameters for sequencing up to 10kb plasmids). The PCR reaction was carried out if a () thermal cycler using the following program:

Step 1	Denature	96°C	30s
Step 2	Anneal	50°C	15s
Step 3	Extension	60°C	4mins
Step 4	Return to step 1	x 25	
Step 5	Chill	4°C	Forever

The primers Cre 5' and Cre 3' were used to amplify the GFPCre sequence in the final vector:

Cre 5': ATCCGAAAAGAAAACGTTGA
Cre 3': ATCCAGGTTACGGATATAGT

2.8 : Flow cytometry

2.8.1 : Surface staining of DC, T cells and LK cells

For flow cytometry, cell suspensions were washed once in PBS/EDTA and stained in ice cold FACS buffer for 30mins. This procedure was repeated when secondary reagents were used. Data were collected on a FACS Calibur

cytometer (Becton Dickinson, Mountain View, CA) and analysed using FlowJo software (Treestar, San Carlos, CA) to gate on appropriate populations. All antibodies used were from Pharmingen unless otherwise indicated.

Antigen	Clone/Isotype
CD11c	HL3 / Armenian Hamster IgG1
CD11b	M1/70 / Rat IgG2b
CD16/32	2.4G2 / Rat IgG2b
Jagged-1	72017 Mouse IgG1 (R&D systems)
CD4	RM4-5 / Rat IgG2a
CD25	3C7 / Rat IgG2b

2.8.2 : Intracellular staining for DC cytokines

DC were stimulated as described in section 2.5.1. DC harvested at the end of the culture period were stained for CD11c for 20 minutes on ice (as described above). After washing in ice-cold FACS buffer, cells were resuspended in "Fix and Perm" Reagent A (Caltag) for fixation and incubated at room temperature for 20mins. Cells were washed once in FACS buffer and then resuspended in "Fix and Perm" Reagent B (Caltag) containing anti-cytokine antibodies and incubated for 20 minutes at room tempterature. If secondary reagents are used then after washing in FACS buffer, cells are suspended in "Fix and Perm" Reagent B containing the secondary reagent. Cells were washed once in FACS buffer and resuspended for acquisiton. All anti-cytokine antibodies are from Pharmingen unless otherwise stated.

Cytokine	Clone/Isotype	Concentration
IL-12 p40	C15.6 / Rat IgG1	1μg/ml
TNFα	MP6-XT3 / Rat IgG1	5μg/ml
IL-6	MP5-20F3 / Rat IgG1	5μg/ml
IL-2	JES6-5H4 / Rat IgG2b	5μg/ml
IL-10	SXC-1 / Rat IgM	5μg/ml
IFNγ	XMG1.2 / Rat IgG1	1μg/ml

IL-17	TC11-18H10.1 / Rat IgG1	1μg/ml
IL-4	11B11 / Rat IgG1	5μg/ml

An identical protocol was used for the intracellular staining of T cell cytokines after restimulation with PMA/Ionomycin in the presence of monensin (described in section 2.6)

2.8.3 : Intracellular staining for FoxP3

T cells were surface stained for CD4 and CD25 as in 2.8.1. Cells are washed in ice cold FACS buffer and resuspended in eBiosceince (Boston, MA) Fixation/Permeabilisation solution (made up to 1x from 4x concentrate with diluent). Cells are incubated at 4°C for 30mins to overnight. After this period cells were washed once in FACS buffer and once in eBioscience Permabilisation buffer (made up from 10x concentrate with ddH_2O). Cells are resuspended in Permeabilisation buffer containing PE-anti-FoxP3 (eBiosceince) and 2.4G2 (Cancer Research UK monoclonal antibody service) and incubated at 4°C for 30mins. Cells were then washed and resuspended for acquisition.

2.8.4 : Intracellular staining for Phospho-MAP kinases

BMDC are enriched for CD11c and plated at 5 x 10^5 per well of a 24 well plate in R10 supplemented with GM-CSF. Cells are rested for 3hrs to overnight at 37°C, 5% CO2. Stimuli are added for the indicated lengths of time (where chemical inhibitors are used, these are added 30minutes before the stimuli and maintained throughout the experiment). To halt signalling cells are fixed by adding parafolmaldehyde to 2% then harvesting the cells and resuspending them in PBS containing 2% paraformaldehyde. Cells are fixed for 20mins at room temperature. After washing twice in FACS-buffer cells are resuspended in "Fix and Perm" Reagent B (Caltag) containing the appropriate anti-phospho-MAP Kinase or isotype control and incubated at room temperature for 20mins. Cells are then washed in FACS-buffer and resuspended in "Fix and Perm" Reagent B (Caltag) containing the appropriate secondary antibody. If a tertiary reagent is

required (such as fluorescently labelled streptavadin) the intracellular staining step is repeated after which cells are resuspended for acquisition. Relevant isotype control stainings were always carried out in parallel to demonstrate that background staining does not change with DC stimulation (data not shown).

2.9 : Sandwich ELISAs for cytokines

All ELISAs are carried out in MAXISORP immunoplates (Nunc 4-39454). Capture antibodies are diluted into ELISA coating buffer and 50µl pipetted per well of the 96-well flat-bottom immunoplate. Plates sealed with parafilm are incubated for 6hrs to overnight at 4°C in a humidified chamber. Plates are then washed 3 times with ELISA wash and blocked for 2hrs with 200µl per well of ELISA block. ELISA block is disgarded and 50µl of experimental supernatants or serial dilutions of the test cytokine are added per well. Plates are sealed again with parafilm and incubated overnight at 4°C. Plates are washed 6 times in ELISA wash. The detection antibody is diluted in ELISA block and 100µl added per well. Plates are incubated for 1hr at room temperature and then washed 6 times with ELISA wash. Extravadin-Alkaline phosphatase (Sigma) is diluted 1:5000 in ELISA block and 100µl is added per well. Plates are incubated for 1hr at room temperature and washed 6 times with ELISA block. ELISA substrate is made up using the Sigma-fast pNitrophenyl Phosphate tablet sets and 100µl is added per well. A_{405} is measured at 15mins, 1hr and 2hrs (using a SpectraMax 190, Molecular Devices, Wokingham, UK) and Softmax Pro software (Molecular Devices Corp). Readings where measurements of experimental cytokine concentrations within the linear range of readings for the standard curve were exported to Excel (Microsoft) for further analysis. Error bars represent 1 standard deviation.

Cytokine	Capture Antibody	Detection Antibody	Top concentration of standard
IL-2	JES6-1A12 4µg/ml	JES6-5H4 Biotin 1µg/ml	5ng/ml
IL-4	11B11 2µg/ml	MM-450D Biotin (Endogen) 0.25µg/ml	5ng/ml
IL-5	TRFK5 2µg/ml	TRFK4 Biotin 2µg/ml	2ng/ml

IL-6	MP5-20F3 4μg/ml	MP5-32C11 Biotin 1μg/ml	5ng/ml
IL-10	JES5-2A5 4μg/ml	SXC-1 Biotin 1μg/ml	25ng/ml
IL-12 p40	C15.6 2μg/ml	C17.8 Biotin 1μg/ml	10ng/ml
IL-12 p70	9A5 5μg/ml	C17.8 Biotin 1μg/ml	10ng/ml
IL-17	555068 2μg/ml	666067 Biotin 1μg/ml	10ng/ml
IL-23	G2308 (eBiosceince) 2μg/ml	C17.8 Biotin 1μg/ml	8ng/ml
IFNγ	R4-6A2 5μg/ml	XMG1.2 Biotin 0.5μg/ml	300U/ml
TNFα	G281-2626 5μg/ml	MP6-XT3 Biotin 1μg/ml	2ng/ml

2.10 : Western Blotting

BMDC were lysed in SDS lysis buffer (2xSDS buffer: 125mM Tris-HCL pH 6.8, 4% w/v SDS, 2% glycerol, 0.02% w/v Bromophenol blue, 2% βmercaptoethanol). Samples were sonicated, boiled for 5mins then centrifuged at 13000rmp for 5mins. Proteins were separated on 10% Tris-glycine pre-cast mini-gels (Novex). A Transblot semi-dry transfer cell was used with methanol-transfer buffer (48mM Tris-base, 39mM Glycine, 1.3mM SDS, 20% MeOH) was used to transfer proteins onto Optitran BA-S83 Reinforced Nitrocellulose (Schleicher & Schuell, Whatman, Brentford, UK).

Ponceau red staining to check transfer and loading, was carried out at this stage. briefly, the membrane was incubated with 5ml Panceau Red staining solution (Sigma) for 3 minutes at room temperature with shaking. The blot was then washed for 3 minutes, or until a clear result was obtained, in 15ml of TBST.

Membranes were blocked with TBST/10% milk (10% dried skimmed milk powder, 1% Tween-20, 20mM Tris, 136mM NaCl, pH 7.6). Anti-phospho-ERK (Cell Signalling technology) and anti-phopsho-p38 MAPK (Cell Signalling technology) were diluted 1:1000 in TBST/10% milk and membranes were incubated overnight at 4°C with shaking. The nest day membranes were washed 3 x for 5minutes in 15ml TBST (1% Tween-20, 20mM Tris, 136mM NaCl, pH 7.6). HRP-goat-anti-rabbit Ig was diluted 1:5000 in TBST/10% milk and membranes were incubated for 1hr at room temperature with shaking. Membranes were washed 3x in TBST then incubated for 5 minutes with Supersignal WestPico Chemiluminsecent Substrate (Pierce, Perbio, Rockford, IL). The membrane was wrapped in saran wrap and exposed to X-ray film (Pierce) for 30s to 2hrs before developing.

2.11 : PCR

2.11.1 : Isolation of DNA from cells and tissues

Harvested cells were pelleted by centrifugation and resuspended in 500µl digestion buffer (100mM Tris-HCl,, pH 8.5, 200mM NaCl, 5mM EDTA, 0.2% SDS and Proteinase K 100µg/ml (QIAGEN)). Samples were digested at 55°C for 5hrs with shaking and pipetted to mix. 500µl of Isopropanol was added and the mixture was incubated overnight at -20°C. DNA was pelletted by centrifugation of 15mins at high speed and washed once in 70% EtOH. The pellet was then air-dried and resuspended in 20µl ddH_2O.

Tissue samples (for example tail or ear snips from mice) were resuspended in an identical digestion buffer and incubated at 55°C for 5hrs. After this time the tubes were centrifuged at 13000 rpm for 15minutes to pellet out insoluble/indigestible components. Supernatant was transferred to a fresh 1.5ml tube and 500µl of isopropanol added. DNA was precipitated as for extraction from cells.

2.11.2 : Typing of Jagged-1-Flox mice and cells

Jagged1-Flox/Flox mice were bred from heterozygous parent mice (a kind gift of Dr F Watt and Dr S. Etracht, Originally made by Dr K. Hozumi). Offspring

were typed by tail-snip, and DNA was extracted as explained above. In order to type for homozygous mice primers were used that generated a 389bp product with the wild-type allele and a 545pb product from the floxed-allele (Primers listed as 5JagA and 3Jag1 below).

To detect deletion of the floxed allele, cells expressing, or not expressing Cre recombinase were purified by cell sorting and DNA extracted as above. A duplex PCR was carried out using one 5' primer and two 3' primers, one of which binds between the two LoxP sites (giving a band of 229bp from undeleted cells) and the other of which binds downstream of both LoxP sites (giving a band of 333bp only when the intervening DNA has been excised by Cre). These primers are listed below as 5JagA, 3JagB and 3JagC.

All PCR reactions were performed using Cancer Research UK in-house Taq polymerase and 10x PCR buffer. Nucleotides were purchased from Invitrogen and peptides were synthesised by Sigma.

PCR reactions were carried out using a Dyad DNA engine running the following program:

Step 1: Denature	94°C	45s
Step 2: Anneal	58°C	60s
Step 3: Elongation	72°C	60s
Step 4: Repeat	To Step 1	34 x

PCR products were mixed 1:10 with DNA loading buffer (Sigma) and run on 2% Agarose gels containing Ethidium Bromide. DNA was visualised using an IMAGO Compact Imaging System (B &L Systems, Maarssen, the Netherlands)

Primers:

Name	Sequence
5JagA	TGAACTCAGGACAGTGCTCT

3Jag1	GTTTCAGTGTCTGCCATTGC
3JagB	CTAGACTCGAGGAATTCCGA
3JagC	ATAGGAGGCCATGGATGACT

2.11.3 : Isolation of RNA from cells

Cells for analysis were pelleted by centrifugation at 1000g for 5min (Beckman Coulter Allegra X-15R Centrifuge, 1500rpm). The supernatant was discarded and pellet resuspended in 1ml TRIzol (Invitrogen)(<10^7 cells per sample). Samples were incubated at room temperature for 5min then 200µl Choloform was added. Tubes were shaken vigorously by hand for 20seconds then incubated for a further 3min at room temperature. The samples were centrifuged at 12000g, 4°C for 15min. The upper phase was transferred to a new tube (approx 600µl) and 500µl Isopropanol was added. Samples were briefly mixed by inversion then incubated at room temperature for 10min. Precipitated RNA was pelleted by centrifugation at 12000g, 4°C for 15mins. The supernatant was discarded and the pellet was washed with 500µl 70% Ethanol followed by centrifugation at 7500g, 4°C for 5min. Supernatant was aspirated and the pellet was dried and then resuspended in 10mM Tris pH8.0 at 37°C.

2.11.4 : Production of cDNA

cDNA is produced using SuperScript II according to the manufacturer's instructions. Briefly, 20µl Extracted RNA is incubated with 2µl (50µg/ml) Random hexamers at 65°C for 5 minutes. The mixture is chilled on ice then 18µl of a "master-mix" containing 2µl 10mM dNTP (Invitrogen), 8µl 5 x first strand buffer (part of SuperScript II kit, Invitrogen), 4µl of 0.1M DTT (part of SuperScript II kit, Invitrogen), 3.5µl of ddH2O and 0.5µl SuperScript II is added to each tube and mixed by pipetting. The mixture is then incubated at 42°C for 1hr followed by 70°C for 15minutes to destroy the enzyme.

2.11.5 : Real-time PCR

40µl of cDNA produced as above was diluted to 100µl and 5µl added to each well of a Thermo-Fast 96 Detection Plate (ABgene, Epsom, UK). RT-PCR was carried out either using SYBR-green primers or using Taqman primers and probes (VIC-MGB/FAM-MGB or VIC-TAMRA/FAM-TAMRA).

SYBR-green analyses were carried out using the Platinum SYBR Green qPCR Supermix-UDG with ROX (Invitrogen). Primers were added to the 2x mix to a final concentration of 100nM and this 15µl of this mix was added per well of the 96 Detection Plate.

Taqman analyses were carried out using 2 x Taqman Universal PCR mastermix (Applied Biosystems). Primer/probe sets uses to generate the data present in this thesis were ordered as "pre-developed assay reagents" from Applied Biosystems and used according to the manufactures instructions.

In all cases, mRNA levels are normalised to 18S ribosomal RNA levels using Applied Biosystems PDAR for 18S containing either a VIC-MGB or VIC-TAMRA probe as appropriate to the other reactions being carried out.

After pipetting 15µl of PCR mix into the plate, the plate was sealed using an Optical Adhesive Cover (Applied Biosystems, Foster City, CA) and analysed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) and analysed using SDS 1.9.1 (Applied Biosystems) and Microsoft Excel.

Primers used:

Gene name	Primer name	Primer sequence
IL-2	MN-IL2-1F	CCTAGAGCAGGATGGAGAATTACA
	MN-IL2-1R	TCCAGAACATGCCGCAGAG
IL-6	muIL-6f	GTTCTCTGGGAAATCGTGGA

	muIL-6r	TGTACTCCAGGTAGCTATGG
IL-23 p19	MN-IL12p19-1F	TGCTGGATTGCAGAGCAGTAA
	MN-IL12p19-1R	GCATGCAGAGATTCCGAGAGA
IL-10	MN-IL10-2F	ATGCTGCCTGCTCTTACTGACTG
	MN-IL10-2R	CCCAAGTAACCCTTAAATCCTGC
TNFα	MN-TNFa-1F	CATCTTCTCAAAATTCGAGTGACCA
	MN-TNFa-1R	TGGGAGTAGACAAGGTACAACCC
Jagged1	SL208	AACGACCGTAATCGCATCGT
	SL209	TATCAGGTTGAATAGTGTCATTACTG GAA
Jagged2	SL210	AAGCCGTGTGTAAACAAGGATGT
	SL211	CCCTGCCAGCCGTAGCT
DLL1	SL202	GCAACCCCATCCGATTCC
	SL203	GAGAGTCTGTATGGAGGGCTTCA
DLL4	SL204	AAGAAAGTAGACAGGTGTACCAGCAA
	SL205	CGGCTTGGACCTCTGTTCAG

IL-12 p40, IL-12 p35, IL-2, IL-10, and 18S were assessed using Taqman PDARs (Applied Biosystems).

3 Chapter 3: Development of Methods for the Study of Cell Signalling in Dendritic Cells

3.1 : Introduction

3.1.1 : Use of Flow cytometry in cell signalling research

Flow cytometry can be carried out on small numbers of cells and using mixed cell populations. In addition, it gives an accurate quantitative reading of antibody staining on a per-cell basis. This makes flow cytometry an ideal technique for the study of cell signalling in primary dendritic cells where only 10^6-10^7 cells can routinely be obtained per mouse, and where 100% pure populations are rarely obtained (as purified in [205] [90]).

Two papers published in 2001 use phospho-specific antibodies to asses c-Jun and ERK phosphorylation [206], [207]. The paper from Chow et al examines ERK activity in human peripheral blood T lymphocytes with the aim of examining signalling in the blood T cells of patients undergoing chemotherapy, i.e. where very few cells can be obtained for analysis. Zell et al used the technique to examine signalling in TCR-transgenic T cells *in vivo* after immunisation with peptide.

Whilst Chow et al used methanol permeabilisation [206], Zell et al used 0.5% Saponin [207] in order to stain intracellular proteins. Both groups used the same anti-phospho-ERK rabbit polyclonal, available through New England Bioscience or Cell Signaling Technology, which works well on both mouse and human samples. The technique is therefore broadly applicable in mouse and human cells and two different fixation and permeabilisation methods can be used successfully (method from Zell et al available in [208]).

The panel of available phospho-specific antibodies available and validated now includes many of the MAP kinase cascade components, PI3 kinase targets, STAT proteins, antigen-receptor signalling components and more. Use of these antibodies directly conjugated to fluorophores rather than with secondary

reagents decreases the strength of the specific signal generated but allows large numbers of different phospho-specific antibodies to be combined in the analysis of signalling networks [209].

Previous studies of MAP kinase signalling in Dendritic cells using classical biochemical techniques tend to use heroic quantities of primary cells [124, 210]. The use of FACS requires roughly 10-fold fewer cells per sample increasing the feasibility of these studies and providing further information such as per-cell measurements within mixed or asynchronous populations, and correlation of different signalling pathways on a per-cell basis. The first part of this chapter describes the optimisation of conditions for analysis of phospho-MAP kinases in murine dendritic cells.

3.1.2 : Genetic manipulation of signalling pathways in dendritic cells

In order to study signalling in dendritic cells and its outcomes we would like to be able to manipulate particular pathways, or dendritic cell proteins induced as a results of signalling through known pathways. Ideally we would like to do this acutely to avoid effects on DC development and additionally, to get around the problems associated with embryonic lethality of some germ-line modifications (e.g. MKK4[211], Net [199]). Retroviruses provide an efficient technique for the stable transduction of actively cycling primary cells (reviewed in [212]).

Dendritic cells themselves are non-dividing therefore retroviral transduction must target dividing precursors of dendritic cells. This has proved very successful in the GM-CSF BMDC system, where cycling bone marrow precursors are amenable to transduction [213] [214]. Although the exact reagents and vectors used vary between labs, the general principle uses a recombinant vector in which a gene of interest is cloned between two retroviral LTRs. Non-replicating, but infective retrovirus is produced by transfecting the vector into a packaging cell line stably expressed retroviral gag, pol and env genes [204, 215].

PhoenixECO cells (provided by G.P. Nolan, Standford, CA) are a widely used retrovirus packaging cell-line. These are 293 T cells stably transfected with gag-pol and env sequences based on Moloney Murine Leukaemia Virus (MMuLV). Retroviral genes are under the control of constitutive non-viral promoters to achieve high-levels of expression, and are separated in different constructs to minimise the likelihood of recombination that could generate replicative virus. Recombinant retroviral vectors transfected into Phoenix cells transcribe the engineered insert and two Long Terminal Repeat sequences (LTRs) that allow packaging of the retroviral RNA by the gag-pol and env gene products already present in the cells. The packaged retroviruses contain RNA of your gene or interest flanked by LTRs, but no nucleic acid for gag, pol or env. This is sufficient to introduce the gene into the target cells, for reverse transcription to take place and for the resulting DNA to be integrated into the host genome but is insufficient for further viral production (detailed in [204, 215] [212] and at http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html).

Retroviral transduction has now been used successfully in a range of *in vitro* studies such as complementation of knock-out phenotypes (for example re-expressing mutated versions of CD40 in CD40-/- DC [216]). In addition, transduction of bone marrow has been successfully employed clinically to replace the defective common-γ-chain gene in individuals with X-linked SCID [217].

In the second section of this chapter I describe the optimisation of BMDC transduction with the aim of expressing Cre-recombinase in dendritic cells. Cre recombinase is a bacterial enzyme that releases concatentated bacterial chromosomes by site-specific recombination between LoxP sites (reviewed in [218]). Cre will also catalyse recombination between LoxP-sites inserted into mammalian genomes, resulting in excision of the intervening DNA if the LoxP sites are in the same orientation, or reversal of the intervening DNA is the LoxP sites are in opposite orientations (reviewed in [218]). A large number of knock-in mice have been produced in which same-orientation LoxP sites flank genes of interest. An efficient retroviral Cre could be used to rapidly produce knock-

out dendritic cells *in vitro* using bone marrow from any of these mice, making this an extremely useful tool.

3.2 : Optimisation of protocols to study signalling in dendritic cells

3.2.1 Flow cytometry can be used to assess MAP kinase phosphorylation

As discussed in the introduction to this chapter, dendritic cells are rare and cumbersome to isolate *ex vivo*. To get around this problem I developed an intracellular staining protocol to assess MAP kinase signalling by FACS. From the scientific literature [206], [207], and in discussion with Cell signalling technology and Pharmingen I obtained antibodies that gave good staining of phosphorylated p38 MAP Kinase, phosphorylated ERK1/2, and phospho-c-Jun.

The basic protocol developed (and summarised in Chapter 2, materials and methods) is as follows. BMDC are enriched for CD11c expression using MACS. Enriched cells are plated at a relatively high density in the presence of GM-CSF and allowed to rest overnight at 37°C. Inhibitors, when used, are added 30mins before the stimulus to allow for passive diffusion into cells. Stimuli are added for indicated times, when the reactions are terminated by the addition of paraformaldehyde to 2% final concentration. Cells are then harvested and resuspended in fresh fixative ("Fix and Perm reagent A"Caltag) for 20 minutes. Staining for surface markers is carried out in PBS/1%FCS/0.05M EDTA, followed by staining for phospho-MAP kinases using "Fix and Perm" reagent B as the staining solution.

The validation of these stainings is shown in Figure 3-1. In part A, intracellular staining (using the final protocol detailed in chapter 2.8.4) for Phospho-ERK or Phospho-p38 is assessed in the presence of 2 different MEK inhibitors, UO126 and PD98059 [219] [220]; or the p38 inhibitor SB203580. When BMDC from C57BL/6 mice are stimulated with zymosan for 30mins there is a significant increase in both phospho-ERK and phospho-p38 staining. In the presence of zymosan plus UO126 or PD98059, levels of phospho-ERK staining do not

increase above those in unstimulated cells, whereas phospho-p38 staining looks identical to that in cells stimulated with zymosan alone. The staining therefore shows specificity for phospho-ERK. BMDC stimulated with zymosan in the presence of SB203580 show diminished (although not abolished) phospho-p38 staining. Reassuringly there is no effect of SB203580 on phospho-ERK staining.

The specificity of phospho-c-jun staining was tested in separate experiments. BMDC were stimulated with CpG in the presence or absence of SP600125, an inhibitor of JNK [221] (3-1B). SP600125 effectively reduces the level of phospho-c-jun to that observed in unstimulated cells. However, variable effects of SP600125 were seen on the p38 and ERK pathway both by FACS and by western blotting so whilst this is suggestive of a specific staining, further evidence is required to determine true specificity.

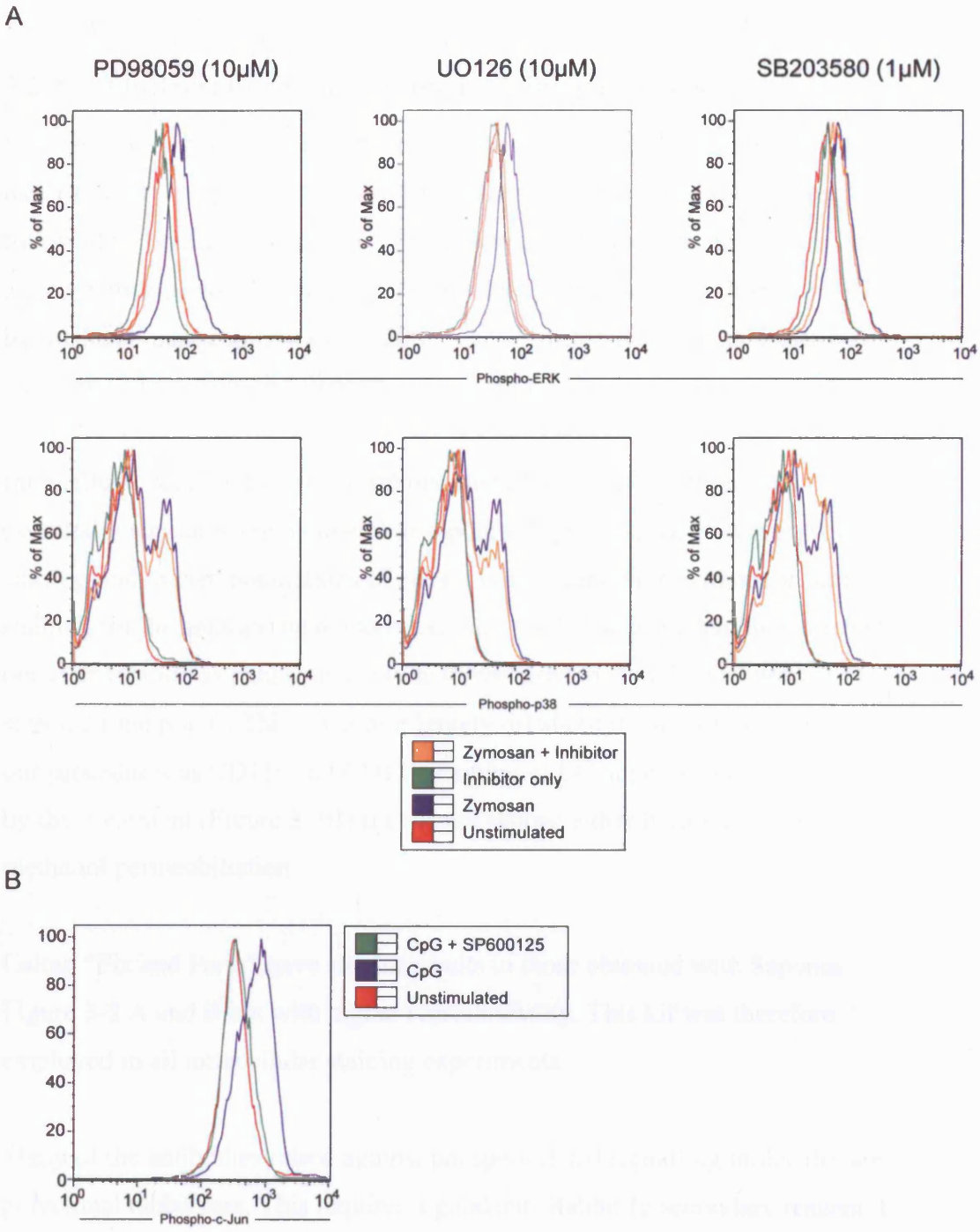


Figure 3-1: Assessment of MAP Kinase activation by FACS. C57BL/6 BMDC were pre-treated for 30mins with 10μM UO126, 10μM PD98059 or 1μM SB203580. After 30minutes stimulation with 10μg/ml CpG cells were fixed in 2% PFA and assessed for ERK and p38 MAPK phosphorylation, as described in chapter 2.

3.2.2 : Optimisation of intracellular staining protocols

Several protocols for staining of intracellular MAP kinases are published. The paper of [208] successfully employs formaldehyde fixation of splenocytes followed by permeabilisation in 0.5% Saponin. The Nolan group has published an alternative protocol in which paraformaldehyde is used as a fixative followed by methanol permeabilisation at -20°C. The 3rd method investigated uses a kit made by Caltag ("Fix and Perm").

Intracellular staining for the phosphorylated MAP kinase epitopes was essentially similar using methanol or saponin (Figure 3-2A). However, when starting with mixed populations of cells it is also important to maintain surface staining for the appropriate markers. Surface marker staining had to be carried out after fixation as paraformaldehyde to was used to stop the reactions at selected time points. This constraint largely ruled-out the use of methanol for our procedures as CD11c and CD11b staining of DC appears to be eliminated by this treatment (Figure 3-2B) if cells are stained either before or after methanol permeabiliztion.

Caltag "Fix and Perm" gave similar results to those obtained with Saponin Figure 3-2 A and B but with higher reproducibility. This kit was therefore employed in all intracellular staining experiments.

Many of the antibodies raised against phosphorylated signalling molecules are polyclonal rabbit sera. This requires a good anti-Rabbit Ig secondary reagent. I tested two such antibodies, a goat-anti-rabbit Ig from Caltag which was not cross-absorbed, and a donkey-anti-rabbit Ig from Jackson that was comprehensively cross-absorbed. BMDC unstimulated, or stimulated for 30mins with CpG were used to determine which combination of antibodies gave the best staining. Despite not being cross-absorbed against rat IgG, the Caltag goat-anti-rabbit antibody only gave reliable staining in the presence of rat-anti-mouse Fc receptor blocking antibody (2.4G2). The donkey-anti-rabbit antibody

from Jackson gave good staining with or without Fc receptor blocking suggesting this is a more reliable staining reagent and this was used in subsequent experiments for detecting rabbit polyclonal primary antibodies (Figure 3-2 C).

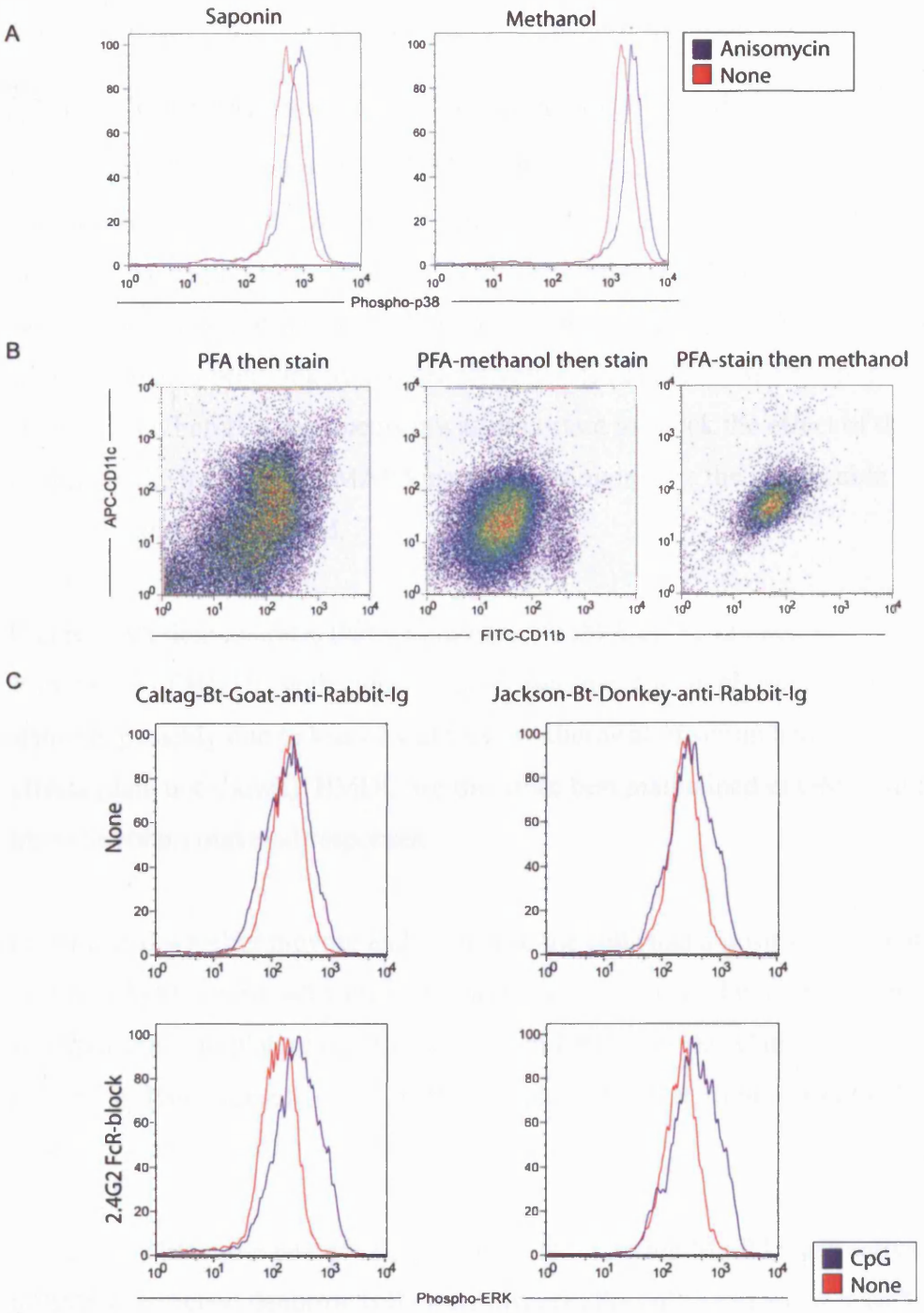


Figure 3-2: Optimisation of Intracellular staining protocol. A) C57BL/6 BMDC were stimulated for 5mins with 5μg/ml Anisomycin before fixation in 2% PFA. Cells were the permeabilised in 90% Methanol for 5 minutes at -20°C or by washing in 0.5% Saponin FACS buffer. Staining for phospho-p38 MAPK was assessed flow cytometry. B) C57BL/6 BMDC were fixed in 2% PFA

then stained directly for APC-anti-CD11c and FITC-anti-CD11b and then left untreated or subsequently treated with ice-cold 90% Methanol for 5 minutes at -20°C. A third group were fixed in PFA, then permeabilised in Methanol and then stained with APC-anti-CD11c and FITC-anti-CD11b. C) B6 BMDC were stimulated with 1g/ml CpG DNA for 30minutes before fixation and permeabilisation. These cells were then stained with Rabbit-anti-phospho-ERK, followed by Caltag or Jackson biotin-anti-Rabbit-Ig and PE-streptavadin.

3.2.3 : Optimisation of BMDC stimulation protocol

A potential pitfall of working with MAP kinases is that they are activated by a very broad range of stimuli, for example changes in adhesion triggered by re-plating cells, responses to serum [222] and responses to GM-CSF [223]. Dendritic cells do not survive well in the absence of serum or GM-CSF, both of which have also been linked to ERK activation [222] [223]. As DC are purified and re-plated before experiments it was important to check the effect of these factors on the induction of MAP kinase activation in case the measurable window could be improved.

