Investigation of type-I IFN regulated gene expression in murine dendritic cells

by

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Abstract

Dendritic cells (DCs) provide a vital link between the innate and adaptive immune systems, allowing for detection of invading pathogens and the rapid initiation of an appropriate response. Until recently, research on type-I interferons (IFN-I) has focused on their function as viral inhibitors. However, it is now evident that IFN-I also has multiple roles in immune regulation, including the alteration of DC function. IFN-I has been shown to enhance T and B cell responses *in vivo* through stimulation of DCs, but the mechanisms by which IFN-I acts on DCs to produce these effects are unclear. We have investigated how gene expression is altered in IFN-I treated DCs, with the aim of identifying IFN induced genes which enhance the ability of DCs to initiate and sustain an effective immune response. Murine splenic $CD11c^+$ DCs or bone marrow-derived DCs were isolated and cultured either alone or in the presence of IFN-I, and two techniques were employed for discovery of differentially expressed genes: representational difference analysis (RDA) and microarray analysis. We identified IFN-I induced genes involved in diverse aspects of cell function, including transcription factors, signaling molecules, co-stimulatory molecules, cytokines and chemokines. For further study, we selected genes which encode putative cell surface proteins or which share homology with proteins involved in adaptive immune functions. Investigation of a putative chemoattractant receptor, Gpr33, showed that expression of this molecule was upregulated in DCs in response to IFN-I both *in vitro* and *in vivo*. We attempted to generate antibodies against Gpr33 and have studied mRNA expression by real-time PCR. Gpr33 mRNA expression was shown to be upregulated by IFN-I in all splenic DC subsets, but was expressed in the $CD8^+$ subset at levels more than 40 fold higher than the $CD4^+$ subset, indicating that it may specifically be involved in the migration of this subset.

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Chapter 1: Introduction

1.1 Overview

Dendritic cells (DCs) are key components of the immune system. In the peripheral organs, DCs are deployed at potential sites of infection, where they are able to capture both self and foreign antigens. DCs directly detect pathogens through specialised receptors but can also detect the presence of an infection indirectly, through receptors for inflammatory cytokines released by neighbouring cells. One such group of cytokines is type I interferon (IFN-I). IFN-I plays an important role in anti-viral defence and can modulate both the innate and adaptive arms of the immune system. In fact, DCs themselves are a major source of IFN-I during infections and its expression can be induced by the detection of pathogens.

Both pathogen-derived signals and inflammatory cytokines can induce DC maturation. This triggers their migration to the lymph node, where they can contact circulating T cells. Before leaving the periphery, DCs release inflammatory cytokines, allowing the recruitment and activation of innate effector cells important for early control of the infection. DC maturation is also essential for prolonged presentation of antigens and delivery of co-stimulatory molecules necessary for naïve T cell activation and proliferation. However, DCs presenting antigen in the absence of co-stimulation are important in promoting tolerance to peripheral antigens. Importantly, DCs are not simply messengers alerting T cells to the presence an infection, but translate information regarding the nature of an infection, through expression of cell surface molecules and release of cytokines. These act as a third signal to T cells and determine the type of T cell response initiated, necessary for effective elimination of the pathogen. DCs also interact with B cells in the lymph node and can enhance their proliferation, as well as enhancing antibody production and isotype switching.

DC maturation is a complex and closely regulated process and consists of many different facets which could effect the outcome of an immune response. We now review this process and focus on the relationship between DCs and IFN-I.

1.2 Dendritic Cells

1.2.1 Identification of dendritic cells as potent antigen presenting cells

Characterisation of dendritic cells (DCs) began just over 30 years ago with the identification of a morphologically distinct splenic cell type featuring long dynamic cytoplasmic projections (Steinman *et al.*, 1973). These cells were subsequently identified as the essential accessories for the initiation of a primary antibody response by T and B cells (Inaba *et al.*, 1983), and were established as potent stimulators of the mixed leukocyte reaction (MLR)(Steinman *et al.*, 1985). Following this, it was shown that antigen-pulsed DCs injected into naïve mice were able to initiate $CD4^+$ T cell response in the draining lymph node (Inaba *et al.*, 1990). DCs are present in various forms and are found in non-lymphoid organs such as the skin, gut, liver and lungs, as well as in the lymphoid organs and in the circulation (Steinman, 1991). DCs are highly specialised antigen presenting cells, expressing abundant major histocompatability complex (MHC) class I and class II molecules on their cell surface, in addition to adhesion molecules which promote homing and interactions with T cells (Steinman, 1991).

DC function can be divided into three separate phases: 1) they act as sentinels of the immune system capturing antigen in the peripheral tissues and, in the context of an infection, receive signals which initiate their activation, 2) on activation they undergo a process of maturation, during which time they migrate to the lymph nodes and 3) on reaching the lymph node they carry out their unique function of inducing a primary immune response.

1.2.2 Antigen capture, processing and presentation

1.2.2.1 Antigen capture

After differentiation from bone marrow progenitors DC precursors migrate to the non-lymphoid tissues where they reside as immature DCs awaiting the arrival of foreign antigen (Banchereau *et al.*, 2000). These immature DCs are highly efficient at capturing antigens and employ several mechanisms for this. Particles, microbes and fragments of apoptotic and necrotic cells can be taken up by phagocytosis (Reis e Sousa *et al.*, 1993; Albert *et al.*, 1998b; Inaba *et al.*, 1998). DCs also use macropinocytosis, a process involving uptake of extracellular fluids by membrane ruffling, which leads to the concentration of solutes in the MHC class II compartment (Sallusto *et al.*, 1995). Receptor-mediated endocytosis is enabled by the expression of C-type lectin receptors, such as the mannose receptor (Sallusto *et al.*, 1995; Engering *et al.*, 1997) and DEC-205 (Jiang *et al.*, 1995), in addition to FcγR (Sallusto *et al.*, 1994) and FcεR1 (Maurer *et al.*, 1995). Using these different mechanisms the amount of antigen available for presentation by DCs is greatly increased. For example uptake via the mannose receptor leads to a 100-fold increase in the presentation of soluble antigen (Engering *et al.*, 1997). Phagocytosed particles from necrotic or apoptotic cells are presented more than 1000 times more effectively than pre-processed peptide (Reis e Sousa *et al.*, 1993; Albert *et al.*, 1998b; Inaba *et al.*, 1998). When DCs are exposed to maturation stimuli antigen macropinocytosis is transiently stimulated, enhancing presentation by MHC class I and class II molecules (West *et al.*, 2004). Following this, DCs down-regulate receptors involved in antigen uptake and lose their phagocytic ability (Reis e Sousa *et al.*, 1993; Sallusto *et al.*, 1994; Sallusto *et al.*, 1995; Albert *et al.*, 1998a).

1.2.2.2 MHC class II presentation pathway

DCs contain specialized machinery necessary for antigen processing (Figure 1.1). MHC class II-rich compartments (MIICs), to which exogenously acquired antigens are targeted, are lysosome-related intracellular compartments where MHC class II molecules are constantly accumulated (Kleijmeer *et al.*, 1995). The class II molecules are associated with the invariant chain which targets them to the endosomal-lysosomal pathway but prevents binding of peptides. The MIICs contain HLA-DM or H-2M molecules which promote the removal of the invariant chain and enhance peptide binding to MHC class II molecules (Cresswell, 1996). On DC maturation the invariant chain is degraded by the cysteine protease cathepsin S, allowing loading of the antigen onto MHC class II and export to the cell surface. The regulation of this process is regulated by a cathepsin S inhibitor, cystatin C, which in mature DCs is expressed at lower levels and is no longer localised in the MIICs (Pierre *et al.*, 1998). During DC maturation there is a period of rapid class II synthesis. Unlike in immature DCs, where class II molecules are rapidly internalised and recycled, the MHC II-peptide complexes are stably expressed on the surface for days, providing a window for prolonged $CD4^+$ T cell stimulation (Cella *et al.*, 1997; Pierre *et al.*, 1997; Winzler *et al.*, 1997). DC-LAMP, a marker of mature human DCs is expressed transiently in the MIIC and was suggested to have a role in MHC class II-restricted antigen presentation (de Saint-Vis *et al.*, 1998). However, more recently it was found that the mouse homologue of DC-LAMP is not expressed in activated DCs (Salaun *et al.*, 2003).

1.2.2.3 MHC class I presentation pathway

DCs also present MHC class I-restricted antigens to $CD8^+$ T cells. The majority of antigens are loaded onto MHC class I molecules through an endogenous pathway, a process which has been extensively reviewed (Pamer *et al.*, 1998; Rock *et al.*, 1999). The class I molecules are

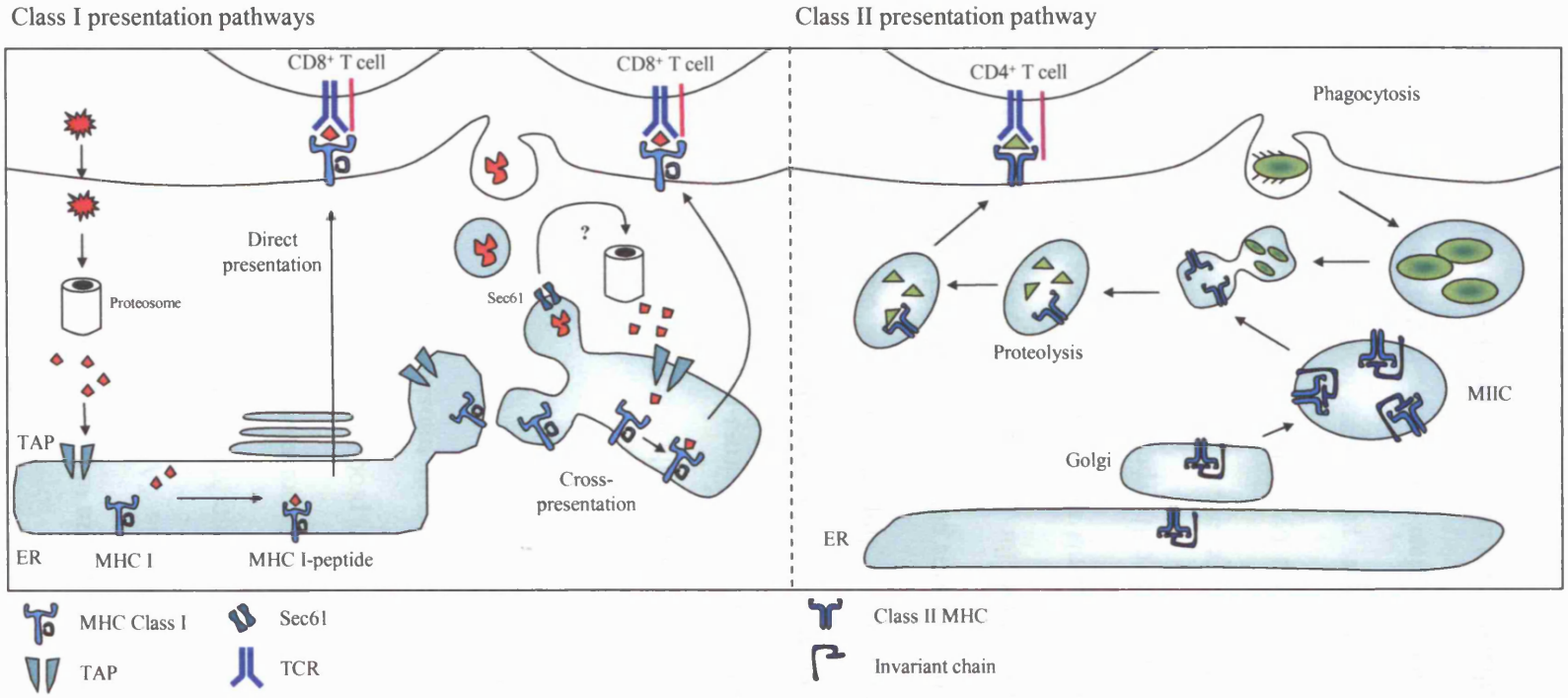


Figure 1.1 Processing of antigens for presentation to CD4$^+$ and CD8$^+$ T cells Antigens entering the cell through exogenous pathways are contained in phagosomes or endosomes which then fuse with the MIICs. After removal of the invariant chain and proteolysis of the antigen, peptides are loaded onto the MHC II molecules and transportated to the cell surface for presentation to CD4$^+$ T cells. Antigens present in the cytosol such as the products of replicating viruses are degraded by the proteosome and transported into the endoplasmic reticulum (ER) via TAP. The peptides are loaded onto MHC I molecules and exported to the cell surface for presentation to CD8$^+$ T cells. Exogenously aquired antigens can also gain access to the class I pathway possibly through fusion with ER-derived vesicles and transport to the cytosol by Sec61.

assembled in the endoplasmic reticulum (ER) and are retained there through interactions with various chaperone proteins and other associated proteins including tapasin. Cytosolic proteins derived from the host or intracellularly replicating pathogens are ubiquitin-conjugated, which targets them to proteosomes for degradation. The peptides generated are then transported to the ER via TAP1/2 transmembrane transporters where they are trimmed into 8-10 mers and loaded onto MHC class I molecules. In the absence of infection a large proportion of the host peptides presented on MHC I molecules are thought to be derived from defective ribosomal initiation products (DRiPs). These proteins are relatively unstable for several reasons such as errors in translation and mis-folding and are rapidly targeted for degradation (Yewdell *et al.*, 2001).

1.2.2.4 Cross-presentation

An important property of DCs is their ability to present exogenous antigens on MHC class I molecules, a process known as cross-presentation (Heath *et al.*, 2004). Several types of antigen may be cross-presented, including soluble proteins, immune complexes, intracellular bacteria and parasites. Importantly, DCs can cross-present cellular antigens acquired through uptake of apoptotic cells, virally-infected cells and tumour cells (Heath *et al.*, 2004). This mechanism is vital for generation of cytotoxic lymphocyte (CTL) responses against tumour antigens and against viruses which do not directly infect DCs. Cross-presentation does not occur in most cell types where expression of antigen on class I molecules is restricted to antigens present in the cytoplasm, ensuring that only infected cells are recognised and destroyed by CTLs. The mechanism by which antigens present in the phagosomes or endosomes gain access to the cytosol for proteolysis and consequently into the ER for loading onto class I molecules is still unclear. The current model is based on the discovery that phagosomes can fuse with ER-derived vesicles (Figure 1.1) (Ackerman *et al.*,

2003; Guermonprez *et al.*, 2003; Houde *et al.*, 2003). The resulting compartment contains MHC class I molecules in addition to the machinery required for peptide loading. It is thought that antigens can be transported into the cytosol via a mechanism which involves the Sec61 complex, which is known to 'translocate' proteins into the cytosol from the ER (Wiertz *et al.*, 1996). Proteosomes closely associated with the phagosome would then degrade the antigens before transport of peptides back into the phagosome via TAP (Ackerman *et al.*, 2004; Heath *et al.*, 2004). In agreement with this model, it was shown that class I molecules contain a residue in their cytoplasmic tail which is necessary for their trafficking to the phagosome (Lizee *et al.*, 2003). This residue is essential for cross-presentation but dispensable in the endogenous pathway. Cross-presentation will be discussed again, in the context of DC subsets (section 1.2.7.3.3) and type I IFNs (section 1.3.8.4).

1.2.3 Innate activation of DCs

1.2.3.1 Activation by Toll-like receptors

1.1.3.1.1 Toll-like receptor recognition of pathogen components

In addition to presenting pathogen-derived peptides in association with MHC molecules, DCs can migrate to the lymph node (LN) and express co-stimulatory molecules in order to stimulate naïve T cells. This maturation process must be tightly regulated to avoid the generation of self-reactive T-cells. Therefore a system has evolved whereby DCs only become activated in the presence of signals associated with tissue damage or infection. DCs respond directly to pathogens through pathogen recognition receptors (PRRs) which recognize a conserved set of microbial molecular patterns known as pathogen-associated molecular patterns (PAMPs) (Janeway, 1989). The largest group of PRRs identified to date are the Toll-like receptors (TLRs) with at least 10 members in most mammalian species

(Iwasaki *et al.*, 2004). TLRs 1-9 are conserved between the human and mouse, however TLR10 appears to be non-functional in mice, while in humans the presence of a stop codon in the TLR11 gene results in a lack of its production (Zhang *et al.*, 2004; Takeda *et al.*, 2005). Various components of infectious agents are recognized by TLRs, and these interactions are summarised in Table 1.1 (Takeda *et al.*, 2005; Yarovinsky *et al.*, 2005). TLR2 recognises a wide range of microbial components, which can be explained by its ability to associate with either TLR1, TLR6 and dectin-1. Many of the TLRs (TLR3, 7, 8 and 9) are not expressed on the cell surface, but instead are present in phagosomal or endosomal compartments and contact their ligands after ingestion and degradation of the microbe by phagocytosis or receptor-mediated endocytosis (Takeda *et al.*, 2005).

Table 1.1. Recognition of pathogen components by TLRs

TLR family member	Organism	Ligand
TLR2	$Gram^+$ bacteria	Peptidoglycan
		Lipotecheicoic acids
	Staphylococcus	Modulin
	Mycobacteria	Lipoarabinomannan
	Yeast	Zymosan
	Trypanosoma cruzi	GPI anchors
	Treponema maltoplilum	Glycolipids
	Non-entero-bacteria	Lipopolysaccaride (LPS)
TLR3	Viruses	Double-stranded RNA
TLR4	$Gram^-$ bacteria	LPS
	$Gram^+$ bacteria	Lipotecheicoic acids
TLR5	Bacteria (with flagella)	Flagellin
TLR7	Viruses	Single-stranded RNA
	(synthetic)	Imidazoquinoline compounds
TLR8	Viruses	Single-stranded RNA
	(synthetic)	Imidazoquinoline compounds
TLR9	Bacteria	Unmethylated CpG DNA
TLR11	Uropathogenic bacteria	Unknown
	Toxoplasma gondii	Profilin

1.2.3.1.2 TLR signaling pathways

Toll-like receptors (TLRs) signal through a pathway that leads to activation of NF-κB and the induction of an inflammatory response (Medzhitov, 2001). Activation of DCs through TLRs triggers fundamental changes necessary for stimulation of adaptive immune responses,

firstly, the alteration of chemokine receptor expression (Dieu *et al.*, 1998; Sallusto *et al.*, 1998c) allowing migration to the draining LN. Secondly, they induce DC maturation, resulting in increased expression of co-stimulatory molecules (Medzhitov, 2001). TLR ligation also influences cytokine secretion, and induces Th1-polarising cytokines such as IL-12 or if appropriate, cytokines leading to Th2 development (Pulendran, 2005). The signaling pathways of the TLRs have distinct components which lead to divergent responses. For instance, signaling through TLR3 and TLR4 leads to the induction of type I interferons (IFN-I) whereas signaling through TLR2 does not (Doyle *et al.*, 2002; Hoshino *et al.*, 2002; Toshchakov *et al.*, 2002). Signaling through TLR7 and TLR9 also leads to the induction of IFN-I, but occurs through a distinct mechanism to TLR3/4 induction (Hemmi *et al.*, 2002; Hoshino *et al.*, 2002). Interestingly, ligation of the same TLR on different types of DC can induce expression of different cytokines. Ligation of TLR7 and TLR9 triggers production of IFN-α by plasmacytoid DCs and production of IL-12 by $CD11c^+$ DCs, whilst co-stimulatory molecule expression is enhanced in both DC subsets (Ito *et al.*, 2002; Hemmi *et al.*, 2003).

TLRs share a common signaling pathway which utilizes the adaptor protein MyD88 and leads to the activation of the AP-1 transcription factors and NF-κB (Figure 1.2). The production of inflammatory cytokines such as TNF-α and IL-12p40 in response to all TLR ligands is dependent on MyD88 (Takeda *et al.*, 2005). A second adaptor TIRAP/Mal is essential for MyD88-dependent signaling through TLR4 and TLR2, but not TLR3, TLR5, TLR7 or TLR9 (Horng *et al.*, 2002; Yamamoto *et al.*, 2002a). In addition to the MyD88-dependent pathway, TLR4 and TLR3 utilize a second pathway which acts through an adaptor known as the Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing IFN-β (TRIF) which associates with IRF3 and can induce IFN-β expression (Diebold *et al.*, 2003; Oshiumi *et al.*, 2003).

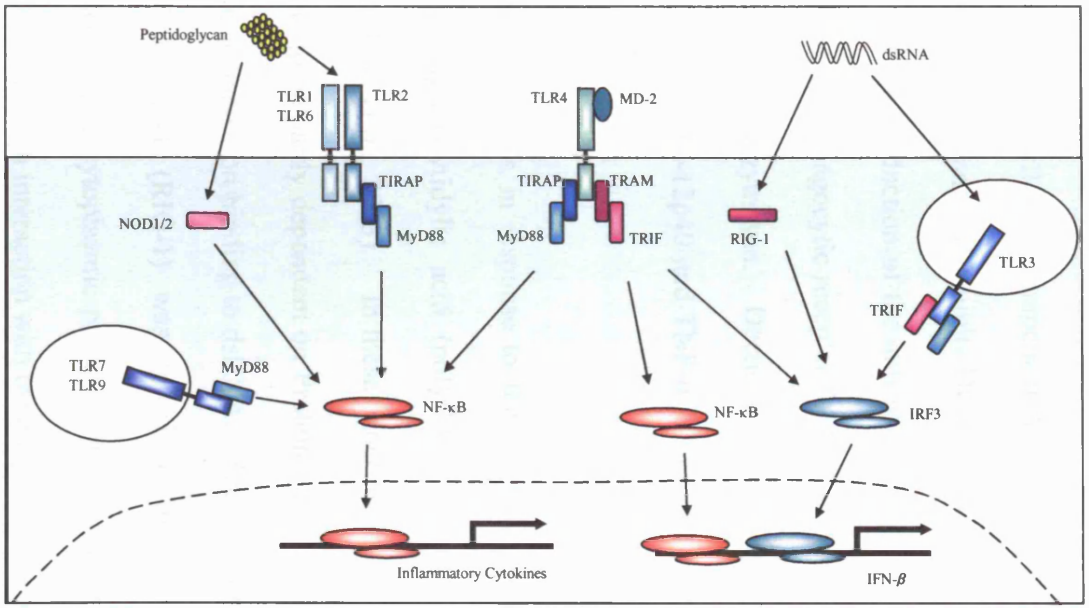


Figure 1.2 Pathogen recognition receptor signaling pathways (Adapted from Takeda 2003) Pathogen components are recognised by TLRs on the cell surface or within endosomal compartments or by non-TLR receptors in the cytosol. Signaling through TLRs occurs via two distinct pathways, which utilise different adaptor molecules and lead to the activation of either NF-κB or IRF3 and the induction of inflammatory cytokines.

1.2.3.2 Non-TLR recognition of pathogens

DCs can recognise PAMPs through receptors other than TLRs. C-type lectins are also thought to act as PRRs, including DC-SIGN which captures and internalizes *mycobacterium tuberculosis* through the cell wall component ManLAM (Geijtenbeek *et al.*, 2003). In contrast to TLR ligation, ManLAM inhibits DC maturation in response to lipopolysaccaride (LPS), and induces the production of the anti-inflammatory cytokine IL-10. A second C-type lectin, dectin-1, is a phagocytic receptor for β-glucan containing particles including the yeast cell wall component zymosan. Dectin-1 acts in a synergistic manner with TLR2 to induce the production of IL-12p40 and TNF-α in response to zymosan (Brown *et al.*, 2003; Gantner *et al.*, 2003).

Induction of IFN-I by DCs in response to the synthetic double stranded RNA (dsRNA) polyriboinosinic polyribocytidylic acid (poly(I:C)) can occur through TLR-independent mechanisms (Diebold *et al.*, 2003). In these experiments, the production of IFN-I, but not IL-12, was found to be partly dependent on Protein kinase R (PKR), which is present in the cytoplasm and is activated on binding to dsRNA. Recently a molecule encoded by the gene Retinoic acid-inducible-1 (RIG-1) was found to mediate TLR3-independent dsRNA recognition. RIG-1 is a cytoplasmic protein which can interact with dsRNA and induces expression of IFN-I through interaction with the adaptor molecule CARDIF (also named IPS-I and MAVS) (Yoneyama *et al.*, 2004; Kawai *et al.*, 2005; Meylan *et al.*, 2005; Seth *et al.*, 2005). CARDIF recruits IKK kinases, leading to the activation of IRF3 and NF-κB (Meylan *et al.*, 2005). In addition, an intracellular mechanism for TLR-independent detection of bacteria has been described, in which the nucleotide-binding oligomerisation domain (NOD) family of proteins appear to play an important role (Chamaillard *et al.*, 2003; Girardin *et al.*, 2003a; Girardin *et al.*, 2003b).

1.2.3.3 DC activation by endogenous signals

DCs can also be activated indirectly, through inflammatory cytokines produced locally by infected cells. For example virally-infected cells produce IFN-α, a potent DC activating factor (discussed in detail in section 1.3.8). IFN-I induces the production of IL-15 from DCs and increases expression of the IL-15 receptor, resulting in an autocrine loop where IL-15 itself can activate DCs (Mattei *et al.*, 2001). DC maturation can also be induced *in vitro* by TNF-α, granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-1 (Heufler *et al.*, 1988; Caux *et al.*, 1992; Sallusto *et al.*, 1994; Winzler *et al.*, 1997). In addition, ligation of chemokine receptors appears to mediate aspects of DC activation, since stimulation of the CCR5 receptor results in the production of IL-12 (Aliberti *et al.*, 2000). It has recently been shown, that although indirect activation of DCs by inflammatory mediators can induce their maturation in the context of antigen presentation and ability to enhance T cell expansion, direct stimulation through TLRs is necessary for IL-12 production and T helper cell polarisation (Sporri *et al.*, 2005). However, the TLR ligands used in this model were LPS and CpG which do not induce high levels of type I IFNs compared to the TLR3 ligand poly(I:C) (Vanessa Durand, personal communication).

In the absence of infection, DCs can be activated by endogenous signals such as mechanical stress and signals from stressed, virally-infected or necrotic cells (Gallucci *et al.*, 1999) as was originally proposed by Matzinger's 'danger model' (Matzinger, 1994). This activation of DCs is due to components which are released by necrotic cells such as heat shock proteins, ATP and UTP which all act by triggering receptors on the DC cell surface (Coutinho-Silva *et al.*, 1999; Basu *et al.*, 2000; Schnurr *et al.*, 2000). Finally, crystalline uric acid, which is an end production of RNA and DNA degradation, induces DC maturation and enhances the generation of $CD8^+$ T cell responses *in vivo*. Removal of uric acid severely reduced the

$CD8^+$ T cell response elicited by damaged cells *in vivo*, indicating that it is the major stimulant of immunity released by necrotic cells (Shi *et al.*, 2003).

1.2.4 Induction of innate effector cells by DCs

When immature DCs encounter pathogens such as viruses they secrete cytokines at the site of infection and in this way they can activate other cells of the innate immune system such as natural killer (NK) cells (Degli-Esposti *et al.*, 2005). DCs secrete IL-12 (Reis e Sousa *et al.*, 1997), which is known to promote IFN-γ production by NK cells (Orange *et al.*, 1996a; Orange *et al.*, 1996b), and IFN-I, which is necessary for NK cell cytotoxity and contributes to but is not essential for IFN-γ production (Orange *et al.*, 1996b; Kamath *et al.*, 2005). In addition, IL-2, which is also produced by DCs, has recently been shown to be necessary for the efficient production of IFN-γ by NK cells (Granucci *et al.*, 2004). Moreover, DCs induce the expansion of NK cells, which in one model of murine viral infection was shown to be dependent on IL-12 and IL-18 (Andrews *et al.*, 2003). In addition to cytokine secretion, direct cell-cell contact has been shown to be necessary for DC induction of NK cell cytolytic activity and maximal IFN-γ production (Fernandez *et al.*, 1999; Fernandez *et al.*, 2002; Borg *et al.*, 2004; Kamath *et al.*, 2005). Notably the NK:DC interaction is reciprocal; NK cells promote the maturation of DCs and secretion of IL-12 (Gerosa *et al.*, 2002).

1.2.5 DC migration

1.2.5.1 Chemokines and their receptors

The migration of leukocytes from the bloodstream and lymphatics to peripheral tissues and lymphoid organs, and their localisation to compartments within the tissues, is regulated by chemokines, chemokine receptors and adhesion molecules. Chemokines are low molecular weight cytokines, which, in the large majority of cases, are secreted from cells and so can exert their effects in an autocrine or paracrine manner. Chemokines can be divided into four

sub-families according to the organisation of a cysteine-containing motif in their amino acid sequence and are known as the CC, CXC, C and CX_3C subfamilies. CXCL16 and CX_3C can adopt both secreted and cell bound forms, in which case they are thought to act as adhesion molecules (McColl, 2002). Chemokines act by binding to seven transmembrane domain G-protein coupled receptors (GPCRs) on the cell surface. This initiates a signaling cascade, triggering changes in cell morphology and adhesion molecule expression, culminating in their migration. Both chemokines and their receptors can be described as constitutive or inflammatory, reflecting their involvement in leukocyte trafficking under resting conditions or during an inflammatory response. Distinct patterns of chemokine receptor expression are associated with stages of DC maturation, allowing them to respond to different chemokines which direct their movement during an inflammatory response. Expression of chemokine receptors is regulated by both gene expression and desensitization (Sallusto *et al.*, 1998c). The chemokine receptors expressed by DCs are shown in Table 1.2.