Figure 3-3A demonstrates that withdrawal of GM-CSF 12 hrs before stimulation of BMDC with zymosan decreased the shift in phospho-ERK staining, possibly due to loss of viability. Withdrawal of serum had identical effects (data not shown). BMDC are therefore best maintained in GM-CSF at all times to obtain maximal responses.

I next tested whether moving and re-plating the cells had a positive or negative effect on MAP kinase activation. If BMDC were stimulated with zymosan simultaneously to plating out, the increase in ERK phosphorylation was negligible. However, after 3hrs, ERK phosphorylation at 30mins was easily observed (Figure 3-3B).

In order to obtain the best possible window for studying MAP kinase activation in BMDC enriched dendritic cells were always allowed to rest for at least 3hrs or overnight in the presence of GM-CSF before stimulation.

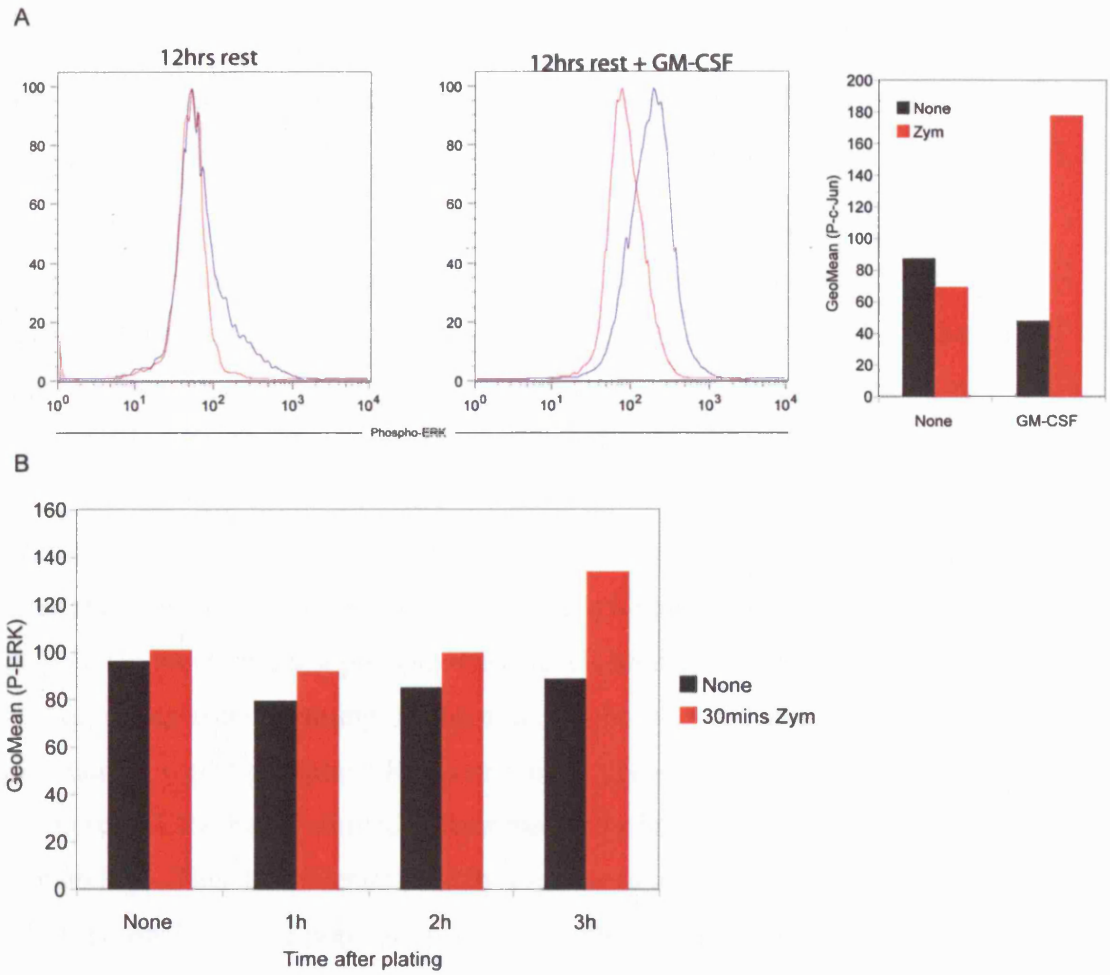


Figure 3-3: Optimisation of BMDC stimulation protocol. A) BMDC were isolated and MACS enriched for CD11c before plating for 12hrs in R10, or R10 containing GM-CSF. ERK phosphorylation induced by 30mins stimulation with zymosan 100µg/ml (blue line) is compared to resting levels (red line) by flow cytometry. Geometric mean fluorescence in FL2 is plotted for cells rested in the absence (None) or presence (GM-CSF) or GM-CSF. B. C57BL/6 BMDC were MACS enriched for CD11c then plated out in R10 containing GM-CSF for the indicated times. After these times zymosan was added to 100µg/ml for 30minutes, then signalling was stopped by addition of PFA to 2%. Phospho-ERK staining was assessed by flow cytometry and Geometric mean fluorescence calculated and plotted

3.2.4 : Assessing MAP kinase activation in mixed cell populations and *in vivo*

The ability to measure MAP kinase activation on a per cell basis is very powerful because it allows measurements to be made in mixed populations [208] and it allows precise correlation of the states of different MAP kinases in any one particular cell [224]. Some examples that I have tested are shown in Figure 3-4.

Part A shows double staining for phopsho-p38 and phospho-ERK. 30minutes after zymosan addition to the cultures, the cells lie on an almost precise diagonal, with the highest phospho-ERK levels seen in cells that also have the highest phospho-p38 staining. By 60 minutes phospho-p38 staining is maintained, whilst phospho-ERK is starting to decrease. By 150 minutes, levels of phospho-ERK have returned to near baseline whilst phospho-p38 levels remain high. This demonstrates that in response to a saturating dose of zymosan all of the BMDC in the population activate both ERK and p38 MAP kinase pathway and that in all cells the p38 MAP kinase signal is more prolonged that the ERK signal.

Another use has been to establish whether MAP kinase activation is a direct or indirect effect of pattern recognition signalling. TLR9-/- BMDC are mixed with wild type congenic (B6.SJL CD45.1) BMDC which can be distinguished by staining with an anti-CD45.1 antibody. When the mixed BMDC population is stimulated with the TLR9-ligand CpG for 30 minutes, c-Jun phosphorylation, as assessed by intracellular flow cytometry, is only observed in the TLR9-sufficient CD45.1 positive cells (Figure 3-4 B). This indicates that at least at early time points, c-Jun phosphorylation is a direct effect of TLR signalling.

A slightly trickier situation in which the ability to gate on individual cell populations and assess the state of MAP kinases within the gated cells could become very useful (elegantly demonstrated in [208]) is elucidation of

signalling *in vivo*. In Figure 3-4C BALB/C female mice were injected with CpG or PBS alone, or were just pricked with a syringe needle. The mice were euthanised after 30minutes and spleens were immediately mashed into paraformaldehyde. Fixed splenocytes were then stained for phospho-c-Jun. The interpretation of these data is highly complex with staining levels going both up and down after CpG stimulating in different spleen DC. Of note, there is a small percentage of phospho-c-Jun positive cells in the splenic DC of mice which have been pricked with a needle. Whether this represents simple mouse to mouse variation or a genuine neuroendocrine effect on spleen DC would require considerable further experimentation and will not be discussed further.

It is therefore clear that flow cytometry can be used to assess MAP kinase phosphorylation status in a quantitative manner and on a per-cell basis. In addition, the ability to correlate surface marker expression with phospho-epitope staining allows the study of signalling within mixed cell populations.

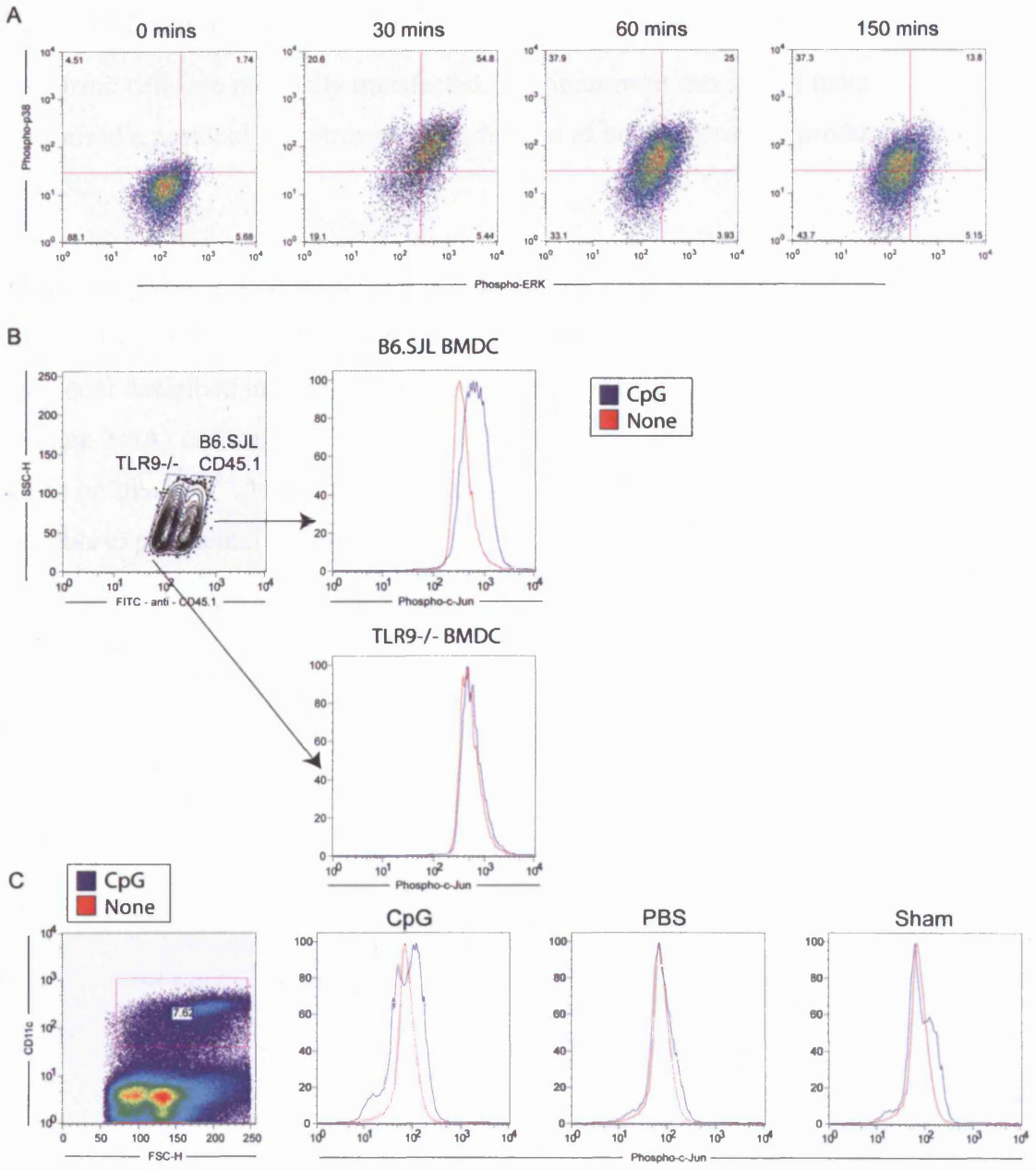


Figure 3-4: Applications of Intracellular staining of phospho-epitopes. A) C57BL/6 BMDC were stimulated with 100µg/ml of zymosan for the indicated times before fixation and staining using the standard protocol. After first staining with purified anti-phospho-ERK, and PE-anti-mouse IgG1, cells were blocked in purified mouse IgG1. Alexa647-conjugated anti-phospho-p38 (Pharmingen) was then used to stain for p38-phosphorylation. B) C57BL/6.SJL CD45.1 congenic BMDC were mixed 1:1 with TLR9-/- BMDC on a C57BL/6 background. The DC were then stimulated with 10µg/ml CpG DNA and c-Jun phosphorylation assessed by flow cytometry. C) 200µl of 25µg/ml CpG, or PBS alone was injected intravenously into age and sex-matched Balb/c mice, or a mouse was pricked with the syringe needle but nothing injected (sham). Spleens were removed after 30minutes, disrupted and cells fixed in 2% PFA. c-Jun phosphorylation was assessed by flow cytometry.

3.2.5 : Retroviruses can be used to efficiently transduce BMDC

Dendritic cells are not easily transfected. To circumvent this issue I have optimised a protocol for retroviral transduction of bone marrow to produce BMDC.

Using the pMSCV-EGFP vector ecotropic Phoenix cell lines were constructed that stably produce MSCV-EGFP retrovirus with the MLV envelope protein (protocol described in 2.3.1). Using these cells to transduce GM-CSF BMDC (Figure 3-5A) or Flt3L BMDC (Figure 3-5B) gave an average of 50% (GM-CSF) or 20% (Flt3L) transduction as assessed by GFP expression, making this a feasible experimental system.

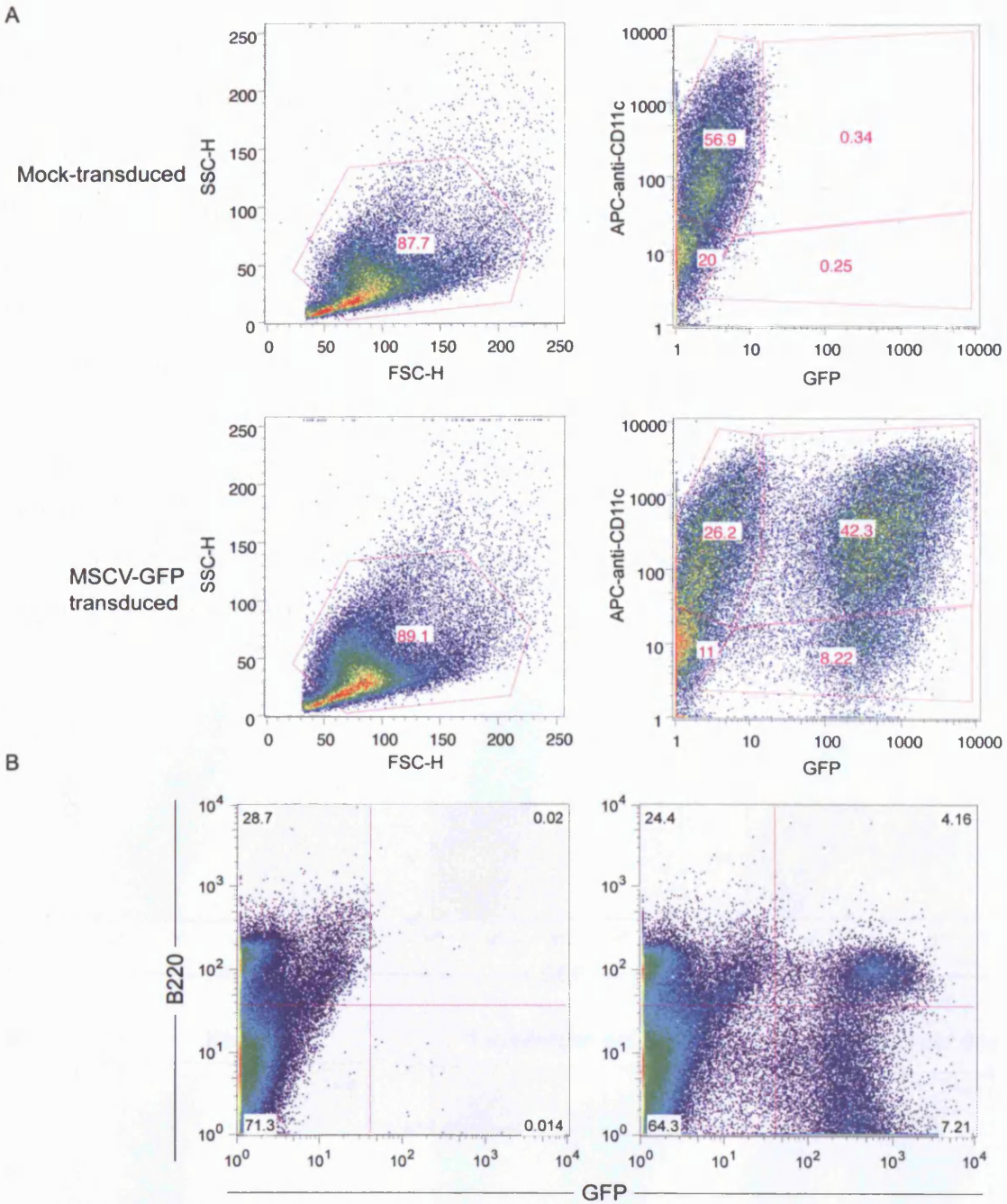


Figure 3-5: Retroviral transduction of GM-CSF (A) and FLT3L (B) BMDC. Bone marrow was transduced with MSCV-EGFP produced from stably transduced phoenicECO cells as described in chapter 2. GFP expression is assessed by FACS on day 5 of GM-CSF BMDC culture or day 10 of Flt3L BMDC culture.

3.2.6 : Amount of virus added correlates well with transduction efficiency

The amount of virus added to each transduction correlates well with the percentage transduction obtained. This parameter was the major determinant of transduction efficiency compared to all other parameters tested. Figure 3-6A shows that when 10, 20 or 50% of the BMDC supernatant was replaced with neat viral supernatant before spinfection the percentage transduction increases accordingly. In order to maximise percentage transduction, 100% of supernatant is routinely replaced before spinfection. Paradoxically, spinfecting twice per day does not increase transduction efficiency despite exposing cells to twice as much virus (figure 3-6B).

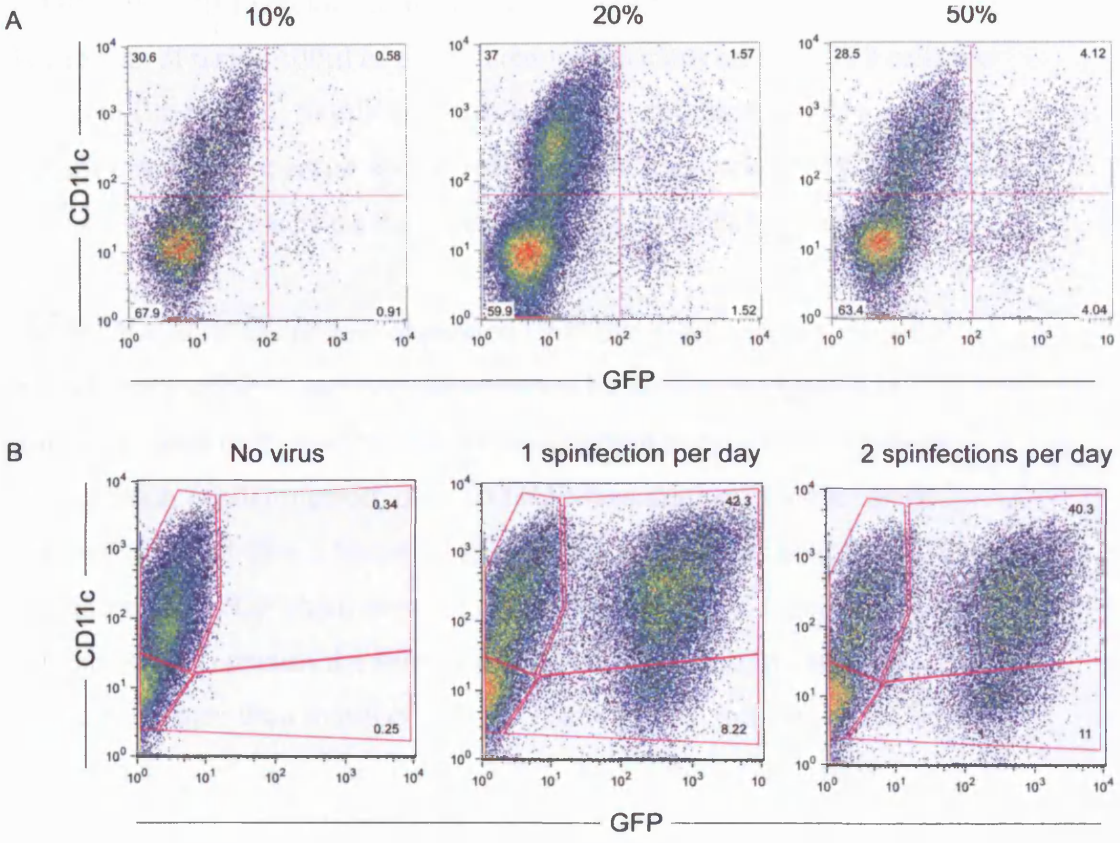


Figure 3-6: Increasing amounts of virus correlate with increased percentage transduction. A) Spinfections of GM-CSF BMDC were carried out as detailed in chapter 2 with 10, 20 or 50% of the supernatant replaced with virus before spinfection, rather than the standard 100%. Percentage of GFP+ cells was assessed on day 5. B) Spinfections of GM-CSF BMDC were

carried out as detailed in chapter 2, or repeating each spinfection 3hrs later on the same day. Percentage of GFP+ cells was assessed on day 5.

3.2.7 : Optimisation of retrovirus production protocol

As the amount of virus added before spinfecting bone marrow is a major determinant of efficiency, it is critical that high titre viruses are produced. Three different methods were tried for the production of retroviruses from two different retroviral vectors that I was interested in using. The two packaging cell lines employed were PhoenixECO cells (from Dr G Nolan, Stanford, CA) or GP2-293 cells (Invitrogen). Transient transfections of the retroviral vectors (either pMSCV-GFPCre or pBabe-DLL1-puro) into Phoenix or GP2-293 cells were compared to "ping-pong" production from a stable Phoenix cell line (described in 2.3.1). A VSV-G expression plasmid was included in transfections of GP2-293 cells to provide a functional envelope protein for viral packaging. The results of titring 100µl of the 48 hour supernatants on NIH-3T3 cells are shown in figure 3-7A. Stably expressing Phoenix cell lines reliably gave the highest titre virus for either vector, with transient transfection of GP2-293 cells giving a slightly higher titre than transient transfection of Phoenix cells.

The MLVA envelope protein expressed by Phoenix cells makes virus that is insufficiently stable to survive concentration by ultracentrifugation (a commonly used method with VSV-G pseudotyped retroviruses). However, using a much gentler method, over 10-fold concentration of virus can be obtained (figure 3-7B). 1.5ml viral supernatant is spun for 1hr at 4°C, 13000rpm in a microfuge, after which time the top 1.45ml of virus is removed. The remaining 50µl contains the same infectivity as the full 1.5ml starting preparation when used to infect NIH-3T3 cells *in vitro* indicating that where very high titres of viruses are required then this method can be used.

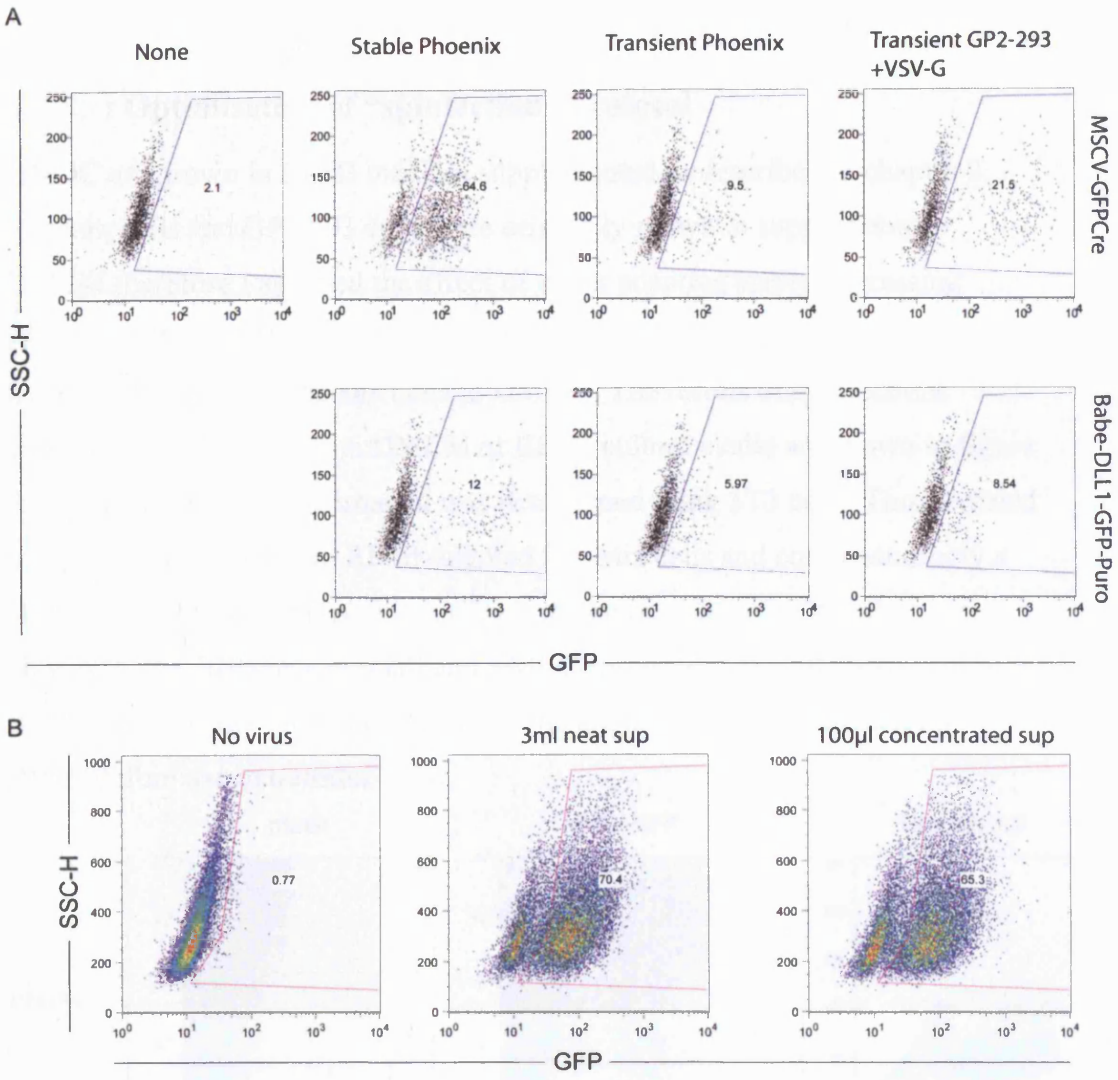


Figure 3-7: Optimal virus production from stably-transduced Phoenix cells: A) pMSCV-GFPCre or pBabe-DLL1-GFP-Puro were used to produce stable phoenix cells via the "ping-pong" method described in chapter 2, or to carry out transient transfections of Phoenix or GP2-293 cells. Retroviral supernatants obtained were used to infect NIH-3T3 fibroblasts and GFP levels assessed 48hrs after infection. B) 3ml of supernatant from phoenix cells stably producing MSCV-GFPCre was added directly to NIH-3T3 cells or was first centrifuged for 1hr at 4°C in two 1.5ml tubes, after which the top 1.45ml from each tube was discarded and the remaining 100µl was added to the NIH-3T3 cells. Percentage GFP+ cells was assessed at 48hrs post-infection.

3.2.8 : Optimisation of "spinfection" protocol

BMDC are grown in RPMI medium supplemented as described in chapter 2. Phoenix cells and GP2-293 cells were originally grown in supplemented DMEM therefore I assessed the effect of either adapting stably-expressing Phoenix cells into RPMI and growing BMDC as usual, versus growing BMDC in DMEM and using Phoenix cells in DMEM. The results of spinfections carried out exclusively with DMEM or RPMI culture media are shown in figure 3-8. The titre of viral supernatant was determined using 3T3 cells. This revealed a slightly lower titre from RPMI-adapted Phoenix cells and correspondingly a slightly lower % of GFP-positive, CD11c-positive cells in the RPMI cultures. However, the difference is small and BMDC responses are well characterised in RPMI-based media, therefore Phoenix cells are now routinely adapted into RPMI before use in transductions.

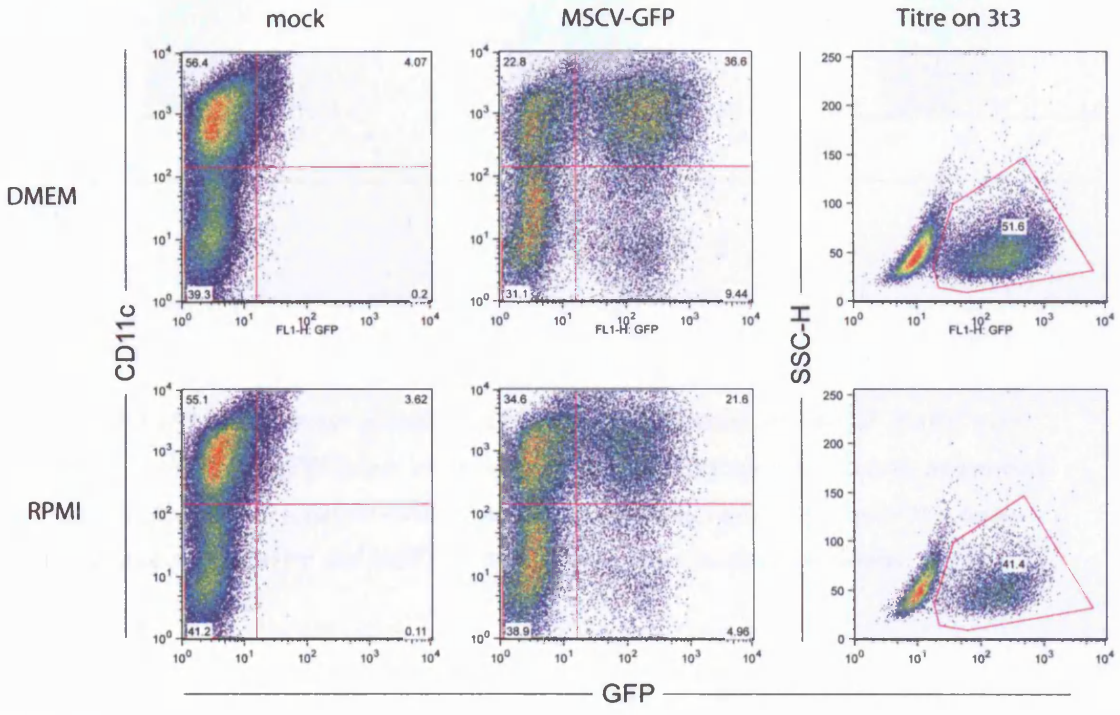


Figure 3-8: Tissue culture medium does not affect GM-CSF BMDC transduction efficiency. Phoenix cells stably-producing MSCV-GFP were grown in D10 or adapted into R10 and used to produce transduced GM-CSF BMDC in the respective media. GFP% is assessed at day 5 of culture.

3.2.9 : Polybrene improves retroviral transduction

It has previously been reported that inclusion of polycations such as polybrene can dramatically improve retroviral transduction (reviewed in [212]). The exact mechanism by which this works is unclear but is generally assumed to favour the close association of virus particles and cells. Figure 3-9 demonstrates an increase in the percentage GFP+ cells achieved by adding 2µg/ml polybrene to the retroviral supernatants before spinfection without a loss in total CD11c+ cells. Polybrene therefore appears to improve retroviral transduction of BMDC.

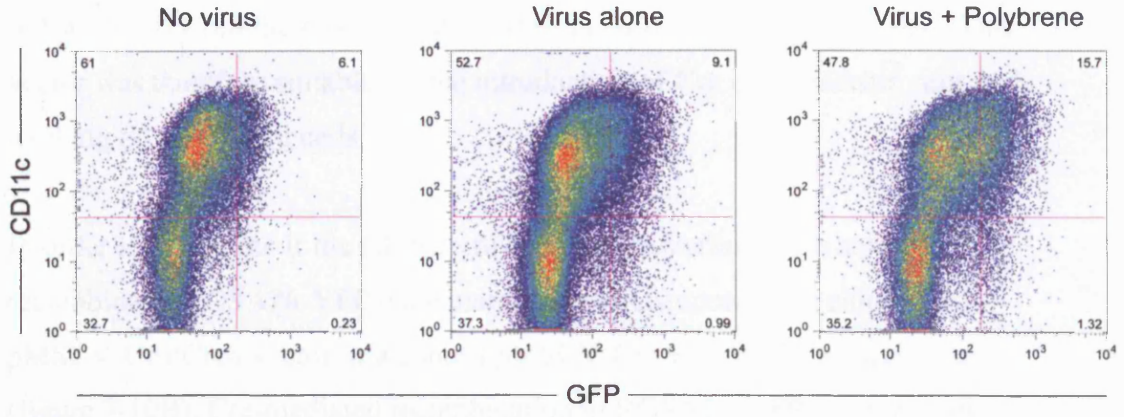


Figure 3-9: Polybrene improves transduction efficiency. Spinfections of GM-CSF BMDC were carried out with MSCV-GFPCre-neo produced from stably transduced Phoenix cells as detailed in chapter 2. Either viral supernatant alone or viral supernatant containing 4µg/ml Polybrene was used in each spinfection and %GFP-positive cells assessed on day 5 of culture.

3.2.10 : pMSCV PGK promoter-driven expression is much stronger than LTR-driven expression

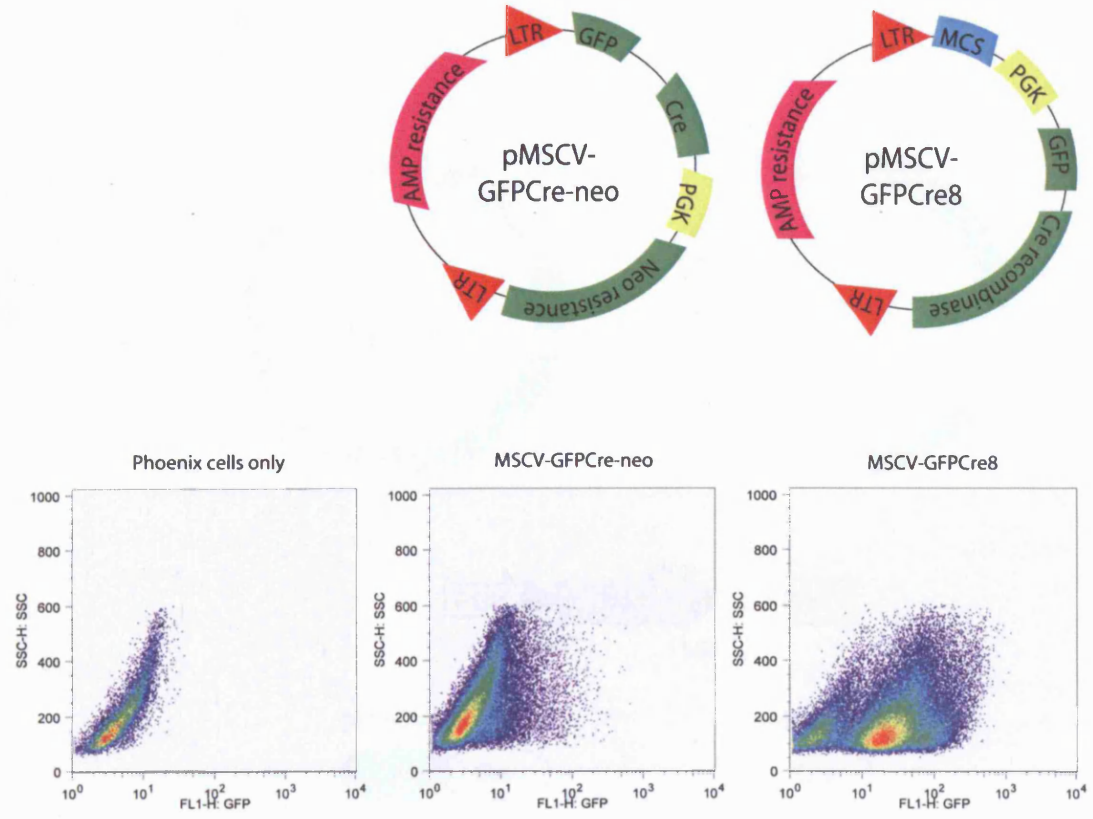
The initial vector I obtained for expression of GFPCre (a kind gift from Dr K Deinhardt, Cancer Research UK, London UK) used the viral LTR promoter to drive expression. This gave very weak expression when compared to another vector expressing GFP from the downstream PGK promoter of pMSCV. I therefore re-cloned the GFPCre protein downstream of the PGK promoter, generating the pMSCV-GFPCre8 vector. When phoenix cells expressing the original pMSCV-GFPCre-neo vector were compared to those expressing pMSCV-GFPCre8 there is a 1 to 2 log shift in GFP levels (figure 3-10A). This vector was therefore suitable for the introduction of Cre into dendritic cells and tracking of transduced cells.

In order to determine if the GFPCre fusion protein worked as an active Cre-recombinase, ROSA26-YFP bone marrow was transduced with either the pMSCV-GFPCre8 vector or a control pMSCV-Cre with no GFP expression (figure 3-10B). Cre-mediated recombination in ROSA26-YFP bone marrow results in YFP expression. GFP and YFP fluorescence were measured in day 5 transduced BMDC using flow cytometry. The emission spectra of GFP and YFP overlap significantly therefore it was not possible to fully compensate between the two channels using the equipment available. However, no GFP+ or YFP+ cells were detected from untransduced C57BL/7 BMDC or ROSA26-YFP BMDC. After transduction, GFP expression was obvious in the C57BL/6 BMDC transduced with GFPCre but not Cre alone. YFP+ cells could be detected in the ROSA26-YFP BMDC transduced with either virus indicating that the GFP-Cre was functional (Figure 3-10B).

In further proof that pMSCV-GFPCre8 produces retrovirus capable of mediating recombination between LoxP-sites, figure 3-10C shows the results of a representative transduction of Jagged-1-Flox bone marrow. Jagged-1-floxed mice were generated by Dr Katsuto Hozumi and were a kind gift of Dr Soline

Estrach (Keratinocyte Laboratory, Cancer Research UK, London Research Institute, UK). The mice are homozygous for a knock-in of the construct shown in part C, such that the exons encoding the DSL-domain of Jagged-1 are flanked by LoxP sites. Introduction of Cre into cells from the Jagged-1 flox mice mediates deletion between the LoxP sites that can be monitored using a duplex PCR (primers marker a, b and c on the construct). Bone marrow from Jagged-1-Flox mice can be successful used in transductions with virus from pMSCV-GFPCre8 to generate BMDC. GFP-positive and GFP-negative DC were FACS-sorted on day 5 of culture and genomic DNA produced. The duplex PCR described above was carried out and the results are shown in figure 3-10D. The PCR product at 333bp can only be obtained if Cre-mediated excision has taken place, whereas the 229bp band can only be obtained if the Floxed construct is still present. Figure 3-10D shows that only 'deleted' allele is detected in sorted GFPCre-positive cells, and only 'floxed' allele is detected in sorted GFP-negative cells indicating that the GFPCre protein produced from PSCV-GFPCre8 is functional and can be used to track transduced and recombined cells. It should be noted that deleted allele was sometimes detected at quite significant levels in the sorted GFP-negative population, probably due to the presence of cells that had ceased to express GFPCre or which expressed at very low levels sufficient for recombination but not sufficient to give measurable GFP fluorescence.

A


LTR
GFP
Cre
PGK
Neo resistance
LTR
AMP resistance
pMSCV-
GFPCre-neo
LTR
MCS
PGK
GFP
Cre recombinase
LTR
AMP resistance
pMSCV-
GFPCre8
Phoenix cells only
MSCV-GFPCre-neo
MSCV-GFPCre8
SSC-H: SSC
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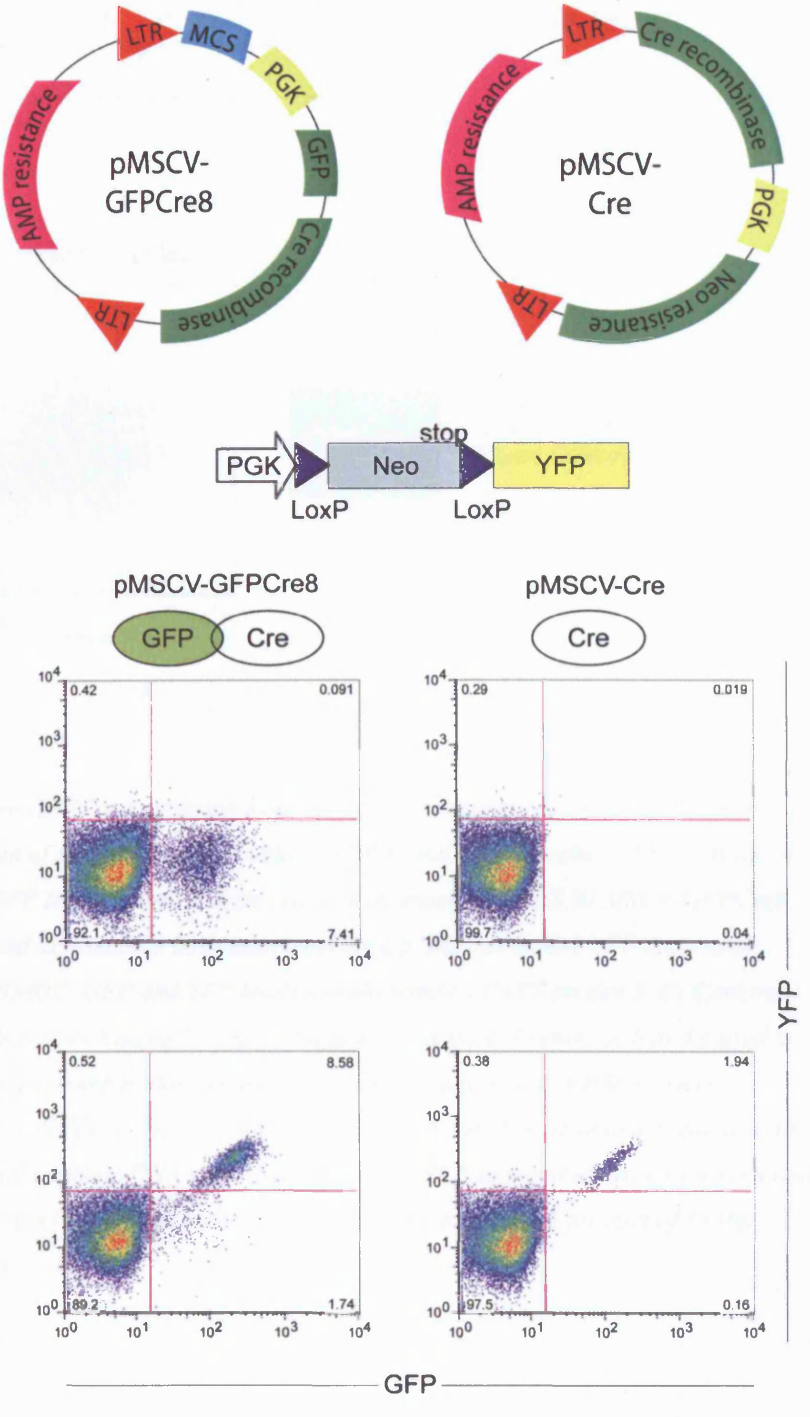
B


LTR
MCS
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Cre recombinase
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AMP resistance
pMSCV-
GFPCre8
LTR
Cre recombinase
PGK
Neo resistance
LTR
AMP resistance
pMSCV-
Cre
PGK
Neo
stop
YFP
LoxP
LoxP
pMSCV-GFPCre8
GFP
Cre
pMSCV-Cre
Cre
C57BL/6
Wild-type
ROSA-YFP
0.42
0.091
92.1
7.41
0.29
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YFP
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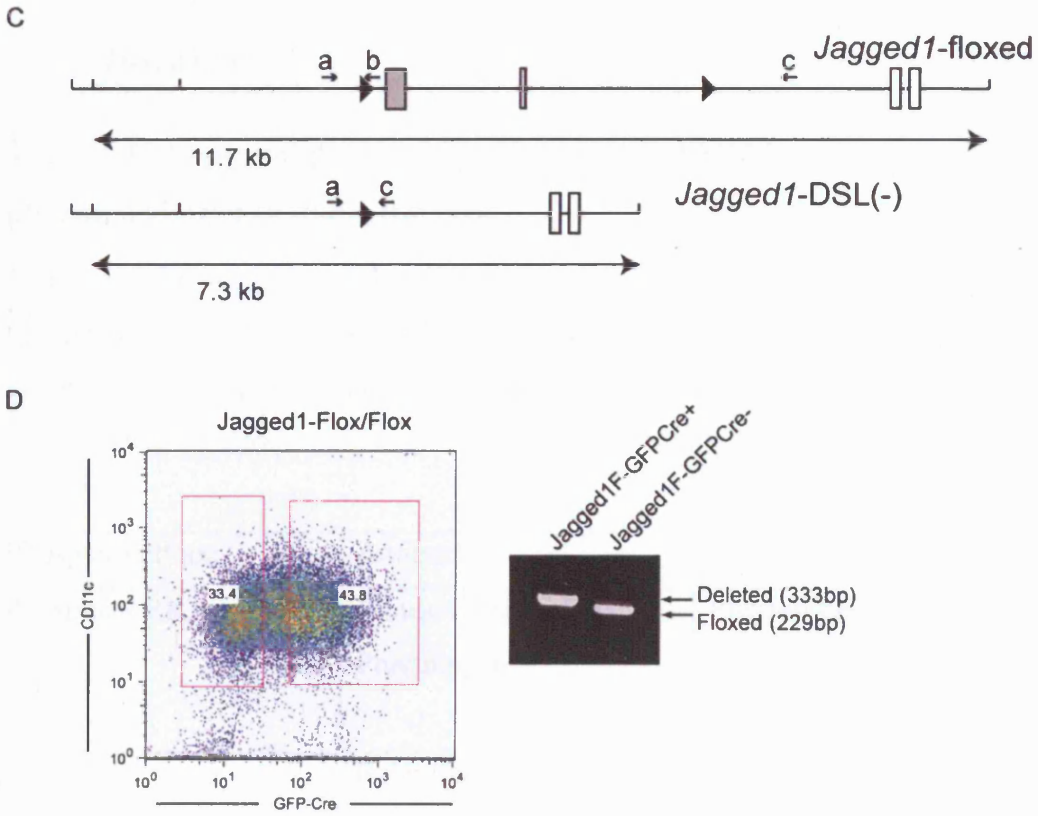


Figure 3-10: Stronger GFPCre-expression from the pgk-promoter of MSCV. A) GFPCre was re-cloned downstream of the pgk promoter within pMSCV and used to make stably transduced Phoenix cell lines. GFP levels in the Phoenix cells are assessed by FACS.B) MSCV-GFPCre8 or MSCV-Cre are used to transduce bone marrow from C57BL/6 or ROSA-YFP mice. and produced GM-CSF BMDC. GFP and YFP levels are assessed by FACS on day 5. C) Construct inserted into the endogenous Jagged-1 locus of Jagged-1-flox mice. Primers a, b and c used in duplex PCR to monitor recombination are shown. D) Day 5 Jagged-1-flox BMDC were transduced with MSCV-GFPCre8 as described in chapter 2.4. GFP-positive and negative cells were FACS-sorted and genomic DNA was isolated. Duplex PCR to monitor was carried out and run on a 2% agarose gel (right-hand panel). Deleted allele yields a PCR product of 333bp, Floxed yields a product at 229bp.

3.3 : Discussion

3.3.1 : The optimal protocol for analysis of MAP kinase phosphorylation in dendritic cells

The aim of the experiments described in sections 3.2.1 to 3.2.4 was to maximise the specific signal for phosphorylated signalling molecules as measured by flow cytometry. A group of antibodies that gave specific staining with reasonable shifts is shown below:

Phospho-eptiope	Antibody	Secondary reagent
Phospho-ERK	Mouse IgG1, Pharmingen	Pharmingen rat-anti-mouse IgG1 OR directly conjugated
	Rabbit polyclonal, Cell Signaling Technology	Jackson, Donkey-anti-rabbit (cross-adsorbed)
Phospho-p38 MAPK	Mouse IgG1, Pharmingen	Directly conjugated to Alexa 647
	Rabbit polyclonal, Cell Signaling Technology	Jackson, Donkey-anti-rabbit (cross-adsorbed)
Phospho-JNK	Rabbit polyclonal, Cell Signaling Technology	Jackson, Donkey-anti-rabbit (cross-adsorbed)
Phospho-c-Jun	Mouse IgG1 (KM-1), Santa Cruz	Pharmingen rat-anti-mouse IgG1

There are now a large number of phospho-specific antibodies being marketed as suitable for flow cytometry and many more will be relevant to DC research. This list will be expanded as different reagents are required in the future.

"Fix and Perm" (Caltag) is the best method for intracellular staining of dendritic cells, both for preservation of surface marker staining and for ease of use and reliability. Interestingly Krutzik et al [225] report that although they find there is a loss of almost 1 log between positive and negative populations staining for

CD11b or CD11c in methanol-fixed splenocytes the populations are still clearly discernable. This appears to relate to a wider separation of their populations of interest in unfixed cells as positive and negative populations become indistinguishable after methanol treatment in my hands.

It is very clear that physical manipulation of dendritic cells affects both their basal level of phosphorylated MAP kinases and their ability to activate MAP kinase cascades in response to stimuli (figure 3-3). In order to measure the response to stimuli it is therefore critical to rest cells after purification and re-plating. DC maintained in serum and GM-CSF during the resting period respond optimally to PAMPs, most likely because removal of either of these factors compromises DC viability.

3.3.2 : Possible applications for flow cytometric analysis of signalling in dendritic cells

The ability to assess MAP kinase activation in individual DCs allows these techniques to be applied to numerous situations. The examples shown in figure 3-4 demonstrate that this can be used to examine multiple MAP kinase pathways simultaneously, can be used to examine DC responses in mixed populations separable by a congenic markers, and can be used to examine MAP kinase status in DCs after *in vivo* stimulation. All of these areas could be investigated much more thoroughly – it would be particularly interesting to work out what signalling events occur after *in vivo* stimulation and the differences in signalling when a PAMP is injected versus saline alone or sham injection as this would provide clues as to the necessary signals for dendritic cell maturation *in vivo*, and would discern whether the signalling response to sham injection was a real phenomenon. Eventually, this may allow comprehensive analysis of signalling networks activated in DC by particular stimuli. The differences in mice genetically deficient in signalling molecules or cell populations could provide multiple insights into the *in vivo* control of DC phenotype.

In addition to this, flow cytometry can now routinely be used as a quick and simple assay for signalling in situations in which western blotting may previously have been used. Throughout chapter 5 of this thesis ERK phosphorylation in BMDC is monitored by flow cytometry and the simplicity with which quantitative data is obtained without the need to normalise for protein loading etc. provided a powerful research tool.

3.3.3 : Optimal protocol for retroviral transduction of GM-CSF and Flt3-L BMDC

BMDC can be efficiently transduced with MSCV-based retroviruses. The optimal protocol is described in section 2.4. The main limit of efficiency of retroviral transduction of DC is the titre of retrovirus produced. The most reliable virus production method appears to be the construction of stable PhoenixECO lines (described in section 2.3.1), which routinely allow 50 to 90% transduction of dendritic cells.

Improvement of percentage transduction can be achieved by including polybrene in the transduction reactions.

3.3.4 : Expression of Cre-recombinase in dendritic cells

The original aim of optimising retroviral transduction of DC was to produce dendritic cells expressing Cre-recombinase. A GFPCre fusion protein was used such that Cre-positive cells could be positively identified even in situations where there is strong selection against recombined DC. MSCV vectors in which the gene of interest are expressed from the LTR promoter and GFP is expressed from the pgk promoter were therefore not optimal as selective silencing of the LTR could lead to selection of transduced cells that were not expressing Cre. It is interesting that expression from the pgk promoter of pMSCV is much stronger than expression from the LTR, both in Phoenix cells (293T cells) and BMDC (figure 3-10A). The reasons for this are not clear but may involve selective LTR silencing or lack of positive transcriptional activators. Regardless

of the reasons, it was obviously better to express GFPCre from the pgk promoter of this vector. This produced a virus that induced efficient recombination in Rosa26-YFP and Jagged-1-Flox bone marrow.

A disadvantage to this system is that GFP-negative cells cannot necessarily be used as negative controls in this system as the GFP signal from the GFPCre fusion protein is still dim and cells expressing low amounts of GFPCre overlap very significantly with the untransduced population. The correct controls are therefore transduced cells differentiated from control bone marrow.

The applications of a Cre retrovirus system are wide-ranging. There are a large number of mice available that have LoxP-sites knocked-in around a gene of interest or which are transgenic for a construct in which expression of a gene of interest is controlled by Cre-mediated excision of an upstream cassette. Bone marrow from any of these animals can be used to rapidly generate knock-out dendritic cells *in vitro*. This is used in chapter 6 of this thesis to make dendritic cells lacking Jagged-1.

An extension of this work could be used to transduced the splenic precursors of conventional DC recently described by Naik et al [87]. These cells, once transduced, could be re-introduced into mice to quickly generate knock-out splenic DC, although these would then need to be differentiated from endogenous DC for analysis.

The ability to assess cell signalling in DC by FACS and to generate knock-out or transduced DC are useful tools for the study of DC signalling and effector function, as will be described in subsequent chapters.

4 Chapter 4: Zymosan recognition activates signalling via Syk to induce high levels of IL-2, IL-10 and Jagged-1: Dectin-1 is a putative β-glucan-binding PRR.

4.1 : Introduction

Zymosan is known to stimulate DC maturation, both in terms of surface marker upregulation and in the induction of IL-10, IL-12 p40 and very low levels of IL-12 p35/IL-23 p70 [96]. I extended these observations to other candidate markers of dendritic cell activation and analyse kinetics of cytokine production. The second part of this chapter addresses the role of different receptors for zymosan in signalling for dendritic cell activation.

4.1.1 Pattern recognition receptors and zymosan

In the context of dendritic cells "PRRs" can be defined very strictly as receptors that signal for dendritic cell maturation resulting in differentiation (not just proliferation) of naïve T cells into effector T cells [77]. This is a particularly important distinction in the case of zymosan recognition as several receptors such as the Mannose Receptor [46], and Complement Receptors (with and without complement fixation) [45] [226] can bind zymosan. However, there is limited evidence that these receptors alone are sufficient to induce full DC maturation and cytokine production.

As discussed in chapter 1, zymosan is a particulate cell-wall extract of Saccharomyces cerevisiae and a good, if complex, source of yeast PAMPs such as β-glucans [39]. TLR2/6 heterodimers and Dectin-1 are both implicated in cytokine production in response to zymosan in macrophages [55, 56], and Dectin-1 is known to specifically recognise the β-glucan component of zymosan [55, 56] [57]. At the point when this project was initiated it was known that

dendritic cells produced IL-12 in response to zymosan in a TLR2 and MyD88-dependent manner, whilst IL-10 was produced independently of both TLR2 and MyD88 [205]. This was particularly intriguing as this TLR-independent production of IL-10 was one of the first indications of bona fide pattern recognition at the cell surface that was not dependent on Toll-like receptors. We set out to further investigate the TLR-dependence of cytokine production in response to zymosan.

4.1.2 : Production of cytokines in response to zymosan

The cocktail of cytokines produced by DCs is carefully regulated and depends on the stimulus (or stimuli) received and the type of DC stimulated (for a detailed discussion see Chapter 1.3.3 and 1.3.4 and references therein). In this study, only GM-CSF BMDC have been considered and the patterns of cytokine production reflect the signature of stimulation via particular PRRs in this cell-type. Necessarily this will differ slightly from the *in vivo* responses of other DC subsets to zymosan, with the possible exception of "inflammatory DC" suggested to be the *in vivo* counterpart of GM-CSF BMDC [87].

Zymosan induces the production of very high levels of IL-10 (up to 20ng/10^5 cells after 18hrs of stimulation – data presented within). IL-10 production is also observed with TLR2, TLR4 and TLR9 ligands [227] [228] [229]. The production of a cytokine typically associated with anti-inflammatory properties by a pathogen-associated stimulus is intriguing although the role of IL-10 production specifically by dendritic cells is unknown.

Recently it was reported that zymosan induces very high levels of IL-2 production from dendritic cells [145]. The striking difference between TLR and zymosan-induced IL-10 and IL-2 levels (demonstrated in this chapter) hints at profound differences between the recognition of zymosan and less complex TLR ligands and provides an assay in which dendritic cell recognition of zymosan can be tested.

4.1.3 : Dectin-1: the MyD88-independent receptor for zymosan?

Dectin1 is a type II membrane protein with an extracellular C-type lectin domain and an intracellular ITAM-like motif [52]. This receptor is essential for the nonopsonic uptake of zymosan by macrophages [60]. In addition, several pieces of evidence suggest that Dectin-1 has functions beyond phagocytosis. The ITAM-tyrosines of Dectin-1, are required to enhance TLR2-dependent TNFα secretion in RAW264.7 cells in response to zymosan [230, 231]. In addition, an oxidative burst in response to zymosan could be triggered in macrophages lacking TLR2 [230, 231]. This suggests that signalling capacity of Dectin-1 is required for it to enhance TLR2-mediated recognition of zymosan, and that Dectin-1 may be able to signal for innate immune cell activation independently of TLR2.

Dectin-1 is expressed on myeloid cells including splenic dendritic cells, macrophages and neutrophils [232].

4.1.4 : ITAM signalling in dendritic cells

Underhill et al demonstrate that the cytosolic tail of Dectin-1 is tyrosine phosphorylated on zymosan binding in macrophages [233] and sequence analysis has identified motif in the cytoplasmic tail of Dectin-1 which bears similarity to an ITAM. This led us to hypothesise that ITAM signalling, as occurs downstream the B cell receptor of Fc-Receptors, may be playing a role in dendritic cell responses to zymosan.

Syk mediates ITAM-dependent signalling in dendritic cells [234] and can signal downstream of the FcγReceptor for the induction of IL-12 p40, indicating that Syk can signal for DC maturation [234]. The same study found that Syk-deficient DC responded normally to TLR-ligands. The ITAM-like motif in Dectin-1 contains an extra amino acid residue in the N-terminal YXXL (see figure 1-5). This suggests that the interaction of Syk with Dectin-1 may differ from Syk-activation induced downstream of consensus ITAM-containing receptors. Atypical modes of Syk activation have been proposed, in particular

tyrosine-independent Syk activation in response to Integrin signalling [235]. The unusual ITAM pattern in the cytoplasmic tail of Dectin-1 may be sufficient for Syk recruitment and activation. My results, together with those by others in the Immunobiology lab, led to the demonstration that this is indeed the case [146].

The aim of work described in the chapter was to describe more fully the activation of DC stimulated by zymosan and to examine the roles of different potential PRRs (namely Dectin-1 and TLR2) in signalling for this activation.

4.2 : Results - Syk-dependent cytokine and Notch-ligand production

4.2.1 : Zymosan induces high levels of IL-2, IL-10, IL-12p40, IL-12 p35, IL-6 and TNFα from DC

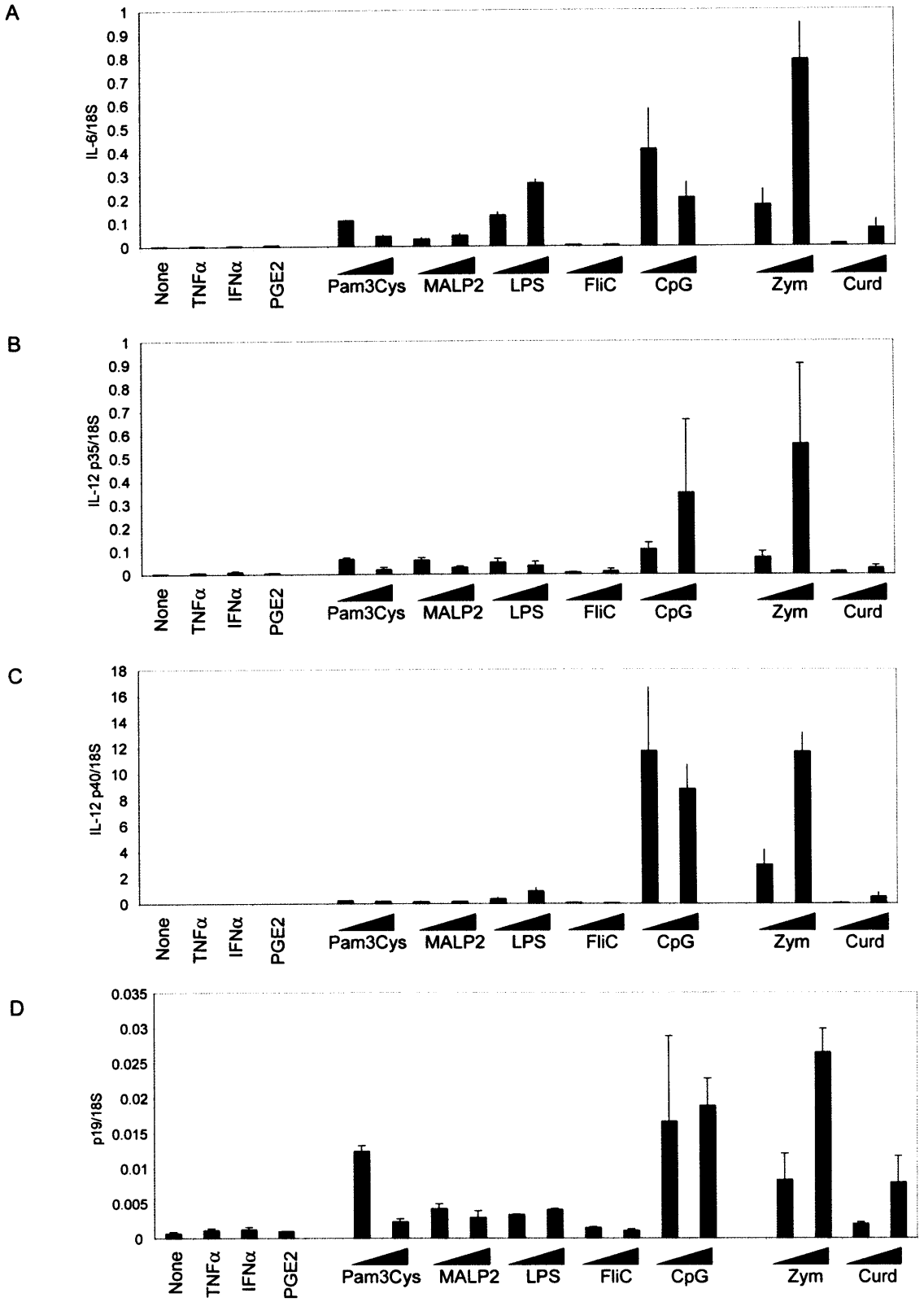
Previous work suggests that zymosan is particularly potent at inducing IL-10 and relatively poor at inducing IL-12 p70 from splenic DC [205]. To extend this observation, BMDC were screened for cytokine mRNA upregulation in response to a panel of inflammatory mediators, conventional TLR ligands, and β-glucan-rich stimuli (zymosan and curdlan). TNFα, and IFNα were chosen as inflammatory stimuli as these have been reported to promote the upregulation of costimulatory molecules and MHC II on DC but are not thought to induce cytokine production, or to be sufficient for the instruction of T cell differentiation [105] [101]. ProstaglandinE2 has been implicated in induction of DC maturation leading to Th2 priming [236]. The TLR ligands used are Pam3Cys (TLR1/2), MALP2 (TLR2/6), LPS (TLR4), Flagellin (FliC, TLR5) and CpG (TLR9) (reviewed in [22]) as these are known to induce differential patterns of cytokine production from DC. Of these, MALP2 signals through the same TLR combination as zymosan [59] [237]. All microbial stimuli are used at a previously established sub-saturating dose and one saturating dose.

Cytokine production was initially monitored by RT-PCR analysis due to the high sensitivity and broad dynamic range, as well as simplicity of screening several different cytokines. A 3hr time-point was chosen for analysis as being early in the response, this minimises the effects of major autocrine signals such as IL-10.

Results are shown in Figure 4-1A-G. The level of cytokine mRNA at 3hrs varies significantly between BMDC stimulated with different ligands, reflecting the flexibility of dendritic cell maturation. There is no induction of cytokines by stimulation with TNFα, PGE2 or IFNα, as would be expected from published

work. Among the TLR ligands tested, CpG induces the highest levels of IL-6, IL-12 p35 and p40, IL-23 p19, IL-2 and TNFα. However, LPS induces higher levels of IL-10 mRNA. Flagellin does not induce significant levels of any of the cytokines tested and TLR2 ligands are consistently less potent in cytokine production than either CpG or LPS. Zymosan is a ligand for TLR2/6. Interestingly, zymosan induces much higher levels of all of the cytokines tested than the purified TLR2/6 ligand (MALP2), inducing significantly higher levels of IL-10, IL-2 and TNFα mRNA than even CpG (figure 4-1 E-G) and comparable levels of IL-6, IL-12 p40, IL-12 p35 and IL-23 p19 to those seen with CpG. This suggests that zymosan can act in a similar way to a strong TLR agonist in the induction of IL-6, IL-12 and IL-23. The extremely potent induction of IL-2, IL-10 and TNFα further implies that zymosan can bias cytokine induction in a manner rarely observed as a result of pure TLR stimulation. With respect to this observation, the highly purified β-glucan, Curdlan, which is recognised by DC in a Dectin-1-dependent manner (Salomé LeibundGut-Landmann, Caetano Reis e Sousa, manuscript in preparation) does not induce high levels of IL-6, or IL-12 but is sufficient to induce moderate levels of IL-2 and IL-10 implying that β-glucan recognition is sufficient for cytokine induction and that it couples particularly well to cytokines that show strong induction with zymosan, as compared to the purified TLR agonists.

To confirm that mRNA levels for the cytokines tested correlate with levels of cytokine produced at the protein level, BMDC were stimulated with maximal doses of zymosan or CpG and production of cytokines was measured by ELISA after 18hrs (figure 4-1H). This reveals that, as for mRNA levels, zymosan induces markedly higher levels of IL-2 and IL-10 and slightly higher levels of TNFα from BMDC than CpG stimulation. CpG DNA is however a slightly stronger stimulus for IL-12 p40 protein production, suggesting that the mRNA data is largely in agreement with protein measurements.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IFNα
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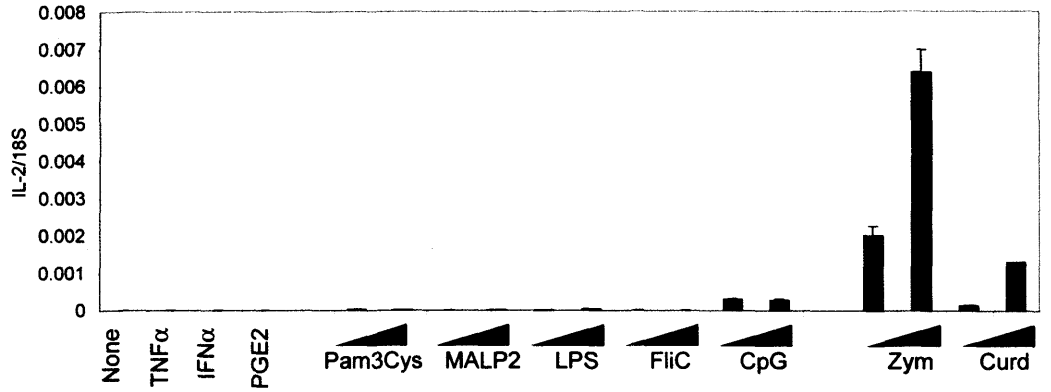
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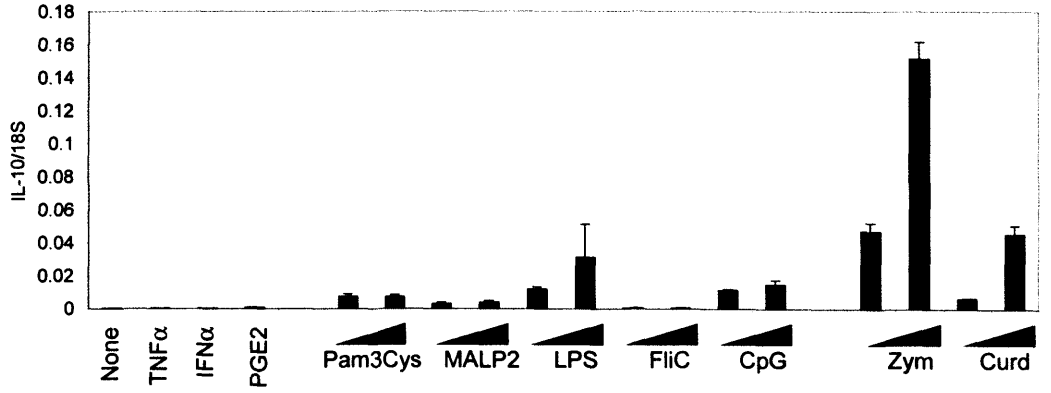
F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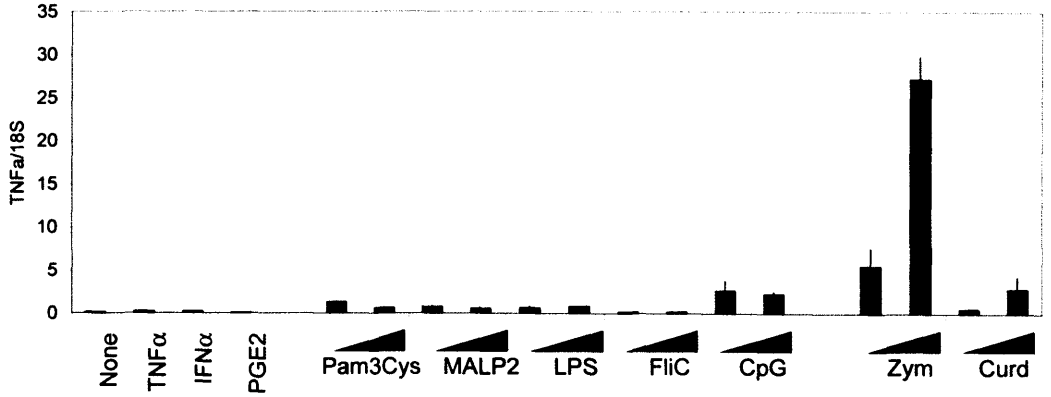
Curd

G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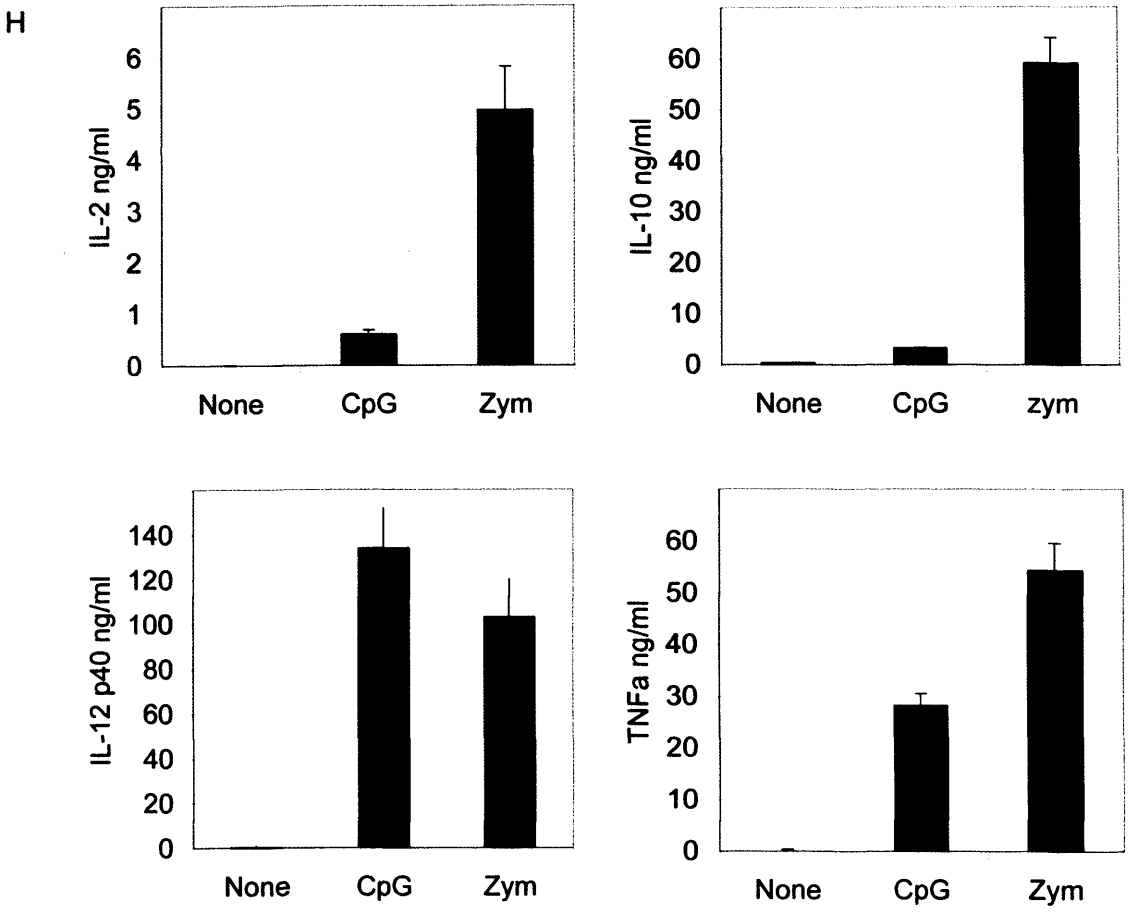


Figure 4-1: Cytokine upregulation in BMDC stimulated with inflammatory or microbial stimuli A-G: C57BL/6 BMDC (5 x 10^5 per well of a 24 well plate) were stimulated with TNFα 50ng/ml, IFNα 5000U/ml, ProstaglandinE2 (PGE2) 1nM, 0.1nM Pam3Cys 2, 10μg/ml; MALP2 0.2, 1μg/ml; LPS (highly purified from Salmonella) 1, 5μg/ml; Flagellin (FliC) 0.2, 1μg/ml; CpG 1668 0.5, 2,5μg/ml; Zymosan (Zym) 20, 100μg/ml; Curdlan (Curd) 20, 100μg/ml. After 3hrs cells were lysed in Trizol (Invitrogen) and RNA was extracted for RT-PCR and Q-PCR analysis using SYBR-green (IL-6, IL-2, TNFα, IL-23 p19) or Taqman (IL-10, IL-12 p35, IL-12 p40). Results are normalised to 18S rRNA. Data is representative of 3 separate experiments. H: BMDC were plated out in complete medium supplemented with GM-CSF and stimulated with 1μg/ml CpG DNA or 25 μg/ml of zymosan. Cytokine production was determined by ELISA after 18hrs of culture. Data is representative of over 8 independent experiments.