Table 1.2 Chemokine receptor expression by DCs

Receptor	Ligands	Original name
CCR1	CCL3	MIP-1α
	CCL5	RANTES
	CCL7	MCP-3 (MARC)
CCR2	CCL2	MCP-1 (JE)
	CCL8	MCP-2
	CCL13	MCP-4
CCR4	CCL17	TARC
	CCL22	MDC (ABCD-1)
CCR5	CCL3	MIP-1α
	CCL4	MIP-1β
	CCL5	RANTES
CCR6	CCL20	MIP-3α/LARC/Exodus-1
CCR7	CCL19	MIP-3β/ELC/Exodus-3
	CCL21	6Ckine/SLC/Exodus-2
CCR8	CCL1	I-309
CXCR1	CXCL8	IL-8
	CXCL6	GCP-2
CXCR3	CXCL9	Mig
	CXCL10	IP-10 (Crg-2)
	CXCL11	I-TAC
CXCR4	CXCL12	SDF-1

1.2.5.2 Migration of immature DCs

Circulating DC precursors express high levels of CCR2, and are thought to be targeted to the epithelium by the ligand CCL13, produced by basal epithelial cells at the contact of blood vessels (Vanbervliet *et al.*, 2002). Likewise, the expression of CCR6 by Langerhans cells (LCs), the resident population of skin DCs, enables their migration to the epidermis in response to MIP-3α/CCL20 (Charbonnier *et al.*, 1999). In the mouse, CCR6 is necessary for DC localisation in the Peyer's patches and the maximal induction of humoral immunity in the mucosal tissues (Cook *et al.*, 2000). In addition to its role in DC homeostasis in the peripheral tissues, CCR6 also directs DCs to sites of inflammation. Although epithelial cell lines constitutively produce low levels of the CCR6 ligand CCL20, primary cultures do not, and keratinocytes produce high levels of CCL20 only after stimulation by inflammatory cytokines (Dieu-Nosjean *et al.*, 2000). Likewise, the importance of CCR2 in the recruitment of skin DC precursors into inflamed skin has been demonstrated in the mouse using $CCR2^{-/-}$ bone marrow chimeras. Recruitment of LCs into the skin in the steady-state was unaffected, indicating that other chemoattractants are involved in this process (Merad *et al.*, 2002). *In vitro*, mouse and human immature DC express receptors for CCR1, CCR2 and CCR5, and respond to their ligands which include CCL3, CCL4, CCL5, CCL7 and CCL13, which are expressed in inflamed tissues (Sozzani, ; Sozzani *et al.*, 1997; Sallusto *et al.*, 1998c; Vecchi *et al.*, 1999). The importance of CCR1 and CCR5 in recruitment of DCs to airway epithelium has been demonstrated in the rat, where administration of a CCR1 and CCR5 antagonist resulted in a reduction of DC numbers in resting airway epithelium and prevented DC influx after inhalation of heat-killed bacteria (Stumbles *et al.*, 2001). Immature DCs also express functional CXCR4, the receptor for CXCL12 which is constitutively produced in both lymphoid and non-lymphoid tissues (Sozzani *et al.*, 1997).

Some of the first molecules identified as inducers of DC migration were classical chemotactic agonists including formylated peptides and complement cleavage products (Sozzani *et al.*, 1995; McWilliam *et al.*, 1996). Formylated peptides originate from endogenous sources, such as mitochondrial proteins of ruptured cells, as well as from the proteins of invading pathogens (Le *et al.*, 2002), and so are involved in targeting DCs to sites of tissue damage or infection.

1.2.5.3 Migration of mature DCs

1.2.5.3.1 Chemokine receptor expression

At the site of infection in the peripheral tissues DCs are exposed to both microbes and inflammatory cytokines, triggering their maturation. Part of this program of maturation includes a complete switch in chemokine receptor expression, resulting in migration to the secondary lymphoid organs where they localize in the T-cell areas (Banchereau *et al.*, 2000). Firstly, DCs downregulate CCR1 and CCR5 from the cell surface, which occurs within one to two hours of initial stimulation (Sallusto *et al.*, 1998c). This rapid downregulation is not due to changes in mRNA levels which remain unaffected, but is mediated by chemokines released by the DC after exposure to maturation stimuli. These bind to their cognate receptors and trigger their internalization (Sallusto *et al.*, 1998c). Maturing DCs also lose responsiveness to formylated peptides (Sozzani *et al.*, 1998). Next, after three to four hours, DC upregulate CCR7 and acquire responsiveness to its ligands (Dieu *et al.*, 1998; Sallusto *et al.*, 1998c; Vecchi *et al.*, 1999), a feature essential for their migration to the LN. The ligands for CCR7, CCL19 and CCL21, are expressed constitutively in the high endothelial venules (HEVs) lining the draining lymphatics (Saeki *et al.*, 1999; Baekkevold *et al.*, 2001) and in the T cell zones of the lymph nodes (Willimann *et al.*, 1998; Luther *et al.*, 2000), and so can direct migration both out of the tissues and into specific regions of the LN. This is possible

because CCR7 is not susceptible to ligand-induced downregulation and its expression is maintained on mature DCs for extended periods (Sallusto *et al.*, 1999b). By *in situ* hybridisation, it was shown that the majority of CCL19 and CCL21 in the T cell zones of lymphoid organs is produced by stromal cells, with DCs contributing to the production of CCL19 (Ngo *et al.*, 1998; Luther *et al.*, 2000). The requirement for CCR7 expression for migration of activated skin DCs into the draining LN has been shown in CCR7 deficient mice (Forster *et al.*, 1999) and by injection of CCR7 deficient DCs into wild-type mice (Martin-Fontecha *et al.*, 2003). Pre-injection with DCs or with the inflammatory mediators TNF or IL-1β, known to be released by maturing DCs, increased their migration, and was due to increased expression of CCL21 by lymphatic endothelial cells (Martin-Fontecha *et al.*, 2003). In mice with defects in the production of CCL19 and CCL21, DCs fail to accumulate in the spleen and LN (Gunn *et al.*, 1999). Since CCR7 is also expressed on naïve and central memory T cells (Sallusto *et al.*, 1999a), this receptor co-ordinates the convergence of mature antigen-loaded DCs and circulating T cells.

Recently a second chemokine receptor, CCR8, has been implicated in the migration of DCs to the lymph node. In CCR8 deficient mice, recruitment of monocyte-derived DCs to the lymph nodes was reduced by fifty percent, although this was not as severe as the reduction seen in CCL19/21 deficient mice (Qu *et al.*, 2004). In addition, HCR (human chemokine receptor)/CCRL2 is an orphan chemokine receptor whose expression is induced in maturing DCs (Migeotte *et al.*, 2002). This receptor is reportedly induced in maturing DCs prior to CCR7, suggesting a role in the early phase of DC departure from the peripheral tissues (Sozzani). The role of CXCR4 in DC migration is unclear, since both immature and mature DC have been shown to respond to its ligand, CXCL12, *in vitro* (Delgado *et al.*, 1998; Vecchi *et al.*, 1999; Penna *et al.*, 2002).

1.2.5.3.2 Regulation of migration by adhesion molecules

In addition to changes in chemokine receptor expression during DC maturation, there is a loss of adhesive structures, accompanied by cytoskeleton reorganisation, which confers high motility (Winzler *et al.*, 1997). Adhesion molecule expression also changes. For example, LCs express E-cadherin, which retains them in the epidermis. On maturation E-cadherin expression is lost, allowing migration out of the skin (Tang *et al.*, 1993). Also involved in DC migration from the skin to the lymph nodes are the α6 integrins, which are involved in the initial migration from the epidermis (Price *et al.*, 1997). Although ICAM-1 expression is induced during DC maturation, it appears that ICAM-1 expressed on lymphatic endothelium is more important for DC migration into the draining LN (Xu *et al.*, 2001). DCs differentially regulate CD44 isoforms during their migration and blocking of specific CD44 isoforms prevents their migration to T cell zones and ability to induce a delayed type hypersensitivity reaction (Weiss *et al.*, 1997). In contrast, the adhesion molecule JAM-A, expressed on DCs and intercellular junctions of endothelium, appears to negatively regulate DC migration. In JAM-A deficient mice there was increased migration to the lymph nodes in a contact sensitisation assay. This effect was DC-specific, since injection of JAM-A deficient DCs into wild-type mice also resulted in enhanced contact hypersensitivity (Cera *et al.*, 2004). Similarly, *in vivo* migration of DCs is increased in SPARC (secreted protein, acidic and rich in cysteine) deficient mice, in this case as a result of secretion from the tissue environment which altered the structure of the extracellular matrix (Sangaletti *et al.*, 2005). Finally, Langerhans cells produce digestive enzymes such as metalloprotease-2 and -9, which facilitate their movement through the basement membrane and extracellular matrix (Ratzinger *et al.*, 2002).

1.2.6 DCs and the control of adaptive immunity

1.2.6.1 DC regulation of T cell responses

1.2.6.1.1 Initiation of T cell responses

A critical function of DCs is their ability to prime antigen-specific naïve T cells. It has been shown that DCs pulsed with a soluble antigen and administered to naïve mice can induce the formation of antigen responsive $CD4^+$ T cells in the draining LN (Inaba *et al.*, 1990). Importantly it was demonstrated that it was the injected DCs, and not host DCs that presented the antigen. In contrast, antigen-pulsed spleen cells, of which sixty percent are B cells, were found to be extremely inefficient in their ability to prime naïve T cells. Further evidence showing that DCs are the main antigen presenting cells (APC) *in vivo* comes from studies showing direct interactions between antigen-bearing DCs and antigen specific T cells *in situ*. In these experiments, fluorescently labeled ovalbumin was taken up by DCs either *in vitro* or *in vivo* and ovalbumin-specific T cells were found clustered around the fluorescent DCs in the LN (Ingulli *et al.*, 1997; Byersdorfer *et al.*, 2001). As well as being able to prime naive $CD4^+$ T cells DCs also appear to function in regulating their homeostasis (Brocker, 1997).

DCs have also been shown to be the essential APC capable of stimulating the proliferation of naïve $CD8^+$ T cells in the MLR (Inaba *et al.*, 1987). A number of studies have shown that DCs can generate CTL responses *in vivo*, where the injected DCs expressed viral antigens or had been pre-loaded with viral or tumour antigens (Banchereau *et al.*, 2000). Often $CD4^+$ help is required for $CD8^+$ T cell activation, although it has been shown that DCs can also generate a CTL response directly, without $CD4^+$ help (Young *et al.*, 1990). Presentation of antigens by DCs also seems to be necessary for the maintenance of CTL-mediated immunity against viruses (Ludewig *et al.*, 1999).

1.2.6.1.2 Antigen presentation

During their migration, DCs undergo a process of maturation, developing the machinery necessary for presentation of antigen to naïve T lymphocytes and their efficient activation. Naïve T lymphocytes continuously recirculate through the LNs where they can contact DCs and survey for specific antigen. As discussed earlier, DCs acquire and process antigens for presentation on class I and class II MHC molecules. During maturation DCs increase the synthesis of class II molecules and decrease their degradation, leading to rapid accumulation of MHC-peptide complexes on the cell surface (Cella *et al.*, 1997). DCs also increase the rate of synthesis of class I molecules by approximately 10 fold after exposure to a maturation stimulus (Cella *et al.*, 1999b). The level of peptide-MHC complexes available for interaction with the T cell-receptor on antigen-specific T cells, known as signal one, is a crucial factor in determining the intensity of the signal delivered to the T cell. Another factor determining the amount of signal received by the T cells is the length of contact time between the peptide-MHC and the T cell receptor (TCR) (Lanzavecchia *et al.*, 2001). This contact occurs in molecular structures known as the immunological synapse which are formed by interactions between adhesion molecules such as CD58 (LFA-3) and CD54 (ICAM-1).

1.2.6.1.3 DC co-stimulatory molecules

Mature DCs express high levels of the co-stimulatory molecules CD40, CD80 and CD86 which deliver a second signal to the T cell, an essential requirement for activation of naïve T cells (Banchereau *et al.*, 2000). DCs also express several other cell surface receptors which mediate DC-T cell interactions. These include RANK (receptor activator of NF-kappaB), whose expression on DCs can be induced by CD40 signaling (Anderson *et al.*, 1997). Like CD40L the ligand for RANK, named TRANCE (TNF-related activation-induced cytokine),

is expressed on activated T cells (Josien *et al.*, 1999) and can enhance DC survival and their ability to stimulate T cell proliferation, although unlike CD40L, it does not appear to induce CD80/86 expression (Anderson *et al.*, 1997; Wong *et al.*, 1997; Josien *et al.*, 1999). CD40, TNF receptor (TNFR) and RANK are related molecules belonging to the family of TNFRs and all signal through TRAF2 which results in the activation of NF-κB pathway (Rothe *et al.*, 1995; Wong *et al.*, 1997). NF-κB regulates the transcription of many genes involved in immune and inflammatory responses and can inhibit cell death (Baeuerle *et al.*, 1994). In fact, TRANCE has been shown to act cooperatively with CD40L and TNF-α in promoting DC survival (Josien *et al.*, 1999).

Several other TNFRs expressed by DCs are involved in T cell co-stimulation (Table 1.3) (Watts, 2005). After CD40 ligation, but not LPS stimulation, DCs upregulate OX40 ligand (OX40L) which signals activated T cells expressing OX40 and ultimately leads to accumulation of $CD4^+$ T cells in the B cell follicles (Brocker *et al.*, 1999). OX40L on DCs can contribute to T cell expansion, and expression of OX40 on T cells is associated with their long term survival *in vivo*, consistent with a role in T cell memory (Watts, 2005). Another DC cell surface molecule, 4-1BBL, can act on both $CD4^+$ and $CD8^+$ T cells, although its prominent role appears to be in directing $CD8^+$ T cell responses, since anti-4-1BB antibodies preferentially activate $CD8^+$ T cells *in vivo* (Shuford *et al.*, 1997) and 4-1BBL-deficient mice have reduced $CD8^+$ T cell proliferation and impaired CTL responses in certain viral infections (DeBenedette *et al.*, 1999; Tan *et al.*, 1999). Finally, members of the C-type lectin family of receptors appear to have dual roles in the immune system, and have been implicated in both pathogen recognition and cell-cell interactions. For instance the DC-associated lectin-1 (DCAL-1), which has a putative carbohydrate recognition domain, was

shown to act as a co-stimulatory molecule, enhancing the production of IL-4 by $CD4^+$ T cells (Ryan *et al.*, 2002).

Table 1.3 Expression of co-stimulatory molecules on DCs (Watts 05)

Receptor	Expression	Ligand	Expression
CD80	DC (enhanced on maturation), activated monocytes and B cells	CD28	T cells
CD86		CD28	
TNF/TNFR family members			
CD40	B cells, DCs, activated T cells	CD40L	Activated T and B cells
RANK	Maure DCs	TRANCE	Memory T cells
OX40L	Activated T cells, B cells and DCs	OX40	Activated T cells
4-1BBL	Activated macrophages, DCs and B cells	4-1BB	Activated T cells DC subsets, NK cells
LIGHT	Activated T cells, immature DCs	HVEM	Resting T cells monocytes and immature DCs
GITRL	B cells, macrophages and BMDC	GITR	T cells – enhanced by activation, Tregs

1.2.6.1.4 Activation of DCs by CD40 ligation

The interaction between DCs and T cells is a reciprocal one, that is, T cells also provide signals to the DCs which enhance their maturation and function. DCs express receptors which allow them to respond to T cells, the most well characterised of which being CD40. CD40 ligand (CD40L/CD154) is expressed by activated, but not resting, T cells as well as activated B cells (Quezada *et al.*, 2004). Ligation of CD40 promotes DC survival and increases their expression of MHC and co-stimulatory molecules (Caux *et al.*, 1994; Sallusto *et al.*, 1994). $CD4^+$ T cell help was originally thought to be mediated via IL-2 secretion. However this would require the close proximity of two antigen specific T cells in association with the APC, likely to be a rare event. The maturation signal provided by CD40 ligation enables the DC to stimulate $CD8^+$ killer T cells, and provides a mechanism whereby $CD4^+$ T helper cells can mediate help during CTL generation (Bennett *et al.*, 1998; Ridge *et al.*, 1998;

Schoenberger *et al.*, 1998). These studies showed that stimulation of CD40 could reconstitute CTL responses in mice lacking $CD4^+$ T cells. However the requirement for activation through CD40 was not absolute, since generation of CTL responses could also be initiated by DCs stimulated by other activation signals such as influenza infection (Ridge *et al.*, 1998). Ligation of CD40L by human monocyte-derived DCs was shown to be the most effective stimulus for induction of IL-12. LPS (*Salmonella*), TNF-α and other pathogens tested had little or no effect on IL-12 production (Cella *et al.*, 1996). In contrast to these findings Sousa *et al.* demonstrated that mouse splenic DCs can produce IL-12p40 after exposure to a *Toxoplasma gondii* antigen (STAg) *in vivo*, and that the production of IL-12 was unaffected in CD40L deficient mice (Reis e Sousa *et al.*, 1997). They subsequently showed that CD40 stimulation alone did not lead to the production of IL-12 *in vivo* and that for maximal levels of production both microbial stimulation and CD40 signaling were required. Microbial stimulation led to increased expression of CD40, suggesting that the inability of the DCs to produce IL-12 in response to a CD40 agonist was due its absence (Schulz *et al.*, 2000). Studies carried out on human monocyte-derived DCs *in vitro* have shown that both IFN-γ and CD40 ligation are required for IL-12 production, and that LPS requires a second signal from either IFN-γ or CD40 ligation for maximal IL-12 production (Hilkens *et al.*, 1997; Snijders *et al.*, 1998).

1.2.6.1.5 DC control of Th1/Th2 balance

The delivery of co-stimulatory signals allows T cells to differentiate between antigens which are associated with danger from those which are host-derived, against which it would be detrimental to mount a response. DCs can also provide T cells with information concerning the nature of the pathogen they have encountered and thereby aid in determining the Th1/Th2 polarisation of the response (Kalinski *et al.*, 1999a). The polarisation of $CD4^+$ T cells

towards a Th1 or Th2 phenotype is critical in the control of pathogens. Th1 cells are characterised by secretion of IFN-γ and are important in the cell-mediated control of viral infection, while Th2 cells produce IL-4 and are responsible for the control of extracellular parasites. Soluble factors released by the DCs are the most well characterised influencers of the Th1/Th2 balance, of which IL-12 appears to be the most important in inducing a Th1 response (Trinchieri, 2003). IL-12 produced by DCs was first shown to be necessary for development of Th1 cells *in vitro* (Macatonia *et al.*, 1995). Subsequently it was shown that *in vivo*, DCs in the spleen were the main cell type responsible for IL-12 production in response to microbial stimulation (Reis e Sousa *et al.*, 1997). IL-12 acts synergistically with CD86/CD28 interactions in the stimulation of T cell proliferation and production of IFN-γ (Kubin *et al.*, 1994; Murphy *et al.*, 1994). Further Th1-inducing molecules expressed by DCs include IFN-I (discussed in section 1.3.2), the IL-12 family members IL-23 and IL-27 and intercellular adhesion molecule 1 (ICAM-1) (Trinchieri, 2003). In addition to Th1-biasing molecules, DCs produce the chemokine CCL2 which acts on both Th1 and Th2 cells (Sallusto *et al.*, 2000), and express the co-stimulatory molecule OX40L which induces the differentiation of naive $CD4^+$ T cells into high IL-4 producing effector cells (Ohshima *et al.*, 1998).

The major contributing factor determining the Th1/Th2 polarising potential of DCs is thought to be the environment in which they are first activated, which conditions the DCs to express molecules promoting either Th1, Th2 or regulatory T cell development. As discussed previously a large array of both pathogen and host-derived factors can influence DC maturation, and these can also influence the phenotype of the mature DC. Endogenous inflammatory mediators can instruct DCs to become Th1 or Th2 cell-promoting effector DCs. IFN-γ at can augment the production of IL-12 on re-stimulation of LPS-matured DCs,

and is most effective if added at the initiation of activation (Vieira *et al.*, 2000). Conversely, the addition of prostaglandin E_2 has the opposite effect, inhibiting IL-12 production and conferring a Th2 polarising function on the DCs (Kalinski *et al.*, 1998; Vieira *et al.*, 2000). Since this study showed that the IL-12 producing capacity of the matured DCs could not be altered it appears that stimuli received by immature DCs are the most potent at inducing DC polarisation. An alternative model is that it is the DC subset which influences the Th1/Th2 balance since the DC subsets differ in their capacity to produce Th polarising cytokines (discussed in section 1.2.7.3).

Signaling through TLRs has so far mainly been shown to lead to the development of a Th1 response. For example, signaling through TLR7 or TLR9 by the ligands R-848 and CpG DNA respectively, results in production of either IL-12, IFN-α or both (Hemmi *et al.*, 2000; Krug *et al.*, 2001; Ito *et al.*, 2002). Maturation of DCs by dsRNA, which is the major ligand for TLR3, results in DCs with the capacity to polarize naive T cells towards a Th1 phenotype (Cella *et al.*, 1999b; de Jong *et al.*, 2002). Surprisingly however, this induction of a Th1 response has been shown in the absence of IL-12 production and was not significantly inhibited by anti-IL-18 or IFN-α antibodies (de Jong *et al.*, 2002). Stimulation by the TLR4 agonist, LPS, does induce IL-12 production and thereby also conditions DCs to bias towards a Th1 response (Cella *et al.*, 1999b). However the kinetics of DC activation also influence the response in this case, since after longer periods of LPS stimulation DCs lose their ability to produce IL-12 and preferentially induce Th2 polarisation (Langenkamp *et al.*, 2000). In addition, the amount of antigen presented by DCs may influence the outcome, since stimulation in the presence of high doses of antigen preferentially induces a Th1, and lower doses a Th2 response (Boonstra *et al.*, 2003). Finally, the dose and source of LPS have been

demonstrated to have an effect on the Th1/Th2 bias (Pulendran *et al.*, 2001; Eisenbarth *et al.*, 2002).

Several pathogen components induce Th2 cell-promoting effector DCs, including ES-62 glycoprotein (*Acanthocheilonema viteae*), soluble egg antigens (*Schistosoma mansoni*), and cholera toxin (Gagliardi *et al.*, 2000; Whelan *et al.*, 2000; de Jong *et al.*, 2002), although it is still unclear which PRRs mediate this effect. Finally, pathogens can initiate the development of T regulatory cells, which could be beneficial to both the pathogen and the host, in preventing chronic inflammation (Kapsenberg, 2003).

Another important concept in the initiation of Th1 and Th2 responses by DCs is the apparent ability of different subsets of DCs to preferentially induce one or other of the responses. This will be discussed in section 1.2.7.3.1.

1.2.6.1.6 DC induction of T cell tolerance

As well as their fundamental role in inducing immunity to pathogens, DCs can also induce tolerance to self and to non-infectious commensals. The original concept of tolerance induction was that in the absence of inflammation immature DCs presenting antigen to T cells would not deliver the necessary co-stimulatory signals, resulting in the generation of anergic T cells. Mature DCs, on the other hand, would be able to deliver the co-stimulatory "signal 2" and induce T cell proliferation and differentiation (Gad *et al.*, 2003). However this model does not explain how DCs would make contact with the T cells, since immature DCs require an activation signal to migrate from the peripheral tissues to the LN (Banchereau *et al.*, 2000). It has been shown that mice exposed to ovalbumin in the absence of infection are able to present ovalbumin in the draining LN, leading to tolerance (Brimnes *et al.*, 2003).

Since the ovalbumin was transported to the LN in levels comparable to that in virally infected mice, a system must exist enabling the trafficking of DCs to the LN in the steady state. Mature DCs can induce $CD4^+$ T cell tolerance (Gad *et al.*, 2003), further illustrating that tolerance induction by DCs is not restricted to immature DCs.

It is likely that cells in the local environment can deliver a tolerizing signal to DCs. For example, keratinocytes in the skin produce TGF-β which can prevent LC maturation. In addition, immature LCs could be found in the LN suggesting that full maturation is not necessary for their migration (Geissmann *et al.*, 1999). Production of IL-10 in the Peyer's patch and respiratory tract may skew the T cell response towards a Th2 response, thereby limiting an inflammatory T cell response (Iwasaki *et al.*, 1999). There also appears to exist a population of DCs which are loaded with tissue antigens and can migrate to the LN under steady state conditions (Huang *et al.*, 2000; Hemmi *et al.*, 2001). Although these DCs express co-stimulatory molecules at a level similar to mature DCs, they are unlikely to produce IL-12 or other pro-inflammatory cytokines. It is possible that self-antigens transported to the LN in this way can be delivered to tolerizing DCs within the LN (Carbone *et al.*, 2004). Certain maturation stimuli can induce tolerogenic DCs which upregulate MHC class II and co-stimulatory molecules but lack production of inflammatory cytokines. These include lactobacilli from gut flora, apoptotic cells and TNF-α (Gad *et al.*, 2003).

DCs can induce tolerance by a number of mechanisms. Firstly, they can induce the apoptosis of T cells via ligation of Fas ligand or TRAIL (TNF-related apoptosis inducing ligand) (Suss *et al.*, 1996; Fanger *et al.*, 1999). Secondly, DCs can induce T cell anergy, likely as a result of their incomplete maturation or by negative regulation, for example through CTLA-4 (cytotoxic T-lymphocyte-associated protein 4) and PD-1 (programmed cell

death 1) (Perez *et al.*, 1997; Latchman *et al.*, 2001). These anergic T cells show a lack of antigen-specific proliferation and reduced production of IL-2 and IFN-γ (Schwartz, 2003). Finally, DCs may be able to induce the differentiation of regulatory T cells (Tregs) (Smits *et al.*, 2005). These T cells are also anergic and fail to secrete IL-2 on TCR ligation . Tregs can suppress both $CD4^+$ and $CD8^+$ T cell responses by an antigen specific mechanism, probably explained by their ability to inhibit DC function and mediate anti-proliferative effects on T cells. The induction of tolerogenic DCs is linked to the activation of NF-κB. TLR and CD40 ligation result in sustained NF-κB activation, whilst culturing with Tregs does not (Chang *et al.*, 2002).

To demonstrate the capability of DCs to induce tolerance in the steady state, Steinman *et al.*, used a system where antigen could be targeted to DCs *in vivo* via the endocytic receptor DEC-205 (Hawiger *et al.*, 2001). Adoptively transferred TCR transgenic $CD4^+$ and $CD8^+$ T cells went through an initial stage of proliferation which was followed by their deletion. The remaining T cells were unresponsive to subsequent challenge (Hawiger *et al.*, 2001; Bonifaz *et al.*, 2002). Finally, Probst et al. used the cre/lox system where expression and presentation of a lymphochoriomeningitis virus (LCMV)-derived CTL epitope was induced in $CD11c^+$ DCs by injection of tamoxifen. Presentation of the epitope by resting DCs resulted in tolerance which could not be broken by subsequent viral infection (Probst *et al.*, 2003).

1.2.6.2 DC regulation of B cell responses

DCs are also important regulators of B cell responses. The critical role of DCs in initiating T cell-dependent antibody responses has been demonstrated *in vitro* and *in vivo* (Inaba *et al.*, 1983; Sornasse *et al.*, 1992). DCs have been shown to enhance the proliferation and Ig

production of CD40L-activated B cells as well as inducing an IgA isotype switch (Dubois *et al.*, 1997; Fayette *et al.*, 1997). These studies suggested that DCs could act directly on B cells and that this effect was mediated by soluble factors, in part by TGF-β. IL-12 was found to be an essential DC-derived factor involved in the differentiation of naive B cells into IgM plasma cells, and synergised with IL-6/soluble IL-6Rα in driving naive B cell proliferation (Dubois *et al.*, 1998). IL-2 is also known to augment B cell proliferation *in vitro* (Arpin *et al.*, 1995).

Recently the involvement of two DC secreted factors in T cell independent antibody responses has come to light. These are known as BAFF (B cell activating factor of the TNF family) and APRIL (a proliferation-inducing ligand), which bind to three receptors expressed on B cells and activate a CD40-like pathway (Litinskiy *et al.*, 2002). BAFF binds to BAFF-R, TACI (transmembrane activator and CAML interactor) and with lower affinity to BCMA (B cell maturation antigen), whilst APRIL binds with high affinity to BCMA and lower affinity to TACI but not to BAFF-R (Schneider, 2005). BAFF plays an essential role in B cell survival (Schneider, 2005). Whilst the presence of APRIL does not appear to be critical for B cell survival, it does support the survival of plasma cells (O'Connor *et al.*, 2004). BAFF-R knockout mice, like BAFF-deficient mice, display a dramatically reduced population of mature B cells, although they are capable of T cell independent antibody responses (Shulga-Morskaya *et al.*, 2004). In contrast, TACI knockout mice have increased splenic B cell numbers while antibody responses to T cell independent antigens were almost completely abolished (von Bulow *et al.*, 2001). Both BAFF and APRIL have been shown to induce isotype switching in a CD40-independent manner, and APRIL seems to be especially important in mediating IgA class switching (Litinskiy *et al.*, 2002; Castigli *et al.*, 2004). Therefore signaling through the BAFF and APRIL receptors initiates distinct components of

B cell function. BAFF has also been identified as a T cell co-stimulatory factor, inducing IFN-γ and IL-2 secretion from responding T cells and increasing their proliferation (Huard *et al.*, 2001; Huard *et al.*, 2004).

CD38, which is expressed on both T and B cells, is important in modulating T cell dependent antibody responses (Cockayne *et al.*, 1998), and recently a novel ligand (CD38L) was identified on DCs (Wykes *et al.*, 2004). Stimulation with CD38 enhanced maturation *in vitro* and led to diminished IgG2a responses *in vivo*.

Activated B cells expand and differentiate into plasma cells and memory B cells in a microenvironment known as the germinal centre (GC). It is in the GC where mature B cells undergo Ig gene somatic hypermutation, which increases the antibody affinity for antigen, and Ig heavy chain class switching, which modulates the antibody effector functions by substituting the constant region of IgM with that of IgG, IgA or IgE. A distinct set of DCs, follicular DCs (FDCs) are responsible for sustaining activated B cell viability, growth and differentiation in the germinal centres (Park *et al.*, 2005). These cells appear to belong to a different cell lineage from the DCs typically associated with the initiation of T cell responses since they are not derived from bone marrow progenitors and have a unique pattern of cell surface receptors (Banchereau *et al.*, 1998). FDCs do not internalise and process antigen. Instead they retain antigens on the cell surface bound to Fc and complement receptors (Park *et al.*, 2005). Two cytokines produced in the germinal centres by DCs and T cells, IL-15 and IL-2 respectively, augment B cell proliferation and antibody production (Armitage *et al.*, 1995; Arpin *et al.*, 1995). IL-15, along with B cell receptor ligation, is necessary for class switching induced by BAFF/APRIL (Litinskiy *et al.*, 2002) and acts in a cell bound form since it can be detected in a biologically active form on the cell surface of FDCs where it is

likely to be captured by IL-15Rα (Park *et al.*, 2004a). Also produced by FDCs, the molecule 8D6 appears to be important for plasma cell formation but not memory B cell expansion (Zhang *et al.*, 2001). Another population of DCs present in the GC, termed the germinal centre DC (GCDC), has been identified and are clearly distinct from FDCs (Grouard *et al.*, 1996). GCDCs can induce B cell proliferation and induce isotype switching of naive B cells preferentially towards IgG1 (Dubois *et al.*, 1999).