4.2.2 : Zymosan-stimulation induces IL-10, IL-2, IL-12 p40 and TNFα from overlapping sets of dendritic cells but with different kinetics.

It is intriguing that dendritic cells make cytokines such as IL-10, IL-2, IL-12 p40 and TNFα to high levels despite the proposed disparate functions of these cytokines. As RNA analysis averages mRNA levels over the entire population of cells present, it is possible that the cytokines are being made by subsets of dendritic cells or even that the cytokines are being produced in sequence by individual cells, but out of phase with each other in the population, leading to the observed results in figure 4-1.

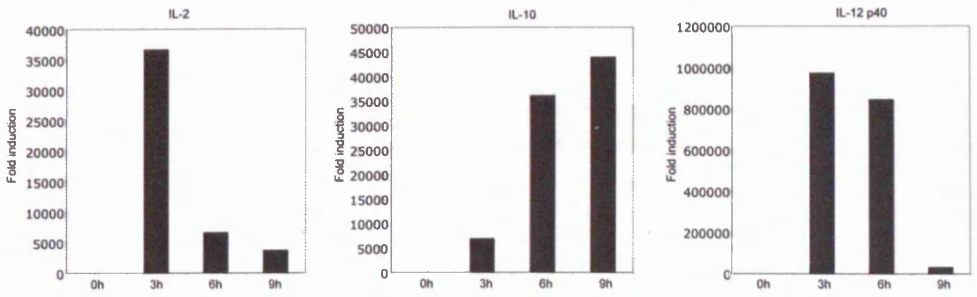
The kinetics of mRNA upregulation for IL-2, IL-10 and IL-12 p40 was determined up to 9hrs post-stimulation. Data shown in figure 4-2A reveals that IL-2 and IL-12p40 mRNA is induced rapidly after zymosan stimulation with maximal mRNA levels found at the 3hr time-point. IL-2 levels have decreased dramatically by 6hrs whilst IL-12 p40 levels are sustained. Conversely, the highest IL-10 mRNA levels are found at 9hrs, indicating a much slower induction.

To really determine if dendritic cells were producing multiple cytokines or if there were sub-populations of cytokine-producing cells I employed intracellular staining for cytokines after BrefeldinA treatment to measure cytokine production at the protein level on a per-cell basis. BrefeldinA disrupts ER to Golgi transport leading to fragmentation of the Golgi apparatus [238, 239] and the accumulation of newly synthesised cytokines in the secretory pathway. Initially, dendritic cells were treated with BrefeldinA from 3 to 9hrs after zymosan stimulation followed by fixation and staining for IL-2, IL-10, IL-12 p40 and TNFα. Figure 4-2B clearly shows IL-2 production uniquely by cells that also make the highest levels of IL-12p40 and TNFα. Some IL-10 producing cells are positive for IL-12 and IL-2 although the overlap here is partial. There is a stronger correlation between TNFα production and IL-10 production. At a

low frequency, there are cells in this experiment that stain positively for all 4 cytokines, i.e. are IL-2 positive and IL-10 positive. Since IL-2 production only occurs from cells expressing the highest levels of IL-12 p40 and TNFα this demonstrates that it is possible for a single dendritic cell to produce IL-2, IL-10, IL-12 p40 and TNFα in response to zymosan.

Given the 6hr period of BrefeldinA treatment employed in generating figure 4-2B it is possible that the cytokines are being produced in sequence, rather than simultaneously. As demonstrated in 4-2A, the kinetics of IL-10 and IL-2 mRNA induction show the greatest differences, making these good candidates for sequential production. I analysed IL-2 and IL-10 intracellular staining over a crude time-course of BrefeldinA treatment to look for evidence of dendritic cells making first IL-2, followed by IL-10. If BrefeldinA is included in the cultures from 3 to 6hrs and intracellular staining is performed at this time-point, IL-2 producers are found, with very few IL-10-positive cells. However, if BrefeldinA is added from 6 to 9 or 9 to 12 hours, predominantly IL-10 producing cells are detected. Double positive cells are only detected at a measurable level if BrefeldinA is present from 3 to 9hrs of culture, over the period at which IL-2 production ceases and IL-10 production commences. Whilst there are caveats to this experiment associated with toxicity of BrefeldinA and the disruption of autocrine and paracrine signalling during the period in which BrefeldinA is present in the cultures this is a hint that whilst the same DC that have recognised zymosan can in fact produce both IL-2 and IL-10 they do so in a sequential manner. It is also probable that there are DC present that have made either IL-10 or IL-2 as the percentage of double positive cells is low. The regulation of cytokine production over time is an interesting phenomenon which may explain the ability of DC stimulated with factors that lead to IL-10 production to induce robust T cell responses despite production of a cytokine typically associated with tolerance.

A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IL-12 p40
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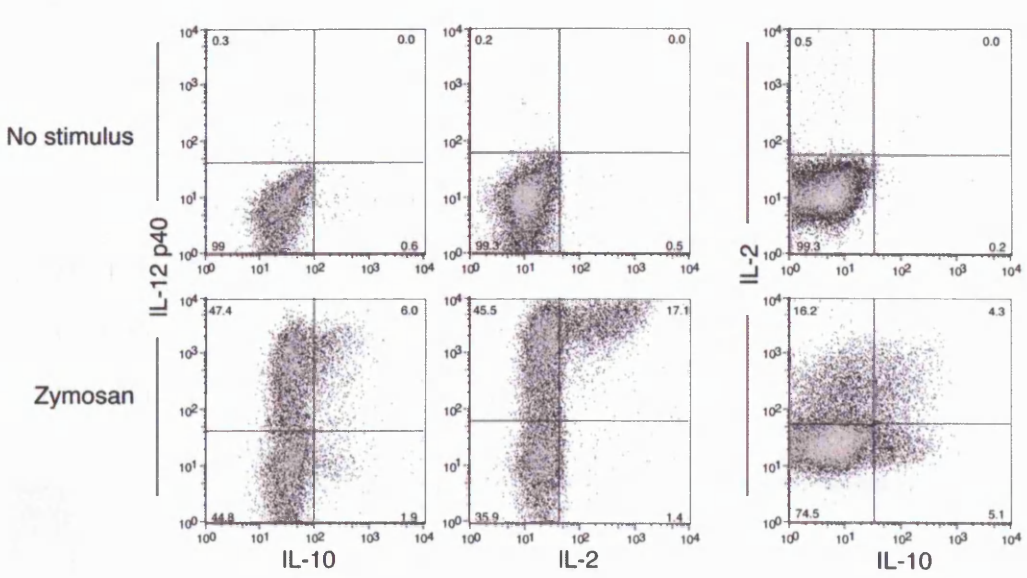
6h
9h

B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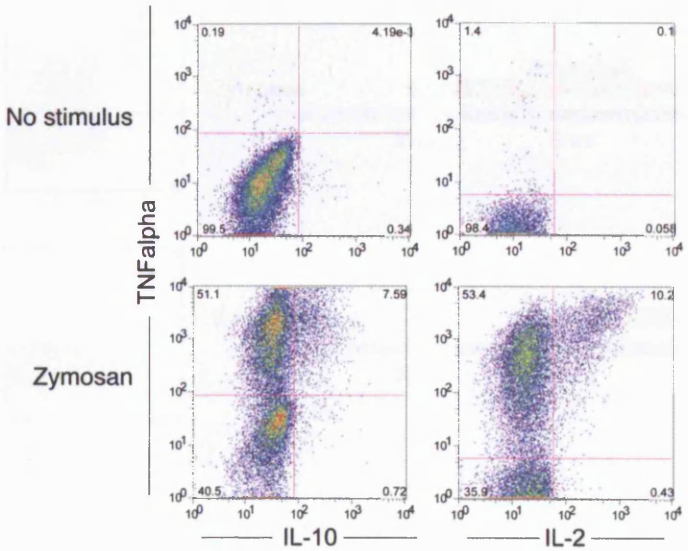
IL-10
IL-2
IL-2
IL-10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Zymosan
TNFalpha
IL-10
IL-2

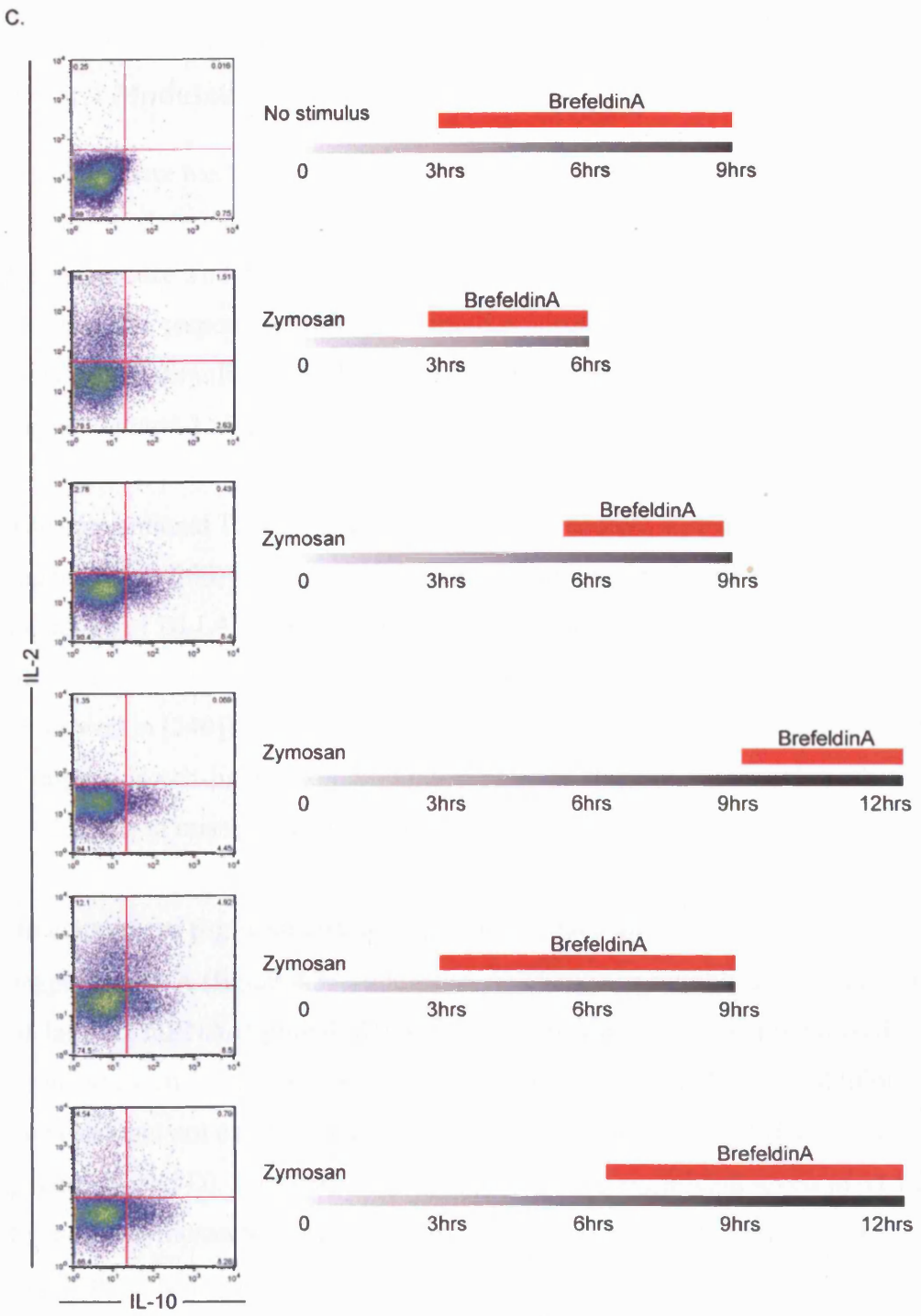


Figure 4-2: Single BMDC can produce IL-12 p40, IL-2, TNF and IL-10. A. C57BL/6 BMDC (5 x 10^5 per well of a 24 well plate) were stimulated with 50μg/ml Zymosan for the given lengths of time before lysis in Trizol and RNA extraction. B. C57BL/6 BMDC (10^6 per well of a 24 well plate) were left unstimulated or stimulated with 50μg/ml zymosan for 3hrs, before adding Brefeldin A to a final concentration of 5μg/ml. 6hrs later cells were recovered and stained for intracellular cytokines C. As part B with alterations is the timing of Brefeldin A addition and cell isolation as marked. Data is representative of 3 separate experiments

4.2.3 : Modulation of Notch-ligands by microbial stimuli

Recently there has been a resurgence of interest in Notch-ligand expression by DC and modulation of T cell differentiation with the proposal that Jagged-ligands induce Th2 differentiation [160]. Detailed analysis of Notch-ligand regulation in response to different stimuli is currently lacking, therefore I tested whether the stimuli used in 4-1 could also modulate mRNA levels for the Notch ligands Jagged-1, Jagged-2, DLL1 and DLL4.

The conventional TLR2, TLR4 and TLR9 ligands give poor induction of both Jagged1 (< 2-fold) and Jagged2 (decrease from unstimulated) and strong induction of DLL4 (figure 4-3 A, B and D). This correlates well with the ability of these stimuli to induce robust Th1 responses when used as adjuvants (reviewed in [240]). The TLR5 ligand, Flagellin, dose not lead to any noticeable change in Notch-ligand expression. It is possible that the 3hr snapshot provided by this screen misses later events in this case.

In contrast the β-glucan-containing ligands induce up to 4-fold induction of Jagged1 mRNA (figure 4.3A) and either no change or roughly 2-fold induction of Jagged2 mRNA (figure 4-3B) which may correspond to the more mixed responses seen when zymosan is used as an adjuvant [90] [175]. In addition, zymosan, but not curdlan, is a good stimulus for induction of DLL1 and DLL4 (figure 4C and D). This may reflect the presence of significant levels of TLR2 ligands in zymosan that are absent from curdlan [Dr Salomé LeibundGut-Landmann, manuscript in preparation].

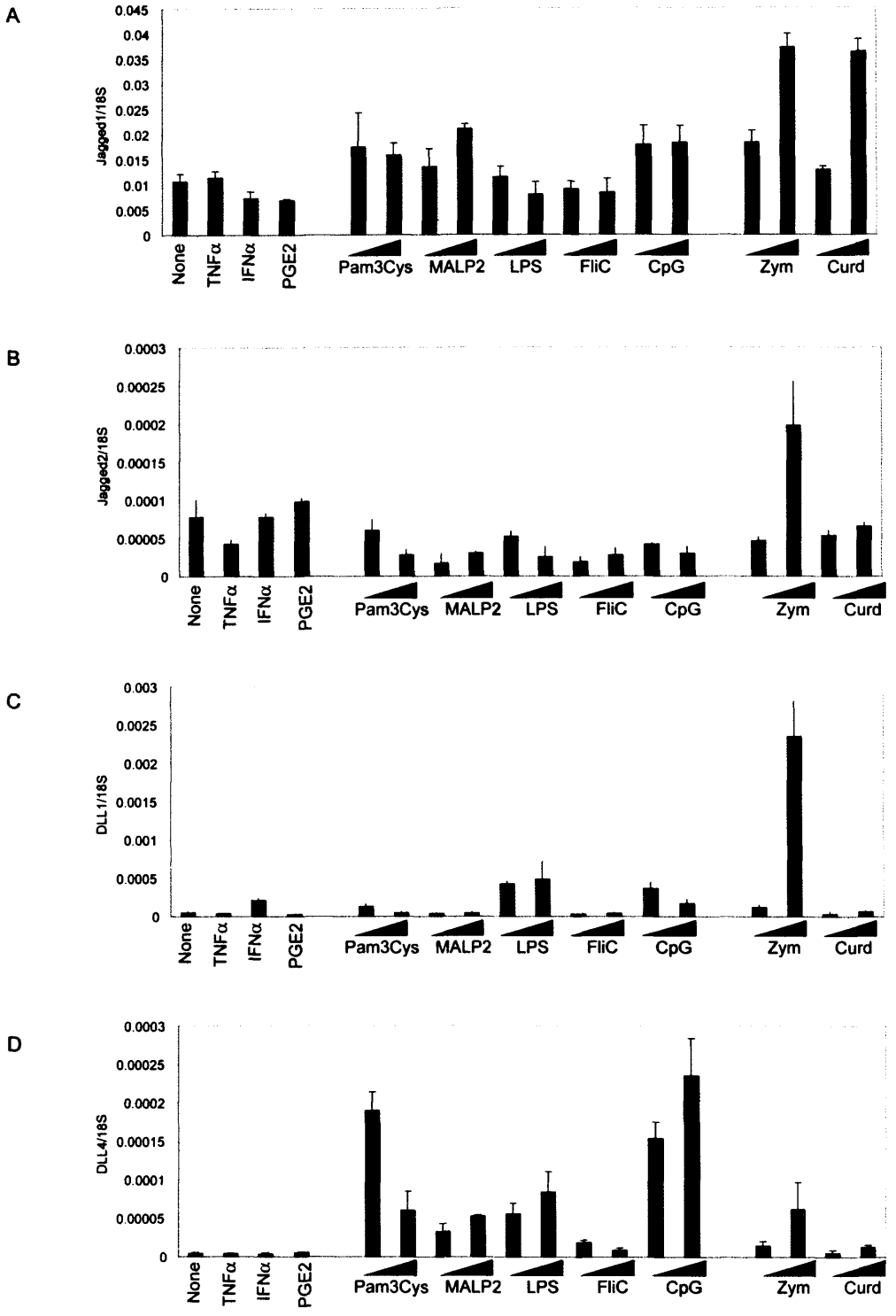


Figure 4-3: Upregulation of Notch-ligands by inflammatory and microbial stimuli. C57BL/6 BMDC (5 x 10^5 per well of a 24 well plate) were stimulated with TNFα 50ng/ml, IFNα 5000U/ml, ProstaglandinE2 (PGE2) 1nM, 0.1nM;, Pam3Cys 2, 10μg/ml; MALP2 0.2, 1μg/ml; LPS (highly purified from Salmonella) 1, 5μg/ml; Flagellin (FliC) 0.2, 1μg/ml; CpG 1668 0.5, 2,5μg/ml; Zymosan (Zym) 20, 100μg/ml; Curdlan (Curd) 20, 100μg/ml. After 3hrs cells were lysed in Trizol (Invitrogen) and RNA was extracted for RT-PCR and Q-PCR analysis using

SYBR-green. Results are normalised to 18S rRNA measured by Taqman. Data is representative of 3 separate experiments

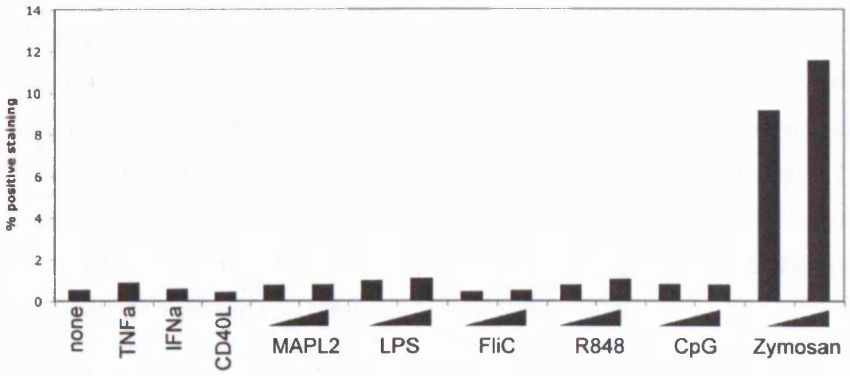
4.2.4 : Zymosan up-regulates Jagged1 at the cell surface

Little is known about the relationship between mRNA levels for Notch-ligands and protein levels found at the cell surface where they can interact with Notch. To establish what my mRNA data represented on a per cell basis I developed a flow-cytometry protocol to stain for Jagged1. Using this method the induction of Jagged1 by zymosan is even more striking (figure 4-4A) with 12% positive cells at 24hrs after stimulation compared to 1-2% after TLR stimulation.

It was necessary to validate the specificity of staining for Jagged1. Jagged-1-floxed mice were generated by Dr Katsuto Hozumi (See figure 3-10C). Bone marrow from these mice was transduced with a retrovirus expressing a Cre-recombinase-GFP fusion protein (MSCV-GFPCre8) and GM-CSF BMDC were grown out. The resulting DC population appeared to be around 60% GFPCre-positive (figure 4-4B) and bulk populations were used for these assays. Remaining cells were sorted on the basis of GFP-expression (for a separate experiment detailed in chapter 6) and a fraction was treated to extract genomic DNA. A duplex PCR for recombination was carried out on cells and is shown in figure 4-4B. All GFP-positive cells have fully deleted Jagged-1. However, around half of the sorted GFP-negative cells are also positive for the recombined allele suggesting that overall transduction was roughly 70-80%. When the unsorted, bulk populations were stimulated with zymosan for 6hrs or were left unstimulated it is clear that Jagged-1 staining is decreased in "Jagged1-/-" BMDC in both the unstimulated and stimulated samples. In addition, there is no significant increase in Jagged-1 staining after zymosan stimulation in the Jagged1-/- cells, with the exception of a small shift in the side population, likely corresponding to the c 20% non-recombined cells remaining (figure 4-4B).

An interesting aspect of Jagged1 staining is that at saturating doses of zymosan only a fraction of the cells are ever found to be positive (up to around 50%) with the negative population precisely overlaying isotype control staining. Jagged1-positive cells are present by 4hrs with the highest percentages found after 6hrs of stimulation (figure 4-4C) that then persist or drop slightly by 24hrs. There are many possible reasons for this non-uniform staining, ranging from inefficency of the staining, through stochasticity of signalling induced by zymosan, to a role of Notch signalling in trans between DC leading to shedding or internalisation of the notch ligands.

A.



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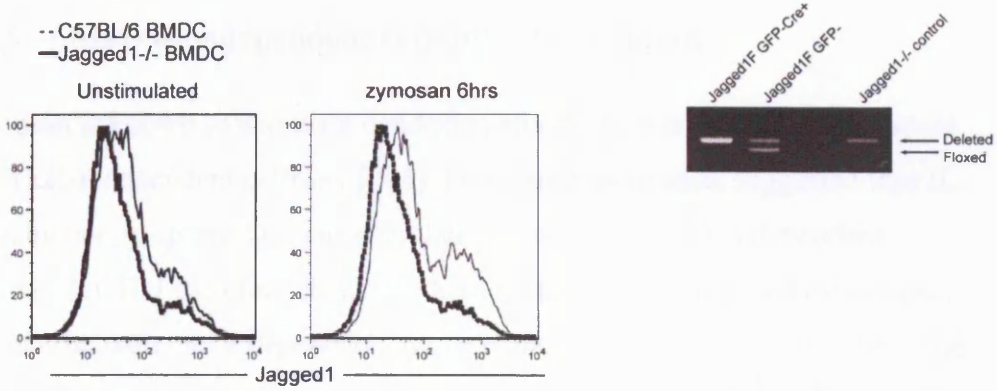

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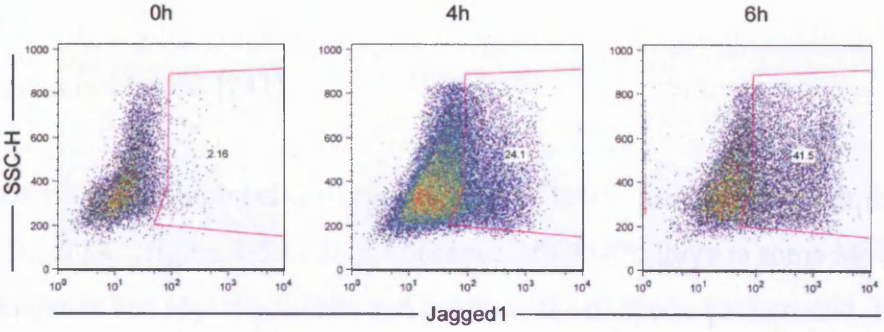


Figure 4-4: Upregulation of Jagged-1 production by Zymosan stimulation of BMDC. C57BL/6 BMDC (5 x 10^5 per well of a 24 well plate) were stimulated with TNFα 50ng/ml, IFNα 5000U/ml, CD40L-trimers 1μg/ml; MALP2 0.2, 1μg/ml; LPS (highly purified from Salmonella) 1, 5μg/ml; Flagellin (FliC) 0.2, 1μg/ml; R848 0.2, 1 nM; CpG 1668 0.5, 2,5μg/ml; Zymosan (Zym) 20, 100μg/ml, at 37°C overnight in the presence of GM-CSF. BMDC were recovered and stained for surface Jagged1. The percentage of CD11c+ cells that are Jagged1-positive (as determined by isotype control staining) is shown in part A. Data is representative of 2 separate experiments. Part B: Bone marrow from Jagged1-flox or littermate controls was transduced with MSCV-GFPCre8 (as described in section 2.4) to generate Jagged1-/- cells. Resulting control or "Jagged-/-" BMDC (5 x 10^5 per well of a 24 well plate) were cultured for 6hrs in the presence (zymosan) or absence (no stimulus) of 100μg/ml zymosan. Recovered cells were stained for Jagged1. Data is represented as histograms of Jagged1 staining. BMDC were 60% GFP-positive as shown in bottom right-hand plot. GFP positive and negative fractions of these cells were separated by FACS sorting and genomic DNA was extracted. This was analysed for the presence of recombined alleles using a duplexed PCR (described in chapter 2.11).Products obtained were run on a 2% agarose gel and imaged. Part C: C57BL/6 BMDC were stimulated with 100μg/ml zymosan for 0, 4 or 6hrs before surface staining for Jagged1. Data is representative of 4 separate experiments.

4.2.5 : MyD88-independent responses to zymosan

Zymosan is known to stimulate dendritic cells through both a TLR-dependent and TLR-independent pathway [205]. Previous experiments suggested that IL-10 induction in spleen DC was stimulated through the TLR-independent pathway [205]. I therefore checked whether other factors induced by zymosan stimulation were more dependent on one pathway or the other in BM-DC. The TLR component of zymosan is recognised through TLR2/6 heterodimers [59]. MyD88 is absolutely required for TLR2/6 to signal (reviewed in [28]), therefore TLR2/6 signalling can be effectively eliminated using dendritic cells genetically deficient in MyD88 [241].

MyD88-/- DC stimulated with zymosan and CD40L produce as much IL-10 as C57BL/6 DC (figure 4-5A). In the absence of CD40L there is some MyD88-dependence but MyD88-/- cells still produce IL-10 above background. IL-2 production shows a partial decrease in MyD88-/- DC cultures, again, not to zero

and is unaffected by CD40L costimulation (figure 4-5A). IL-12 p40 demonstrates a similar partial decrease in MyD88-/- cultures stimulated with zymosan in the absence of CD40L-fibroblasts. However, in the presence of CD40L fibroblasts, IL-12 p40 production cannot be increased by zymosan stimulation (figure 4-5A). It should be noted that CpG stimulation, as an example of a strong IL-12-inducing stimulus, further increases IL-12 p40 levels above the background achieved with CD40 ligation [242].

During the course of these experiments it was observed that GM-CSF and CD40L had differing abilities to amplify the production of IL-2, IL-10 and IL-12 p40. GM-CSF efficiently amplifies IL-2 induction in response to zymosan, leaving IL-10 and IL-12p40 unaffected. Conversely CD40L strongly amplifies IL-10 and IL-12 production but has only a limited effect on IL-2 (figure 4-5B). The regulation of cytokine production clearly involves multiple pathways that can synergise or not depending on their nature (figure 4-5A and B, [32, 33]). It is possible that this goes some way to explaining the variation in MyD88 dependence of IL-10 and IL-12 p40 induction between experiments (production of these cytokines is much less affected by MyD88-deficiency in 4-5B than in 4-5A!) although the actual source of this variation has been elusive.

Despite some variability, the important observation remains that in the absence of TLR signalling, recognition of zymosan can lead to the production of significant levels of IL-10 and IL-12 p40 and low levels of IL-2 implying the existence of a non-TLR receptor that is capable of coupling microbial recognition to cytokine production by dendritic cells.

MyD88-/- BMDC also up-regulated Jagged1 at the mRNA and protein level in response to zymosan demonstrating that this component of DC maturation can also be induced by TLR-independent pattern-recognition (figure 4-5C and D).

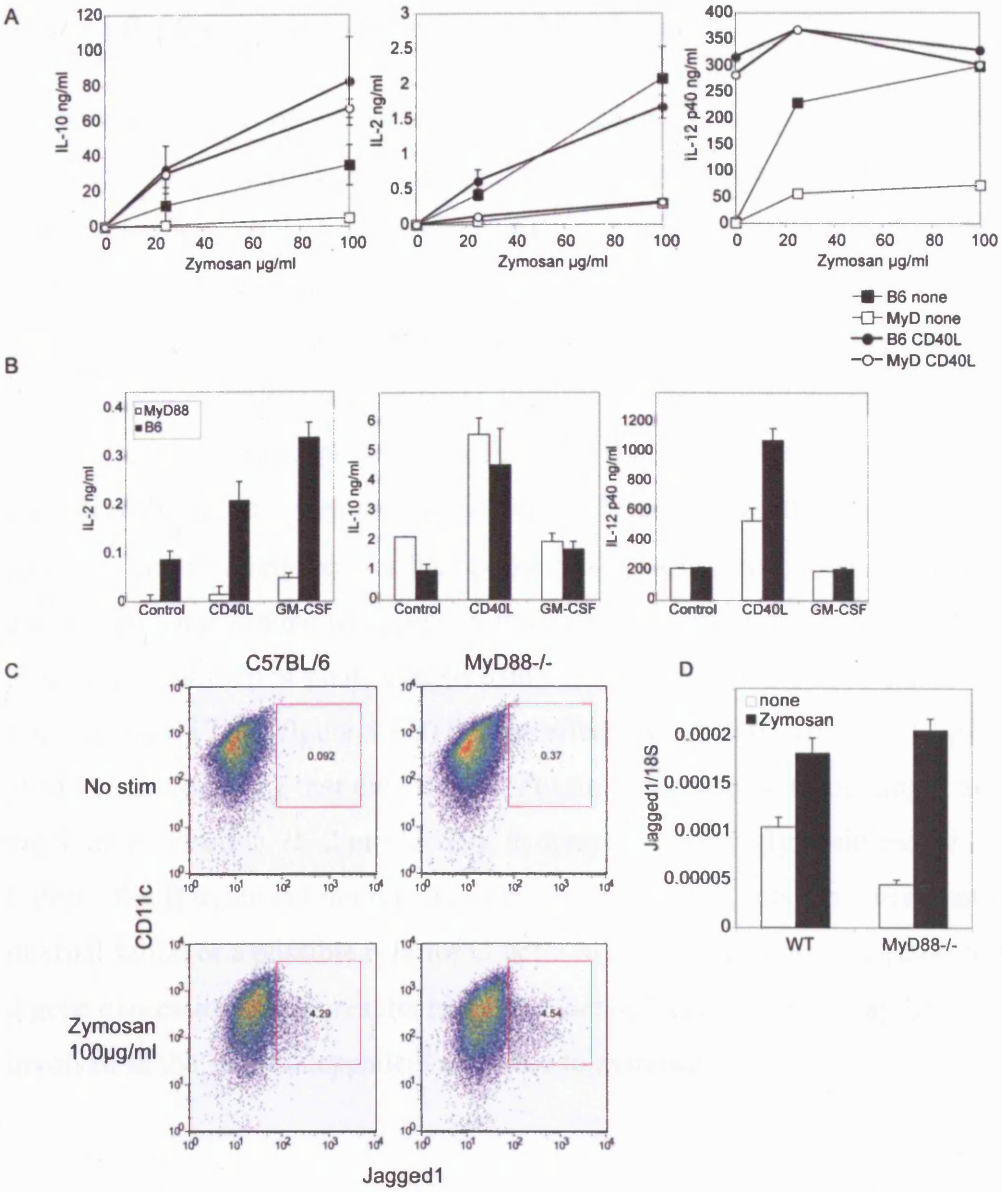


Figure 4-5: IL-10 and Jagged-1 upregulation in response to zymosan can occur independently of TLR signalling. C57BL/6 or MyD88-/- BMDC were enriched to >95% purity using MACS. 10^5 BMDC were plated per well of a flat-bottomed 96 well plate in the presence or absence of CD40L-expressing fibroblasts. After 24hrs cells were freeze-thawed and cytokine concentrations determined by sandwich ELISA. B: Cells handled as in part A were cultured with 25µg/ml zymosan in the presence of CD40L fibroblasts or GM-CSF. Cytokine production at 24hrs was determined by sandwich ELISA. C: BMDC as in A and B were stimulated for 6hrs with 100µg/ml of zymosan. BMDC were stained for Jagged1 and analysed by flow cytometry. All data shown is representative of more than 3 experiments.

4.2.6 : β-glucan recognition in cytokine induction

As discussed in section 4.1.4 the β-glucan receptor Dectin-1 is known to play an important role in zymosan recognition by macrophages [230, 231]. To determine whether a β-glucan receptor such as Dectin-1 might be responsible for the TLR-independent response to zymosan we performed inhibition experiments using a known inhibitor of Dectin-1, glucan phosphate [230]. When glucan phosphate was added to cultures of BMDC and zymosan it could partially inhibit production of IL-10, IL-2 and IL-12 p40 (figure 4-6A). The interpretation of these data is complicated by the possibility that β-glucan receptors could be required for the uptake of zymosan before recognition in the endosomal compartment by receptors with other specificities, such as TLR2. However, inhibition of phagocytosis using the actin-polymerisation inhibitor Latrunculin B [243] (figure 4-6B) had no effect on either IL-10 or IL-12 p40 production suggesting that the effect of glucan phosphate was not simply to block internalisation. IL-2 production, in contrast, is strongly inhibited by Latrunculin B treatment but it is unclear whether this reflects a requirement for internalisation or a possible role for G-actin in regulating signalling [244] to IL-2 gene expression. These results hint that a beta-glucan receptor may be involved in the TLR-independent response to zymosan.

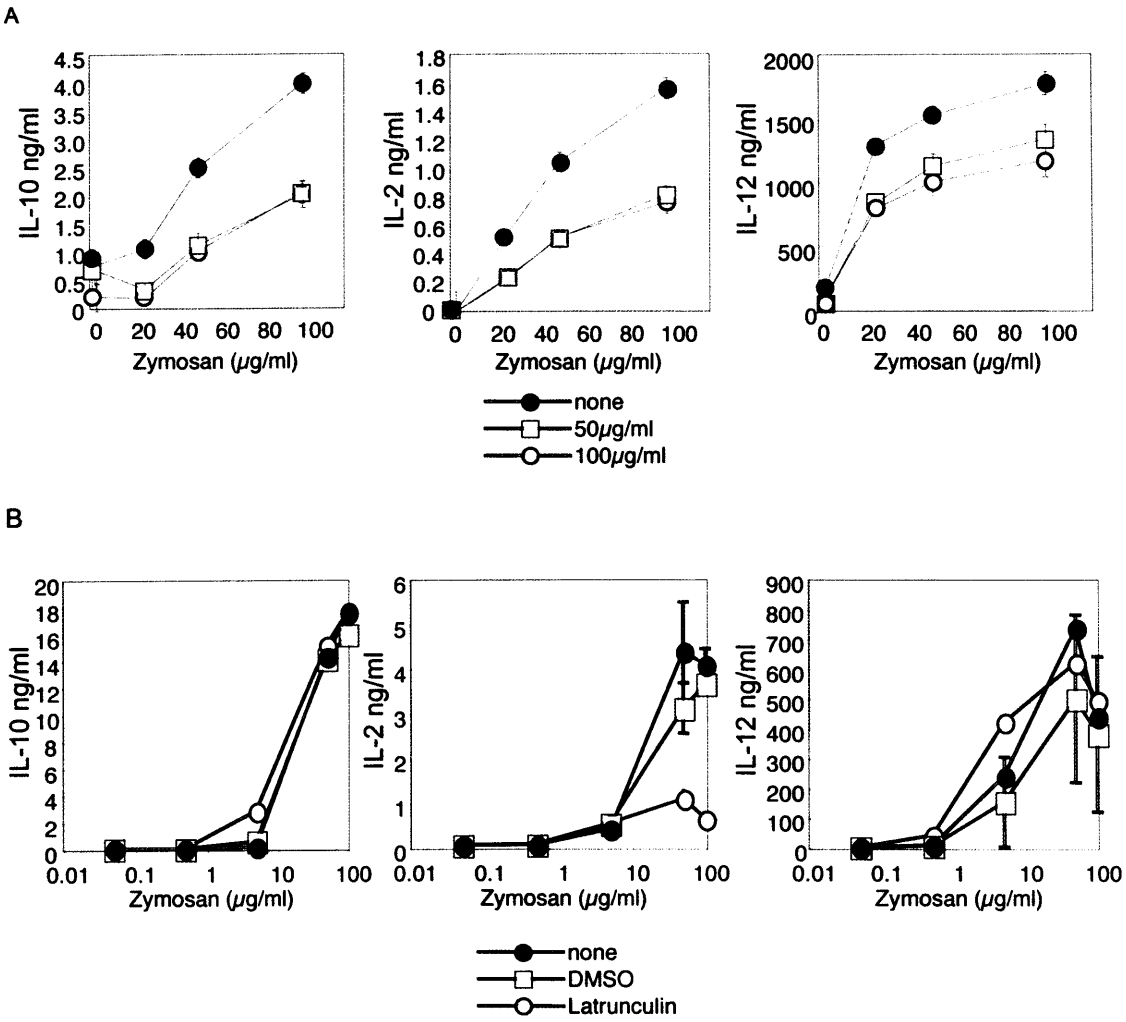


Figure 4-6: IL-10 production induced by zymosan is inhibitable with soluble Glucan phosphate, but not Latrunculin B. A) C57BL/6 BMDC (10^5 per well of a flat-bottomed 96 well plate) were pre-treated for 30mins with 50 or 100μg/ml of glucan phosphate. Indicated concentrations of zymosan were added and cells were cultured at 37°C overnight. Freeze-thawed supernatants were assayed for cytokines by sandwich ELISA. B) Bulk C57BL/6 BMDC (Unenriched by MACS; 2 x 10^5 per well) were cultured with the indicated concentration of zymosan in the presence or absence of 10μM Latrunculin B or vehicle (DMSO) alone. Cytokine concentration in the supernatants after overnight culture was determined by sandwich ELISA. Data is representative of 3 independent experiments

4.2.7 : Dectin-1: A β-glucan receptor with a HemITAM.

β-glucan recognition appears to play a role in zymosan recognition by BMDC making Dectin-1 a good candidate receptor for zymosan on DC. As discussed, the 30ααα intracellular tail contains what initially looked like a putative ITAM motif (figure 4-7A) [52]. The presence of a possible ITAM was an exciting clue

that the signalling initiated by Dectin-1 may be similar to conventional ITAM signalling leading to the activation of Syk or Zap70 [245] [246]. Dectin-1 is widely expressed in myeloid cells [232], as is Syk, whilst Zap-70 is largely restricted to the T cell lineage [247].

As we wanted to define whether Dectin-1 signals for cytokine production rather than presents zymosan to other receptors for recognition we carried out gain-of-function experiments. Neil Rogers (Immunobiology Lab, Cancer Research UK, London [146]) first established that the LK B cell hybridoma line was unresponsive to zymosan but could produce IL-2 and IL-10 on BCR cross-linking. LK cells expressing full-length Dectin-1 but not Dectin-1 lacking the intracellular tail could respond to zymosan by making IL-2 and IL-10 indicating that the cytoplasmic domain of Dectin-1 was critical for its function [146]. This requirement was not at the level of Dectin-1 binding as cells expressing truncated Dectin-1 bound zymosan as efficiently as cells expressing wild-type Dectin-1 [146]. Given that IL-10 production is independent of phagocytosis, as determined by Latrunculin B inhibition, these data suggest that Dectin-1 plays a direct signalling role in the induction of cytokines.

In macrophages the intracellular tail of Dectin-1 becomes tyrosine-phosphorylated on zymosan binding [233]. The two tyrosines that could be phosphorylated are tyrosine 3 (in the first YXXXL motif of the ITAM (figure 4-7A) and tyrosine 15 in the second YXXL motif. LK cell lines expressing Dectin-1 in which the two tyrosines were mutated separately or simultaneously to pheylalanine were tested for responsiveness to zymosan. Surprisingly, mutation of tyrosine-3 to phenylalanine (Y3F) had no effect on induction of IL-2 or IL-10 by zymosan (figure 4-7B) or in recruitment of phospho-Syk to the site of zymosan-binding (figure 4-7C, produced by Martijn Nolte and reproduced with kind permission here). Despite requiring only a single YXXL-motif Dectin-1 was still able to recruit phospho-Syk to the phagocytic cup as determined by confocal microscopy. In addition Neil Rogers demonstrated that peptides corresponding to the Dectin-1 intracellular tail mutants required only the membrane-proximal tyrosine-15 to immunoprecipitate purified Syk [146].

Together, this data implies that Dectin-1 can signal through Syk using a novel half-ITAM or "hemITAM" motif and can induce cytokine production in a B cell hybridoma.

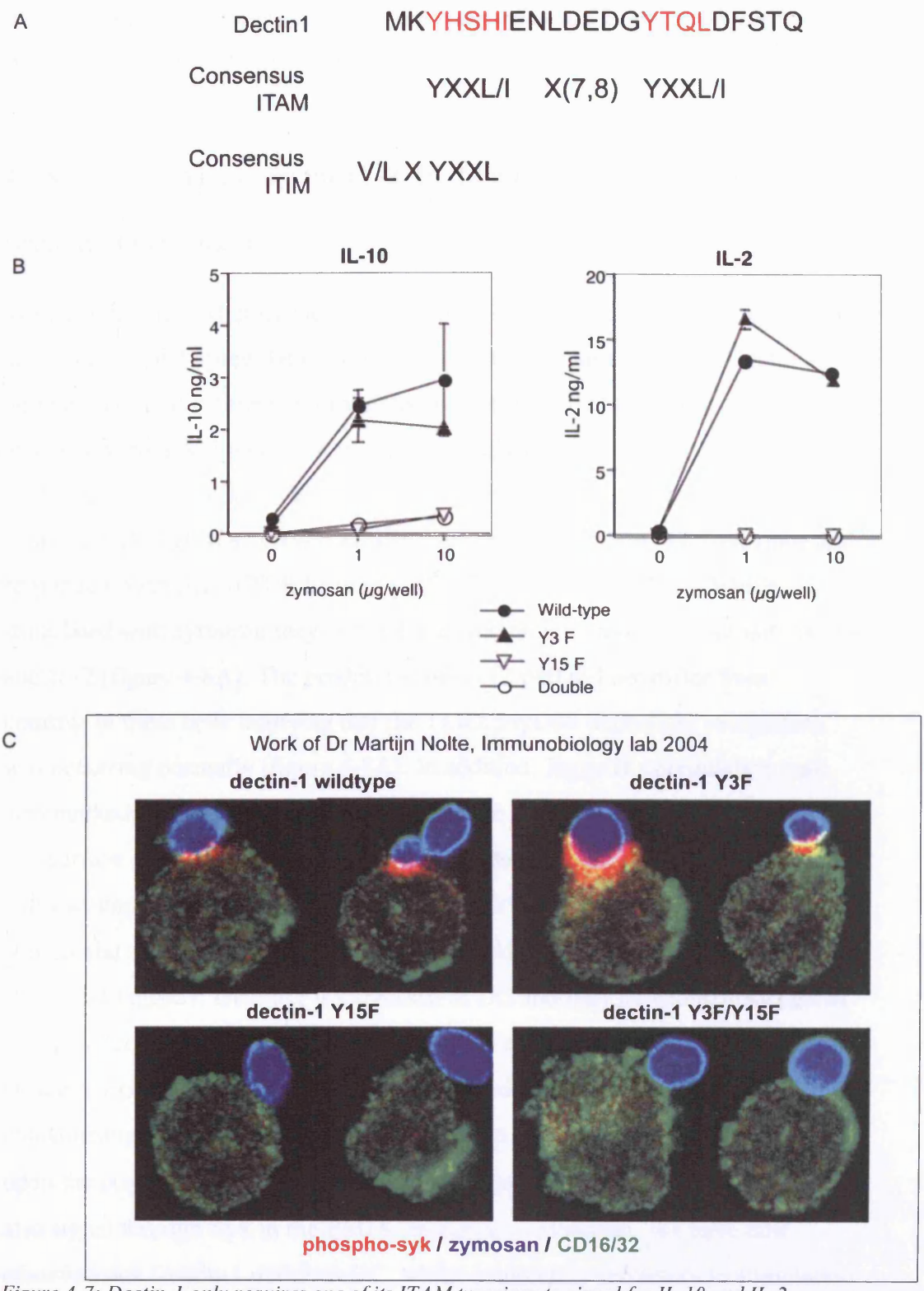


Figure 4-7: Dectin-1 only requires one of its ITAM tyrosines to signal for IL-10 and IL-2

production. A) Sequence of the intracellular tail of Dectin-1 compared to a standard ITAM or ITIM sequence. B) LK cells expressing either wild-type Dectin-1 or the indicated Dectin-1 mutants (10^5 per well) were stimulated with the indicated concentrations of zymosan. Levels of IL-10 in the supernatants after overnight culture were determined by sandwich ELISA. C) WORK OF DR M NOLTE. The same LK cells as in (B) were stimulated with Cy5 zymosan (blue) for 5 min then fixed and stained for phospho-Syk (red) and CD16/32 (green). Images are optical sections through representative cells.

4.2.8 : Syk is necessary for IL-2, IL-10 and Jagged1 induction in response to zymosan

We wanted to test whether Dectin-1 might be signalling for cytokine production and Notch-ligand upregulation in DC. As we did not have access to Dectin-1-deficient DC at this time we formulated a hypothesis that since Dectin-1 could associate with Syk, loss of Syk in dendritic cells should abrogate Dectin-1 signalling. Chimeric mice were made using Syk-/- foetal liver or womb-mate controls. Syk-/- BMDC grew normally from the bone marrow of these mice and responded normally to TLR ligation [146]. However, when Syk-/- BMDC were stimulated with zymosan they showed a drastic reduction in levels of both IL-10 and IL-2 (figure 4-8A). The production of IL-12 p40 did not differ from controls in these cells implying that the TLR2/MyD88 dependent recognition was occurring normally (figure 4-8A). In addition, Jagged1 upregulation was also markedly reduced in Syk-/- BMDC at both the level of mRNA induction and surface staining (figure 4-8B). Syk-dependent pattern-recognition clearly plays an important role in the response of dendritic cells to β-glucan-containing stimuli and appears to bias DC towards the production of high levels of IL-2, IL-10 and Jagged1. Dectin-1 is expressed in DC and may be acting upstream of Syk to induce signalling. It should however be noted that the effects of Syk-deficiency (figure 4-8) are much more profound than the effects seen by antagonising β-glucan recognition using glucan phosphate (figure 4-6) leaving open the possibility that receptors for components other than β-glucans may also signal through Syk in the BMDC response to zymosan. We have now observed that Dectin-1 deficient DC, whilst completely refractory to stimulation

by the purified β-glucan, Curdlan, are only partially impaired in their response to zymosan (IL-2, IL-10 and IL-12 p40 induction at the protein level. Salomé Leibundgut, manuscript in preparation) adding weight to the hypothesis that further Syk-dependent receptors may be involved.

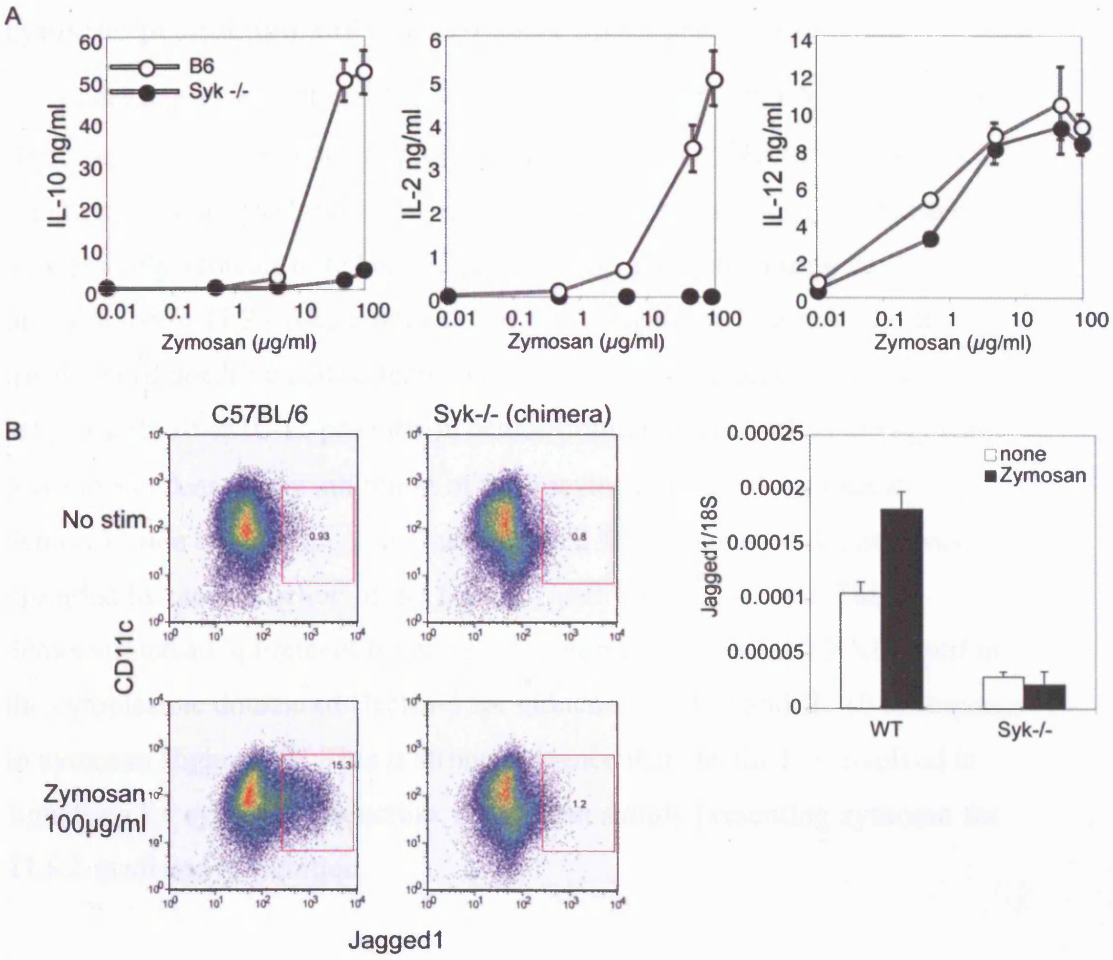


Figure 4-8: IL-2, IL-10 and Jagged-1 induction in response to zymosan is dependent on signalling through Syk. A: syk-/- or control BMDC (10^5 per well) were cultured with the indicated doses of zymosan for 24hrs. Cytokine concentrations in freeze-thawed supernatants were determined by sandwich ELISA. B: syk-/- or control BMDC (10^6 per well of a 24 well plate) were stimulated with 100μg/ml zymosan for 6hrs. Cells were recovered and stained for Jagged1. All data is representative of 3 independent experiments.

4.3 : Discussion

4.3.1 Dectin-1 is a Pattern Recognition Receptor capable of inducing cytokine production and Notch-ligand upregulation in DC

I have demonstrated that small soluble β-glucans inhibit the BMDC cytokine response to zymosan, hinting that the β-glucan receptor, Dectin-1 may play a role in this induction (figure 4-6). Blocking experiments cannot differentiate between requirements to tether and phagocytose zymosan, aiding its presentation to TLR2/6 heterodimers, or actual signalling. Latrunculin B treatment of dendritic cells effectively blocks zymosan phagocytosis without affecting IL-10 or IL-12 production demonstrating that the effect of β-glucan was independent of any inhibition of phagocytosis (figure 4-6). Further demonstration that Dectin-1 can indeed signal for cytokine production was provided by reconstruction of the Dectin-1 pathway in LK cells. This demonstrated a requirement for an intact membrane-proximal YXXL motif in the cytoplasmic domain of Dectin-1 for induction of IL-2 and IL-10 in response to zymosan (figure 4-7). This is strong evidence that Dectin-1 is involved in signalling for cytokine production, rather than simply presenting zymosan for TLR2-mediated recognition.

Further evidence that a zymosan-binding receptor was capable of signalling for cytokine production is revealed in the upregulation of IL-10 and Jagged-1 in response to zymosan in MyD88-deficient DC (figure 4-5). We hypothesised that Dectin-1 may be a TLR-independent PRR involved in zymosan (and therefore yeast) recognition by DC.

The ITAM-like sequence in the short intracellular tail led us to investigate signalling through Syk. Tyrosine-phosphorylation of Dectin-1 has been demonstrated previously [233] suggesting that the ITAM may be functional. Martijn Nolte elegantly demonstrated that Dectin-1 can recruit phospho-Syk to zymosan-containing phagocytic cups (figure 4-7). In the LK cell system this

requires the presence of tyrosine-15 in the intracellular tail but not tyrosine-3, i.e. only the membrane-proximal half of the ITAM is required for Syk recruitment [146]. Interaction between purified recombinant Syk and synthetic peptides of the entire Dectin-1 intracellular domain, with mutations in the ITAM tyrosines confirmed the requirement for tyrosine-15 but not tyrosine-3. Half-ITAMs or "HemITAMs" appears to be a novel signalling motifs capable of binding and activating signalling through Syk (figure 4-7, [146]).

There is prcedent for non-ITAM signalling through Syk. In a reconstruction of proximal B cell Receptor signalling in Drosophila S2 Schneider cells, a BCR in which the only one ITAM tyrosine remained could induce low level phosphorylation of SLP-65 via Syk that was absent if both tyrosines were mutated [248]. However, compared to the activity of Syk in the presence of 2 full ITAMs, this induction is marginal suggesting there may be other facets of the Dectin-1 tyrosine motif that favour Syk recruitment. Intriguingly it has previously been noted that the N-terminal SH2 domain of Syk does not bind efficiently to phospho-ITAMs in isolation whilst the C-terminal SH2 domain binds with moderate affinity [249]. The N-terminal SH2 domain and adjacent linker can bind to the intracellular tails of β1, β2 and β3 integrins independently of tyrosine phosphorylation [235]. This demonstrates that non-ITAM motifs can bind and activate Syk and hints that similar interactions could play a role in Dectin-1-Syk interactions.

Dendritic cells deficient for Syk are markedly impaired in their responses to zymosan, in particular in the induction of IL-10, IL-2 and Jagged1, whilst IL-12 p70 production is unaffected (figure 4-8, [146]). This is evidence in favour of the involvement of Dectin-1 signalling in zymosan recognition by DC, although other Syk-activating receptors may also be involved.

A critical observation in this series of experiments is that neither removal of MyD88 nor removal of Syk leads to a complete loss of production in any cytokine tested, although there are quantitative decreases (sometimes pronounced, figure 4-5, 4-8). This has led us to conclude previously that IL-10

induction is Syk-dependent whilst IL-12 induction is TLR-dependent [146]. On closer inspection this turns out to be a simplification and the majority of cytokines induced by zymosan can be induced, at least to low levels, by either signalling pathway. This data correlates with recent data generated using a purified Dectin-1 agonist that signals exclusively through Dectin-1 and Syk and can lead to the induction of IL-2, IL-10, IL-23, IL-12 p40, and IL-6 (Salomé LeibundGut Landmann, manuscript in preparation). A Syk-dependent pathway for pattern recognition in DC exists. β1,3 and β1,6-glucans are essential structural components of yeast cells walls. Dectin-1 can recognise these conserved pathogen-associated molecules and signal into dendritic cells for maturation and cytokine production, fulfilling both of the original criteria for the identification of bona fide "Pattern Recognition Receptors".

4.3.2 Zymosan induces high levels of IL-2, IL-10 and Jagged-1

What is the significance of the very high IL-2, IL-10, Jagged1 and TNFα production by zymosan and curdlan-stimulated DCs (demonstrated in figure 4-1)? So far the role of these cytokines in instructing T cell differentiation has not been conclusively demonstrated. Several possibilities exist. Firstly, T cells and dendritic cells are not the only cells in the body and some DC cytokines may be required to act on cells other than T cells. It is interesting to note that intracellular staining of DC demonstrates that IL-2 production only occurs from cells also making high levels of IL-12 p40 and TNFα (figure 4-2) suggesting that at least at some point these cytokines are produced together. IL-10, on the other hand appears to be produced after IL-2 production has ceased (figure 4-2) suggesting that the targets of these two cytokines are different, or that the timing of cytokine signals is an important factor in programming T cell differentiation. Pulse-chase experiments on antigen-processing and presentation suggest that antigen-presentation can be detected (using the C4H3 antibody to detect MHC-class II-HEL complexes) by around 3-4hrs after protein uptake *in vitro* [250], roughly coincident with the start of IL-2 and IL-12 p40 production (figure 4-2).

The ability of GM-CSF to amplify IL-2 but not IL-10 or IL-12 production is additionally intriguing (figure 4-5). It is unclear whether this simply represents differences in the signalling requirements of the different cytokines or whether feedback amplification by different cell types promotes the delivery of DC cytokines in cis.

This work helped us to understand how signalling via a Syk-dependent, TLR-independent PRR activated by zymosan produces a specific cytokine and Notch-ligand signature in DCs. In addition, I have demonstrated that Dectin-1 is capable of signalling, via Syk and a novel HemITAM motif for the induction of IL-2 and IL-10. This raises two sets of interesting questions, firstly in the cell signalling activated in response to zymosan and secondly in the physiological role of Dectin-1 ligation in DC maturation and T cell priming. In the next chapter I will discuss the some advances on dissecting the signalling downstream of zymosan recognition.

5 Chapter 5: Signals linking Syk activation to the production of high levels of IL-2 and IL-10

5.1 : Introduction

Zymosan induces signalling through Syk, at least in part by binding to Dectin-1. Syk is essential for the induction of IL-2 and IL-10 in response to zymosan. What is going on downstream of Syk to lead to the strong induction of these cytokines?

5.1.1 Signalling in response to zymosan: What do we know?

As discussed in chapter 4, zymosan can induce signalling through Dectin-1/Syk and through TLR2/MyD88. Whilst the TLR/MyD88 signalling pathway is now quite well-defined (reviewed in [23]), the half-ITAM motif used by Dectin-1 is novel and therefore research into the signalling induced is only just beginning.

Classical ITAM signalling through Syk/ZAP-70 can activate all of the MAP kinase pathways as well as NF-κB and calcium signalling [251]. Recruitment of Syk/ZAP-70 to ITAMs results in tyrosine-phosphorylation of Syk and subsequent recruitment of downstream signalling components [252] including two adaptor proteins, LAT and SLP-76. These act as scaffolds for the activation of PLCγ, Sos/Grb2, Vav, etc. Ras can be activated by Grb2/Sos associated with LAT; by activation of RasGRP (Ras guanyl nucleotide-releasing protein) downstream of PLCγ; or via PKC (Reviewed in [251]). In addition, Vav appears to be necessary to couple ZAP-70 activation to ERK activation and calcium mobilisation in murine T cells [253].

Signalling to the MAP kinases triggered by cross-linking of the high affinity IgE receptor on Mast cells proceeds via an almost identical pathway using Syk, rather than ZAP-70 as the non-receptor tyrosine kinase (reviewed in [254]). B cell receptor signalling through Syk is very similar to T cell receptor signalling, with a few notable differences. There is a B cell homologue of LAT, called NTAL/LAB which is phosphorylated by Syk and recruits Grb2 to enhance BCR-induced ERK activation (reviewed in [255]). However, unlike LAT, NTAL does not associate with PCLγ suggesting there may be a further adaptor in B cells that links to PLCγ and calcium mobilisation.

ITAM signalling to NF-κB has recently been elucidated. Mice deficient in Bcl10, CARMA1 or MALT1 all have severe defects in TCR-induced NF-κB activation (reviewed in [256, 257]). On TCR ligation PKCθ, Bcl10, MALT1 and CARMA1 are recruited into lipid rafts. Oligomerised Bcl10 and MALT1 form an E3 ubiquitin ligase which together with the E2-ubiquitin-conjugating complex UBC13 and MMS2 catalyse the K63 poly-ubiquitination and activation of NEMO and therefore NF-κB [256, 257]. Intriguingly, MALT1-deficient mice also show a defect in p38 MAP kinase and JNK signalling on PMA/ionomycin treatment of T cells, although the link between MALT1 and MAP kinase signalling is currently unclear[256]. Contradictory results have been reported for B cell signalling in the MALT-1 knock-out although B cell development is impaired suggesting a similar signalling machanism is likely to play a role (reviewed in [258] [259]). PKCθ is not expressed in B cells therefore a PKCβ is instead required for CARMA1 recruitment (reviewed in [258]).

5.1.2 Activation of NF-κB downstream of Dectin-1

A recent publication by Gross et al [147] elegantly demonstrates that NF-κB activation downstream of Dectin-1 is achieved by a homologous pathway to that downstream of the TCR. In place of CARMA1, Dectin-1 signalling uses CARD9, which lacks the membrane guanylate kinase domain found in CARMA1 but is sufficient to form an active E3 ubiquitin ligase complex with MALT1 and Bcl10 leading to NF-κB activation. Dendritic cells deficient in

CARD9 show a severe defect in IL-10 production in response to zymosan stimulation [147].

5.1.3 Signals associated with IL-2 and IL-10 induction in DC

IL-2 induction in DC, like IL-2 induction in T cells, can be inhibited by cyclosporin A [260]. This inhibitor targets calcineurin, preventing the calcium-induced dephosphorylation, and activation of NFAT [261]. However, cyclosporine has also been reported to affect calcium-dependent signals leading to AP-1 transcriptional activation [262].

Cyclosporin A is an extract from *Tolypocladium inflatum Gams* a member of the Cordyceps family of parasitic fungi (predominantly entemoparasites) isolated from Norwegian soil under certain conditions [261]. The function of the peptide in intact *Tolypocladium inflatum Gams* is unknown but although NFAT is restricted to vertebrates, calcineurin is present in both C. elegans and Drosophila [263] and it is an intriguing possibility that Cyclosporin A may inhibit evolutionarily conserved anti-fungal innate responses in its host organism.

IL-2 production is best characterised in T cells. ERK, JNK and calcium signalling are required for T cell IL-2 production [262, 264, 265]. The IL-2 promoter contains AP-1, NFAT and NF-κB binding sites, all of which are necessary for the efficient induction of IL-2 [266]. IL-2 is also known to be regulated post-transcriptionally at the level of mRNA stability [132]. As discussed in chapter 1, this appears to require p38 MAP kinase signalling to re-localise mRNA bound to TTP away from stress-granules where it is otherwise rapidly degraded [126, 132].

In dendritic cells IL-2 is known to be induced by microbial stimuli and to require autocrine IL-15 [133].

A number of reports now associate high levels of IL-10 production by DC with robust ERK activation [267] [175] [94]. c-Fos is implicated as a downstream

target of ERK required for IL-10 induction [94]. It has recently been suggested that in macrophages ERK activation is essential for Histone H3 phosphorylation and chromatin modification in the IL-10 promoter allowing transcription factor binding although whether this is also the case in DC is unknown [268].

Further factors required for the inducible transcription of IL-10 are IRF1 downstream of type I IFN signalling [269]; Sp-1 downstream of p38 MAP Kinase signalling [270] and STAT3, which is required for IL-10 induction by LPS [229] [269] although the mechanism of STAT3 activation in this case is unclear.

5.1.4 The ERK pathway is a strong candidate for modulating zymosan-induction of IL-2 and IL-10

A large number of signalling components are likely to be activated downstream of Dectin-1 and will play a role in modulating the transcription and translation of IL-2 and IL-10. I have therefore started to investigate the possible signalling by examining a good candidate pathway. Published data supports a role of ERK signalling in the induction of these cytokines in a variety of cell types [262, 264, 265] [267] [175] [94].

Zymosan has been used as a strong stimulus for ERK activation in macrophages for some time [271, 272] and both TLR signalling and classical ITAM signalling can couple to ERK activation (reviewed in [251] [23]). Interestingly, TLR2 induced ERK activation is implicated in the ability of TLR2-ligands to induce IL-10 [136], despite the observation that TLR signalling is dispensable for IL-10 production in response to zymosan (Chapter 4, [96] [146]). In macrophages, ERK activation downstream of TLR2 requires the proteasomal degradation of NF-κB1 p105, which releases active TPL2 to phopshorylate and activate MEK1/2 [135]. Targeting of NF-κB1 p105 for proteasomal degradation is achieved through activation of the IKK complex [135].

Experiments discussed in this chapter aim to examine the role of ERK signalling in dendritic cell responses to zymosan. The data presented here demonstrate that zymosan is a strong stimulus for ERK activation and that ERK inhibitors decreased IL-10 and IL-2 induction in response to zymosan.

5.1.5 What are the candidate downstream targets of ERK signalling?

Many diverse ERK targets have been identified, many of which are direct transcriptional activators, but also including proteins associated with control of mRNA processing, translation, cytoskeletal function, cell cycle progression and organelle-specific functions [273]. c-Fos has previously been implicated as a target of ERK required for IL-10 production [136]. However, c-Fos upregulation, rather than the direct phosphorylation of c-Fos was required indicating a possible role for an intermediate transcription factor. Growth-factor induced c-Fos transcription requires ERK signalling to phosphorylate and activate the Ternary Complex Factor Elk-1, leading to association with Serum Response Factor and transcriptional activation from the Serum Response Element in the c-fos promoter [274]. There are two other TCF family members Sap-1 and Net, both of which are direct targets of ERK (reviewed in [275]). LPS signalling in macrophages induced TNFα expression via the ERK pathway. This requires the phosphorylation of Elk-1 by ERK and subsequent transcription of Egr-1 [276].

The ERK cascade can also activate a number of kinases, including p90 Ribosomal S6 Kinase (RSK)1, RSK2, RSK3, RSK4, MNK1, MNK2, MSK1 and MSK2. These can phosphorylate numerous transcriptional regulators such as Jun, c-Fos and Nur77. MSK1/2 can phosphorylate CREB at Ser133, as well as the NF-κB p65 isoform and STAT3 (Reviewed in [273]). MNK1/2 are implicated in control of translation through their ability to phosphorylate eIF4E. The physiological consequences of eIF4E phosphorylation remain to be definitively investigated, with both increased and decreased translation reported.