1.2.6.3 Expression of chemokines by DCs

As well as responding to chemokines, DCs are capable of producing many chemokines which assist in the migration of leukocytes to the site of infection and to the secondary lymphoid organs. Several studies on human *in vitro* derived DCs have characterised the expression of chemokines during different stages of maturation. Activated DCs transiently express several inflammatory chemokines; CCL3 (MIP-1α), CCL4 (MIP-1β) and IL-8 are rapidly produced at high levels after DC activation but only remain for a few hours, whilst CCL5 (RANTES) is expressed in a more sustained fashion (Sallusto *et al.*, 1999b). Similar results were seen in mouse LC, where CCL3 and CCL4 levels were elevated 6h after stimulation, whereas CCL5 production increased dramatically after 48h (Fujita *et al.*, 2004). In addition, maturation of mouse BM-derived DCs resulted in the upregulation of mRNA encoding CCL2, CCL3, CCL4, CCL5, CXCL1 and CXCL2 (Chen *et al.*, 2002).

The chemokines CCL17 (TARC), CCL18 (PARC/DC-CK1) and CCL19 (ELC) are expressed by immature DCs and are upregulated by maturation stimuli, but only at later time points (Sallusto *et al.*, 1999b; Vissers *et al.*, 2001). This indicates that these chemokines are important under resting conditions in attracting cells to peripheral tissues, and also at later time points during an inflammatory response, by which time the DCs are likely to have

migrated to the LN. In addition, CCL19 but not CCL18 expression was further enhanced by CD40 ligation (Vissers *et al.*, 2001), consistent with its role for maximising DC-T cell interactions. More recently, CCL20 was shown to be induced during DC maturation whereas expression of CCL18, a chemokine active on naive T and B cells and immature DCs, was suppressed (Adema *et al.*, 1997; Vulcano *et al.*, 2003). CCL22 (MDC) has also been shown to be induced by maturation stimuli in both humans and mice (Sallusto *et al.*, 1999b), although a later study reported constant levels of CCL22 during maturation (Vissers *et al.*, 2001). Mice do not express the same repertoire of chemokines as humans. For example, there appears to be no functional IL-8 or CCL18 in mice (Rollins, 1997).

CXCL9 (MIG), CXCL10 (IP-10) and CXCL11 (I-TAC), which all act on the receptor CXCR3, are produced by DCs and are induced by stimulation with IFN-γ or LPS (Meyer *et al.*, 2001). These chemokines assist in the attraction of resting memory and activated T cells and their retention in secondary lymphoid organs (Cole *et al.*, 1998; Rabin *et al.*, 1999; Yoneyama *et al.*, 2002). CXCL9 has also been shown to attract B cells and so would act to maximise interactions between DCs, T and B cells (Park *et al.*, 2002). Therefore the expression of chemokines is also regulated during DC maturation. This makes physiological sense, since on initial DC activation release of inflammatory cytokines would mediate the recruitment of monocytes and immature DCs into the site of infection. The delay in expression of chemokines such as CCL19 would allow for the time taken to migrate to draining LN where they would then have the capacity to attract T cells.

DCs can also influence the homing patterns of T cells. For example, stimulation of T cells with DCs from gut-associated lymphoid tissues imprints an ability to migrate back to the gut. This was recently shown to be mediated by the vitamin A metabolite, retinoic acid, which is

produced by mesenteric LN and Peyer's patch DCs and enhances expression of the gut-homing receptors α4β7 and CCR9 by responding T cells (Iwata *et al.*, 2004).

1.2.7 DC subsets

1.2.7.1 Murine DC subsets and their origins

Murine lymphoid tissue resident DCs can be segregated into five subsets depending on expression of CD4, CD8, CD11b (the integrin α_M chain of Mac-1) and CD205 (DEC205, originally NLDC-145). Three of these subsets are present in the spleen, with an additional two DC subsets existing in the LN (Shortman *et al.*, 2002)(Table 1.4). These additional LN DC subsets are the $CD4^-CD8\alpha^-CD205^+CD11b^+$ DCs which are found in all LNs and are thought to be a mature form of tissue interstitial DCs, while the $CD4^-CD8\alpha^{lo}CD205^{hi}CD11b^+$ DCs are only found in the skin-draining LN and express langerin, a characteristic LC marker (Henri *et al.*, 2001). These DCs also express high levels of MHC II, CD40, CD80 and CD86, and therefore it is likely that these are mature LCs which have arrived at the LN after activation at the periphery. The majority of work examining DC subset function has been on DCs divided on the basis of CD8α expression only, and so will be referred to simply as the $CD8^+$ and $CD8^-$ subsets.

Table 1.4 DC subsets (adapted from Anjuere et al., 1999; Shortman et al., 2002)

	$CD4^-CD8\alpha^{hi}$ $CD205^{hi}$ $CD11b^-$	$CD4^+CD8\alpha^-$ $CD205^-$ $CD11b^+$	$CD4^-CD8\alpha^-$ $CD205^-$ $CD11b^+$	$CD4^-CD8\alpha^-$ $CD205^+$ $CD11b^+$	$CD4^-CD8\alpha^{lo}$ $CD205^{hi}$ $CD11b^+$
% total DCs in:					
Spleen	23	56	19	<4	<1
Thymus	70				
Mesenteric LN	19	4	37	26	<4
Skin-draining LN	17	4	17	20	33
Peyer's Patch	70			10	20

DCs were originally thought to be derived from myeloid precursors, and it was shown that human DCs could be derived *in vitro* from monocytes (Sallusto *et al.*, 1994). However, DC

development is more complex than this, since it has also been shown that DCs can develop from $CD4^{lo}$ thymic precursors which are devoid of myeloid reconstitution potential, leading to the idea that some DCs could be of lymphoid origin (Ardavin *et al.*, 1993). One of the questions concerning DC subsets was whether they represent different stages of a single developmental lineage or whether they are derived from entirely separate lineages. After showing that thymic precursors only developed into $CD8^+$ DCs (Wu *et al.*, 1996), it was thought that the $CD8^+$ DC subset was derived from lymphoid-restricted precursors and the $CD8^-$ DCs from myeloid-restricted precursors. However, it is now clear that both $CD8^-$ and $CD8^+$ DCs can be produced from either lymphoid or myeloid-restricted precursors (Martin *et al.*, 2000; Traver *et al.*, 2000). Nevertheless, there is some evidence from studies on knockout mice that the DC subsets are products of separate developmental pathways. In mice lacking the transcription factors RelB (a subunit of NF-κB) or PU.1, there is a defect in the differentiation of $CD8^-$ DCs but normal $CD8^+$ DC development (Wu *et al.*, 1998; Guerriero *et al.*, 2000). Conversely, in mice deficient in the IFN consensus sequence binding protein (ICSBP) or Id2, differentiation of the $CD8^+$ DCs is affected while maturation of both $CD8^-$ and $CD8^+$ DCs is defective (Schiavoni *et al.*, 2002; Aliberti *et al.*, 2003; Hacker *et al.*, 2003). Therefore it appears that RelB and PU.1 play a role in development of $CD8^-$ DCs while ICSBP and Id2 are important for $CD8^+$ DC development. Although $CD8^+$ DCs were suggested to originate from $CD8^-$ DCs in experiments where $CD8^-$ DCs injected into mice appeared to differentiate into CD8 expressing DCs (del Hoyo *et al.*, 2002), this was subsequently ruled out in subsequent experiments by Naik *et al.* (Naik *et al.*, 2003).

In 1999 the plasmacytoid DCs (pDCs) of human peripheral blood were definitively characterised as the main producers of type I IFNs after microbial stimulation (Cella *et al.*, 1999a; Siegal *et al.*, 1999). Mouse pDCs were subsequently identified, and share the

phenotype and functional characteristics of human pDCs (Asselin-Paturel *et al.*, 2001; Bjorck, 2001; Nakano *et al.*, 2001). However, unlike human pDCs they express B220, Ly6C and intermediate levels of CD11c. Freshly isolated pDCs are poor inducers of T cell proliferation, which can be explained by their low expression of MHC class II and co-stimulatory molecules (Grouard *et al.*, 1997; Asselin-Paturel *et al.*, 2001). On activation, pDCs upregulate MHC class II and co-stimulatory molecule expression, and are able to stimulate $CD4^+$ and $CD8^+$ T cell proliferation, although not as efficiently as $CD11c^+$ DCs (Grouard *et al.*, 1996; Cella *et al.*, 2000; Asselin-Paturel *et al.*, 2001; Fonteneau *et al.*, 2003). pDCs were initially thought to be inducers of Th2 differentiation, however this is not consistent with their IFN-I producing ability, and they are now known to be potent Th1 inducers (Cella *et al.*, 2000; Boonstra *et al.*, 2003; Krug *et al.*, 2003).

1.2.7.2 DC subset localization

DC subsets reside in distinct anatomical locations within the lymphoid organs. In the spleen, $CD8^+$ DCs are concentrated in the T cell-rich periarteriolar lymphatic sheaths (PALS), whereas the $CD8^-$ DCs are found mainly in the marginal zones but can migrate to the PALS after stimulation by microbial products, for example LPS (De Smedt *et al.*, 1996) or a *Toxoplasma gondii* extract (STag)(Reis e Sousa *et al.*, 1997). Corresponding subsets exist in the Peyer's patch. Again, the $CD8^+$ subset is localised in the T-cell rich areas and the $CD8^-$ subset in the subepithelial dome where they would be positioned for antigen uptake (Kelsall *et al.*, 1996). As seen in the spleen $CD8^-$ DCs have been shown to migrate into the T-cell rich areas of the Peyer's patch in response to microbial stimuli (Iwasaki *et al.*, 2000). The subsets also have different trafficking properties *in vivo*; when injected into the skin of mice, the $CD8^+$ subset is not able to migrate to the LN, whilst the $CD8^-$ subset can (Ruedl *et al.*, 1999; Smith *et al.*, 1999). Similarly, Colvin *et al.* demonstrated that although both DC

subsets, when matured overnight in the presence of GM-CSF, are detected in the draining LN 24 hours after sub-cutaneous injection, the $CD8^-$ DCs are found in much lower numbers and disappear at an earlier time point (Colvin *et al.*, 2004b). The reason for this inefficient migration may be that the $CD8^+$ DC subset is normally resident in the lymphoid tissues and absent from peripheral tissues, since several studies implicate the existence of a system where antigen is transferred from migratory $CD8^-$ DCs arriving from peripheral tissues, to $CD8^+$ DCs in the LN for presentation to T cells (Vermaelen *et al.*, 2000; Scheinecker *et al.*, 2002; Allan *et al.*, 2003).

1.2.7.3 Subset specialisation

1.2.7.3.1 Induction of T helper responses

Both the $CD8^+$ and $CD8^-$ DC subsets share the ability to prime $CD4^+$ and $CD8^+$ T cell responses *in vivo* (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999; Ruedl *et al.*, 1999; Smith *et al.*, 1999). However it appears that the $CD8^+$ DC subset preferentially induces CTL responses, while the $CD8^-$ subset preferentially induces $CD4^+$ T cell responses (Yoneyama *et al.*, 2005). As discussed earlier, DCs can adopt a Th1 or Th2 polarizing function depending on environmental factors encountered during their immature stage. This was further complicated by observations suggesting that the DC subsets can differentially regulate Th1 and Th2 development. The DC subsets appear to differ in their ability to respond to pathogen components and in their subsequent cytokine production, thereby promoting different types of responses. Pulendran *et al.* made use of Flt-3 ligand-treated mice, in which numbers of both $CD8^+$ and $CD8^-$ subsets are substantially increased. Injection of the $CD11b^+$ subset (which corresponds to the $CD8^-$ subset) resulted in T cells which produced higher levels of Th2 cytokines than after injection of the $CD11b^-$ ($CD8^+$) subset, whilst levels of IFN-γ and IL-2 induced by the two subsets were similar (Pulendran *et al.*, 1999). In a

similar set of experiments using normal mice, antigen pulsed $CD8^-$ DCs promoted the production of IL-4, IL-5 and IL-10 while $CD8^+$ DCs promoted the production of IL-2 and IFN-γ (Maldonado-Lopez *et al.*, 1999). The ability to promote Th1 cytokine production was attributed to IL-12 p70 production, which has been shown to be produced mainly by the $CD8^+$ DC subset (Reis e Sousa *et al.*, 1997; Maldonado-Lopez *et al.*, 1999). By injecting $CD8^+$ DCs from IL-12 deficient mice, it was shown that the production of IFN-γ was severely diminished. Likewise, $CD8^-$ DCs from IL-10 deficient mice have a reduced capacity to elicit a Th2 response (Maldonado-Lopez *et al.*, 1999; Maldonado-Lopez *et al.*, 2001).

1.2.7.3.2 Responses to TLR agonists

As discussed earlier, DCs express TLRs which recognize specific conserved motifs on pathogens, and stimulation through TLRs can influence the type of response elicited. Although DCs do exhibit a considerable amount of plasticity, there does appear to be restraints on this flexibility. Stimulation with zymosan inhibits the production of IFN-γ from responding T cells by both DC subsets, although IL-4 production was only induced by the $CD8^-$ subset. In contrast, stimulation with STag induced a dramatic Th1 profile by $CD8^+$ DCs but not by $CD8^-$ DCs (Manickasingham *et al.*, 2003). Again, this can be explained by the preferential induction of IL-12 by the $CD8^+$ subset in response to STag (Reis e Sousa *et al.*, 1997; Aliberti *et al.*, 2000; Schulz *et al.*, 2000). In addition, IL-12 has been shown to be preferentially expressed by the $CD8^+$ subset in response to *E.coli* LPS, whilst IL-10 is preferentially produced by the $CD8^-$ subset in response to Pam-3-Cys (a TLR2 stimulus) (Pulendran *et al.*, 2001; Dillon *et al.*, 2004). This responsiveness can not clearly be attributed to TLR2 and TLR4 expression (Table 1.5 (Boonstra *et al.*, 2003; Edwards *et al.*, 2003b)). In addition, both *E.coli* LPS and Pam-3-Cys induced expression of co-stimulatory

molecules on both DC subsets, indicating that although both subsets can respond to these ligands they differ in the intracellular signaling pathways which regulate cytokine production. These results are consistent with the expression of TLR4 on both subsets, which has been demonstrated by semi-quantitative PCR (Edwards *et al.*, 2003b). However in a separate study, TLR4 was only found to be expressed on $CD11b^+$ BM-derived DC (BM-DC), and only these DCs produced IL-12 in response to LPS (Boonstra *et al.*, 2003). In contrast to $CD8^-$ DCs, $CD8^+$ DCs do not express TLR5 or TLR7 and fail to produce IL-12 or upregulate co-stimulatory molecules in response to TLR7 agonists (Edwards *et al.*, 2003b).

Human DC subsets also express different combinations of TLRs; myeloid DCs express TLR4 but not TLR9 and correspondingly they respond to LPS but not CpG by expressing IFN-α. This is in contrast to pDCs which express TLR9 and produce IFN-α in response to CpG (Jarrossay *et al.*, 2001; Kadowaki *et al.*, 2001). Interestingly, two different forms of CpG induced the production of different cytokines from pDCs; CpG 2006 induced IL-12 whereas CpG 2216 induced IFN-α production, although both induced upregulation of CCR7 (Jarrossay *et al.*, 2001). Therefore it appears that different ligands for the same TLR activate different signaling pathways and consequently induce different responses. Overall, it seems that several mechanisms can determine the response of DCs to pathogens, including the nature of the pathogen itself and the expression of TLRs by the DC subsets. Although DC subsets appear to have an intrinsic bias in the type of response they initiate, they also retain a degree of functional plasticity and can respond rapidly to environmental signals. In this way, an appropriate immune response can be mounted.

Table 1.5 Expression of TLRs on DC subsets (adapted from Iwasaki et al., 2004)				
	$CD8^-CD4^+$	$CD8^+CD4^-$	DN	pDC
TLR1	++	++	++	++
TLR2	++	++	++	++
TLR3	-	++	++	-
TLR4	++/-	++/-	++/-	++/-
TLR5	++	-	++	+
TLR6	++	++	++	+
TLR7	++	-	++	++
TLR8	++	++	++	++
TLR9	++	++	++	++

1.2.7.3.3 CTL responses, cross-priming and cross-tolerance

$CD8^+$ DCs have been shown to be the principal DC subset involved in priming MHC class I-restricted CTL immunity against a number of cytolytic viruses including herpes simplex virus-1 (Smith *et al.*, 2003), as well as a non-cytolytic virus (LCMV), and an intracellular bacterium (Listeria monocytogenes) (Belz *et al.*, 2005). The $CD8^+$ and $CD8^-$ DC subsets also differ in their ability to cross-present exogenous antigen in association with MHC class I (den Haan *et al.*, 2000). Although cross-presentation was originally thought to be restricted to the $CD8^+$ subset, it was subsequently shown that the $CD8^-$ subset could cross-present an ovalbumin-immune complex, although this was dependent on their activation via the Fcγ receptor (den Haan *et al.*, 2002). The efficiency of the $CD8^+$ DCs cross-presenting capacity is enabled by their ability to capture apoptotic cells (Iyoda *et al.*, 2002; Schulz *et al.*, 2002)

Several studies have highlighted the importance of $CD8^+$ DCs in tolerance. Work by Suss *et al.* showed that $CD8^+$ DCs induced a weak $CD4^+$ T cell response compared to $CD8^-$ DCs, which was associated with T cell apoptosis (Suss *et al.*, 1996). $CD8^+$ DCs also prevented proliferation of $CD8^+$ T cells by inhibiting their IL-2 production (Kronin *et al.*, 1996). The ability of $CD8^+$ DCs to capture cell-associated antigens (Iyoda *et al.*, 2002), and to induce the deletion of reactive $CD8^+$ T cells (Belz *et al.*, 2002) is consistent with a capacity for inducing

cross-tolerance. The decision by $CD8^+$ DCs to induce T cell immunity or tolerance is dictated by the licensing signal delivered by $CD4^+$ T helpers or microbial stimuli (Bennett *et al.*, 1997).

1.3 Type I Interferons

1.3.1 IFN-I subtypes

Interferon (IFN) was first identified more than 50 years ago as an "inhibitory factor" of viral replication (Isaacs *et al.*, 1957). IFNs are split into the acid-resistant type I IFNs and the acid-labile type II IFNs (now known as IFN-γ). The type I IFNs (IFN-I) belong to a family of closely related cytokines and consist of the products of numerous IFN-α genes, a single IFN-β gene (in mice and humans) and several other genes that are expressed variably in different species (IFN-ω, -τ, -κ, -ε, -δ and limitin) (De Maeyer *et al.*, 1998). In mice, 14 IFN-α genes have been identified (van Pesch *et al.*, 2004), in addition to IFN-κ (Vassileva *et al.*, 2003), limitin (Oritani *et al.*, 2001) and IFN- ε1 (Hardy *et al.*, 2004) and are clustered on chromosome 4.

All of the type I IFNs tested act on a common cell surface receptor, the IFN-I receptor (IFN-IR) (Uze *et al.*, 1995; Oritani *et al.*, 2001), which has brought into question whether they are able to mediate distinct biological effects. Varying anti-viral activities of human IFN-I subtypes have been reported (Foster *et al.*, 1996) and recently the murine IFN-α subtypes were found to vary widely in their anti-viral and anti-proliferative effects. IFN-α11, -α12 and -β were 9-18 fold more active as anti-viral factors, and 44-197 more effective as anti-proliferative factors than IFN-α1 (van Pesch *et al.*, 2004). The correlation between anti-

viral and anti-proliferative effects suggests a lack of functional specialisation for the subsets at least in this respect. Studies using global gene expression analysis have identified additional genes induced by IFN-β compared to IFN-α2. Again, however, these differences could be due to the specific activity of the IFNs, i.e. quantitative rather than qualitative differences (Der *et al.*, 1998; da Silva *et al.*, 2002). There is evidence that IFN subtypes differ in their ability to activate other cells of the immune system, since human IFN-α7 has anti-viral activity but is unable to activate NK cells (Ortaldo *et al.*, 1984). In addition, human IFN-α8 was shown to induce B cell proliferation at concentrations up to 100-fold lower than other IFN subtypes. IFN-α8 appeared to be able to activate proliferation via a distinct pathway from that leading to transcription of IFN stimulated genes (ISGs), since no difference in subtype induction of ISGs was detected (Hibbert *et al.*, 1999). Similarly CXCL10 expression by DCs is differentially regulated by IFN-α subtypes, since even at 10-fold lower concentrations, IFN-α2 induced both CXCL10 mRNA and protein to significantly higher levels than IFN-α1, in contrast to other ISGs which were induced to comparable levels (Hilkens *et al.*, 2003). Recently, the migration of T cells was shown to be induced by IFN-α2 but not IFN-α8. This was reflected in the regulation of ISGs, since certain ISGs were induced by IFN-α2 but not IFN-α8 (Foster *et al.*, 2004).

1.3.2 IFN-I expression

1.3.2.1 The IFN-I producing cells

Although many cell types can produce IFN-I in response to viral infection, a type of cell originally known as the "natural IFN-producing cell" is considered to be the major source of IFN-I. As discussed in section 1.2.7.1 the plasmacytoid DCs (pDCs) are the natural IFN-producing cells which have been identified in both human and mouse (Siegal *et al.*, 1999;

Asselin-Paturel *et al.*, 2001). In humans, pDCs produced 200 –1000 times more IFN-I than peripheral blood mononuclear cells after challenge with UV-inactivated herpes simplex virus (Siegal *et al.*, 1999). Similarly, murine pDCs were shown to be the main source of IFN-I, since *in vivo* depletion of cells expressing the pDC marker Ly6G/C resulted in the dramatic inhibition of IFN-α production from spleen cells stimulated with influenza and in the serum of cytomegalovirus-infected mice (Asselin-Paturel *et al.*, 2001).

pDCs express a number of TLRs which allow them to sense pathogens and initiate the production of IFN-I (Table 1.5), including TLR7 and TLR9 which account for their ability to respond to ssRNA viruses and bacterial DNA (Diebold *et al.*, 2004; Heil *et al.*, 2004; Iwasaki *et al.*, 2004; Lund *et al.*, 2004). After activation by CD40 or virus infection pDCs develop into cells with the properties of mature DCs; they develop long dendrites, lose their phagocytic capacity, express mature DC cell surface markers and have increased T cell stimulatory activity (Grouard *et al.*, 1997; Cella *et al.*, 2000; Asselin-Paturel *et al.*, 2001). CpG also activates pDCs; stimulation with CpG 2216 induces high levels of IFN-α but has minimal effect on MHC II and co-stimulatory molecule expression, whilst for CpG 2006 the opposite is true (Kerkmann *et al.*, 2003). $CD11c^+$ DCs are also able to produce IFN-I in response to viruses, but the levels of IFN-I produced were initially shown to be considerably lower than that from pDCs (Cella *et al.*, 1999a; Kadowaki *et al.*, 2000; Asselin-Paturel *et al.*, 2001). However, during LCMV infection of mice, IFN-I production is not dependent on pDCs (Dalod *et al.*, 2002), and in fact $CD11c^+$ DCs can produce high levels of IFN-α in response to an LCMV strain which infects the $CD11c^+$ DCs themselves (Diebold *et al.*, 2003). Indeed classical $CD11c^+$ DCs could produce IFN-I to the same magnitude as pDCs when poly(I:C) was delivered intracellularly.

1.3.2.2 Induction of IFN-I expression

One pathway of IFN-I induction is through TLRs, as discussed in section 1.2.3.1.2. TLRs are known to signal through both MyD88-dependent and independent pathways (Figure 1.2). TLR3 and TLR4 utilise an alternative pathway which is dependent on the adaptor molecule TRIF, and blocking its function using a dominant negative TRIF inhibits the TLR3 dependent activation of the IFN-β promoter (Yamamoto *et al.*, 2002b). TRIF is essential for the activation of IFN regulatory factor 3 (IRF-3), a key transcription factor in regulating IFN-I expression (Takeda *et al.*, 2005) and this activation is now known to be mediated by two kinases, IκB kinase ε (IKKε) and TANK binding kinase 1 (TBK1). IKKε and TBK1 were shown to be essential for the phosphorylation of IRF3, and the equally important IFN-gene transcription factor, IRF7 (Fitzgerald *et al.*, 2003; Sharma *et al.*, 2003).

In addition, dsRNA can induce IFN-I through a TLR3-independent mechanism; in the case of BM-derived $CD11c^+$ DCs, PKR appears to be more important for IFN-I induction (Diebold *et al.*, 2003). Cellular lectin receptors, especially the mannose receptor are also able to induce IFN-I expression (Milone *et al.*, 1998), for which the activating ligands are likely to be viral glycoproteins present on the surface of enveloped viruses (Malmgaard, 2004). Finally, it should be noted that signals from the host can also induce IFN-I production: CD40L on activated T cells interacts with CD40 on DCs inducing them to produce high levels of IFN-I (Foster *et al.*, 2000).

These observations therefore provide a mechanism for induction of IFN-I by TLR3 or TLR4 ligation. However other TLR ligands such as those binding TLR7 and TLR9 which do not utilise the TRIF pathway are capable of inducing IFN-I expression. Indeed there is no evidence for a TRIF pathway of IFN-I induction in pDCs, and induction of IFN-α through TLR7 and TLR9 occurs through an MyD88-dependent pathway (Colonna *et al.*, 2004).

Recently, IRF7 was shown to interact with MyD88 and tumour necrosis factor receptor-associated factor 6 (TRAF6). Furthermore IRF7 activation and translocation to the nucleus by TLR9 ligand was dependent on both MyD88 and TRAF6 (Kawai *et al.*, 2004). Unlike most cells, which inducibly express IRF7 after IFN-I stimulation, pDCs constitutively express IRF7 (Izaguirre *et al.*, 2003), and so this mechanism may enable pDCs to rapidly produce IFN-α in response to microbes. In agreement with this, it has recently been shown that pDCs from IRF7 knockout mice are unable to produce IFN-I and that this results in impaired $CD8^+$ T cell responses when CpG was used as an adjuvant (Honda *et al.*, 2005). In addition, IRF5 appears to be a critical mediator of IFN-I induction by TLR7 signaling, and its activation is also dependent on MyD88 and TRAF6 (Schoenemeyer *et al.*, 2005).

IFN regulatory factors (IRFs) are a family transcription factors which regulate the transcription of both the IFN genes and IFN-stimulated genes. Nine mammalian IRFs have so far been identified, of which two closely related IRFs, IRF3 and IRF7, play a key role in IFN-I gene induction (Taniguchi *et al.*, 2001). Their importance in IFN-I induction has been demonstrated in knock-out mice: in IRF3 deficient mice virally induced IFN-I is severely disrupted, whilst in IRF3 and IRF9 double knockouts, in which IRF7 cannot be induced, IFN-I mRNA induction is completely abolished (Sato *et al.*, 2000). IRF3 is constitutively expressed in the cytoplasm and is not induced by viral infection or IFN treatment (Au *et al.*, 1995). However, during a viral infection, phosphorylation of IRF3 allows its translocation to the nucleus where it initiates transcription of IFN-β and IFN-α4 (Sato *et al.*, 1998b). In contrast to IRF3, which is constitutively present in the cytoplasm, expression of IRF7 is dependent on IFN-I signaling in most cells (Marie *et al.*, 1998; Sato *et al.*, 1998a) but like IRF3, translocation of IRF7 to the nucleus is dependent on virally induced phosphorylation

(Sato *et al.*, 1998a). Once in the nucleus IRF7 then maximizes IFN-I production through transcription of IFN-β and further IFN-α subtypes (Marie *et al.*, 1998).

IRF3 and IRF7 are therefore essential components controlling a positive feedback loop which amplifies the expression of IFN-I only in the continued presence of infection (Figure1.3). However pDCs appear to able to bypass the need for IRF3 activation and the initial stage of low IFN-I production by their constitutive IRF7 expression, which allows them to rapidly produce high levels of IFN-I. In addition, IFN-β and IFN-α4, which are induced early after viral infection and released by the infected cell, can then act on neighbouring cells and induce the expression of other IFN-α subtypes. This amplification mechanism also provides a reason for the large number of IFN-α genes, that is, the levels of IFN-I could be more easily controlled by having a number of genes which respond to different transcription factors (Levy *et al.*, 2002).