There is a correlation between eIF4E phosphorylation and decreased 5'-cap-dependent translation (Reviewed in [273]).

The ERK pathway can also regulate protein expression downstream of transcriptional activation. RSK1 can phosphorylate glycogen synthase kinase 3β within polyribosomes. This inhibits the kinase activity of RSK1 and results in increased protein translation through eIF2B (Reviewed in [273]). MNKs can bind and phosphorylate hnRNP A1, decreasing its specific interaction with the 3'AU-rich element in TNFα mRNA and stabilising TNFα mRNA [277].

Experiments presented in the latter half of this chapter explore the involvement of possible signalling events downstream of ERK in controlling DC activation by zymosan.

5.2 : Results - ERK activation is necessary for IL-2 and IL-10 induction

5.2.1 : Zymosan recognition activates the ERK pathway in BMDC

The ERK pathway has previously been reported to play a role in the control of IL-10 production by TLR2 ligands and zymosan [94, 136] [175]. Figure 5-1A shows a western blot of phospho-p38 MAP Kinase and phospho-ERK over a short time-course of zymosan stimulation. Whilst there is some background ERK phosphorylation this is increased to a maximum level by 15 to 20 minutes. p38 MAP kinase activation in contast appears to be induced by 15minutes and then remains stable up to 40 minutes after stimulation. FACS for phospho-ERK demonstrates a robust shift in specific antibody staining 30minutes after zymosan stimulation (figure 5-1B). As demonstrated in chapter 3, increased FACS-staining for phospho-ERK after zymosan-stimulation is abolished by pre-treatment with the ERK inhibitor UO126, supporting the specificity of staining (Figure 3-1B). To determine if Dectin-1 is capable of activating the ERK pathway LK cells transfected with wild-type or tyrosine-mutated Dectin-1 were used. Zymosan stimulation could induce ERK activation by FACS in the LK cells expressing wild-type Dectin-1 but not in cells expressing the mutant receptor that cannot signal (Figure 5-1C). Therefore zymosan recognition by DC results in robust ERK activation, and Dectin-1 can initiate a signalling cascade that results in ERK activation.

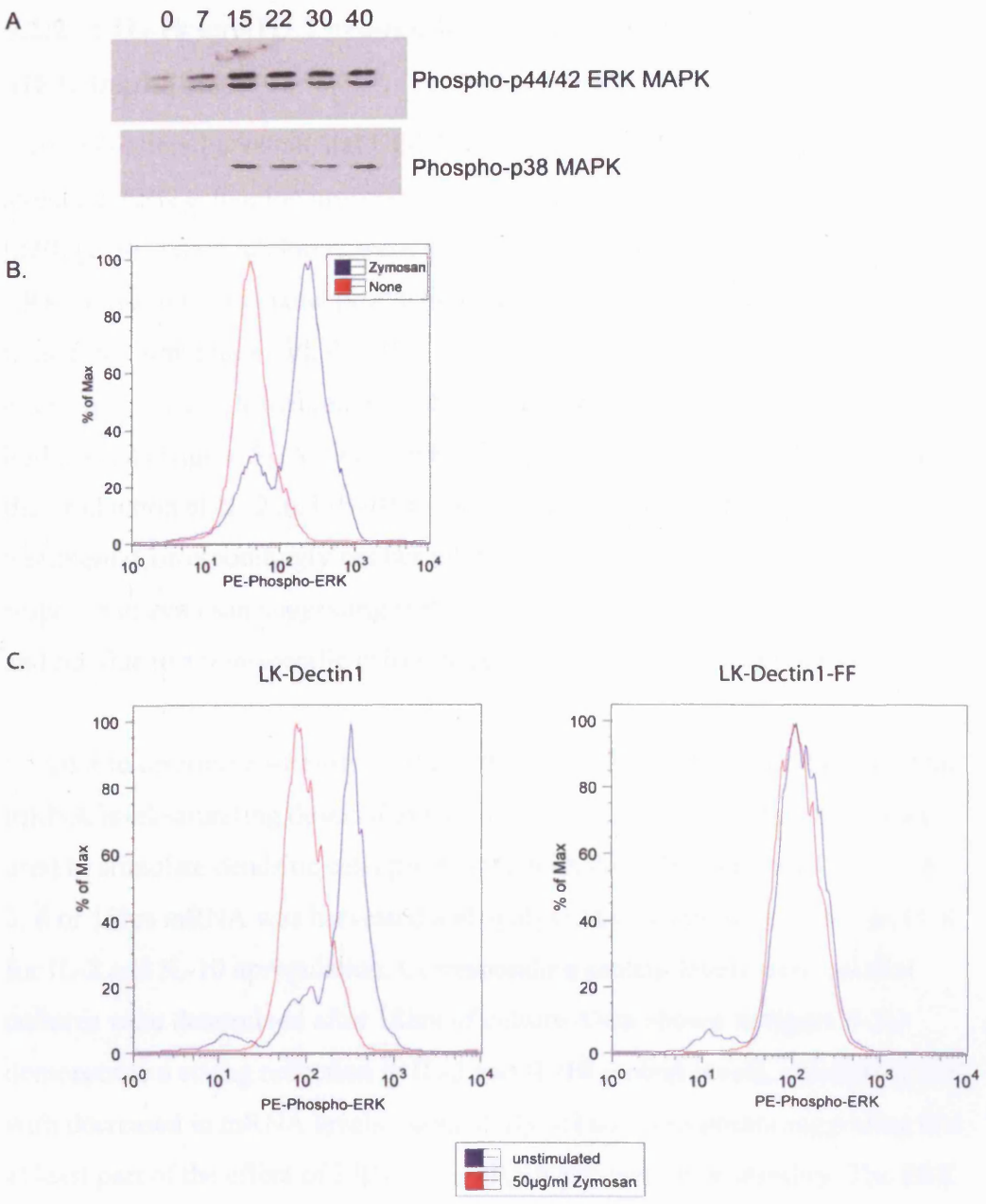


Figure 5-1: Stimulation of BMDC with zymosan results in ERK activation. A) C57BL/6 BMDC were plated at 10^6 per well of a 24 well plate and stimulated with 100µg zymosan for the indicated time-periods. Signalling was halted by addition of SDS-sample buffer. Samples were run on a 10% Tris-glycine gel, blotted onto nitrocellulose and probed for phospho-ERK and phospho-p38. B) BMDC as in (A) were stimulated for 30minutes with 100µg zymosan then fixed and stained for phospho-ERK before analysis by flow cytometry. C) LK cells stably expressing Dectin-1 of Dectin-1-FF were stimulated with 100µg/ml zymosan for 30 minutes before fixation and analysis of levels of phospho-ERK by flow cytometry.

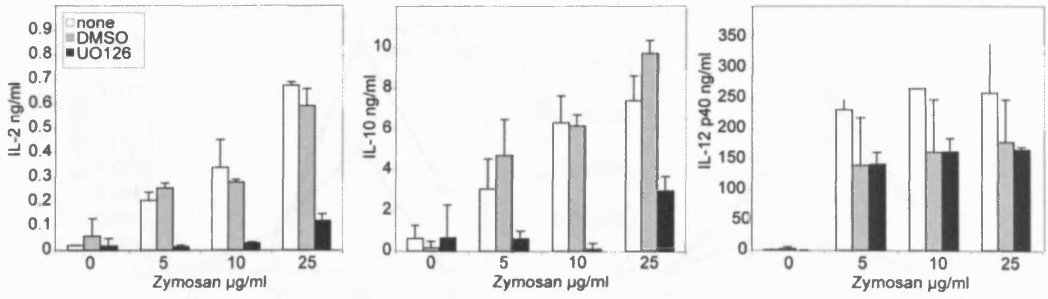
5.2.2 : IL-10 and IL-2 production is decreased in the presence of MEK inhibitors

MEK inhibitors PD98059 and UO126 are unrelated chemical compounds that attenuate ERK activation upstream of phosphorylation and activation of ERK [220] [219]. These inhibitors were therefore used to assess the contribution of ERK activation to cytokine production induced by zymosan. BMDC were pre-treated for 30mintues with the ERK inhibitors before addition of zymosan and over-night culture. Measurements of cytokines in the supernatants showed that both UO126 (figure 5-2A) and PD98059 (figure 5-2B) significantly decreased the production of IL-2 and IL-10 across the full dose-response of zymosan-treatment. Correspondingly neither inhibitor affected IL-12 p40 production in response to zymosan suggesting that the effect of the compounds was specific and not due to a non-specific effect on receptor expression or cell survival.

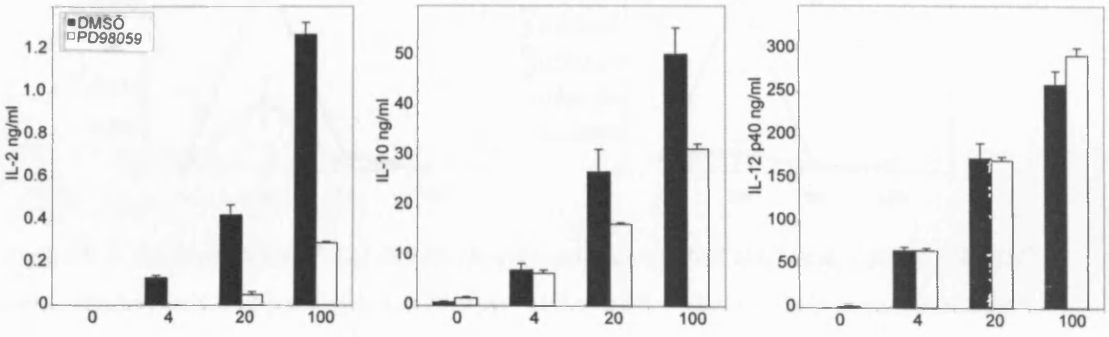
In order to determine whether UO126 affected IL-2 and IL-10 production at the mRNA level saturating doses of zymosan or the TLR2-ligand Pam3Cys were used to stimulate dendritic cells pre-treated with the ERK inhibitor UO126. At 3, 6 or 18hrs mRNA was harvested and analysed by quantitative real-time PCR for IL-2 and IL-10 upregulation. Corresponding protein levels from parallel cultures were determined after 18hrs of culture. Data shown in figure 5-2D demonstrate a strong reduction in IL-2 and IL-10 protein levels, correlating well with decreased in mRNA levels, particularly at later time-points suggesting that at least part of the effect of ERK is on mRNA production or stability. The ERK pathway therefore makes an important contribution to induction of both IL-2 and IL-10. These data also reveal that whilst the levels of mRNA induced by Pam3Cys and zymosan are very different, corresponding to the induction of protein, the peak of cytokine mRNA production occurs at similar time-points (i.e. at 3hrs of IL-2 and at 6hrs for IL-10). Pam3Cys induced IL-2 and IL-10 mRNA appears to be more strongly inhibited by UO126 than zymosan induced mRNA levels, suggesting that TLR2/Pam3Cys-induced IL-2 and IL-10 may be more heavily dependent on ERK signalling than that induced by zymosan.

In order to determine the contribution of ERK signalling to Dectin-1 dependent responses LK cells expressing wild-type Dectin-1 were pretreated with the ERK inhibitor, followed by 18hrs stimulation with zymosan. Measurements of cytokines in the supernatant revealed that UO126 will also inhibit IL-2 and IL-10 production by Dectin-1-expressing LK cells stimulated with zymosan (figure 5-2C) suggesting that ERK may be a crucial link between Dectin-1 and the production of these cytokines.

A



B



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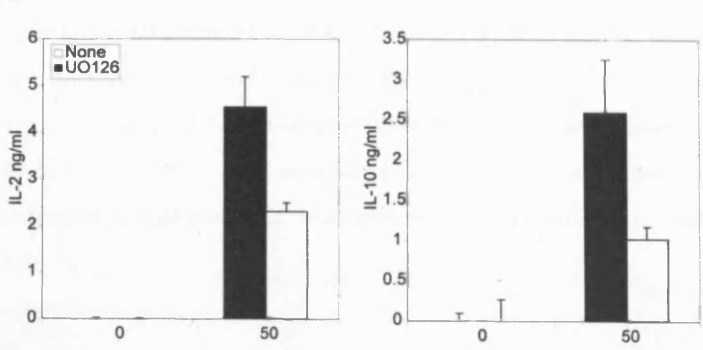


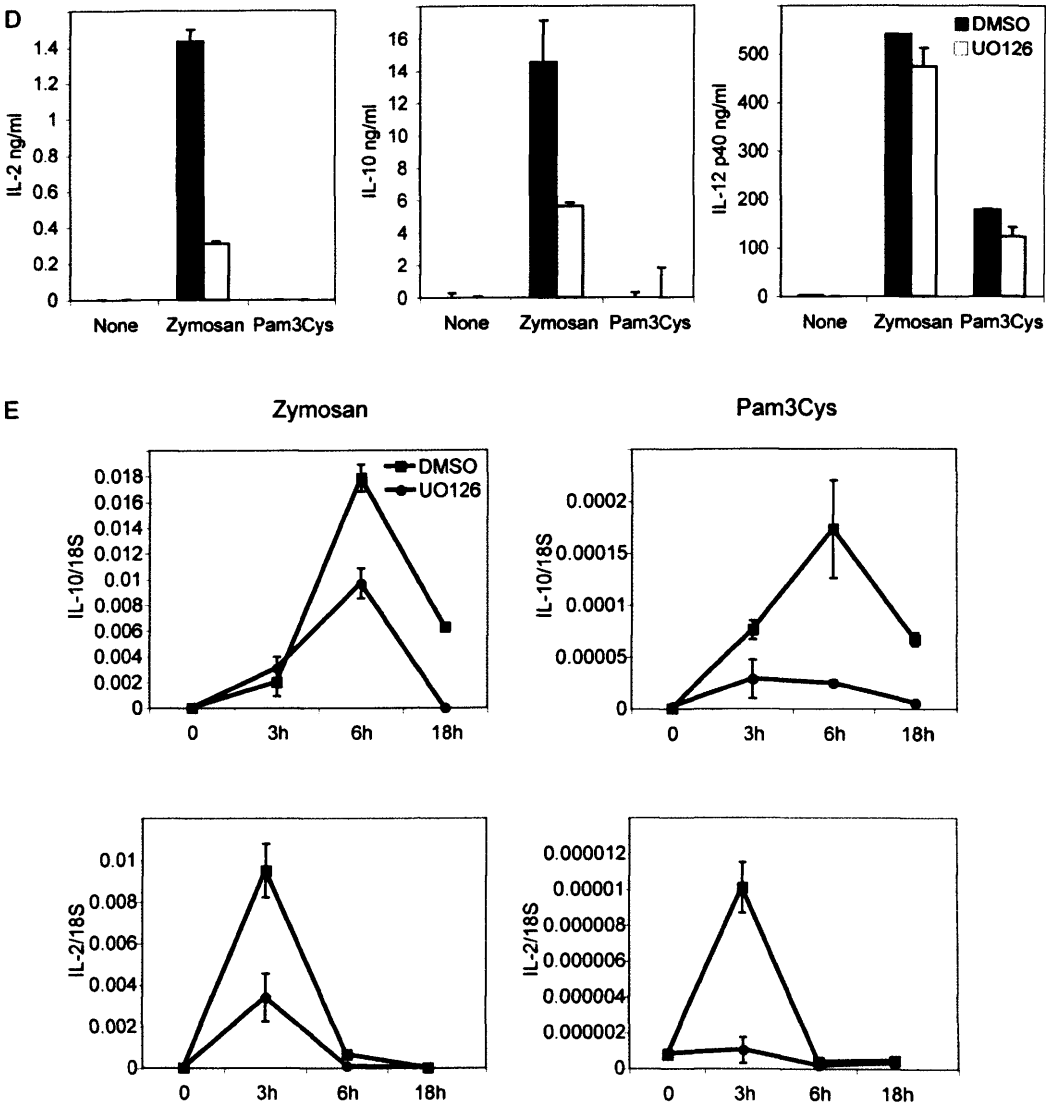


Figure 5-2: Zymosan-induced IL-2 and IL-10 is diminished by MEK inhibitors. C57BL/6 BMDC were enriched for CD11c and plated at 10^5 per well of a 96 well plate. Cells were pre-treated for 30 minutes with 10μM UO126 (A) or 10μM PD98059 (B) before stimulation with the indicated doses of zymosan. Cytokine secretion was determined after 18hrs using sandwich ELISA. C) LK cells expressing full-length Dectin-1 were pre-treated with 10μM UO126 before stimulation with 100μg/ml zymosan. Cytokine production was measured after 18hrs of stimulation by sandwich ELISA. D) and E) C57BL/6 BMDC were pre-treated for 30minutes with UO126 then stimulated with 100μg/ml zymosan or 10μg/ml Pam3Cys. For part D) supernatants were collected after 18hrs and used to determine cytokine production by sandwich ELISA. For part E), cells were harvested at the indicated time points and mRNA extracted and subjected to real-time PCR to determine mRNA levels of IL-2 and IL-10.

5.2.3 : Zymosan-induced ERK-activation is Syk-dependent

Experiments described in 5.2.1 and 5.2.2 using Dectin-1 LK cell lines suggest that Dectin-1 can induce ERK activation on zymosan binding. Data presented in chapter 4 (and published [146]) demonstrate that this receptor couples to downstream events by signalling through Syk. I set out to determine whether ERK activation in BMDC in response to zymosan was triggered by the TLR2/MyD88 pathway or the Dectin-1/Syk pathway.

C57BL/6 or MyD88-/- Dendritic cells were stimulated with a saturating dose of zymosan (100μg/ml) for the indicated times before harvesting for analysis. As can be seen in figure 5-3A, dendritic cells deficient in MyD88 shown essentially normal ERK activation in response to zymosan. Geometric mean-fluorescences were calculated for phospho-ERK staining by FACS over a 2hr time-course of zymosan stimulation. Figure 5-3A shows this data plotted against time, demonstrating near identical kinetics and amplitudes of ERK activation induced by zymosan in MyD88-sufficient and deficient cells. This implies that the TLR2 pathway is not necessary for ERK activation in response to zymosan. In order to confirm this data using classical biochemistry wild-type or MyD88-deficient BMDC were stimulated with zymosan for 30 minutes then lysed into SDS-containing sample buffer and proteins resolved on a 10% Tris-glycine gel. Western blotting for phospho-ERK in BMDC lysates reveals that ERK-phosphorylation in response to zymosan is normal in MyD88-deificent cells. A low molecular weight β-glucan, Glucan Phosphate, here fails to induce measurable ERK activation in B6 or MyD88-/- BMDC.

When Syk-/- BMDC were assessed in the same system (Figure 5-3B) Syk-/- cells failed to induce any ERK activation above background on zymosan stimulation. Again, BMDC were stimulated with zymosan over a 2hr time-course and ERK phosphorylation was analysed by FACS. Syk deficient cells show no positive phospho-ERK staining at any time-point after zymosan stimulation indicating that ERK activation is completely blocked rather than just

delayed in these cells. However, Syk-deficient cells stimulated with the TLR2-ligands Pam3Cys (figure 5-3C) or MALP2 (data not shown) stained for phospho-ERK similarly to or better than wild-type cells demonstrating that there was no intrinsic defect in ERK activation in Syk-/- BMDC.

These results demonstrate that ERK activation downstream of zymosan recognition is dependent on Syk; and IL-2 and IL-10 induction are dependent on both Syk and ERK. Syk-signalling to ERK may therefore be crucial for the induction of IL-2 and IL-10 in the BMDC response to zymosan.

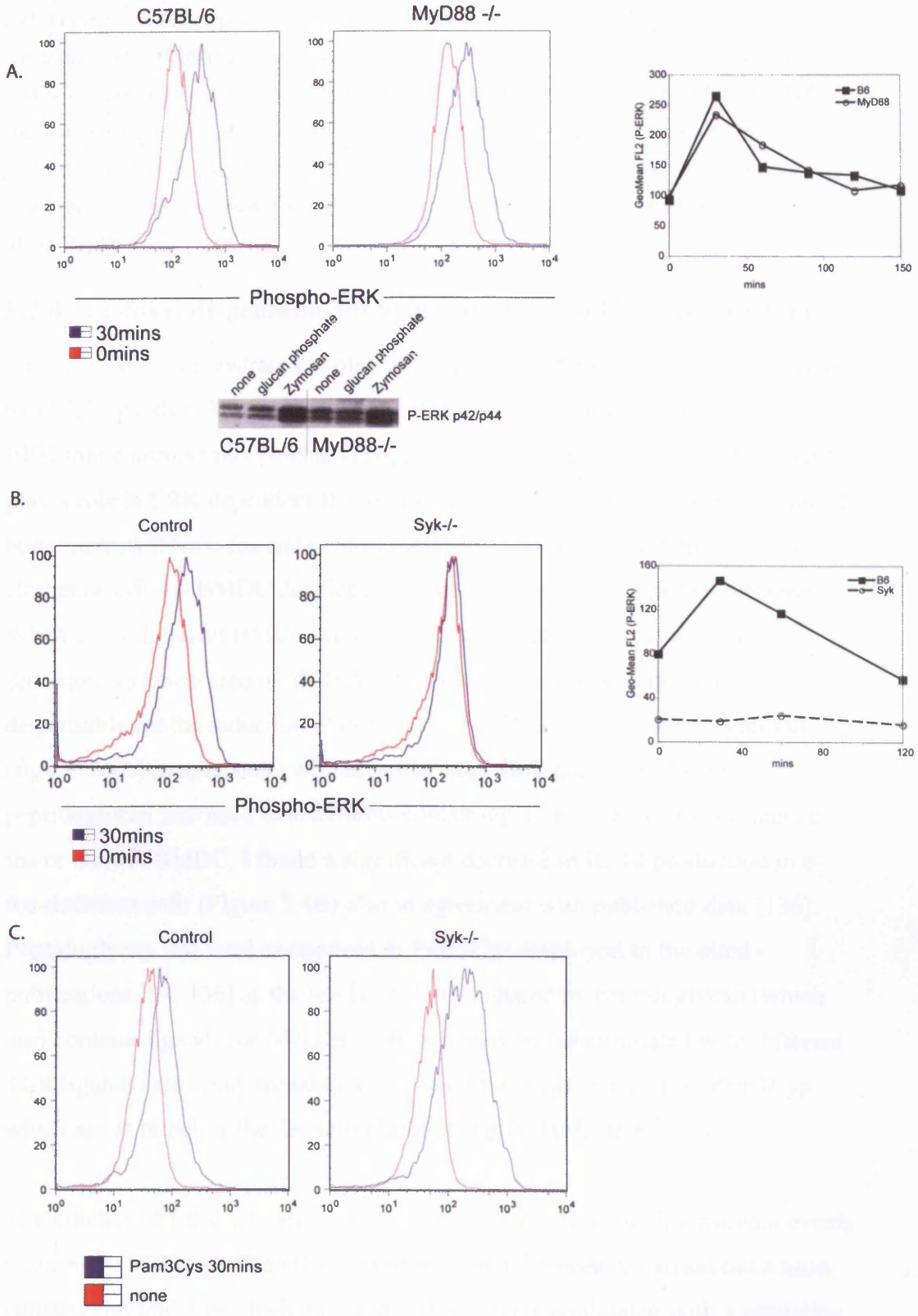


Figure 5-3: ERK activation downstream of zymosan recognition requires Syk and is MyD88-independent. A) B6 or MyD88-/- BMDC were stimulated with 100μg/ml zymosan for 30minutes (LH panel) or for the indicated time points (RH panel) before fixation and assessment of ERK phosphorylation by flow cytometry or western blotting (loading control determined by Ponceau-

red staining of blotted membrane – data not shown). Geometric mean fluorescence was calculated for >10000 events per time-point. B) B6 or Syk-/- BMDC were stimulated with 100μg/ml zymosan for 30minutes (LH panel) or for the indicated time points (RH panel) before fixation and assessment of ERK phosphorylation by flow cytometry. Geometric mean fluorescence was calculated for >10000 events per time-point. C) B6 or Syk-/- BMDC were stimulated with 10μg/ml Pam3Cys for 30 minutes before fixation and assessment of ERK phosphorylation.

5.2.4 : c-fos is dispensable for zymosan-induced IL-10 production

c-fos has been demonstrated to play a role in the induction of IL-10 in response to TLR2 ligands [136] [94], and is suggested to be the downstream target of ERK that controls this cytokine [136] [94]. To investigate whether c-fos might play a role in ERK dependent IL-10 production induced by zymosan, I obtained bone marrow from c-fos and control foetal-liver-reconstituted bone marrow chimeras. c-fos-/- BMDC developed normally and yielded expected numbers. When c-fos-deficient BMDC were stimulated with zymosan and cytokine secretion was measured by ELISA, I found that c-fos was completely dispensable for the induction of either IL-10 or IL-2 in response to zymosan (figure 5-4A) in agreement with recently published data [175]. When peptidoglycan was used as a stimulus containing TLR2-ligands to stimulate c-fos or control BMDC, I found a significant decrease in IL-10 production in c-fos-deficient cells (Figure 5.4B) also in agreement with published data [136]. Peptidoglycan was used as opposed to Pam3Cys employed in the cited publications [94, 136] as the levels of IL-10 induced by peptidoglycan (which also contains ligands for NOD proteins and may be contaminated with different TLR ligands) are easily measurable compared to those induced by Pam3Cys which are at or below the detection limit of our IL-10 ELISA.

The kinetics of ERK activation can have profound effects on downstream events induced by ERK signalling (Reviewed in [144]). I therefore carried out a time-course experiment in which wild-type BMDC were stimulated with a satutating dose of Pam3Cys or Zymosan. ERK phosphorylation was measured by FACS at each time point and geometric means of the fluorescence were determined. Plotting these values against time demonstrates almost identical kinetics of ERK

activation induced by the two ligands (figure 5-4C), despite the differences in upstream activators. Zymosan reproducibly induces a slightly higher peak amplitude of ERK activation although it is unclear whether this would be sufficient to explain the differences between IL-2 and IL-10 levels observed.

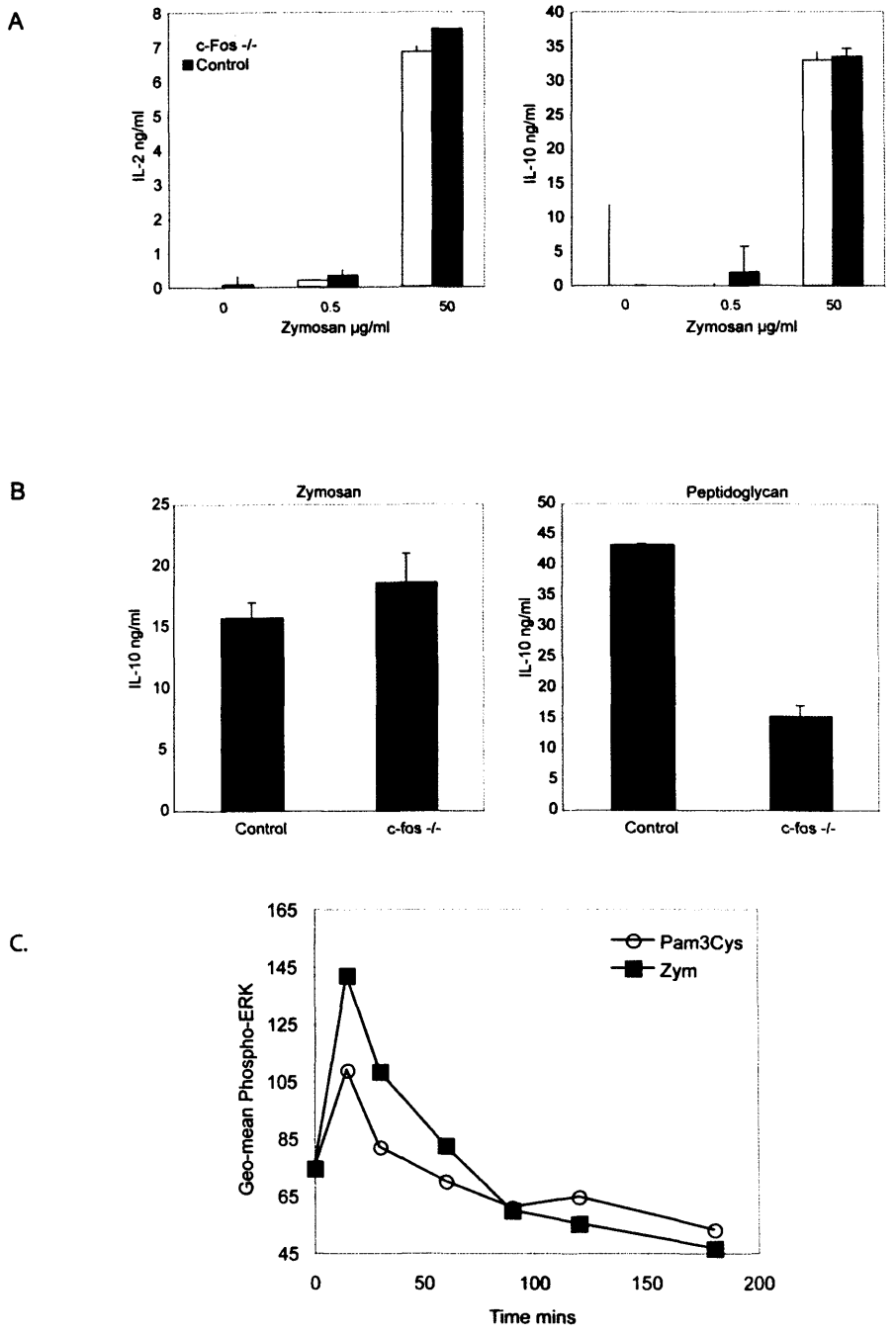
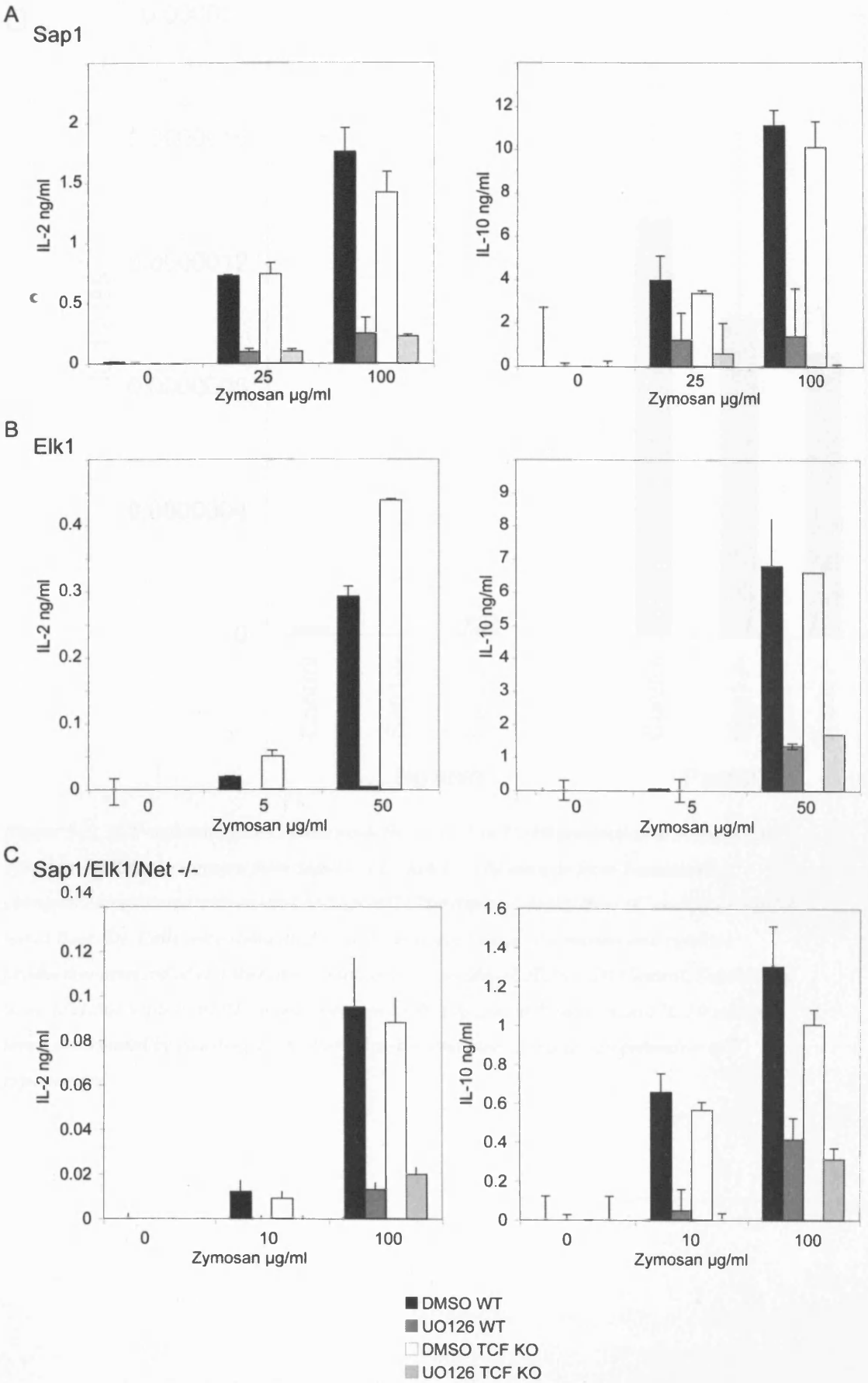


Figure 5-4: c-fos-deficient BMDC show no defect in IL-2 or IL-10 production in response to zymosan. A) BMDC were grown from the bone marrow of chimeras which had been reconstituted with c-fos-/- or littermate control foetal liver. c-fos-/- and WT BMDC were stimulated with the indicated doses of zymosan and cytokine production assessed by ELISA 18hrs later. B) c-fos-/- and WT BMDC, as in (A) were stimulated with 0.5µg/ml Zymosan or 50µg/ml peptidoglycan. Cytokine production was analysed by ELISA 24 hrs later. C) C57BL/6 BMDC were stimulated with 100µg/ml zymosan or 10µg/ml Pam3Cys for the indicated times before fixation and analysis of phospho-ERK levels by flow cytometry. Geometric means were calculated from >10000 events.

5.2.5 : TCF family members are not required for IL-10 or IL-2 production in response to zymosan or Pam3Cys

The Ternary Complex Factor (TCF) family comprises of 3 transcription factors, Sap-1, Elk-1 and Net,, that form transcriptional activators or repressors in complex with Serum Response Factor (SRF) [278]. TCFs are direct targets of ERK phosphorylation and both Sap-1 and Elk1 have been implicated in the upregulation of c-fos after ERK pathway activation [278] [274]. I used BMDC deficient either in Sap-1, Elk-1, or all three TCFs to determine whether differences in TCF usage could account for the differential requirement of c-fos downstream of Pam3Cys or zymosan recognition. To confirm that any effects observed were downstream of ERK, the effects of the MEK-1 inhibitor UO126 on cytokine production by each genetype of cells was assessed. Neither the Sap-1-/-, the Elk-1-/- nor the triple -/- cells showed any defect in IL-10 or IL-2 production in response to zymosan (Figure 5-5A-C). In addition all 3 genotypes of cells were stimulated with Pam3Cys and cytokine mRNA levels determined by quantitative RT-PCR. This demonstrated that the TCFs are also not required for IL-2 or IL-10 upregulation by Pam3Cys (Figure 5-5D). Although the TCF-family members are expressed in BMDC they do not appear to play a non-redundant role in signalling downstream of the pattern-recognition receptors tested in these experiments.