1.3.3 IFN-I signaling pathways

1.3.3.1 Signaling through the JAK/STAT pathway

The IFN-IR is a hetereodimer composed of IFNAR1 and IFNAR2 subunits, which interact with the Janus family member tyrosine kinases (JAK), JAK1 and tyrosine kinase 2 (TYK2), respectively. Several signaling pathways are initiated through binding of IFN-I, consistent with their pleiotropic biological effects, and their ability to induce transcription of hundreds of genes (Der *et al.*, 1998). The first IFN-I signaling pathway to be identified was the JAK-STAT signaling pathway (Figure 1.4). Once IFN-I binds to its receptor a tyrosine phosphorylation cascade is initiated, involving autophosphorylation and activation of JAK1 and TYK2 (Velazquez *et al.*, 1992; Silvennoinen *et al.*, 1993), and leads to the phosphorylation of STAT1 and STAT2 (signal transducers and activators of transcription 1

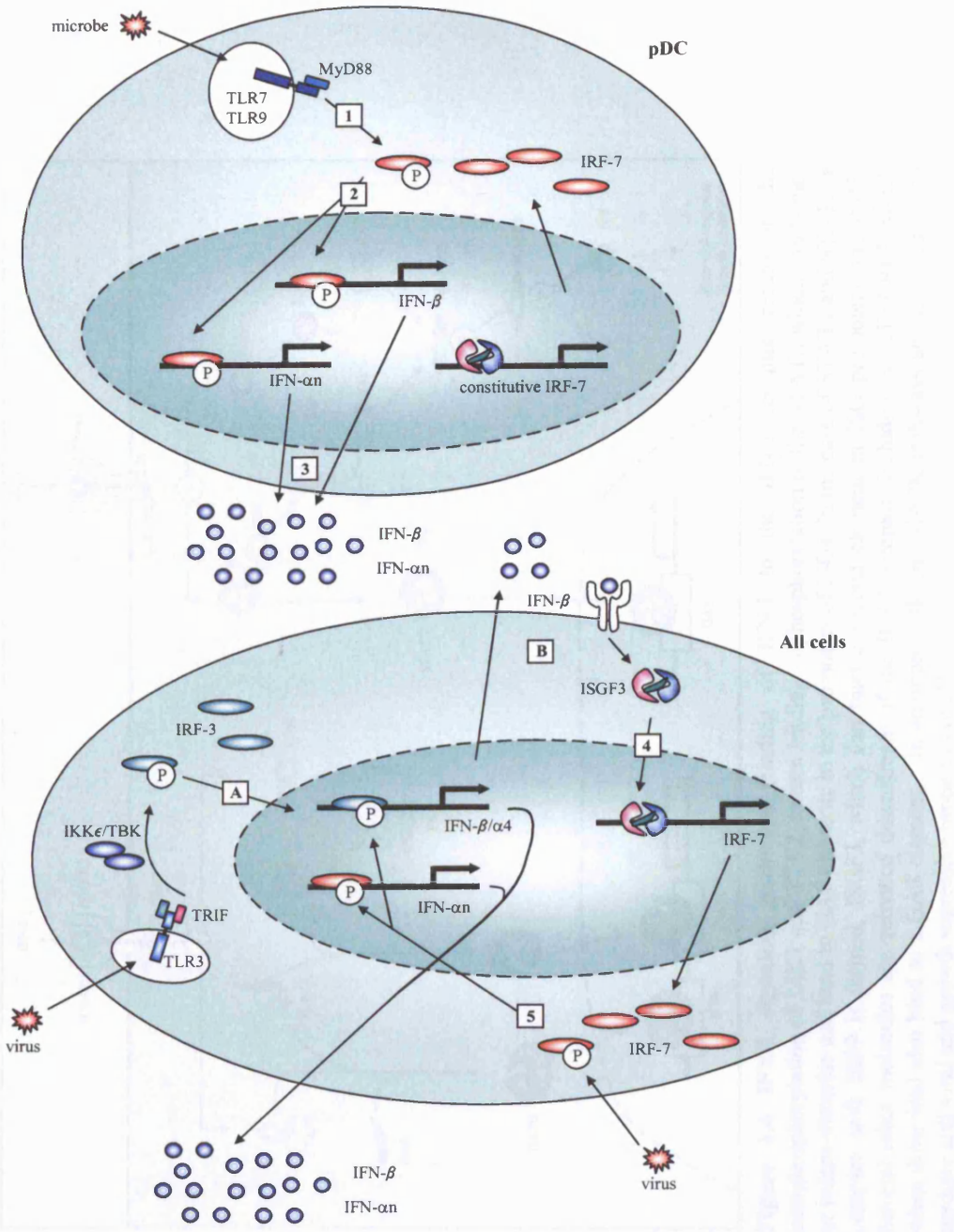


Figure 1.3 Induction of IFN-I expression In pDCs IRF7 is constitutively expressed and can be activated via TLR signaling (1). Phoshorylated IRF-7 can then tranlocate the nucleus (2) and induce the production of high levels of IFN-I (3). This can then act on neighbouring cells to induce the expression of IRF-7 (4), which in the presence of infection will be phoshorylated and induce IFN-I expression (5). In the absence of pDCs, most cell types require virally activated IRF3 for the initial stage of IFN-I production which induces production of IFN-β and IFN-α4 (A) and acts in a positive feedback loop (B) in the induction of IRF-7 expression and consequently high levels of IFN-I production.

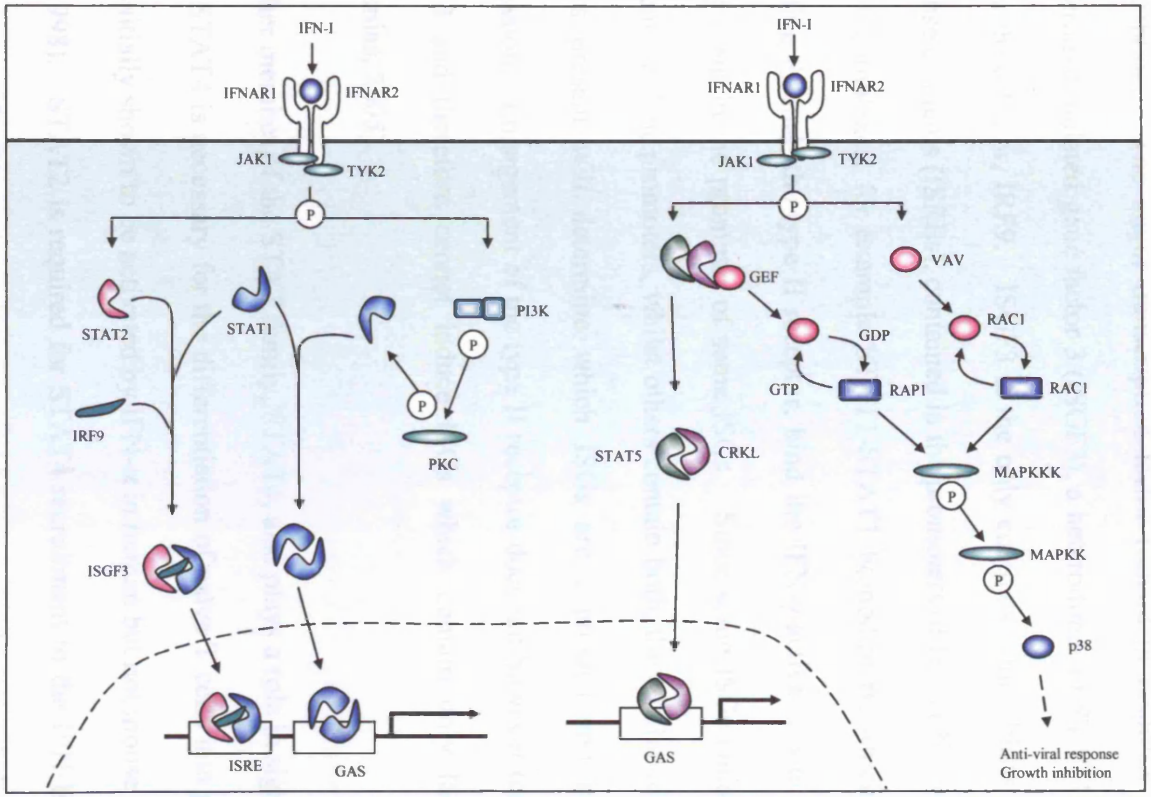


Figure 1.4 IFN-I signaling pathways Binding of IFN-I to the IFN-I receptor results in the autophosphorylation of JAK1 and TYK2, in turn leading to phosphorylation of the STATS which can homo- or hetero-dimerise and bind to GAS elements in the promoters of ISGs. In the case of STAT1 and STAT2, a complex with IRF9 is formed, ISGF3, which can bind a second element in ISG promoters, the ISRE. Several other molecules are activated downstream of the IFN-I receptor, including STAT5/CRKL which when dimerised also bind to a GAS element. In addition PI3K and MAPK pathways are activated which mediate anti-viral and growth supressive properties of IFN-I.

and 2) (Stark *et al.*, 1998). The phosphorylated STATs can then homo- or heterodimerize and translocate to the nucleus where they bind to specific DNA sequences and stimulate transcription. The major transcription factor formed in response to IFN-I is known as interferon-stimulated gene factor 3 (ISGF3), a heterodimer of STAT1 and STAT2 combined with a third factor, IRF9. ISGF3 is the only complex which binds to interferon regulated response elements (ISREs), contained in the promoters of ISGs (Platanias, 2005). All other STAT complexes, for example STAT1-STAT1 homodimers, which are also formed after signaling through the type II receptor, bind the IFN-γ-activated site (GAS) element that is also present in the promoter of some ISGs. Since some ISGs contain only GAS or ISRE elements in their promoters, whilst others contain both, the combinations of activated STAT dimers present will determine which ISGs are expressed and influence their optimal expression. Engagement of the type II receptor does not however result in the formation of ISGF3 and therefore cannot induce ISGs which contain only ISRE in their promoter (Platanias, 2005).

Another member of the STAT family, STAT4, also plays a role in signaling through the IFN-IR. STAT4 is necessary for the differentiation of naïve T cells into polarized Th1 cells and was initially shown to be activated by IFN-α in human but not mouse $CD4^+$ T cells (Rogge *et al.*, 1998). STAT2 is required for STAT4 recruitment to the IFN-IR (Farrar *et al.*, 2000b) and murine STAT2 carries a disruption which prevents this association (Farrar *et al.*, 2000a). However, more recent studies have demonstrated that IFN-I can activate STAT4 in mouse cells, which presumably occurs through a different pathway from that in humans (Freudenberg *et al.*, 2002; Nguyen *et al.*, 2002). The delicate balance of STAT activation can influence the cellular response, since IFN-α and IFN-β mediated induction of STAT4 is

required for IFN-γ production during viral infections, whereas STAT1 negatively regulates IFN-γ production (Nguyen *et al.*, 2000; Nguyen *et al.*, 2002).

1.3.3.2 CRK protein mediated IFN-I signaling

A second IFN-signaling pathway acts through the CRK family of adaptor proteins, which has three members, CRKL, CRKI and CRKII (Platanias, 2005). These adaptors also interact with TYK2 and can be activated by both IFN-I and IFN-γ (Ahmad *et al.*, 1997; Alsayed *et al.*, 2000). The SH3 domain of CRKs interacts with guanine-nucleotide-exchange factors (GEFs) through which RAP1, a small GTPase, is activated (Feller, 2001). RAP1 has several biological functions, including growth inhibition (Platanias *et al.*, 1999) and regulation of mitogen-activated protein kinase (MAPK) signaling cascades (Bos *et al.*, 2001). In addition, during IFN-I signaling CRKL forms a complex with activated STAT5 which can translocate to the nucleus and bind a GAS element (Fish *et al.*, 1999). The STAT-CRKL complex seems to be critical for transcription from promoters containing GAS elements, since defects in either STAT5 or CRKL results in the loss of IFN-I dependent transcription via GAS elements (Lekmine *et al.*, 2002; Uddin *et al.*, 2003).

1.3.3.3 MAPK mediated IFN-I signaling

Of the MAPK pathways, those involving p38 appear to be the most important in mediating IFN-mediated signals (Platanias, 2005). One pathway for p38 activation is believed to occur through VAV phosphorylation by TYK2, which mediates guanine nucleotide exchange in RAC1 leading to the activation of MAPK kinases that phosphorylate p38 (Platanias, 2005). Inhibition of p38 blocks IFN-α-dependent transcription from ISREs, but ISGF3 formation and its binding to ISREs was not prevented, suggesting that p38 acts independently from STATs (Uddin *et al.*, 1999). p38 is also required for IFN-I driven gene transcription through GAS elements, and again this was not the result of a lack of STAT phosphorylation or their

dimerisation (Uddin *et al.*, 2000). In contrast, IFN-γ driven transcription from GAS elements is not p38 dependent (Li *et al.*, 2004). Several kinases are activated by p38, including MAPK-activated protein kinase 2 and 3 (MAPKAPK2 and 3) (Uddin *et al.*, 1999). The activation of MAPKAPK2 is required for transcription of ISGs, including Isg15 (IFN stimulated gene 15) (Li *et al.*, 2005) and for anti-viral properties of IFN-I (Li *et al.*, 2004). p38 has also been shown to be necessary for maximal IFN-I anti-viral activity, as inhibition of p38 results in the loss of protection by IFN-α against encephalomyocarditis virus (Goh *et al.*, 1999) and hepatitis C virus (Ishida *et al.*, 2004). Finally, the p38 signaling pathway is involved in the growth suppressive effects of IFN-I (Mayer *et al.*, 2001).

1.3.3.4 PI3K in IFN-I signaling

The phophatidyl-inositol 3-kinase (PI3K) signaling pathway is induced by both IFN-I and IFN-γ, in addition to various other cytokines, hormones and growth factors (Platanias, 2005). IFN-I induces the phosphorylation of PI3K via the insulin receptor substrate (IRS) family of multi-site docking proteins (Uddin *et al.*, 1995), a distinct mechanism from that of IFN-γ. Phosphorylated PI3K can activate various downstream effectors, including protein kinase C (PKC) isoforms, of which PKC-δ is important in IFN-signaling as it acts as a serine kinase for STAT1 (Uddin *et al.*, 2002). Another downstream effector of PI3K, AKT, mediates anti-apoptotic and pro-survival signals (Vivanco *et al.*, 2002).

1.3.3.5 IFN induction of mRNA translation

Finally, IFN-signaling can regulate the translation of ISG mRNAs. One regulator of this translation is mammalian target of rapamycin (MTOR), whose activation is dependent on IFN-signaling through PI3K (Lekmine *et al.*, 2003). In turn, MTOR is necessary for the de-

activation of the repressor of translation EIF4E- binding protein (EIF4EBP), allowing its dissociation from EIF4E, and the initiation of translation (Hay *et al.*, 2004).

1.3.4 IFN-I induces anti-microbial immunity

1.3.4.1 IFN-I and the anti-viral state

As mentioned earlier, interferons were first identified because of their ability to impart an anti-viral state on cells (Isaacs *et al.*, 1957). They are able to block viral replication at any stage, from entry and uncoating (eg simian virus 40) through to assembly and release of the mature virion (retroviruses and vesicular stomatitis virus)(Stark *et al.*, 1998). The importance of IFN-I in anti-viral responses has been illustrated in mice with deletions in the IFN-I receptor genes (Muller *et al.*, 1994). IFN-IR knockout mice are extremely susceptible to a number of viruses including LCMV, vaccinia virus (VV) and vesicular stomatitis virus (VSV), which is largely due to their inability to inhibit viral replication (Muller *et al.*, 1994). In contrast, loss of the IFN- II receptor signaling pathway did not affect the susceptibility to VSV, and LCMV titers were maintained at much lower levels compared to that found in IFN-IR knockout mice whereas susceptibility to VV was equally profound in both types of knockout mice (Muller *et al.*, 1994). Therefore IFN-γ only appears to be necessary for the control of certain viral infections. Cells can respond to IFNs by the initiation of several mechanisms which give rise to an anti-viral state and are discussed next.

One of the proteins induced by IFNs is the protein kinase R (PKR), which, when activated by dsRNA, leads to the phosphorylation of eIF2 (elongation initiation factor 2). eIF2 is required for protein translation and its phosphorylation renders it inactive, thereby halting all translation in the virally infected cell (Stark *et al.*, 1998). Experiments in PKR-deficient mice highlighted the importance of PKR in viral infection, since poly(I:C) or IFN-γ treatment

extended the survival of wild-type but not PKR-deficient mice after encephalomyocarditis virus (ECMV) infection. However, IFN-α also utilises separate anti-viral mechanisms since the IFN-α-induced anti-viral response against ECMV was the same in wild-type and PKR-deficient mice (Yang *et al.*, 1995). The IFN-inducible 2-5A synthetases constitute a multi-enzyme family, which like PKR, is stimulated by dsRNA and produces short 2',5'-oligoadenylates (2-5A) that activate RNaseL (Kerr *et al.*, 1978). This results in the extensive cleavage of single-stranded RNA (ssRNA) (Wreschner *et al.*, 1981) and therefore also prevents translation. Studies using RNaseL deficient mice have shown the importance of the 2-5A system in the anti-viral properties of IFN-α against ECMV (Zhou *et al.*, 1997).

Another important group of IFN induced proteins are the Mx proteins, high abundance GTPases which restrict RNA virus replication (Stark *et al.*, 1998). Although their mode of action is not completely understood, they act primarily at the level of transcription and are thought to interfere with the viral polymerases (Stranden *et al.*, 1993). However, they also act at other stages of viral replication. In mice, Mx1 associates with distinct nuclear domains, known as PML bodies, and interferes with the transcription of RNA viruses having a nuclear replication phase, such as influenza (Staeheli *et al.*, 1986b; Engelhardt *et al.*, 2004). The human Mx protein, MxA, operates via a different mechanism, since it is located in the cytoplasm and inhibits a step of influenza replication that follows primary transcription (Pavlovic *et al.*, 1992). In contrast to other ISGs, Mx1 expression is not constitutive and can only be induced by IFN-I via the JAK-STAT signaling pathway and not directly by viruses or dsRNA (Simon *et al.*, 1991; Bazzigher *et al.*, 1992; Dupuis *et al.*, 2003).

Table 1.6 Mechanisms of IFN-induced viral inhibition (Weber et al., 2004)		
Protein	**Mechanism**	**Viruses known to inhibit**
ISG20	3'-5' exonuclease that degrades ssRNA	Vesicular stomatitis virus
P56	Inhibits translation via eukaryotic translation initiation factor 3e (eIF3e)	Hepatitus C virus
RNA-specific adenosine deaminase (ADAR 1)	dsRNA-editing enzyme: destabilizes dsRNA structure	Unknown
Promyelocytic protein (PML)	Organizer and main component of PML nuclear bodies, which contain various IFN-induced protein involved in apoptosis and the anti-viral state (ISG20, Daxx and p53)	Vesicular stomatitis virus Influenza A virus LCMV Human foamy retrovirus
Guanylate binding protein-1 (GBP-1)	GTPase – unknown mechanism	Vesicular stomatitis virus

The three pathways discussed above are the best characterised anti-viral mechanisms induced by IFNs. However, it is certain that other pathways do exist. In one study, mice were generated which were deficient in all three pathways, carrying no functional RNaseL, PKR or Mx1 genes. These mice showed increased survival times after EMCV infection when treated with IFN (Zhou *et al.*, 1999). Additional IFN-induced anti-viral proteins have been identified, and their properties are summarised in table1.6.

1.3.4.2 Role of IFN-I in non-viral infections

Although IFN-I is known primarily for its anti-viral properties, it has many effects on the immune system including defence against bacterial and protozoan infections. Bacteria can induce the expression of IFN-I through TLR4 (LPS of gram-positive bacteria) and TLR9 (non-methylated bacterial CpG DNA). In addition there appears to be an alternative mechanism for IFN-I induction by bacterial components, since IFN-β production during *Listeria monocytogenes* (*L. monocytogenes*) infection is dependent on IRF3 but not TLRs or the TRIF adaptor (Stockinger *et al.*, 2004). A large number of non-viral pathogens induce IFN-I expression including bacteria such as *L. monocytogenes*, protozoa, such as *Leishmania*

major and *Toxoplasma gondii*, and the eggs of the helminth *Schistosoma mansoni*, indicating the importance if IFN-I in controlling these infections (Bogdan *et al.*, 2004). Although IFN-I does have a protective effect against many of these pathogens, it can be detrimental during certain infections. For example increased resistance against *L. monocytogenes* has been seen in mice deficient in IRF3 or signaling through the IFN-IR (O'Connell *et al.*, 2004). IFN-I does contribute to a direct anti-microbial effects as it is necessary for nitric oxide synthesis induced by TLR signaling (Decker *et al.*, 2005). Several members of the IFN-γ inducible family of p47 GTPases, which have microbicidal properties, are also induced by IFN-I (Taylor *et al.*, 2004). However to a large extent, the anti-bacterial properties of IFN-I are likely to be indirect, that is by inducing the production of IFN-γ or by inducing DC maturation (Decker *et al.*, 2005).

1.3.5 IFN-I, cell cycle control and apoptosis

IFN-I also controls microbial infections by growth suppression and apoptosis of infected cells, which is also important for the elimination of cancer cells. IFN-α treatment inhibits the cell cycle kinases, which results in loss of phosphorylation of the retinoblastoma protein that is necessary for the release of transcription factors such as E2F and cell cycle progression (Chawla-Sarkar *et al.*, 2003). IFN-I mediated apoptosis involves TRAIL and Fas, which on binding to their respective receptors induce signaling through the Fas associated death domain (FADD) and caspase-8 (Chawla-Sarkar *et al.*, 2003). The regulators of IFN-I expression have been linked to apoptosis: IRF1 is a mediator of both cell cycle arrest and DNA-damage-induced apoptosis (Tanaka *et al.*, 1994), whilst over expression of IRF3 is a potent inducer of apoptosis (Heylbroeck *et al.*, 2000). In addition to TRAIL, Fas, caspase-4 and caspase-8, several other ISGs involved in apoptosis and growth inhibition have been identified by gene microarray studies (Der *et al.*, 1998; de Veer *et al.*,

2001). These include the anti-viral factors PKR, PML and 2-5A oligoadenylate/RNase L, as well as several more recently identified genes known as the regulators of IFN-induced death (RIDs) (Chawla-Sarkar *et al.*, 2003). Over expression of the ISG galectin-9, an eosinophil chemoattractant involved in cell-cell attraction and adhesion, sensitizes to Fas-induced apoptosis (Saita *et al.*, 2002). Finally, IFN-I inducible phospholipid scramblase inhibits tumour growth (Silverman *et al.*, 2002).

1.3.6 IFN-I control of innate immunity

1.3.6.1 Macrophages

IFN-I has been shown to augment the effector function of macrophages in several ways. For instance, IFN-I stimulates antibody-dependent cytotoxicity (Ralph *et al.*, 1988), is necessary for complement receptor mediated phagocytosis (Sampson *et al.*, 1991) and regulates the production of cytokines by macrophages (Taylor *et al.*, 1998). IFN-I can also induce the expression of the enzyme inducible nitric oxide synthase (iNOS) by macrophages, which catalyzes the production of nitric oxide and helps to mediate their cytotoxic function (Gao *et al.*, 1998). Finally, an IFN-I dependent, IL-12 independent mechanism of IFN-γ production has been found in *Chlamidia*-infected macrophages (Gigliotti Rothfuchs *et al.*, 2001).

1.3.6.2 NK cells

IFN-I and IL-12 appear to have complementary roles in NK cell activation; whereas IFN-I is necessary for NK cell blastogenesis and cytotoxicity but not IFN-γ production, IL-12 is required for IFN-γ production but not cytotoxicity or blastogenesis (Biron *et al.*, 1984; Biron *et al.*, 1996; Orange *et al.*, 1996a). Induction of NK cell cytotoxicity by IFN-I has been shown to be dependent on STAT1 (Lee *et al.*, 2000). In addition, IFN-I has been shown to negatively regulate IFN-γ production by NK and T cells in response to virus infection, an

effect which is again mediated by STAT1 (Nguyen *et al.*, 2000); STAT1 activation renders the NK and T cells unresponsive to IL-12 and TCR stimulation. However, in the absence of STAT1 IFN-I can stimulate IFN-γ production. Therefore, STAT1 may function to inhibit IFN-γ production early on in the innate response to viral infection. Later, STAT1 activation appears to be regulated to allow IFN-I induction of IFN-γ in T cells at the peak of the adaptive response. Indeed, after 8 days of LCMV infection when adaptive immune responses would be initiated, production of IFN-γ could be induced (Nguyen *et al.*, 2002). This IFN-γ production was dependent on STAT4, whose phosphorylation could not be induced by IFN-I at the early stages of infection. Both IL-12 and IFN-I induce STAT4 phosphorylation and binding to the IFN-γ gene (Nguyen *et al.*, 2002).

1.3.7 IFN-I control of adaptive immunity

1.3.7.1 T cell responses

In recent years, it has been revealed that IFN-I plays a crucial role in the development of adaptive immune responses. All IFNs have the ability to enhance expression of MHC class I proteins and so can promote the development of $CD8^+$ T cell responses (Boehm *et al.*, 1997). The transcription factor IRF1 is known to drive this expression and mutations in either the IFN receptors, STAT1, PKR or IRF1 results in the cells being unable to upregulate MHC class I in response to IFN-I (Chang *et al.*, 1992; Reis *et al.*, 1992). Although both type I and type II IFNs can upregulate MHC I expression, IFN-I was shown to inhibit IFN-γ upregulation of class II expression in macrophages (Boehm *et al.*, 1997).

Live viruses are more effective than killed virus or virus components at generating long term immune memory. One explanation for this is the production of IFN-I following viral infection. IFN-I has been shown to trigger bystander $CD8^+$ T cell proliferation and has also

been implicated in the maintenance of $CD44^{hi}$ $CD8^+$ memory cells (Tough *et al.*, 1996). In these experiments, mice were injected with either LCMV or poly(I:C), an inducer of IFN-I, after which 70-90% of $CD44^+$ $CD8^+$ T cells went through cell division. This is a much higher proportion than that which could be accounted for by antigen-specific T cells and therefore could represent a mechanism by which the memory T cell pool is maintained. Injection of LPS or CpG DNA has also been shown to induce T cell activation and proliferation of $CD44^+$ $CD8^+$ T cells, an effect which is abolished in IFN-IR-deficient mice (Tough *et al.*, 1997; Sun *et al.*, 1998). This is likely to be mediated by DCs, which produce IFN-I after stimulation by LPS or CpG DNA. The IFN-I was acting directly on the T cells, since IFN-RI-deficient T cells could not be activated by IFN-I in the presence of normal APCs, and purified T cells could be activated by IFN-I itself. IFN-I is also important in T cell survival during an infection, and is able to prevent the death of antigen-activated, but not resting T cells (Marrack *et al.*, 1999). In support of observations by Sun *et al.*, IFN-I was shown to act directly on the T cells, as shown by increased survival of normal but not IFN-IR-deficient T cells in response to IFN-I *in vitro*.

In humans, IFN-I can induce the expression of the IL-12 receptor β2 chain on $CD4^+$ T cells, thereby enabling them to respond to IL-12 and differentiate into Th1 cells (Rogge *et al.*, 1997). Studies in mice had shown that IFN-α could not substitute for IFN-γ in inducing IFN-γ production by $CD4^+$ T cells in response to IL-12, but could inhibit their IL-4 production (Wenner *et al.*, 1996). Indeed, IFN-I does not induce Th1 differentiation as strongly as IL-12. Whereas IL-12 induces sustained STAT4 phosphorylation (>48h), IFN-α induction of STAT4 was only transient (<4h) and could be explained by the loss of cell surface IFN-I receptors (Athie-Morales *et al.*, 2004). Although it was initially thought that IFN-I could act directly on human, but not mouse T cells to drive Th1 development via the

activation of STAT4 (Rogge *et al.*, 1998), it was subsequently shown that IFN-I, in synergy with IL-18, could induce IFN-γ via a STAT4 dependent mechanism in mice (Freudenberg *et al.*, 2002; Nguyen *et al.*, 2002).

IL-12 is important in promoting NK and Th1 cell IFN-γ responses in parasite and bacterial infections. However, IL-12 is not induced in all viral infections, for example LCMV, whereas IFN-I levels are highly elevated 2-3 days after infection (Orange *et al.*, 1996b). In addition, IFN-I can negatively regulate IL-12 expression (Cousens *et al.*, 1997). In fact both IFN-I and IL-12 dependent pathways were shown to be important for the induction of IFN-γ from $CD8^+$ T cells, and in the control of LCMV infection (Cousens *et al.*, 1999). Therefore the balance between IFN-I and IL-12 seems to be important for the control of different infections.

IFN-I can also inhibit T cell function since it can reduce T cell proliferation (Sun *et al.*, 1998). However IFN-I is only able to exert its anti-proliferative effect on naive and not activated T cells (Dondi *et al.*, 2003). Inhibition of naive T cell proliferation may be overruled in the presence of DCs, on which IFN-I has a strong adjuvant effect (Tough, 2004).

1.3.7.2 Humoral immune response

Studies on the role of IFNs in the humoral immune response have mainly been focused on IFN-γ. It now appears though, that IFN-I may induce many of the same effects. IFNs are known to affect three aspects of B cell function; development and proliferation, Ig secretion and Ig heavy chain switching (Stark *et al.*, 1998). Ig heavy chain switching is important in tailoring the immune response to different pathogens as the different isotypes promote distinct effector functions in the host. Injection of IFN-α into mice enhances IgG2a secretion whilst suppressing IgE secretion (Finkelman *et al.*, 1991). In addition, in mice

deficient for both type I and type II receptors IgG2a is not produced in response to LCMV infection. This contrasts with normal IgG2a levels in single knockout mice suggesting that IFN-I and IFN-γ share a redundant role in Ig class switching (van den Broek *et al.*, 1995). IFN-I has been shown to upregulate the B cell activation markers CD69, CD86 and CD25 and to enhance responses to B cell receptor ligation (Braun *et al.*, 2002). In addition, IFN-I promoted survival of mature B cells and resistance to Fas-mediated apoptosis *in vitro*. In another study, co-injection of soluble antigen and IFN-I resulted in long lasting antigen-specific antibodies and the generation of memory B cells (Le Bon *et al.*, 2001). Finally, IFN-I and IL-6 secreted by pDCs induces the generation of plasma cells and their differentiation into Ig-secreting plasma cells, respectively (Jego *et al.*, 2003).

1.3.8 The immunomodulatory effect of IFN-I on DCs

1.3.8.1 IFN-I induces DC differentiation and maturation

DCs are highly dynamic cells and can rapidly transform themselves in response to environmental factors in order to carry out the distinct functions required of them. As already discussed, the factors that regulate these transformations include cytokines, chemokines and danger signals from pathogens or distressed cells. Several groups have demonstrated the ability of IFN-I to accelerate the differentiation and maturation of human *in vitro*-derived DCs. In one system, immature human DCs generated from $CD34^+$ progenitors in the presence of GM-CSF, TNF-α and IL-4 spontaneously matured into activated DCs in 14 days (Luft *et al.*, 1998). When IFN-I was added the DCs matured into activated DCs in only 3 days, an effect which could not be duplicated with any other cytokine tested. These DCs had upregulated MHC I and the co-stimulatory molecules CD80 and CD86. In addition, DCs matured in the presence of IFN-α2a upregulated class II expression and expressed the human DC lineage-associated antigens CD83 and CMRF44. The DCs were

functionally mature as they had high stimulatory capacity in the MLR. Likewise, maturation of DCs directly isolated from human blood can be induced by IFN-I (Ito *et al.*, 2001).

The development of phenotypically and functionally mature DCs from peripheral blood monocytes after culture with IFN-α and GM-CSF has also been reported (Paquette *et al.*, 1998; Radvanyi *et al.*, 1999; Santini *et al.*, 2000). This effect could be enhanced by the addition of IL-4 (Paquette *et al.*, 1998) and is superior to treatment with IL-4/GM-CSF alone (Santini *et al.*, 2000). In contrast to the experiments by Luft and Radvanyi (Luft *et al.*, 1998; Radvanyi *et al.*, 1999), this DC maturation appeared to be independent of TNF-α. IL-4/GM-CSF treated DCs could revert back to cells of macrophage-like morphology after cytokine removal. In contrast, the monocytes matured in the presence of IFN-α had reached a more advanced stage of maturation and expressed CD83, which was irreversible after removal of the cytokines (Santini *et al.*, 2000). Overproduction of IFN-α in vivo as seen in patients with systemic lupus erythematosus (SLE), appears to affect DC development since monocytes from these patients could function as APCs. Serum from these patients could also be used to induce the differentiation of monocytes into cells with mature DC characteristics able to induce $CD4^+$ T cell proliferation, which was inhibited by neutralising antibodies against IFN-α. Therefore IFN-α-maturation of DCs presentating antigens from captured apoptotic cells could drive the production of auto-reactive T and B cells as seen in SLE (Blanco *et al.*, 2001). Finally, DCs able to initiate proliferation of naive $CD4^+$ T cells can be generated from human monocytes in the absence of GM-CSF using a combination of IL-3 and IFN-β (Buelens *et al.*, 2002).

Some studies have reported the inhibition of DC development from monocytes in the presence of IFN-I (Bartholome *et al.*, 1999; McRae *et al.*, 2000; Wiesemann *et al.*, 2002).

This may be due to differences in culture conditions, since IL-4, which can suppress transcription of ISGs in monocytes (Larner *et al.*, 1993), was also present in the monocyte cultures. The stage of differentiation or maturation at which DCs are exposed to IFN-I may also determine how they respond. Indeed, when IFN-β was added at the initial stage of monocyte-derived DC (MoDC) differentiation it had an inhibitory effect on DC function, whilst addition at a later stage (at 7-10 days of culture) had the opposite effect and enhanced their T cell stimulatory capacity (Wiesemann *et al.*, 2002).

In mice, IFN-α can activate resting BM-derived DCs in the absence of any other stimulating factors (Gallucci *et al.*, 1999). Recently, splenic DCs have also been shown to mature in response to IFN-I both *in vitro* and *in vivo* (Montoya *et al.*, 2002). In this study it was also shown that both BM-derived DCs and splenic DCs secrete IFN-I, which could activate DCs in an autocrine manner. Another study highlighted the importance of IFN-I during the maturation of mouse DCs stimulated by CpG DNA *in vitro* (Cho *et al.*, 2002). Stimulation of mouse BM-derived DCs with CpG DNA resulted in upregulation of MHC class I and co-stimulatory molecules. However, among BM-derived DCs from IFN-IR$^{-/-}$ mice only a subset of DCs up-regulated co-stimulatory molecules, and no upregulation of MHC I molecules could be detected in response to CpG DNA.

Although IFN-I is necessary for the full maturation of DCs by CpG or dsRNA, it does not appear to be sufficient, since maturation of DCs induced by IFN-β was not as complete as that seen with poly(I:C) (Honda *et al.*, 2003). Due to the kinetics of co-stimulatory molecule and inflammatory cytokine induction seen in wild-type and IFN-IR$^{-/-}$ mice, these authors suggested that stimulation of DC maturation by dsRNA occurs in two phases; dsRNA induces expression of IFN-I, inflammatory cytokines and CCR7, and IFN-I augments cytokine expression and induces effective DC maturation in the late phase. A recent report

has shown that although IFN-I produced by HSV infected MoDCs could activate uninfected DCs, it was not necessary for the maturation of the infected DCs themselves (Pollara *et al.*, 2004). Similarly, in IFN-IR$^{-/-}$ mice lower levels of costimulatory and MHC molecules are induced following LCMV infection compared to wild-type mice. DCs isolated from IFN-IR$^{-/-}$ mice after LCMV infection could stimulate proliferation of naive T cells but not as efficiently as wild-type controls (Montoya *et al.*, 2005). Therefore a degree of maturation had occurred by an independent IFN-I pathway.

There appears to be a differential requirement for IFN-I signaling for maturation in response to TLR ligands. Defects in maturation caused by loss of IFN-I signaling are less severe when the initial stimulus is LPS or CpG compared to poly(I:C) (Honda *et al.*, 2003). Likewise, maturation of DCs *in vivo* after viral infection or injection of R848 or CpG is suppressed in IFN-IR$^{-/-}$ mice, although maturation in response to LPS remains intact (Honda *et al.*, 2003; Asselin-Paturel *et al.*, 2005b). This is consistent with earlier reports showing the varying dependence on IFN-I signaling for induction of CD40 expression through TLR4 and TLR9 stimulation (Hoshino *et al.*, 2002).

DCs also regulate their own responsiveness to IFN-I during maturation. This has been demonstrated in human MoDCs, which after LPS-induced maturation down-regulated the expression of both type I IFN receptor chains (Gauzzi *et al.*, 2002). Likewise, regulation of genes involved in IFN-I signaling and production appears to be essential for the correct development of DCs. Recent studies revealed that development of CD11c^{+} CD8^{+} DCs and pDCs is dependent on an IRF family member which regulates expression of IFN-responsive genes, the IFN consensus sequence binding protein (ICSBP)/IRF-8 (Schiavoni *et al.*, 2002; Aliberti *et al.*, 2003; Tsujimura *et al.*, 2003). The few CD8^{+} DCs that develop in ICSBP-deficient mice are unable to mature in response to LPS or poly IC (Schiavoni *et al.*, 2002).

In addition, the $CD8^-$ subset of DCs in these mice also have impaired function and are unable to mature after TLR signaling (Aliberti *et al.*, 2003). $ICSBP^{-/-}$ DCs are also unable to produce IFN-α, IL-12p40 or IL-15 (Schiavoni *et al.*, 2002; Tsujimura *et al.*, 2003). In contrast, IRF2-deficient mice display a loss of the $CD8^-$ DC subset (Honda *et al.*, 2004). This defect has been attributed to excessive IFN-I signaling which in normal mice is regulated by IRF2 via the inhibition of ISGF3. These studies highlight the importance of IFN-regulated genes for the development and function of DCs. However, in IFN-IR knockout mice there is no apparent deficiency in DC development, so IFN-I may not be required for DC development *in vivo*.

Finally, treatment of pDCs with IFN-I can maintain their survival, although unlike stimulation with viruses, does not induce their differentiation (Kadowaki *et al.*, 2000; Ito *et al.*, 2001). It was subsequently shown that IFN-I is necessary for dsRNA induced differentiation of bone marrow pDCs into $CD11c^+$ $CD11b^+$ DCs (Zuniga *et al.*, 2004).

It is clear therefore, that the effect of IFN-I on DC maturation is dependent on a complex set of factors, including the stage of maturation, the maturation stimulus, the DC subset and the influence of other cytokines present during their differentiation and maturation.

1.3.8.2 IFN-I enhances chemokine and cytokine secretion by DCs

IFN-I induces the upregulation of MHC and co-stimulatory molecules by DCs which results in an increased ability to stimulate antigen-specific T cell proliferation (Luft *et al.*, 1998; Paquette *et al.*, 1998; Wang *et al.*, 1999). In addition, IFN-I can enhance the ability of DCs to attract T cells. IFN-α2a-treatment of MoDCs induces the secretion of the chemokines CXCL9 (MIG) and CXCL10 (IP-10) as well as the upregulation of CXCL11 (I-TAC) mRNA (Padovan *et al.*, 2002). Furthermore, memory and activated $CD8^+$ T cells show increased

chemotaxis in response to medium from IFN-I treated DCs which is inhibited by blocking the CXCR3 receptor. In addition, expression of the chemokine DC-CK-1, involved in the recruitment of naive T cells, has been shown to be expressed in MoDCs derived *in vitro* with GM-CSF combined with IFN-I but not IL-4 (Parlato *et al.*, 2001).

MoDCs derived in the presence of IFN-I produce elevated levels of IL-15 compared to MoDCs generated in GM-CSF and IL-4 (Santini *et al.*, 2000). In addition IFN-I has been shown to induce expression of IL-15 by mouse splenic DCs after treatment with IFN-I either *in vivo* or *in vitro* (Mattei *et al.*, 2001). Interestingly, IFN-I also induced the expression of the IL-15 receptor (IL-15R) by splenic DCs, which were shown to respond to IL-15 by upregulating co-stimulatory molecules. Furthermore DCs from IL-15-treated mice were better stimulators of $CD8^+$ T cell proliferation, an effect which was reduced in DCs from IFN-$IR^{-/-}$ mice suggesting that IFN-I could enhance the response of DCs to IL-15. The importance of DC stimulation by IL-15 in the initiation of $CD8^+$ T cell responses *in vivo* has subsequently been demonstrated (Ruckert *et al.*, 2003), and has also recently been shown to regulate DC production of IL-2 (Feau *et al.*, 2005). IL-15 shares an overlapping yet distinct set of biological functions with IL-2, explained in part by the composition of the IL-15 receptor, which contains the β and γ subunits of the IL-2 receptor but has a unique α subunit (Fehniger *et al.*, 2001). IL-15 also acts directly on T cells, and has been shown to act as a survival factor for both naive and memory $CD8^+$ T cells (Berard *et al.*, 2003) as well as being an inducer of memory $CD8^+$ T cell proliferation (Zhang *et al.*, 1998).

There is conflicting evidence on the effect on IFN-I on IL-12 production by DCs. IFN-I regulates the production of IL-12 during viral infections, as shown by elevated IL-12 levels after *in vivo* neutralisation of IFN-I during murine cytomegalovirus (MCMV) infection and in IFN-$IR^{-/-}$ mice during LCMV infection (Cousens *et al.*, 1997). Several reports have

indicated that monocyte-derived DCs generated in the presence of IFN-I secrete reduced levels of IL-12 compared to monocyte-derived DCs generated with GM-CSF/IL-4 alone (Bartholome *et al.*, 1999; McRae *et al.*, 2000; Huang *et al.*, 2001b). In these cases the DCs showed an inability to support $CD4^+$ T cell production of IFN-γ. Ito *et al.* also reported that IFN-α did not induce IL-12p70 production by human blood DCs, and instead augmented IL-10 production (Ito *et al.*, 2001). However treatment of immature MoDCs with IFN-α2a augments the production of IL-12 by CD40 ligation or LPS stimulation (Luft *et al.*, 2002; Pollara *et al.*, 2004). Furthermore, addition of IFN-I during LPS or CD40L stimulation of immature MoDCs increases IL-12 production, but inhibits CD40L-induced IL-12 production by mature DCs (Heystek *et al.*, 2003). In fact production of IL-12 appears to be strictly controlled during DC maturation since it had previously been demonstrated that mature DCs were limited in their ability to produce IL-12, and were unresponsive to IFN-γ or bacterial stimulation (Kalinski *et al.*, 1999b). There appears to be a tight cross-regulation between IFN-I and IL-12 production, which may be important for limiting overproduction of either of the cytokines. For example during infection of mice with MCMV, pDC production of IFN-I limits IL-12 production by $CD11c^+$ DCs whilst enhancing IFN-I levels (Dalod *et al.*, 2002). In addition, IFN-I can interfere with the ability of IFN-γ to induce IL-12 even under conditions where IFN-I would normally enhance IL-12 (Heystek *et al.*, 2003).

A recent study has demonstrated the key role of IFN-I in IL-12 production by DCs in response to TLR stimulation (Gautier *et al.*, 2005). The TLR7 ligand R848 was shown to synergise with poly(I:C) and LPS in IL-12 p70 and p40 production by mouse BM-derived DCs. The amount of IL-12p70 but not p40 was dramatically reduced in IFN-IR deficient DCs, in addition to reduced levels of IL-12p35 mRNA. Likewise addition of a neutralizing anti-human IFN-IR antibody partially blocked IL-12p70 but not p40 secretion from MoDCs

in response to TLR stimulation. These results suggest that IFN-I increases IL-12p70 secretion through regulation of p35 production. However in other studies, the production of IL-12p40 was severely impaired in IFN-IR$^{-/-}$ mice after viral infection (Honda *et al.*, 2003).

The Th1 inducing ability of IFN-I may result from the induction of cytokines other than IL-12 from DCs. Nagai *et al.* showed that maturation of MoDCs with TNF-α and IFN-β augmented IL-18 production and increased levels of the IL-23 p19 subunit. In addition, DCs matured in the presence of IFN-β but not with TNF-α alone were partially dependent on IL-18 for inducing IFN-γ production by responding T cells (Nagai *et al.*, 2003). The ability of pDCs to induce Th1 polarisation has also been shown to be independent of IL-12 and only partially dependent on IFN-β and IL-18; addition of a combination of anti-IFN-β, anti-IL-18 receptor and anti-IL-12 did not abrogate T cell IFN-γ production during stimulation with pDCs (Krug *et al.*, 2003).

1.3.8.3 IFN-I induces DC migration

The migration of DCs is dependent on their ability to respond to chemokines released by cells in the surrounding environment and by the DCs themselves. Early experiments indicated that IFN-I could induce the migration of DCs from the skin (Luft *et al.*, 1998). Expression of certain chemokines and their receptors by DCs are enhanced by stimulation with IFN-I; CCR5 expression is elevated on MoDCs derived in the presence of IFN-I and correspondingly they respond more strongly to CCL5 (RANTES), CCL3 (MIP-1α) and especially CCL4 (MIP-1β) compared to MoDCs generated with IL-4 (Parlato *et al.*, 2001). Mature DCs are known to lose their responsiveness to inflammatory cytokines and become responsive to CCL19 (MIP-3β) and CCL21 (6Ckine) due to upregulation of their receptor CCR7. Both the expression of CCR7 and its ligand CCL19 were induced in IFN-generated

DCs (Parlato *et al.*, 2001). Consequently these DCs migrated in response to CCL19 whereas IL-4 derived DCs did not. Since CCL19 is thought to play a key role in homing of mature DCs to the LN, this suggests a role for IFN-I in DC trafficking. In contrast, treatment of immature MoDCs with IFN-I did not result in expression of functional CCR7 even though gene expression was induced (Parlato *et al.*, 2001; Luft *et al.*, 2002), implying that IFN-I is not an influencing factor in the migration of mature DCs. In support of this, lack of IFN-I signaling in the mouse did not effect the expression of CCR7 or migration of $CD11c^+$ DCs into the T cell zone of the spleen after viral infection or injection of CpG (Honda *et al.*, 2003; Asselin-Paturel *et al.*, 2005b). However, migration of pDCs does appear to be dependent on IFN-I. In resting conditions, pDCs are scattered through the T cell area and red pulp of the spleen. After injection of TLR7 or TLR9 ligands (but not TLR4 ligands), pDCs form clusters within the marginal zone and T cell area. However in IFN-$IR^{-/-}$ mice, this clustering does not occur, and *ex vivo* pDCs from CpG-treated mice were impaired in their migration in response to CCL21 (Asselin-Paturel *et al.*, 2005a).

1.3.8.4 IFN-I enhances immunity in vivo

Finally, IFN-I has also been shown to enhance the immunostimulatory capacity of DCs *in vivo* (Figure 1.5). MoDCs generated in the presence of IFN-I can induce a strong primary antibody response against HIV-1 antigens when inoculated into severe combined immunodeficiency (SCID) mice reconstituted with human peripheral blood leukocytes (Santini *et al.*, 2000; Parlato *et al.*, 2001). Similarly, Le Bon *et al.* showed that co-injection of poly(I:C) and antigen resulted in highly elevated antigen-specific antibody titres of all IgG subclasses, an effect which was severely diminished in IFN-IR knockout mice (Le Bon *et al.*, 2001). Injection of IFN-I also enhanced levels of antigen-specific antibodies, and its activity was comparable to that of the potent adjuvant, complete Freund's adjuvant (CFA). In fact,

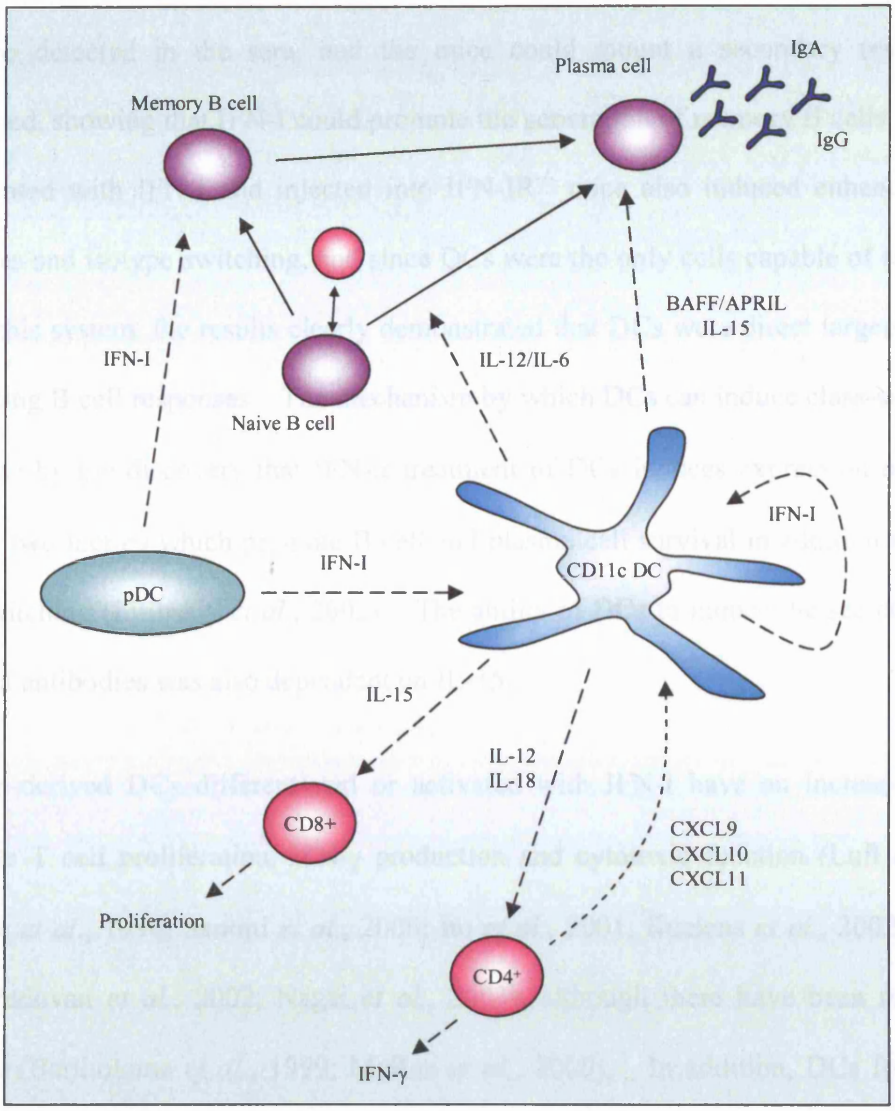


Figure 1.5 IFN-I enhances the immunostimulatory capacity of DCs IFN-I produced by pDCs or CD11c$^+$ DCs induces the production of cytokines by DCs which regulate T and B cell function. These include IL-12, which promotes IFN-γ production by CD4 T cells and differentiation of naive B cells into plasma cells (in co-operation with IL-6 also produced by DCs). BAFF and APRIL promote isotype switching, which is also dependent on IL-15. In addition, IL-15 induces CD8$^+$ T cell proliferation. IFN-I also enhances the production of chemokines which aid in the attraction of T cells.

the ability of CFA to induce high titers of IgM, IgG2 and IgG3 antibodies was dependent on IFN-I. Six months after immunisation with antigen and IFN-I, antigen-specific antibodies could be detected in the sera, and the mice could mount a secondary response when challenged, showing that IFN-I could promote the generation of memory B cells. Wild-type DCs treated with IFN-I and injected into IFN-IR$^{-/-}$ mice also induced enhanced antibody responses and isotype switching, and since DCs were the only cells capable of responding to IFN in this system, the results clearly demonstrated that DCs were direct targets of IFN-I in stimulating B cell responses. The mechanism by which DCs can induce class-switching was explained by the discovery that IFN-α treatment of DCs induces expression of BAFF and APRIL, two factors which promote B cell and plasma cell survival in addition to promoting class switching (Litinskiy *et al.*, 2002). The ability of DCs to induce the secretion of class-switched antibodies was also dependent on IL-15.

In vitro-derived DCs differentiated or activated with IFN-I have an increased ability to stimulate T cell proliferation, IFN-γ production and cytotoxic function (Luft *et al.*, 1998; Paquette *et al.*, 1998; Santini *et al.*, 2000; Ito *et al.*, 2001; Buelens *et al.*, 2002; Luft *et al.*, 2002; Padovan *et al.*, 2002; Nagai *et al.*, 2003), although there have been reports to the contrary (Bartholome *et al.*, 1999; McRae *et al.*, 2000). In addition, DCs from IFN-IR$^{-/-}$ mice are less efficient stimulators of CD4^{+} and CD8^{+} T cell proliferation (Montoya *et al.*, 2002). The ability of CpG DNA to induce cross-priming and IFN-γ production against a soluble antigen *in vivo* was found to be partially dependent on IFN-I (Van Uden *et al.*, 2001; Cho *et al.*, 2002). In addition, cross-priming induced by LCMV infection was abolished in IFN-IR$^{-/-}$ mice (Le Bon *et al.*, 2003), while co-injection of IFN-α and ovalbumin resulted in antigen-specific CD8^{+} T cell proliferation, IFN-γ production and cytotoxicity through a mechanism that was independent of CD4^{+} T cells and CD40 licensing. Notably, the CD8^{+} T

cells primed in the presence of IFN-I are functional *in vivo*, since co-injection of IFN-α and ovalbumin was shown to result in protection against a recombinant vaccinia virus expressing ovalbumin. Similar to findings on the induction of antibodies by IFN-I, IFN-I could act directly on DCs to induce cross-priming, since wild-type DCs treated with IFN-I and injected into IFN-IR$^{-/-}$ resulted in antigen-specific IFN-γ production.

1.3.8.5 Conclusions

The relationship between IFN-I and DCs acts as a bridge between the innate and adaptive immune systems. Pathogen-associated molecules can induce large amounts of IFN-I which is essential for the full maturation of DCs during certain infections. In addition, DCs themselves are a major source of IFN-I. The presence of IFN-I during DC maturation may affect the outcome of the immune response by conditioning the DCs to become Th1 inducers. Alternatively IFN-I could simply act to augment DC maturation thereby promoting a stronger immune response. It is apparent that IFN-I influences many facets of DC biology: regulation of cell surface molecules which mediate migration or interactions with T cells, chemokines which attract T cells, and the release of cytokines which act on T and B cells. Altogether, these result in promotion of the adaptive immune response. Further knowledge of these mechanisms may be applied in the development of vaccines and therefore the DC-IFN-I relationship is worthy of detailed investigation.

1.4 Objectives

Our interest in the effect of IFN-I on DCs stemmed from experiments in which *in vivo* administration of either poly(I:C) or IFN-I itself were shown to enhance both antibody production and isotype switching in response to antigen (Le Bon *et al.*, 2001). IFN-I was shown to be acting directly on DCs in stimulating the increase in titers of all four IgG subclasses specific for co-injected antigen. As discussed, IFN-I also enhances the ability of DCs to stimulate $CD4^+$ and $CD8^+$ T cell proliferation, as well as promoting cross-priming *in vivo* (Le Bon *et al.*, 2003).

It is clear that IFN-I plays an important role in modulating several aspects of DC function. In order to investigate this further, we decided to study how gene expression in DCs is affected by treatment with IFN-I. We hoped to identify genes involved in the adaptive immune response, specifically, genes encoding co-stimulatory molecules, cytokines, chemokines or their receptors.

The aims of this study were to;

1) identify genes regulated by IFN-I in murine DCs
2) verify regulation of genes by IFN-I using methods such as quantitative-PCR
3) functionally characterise novel genes with potential roles in DC biology by examining protein expression and test functional properties using assays appropriate for their predicted function

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Type-I IFN

2.1.1.1 IFN-α/β

IFN-α/β was obtained from Filippo Belardelli (Istituto Superiore di Sanita, Rome) and had been prepared in the C243-3 cell line following a method adapted from Tovey et al. (Tovey *et al.*, 1974). Briefly, confluent cells were primed by the addition of 10U/ml IFN in Eagle minimum essential medium (MEM) containing 10% FCS and 1mM sodium butyrate. After 16 hours of culture at 37^0C, C243-3 cells were infected by Newcastle disease virus in MEM plus 0.5% FCS plus 5mM theophylline. At 18 hours after infection, culture supernatant was collected and centrifuged at 1500 rpm for 10 minutes. The supernatant was adjusted to pH 2.0 and kept at 0^0C for six days. IFN was concentrated and partially purified by ammonium sulphate precipitations and dialysis against PBS, followed by further dialysis for 24 hours at 4^0C against 0.01 M percloric acid and then against PBS, before being tested for any possible residual toxicity on a line of L1210 cells resistant to IFN. These partially purified IFN preparations had a titer of at least 2 x 10^7 U/mg protein and were endotoxin free, as assessed by the *Limulus* amebocyte assay. The IFN was constituted of 75% IFN-β and 25% IFN-α as evaluated by neutralisation assays using mouse antibodies to IFNs (Belardelli *et al.*, 1987).

2.1.1.2 IFN-α4

IFN-α4 was obtained from Cornelia Rossmann (Acambis, Cambridge, UK) and Dirk Gewert (Biolauncher, Cambridge, UK). Briefly, the mouse gene encoding IFN-α4 was cloned into the pEE12 plasmid (Celltech, Slough, UK). After amplification in *E.coli*, pEE12 encoding

IFN-α4 DNA was used to transfect NSO mouse myeloma cells. A single colony of cells expressing high levels of IFN-α4 was selected, and grown for 10-15 days in serum-free medium supplemented with Cholesterol Lipid Concentrate (Invitrogen, Paisley, UK). IFN-α4 content of the supernatant was assayed by inhibition of the cytopathic effect of vesicular stomatitis virus on L cells. The IFN-α4 had an activity of 2×10^6 U/ml.

2.1.2 Equipment

Item	Supplier
Eppendorf 5417R centrifuge	Eppendorf
Allegra 6R centrifuge	Beckman
Storm 860 phosphorimager	Molecular Dynamics
MoFlo MLS	DakoCytomation, Colorado
FACSCaliber flow cytometer	Becton Dickenson, UK
MiniMACS, MidiMACS Cell Separation Units	Miltenyi Biotech, UK
ABI prism 377 DNA sequencer	Perkin-Elmer
ABI prism 7700 sequence detector	Perkin-Elmer
GeneAmp 9700 PCR thermal cycler	Perkin-Elmer
SpectraMAX 340, 96-well plate spectrophotometer	Molecular Devices, UK
UV Stratalinker 2400	Stratagene
Gene Pulser II	Biorad
Dynabead magnetic particle concentrator	Dynal
Agilent 2100 Bioanalyser	Agilent
GeneChip fluidics station 450	Affymetrix
GeneChip scanner 2000	Affymetrix

2.1.3 Chemicals

Chemical	Supplier
Acetonitrile	Sigma
Ammonium acetate	BDH AnalaR
Ammonium sulphate	Sigma
B-mercapto ethanol	Sigma
Bromophenol blue	Sigma
Chloroform	Sigma
Chloroform/isoamyl alcohol (24:1)	Sigma
Dithiothreitol (DTT)	Invitrogen
Ethanol	Sigma
Ethanolamine	sigma
Glacial acetic acid	Sigma
Glycerol	BDH AnalaR
Glycine	Sigma
Isopropanol	Sigma
Magnesium chloride	Sigma
Methanol	BDH AnalaR

Chemical	Supplier
Milk powder	Tesco
Mineral oil	Sigma
NP40 (RIPA buffer)	Sigma
Paraformaldehyde	Sigma
Phenol/chloroform/isoamyl alcohol(25:24:1)	Sigma
Potasium chloride	Sigma
Tris HCl/base	Sigma
Tween-20	Sigma
Saponin	Sigma
Sodium acetate	Sigma
Sodium carbonate	Sigma
Sodium (H) carbonate	Sigma
Sodium chloride	Sigma
Sodium Dodocyl sulphate	Gibco
Sodium deoxycholate	Sigma
Sodium hydroxide	Sigma

2.1.4 Tissue culture and cell isolation reagents

Reagent	Supplier
Antibiotic-antimycotic solution	Gibco
Dimethyl sulphoxide	Sigma
Phosphate buffered saline	Gibco
Feotal Bovine Serum (FBS)	Gibco
Feotal Bovine Serum (endotoxin-free)	Harlan
Iscoves MOD.DMEM medium	Gibco
RPMI 1640	Gibco
Glasgow MEM	Gibco
Dulbecco's MEM	Gibco
Hanks buffered salt solution	Gibco
Bovine serum albumin	Sigma
Tryptose phosphate broth	Media services, IAH, Compton
Guinea Pig Complement	Cedarlane Laboratories
Collagenase type III, 117U/mg	Lorne Laboratories, UK
Dnase, 200-400 Kunitz U/mg)	Sigma
Nycoprep 1.077 A	Nycomed
β-mercaptoethanol	Gibco
Accutase	PAA labs
Red blood cell lysing buffer	Sigma
Recombinant mouse GM-CSF	R&D Systems
DMSO	Sigma
Dynabeads M-450 Sheep anti-Rat IgG	Dynal
FITC microbeads	Miltenyi Biotech
CD4 microbeads	Miltenyi Biotech
CD8a microbeads	Miltenyi Biotech
CD19 microbeads	Miltenyi Biotech
DX5 microbeads	Miltenyi Biotech
Plasmacytoid DC isolation kit	Miltenyi Biotech
MACS LS columns	Miltenyi Biotech
MACS MS columns	Miltenyi Biotech

2.1.5 Molecular Biology reagents

2.1.5.1 General reagents

Reagent	*Supplier*
Isolation and quantification of RNA	
Trizol reagent	Life Technologies Ltd
Glycogen	Boehringer Mannheim
RNAse-free water	Ambion
Oligotex mRNA isolation kit	Qiagen Ltd
RNase-free DNase kit	Qiagen Ltd
Ribogreen RNA quantification kit	Molecular probes
Agarose Gel Electrophoresis	
Agarose	BioRad
Ethidium bromide	Gibco
10 x Tris-Acetate-EDTA buffer	Invitrogen
6x Gel Loading Dye	Abgene
Low DNA mass ladder	Invitrogen
1kb plus Ladder	Invitrogen
PCR/RT-PCR/qPCR	
10 x PCR buffer	Invitrogen
dNTPs	Invitrogen
50mM $MgCl_2$	Invitrogen
Taq DNA polymerase	Invitrogen
Reverse transcriptase qPCR kit	Oswel Research Products, UK
Reverse-iT 1st strand synthesis kit	Abgene
Sequencing	
Big dye terminator kit	PE Applied Biosystems, UK
Long Ranger Singel Gels	Flowgen, UK
Loading buffer	PE Applied Biosystems, UK

2.1.5.2 Representational Difference Analysis

2.1.5.2.1 Buffers

5 X RT2 Second Strand Buffer: 100 mM trisHCl (pH 7.5), 500 mM potassium chloride, 25 mM magnesium chloride, 50 mM ammonium sulphate , 50 mM DTT, 250 ng/ml BSA (non-acetylated, Ambion) and 225 µl of water

5 x PCR buffer: 335 mM trisHCl, pH 8.9, 20 mM $MgCl_2$, 80 mM $(NH_4)_2SO_4$, 166 µg/ml BSA

EE x 3 buffer: 30 mM EPPS (Sigma), pH 8.0 at 20°C; 3 mM EDTA

2.1.5.2.2 Additional reagents

OligodT primer, 50 ng/µl (Promega) βNAD (Boehringer Mannheim), ATP (Invitrogen), EPPS, (Sigma)

2.1.5.3 Cloning and transfections

Reagent	Supplier
Cloning	
QIAquick PCR purification kit	Qiagen Ltd
Qiagen gel extraction kit	Qiagen Ltd
QIAgen plasmid mini/midi/maxi kits	Qiagen Ltd
QIAgen plasmid endo-free maxi kit	Qiagen Ltd
pBluescript II KS	Stratagene
pcDNA6/V5-His (C)	Stratagene
Luria broth agar plates, 100µg/ml ampicillin	Media services, IAH, Compton
Luria broth	Media services, IAH, Compton
Ampicillin	Media services, IAH, Compton
XL1-Blue-MRF' competent cells	Stratagene
One shot TOP10 competent cells	Invitrogen
Isopropylthio-β-galactoside (IPTG)	Invitrogen
X-gal	Invitrogen
FLAG mammalian N-terminal expression system	Sigma
Transfections	
Effectene	QIAgen
Blasticidin	Invitrogen
G418	Invitrogen
Mammalian cell lines	
BHK-21	
BAF/3	Gift from Mike Hubank, ICH, UK
WEHI	Gift from Mike Hubank, ICH, UK

2.1.5.4 Microarray analysis

Reagent	Supplier
RNeasy mini kit	Qiagen Ltd
cDNA synthesis system	Roche Lewes
Genechip Sample Cleanup Module	Affymetrix, UK
Enzo BioArray high yield RNA transcript labeling kit	Affymetrix, UK
Murine genome U74Av2 array	Affymetrix, UK
Murine genome 430 2.0 array	Affymetrix, UK

2.1.5.6 Southern/Western Blotting

Reagent	Supplier
Southern blotting	
20 x Sodium chloride-Sodium citrate buffer	Invitrogen
Rapid-Hyb buffer	Amersham
Hybond N+ membrane	Amersham
PCR DIG probe synthesis kit	Roche
DIG wash and block buffer set	Roche
Hyperfilm ECL	Amersham
Microarray analysis	
RNeasy mini kit	Qiagen Ltd
cDNA synthesis system	Roche Lewes
Genechip Sample Cleanup Module	Affymetrix, UK
Enzo BioArray high yield RNA transcript labeling kit	Affymetrix, UK
SDS-PAGE/Western Blot	
Biorad ready gel, 10% Tris-HCl	Biorad
Protease Inhibitor cocktail	Sigma
Phenylmethylsulphonyl flouride (PMSF)	Sigma
Tris/Glycine/SDS Buffer	Biorad
Wide range colour marker	Sigma
Hybond C Super nitrocellulose membrane	Amersham
ECL western blotting detection reagent	Amersham
Anti FLAG M2	Sigma
Anti FLAG M2	Sigma
Mouse IgG1	Amersham
ECL donkey anti-rabbit HRP linked $F(ab')^2$ fragment	Amersham

2.1.5.7 Enzymes

Enzyme	Concentration	Supplier
RNase Inhibitor	40 U/µl	Promega
DNase I (RNase-free)	10 U/µl	Roche
Superscript II	200 U/µl	Invitrogen
E. coli DNA ligase	10 U/µl	New England Biosciences
RNase H	2 U/µl	Invitrogen
E. coli DNA polymerase	10 U/µl	New England Biosciences
T4 DNA ligase	400U/µl	New England Biosciences
T4 DNA ligase	20U/µl	Promega
Taq DNA polymerase	5 U/µl	Invitrogen
PfuTurbo® DNA polymerase	2.5U/µl	Stratagene
Mung Bean Nuclease	10U/µl	New England Biosciences
Calf Intestinal Alkaline Phosphatase	1U/µl	Promega
*Dpn*II	10U/µl	New England Biosciences
*Bam*H1	10U/µl	Invitrogen
*Eco*R1	10U/µl	Invitrogen
*Hind*III	10U/µl	Invitrogen

2.1.6 Oligos

2.1.6.1 Representational Difference Analysis

All primers were synthesised by MWG-biotech, Germany

Oligo	Sequence 5'- 3'
R-12	GATCTGCGGTGA
R-24	AGCACTCTCCAGCCTCTCACCGCA
J-12	GATCTGTTCATG
J-24	ACCGACGTCGACTATCCATGAACA
N-12	GATCTTCCCTCG
N-24	AGGCAACTGTGCTATCCGAGGGAA

2.1.6.2 Sequencing

Primers were synthesised by MWG-biotech, Germany or by Sigma-genosys, UK.