A
Sap1
IL-2 ng/ml
IL-10 ng/ml
Zymosan µg/ml
B
Elk1
C
Sap1/Elk1/Net -/-
DMSO WT
UO126 WT
DMSO TCF KO
UO126 TCF KO

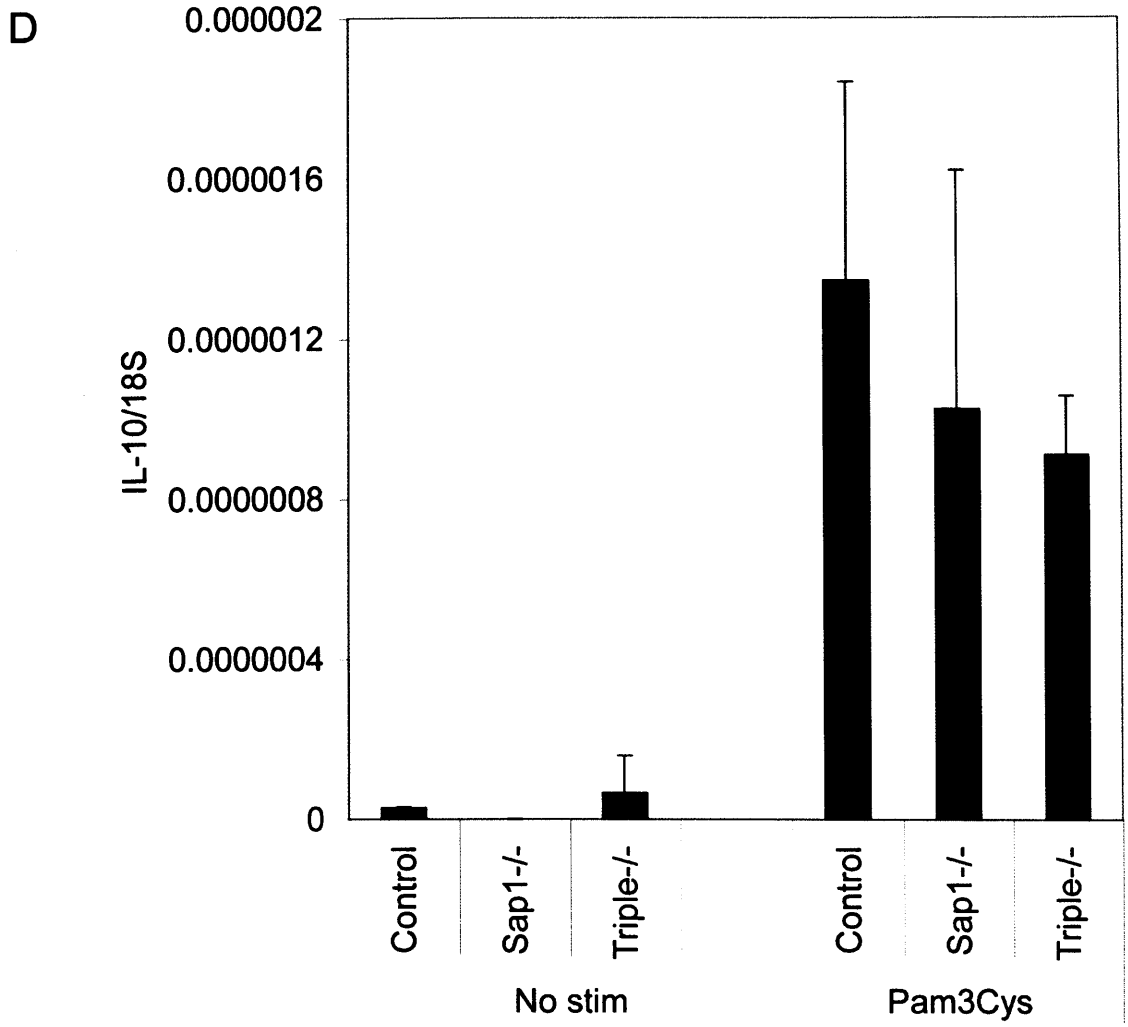


Figure 5-5: TCF-deficient BMDC show no defect in IL-2 or IL-10 production in response to zymosan. BMDC were grown from Sap-1-/- (A), Elk-1-/- (B) mice or from bonemarrow chimeras reconstituted with control or Sap1/Elk1/Net triple -/- foetal liver (C and D) or Sap1-/- foetal liver (D). Cells were stimulated with the indicated doses of zymosan and cytokine production assessed after 18hrs of stimulation using sandwich ELISA. D) Control, Sap1-/- and Sap1/Elk1/Net triple-/- BMDC were stimulated with 10μg/ml of Pam3Cys and IL-10 mRNA levels determined by real-time PCR after 3hrs of stimulation. Data is representative of 3 experiments.

5.2.6 : IL-2 induction is independent of de novo transcription

It is now well documented that induction of IL-2 from T cells after stimulation involves mRNA stabilisation [132]. To determine whether this was also the case in dendritic cells BMDC were treated with the transcriptional poison Actinomycin D [279] and stimulated with zymosan for 3hrs before measurement of mRNA levels by quantitative RT-PCR. ActinomycinD dramatically decreased IL-10 production in response to zymosan but had almost no effect on IL-2 production (Figure 5-6) indicating that de novo transcription is not required for the upregulation of IL-2 mRNA and any effects of signalling on this phenomenon are likely to be at the level of mRNA stabilisation, including the effects of ERK signalling. In contrast, IL-10 and IL-12 p40 appear to be regulated at least partially at the transcriptional level.

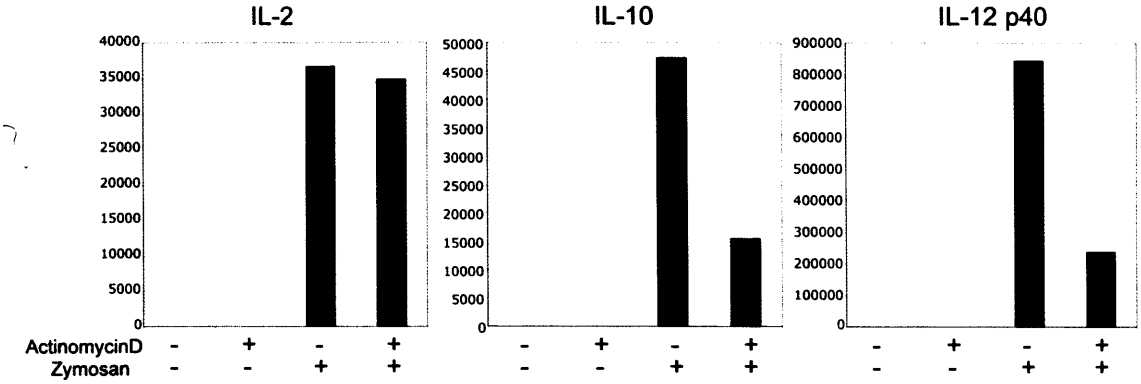


Figure 5-6: IL-2 mRNA upregulation induced by zymosan is independent of de novo transcription. C57BL/6 BMDC were pre-treated for 10minutes with 100nM Actinomycin D before stimulate for 6hrs with 100μg/ml zymosan. mRNA was extracted and subjected to RT-PCR to determine mRNA levels of IL-2, IL-10 and IL-12 p40.

5.3 : Discussion

5.3.1 ERK is required for IL-10 and IL-2 induction

Data described in this chapter demonstrate robust inhibition of zymosan-induced IL-2 and IL-10 production by the ERK inhibitors PD98059 and UO126, both in BMDC and in LK cells (figure 5-2). This correlates well with published data demonstrating a role of the ERK pathway in the production of these cytokines by dendritic cells [267] [175] [94] and T cells [264]. As discussed in the introduction to this chapter, ERK targets can affect transcription, mRNA stability and translation [280]. Measurements of mRNA levels for IL-2 and IL-10 in the presence of the ERK inhibitor indicates that zymosan-induced ERK activation can decrease message-levels for IL-10 and IL-2 (figure 5-2E) suggesting that effects on transcription and mRNA stability may play a role. In the case of IL-2, Actinomycin D inhibition of transcription has almost no effect of mRNA levels at 3 hours (figure 5-6). When combined with the observation that ERK inhibition decreases IL-2 mRNA levels this is highly suggestive ERK mediates IL-2 induction by stabilising IL-2 mRNA. IL-10 mRNA upregulation is significantly inhibited by Actinomycin D suggesting that de novo transcription is playing an important role, therefore ERK could affect both transcription and message stabilisation of IL-10.

5.3.2 ERK activation in response to zymosan requires Syk, but not MyD88

MyD88-deficient BMDC activate ERK signalling identically to wild-type DC in response to zymosan, whereas Syk-deficient BMDC completely fail to activate ERK in response to zymosan stimulation (figure 5-3). ERK signalling in response to zymosan is therefore mediated entirely downstream of novel Syk-dependent PRRs such as Dectin-1, providing further evidence that this mode of pattern recognition is genuinely independent of TLRs.

There is one intriguing facet to this data. Zymosan can signal through TLR2/6 heterodimers and we, and others, have reported that this event is critical for the induction of IL-12 p70 in response to zymosan [59] [96]. However, TLR2 ligation by zymosan is not sufficient to induce ERK activation in the Syk-deficient cells stimulated with zymosan (figure 5-2). In contrast, Syk deficient BMDC stimulated in parallel with the TLR2/1 ligand Pam3Cys or the TLR2/6 ligands MALP2 exhibit ERK activation similar or greater than that seen in wild-type cells (Figure 5-3C). At this time, the reasons for TLR2 coupling to ERK in response to lipopeptides, but not zymosan, is unclear but the observation that TLR2 can signal differently in response to two different ligands is clearly interesting. Possible explanations are either that conformation of the receptor induced by lipopeptides is different to that induced by zymosan or that the microenvironment around TLR2 during lipopeptides and zymosan recognition allows or denies access to particular signalling components.

5.3.3 Differences in transcription-factor usage by TLR and Dectin-1/Syk for IL-10 induction

It appears that c-fos contributes to IL-10 production induced by the TLR ligand Pam3Cys [94, 136] and the complex ligand Peptidoglycan (from S. aureus, figure 5-4). However, c-fos is not required for IL-10 production in response to zymosan (figure 5-4). This is an interesting observation as it suggests that the induction of IL-10 by either TLR2 or a Syk-dependent pattern recognition receptor (such as Dectin-1) requires different factors downstream of ERK activation. Additionally, my data clearly demonstrate that TCF transcription factors (known targets of ERK) are not responsible for ERK-dependent induction of IL-10 by zymosan or Pam3Cys. Assuming the published data demonstrating a requirement for c-fos in TLR2-mediated IL-10 production are correct [94, 136], this suggests that TCFs are not required for c-fos upregulation in response to TLR2 ligands. It is therefore clear that signalling downstream of Syk renders c-fos redundant in the induction of IL-10 implying that the precise mechanism of IL-10 induction downstream of Syk-coupled PRRs and MyD88-coupled PRRs is divergent.

In the papers of Agrawal et al and Dillon et al [94, 136] ERK dependency of c-fos upregulation in TLR2-stimulated DC is only implied from the literature, rather than directly demonstrated. An alternative explanation for the observed results is therefore that ERK signalling plays an identical role in TLR2 and Dectin-1 induced IL-10 production but that TLR2 also requires the induction of c-fos via an alternative pathway. It should also be noted that peptidoglycan-induced IL-10 production (figure 5-4) is known to occur at least partially by signalling via NOD2 (reviewed in [34]) therefore the effects of c-fos-deficiency shown in figure 5-4 could be attributable to either TLR2 or NOD2 signalling.

The kinetics of ERK activation are similar in response to saturating doses of Pam3Cys and saturating doses of zymosan, although the initial peak of ERK activation with zymosan is slightly higher (figure 5-4). Differences in the kinetics of ERK activation therefore cannot explain the differential requirement for c-fos or the large differences in the amount of IL-10 and IL-2 produced in response to saturating doses of the two stimuli. ERK signalling is therefore necessary, but not sufficient, for induction of high levels of IL-10 and IL-2 in response to zymosan. Transcription factors activated downstream of zymosan recognition make c-fos redundant in the induction of IL-10.

My data clearly demonstrate that the ERK pathway is a necessary link between Syk and the production of high levels of IL-2 and IL-10 by dendritic cells stimulated with zymosan. Additionally there must be signalling events triggered via Syk and zymosan recognition, but not by TLR2 and lipopeptide recognition, that lead to the widely different levels of cytokine production observed with the two stimuli. This interesting area of research remains open. A further interesting conclusion from my data is that TLR2 signalling induced by binding to lipopeptides or zymosan is somehow qualitatively different, such that TLR2-ligation by zymosan in the absence of Syk-dependent pattern recognition fails to activate ERK, whilst ERK activation downstream of TLR2-recognition of lipopeptides in Syk-independent.

6 Chapter 6: T cell differentiation induced by zymosan-stimulated BMDC

6.1 : Introduction

Dectin-1 signalling induces a mature BMDC phenotype that is significantly different from that induced by signalling through any TLR (high IL-10, IL-2, Jagged-1 and DLL1 for example). How does this affect T cell differentiation induced by zymosan-stimulated DC? In order to start addressing this question I investigated the effect of zymosan on *in vitro* T cell differentiation assays.

6.1.1 T cell polarisation by zymosan: What do we know?

Dendritic cells that have encountered microbial stimuli display an activated phenotype that primes T cells to best deal with the class of microbe encountered (for a detailed discussion of this see chapter 1). Most well characterised TLR ligands are predominantly viral or bacterial in origin and induce a mature DC phenotype that biases strongly towards Th1 induction (reviewed in [240]). DC stimulated with an extract of shistosome eggs adopt an alternative phenotype that biases towards Th2 induction (reviewed in [281]). Furthermore it has recently been discovered that DC can instruct Th17 differentiation in the presence of TLR ligands and a high density of Tregs [102]. The mature DC phenotype induced by zymosan differs from that of TLR-stimulated DC in the production of IL-10, IL-2 and Jagged-1, all of which have controversial effects on T cell differentiation (as described in chapter 4). We were therefore interested in the ability of zymosan to act as an adjuvant for T cell differentiation *in vitro*.

Previous attempts to assess the adjuvant function of zymosan *in vitro* suggest that zymosan is a relatively weak inducer of Th1 polarisation and that some Th2 differentiation can be induced in the presence of zymosan [90]. A more recent publication observes a decreased proliferation of antigen-specific CD4 T cells

when zymosan is used as an adjuvant *in vitro* although cytokine production is not assessed in these experiments [175]. *In vivo* immunisations with zymosan and antigen again suggest that zymosan is a poor inducer of Th1 responses [175]. However, the majority of zymosan injected I.V is rapidly taken up by neutrophils (Dr M.N Nolte, unpublished observations) such that the authors are primarily considering indirect DC activation [175]. These experiments hint that when compared to highly purified TLR ligands such as CpG DNA or LPS, zymosan induces T helper responses that are less Th1-skewed.

In vitro T cell differentiation assays are to some extent a useful simplification of T cell priming *in vivo* since the cell-types present, antigen-dose and cell contacts can be tightly controlled. However their interpretation is still complex. The requirements for T cell priming *in vitro* are less stringent than corresponding *in vivo* situations. In addition, in the absence of innate stimuli the T cell differentiation induced depends heavily on the antigen-dose [78], APC:T cell ratio [90], starting cell densities, foetal-calf serum batch, T cell purification protocol [90] and the genetic background of the cells used (to name just a few). Care has therefore been taken to assess the adjuvant effects of zymosan over a range of antigen-doses and always to compare to relevant control assays.

6.1.2 Jagged1 and T cell differentiation

A striking observation made in chapter 4 is the upregulation of Jagged-1 by Dectin-1 ligands. As discussed in chapter 1, the role of Notch-ligands in T cell differentiation has been suggested by a plethora of different experiments yielding contradictory results ([160], reviewed in [161]). Jagged-1 presentation by APCs has been suggested to induce the differentiation of naïve T cells into Th2 cells [160] or regulatory T cells [169] [170] in experiments over-expressing Jagged-1 on fibroblasts, splenic DC or B cells respectively. The second part of this chapter presents a preliminary investigation of the role of Jagged-1 on BMDC by selective deletion rather than over-expression.

The data presented aims to address how zymosan can bias T helper differentiation and the part played by Jagged-1, induced on DC by zymosan stimulation, in instructing these T cell responses.

6.2 : Results - Zymosan can act as an adjuvant in vitro

6.2.1 : Zymosan can amplify Th1 or Th17 differentiation *in vitro*

In vitro T cell differentiation assays can be carried out in a variety of different ways, with variable results [78, 90]. In order to avoid issues associated with antigen processing and variation in polyclonal T cell populations, zymosan was first tested in the differentiation of OTII transgenic T cells induced by BMDC presenting cognate peptide. CD4+ T cells were purified from spleen and lymph nodes by lineage depletion, followed by CD4-enrichement using MACS. Purified T cells were labelled with CFSE and cocultured with BM-DC, cognate peptide and innate stimulus. With reference to the experiments of Boonstra et al, I examined the effect of adding zymosan to the cultures over a 4log dose-titration of peptide. As shown in Figure 6-1A, although OTII CD4+ T cells contain very few FoxP3 positive cells compared to the T cells from a C57BL/6 mouse, these T cells expand during coculture with DC, as demonstrated by CFSE dilution, and can be identified by FoxP3 intracellular staining on d5. The FACS profile shown represents data acquired on day 5 of stimulation with 1nM peptide, where expansion of T_{regs} is almost maximal but expansion of FoxP3-negative cells is still minimal and was hence chosen for clarity of staining. A known number of beads (easily gated on by low forward-scatter and high side-scatter) were included in the FACS samples during acquisition to allow calculations of the number of FoxP3-negative and FoxP3-positive cells after 5 days of coculture. Zymosan does not appear to increase the peptide-dose-dependent expansion of FoxP3-negative cells and high doses of zymosan (50μg/ml) mildly suppress proliferation. The effect of peptide dose on proliferation of FoxP3-positive cells is less clear, with almost maximal numbers of T_{regs} obtained with very low peptide doses, particularly in the presence of zymosan. However, the maximal number is very low (less than 1200 per well, compared to >200,000 FoxP3 negative cells) and does not appear to have a major impact on T cell effector function as demonstrated by the minimal Th17 differentiation shown in figure 6-1B.

The production of cytokines from the T cells counted in 6-1A was determined after 48hrs of restimulation on plate-bound anti-CD3. Cytokines secreted into the supernatants were measured by ELISA. As suggested by Boonstra et al [78] there is a significant effect of peptide-dose on the induction of different cytokines. IFNγ production is maximal at 10 to 30nM peptide whilst IL-4 production peaks at 3 to 10nM. Zymosan induces a slight increase in IFNγ production that is not dependent on the peptide dose. However, IL-4 production appears to be unaffected by zymosan. Production of IL-17 in this system is extremely low, although variable between experiments, due to the low numbers of T_{regs} found in OTII TCR-transgenic mice (figure 6-1A). Experiments by Salomé Leibundgut demonstrate that supplementing the OTII populations with OTII-T_{regs} results in robust Th17 induction when Dectin-1 agonists are used as adjuvants (manuscript in preparation).

A second *in vitro* T cell differentiation assay is employed in Figure 6-1C & D. In these experiments total CD4+ T cells were purified from C57BL/6 spleen and lymph nodes. As demonstrated in figure 6-1C, these CD4+ T cells contain a much higher percentage of FoxP3-positive cells, observable both before and after stimulation. CD4+ T cells were cocultured with C57BL/6 or MyD88-/- (back-crossed onto a C57BL/6 background) BMDC and 0.1μg/ml anti-CD3. Addition of zymosan to these cultures increases the recovery of T cells at day 5 through effects of survival and proliferation. B6 BMDC are slightly better stimulators of T cell proliferation than MyD88-/- BMDC in the absence of zymosan although the percentage increase appears similar for both.

IFNγ production appears to be maximal in the absence of innate stimulus when C57BL/6 BMDC are used as APC. However, MyD88-/- BMDC show an increased induction of IFNγ (Th1) and IL-17 (Th17) responses when zymosan is added (figure 6-1D) implying that the TLR-independent, Syk-dependent pattern recognition pathway is capable of promoting both Th1 and Th17 differentiation.

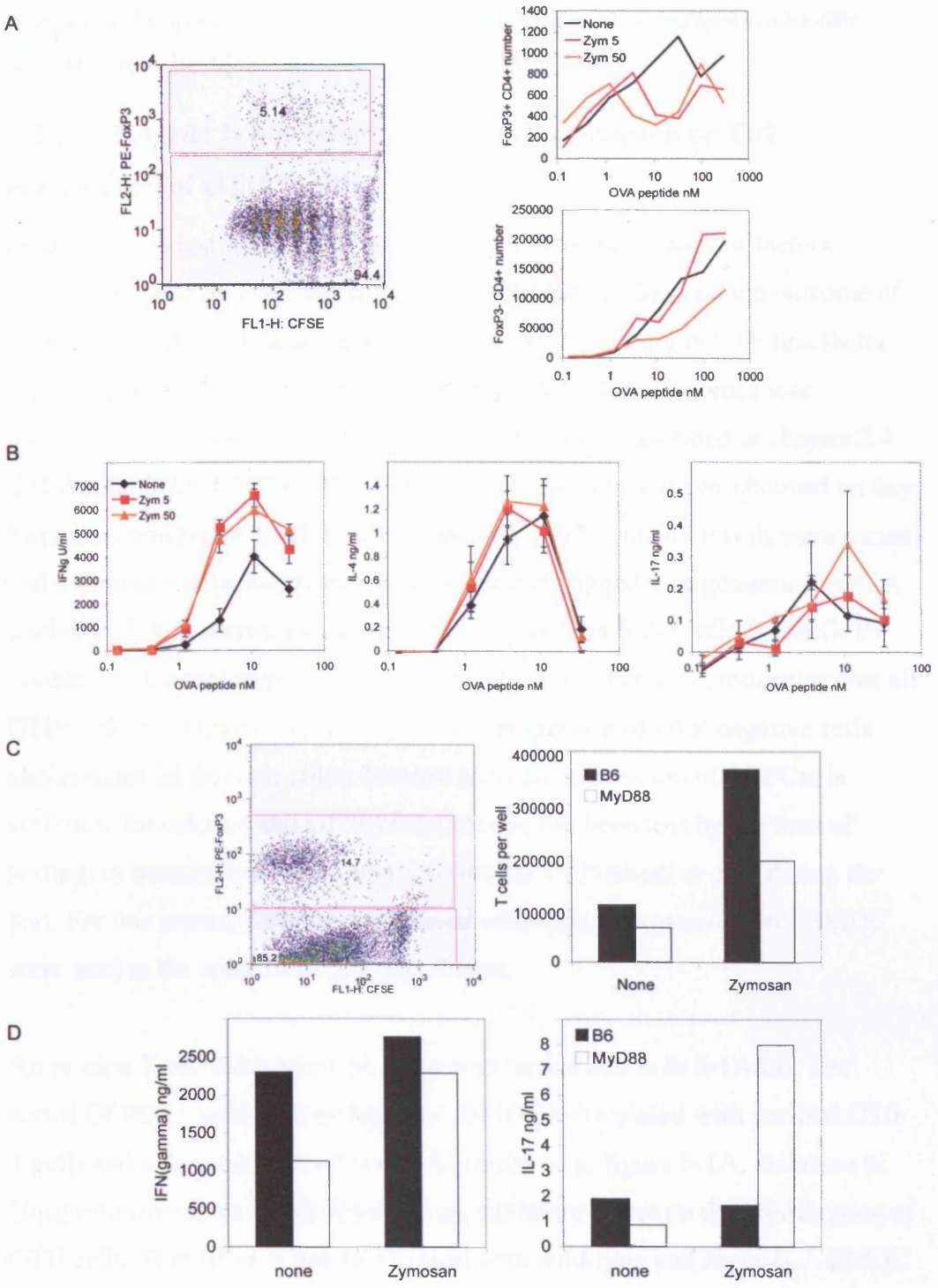


Figure 6-1: Zymosan acts as an adjuvant for in vitro T cell differentiation. A and B) 10^4 BMDC were used to present indicated doses of OVA peptide to 5×10^4 purified, CFSE labelled OTII T cells in the presence of indicated doses of zymosan. After 5 days of stimulation CD4+, FoxP3 positive and negative cells were counted by FACS (A) and total T cells from each well were restimulated on plate-bound anti-CD3. 48hrs later cytokine production was determined by ELISA (B). C and D) C57BL/6 or MyD88-/- BMDC were used to stimulate CD4+ T cells from MyD88-/- spleen and lymph nodes in the present of 0.1µg/ml anti-CD3 and 50µg/ml zymosan.

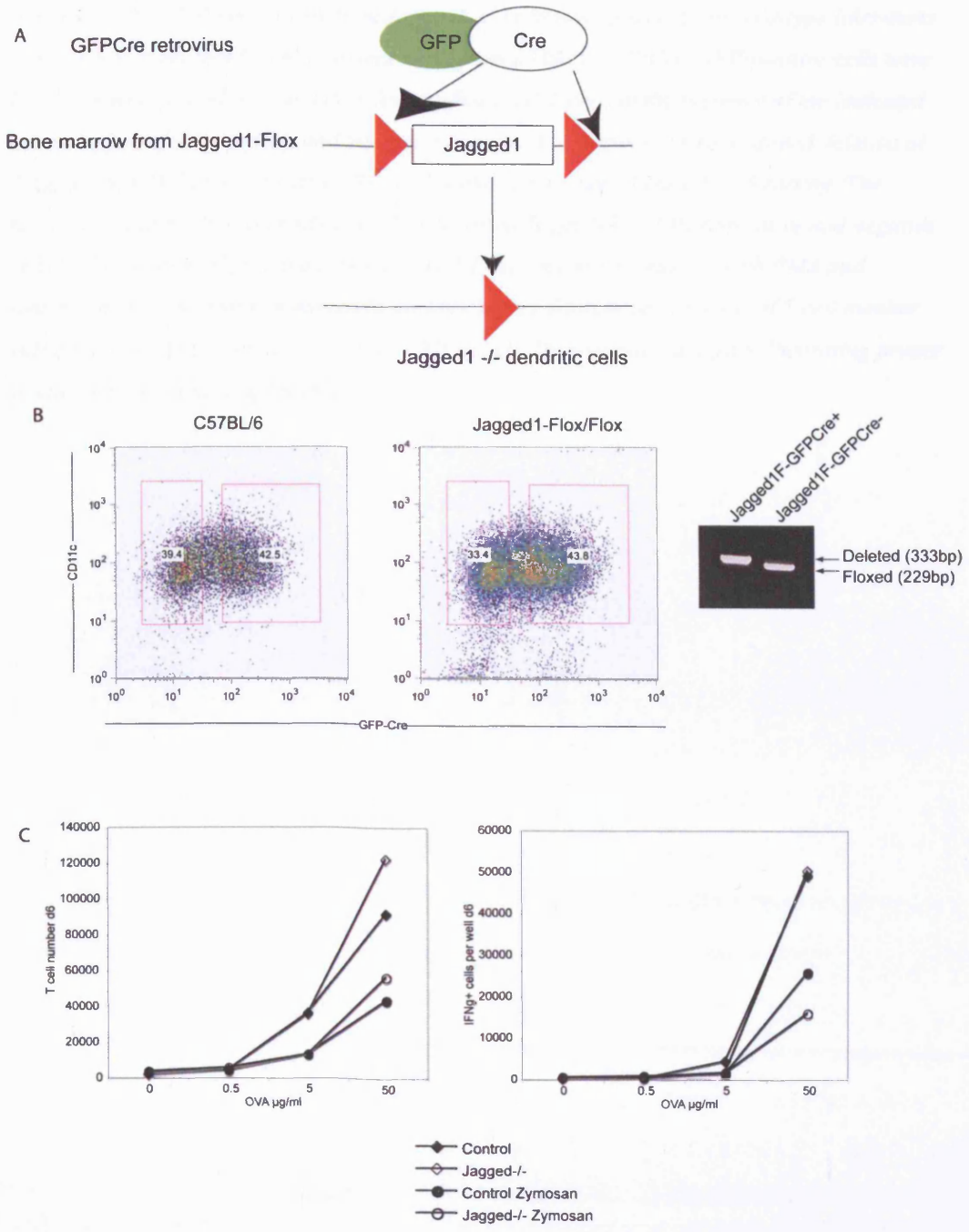
As in part A, T cell number is determined by FACS (C) and cytokine production after 48hr restimulation on anti-CD3-coated plastic is determined by ELISA (D).

6.2.2 : Jagged1 is not essential for T cell expansion or Th1 polarisation of OTII T cells.

I would like to test whether particular factors, or combinations of factors, strongly induced by zymosan stimulation of BMDC influenced the outcome of *in vitro* T cell differentiation assays. This work is on-going but the first factor tested was Jagged-1. Bone marrow from Jagged-1-Flox/Flox mice was transduced with a GFPCre retrovirus (figure 6-2A) as described in chapter 2.4 and chapter 3.2.5-10. The CD11c-enriched BMDC populations obtained on day 5 are shown in figure 6-2B. GFPCre-positive, CD11c-positive cells were sorted and a sample was taken to determine deletion of Jagged-1 in genomic DNA. A duplex PCR was carried out using primers marked in 6-2D. Within the GFP+ Jagged1-flox sorted population only deleted allele is detected, indicating that all GFP+ cells are Jagged1-/-. A considerable proportion of GFP-negative cells also contain the deletion either because transient expression of GFPCre is sufficient for deletion and GFPCre-expression has been lost by the time of sorting, or because low-expressers fall into the GFP-negative gate during the sort. For this reason, GFPCre-transduced wild-type littermate-control BMDC were used as the controls in this experiment.

An *in vitro* T cell differentiation assay was carried out as in 6-1A&B. The sorted GFPCre+ wild-type or Jagged-/- BMDC were plated with purified OTII T cells and a dose-titration of OVA. As observed in figure 6-1A, inclusion of 50μg/ml zymosan as an adjuvant had an inhibitory effect on the proliferation of OTII cells. This effect is nearly identical with wild-type and Jagged1-/- BMDC indicating that upregulation of Jagged-1 is not necessary for this effect under these conditions. After 5 days of culture the OTII T cells were restimulated for 6hrs with PMA/ionomycin in the presence of monensin. The production of IFNγ was assessed by intracellular staining, rather than by ELISA. This reveals fewer T cells producing IFNγ in the cultures where zymosan was used as an adjuvant, in contrast to the IFNγ production measured by ELISA after 48hrs of

restimulation in figure 6-1B. Regardless of discrepancies with previous data, the number of IFNγ producing cells on day 5 of culture is almost identical between wild-type and Jagged1-/- DC, again suggesting that in this particular type of assay Jagged-1 upregulation was not required for the zymosan-mediated effects on Th1 induction. In this experiment, as in that shown in Figure 6-1, there was minimal IL-17 induction, probably due to the very low numbers of T_{regs} present in the starting OTII population that went into the cocultures.



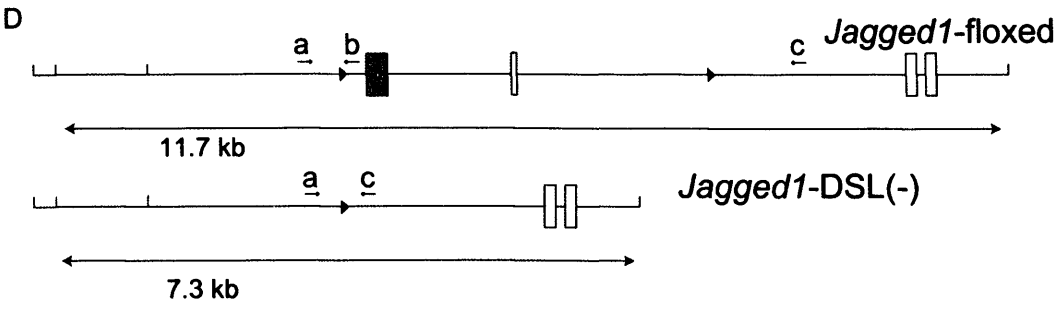


Figure 6-2: Deletion of Jagged-1 does not affect Th1 induction in response to zymosan-stimulated BMDC. Bone marrow from Jagged1-Flox homozygous mice or wild-type litter-mate controls was transduced with retrovirus made from pMSCV-GFPCre. GFP-positive cells were FACS sorted and used as stimulators for purified OTII T cells in the presence of the indicated concentration of OVA protein and 50μg/ml zymosan. A) Diagram of Cre-mediated deletion of Jagged1. B) CD11c+ GFP+ and GFP- cells obtained on day 5 before FACS sorting. The results of a duplex PCR carried out on FACS-sorted Jagged1F, GFPCre-positive and negative cells is shown in the right hand panel. C) OTII T cells were restimulated with PMA and ionomycin in the presence of monensin for 6hrs before fixation and analysis of T cell number (LH panel) and intracellular IFNγ levels (RH panel). D) Schematic diagram illustrating primer binding sites used for Duplex PCR.

6.3 : Discussion

6.3.1 Zymosan is a weak inducer of Th1 responses

T cell expansion of highly purified antigen-specific T cells induced by BMDC is minimally affected by the presence of zymosan (figure 6-1A and B). At high doses of zymosan a slight suppression of T cell expansion is observed, possibly due to physical inhibition of the formation of T:DC conjugates early in the response. However, when polyclonal T cells are stimulated with BMDC and anti-CD3, a high dose of zymosan improves T cell expansion induced by both MyD88-/- and C57BL/6 BMDC (figure 6-1C and D). There are many potential differences between the antigen-specific and polyclonal systems. A notable example is the higher frequency of regulatory T cells that are being optimally stimulated by anti-CD3 treatment in the polyclonal system. DC that have been activated by a microbial stimulus can induce Treg-independent T cell proliferation and Th1 differentiation [173]. It is therefore possible that zymosan increases BMDC/anti-CD3-induced proliferation of polyclonal T cells as innate signals are required to overcome Treg-mediated suppression in this system. However, the nature of T:DC interaction may also be different in anti-CD3-stimulation versus MHC-peptide:TCR interaction.

It is intriguing that MyD88-deficient DC, in the absence of added microbial stimuli, are less potent inducers of Th1 differentiation than C57BL/6 BMDC (figure 6-1C and D). In fact, despite the marked cytokine production by B6 BMDC stimulated with zymosan, there is only a very marginal effect on IFNγ accumulated in the supernatant after 48hrs of T cell restimulation with plate-bound anti-CD3. However, MyD88-/- DC stimulated with zymosan are markedly better inducers of Th1 differentiation than their unstimulated counterparts (figure 6-1C and D). Given the inherent instability of *in vitro* T cell differentiation assays the validity of this observation needs to be confirmed by assessing a full dose-response of anti-CD3 stimulation and including known strong Th1 and Th2 inducing stimuli with which the amount of T cell effector function achieved with zymosan-stimulated BMDC can be compared.

6.3.2 Th17 induction in response to zymosan requires the presence of Tregs *in vitro*

Th17 effector differentiation is only robustly observed for the polyclonal T cell differentiation assays shown in figure 6-1C and D. TGFβ production, promoted by the presence of Tregs in the cocultures is known to drive Th17 differentiation in the presence of inflammatory cytokines [102, 282]. However, IFNγ inhibits Th17 differentiation induced in this manner [102]. MyD88-/- BMDC stimulated with zymosan promote strong Th17 differentiation of polyclonal T cells as measured by IL-17 production on restimulation (figure 6-1D). It is an attractive hypothesis that MyD88-deficient BMDC stimulated with zymosan produce a cytokine cocktail that, possibly along with TGFβ induced by Tregs, biases towards Th17 induction whilst additional factors produced by BMDC in the presence of TLR (or IL-1 Receptor) signalling dampen Th17 differentiation and rather promote Th1 differentiation. Since MyD88-deficient BMDC stimulated with zymosan are predominantly activated via the Syk-dependent pathway (chapter 4, [146]) this could be an indicator that BMDC activated via Syk-dependent PRRs such as Dectin-1 bias naïve T cells towards the Th17 lineage. This hypothesis requires further validation in a variety of models but would link intriguingly with the requirement for IL-17 to clearance of yeast infections such as Candida [191].

6.3.3 Jagged-1-loss does not appear to modify T cell responses to zymosan

BMDC lacking Jagged-1 can be generated in the expected numbers and Jagged-1 deletion can be confirmed by PCR of genomic DNA purified from GFPCre-positive sorted BMDC (figure 6-2A and B). When these cells are tested in their ability to induce T cell differentiation of highly purified naïve OTII T cells they display no apparent defect in the induction of T cell expansion or Th1 polarisation induced in the absence of innate stimuli or in the presence of zymosan (figure 6-2C). From this, we can conclude that in this particular system, Jagged-1 does not play a non-redundant role in T cell expansion or in

Th1 polarisation. However, as demonstrated in chapter 4, zymosan stimulation of BMDC results in upregulation of mRNA levels not only of Jagged-1, but also Jagged-2, DLL1 and DLL4 such that any functional redundancy between the ligands would compensate for loss of Jagged-1 in this system. It is possible that T cell polarisation induced by BMDC activated via Syk-dependent PRRs is only revealed in the absence of TLR signalling, for example the instruction of Th17 differentiation by MyD88-/- BMDC stimulated with zymosan (figure 6-1C and D). A better system in which to test the role of Jagged-1 might therefore be to use MyD88/Jagged-1 doubly deficient BMDC to stimulate polyclonal T cells as in figure 6-2C and D. Alternatively, pure Dectin-1 agonists could be used to selectively ligate Dectin-1 and activate BMDC via Syk. Using either zymosan in MyD88-/- BMDC of purified Dectin-1 agonists may provide a more sensitive system in which to check the effect of Jagged-1 deletion. This remains an interesting question, in particular given the published links between Jagged-1 overexpression and regulatory T cell development [169, 170] and between regulatory T cells and Th17 differentiation [102]. Work being carried out currently, and in the near future will hopefully shed more light on this matter.

7 Chapter 7: Final Discussion and Perspectives

The results presented in this thesis fully support the designation of Dectin-1, signalling via Syk, as a bona fide pattern recognition receptor as originally defined by Charlie Janeway Jr [1]. In addition. the same results help to define the activated DC phenotype induced by signalling through Dectin-1/Syk or similar Syk-coupled receptors and demonstrated a requirement for ERK signalling downstream of Syk. Finally this thesis presents preliminary evidence that zymosan-stimulation of BMDC can increase their ability to induce Th1 and Th17 T cell differentiation *in vitro*.

7.1 Dectin-1 and Pattern Recognition

7.1.1 Is Dectin-1 a PRR?

In his 1989 opinion [1], Janeway proposed the existence of pattern recognition receptors with the following properties:

"*non-clonally distributed... receptors that allow recognition of certain pathogen-associated molecular patterns that are not found in the host... the pattern recognized should be the product of a complex and critical enzymology in the microorganism*".

What evidence do we have that Dectin-1 fulfils these criteria?

Dectin-1 is clearly non-clonally distributed, as demonstrated by Taylor et al [283], i.e. it is expressed on neutrophils, monocyte/macrophage lineage cells, dendritic cells and a subset of Gr-1-positive T cells.

Dectin-1 is a C-type lectin-like receptor recognising β1,3 and β1,6-glucans [57, 60, 230, 231, 284]. β-glucans are an essential structural component of yeast cell walls [61, 62], i.e. are critical for yeast viability and are not synthesised by

mammals, fulfilling the criteria to be defined as a PAMP. It is therefore reasonable to state that Dectin-1 is a PAMP receptor.

In a later review, Medzhitov and Janeway further added:

"*The signals that control the induction of any particular effector function should therefore convey information about the features of the pathogen so that it can stimulate an adaptive immune response that chooses the relevant effector mechanism.*" [285]

The work presented in this thesis contributes to the evidence that Dectin-1 fulfils this last postulate. At the commencement of this project, work in macrophages had implicated Dectin-1 signalling in oxidative burst and TNFα production induced by zymosan [230, 231]. However, production of TNFα and reactive oxygen species by macrophages is not known to be instructive for adaptive immunity.

Dendritic cells are innate immune cells that, when appropriately activated, can potently stimulate the differentiation of naïve T cells into appropriate effectors (recently reviewed in [77]). As discussed in chapter 1, the production of cytokines by DC correlates well with direct pattern recognition and with the instruction of adaptive immunity [101]. We have previously published that MyD88-independent recognition of zymosan resulted in robust IL-10 production by dendritic cells [96] implying the existence of a non-TLR pattern recognition receptor for zymosan. A more thorough examination of cytokine production by MyD88-/- BMDC stimulated with zymosan revealed production not only of high levels of IL-10 but also significant Jagged-1 upregulation (detailed in chapter 4). In addition, moderate levels of IL-2 and IL-12 p40 production were detected. This suggests that signalling through a non-TLR PRR is particularly effective in the induction of IL-10 and Jagged-1, but it is also capable of inducing many of the cytokines previously ascribed to TLR-activated DC, albeit to lower levels. It would appear that MyD88-/- DC activated with zymosan are quantitatively, rather than qualitatively different to DC activated

by TLR ligands. TLRs are well defined as PRRs that convey information about the features of the pathogen for translation into adaptive immunity [286]. This is strong evidence that zymosan is recognised by a true TLR-independent PRR sufficient to induce adaptive immunity.

What is the evidence that this PRR is Dectin-1? Overexpression of Dectin-1 in a B cell hybridoma (LK cells) confers the ability to bind zymosan and to produce IL-2 and IL-10 in response to zymosan (as detailed in chapter 4, [146]). This requires the presence of tyrosine-15 in the intracellular tail of Dectin-1 which, when phosphorylated, can mediate binding of Dectin-1 to the non-receptor tyrosine kinase, Syk (chapter 4, [146]). In BMDC, the observed MyD88-independent IL-10 production, as well as production of high levels of IL-2, requires signalling through Syk (chapter 4, figure 4-8), and is inhibitable with the Dectin-1 antagonist Glucan phosphate (figure 4-6). This strongly suggests that Dectin-1 or a related receptor is acting as a novel β-glucan-sensing PRR sufficient to induce cytokine production from DC.

Conclusive evidence that Dectin-1 can "convey information about the pathogen" and couple innate to adaptive immunity requires demonstration that activation of DC via Dectin-1 alone is sufficient to induce effector T cell differentiation. Very preliminary data presented in chapter 6 suggests that zymosan stimulation of MyD88-/- DC improves the ability of these cells to instruct Th1 and Th17 differentiation. However, further experiments are required to establish exactly how this is occurring.

There is one aspect in which Dectin-1 deviates from the established model of PRRs, in the observation that PRRs are phylogenetically ancient. Dectin-1 and Toll-like receptors appear to have strikingly different evolutionary histories. Dectin-1 is encoded within a large cluster of C-type lectins including the NK cell receptor NKG2D [53]. The C-type lectins within this cluster show remarkably conserved gene structures, indicative of recent gene duplication and diversification (for example TLR1,2 and 6 [21]). The Toll-like receptors, in contrast, are scattered throughout the genome and have more diverse genomic

structures suggesting that they are evolutionarily much more ancient. This correlates with the distribution of Dectin-1 and TLRs throughout the animal kingdom. Proteins showing highly significant homology to TLRs are found in organisms as diverse as man and fruit flies. Dectin-1 homologues, are found only as far back as vertebrates. Although there is no Dectin-1 homologue in flies, β-glucan recognition does occur. GNBP is a soluble protein with homology to the β1,3 glucanase of *Bacillus circulans [66]* which on β-glucan binding induces cleavage of Spaetzle and activation of Toll signalling. At some point in evolutionary history it appears that vertebrates lost the gene for a soluble β-glucan receptor which signals via an indirect PRR, and gained the ability to directly signal for the presence of β-glucans. Therefore the ability to recognise β-glucans has been selected for over aeons of evolutionary history, despite a more recent diversification of the recognition machinery.

7.1.2 Dendritic cell responses to zymosan are the results of signalling through multiple PRRs

My work and that of others has now confirmed that the recognition of zymosan by dendritic cells occurs via both TLR2/6 signalling through MyD88, and Dectin-1 signalling through Syk (chapter 4, [146] [59]. Of note, inhibition of zymosan responses by soluble β-glucan is at best 30-40% in BMDC (figure 4-6). In contrast, production of IL-10 and IL-2 in response to zymosan is almost completely abrogated in Syk-/- BMDC (figure 4-8). Whilst this could simply represent inefficiency of Glucan phosphate as an inhibitor, it is also possible that alternative Syk-dependent PRRs for zymosan are expressed by BMDC. Good candidates include Complement Receptor 3 which has previously been implicated in zymosan binding [45] [226] and contains an integrin β chain that can mediate Syk activation [235]. Whether further receptors are involved or not, it is clear that at least 2 PRRs present on BMDC are engaged by zymosan (chapter 4, [146]). The production of cytokines in response to zymosan is therefore the result of combinatorial PRR signalling, rather than a single pathway. As suggested [32] [33], synergy between different receptors can

dramatically alter the cytokine profile induced compared to ligation of single receptors.

On one hand, this can be seen as an advantage as zymosan provides something approaching the complexity of a model of the interactions between DCs and live pathogenic yeasts. On the potentially synergistic interactions between receptors can amplify small differences between experiments and between different labs and can significantly complicate the interpretation of experiments. Future elucidation of the effects of particular PRRs on DC activation and in DC signalling would benefit from the use of much cleaner ligands such as pure preparations of β-glucans.

We started this project from the observation that DC produce high levels of IL-10 when stimulated with zymosan. Despite the complexity of our chosen system we have successfully identified a receptor that is capable of mediating this effect and have determined that signalling via Syk into BMDC can lead to DC activation and cytokine production that loosely resembles that induced by known PRR signalling occurring via MyD88. As discussed in chapter 1, zymosan is a good source of yeast PAMPs. The robustness of phenotype induced by Syk-deficiency suggests that despite the many possible receptor-ligand interactions that may, or may not be occurring between zymosan and DCs a Syk-dependent PRR such as Dectin-1 is likely to play an important physiological role in recognition of yeasts by dendritic cells.

7.2 Dectin-1 signals for DC activation via Syk and ERK

7.2.1 Dectin-1 couples to Syk via a novel HemITAM motif

The signalling occurring downstream of Dectin-1 was initially suggested by the presence of an ITAM-like motif in the intracellular tail of Dectin-1 [52]. ITAMs couple to the non-receptor tyrosine kinases Syk and ZAP-70 via binding of the two tandem SH2 domains to two phosphotyrosines of the ITAM [246]. As ZAP-70 expression is largely restricted to cells of the T cell lineage, we

examined the role of Syk in DC responses to zymosan and found that Syk-deficient DC showed a severe defect in the induction of IL-2, IL-10 and Jagged-1 in response to zymosan but were unaffected in their production of IL-12 (figure 4-8). Syk has not previously been implicated in PRR signalling although it is required for activation of DC by immune complexes [234] and has been suggested to negatively regulate TLR signalling via TREM1 [287].

The same LK cell model which was successfully employed to demonstrate Dectin-1 signalling for IL-2 and IL-10 production was employed again to investigate Dectin-1 coupling to Syk. LK cell lines were produced in which the intracellular tyrosines of Dectin-1 are individually or doubly mutated to phenylalanine [146]. When I tested these LK cell lines for cytokine production in response to zymosan we were very surprised to observe that only the membrane proximal tyrosine (Y15) was required for IL-2 and IL-10 production. Further work from Neil Rogers, and work by Dr M. N. Nolte (Immunobiology Lab, London Research Institute, London, UK) confirmed that only this single tyrosine was necessary for association of Dectin-1 with Syk [146]. Dectin-1 therefore appears to signal via Syk using an unusual half-ITAM, or HemITAM motif not previously documented (chapter 4, [146]). The ability of Syk to signal through non-classical motifs is not unheard of, but is also slightly controversial. There are reports that intergrin β chains can signal via Syk [235] in a tyrosine-independent manner. However, there is little or no homology between intergrin β chains and the intracellular domain of Dectin-1 (by sequence alignment – data not shown). There is also a single report that suggests a low level of Syk activation can be achieved with a single ITAM tyrosine [248] but this is orders of magnitude less efficient that full ITAM signalling. It is possible that Syk could bind two phosphotyrosines by bridging two Dectin-1 molecules brought into close proximity by β-glucan binding but this remains to be fully investigated.

7.2.2 Zymosan activation of ERK signalling is Syk dependent

It is clear that Syk-dependent DC activation results in the production of high levels of IL-2 and IL-10. Scientific literature indicated that ERK signalling was a strong candidate for mediating the robust induction of IL-2 and IL-10 in response to zymosan. This pathway is known to be activated by zymosan recognition [271, 272], and is implicated in IL-10 production in response to TLR2 ligands Pam3Cys and MALP2 [136]. Accordingly, I found that zymosan induced robust ERK activation in BMDC. ERK inhibitors PD98059 and UO126 both significantly inhibited IL-10 production as well as IL-2 production induced by zymosan. Although TLR2 signalling is known to couple to ERK activation via the TPL2 pathway [135] ITAM signalling via Syk is also a strong inducer of ERK signalling (reviewed in [245]). As zymosan can induce signalling via both MyD88 and Syk it was interesting to assess the contributions of each PRR pathway to ERK activation. Slightly surprisingly, I found that ERK activation induced by zymosan signalling occurs exclusively downstream of Syk, with ERK activation undetectable in Syk-deficient cells stimulated with zymosan (figure 5-3). However, purified TLR2 ligands show stronger ERK activation in Syk-deficient cells than in wild-type BMDC (figure 5-3), possibly due to loss of Syk-dependent inhibitory signalling via the TREM proteins [287].

Clearly zymosan can induce signalling via TLR2 as IL-12 production is MyD88-dependent (figure 4-5). IL-12 production in response to zymosan is unaffected by Syk-deficiency (figure 4-8) and is higher than IL-12 production induced by saturating concentrations of Pam3Cys or MALP2 (figure 4-1). It would therefore appear that TLR2 signalling induced by purified lipopeptides is qualitatively different from TLR2 signalling induced by zymosan. There are numerous possible explanations for this that fall broadly into two categories: 1) TLR2-interactions with its ligand in zymosan induces a different conformation change in the receptor which affects its ability to couple to the pathway for ERK activation and 2) the microenvironment induced by massive receptor clustering in response to zymosan may affect coupling of TLR2 to ERK.

As the TLR2-ligand in zymosan is not defined it is hard to test explanation-1 directly. However, direct coupling of Pam3Cys or MALP2 to zymosan would result in a particle that could activate ERK in a Syk-dependent manner if the nature of the ligand, rather than the membrane microenvironment, was important.

It is an intriguing possibility that receptor microenvironment may play a role in receptor coupling to ERK. Zymosan is a large particulate ligand, compared to the small soluble lipopeptides MALP2 and Pam3Cys, and in addition, β-glucans of zymosan are bound by Dectin-1 with a very high affinity [57] meaning that TLR2 associated with zymosan must be recruited into the membrane patch containing high levels of Dectin-1. Indeed membrane microdomains have been implicated in both TLR and ITAM signalling [288] [108]. The exclusion of factors required for MyD88-coupling to ERK activation from Dectin-1-enriched membrane patches could account for the observed results. A further possibility involving the microenvironment around TLR2 is that zymosan may ligate a Syk-independent inhibitory receptor which actively blocks TLR2 signalling to ERK. Experiments are currently being carried out to test these hypotheses.

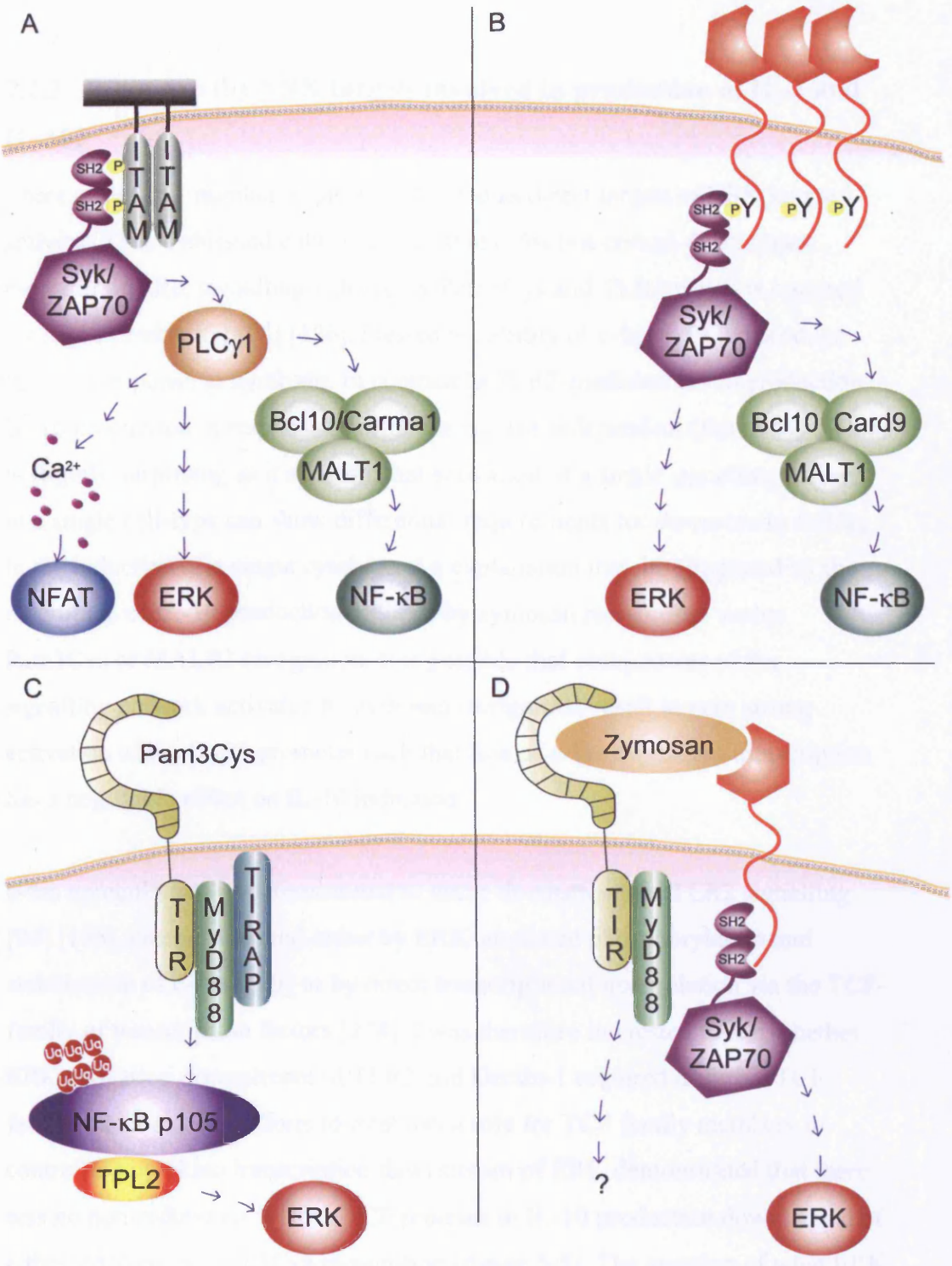


Figure 7-1: Signalling induced by ITAMs, Dectin and TLR2. A) Signalling to NF-κB, NFAT and ERK downstream of classical ITAMs such as those found in antigen receptors. [256, 257] [255]. B) Signalling to NF-κB and ERK downstream of Dectin-1. [147] figure 5-3 C) Signalling to ERK induced by TLR2 ligands Pam3Cys or MALP2. [94, 136] D) Signalling to ERK induced by zymosan (figure 5-3).

7.2.3 What are the ERK targets involved in production of IL-2 and IL-10?

There are a large number of proteins defined as direct targets of ERK kinase activity [273]. Published data suggested that c-fos is a critical downstream mediator of ERK signalling induced by Pam3Cys and TLR2 which is required for IL-10 production [94] [136]. I tested the ability of c-fos-/- DC to produce IL-10 in response to zymosan. In contrast to TLR2-mediated IL-10 production, IL-10 production in response to zymosan is c-fos independent (figure 5-4). This is slightly surprising as it suggests that activation of a single signalling pathway in a single cell-type can show differential requirements for downstream factors in the induction of a single cytokine. An explanation may be suggested by the magnitude of IL-10 production induced by zymosan recognition versus Pam3Cys or MALP2 recognition. It is possible that components of the signalling network activated by zymosan recognition result in very strong activation of the IL-10 promoter such that loss of c-fos dependent transcription has a negligible effect on IL-10 induction.

c-fos upregulation, as demonstrated to occur downstream of TLR2 signalling [94] [136], can be achieved either by ERK-mediated phosphorylation and stabilisation of c-fos [289] or by direct transcriptional upregulation via the TCF-family of transcription factors [274]. I was therefore interested to test whether ERK activation downstream of TLR2 and Dectin-1 required different TCF family members. My efforts to establish a role for TCF family members in controlling cytokine transcription downstream of ERK demonstrated that there was no non-redundant role for TCF proteins in IL-10 production downstream of either zymosan or Pam3Cys recognition (figure 5-5). The question of what ERK targets are required to support IL-2 and IL-10 production therefore remains open. An interesting suggestion comes from work of Lucas et al [268] proposing a role for ERK activation in modification of Histone H3 at the IL-10 promoter allowing binding of STAT3 and Sp1. However, there is an extensive

collection of ERK targets that I have not considered here, many of which may be involved in this process [273].

My experiments suggest that the ERK pathway at least partially acts to control IL-10 and IL-2 production at the mRNA level (figure 5-2). It should be noted that IL-2 mRNA upregulation induced by zymosan stimulation appears to be largely independent of *de novo* transcription (figure 5-6), suggesting that ERK may be playing a role in stabilising IL-2 mRNA, rather than in direct transcriptional activation of the IL-2 gene. Indeed, there is evidence to suggest that ERK signalling could modify the production of secreted proteins at multiple stages in the production process (summarised in figure 7-2) including transcriptional, post-transcriptional and translational events [126, 127, 268, 295, 296]. It is probable that ERK activation downstream of Syk affects IL-10 and IL-2 production at several different levels. Measurements of the effects of ERK inhibition on mRNA half-life will help to establish whether ERK targets mediate mRNA stabilisation in activated DC.

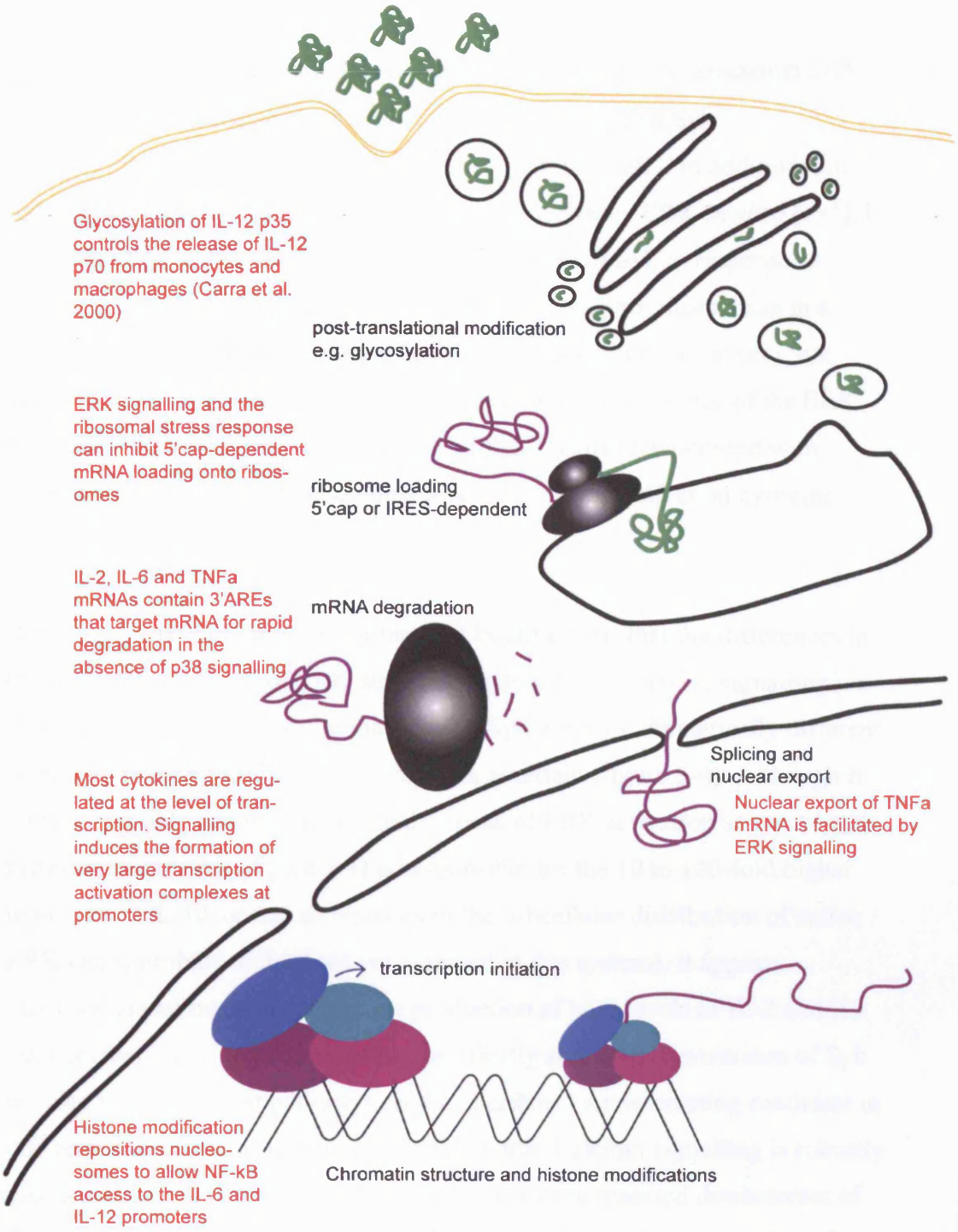


Figure 7-2: Cytokine production can be regulated at multiple stages of mRNA and protein synthesis: chromatin remodelling [268], transcription factor activation [136], mRNA Nuclear export [129], mRNA stability [126, 127], translation initiation [131], post-translational regulation [290]

Experiments on Fcε Receptor signalling in the Rat basophilic leukaemia 2H3 cell line RBL-2H3 suggested that ERK inhibition decreased Syk phosphorylation induced by Fc-receptor cross-linking [291]. In addition, the authors observed direct serine phosphorylation of Syk by ERK *in vitro* [291]. It is possible that whilst ERK is activated downstream of Syk in response to zymosan, it plays an important role in promoting Syk phosphorylation in a positive feedback loop required for initiation of signalling. Examining Syk phosphorylation induced by zymosan recognition in the presence of the ERK inhibitor, and considering the behaviour of Syk-/- cells reconstituted with appropriate Syk mutants would allow assessment of this effect on cytokine production.

During the course of these experiments, it became clear that the differences in kinetics and magnitude of ERK signalling induced by Pam3Cys signalling via TLR2 and Zymosan signalling via Dectin1/Syk were not dramatically different at least as far as per cell measurements can ascertain (figure 5-4). Although it remains possible that the slightly higher peak of ERK activation achieved with zymosan stimulation (figure 5-4) is responsible for the 10 to 100-fold higher induction of IL-10, or that differences in the subcellular distribution of active ERK can contribute to this (not yet assessed in this system), it appears that ERK signalling is necessary for production of high levels of IL-2 and IL-10, but other signalling components specifically activated downstream of Syk are also required. What pathways could contribute? An interesting candidate in this respect is calcium signalling via calcineurin. Calcium signalling is robustly activated by ITAM signalling [292] but has not been reported downstream of TLR ligation. Evidence in support of this is provided by the observation of Granucci et al [145] that IL-2 production is inhibitable with the calcineurin inhibitor, cyclosporin A.

In conclusion, Dectin-1 – a β-glucan receptor that can be activated by zymosan – signals via a novel hemITAM motif to activate ERK (data presented in chapter 4 and 5). TLR2-mediated recognition of zymosan fails to result in ERK

activation (figure 5-3) and the mechanistic explanation of this is currently under investigation. Signalling components required downstream of ERK are elusive but do not include the TCF family members Sap1, Elk1 or Net, or the downstream AP-1 component c-fos (figure 5-4 and 5-5). The absence of c-fos requirement distinguishes between zymosan-induced and TLR2-ligand induced IL-10 production. This suggests that signalling downstream of ERK in zymosan responses requires an alternative range of effectors and can collaborate with alternative signalling pathways not strongly activated by TLR signalling.

7.3 How zymosan recognition by DC couples to adaptive immunity.

Data presented in chapter 6 represent the first tentative steps into study of the effects of zymosan as an adjuvant. This is an important study as it would finally confirm a role of Dectin-1 in translating innate into adaptive immunity and may shed light on how immunity to pathogenic yeasts is established. One of the main challenges in addressing this question is the potential complexity of zymosan itself as a stimulus for DC maturation [39]. In order to consider Syk-dependent DC activation, all experiments would need to be carried out in MyD88-deficient DC (chapter 4), which means that in order to genetically manipulate the system further, for example by knocking-out Jagged-1 we really need to use Jagged-1/MyD88 double knock-out animals produced as the result of time-consuming breeding. A far preferable system would allow selective ligation of Dectin-1 in DC in order to examine the phenotype of mature DC and T cell differentiation induced. On-going work is examining these possibilities.

Work to establish a role for Jagged-1 upregulation induced by Dectin-1/Syk signalling is also on-going. Although there did not appear to be a role for Jagged-1 on Th1 differentiation of naïve OTII-TCR-transgenic T cells (figure 6-2) this may not be the ideal system in which to test this hypothesis, as the window of zymosan-induced increase in Th1 differentiation was very small. A more promising system is to use DC stimulated exclusively via Syk-dependent PRRs in a system where either Th1 or Th17 differentiation can be induced and where DC can interact with regulatory T cells. It is likely that observed effects

will be relatively subtle therefore it is important to establish as robust a system as possible in which to test this hypothesis. The experiments presented so far demonstrate that the system for retroviral introduction of Cre into dendritic cells is feasible and can be used to produce Jagged-1-deleted dendritic cells (figure 6-2).

Further experiments with genetic manipulation or blocking antibodies could investigate the roles of DC-derived IL-2 and IL-10 in T helper differentiation. Extending these observations to *in vivo* T cell priming and yeast infection models would eventually allow us to determine the physiological role of Syk-dependent pattern recognition and would firmly establish Dectin-1 as a novel cell-surface pattern recognition receptor.

7.4 The future

It is intriguing to speculate that the evolution of adaptive immunity required the evolution of direct PRRs such that antigen presentation and "information about the pathogen" [285] could be restricted to single antigen-presenting cells. TLRs have diversified from the original Toll proteins such that they are now thought to bind directly to lipid, peptide and nucleic acid ligands [27] [26]. However, carbohydrate recognition does not appear to have evolved amongst the TLRs despite the requirement for Drosophila Toll signalling downstream of β-glucan recognition by GNBP. Instead this function appears to have been taken over by Dectin-1. Once Dectin-1 has been fully established to be a PRR it would be interesting to determine whether it is one of a family of such PRRs.

The closest homologues of Dectin-1 are other C-type lectins (CLECTs) encoded alongside Dectin-1 within the NK-cell receptor cluster, many of which have been implicated in NK cell function [53]. However, a number of these receptors are orphan receptors and one such, CLEC-2, has recently been found to activate Syk via a HemITAM motif on artificial cross-linking or binding of the snake venom rhodocytin [293]. It is currently unclear whether other genes in the cluster can act like Dectin-1. In addition, it is possible that gene conversion has resulted in use of the HemITAM motif in non-CLECT receptors that are

potential novel Syk-dependent PRRs. As well as identifying full homologues of Dectin-1, it would therefore be interesting to identify genes with regions homologous to the first exon of Dectin-1, which encodes its ability to interact with Syk via the HemITAM motif.

Another interesting questions which, despite our best efforts so far remains unanswered is to identify what Syk-dependent signalling events are responsible for the extremely high levels of IL-10 induced downstream of Dectin-1/Syk signalling. Although ERK plays a role, it is clearly not sufficient as TLR2 signalling induces ERK activation similarly to Dectin-1, but induces 10 to 100-fold less IL-10. Two approaches to this problem are possible. One is to consider strong candidates likely to be activated downstream of Dectin-1 such as calcium signalling/ NFAT [292] or autocrine production of type I interferons [33]. An alternative approach is being carried out elsewhere, examining transcription factors bound to the IL-10 promoter under different conditions (started in [294]). The identity of these transcription factors may lead us to the nature of signalling events required for their induction.

A final area that requires further investigation is the putative link between Th17 differentiation and MyD88-/- DC primed with zymosan. It has been suggested that IL-17 is critical for productive immunity against systemic challenge with *Candida albicans* [191] and more historically, that TGFβ is important in establishing resistance to Candida infection [192]. A link between yeast infection and Th17 induction could also open up new avenues of investigation in the large number of autoimmune conditions in which Th17 effector T cells are responsible for pathology (Crohn's disease [156, 182], EAE [280] etc).

7.5 Concluding Remarks

I have presented evidence to strongly support the designation of Dectin-1 as a bona fide PRR that can function to induce DC cytokine production completely independently of the TLR system. Dectin-1 signals via a novel HemITAM motif which couples pattern recognition to activation of Syk. ERK signalling,

activated downstream of Syk, is required for the production of high levels of IL-2 and IL-10 induced in response to zymosan. Several new techniques (retroviral transduction of bone marrow, assessment of cell signalling by flow cytometry) have allowed the first steps to be made in detailed analysis of signalling induced by the recognition of a complex yeast-derived stimulus (zymosan). Many interesting questions remain. The phenotype of BMDC activated via the Syk-dependent pathway qualitatively resembles that of TLR-stimulated DC, in that IL-12 p40, IL-2, IL-6, TNFα and IL-10 are induced. Intriguingly, there are large quantitative differences in levels of these cytokines produced when compared to cytokine induction induced by TLR-ligands. In particular high levels of IL-10, IL-2 and Jagged-1 suggest that DC activated in this way may bias T cell differentiation towards different T cell effector functions. In addition the observation that TLR2-mediated zymosan recognition is insufficient to induce ERK activation highlights the fact that TLR signalling induced by different ligands or induced from within different receptor complexes can vary qualitatively. Future work will help to provide further insight into the precise mechanisms of yeast recognition by DC and the induction of anti-fungal adaptive immunity.

References

For convenience, the publication by Rogers et al (containing the work described in this thesis) is reproduced in the appendix

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Appendix

Paper containing work described in this thesis (Reference 146)

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Syk-Dependent Cytokine Induction by Dectin-1 Reveals a Novel Pattern Recognition Pathway for C Type Lectins

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