Oligo	Sequence 5'- 3'
M13 –20	GTAAAACGACGGCCAGT
M13 rev	GGAAACAGCTATGACCATG
T3	AATTAACCCTCACTAAAGGG
T7	GTAATACGACTCACTATAGGGC
N-CMV-30 forward	AATGTCGTAATAACCCCGCCCCGT
C-CMV-24 reverse	TATTAGGACAAGGCTGGTGGGCAC
Gpr33 forward	CTGACTGGGAGAGCAAAGAGC
Gpr33 reverse	CCATTGCCCTAGTGTTTGATGC
CCR7 forward	CGGGATGCTGCTGCTCCTATGC
CCR7 reverse	GCATAGGAGCAGCAGCATCC

2.1.6.3 Real time PCR

Probes were obtained from Sigma-genosys, Cambridge, UK. Primers were synthesised by MWG-biotech, Germany

Name		Sequence 5' – 3'
28S	Forward	CGC CGC TAG AGG TGA AAT TCT
	Reverse	CAT TCT TGG CAA ATG CTT TCG
	Probe	ACC GGC GCA AGA CGC ACC AG
Mx1	Forward	GGA ATT ACC AGG GTG GCT GTA G
	Reverse	TGG ATG TAT GTC TTG ATA AGT CTC TTG A
	Probe	ACC AGC CTG CAG ACA TAG GAC GCC
9130002C22Rik	Forward	CAC CAA AAC CCA TGT TCT TCA A
	Reverse	AAG AGG AAT ACA CAA TCA ATA CTC CTG TT
	Probe	AAC AGA ATC AAG CCA TCA CAC AAC AGG AAA TT
AI448571	Forward	GCT AGA ACT TAG CAA AGA GAG GAC AAC
	Reverse	GGC ATG AAT ACT TAT CTG TTA GAA AGA GAA
	Probe	CCC GCC CCC ACC CCA AAT T
Mx1 Intron	Forward	GCA GAG TGA AGA ATC AAC TCT GTT GT
	Reverse	CAG TGA TGC CCC CTC TAT CAG
	Probe	ACC TCT CCA TGG CCA CCA CCC A
Slfn5	Forward	AAC ACG ACG TGA CTA CCC AGA CT
	Reverse	CGC TGA GGA GCC CAG TTT AC
	Probe	TCC AAC CCC TCC CTG ACC TGC A
Slfn4	Forward	GAG CAG AAC ATG GCT AAG GAT AAT G
	Reverse	GCC CAT CAC GAC AGT TCT GA
	Probe	CTG TGA ACA CCC AAC TCA TAG CCC CC
Gpr33	Forward	CAT CAA ACA CTA GGG CAA TGG A
	Reverse	CAG AAG GAA ACC CAG CAA GAA
	Probe	TCA TGC AGC CTG TTT CGT CGG C
Ms4a4c	Forward	CAG GAT CCC TGT CAA TTG CA
	Reverse	CAC AGA GGT GAT AGT GTT CAG AGT TAG A
	Probe	AGT GAA ACC TAC AAA AAG CCT GAT CAT CAG CA
Ms4a6B	Forward	CCC CAC TAC AAT GGC CTG AA
	Reverse	GCC AAA GCC CAG ACC TAC AA
	Probe	CAT TCT GTT TGG TTT TCT AAG AAA GGC CAC CA
IMAGE 4168084	Forward	TTA AGG GTG TCT CCT CCA ACC TT
	Reverse	GTG ACT TTT GCC TTC TTG GAT CTC
	Probe	TCT TTG TGG AGC ACA ACT TTC CTG AGC A
Ppicap	Forward	CGT CAT CAT GAG AGT GGA TGC T
	Reverse	CGA TTC TTC GGG AGT AAA AGT ACC T
	Probe	TGC ATG CCT GTC GTC AGA GAC TTC CT
CCR7	Forward	CCCAAAACGACAGCCAAAA
	Reverse	GGCCCCACATCCCTCACT
	Probe	AAAGTGAGAGGCTGCCACACTTTCCG

2.1.7 Antibodies and FACS reagents

2.1.7.1 Antibodies for FACS staining and sorting

Antibody	Flourochrome	Supplier
FACS sorting		
CD11c	FITC	BD Pharmingen
CD11b	PE	BD Pharmingen
CD4	Cy5	Made in house
CD4	PE	BD Pharmingen
CD8	Cy5	Made in house
CD19	PE	BD Pharmingen
F4/80	FITC	Serotec
NK1.1	PE	BD Pharmingen
MACS sort		
CD4	FITC	BD Pharmingen
CD11c	FITC	BD Pharmingen
FACS analysis		
CD3	PE	BD Pharmingen
CD4	PE	BD Pharmingen
CD8	Cy5	Made in house
CD11b	PE	BD Pharmingen
CD11c	FITC/PE/APC	BD Pharmingen
CD14	PE	BD Pharmingen
CD16/32 (Fc)	Purified	BD Pharmingen
CD19	PE	BD Pharmingen
CD40	Biotinylated	BD Pharmingen
CD45R (B220)	PE	BD Pharmingen
CD80	Biotinylated	BD Pharmingen
CD86	Biotinylated	BD Pharmingen
CD86	Purified	BD Pharmingen
DX5	PE	BD Pharmingen
F4/80	PE	Serotec
GR-1	FITC	BD Pharmingen
H-2D^b	Biotinylated	BD Pharmingen
I-Ab	Biotinylated	BD Pharmingen
NK1.1	PE	BD Pharmingen
Propidium Iodide		Sigma
Rat IgG (rabbit)	FITC	vector
Rat Ig	PE	BD Pharmingen
Rabbit IgG	FITC	Jackson Immunoresearch
Mouse IgG	Biotin	BD Pharmingen
Streptavidin	APC	BD Pharmingen
FLAG M2	None	Sigma
Isotype controls		
Rabbit Ig		Gift from Rob Carter

2.1.7.2 Antibodies for Dynabead Depletions

Antibody specificity	Clone name/supplier
CD2	BD Pharmingen
CD3	KT3
CD4	GK1.5
CD45R (B220)	RA36B2
CD90	T24.1
Gr-1	RB68C5
MAC-1	M1/70
MHC II	TIB120

2.1.8 Immunisations for monoclonal antibody production

Titermax Gold adjuvant, Sigma

Peptides were synthesised by Alta Bioscience, University of Birmingham

Peptide	Sequence
A1	CQNNYIVSTDWESKEB
A2	VSTDWESKEHQTLGQB
A3	ESKEHQTLGQWIHAAB

2.1.9 ELISAs

Reagent	Supplier
IL-1β Immunoassay kit	Biosource
IL-6 Immunoassay kit	Biosource
CCL2 Immunoassay kit	Biosource
CCL4 Quantikine ELISA kit	R&D
CCL5 Immunoassay kit	Biosource
CCL12 Quantikine ELISA kit	R&D
CXCL9 Quantikine ELISA kit	R&D
CXCL10 Detection antibody	R&D
CXCL10 Capture antibody	R&D
CXCL10 Protein	R&D
Anti Rat IgG - peroxidase	Jackson Immunoresearch
Anti Rat IgG – alkaline phosphatase	Jackson Immunoresearch
p-Nitrophenyl phosphate (pNPP) SIGMA FAST tablets	Sigma
o-Phenylenediamine dihydrochloride (OPD) SIGMA FAST tablets	Sigma

2.1.10 Calcium Flux Assay

Ionomycin (Sigma), Indo-1 AM (Molecular Probes, Netherlands)

Peptide	Sequence	Supplier
A5	SLLWLTCRPWEAM	
Annexin 1 (Acl-25)	Ac-AMVSEFLKQAWFIEN EEQEYVQTVK	
HIV-derived F	EGSDTITLPCRIKQFINMWQE	
HIV-derived T20	Ac-YTSLIHSLIEESQNQQEKN EQELLELDKWASLWNWF-NH2	
HIV-derived V3	RIHIGPGRAFYTTKN	
Humanin	MAPRGFSCLLLLTSEIDLPVKRRA	Open Biosystems
I4G10-C	FLPIIASLLGKLL-NH2	
I4S10-C	FLPIIASLLSKLL-NH2	
MMK-1	LESIFRSLLFRVM	
Rana-6	FISAIASMLGKFL-NH2	
T1P	FLPIVGKLLSGLL-NH2	
TA	FLPLIGRVLSGIL-NH2	
W	WKYMVm	
Amyloid β protein	DAEFGHDSGFEVRHQKLVFFAEDVGSNKGAIIG LMVGGVVIA	Bachem AG
Prion protein (106-126)	KTNMKHMAGAAAAGAVVGGLG	Bachem AG
Chemerin (human)	NCBI: Y14838	R&D systems
Chemerin (mouse)	NCBI: NP_082128	R&D systems
fMLF	Formyl-MLF	Sigma

2.2 Methods

2.2.1 Animals and immunisations

2.2.1.1 Mice

C57/Bl6 mice were obtained from the specific pathogen-free unit at the Institute for Animal Health (Compton, UK) or from Harlan UK Ltd (Blackthorn, UK). 200µl of phosphate buffered saline (PBS) or PBS containing 10^5 units IFN-α4 was injected intravenously. Mice were kept under humane conditions at all times and the number of mice used was kept to the minimum necessary for each experiment. Mice were sacrificed by asphyxiation by rising concentrations of carbon dioxide.

2.2.1.2 Rats

Female Lewis rats obtained from Charles River-UK were immunized intraperitoneally or subcutaneously and sacrificed by asphyxiation by rising concentrations of carbon dioxide.

2.2.2 Isolation and purification of murine cell types

2.2.2.1 Splenic Dendritic Cell Isolation

2.2.2.1.1 Digestion and release of DCs

C57/Bl6 spleens were injected with 1ml 1mg/ml collagenase, 0.5mg/ml DNase in RPMI-2.5% FCS and incubated at 37^0C for 25 minutes. The digested spleens were then pushed through a 70µ cell strainer and washed with RPMI-2.5% FCS-2mM EDTA.

2.2.2.1.2 Selection of low density cells

Cells resuspended in 3ml of Nycoprep were layered onto 3ml of Nycoprep. 1ml of FCS was then layered on top and the samples centrifuged at 2800rpm for 25 minutes at room

temperature. The low density fraction was collected and the cells washed with RPMI-2.5% FCS-2mM EDTA.

2.2.2.1.3 CD11c$^+$ DC Isolation

The cells were stained with CD11c-FITC, washed with RPMI-2.5% FCS -2mM EDTA and incubated with anti-FITC MACS beads, washed again and resuspended in 500ul medium/100 million cells. CD11c$^+$ cells were then selected by running through a MACS column and further purified either through a second MACS column or by FACS sorting. Cells purities were routinely >99% CD11c$^+$.

2.2.2.2 Isolation of dendritic cell subsets

Dendritic cells subsets were isolated by one of two methods:

2.2.2.2.1 Depletion/MACS sorting

Spleens were digested with collagenase/DNase and the low density cells enriched as described for the CD11c$^+$ DC isolation. The cells were incubated with a cocktail of rat antibodies specific for cell types to be depleted. This included antibodies specific for T cells (CD90, CD3), NK cells (CD2), B cells (CD45R), granulocytes (GR-1) and macrophages (F4/80). For isolation of the CD4$^-$CD8$^+$ and CD4$^+$CD8$^-$ subsets, anti-CD4 and anti CD8- antibodies were added respectively. The cells were then incubated with sheep anti-rat magnetic beads (Dynal) for 30 minutes at 4^{0}C with rotation. Unwanted cells were then removed by two rounds of contact with a magnetic particle concentrator (Dynal). The cells then went through two rounds of positive selection by MACS, using either CD4 or CD8 MACS beads, following the manufacturers instructions (Miltenyi Biotech). To purify the CD4$^-$CD8$^-$ DC subset, cells which eluted in the negative fractions were positively selected for CD11c expression by MACS sorting. Cell purities were as follows CD11c$^+$CD8$^+$CD4$^-$, 88%, CD11c$^+$CD8$^-$CD4$^+$, >82%, CD11c$^+$CD8$^-$CD4$^+$, >87%

2.2.2.2.2 MoFlo sorting

RDA: MACS sorted $CD11c^+$ DCs were stained with CD11b-PE and sorted on expression of CD11b and CD11c. Isolated cells were >99% $CD11c^+$ and >92% $CD11b^{+/-}$.

Taqman analysis: $CD11c^+$ DCs were stained with CD4-PE and CD8-Cy5 and sorted by FACS on a MoFlo cytometer. The cells were sorted on CD11c expression as well as on CD4 and CD8 expression. Cell purities were as follows $CD11c^+CD8^+CD4^-$, >90%, $CD11c^+CD8^-CD4^+$, >93%, $CD11c^+CD8^-CD4^-$, >96%.

2.2.2.3 Isolation of plasmacytoid DCs

Spleens were digested with collagenase/DNase I and the low density cells isolated. The plasmacytoid DCs were then isolated using a MACS plasmacytoid DC isolation kit (Miltenyi Biotech). This involves a depletion step, where T, B, NK cells and macrophages were labeled and removed by separation on a MACS depletion column. The CD45R positive cells in the remaining population were then labeled and purified by separation on an MS column. To achieve high purity (>90%) the separation on an MS column was repeated.

2.2.2.4 Generation of Bone Marrow-Derived DCs

Bone marrow-derived DCs (BMDCs) were isolated using method based on that of Lutz et. al. (Lutz *et al.*, 1999), and adapted by Brandt et al (Brandt *et al.*, 2003).

2.2.2.4.1 Bone marrow preparation

The femurs and tibiae of C57BL/6 mice were removed and as much flesh as possible cleaned using a scalpel. The ends of the bone were then cut and the marrow flushed out with BMDC media (RPMI containing 10% low-endotoxin FCS, 100 units/ml penicillin, 100μg/ml streptomycin and 0.5mM β-mercaptoethanol) using a 25 gauge needle. The cells were pipetted to obtain a homogenous cell suspension, then filtered through a 70μ strainer and

washed. The red blood cells were lysed for 2 minutes on ice with red blood cell lysis buffer (Sigma) and remaining cells filtered through a 40μ strainer.

2.2.2.4.2 Bone Marrow Cell Culture

Cells were plated into petri dishes (Falcon 1029) at 2 x 10^6 cells/dish in 10ml BMDC media containing 20ng/ml recombinant mouse granulocyte macrophage colony stimulating factor (rm-GM-CSF)(R&D systems). At day three, 10ml BMDC media plus 20ng rm-GM-CSF was added to the dishes. At day six, 10ml of medium was removed from the dishes, centrifuged, and resuspended in 10ml BMDC media plus 10ng/ml rm-GM-CSF before returning to the dish. At day 8 the non-adherent cells were removed. 2ml of accutase (PAA labs) was added to the adherent cells and incubated for 15 minutes at 37^0C. Cells were collected by thorough pipetting.

2.2.2.4.3 Bone marrow DC purification

Adherant bone marrow culture cells were depleted of granulocytes, T and B cells. They were first incubated with rat anti-Gr-1, -B220 and –CD3, washed and incubated with sheep anti-rat magnetic beads (Dynal). Unwanted cells were then removed by two rounds of contact with a magnetic particle concentrator (Dynal). After depletion cells were >95% $CD11c^+$.

2.2.2.5 Isolation of T cells and B cells

Cell suspensions were made in RPMI containing 2.5% FBS by disrupting lymph nodes and spleens from C57BL/6 mice using glass slides. The cell suspensions were incubated with antibodies specific for cell types to be depleted (Table 2.1), followed by incubation with sheep anti-rat magnetic beads (Dynal) at 4^0C with rotation. Unwanted cells were then removed by two rounds of contact with a magnetic particle concentrator (Dynal). The cells

were then either stained with the appropriate antibodies or MACS microbeads and sorted by FACS on a MoFlo cytometer or by MACS sorting respectively. Cell purities of cells isolated from the spleen were as follows: $CD4^+$ T cells, >90%, $CD8^+$ T cells >97% ,B cells, >98%, and from the lymph nodes: $CD4^+$ T cells, >92%, $CD8^+$ T cells >99%, B cells, >99%.

Table 2.1 Staining of cells for negative depletion and sorting

	Negative Depletion		Sorting	
Cell Type	**Specificity**	**Ab Clone**	**Staining for MoFlo sort**	**Staining for MACS sort**
CD8 T cells	MHC II B220 MAC-1 CD4	TIB120 RA36B2 M1/70 GK1.5	CD8-Cy5	CD8a microbeads
CD4 Tcells	MHC II B220 MAC-1 CD8	TIB120 RA36B2 M1/70	CD4-Cy5	CD4-FITC, FITC microbeads
B cells	MAC-1 CD4 CD8	M1/70 GK1.5	CD19-PE	CD19 microbeads

2.2.2.6 Isolation of macrophages

Peritoneal lavages from C57BL/6 mice resuspended in RPMI/2.5% FCS were plated in petri dishes (Falcon 1029) for 3 hours at 37^0C. After this time, cells still in suspension were discarded and adherant cells harvested using a cell scraper. The cells were then stained with F4/80-FITC and sorted on a MoFlo cytometer. Cell purities were greater than 99%.

2.2.2.7 Isolation of natural killer cells

Spleens were digested by DNase/collagenase (as in 2.2.2.1.1) followed by incubation in red blood cell lysis buffer (Sigma) for two minutes at room temperature. The cells were then incubated with DX5 MACS beads and positively selected on a MACS LS column according

to the manufacturers instructions (Miltenyi Biotech). The cells were then stained with NK1.1 PE and sorted on a MoFlo cytometer. Cell purities were greater than 96%.

2.2.3 Treatment of ex vivo isolated cells with type-I IFN

2.2.3.1 Splenic DCs

3-5 x 10^6 cells/ml of purified DCs were cultured in 12ml Sterilin tubes in RPMI-10% low endotoxin FBS, either alone or with 20,000 U/ml IFN-α/β or IFN-α4 at 37^0C in tissue for 2-24 hours.

2.2.3.2 BM DCs

4 x 10^6 cells/ml of purified DCs were cultured in 12ml Sterilin tubes in RPMI-10% low endotoxin FBS, either alone or with 20,000 U/ml IFN-α4 at 37^0C in tissue culture tubes for 2-24 hours.

2.2.3.3 T, B cells

5- 10 x 10^6 cells/ml were cultured in RPMI-10% FCS alone or with 20,000 U IFN-α4/ml in tissue culture tubes (Sterilin), for two hours at 37^0C.

2.2.4 FACS staining

2.2.4.1 Cell surface staining

FACS staining was carried out in 96-well U-bottomed plates. 2 x 10^5 cells were washed in FACS buffer (PBS, 2.5% FCS) and vortexed gently to resuspend the cell pellet. Fc receptors were blocked for 10 minutes on ice, then the cells were washed with FACS buffer. 15μl of diluted antibodies were added before incubating on ice in the dark for 15 minutes.

Cells were again washed before addition of any secondary antibodies which were incubated on ice for 15 minutes. Cells were washed and resuspended in PBS before FACS acquisition. 1 x 10^4 cells were acquired and analysed using CellQuest Pro software (BD Pharmingen).

2.2.4.2 Propidium Iodide staining

Propidium iodide was added to the cells at a final concentration of 2.5μg/ml immediately before FACS acquisition.

2.2.4.3 Intracellular staining

After blocking the Fc receptors cells were washed with PBS and fixed in 2% paraformaldehyde diluted in PBS for 20 minutes on ice. The cells were washed with FACS buffer and made permeable in FACS buffer containing 0.1% saponin for 10 minutes at room temperature. The cells were pelleted and resuspended in antibody diluted in FACS buffer containing 0.1% saponin and incubated for a further 10 minutes at room temperature. The cells were washed in FACS buffer before FACS aquisition.

2.2.4.4 Rat Gpr33 antibody FACS staining

Gpr33 transfected cell lines or DCs (pre-blocked with mouse sera) were incubated with supernatants from the poly- or monoclonal hybridomas. The anti-Gpr33 antibodies were then detected using anti-rat IgG antibodies.

2.2.4.5 Rabbit Gpr33 antibody FACS staining

After blocking the Fc receptors Gpr33 transfected cell lines or DCs were incubated with the purified antibodies raised against the Gpr33 N-terminus (Pacific Immunology). The anti-Gpr33 antibodies were then detected using anti-rabbit IgG-FITC.

2.2.5 Molecular biology techniques

2.2.5.1 RNA isolation

Total RNA was isolated from up to 10^7 cells using Trizol reagent (Invitrogen) following the manufacturers instructions. During the precipitation step, 1 µl of 10 mg/ml glycogen was added as a co-precipitant. The dried pellets were resuspended in RNase-free water. In some cases, RNA was further purified using the RNeasy mini kit (QIAgen), residual DNA was digested on column using the RNase-free DNase kit (QIAgen). The integrity of total RNA was determined by agarose gel electrophoresis and was quantified using the Ribogreen RNA quantification kit (Molecular probes).

2.2.5.2 Representational Difference Analysis (RDA)

RDA was carried out as previously described (Hubank *et al.*, 1999) with minor modifications.

2.2.5.2.1 Isolation of mRNA and preparation of double stranded cDNA

Residual genomic DNA was removed by digestion of total RNA with DNase I before isolation of polyA+ RNA using the Oligotex mRNA isolation kit (QIAgen). The mRNA was reverse transcribed using Superscript II and oligo dT primers, followed by second strand synthesis by *E. coli* DNA polymerase.

2.2.5.2.2 Generation of Representations

Double stranded cDNA was digested with *Dpn*II and ligated to R-adaptors using T4 DNA ligase overnight at 14 °C. Pilot reactions were performed with the R-24 primer under the following cycle conditions: 72°C for 3 min, *Taq* DNA polymerase added, 72°C for 5 min, then 26 cycles: 95°C for 1 min and 72°C for 3 min with final extension at 72°C for 10 min.

After 20, 22, 24 and 26 cycles the reactions were paused and 10 µl aliquots of the product were removed for analysis on a 1.5 % agarose gel. A cycle number that produced a smear ranging in size from 0.2 – 1.5 kb, and containing approximately 0.5 µg of DNA in the 10 µl aliquot was selected for subsequent reactions. For each sample sixteen 100 µl reactions were set up, and the PCR run using the appropriate number of cycles. The reactions were extracted with twice with phenol/chloroform/isoamyl alcohol (P/C/I) and once with chloroform/isoamyl alcohol (C/I). Samples were then precipitated on ice for 20 minutes with one tenth volume 3M sodium acetate (pH 5.3) and one volume of isopropanol. DNA was pelleted, washed with 70% ethanol and resuspended in TE buffer (10mM Tris, 1mM EDTA, pH 8) at a concentration of 0.5mg/ml.

2.2.5.2.3 Generation of Tester and Driver

90 µg (180 µl) of each representation was digested with *Dpn*II, extracted with P/C/I and C/I, precipitated and resuspended in TE at 0.5 mg/ml, to form the driver. The R-adaptors were removed from the final digested product using QIAquick spin columns (QIAgen). 1 µg of spin column-purified DNA was ligated to J-adaptors overnight using a ligation temperature of 12^0C. The ligation was then diluted to 10 ng/µl to generate the J-ligated tester.

2.2.5.2.4 Subtractive hybridisation

20 µl (10 µg) of digested driver representation was mixed with 10 µl of diluted, J-ligated tester representation (0.1 µg), a driver:tester ratio of 100:1. The samples were P/C/I and C/I extracted, and precipitated with 10 M ammonium acetate and 100% ethanol at -70 °C for 10 min. The pellets were washed twice with 70% ethanol and resuspended very thoroughly in 4 µl of EE x 3 buffer and the solution overlaid with 35 µl of mineral oil. The DNA was then denatured for 5 min at 98°C, cooled to 67°C, and immediately 1 µl of 5 M sodium chloride

was added directly into the DNA. The samples were hybridised at 67°C for 20 hours, after which they were diluted to 100µl with TE.

2.2.5.2.5 Generation of the first difference product (DP1)

The subtractions were then amplified by PCR using the following cycle: 72 °C for 3 min, *Taq* DNA polymerase added, 72 °C for 5 min, J-24 primer added, then 11 cycles: 95 °C for 1 min and 70 °C for 3 min, final extension at 72 °C for 10 min. After extraction and precipitation, the PCR products were resuspended in 20µl of TE and digested with Mung Bean Nuclease (MBN) (NEB) to eliminate single stranded DNA.

Amplification to DP1: PCR reactions were set up, using the MBN treated DNA as a template, this time the J-24 primer was added to the master mix. PCR cycle; 95°C for 1 min, cool to 80 °C before addition of Taq DNA polymerase, followed by 18 cycles of 95°C for 1 min and 70°C for 3 min with final extension at 72°C for 10 min. 10 µl of the samples were run on a 1.5% agarose gel next to standards to confirm the presence of products and to estimate the concentration. The samples were extracted with P/C/I and C/I, precipitated and resuspended at 0.5 µg/µl to form the first difference product (DP1).

2.2.5.2.6 Generation of the second difference product (DP2)

To remove the J-adaptors, 2 µg of the difference product was digested with *Dpn*II. 200ng of the digested difference product was then ligated to N-adaptors using T4 DNA ligase at 14^0C. This ligation was then diluted to 1.25 ng/µl. 10 µl (12.5 ng) of N-ligated DP1 was mixed with 20 µl (10 µg) of driver (a driver:tester ratio of 800:1), and the subtraction hybridisation procedure carried out as before. Amplification to a second difference product was carried out as for the first difference product , except using N-primers in place of J-primers and using

an annealing temperature of 72^{0}C. After extraction and precipitation the pellets were resuspended at 0.5 μg/μl in TE to form the DP2.

2.2.5.2.7 Generation of the third difference product (DP3)

The DP2 was digested, ligated to J adaptors and diluted to 1 ng/μl. Hybridisations were set up using driver to tester ratios between 5,000:1 and 40,000:1. The DP3 was generated as before, performing the final amplification for 23 cycles.

2.2.5.3 Cloning

2.2.5.3.1 Cloning of RDA products

*Dpn*II digested RDA products were ligated into the pBluescript II KS vector digested with *Bam*HI, and used to transform XL1-Blue MRF' supercompetent cells.

2.2.5.3.2 Cloning of Gpr33

Primers were designed against Gpr33 cDNA (NCBI Accession NM_008159). The forward primer was engineered to contain a *Hind*III site (5' AGA CAA **AGC TT**T GAG GTA ATT ATG GAT TTG ATC AAC TCC 3') the reverse primer contains an *Eco*R1 site (5' GGA ACT **GAA TTC** CAA TTG AAG AAC AGC AAG GAT TTC TTA 3'). Gpr33 was amplified from IFN-α/β treated DCs cDNA using Pfu Turbo DNA polymerase (Stratagene), digested with *Hind*III and *Eco*R1 and ligated into the pcDNA6/V5-His/ABC vector (Invitrogen). The ligated plasmid DNA was used to transform One shot TOP10 competent cells (Invitrogen). Plasmid DNA containing the correct Gpr33 sequence was used to transfect various mammalian cell lines including BHK-21. Stable transfectants were generated by selection in 10ug/ml blasticidin (Invitrogen).

2.2.5.3.3 Cloning of FLAG-Gpr33/CCR7 constructs

The forward primers were engineered to contain a *Hind*III site (Gpr33) or an *Eco*RI site (CCR7), additional bases were added to allow translation in frame with the N-terminal FLAG tag when cloned into the pFLAG-CMV-4 vector (Sigma). Between the *Hind*III site and the start codon of Gpr33 we modified a stop codon to encode glycine, and changed a hydrophobic isoleucine to asparagine. The CCR7 reverse primer was engineered to contain a *Bam*HI site, whilst a BamHI site exists in Gpr33 shortly after the stop codon. Primers sequences were as follows: Gpr33 forward 5'-TTA CTT **AAG CTT** GGA GGT AAT ATG GAT TTG ATC AAC TCC-3', Gpr33 reverse 5'- GGA ACT GAA TTC CAA TTG AAG AAC AGC AAG GAT TTC TTA-3', CCR7 forward 5'-CTG TGT **GAA TTC** TTA CAG CCC CCA G-3', CCR7 reverse 5'-CAC ATT AA**G GAT CC**T GGG AGA GGT C-3'. Gpr33 was amplified from previously cloned cDNA in the pcDNA6 vector, CCR7 was amplified from $CD8^+$ T cell cDNA. The products were digested and ligated into pFLAG-CMV-4 and used to transform competent bacteria. Plasmid DNA containing Gpr33 or CCR7 cDNA of the correct sequence was used to transfect Baf/3 cells.

2.2.5.3.4 Vector and Insert Preparation

The vector and PCR product to be cloned were digested with the appropriate restriction enzyme(s). If necessary, the vector was treated with calf alkaline phosphatase to prevent the ends re-ligating and extracted once with P/C/I. The vector was purified by running on a 1% agarose gel and DNA recovered by gel extraction. The digested vector and PCR product were then precipitated with 2.5 volumes ethanol and 0.1 volume 3M sodium acetate (pH 5.3).

2.2.5.3.5 Ligation and transformation

Ligations were carried out using T4 DNA ligase (Promega), incubating at room temperature for at least 2 hours. 10 – 100ng of DNA was used to transform XL1-Blue MRF' or TOP10

cells following the manufacturer's protocol (Stratagene/ Invitrogen). The cells were plated onto Luria broth (LB)- agar plates containing 100μg/ml ampicillin (for blue-white colour screening a solution containing 2% X-gal and 40mM IPTG was first spread onto the plates), and incubated overnight at 37^0C. Colonies were screened for the correct inserts by PCR using M13 primers or by restriction digest.

2.2.5.3.6 Preparation of plasmid DNA

Luria broth containing 100μg/ml ampicillin was inoculated with a single colony and incubated overnight at 37^0C , 225 rpm. Plasmid DNA was isolated using QIAgen plasmid mini, midi or maxi kits following the manufacturers instructions.

2.2.5.4 Transfection of mammalian cell lines

2.2.5.4.1 Transfection of the cell line BHK-21

Cells were cultured in Glasgow MEM media containing 7% FBS, 100 units/ml penicillin, 100μg/ml streptomycin and 10% tryptose phosphate broth. Cells were seeded the day before transfection and were 40-80% confluent at the time of transfection. 1μg of the pcDNA6 plasmid (Invitrogen) containing the Gpr33 gene or a control plasmid containing the green flourescent protein gene were transfected into BHK-21 cells using Effectene (QIAgen) according to the manufacturers instructions. BHK-21 cells containing the pcDNA6 plasmid were selected by culturing in medium containing 10μg/ml blasticidin.

2.2.5.4.2 Transfection of the cell line BAF/3

The IL-3 dependent cell line Baf/3 was grown in RPMI1640 containing 10% FBS, 10% WEHI-3B (an IL-3 producing cell line) conditioned media. 20μg DNA was used to transfect 5 x 10^6 cells by electroparation at 260V, 950μF on a Gene Pulser II (Biorad). 24 hours later selection in 1mg/ml G418 was started to generate stable transfectants.

2.2.5.5 RT-PCR

Single-stranded cDNA was synthesised using the reverse-iT first strand synthesis kit following manufacturer's instructions (Abgene). To determine expression of Gpr33 in various cell lines, PCR was carried out using the following primers: Gpr33 forward 5'-GTT TCC TTC TGC CTT TCC-3', reverse 5'-TCA CTG AGC CAT CTC TCC-3' and β-actin forward, 5'-GCC AAC CGT GAA AAG ATG ACC-3', reverse 5'-CGT ACT CCT GCT TGC TGA TCC-3'.

2.2.5.6 Sequencing

Reactions were carried out using the Big Dye Terminator kit (PE applied biosystems) and T3 primers, cycling: 90^0C for 10 minutes, 25 cycles of 50^0C for 5 seconds and 60^0C for 4 minutes. Samples were loaded onto Long Ranger Singel gels (Flowgen) and run on an ABI377 automatic sequencer. Alternatively, samples were air-dried and shipped to MWG for sequencing.

2.2.5.7 Southern blotting

2.2.5.7.1 Transfer of DNA to nylon membranes

DNA was separated by agarose gel electrophoresis. The DNA was denatured by soaking the gel in 1.5M sodium chloride, 0.5N sodium hydroxide for 45 minutes and then neutralised in 1M Tris (pH 7.4), 1.5M sodium chloride for 45 minutes. The DNA was transferred onto Hybond N nylon membranes (Amersham) by capilliary action in 10x sodium chloride-sodium citrate buffer (SSC). After transfer the membrane was rinsed in 6x SSC, dried and the DNA fixed by UV irradiation in a UV Stratalinker 2400 (Stratagene).

2.2.5.7.2 Generation of DIG labeled probes

PCR was carried out on pBluescript plasmids containing the cloned sequences of interest using T3 and T7 primers and the PCR DIG probe synthesis kit (Roche) according to the manufacturers instructions.

2.2.5.7.3 Hybridisation and detection of bound probes

Nylon membranes were pre-hybridised in Rapid-Hyb buffer (Amersham) at 60^0C for 30 minutes, then hybridised for 2.5 hours at 60^0C with probes diluted 1:1000 in Rapid-Hyb buffer. Membranes were washed twice in 2x SSC, 0.1% SDS at 60^0C for five minutes followed by two washes in 0.1x SSC, 0.1%SDS. Membranes were processed using the DIG wash and block buffer set (Roche): equilibrated in washing buffer for one minute and then blocked for one hour at room temperature. Membranes were incubated for 30 minutes with anti-DIG antibody diluted 1:20,000 in blocking solution then washed several times in washing buffer. Chemiluminescent detection was by CPD-Star diluted 1:50 in detection buffer (Roche). The membranes were exposed to chemiluminescent film for varying durations.

2.2.5.8 Western blotting

2.2.5.8.1 Protein lysates

1 x 10^7 cells were lysed in 1ml of a) RIPA buffer (PBS, 1% v/v NP40, 0.1% (w/v) SDS, 0.5% (w/v) sodiumdeoxycholate containing 200 μg/ml PMSF (Sigma) and a 1:500 dilution of a protease inhibitor cocktail (Sigma) or b) lysis buffer B (50mM Tris pH 7.4, 150 mM sodium chloride, 1mM EDTA 1% Triton X-100) containing a 1:500 dilution of a protease inhibitor cocktail. The samples were rotated at 4^0C for at least two hours. Cell debris was removed by centrifugation and the supernatants stored at -20^0C.

2.2.5.8.2 Immunoprecipitations

FLAG-tagged proteins from tranfected cells lysed with lysis buffer B were immuno-precipitated using the anti-FLAG M2 affinity gel (Sigma) according to the manufacturers instructions. The Met-FLAG-BAP control protein was immunoprecipitated in parallel.

2.2.5.8.3 SDS-PAGE and Western Blotting

Samples were separated by denaturing gel electrophoresis and transferred onto a Hybond C membrane. The membranes were blocked in TBS-T/10% dried milk powder for 1 hour. The membranes were incubated with primary antibody for 2 hours, then secondary biotinylated antibody for 1 hour and strepavidin-HRP for one hour, all in TBS-T/1% marvel. The membranes were washed 3 times with TBS-T after each step and all steps were done at room temperature. The detection was done using an ECL detection kit (Amersham pharmacia) and exposure to X-ray film. Detection of FLAG fusion proteins was carried using the method detailed by the manufacturers (Sigma) using the anti-FLAG M2 antibody.

2.2.5.9 Real-Time (quantitative)PCR

Probes and primers were designed using Primer Express 1.0 software (PE Applied Biosystems). We applied additional rules not included in the software: primers containing more than two cytosines or guanines in the five nucleotides at their 3' end were excluded. Probes with an initial 5' guanine (which would act as a quencher) or containing more guanines than cytosines were excluded. The PCR was carried out as a one step reaction using the Reverse Transcriptase qPCR kit from Eurogentec. Probes were obtained from Sigma-Genosys labeled 5' with the reporter fluorophore 6-carboxy-fluorescein (FAM) and 3' with the reporter fluorophore 6-carboxy-N,N,N',N'-tetramethylrhodamine (TAMRA). The probes were used at a final concentration of 100nM, while primer concentrations were

optimized and were between 0.8 and 1.2μM (Table 2.2). Cycling was carried out on an ABI prism 7700 Sequence Detector (PE Applied Biosystems) as follows: cDNA synthesis for 30 minutes at 48^{0}C, hot GoldStar activation for 10 minutes at 95^{0}C followed by 40 cycles of 15 seconds at 95^{0}C and 1 minute at 60^{0}C. Quantification of RNA was based on the cycle number (C_t) at which the change in level of fluorescence from the reporter dye passed a significance threshold. Standard curves were generated using 10-fold dilutions from 10^{-1} to 10^{-5} of total RNA extracted from splenic DCs treated in vitro with type I IFN. To correct for differences in RNA between samples and for the efficiency of the reactions the following equation was used, where m is the slope of the standard curve and DF is the difference factor:

$$\frac{((40- Ct) \times m_{geneX})}{(m_{28S} \times DF)}$$

The DF was calculated by dividing the mean of the 28S C_t for the samples by the mean 28S C_t for all samples. Fold changes were calculated in one of two ways, for chapter 3 and 4 the following equation was used:

$$\frac{(Ct_{sample} - Ct_{control})*10}{m_{genex}}$$

For chapter 5 fold changes were calculated as the difference in corrected C_t values to the log of base 2.

Table 2.2 Taqman primer concentrations and reaction efficiencies

Name	Primer concentration (μM)	m (slope of standard curve)
28S	0.6	3.57
Mx1	0.2	3.87
9130002C22Rik	0.8	2.98
AI448571	0.8	4.80
Mx1 Intron	0.6	3.82
Slfn5	0.2	3.79
Slfn4	0.6	3.26
Gpr33	0.6	4.12
Ms4a4c	0.1	4.62
Ms4a6B	0.2	4.54
IMAGE 4168084	0.6	4.57
Ppicap	0.1	4.20
CCR7	0.6	3.81

2.2.6 Microarray Analysis

2.2.6.1 Processing of samples

2.2.6.1.1 cDNA synthesis

Total RNA was isolated using Trizol reagent. The RNA was quantified and the quality checked on an agarose gel. RNA was then purified using a QIAgen RNeasy kit. cDNA was synthesized using a cDNA synthesis system (Roche) which includes the oligo [(dT)24 T7prom] 65 primer (GGCCAGTGAATTGTAATACGACTCACTATAGGGA GGCGG $(T)_{24}$ VN). First and second strand synthesis were carried out as described in the manufacturers' protocol.

2.2.6.1.2 cRNA synthesis

Double stranded cDNA was purified using the GeneChip Sample Cleanup Module (Affymetrix) before the in vitro transcription reaction. Biotin-labeled cRNA was synthesized using the Enzo BioArray High Yield RNA transcript labeling kit. This kit uses T7 RNA polymerase which binds the T7 promoter site incorporated during cDNA synthesis. The cRNA was purified using the QIAgen GeneChip Sample Cleanup Module and the quality and quantity assessed on the Agilent 2100 Bioanalyser.

2.2.6.1.3 Hybridisation and detection of cRNA transcripts

15 µg of fragmented cRNA was hybridised to the murine U74Av array or the mouse genome 430 2.0 array (Affymetrix) for 16 hours at 45^0C. After hybridisation the transcripts were detected by sequential staining with streptavidin phycoerythrin (SAPE), biotinylated, anti-streptavidin antibody and a second layer of SAPE using the GeneChip fluidics station 450.

2.2.6.2 Data Analysis

2.2.6.2.1 Image acquisition

Arrays were scanned on the GeneChip scanner 2000. Scanned arrays were visualised and analysed using the Affymetrix Microarray Suite 5.0. The initial step of analysis was automatic grid alignment onto the array image for demarcation of probe cells. The software uses the hybridisation pattern of the B2 oligo (a positive hybridisation control spiked into the hybridisation cocktail) to align the grid. The intensity of signal from individual probe cells is then calculated and this information is used to generate an expression analysis file. Each transcript is represented on the arrays as a set of probe pairs which consist of a perfect match probe and a mismatch probe. Signal intensities from the probe pairs is used to determine the overall signal for a given probe set, in addition to a detection call which indicates whether the transcript is present or absent.

2.2.6.2.2 Quality control

During the analysis of the array image file, various parameters were checked to monitor the quality of the array and sample hybridisation. Initial inspection of the array image was carried out to check for irregularities and to verify correct grid alignment. Presence of the B2 oligo hybridisation control around the borders of the array was also checked. The average background of the arrays was between 40 and 70, and raw noise between 1 and 3, both falling within acceptable levels for the scanner type. There were similar levels of background and noise in arrays to be compared. The presence of hybridisation controls (Genechip Eukaryotic Hybridisation control kit) was used to evaluate the sample hybridisation efficiency. RNA sample quality was determined by the 3' to 5' ratio of the probe sets for actin and GAPDH internal control genes. A high 3' to 5' ratio indicates RNA degradation or inefficient cDNA or cRNA transcription resulting in truncated transcripts.

For initial analysis of the arrays global scaling was carried out, which corrects for any differences in overall intensity of the arrays due to assay variables such as hybridisation efficiency. The scaling factors were also taken into consideration when deciding which arrays were suitable for comparison, since large scaling factors (>3 fold) may indicate significant assay variability or sample degradation leading to noisier data.

2.2.6.2.3 Genespring analysis

Data obtained in the Microarray Suite 5.0 was imported into Genespring 7 software (Agilent) as metrics text files. The data was normalised as follows: measurements less than 0.01 were set to 0.01, per chip normalisation was to the 50^{th} percentile, per gene normalisation was to specific samples. With the Genespring software data can be interpreted in a number of ways, and this determines how the data is analysed. In our case, we wished to compare replicates from one treatment compared to another, therefore the interpretation was set up to group the data in this way. The data was then filtered to include only genes which had a detection call of present or marginal in at least 2 out of 6 samples. If gene expression is not detected in either condition any changes in expression seen would not be real. Secondly we filtered out any genes which were not changing in expression by more than 1.5 fold. The filtered gene list was then subjected to a parametric statistical test with a p-value cut-off of 0.05, where the variances were not assumed to be equal.

2.2.6.2.4 Gene clustering

Data normalised in Genespring was exported to Microsoft Excel. The expression data from experiments on splenic DCs came from the murine U74Av2 array whereas the data from BMDCs came from the murine genome 430 2.0 array. In order to link the data from these experiments we used the array comparison files available on the Affymetrix web site to identify cases where probe sets from the U74Av2 array could be directly compared to probe

sets on the 430 2.0 array. The two sets of data were merged in Microsoft Access, which generated a file containing all the data from both experiments, including the cases where data from either experiment had no corresponding data from the other experiment. This file was then transferred back into Excel to make a number of modifications. Firstly, a unique identifier was generated by concatenating the U74Av2 array systematic name with the 430 2.0 array systematic name. The data from each probe set is flagged as present, absent or marginal. In the cases where there was a mixture of these flags from the same probe set we converted the flag to absent. In Microsoft Access, we then filtered the data so that only those probe sets passing the following criteria in at least one of the cell types/timepoints tested would remain: the expression must change at least 1.5 fold between control and IFN-treated samples, have an associated p-value of less then or equal to 0.05 and be flagged as present for the transcripts with higher expression. In Excel, where the expression data was changing significantly, the fold change values were transformed into log base 2. Otherwise the data was given a value of zero. The data was also colour coded depending on the level of expression. The data was then clustered and visualised using the TIGR multi-experiment viewer (Saeed *et al.*, 2003) from the Institute of Genomic Research.

2.2.7 ELISAs for detection of mouse cytokines

ELISAs were carried out on supernatants from cultured DCs using Biosource Immunoassay kits (IL-1β, IL-6, CCL2, CCL5) or Quantikine kits from R & D systems (CCL4, CCL12, CXCL9). These were carried out using the manufacturer's protocols, absorbance read at 450nm and subtracted from absorbance at 540nm to correct for optical imperfections in the plate. CXCL10 was measured using matched antibody pairs following the manufacturer's instructions (R & D systems).

2.2.8 Production and screening of monoclonal antibodies

2.2.8.1 Gpr33 peptides

2.2.8.1.1 Prediction of the structure of Gpr33

A model for the structure of Gpr33 was provided by Darren Flower (Edward Jenner Institute, Compton, UK). Briefly, the sequence of the uncharacterised chemokine-like G-protein coupled receptor (GPCR) was aligned to other representative GPCR sequences using CINEMA (Lord *et al.*, 2002), which gave a one-to-one relationship between the chemokine-like GPCR and bovine rhodopsin. This was used to perform homology modelling of the chemokine-like GPCR using the structure of bovine rhodopsin, as determined (Palczewski *et al.*, 2000), as the template. Homology modelling was undertaken using the modeller system (Fiser *et al.*, 2003).

Since the purpose of this exercise was to establish the probable distribution of residues between intracellular, extracellular, and transmembrane regions, only initial modelling, without subsequent optimisation, was performed. In the absence of extra constraints provided by experiment, only those constraints implicit within the process, i.e. those arising from residue substitution, were used. Regions of the protein which are extracellular were established by visual inspection.

2.2.8.1.2 Peptide synthesis

Peptides were synthesised by Alta Bioscience (University of Birmingham) as octomeric multiple antigenic peptides. Eight identical peptide chains were synthesised, and joined to a common poly-lysine core peptide. The molecular weight of these peptides, (10kDa-15kDa) is sufficient for them to be highly immunogenic without the need to couple to a carrier protein. The peptides were purified by dialysis through a 3kDa membrane.

2.2.8.1.3 Peptide dissolution

A volume of methanol or acetonitrile (50/100µl) was added to 1mg of peptide and left to stand for 5 – 10 minutes at room temperature. 5µl of pH modifying reagent was then added: either ethanolamine (acidic peptides) or glacial acetic acid (basic peptides). After a further 5 - 10 minute incubation at room temperature, a volume of water equal to that used in the initial step was added. The sample was then diluted to an appropriate volume with PBS.

2.2.8.2 Immunisation with Gpr33 peptides

0.5mg of each peptide (A1, A2 and A3) were dissolved into a total volume of 300µl in PBS. An emulsion was then made with an equal volume of Titermax Gold Adjuvant (Sigma) and used to immunize rats either intraperitoneally or subcutaneously. 19 days later, the rats were boosted with the same dose of peptide with Titermax Gold. A final boost consisting of a combination of 0.1mg of each peptide in PBS was given four days prior to sacrifice.

2.2.8.3 ELISAs

Falcon 3912 plates were coated with 10µg/ml of peptide diluted in carbonate buffer (0.5M sodium carbonate, pH 9.6) overnight at room temperature or for two hours at 37^0C. Plates were washed three times with PBS, 0.05% Tween and blocked with a 4% milk solution in PBS for one hour at room temperature (or 45 minutes at 37^0C). Plates were washed three times before addition of test antibodies. Serial 1:2 dilutions of rat sera were made in PBS, 1% milk, and added to the plates. Hybridoma supernatants were added to the plates neat. After one hour at room temperature a 1:500 dilution of anti-rat IgG –HRP or anti-rat IgG-AP in PBS, 1% milk was added, followed by a further one hour incubation. The ELISAs were developed using an OPD or pNPP solution (Sigma) and the reactions stopped with 3M hydrochloric acid or 3 N sodium hydroxide, with absorbance read at 492nm or 405nm respectively.

2.2.8.4 Cell fusion

Fusion of rat spleen cells with myeloma cells was done as originally described by Galfre et al. (Galfre *et al.*, 1979). Cell fusions were carried out by Brenda Jones at the Institute for Animal Health, Compton, UK. 1 x 10^8 spleen cells from the immunized rat were mixed with 1 x 10^8 Y3 myeloma cells and centrifuged at 1200 rpm for five minutes. The cell pellet was resuspended in 1ml of polyethylene glycol (PEG) 1500 followed by slow addition of 10ml RPMI. The cells were centrifuged at 1200 rpm for five minutes and the cell pellet resuspended in 48ml RPMI 20% FCS. The cells were aliquoted at 1ml/well into 48 well plates and 1ml of spleen feeder cells (5 x 10^5/ml) was added. On days one, three, five and seven, 1 ml of the cell cultures was removed and 1ml of hypoxanthine, aminopterin and thymidine (HAT) selection medium was added. After 10-14 days, clones were assayed for production of Gpr33 peptide specific antibodies by ELISA. Positive clones were subcloned by limiting dilution to produce monoclonal hybridomas.

2.2.9 Production of Rabbit Polyclonal Antibodies

Polyclonal antibodies were made by Pacific Immunology, California. The antibodies were raised against the N-terminal region of Gpr33 by immunising rabbits with a 15 amino acid sequence (NSSTHVINVSTSLTN) conjugated to a KLH carrier protein. Gpr33 peptide-specific antibodies were isolated from the serum of immunised rabbits by affinity purification.

2.2.10 Calcium flux assay

Baf/3 cells were washed twice in modified HBSS (containing calcium chloride and magnesium chloride), 1% BSA, and resuspended at 10^6 cells/ml. Indo-1 AM was added to the cells to give a final concentration of 2.5μg/ml. The cells were then incubated at 30^0C in the dark for 30 minutes followed by two washes with modified HBSS. Agonists were added to the cells to give a final concentration of 1μg/ml and calcium mobilization measured on a MoFlo cytometer. Once calcium mobilization reached a plateau 100μl of 25mM EDTA was added.

Chapter 3: Identification of IFN-I-induced genes in splenic DCs by Representational Difference Analysis

3.1 Introduction

Representational difference analysis (RDA) was first developed as a method for analysing the differences between complex genomic DNA populations and can be used to identify changes such as gene rearrangements, which could be the cause of cancers or inherited disease (Lisitsyn et al., 1993). Since we are interested in gene expression we used a modification of this technique, cDNA RDA, which identifies differences in levels of mRNA between cell populations (Hubank et al., 1994). RDA is a polymerase chain reaction (PCR)-based, subtractive hybridization technique used to find genes which are more highly expressed in one cell population, the tester population, compared to another, the driver (Figure 3.1). cDNA generated from the two cell populations is first digested using a restriction enzyme, producing fragments of a relatively homogenous length for efficient amplification which can be ligated to adaptor molecules which serve as primer binding sites. The enzyme used (DpnII) recognizes a 4bp sequence and yields fragments with an average length of 256bp which ensures that the majority of genes will contain at least one amplifiable fragment. These amplified cDNA products are then representative of the original mRNA population. It is essential to maintain the relative proportions of mRNA species throughout the procedure and this achieved by limiting the number of PCR cycles at each stage to keep amplification within the linear range. The representative amplicons (representations) are then digested again to remove the adaptors before re-ligating new adaptors to the tester representation only. The driver and tester populations are hybridized at a ratio of 100:1 to compete out sequences that are present in both the driver and tester populations. Any tester

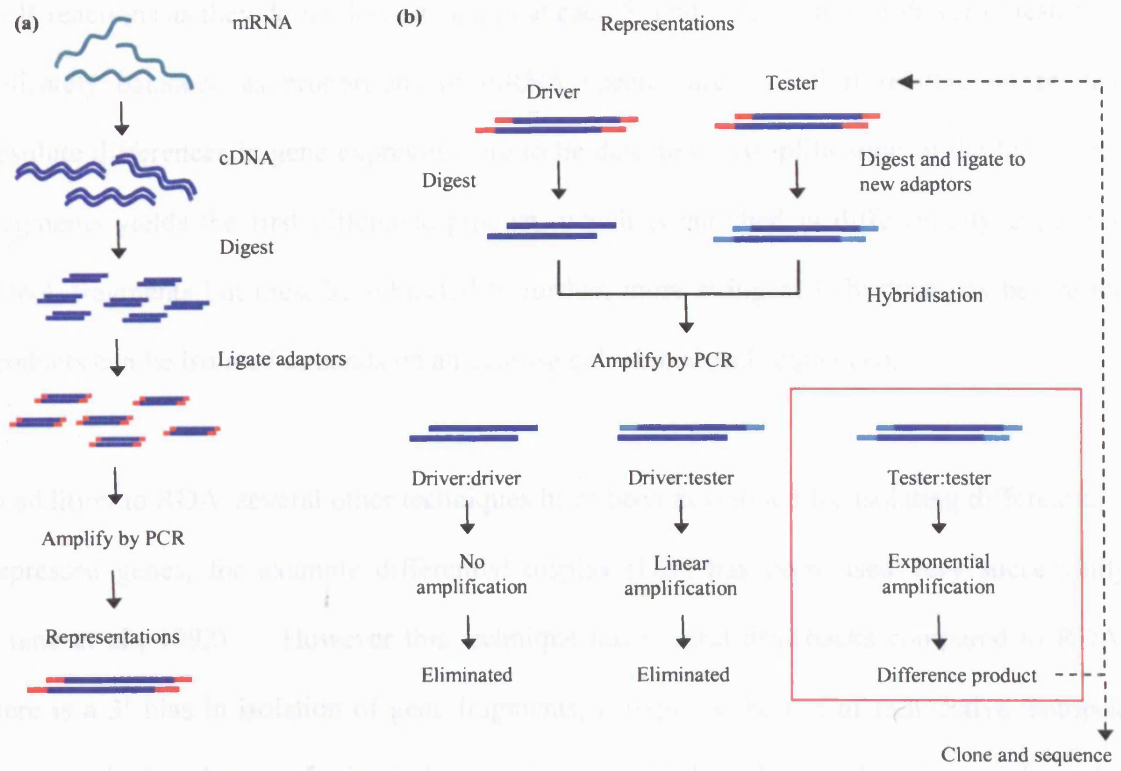


Figure 3.1 cDNA Representational Difference Analysis
(a) mRNA is isolated from two cell populations and reverse transcribed into cDNA. The cDNA is digested and ligated to oligonucleotide adaptors which serve as primers for PCR amplification to generate representations. (b) The adaptors are then removed and replaced by new adaptors on the tester cDNA only. At this stage the cDNA from the tester and driver populations are hybridised together with an excess of driver (100:1). Subsequent PCR will result in the amplification of only those fragments which rehybridise as tester:tester, and so will represent genes which are expressed at elevated levels in the tester population compared to the driver. This process is then repeated, hybridising the difference product to the driver at increasingly higher ratios to produce 2nd and if necessary 3rd difference products. These will contain only differentially expressed genes which can then be cloned and sequenced.

fragments which hybridize to the driver will not be amplified exponentially in subsequent PCR reactions as they do not have adaptors at each 5' end. The ratios of driver to tester are delicately balanced as proportions of mRNA species are critical if relative rather than absolute differences in gene expression are to be detected. Amplification of the hybridized fragments yields the first difference product, which is enriched in differentially expressed cDNA fragments but must be subjected to further, more stringent hybridizations before the products can be isolated as bands on an agarose gel, cloned and sequenced.

In addition to RDA, several other techniques have been developed for isolating differentially expressed genes, for example differential display (DD) has been used very successfully (Liang et al., 1992). However this technique has several drawbacks compared to RDA; there is a 3' bias in isolation of gene fragments, it requires the use of radioactive isotopes, there is a higher degree of mispriming events during PCR and since there is no subtractive step it yields a higher proportion of false positives (Frazer et al., 1997). When isolating cells directly from their in vivo environment (as we have done) it is impossible to obtain an absolutely pure cell population, and this would not be amenable to analysis by differential display.

An important advantage of RDA over other methods is that it is extremely sensitive: genes expressed in less than 1% of the tester population can be identified. Secondly, only small quantities of starting material are needed. It is possible to carry out RDA on just 10^4 to 10^5 cells and so the technique is suitable for study on FACS sorted lymphocytes. Finally, RDA is relatively cheap and uses conventional molecular biology techniques. For these reasons it was initially our preferred method for identifying differentially expressed genes.

cDNA RDA has previously been used to identify gene expression changes induced by cytokines in T cell helper clones (Louahed et al., 1999) and differences between B cell populations (Frazer et al., 2000). The discovery of DC subsets and their divergent roles in immunity has stimulated a search for markers differentially expressed between them. Two molecules, CIRE and FIRE, expressed more highly on the CD8α- DC subset than the CD8α+ subset were identified by RDA (Caminschi et al., 2001a; Caminschi et al., 2001b). Both are cell surface molecules: CIRE, a C-type lectin, shares 57% amino acid identity with human DC-SIGN, the receptor for ICAM-2 and ICAM-3, while FIRE shows homology to G-protein coupled receptors. Both CIRE and FIRE were found to be downregulated on activation and so may be involved during early stages of DC maturation. Tspan-3, another mouse DC transmembrane protein, was identified by RDA (Tokoro et al., 2001). In this case, RDA was used to compare resting and CD40 activated DCs. As with CIRE and FIRE, Tspan-3 expression was downregulated in activated DCs.

We have used RDA to investigate the regulation of gene expression by IFN-I in splenic DCs. By identifying changes in levels of gene expression associated with DC activation, we hoped to gain insights into the mechanisms by which DCs influence the outcome of immune responses. A diverse range of genes were found to be upregulated by IFN-I in splenic DCs, from genes encoding transcription factors involved in regulating the IFN genes themselves to proteins involved in the anti-viral response and antigen presentation. Several expressed sequences of unknown function were also identified.

3.2 Results

3.2.1 RDA on IFN-I-treated CD11c$^+$ DCs : optimization

RDA was used to test the differences in gene expression between untreated DCs (DC 0) and DCs treated with IFN-I (DC IFN). In all RDA experiments the IFN-I used was a combination of IFN-α (25%) and IFN-β (75%). 7 x10^6 CD11c$^+$ DCs were isolated from C57Bl/6 mouse spleens by FACS sorting and were cultured for two hours with or without 2 x 10^4 U/ml IFN-I. This dose of IFN-I approximates that produced locally during a viral infection (Gallucci *et al.*, 1999), which can rapidly reach very high titres. Notably however, different viruses induce amounts of IFN-I which can vary over a 10,000 fold range (Marcus *et al.*, 1998) and human plasmacytoid DCs can produce in the range of 2 - 64 x 10^4 U IFN-I per 10^5 cells on viral stimulation (Siegal *et al.*, 1999). Transcription of IFN-induced genes occurs rapidly: Mx gene transcription is increased 50-fold only 90 minutes after IFN treatment (Staeheli *et al.*, 1986a). We chose to isolate the DCs after a two hour incubation as we wished to use conditions which would reveal genes induced at an early stage after IFN-I exposure. This would increase the chance of identifying genes directly induced by IFN-I and not those induced further downstream.

During the RDA procedure reciprocal subtractions were carried out, that is, we used DC 0 as the driver sample and DC IFN as the tester sample in one experiment and vice versa in another. This allowed us to test for genes both upregulated and downregulated by IFN-I.

In order to generate sufficient quantities of representative cDNA, 26 cycles of amplification of the R-ligated cDNA template was needed. The representations were visualized as a smear of DNA fragments on an agarose gel, and ranged between 200bp and 1.2kb in size, as would

be expected (Figure 3.2). After generation of the first difference product (DP1) there were no obvious bands in the samples, and both the samples (from the reciprocal subtractions either with DC 0 or DC IFN as the tester) appeared very similar. However, the second difference product (DP2) had distinct bands in the sample where DC IFN was used as the tester (Figure 3.3). In the sample with untreated DCs as the tester, we could detect only a very faint smear. To try to eliminate any background and make the banding more distinct we generated a second DP2, with increased stringency. However, since this only resulted in the bands becoming fainter, we used the initial DP2 for further analysis. Individual bands ranging from 300 to 700 bp were isolated from the agarose gel (Figure 3.4), cloned and sequenced.

The nucleotide sequences were run through BLAST searches against the NCBI non-redundant or mouse EST databases (Table 3.1). Although we were unable to clone the 1kb band due to insufficient amounts of DNA, we considered this preliminary RDA to be successful since several of the genes identified had previously been characterized as IFN-inducible. At the time of cloning, three of the inserts (from bands 3, 4 and 6), did not match to known genes in the NCBI database and were either uncharacterized, or classed as "similar to" known genes. For instance, the product cloned from band 3, expressed sequence AI447904, is also known as "similar to interferon activated gene 203 (Ifi203)". Ifi203 itself belongs to a family of IFN induced genes, the Ifi 200 family. Unlike other members of this family, Ifi203 has not been shown to be involved in the anti-proliferative effect of type I IFN (Gribaudo *et al.*, 1999). The function of Ifi203 and "similar to Ifi203" is currently unknown. Verification that these genes were truly differentially expressed and not simply an artifact produced by multiple PCR amplifications, was provided by probing a southern blot of independently isolated cDNA representations, an example of which is shown in Fig 3.5.

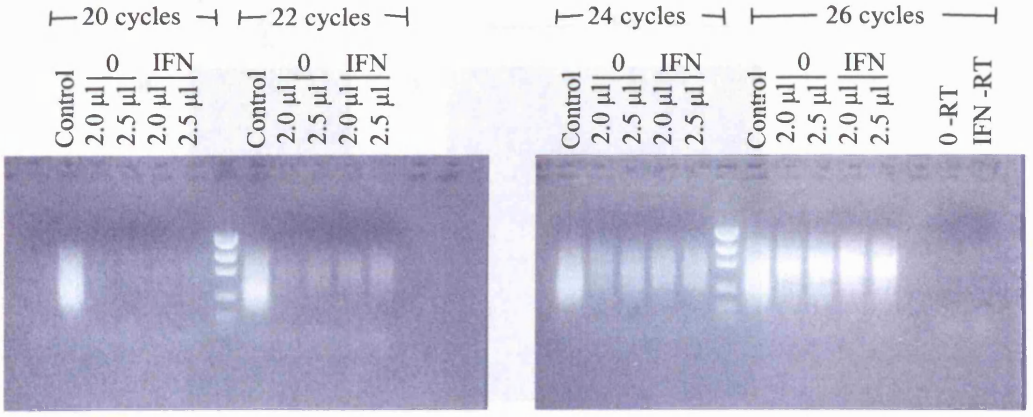


Figure 3.2: Optimisation of PCR conditions for generation of representations
$CD11c^+$ DCs were isolated from the spleens of C57/Bl6 mice and placed in culture for two hours in the presence or absence of IFN-I (2×10^4 U/ml). RNA was isolated and RDA performed, using 2 or 2.5µl of the R-ligated samples for the indicated number of PCR cycles. 0 = cDNA from non-treated DCs; IFN = cDNA from IFN-α/β treated DCs; control = positive control cDNA derived from a cell line (EL4); -RT are negative controls where no reverse transcriptase was added to the cDNA synthesis reactions.

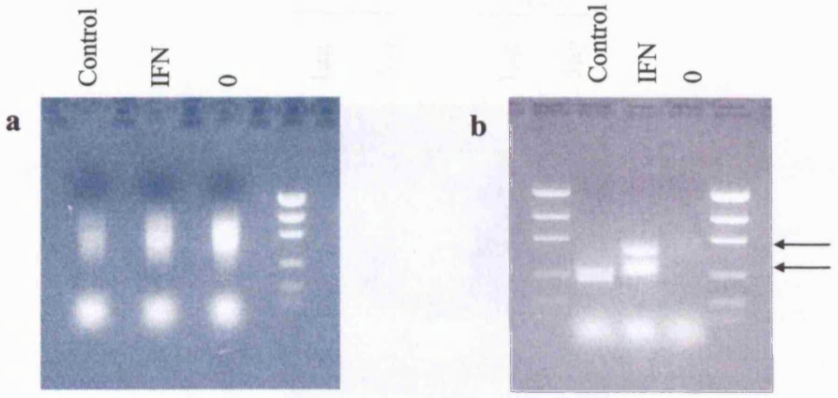


Figure 3.3: Bands representing differentially expressed genes are apparent in the second difference product (DP2) when using DC IFN but not DC 0 as the tester
a, DP1, and **b**, DP2 for RDA analysing gene expression in IFN-I-treated DCs (subtractive hybridisation was carried out at a ratio of 100:1 and 800:1 driver:tester, repectively). IFN = DC IFN used as tester; 0 = DC 0 used as tester. Control = RDA carried out on samples generated from an untransfected (driver) and tranfected (tester) cell line and therefore with known differences in gene expression, as a positive control. The lower bands represent digested adapters. The arrows indicate bands that represent differentially expressed genes in the IFN-treated DCs. Only a faint smear was seen in the reciprocal subtraction with untreated DC as the tester.

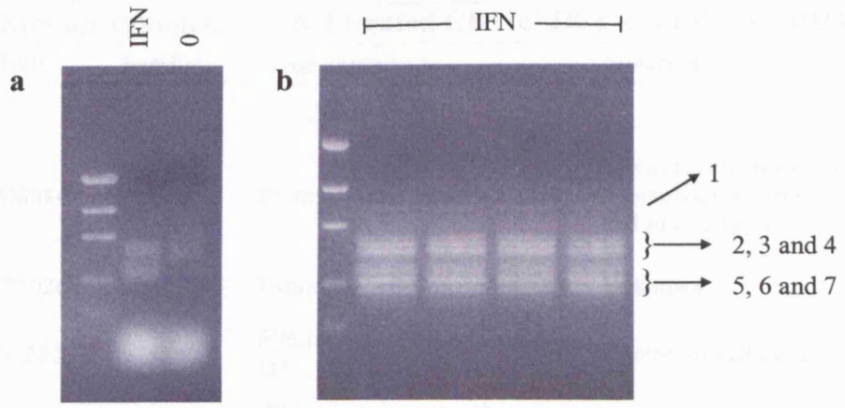


Figure 3.4: Strategy for cloning DP2 generated from RDA on CD11c$^+$ DCs
a, Subtractive hybridisation was repeated using a ratio of 4000:1 driver:tester. The bands were identical to the initial DP2, only fainter. **b**, Bands from the DP2 produced using a ratio of 800:1 driver:tester were separated on an agarose gel at low voltage for several hours. Seven bands were excised from the gel and bands 2 - 7 were cloned.

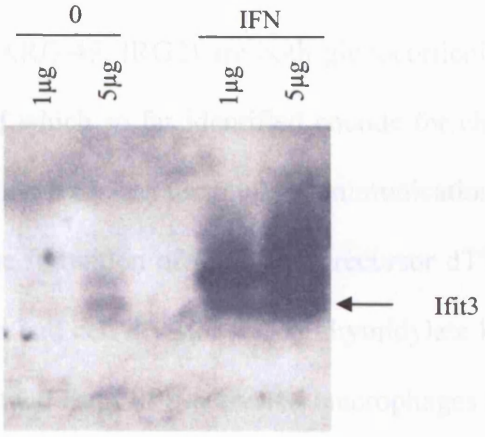


Figure 3.5 Verification of differential gene expression by probing of Southern blots from independently generated cDNA representations. cDNA representations from splenic DCs cultured in media alone or with 2 x 10^4 U IFN-I were blotted onto a membrane and hybridised with a probe specific for the Ifit3 gene fragment identified by RDA. Upregulation of the transcript can clearly be seen in samples treated with IFN-α/β. The probe for Ifit3 also recognizes a band of smaller size from which Ifit2 was cloned.

Table 3.1 Genes upregulated in IFN-I treated CD11c$^+$ DCs identified by RDA				
Band	**Accession**	**Symbol**	**Gene name**	**Function**
1			Not cloned	
2	NM_008884	PML	Promyelocytic leukemia gene	Controls cell proliferation, apoptosis, transcription regulation and tumourigenesis
3	NM_175026	AI447904	Expressed sequence AI447904	Unknown
4	CV_562282		Similar to G1/S specific cyclin D2	Control of cell cycle
5	NM_008332	Ifit2	IFN-induced gene with tetratricopeptide repeats 2	Unknown
6	NM_010501	Ifit3	IFN-induced gene with tetratricopeptide repeats 3	Unknown
7	NM_020557	Tyki	Thymidylate kinase family, LPS inducible member	Nucleotide biosynthesis

The genes Ifit2 and Ifit3 (Interferon-induced gene with tetratricopeptide repeats 2 and 3) neither of which has a known function, were also identified in these initial RDAs. Ifit2 (GARG-39, IFI54) and Ifit3 (GARG-49, IRG2) are both glucocorticoid-attenuated response genes (GARGs), the majority of which so far identified encode for chemokines and so it is possible that Ifit2 and Ifit3 are involved in intercellular communication (Smith *et al.*, 1997). Thymidylate kinase catalyses the formation of the DNA precursor dTTP, a function usually associated with DNA replication and cell division. The thymidylate kinase family member identified here was originally cloned from LPS-activated macrophages (Lee *et al.*, 1995), and may be needed during activation due to the rapid synthesis of mitochondrial DNA.

3.2.2 RDA on DC subsets treated with IFN-I

Splenic DC subsets preferentially inhabit different anatomical areas, the CD8$^+$ DCs in the T-cell rich areas and the CD8$^-$ in the marginal zone (De Smedt *et al.*, 1996; Kelsall *et al.*, 1996; Reis e Sousa *et al.*, 1997), which suggests they may affect different aspects of T and B cell activation. They express different TLRs and therefore have the ability to respond to

different pathogens (Iwasaki *et al.*, 2004). They also produce different cytokines in response to pathogens, thereby stimulating T cell polarization (Hochrein *et al.*, 2001), and in turn require different cytokines to promote this function (Maldonado-Lopez *et al.*, 2001). For these reasons, we wanted to test whether the DC subsets would respond differently to IFN-I.

The $CD8^-$ subset of DCs expresses the myeloid lineage marker CDllb, whereas the $CD8^+$ subset does not. In order to separate these two subsets of DCs we first enriched the $CD11c^+$ population by positive selection on a MACS column, which facilitates and improves the final purity from the following FACS sort on CD11b and CD11c expression (Vremec *et al.*, 2000)(Fig 3.6). The DCs were cultured for two hours with or without 2 x 10^4 U/ml IFN-I and we proceeded with the RDA, carrying out reciprocal subtractions as before. Again, the first difference products appeared as smears with no distinct bands. The second difference product appeared as distinct bands where DC IFN was used as tester and only as a smear in the samples with DC 0 as tester. After going through a third round of subtractive hybridization the results were much improved, with several well defined bands detected in each of the DC IFN samples (Fig 3.7, a and b). A slight background smear persisted.

In each of these RDAs less than ten separate bands representing differentially expressed genes were detected. However, we would expect there to be a far greater number of IFN-α/β induced genes, since greater than 400 human genes are induced by IFN-I (Der *et al.*, 1998). It is possible that amplified products from different genes could be of the same size and therefore would migrate together on a gel. Therefore, we endeavored to sequence at least five separate clones from each band; in some cases up to three different genes were identified in the same band (Table 3.2). We were again unable to clone three of the amplified products (Figure 3.7b) due to insufficient quantities of DNA.

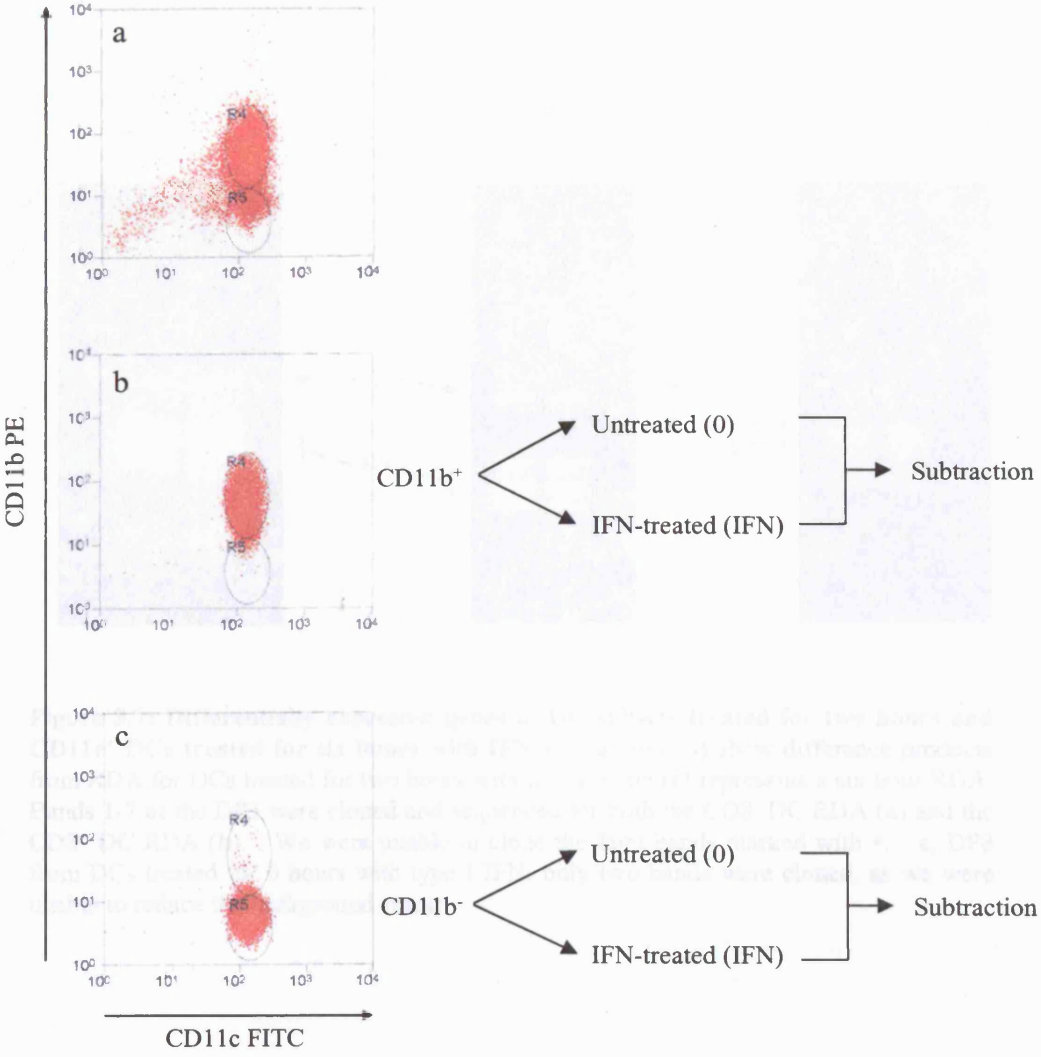


Figure 3.6: Isolation of CD11b$^-$ and CD11b$^+$ DC subsets.
DCs were enriched from splenocytes on a Nycodenz gradient followed by positive selection of CD11c$^+$ cells by MACS. **a**, Purified DCs before cell sort. The sorted DC subsets are **b**, CD11b$^+$ DCs (which corresponds to the CD8$^-$ DC subset) and **c**, CD11b$^-$ DCs (which corresponds to the CD8$^+$ DC subset)

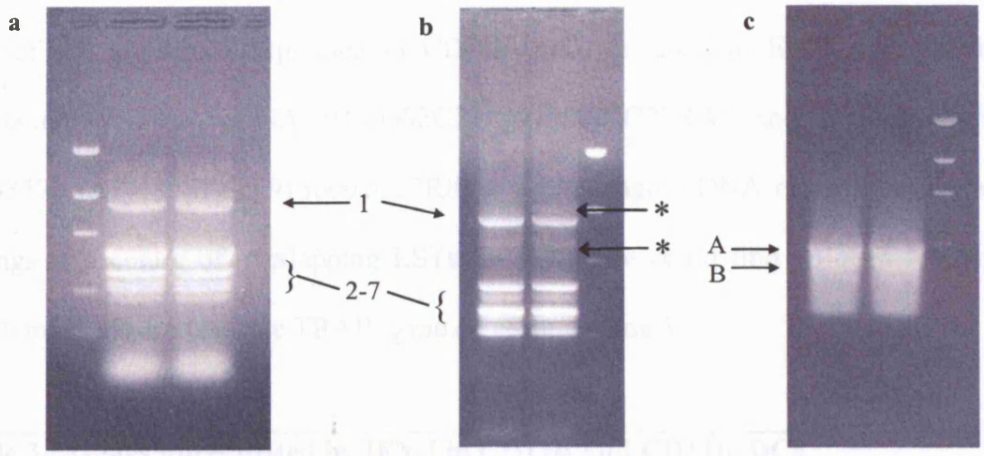


Figure 3.7: Differentially expressed genes in DC subsets treated for two hours and CD11c$^+$ DCs treated for six hours with IFN-I. **(a)** and **(b)** show difference products from RDA for DCs treated for two hours with IFN-I while **(c)** represents a six hour RDA. Bands 1-7 of the DP3 were cloned and sequenced for both the CD8$^-$ DC RDA (**a**) and the CD8$^+$ DC RDA (**b**). We were unable to clone the faint bands marked with *. **c**, DP3 from DCs treated for 6 hours with type I IFN: only two bands were cloned, as we were unable to reduce the background smear.

Two genes appeared frequently in the sequencing results, namely promyelocytic leukemia gene (Pml) and guanylate binding protein 2 (Gbp2). Both of these genes have been characterized as IFN induced, although their precise functions are not well understood.

Two of the sequences expressed in CD11b$^+$ DCs matched to ESTs with no functional annotation: RIKEN cDNA 9130002C22 (9130002C22Rik) and Expressed Sequence AI448571 (AI448571). 9130002C22Rik is a full length cDNA clone whereas AI448571 belongs to a contig of overlapping ESTs (in which we could find no open reading frame) which maps close to murine TRAIL gene on chromosome 3.

Table 3.2 Genes upregulated by IFN-I in CD11b$^+$and CD11b$^-$ DCs

	CD11b$^+$		CD11b$^-$	
Band	**Accession**	**Gene**	**Accession**	**Gene**
1	NM_008884	Pml	NM_008884	Pml
2	NM_008884	Pml	NM_008884	Pml
	NM_010260	Gbp2		
3	NM_010260	Gbp2	NM_021394	No significant matches (Zbp1)
	NM_008884	Pml	NM_010846	No significant matches (Mx1)
4	NM_008884	Pml	NM_008884	Pml
	NM_029000	9130002C22Rik	NM_183201	Schlafen 5
5	AI448571	AI448571	NM_008332	Ifit2
	NM_010260	Gbp2		
6	NM_010501	Ifit3	NM_010501	Ifit3
	AI448571	AI448571	NM_008884	Pml
		X-actin		
7	NM_010501	Ifit3	NM_010501	Ifit3
	NM_008884	Pml	NM_173754	Usp43
	NM_020557	Thymidylate Kinase		

Two sequences from the CD11b$^-$ RDA did not produce any significant matches to sequences on the NCBI non redundant or mouse EST databases. At first it appeared that we had isolated sequence from novel genes, however we subsequently discovered, by carrying out a

BLAST search of the Ensemble database, that the sequences are located within the introns of Mx1 and Zbp. Zbp is a Z-DNA binding protein also known as DLM-1, which is upregulated in macrophages activated by LPS or IFN-γ (Fu *et al.*, 1999).

Finally, a sequence matching to a gene named Similar to hypothetical protein FLJ34922 (*Homo sapiens*) was identified. This gene belonged to a predicted family (Ensemble ENSF00000003059) containing 5 members which all contain an ATP/GTP binding-site motif. Our cloned sequence shared 98% sequence identity with this gene and 65-67% identity with three other members of the family. This region of identity is split into two sections from nucleotides 1-58 and 136-154 of our sequence. Nucleotides 1-58 also matched human genes which are weakly similar to the Schlafen (Slfn) gene members Slfn3 and Slfn4. Since this predicted gene family is located directly next to the Schlafen gene family on chromosome 11, we thought it possible that they were related. Recently this gene has been assigned as Slfn5 (Geserick *et al.*, 2004).

In conclusion, several genes were upregulated in both the CD11b$^-$ and CD11b$^+$ DC subsets, namely Pml, Ifit2 and Ifit3. However, Gbp2 upregulation was detected several times in the CD11b$^+$ DCs but never in the CD11b$^-$ DCs. In these experiments we identified what we believed to be only a small subset of the genes upregulated by IFN-I in the two subsets, and amongst these, we did not identify any genes which could contribute to different localisation or the T cell-polarizing effect of the two subsets.

3.2.3 RDA on DCs treated for 6h with IFN-I

Not surprisingly, many of the genes identified in the RDAs on DCs treated for 2 hours with IFN-I were involved in the innate anti-viral response common to most cell types. However, our aim was to isolate genes which were involved in the DCs ability to regulate adaptive

immune responses. We thought it possible that these genes might be upregulated at a later stage of IFN-stimulation. To investigate this we isolated $CD11c^+$ DCs and cultured for six hours with IFN-I stimulation. Technical difficulties with this RDA meant that we could not amplify clear bands, even after a third subtractive hybridization (Fig 3.7c). The presence of a slight background smear is likely to have produced some false positives in the sequencing results, such as the 18s RNA which is very highly expressed and therefore may not have been completely competed out during the subtractions. However, many of the genes sequenced were known IFN-induced genes (Table 3.3). In addition to genes identified in RDAs carried out on DCs treated for two hours with IFN-I, we found expression of the PI3 kinase γ regulatory subunit p101. PI3 kinase I is a key component in signal transduction from numerous receptors, including cytokine receptors, TLRs and T and B cell receptors, and mediates cell proliferation, survival and migration (Deane *et al.*, 2004). The association of the p101 regulatory protein with PI3 kinase γ is essential for its sensitivity to G-protein coupled receptor (GPCR) activation (Stephens *et al.*, 1997). Two transcription factors were upregulated at 6h: RelB, a component of the transcription factor NFκB and Irf7, which acts in a positive feedback loop by upregulating expression of the IFN-I genes themselves. Encouragingly we also saw upregulation of an MHC II gene, which we would expect to see as the DCs mature. In this experiment we also identified another member of the Slfn gene family Slfn4. The function of several of the genes identified remains elusive and we carried out an Interpro search for conserved domains within the amino acid sequences, in an attempt to assign a putative function for these genes (Table 3.4). This gave us an indication of type of processes these proteins may be involved in. For example, the Myosin 1G protein contains myosin domains which play important roles in both cell motility and organelle transport.

Table 3.3 Genes identified by RDA in DCs after 6h IFN-I treatment

Clone No	Symbol	Function	Accession	Gene Name
Antigen presentation				
B5b, B7a, B8		Antigen presentation	BC031711	MHC class II H2-IA-alpha gene
Signal transduction				
A6	F730038I15Rik	Kinase	AY156924	PI3-kinase γ regulatory subunit p101
B2	CRHSP-24		AK004711	Homologue of calcium-regulated heat stable protein
Transcription/translation				
A5a	RelB	Transcription factor	NM_009046	V-rel oncogene related B
A5b	Rpl9	Inferred protein synthesis	NM_011292	Ribosomal protein L9
A7	Irf7	Transcription factor	NM_016850	IFN regulatory factor 7
B9a	Slu7-pending	Pre-mRNA splicing	AK049178	Step II splicing factor SLU7
B13	18s rRNA		HSRRN18S	18s ribosomal RNA
IFN response				
A10	Ifit3		NM_010501	IFN induced protein with tetratricopeptide repeats 3
B12	Vig1		NM_021384	VHSV induced gene
Ion transport				
A1	Atp5b	ATPase	BC013253	Beta-F1 ATPase, mitochondrial
B3	Fth	Iron homeostasis	NM_010239	Ferritin heavy chain
B5a	Slc25a3	Mitochondrial substrate carrier	BC018161	Solute carrier family 25, member 3
Protein alteration				
A11	Usp18	Protease	NM_011909	Ubiquitin specific protease 18
B11	Sat		NM_009121	Spermidine N1-acetyl transferase
Nucleotide biosynthesis				
B6	Tyki	Kinase	BC057565	Thymidylate kinase family, LPS-inducible member
Thymocyte development				
A2b, A3, A8	Slfn4		NM_011410	Schlafen 4

Table 3.4 Conserved domains present in novel IFN-I induced genes				
Clone No	**Gene Name**	**Accession**	**Interpro/NCBI Domains**	**Interpro**
A2a	RIKEN cDNA 9530019H02 gene	BC056466	Ras GTPase Sigma-54 factor, Small GTP-binding domain	IPR001806 IPR002078 IPR005225
A9a	Epididimal secretory protein (Niemann-Pick type C2)	AB021289	ML E1_DerP2_DerF2	Smart00737 Pfam02221
A12	KIAA0431 protein	BC060631	Zinc finger,C2H2 type	IPR007087
B7b	Similar to RIKEN cDNA 4933437I04 gene	BC031980	Untranslated	
B9b	Lymphocyte cytosolic protein 1	BC022943	Actin-binding, actinin-type Calponin-like actin binding Calcium-binding EF hand	IPR001589 IPR001715 IPR002048
B10	Myosin 1G	AK088011	Calmodulin-binding Myosin head Myosin tail 2	IPR000048 IPR001609 IPR010926

3.2.4 Verification of RDA results by Real-time PCR

To confirm that the results of the RDA experiments were true difference products corresponding to IFN-I induced genes we carried out real-time PCR. Initially we designed probes and primers against the two expressed sequences of unknown function, since we believed that these had not previously been identified as IFN-induced genes. These were 9130002C22Rik and AI448571 identified in the $CD11b^+$ DCs, and the sequence that subsequently was found to be an intron of Mx1 from the RDA on $CD11b^-$ DCs. RNA for these experiments came from freshly isolated $CD11c^+$ splenic DCs that were cultured with or without 20,000 U/ml IFN-I for two hours. The results are summarised in figure 3.8, and confirmed that both 9130002C22Rik and Mx1 were upregulated by IFN-I. However, when we tested for expression of these genes in T and B cells, we found that in some cases IFN-I enhanced the expression even further than in DCs. This was especially true for AI448751 which was not significantly upregulated in DCs compared with 12-14 fold in T cells and for Mx1 (intronic sequence) which was also strongly upregulated in T cells after IFN-I

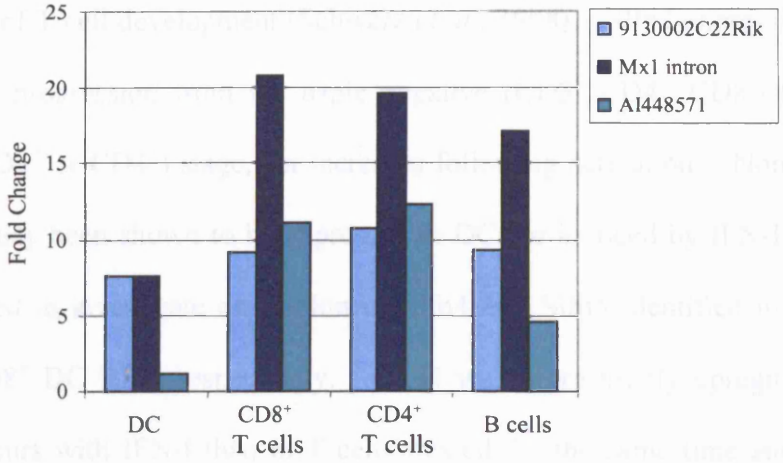


Figure 3.8: The expression of uncharacterised sequences upregulated in IFN-I treated DCs is also elevated in T and B cells following IFN-I stimulation. RNA was isolated from CD11c$^+$ DCs, CD8$^+$ and CD4$^+$ T cells and B cells which had been cultured for two hours with or without IFN-I (2 x 10^4 U/ml). Expression of sequences identified by RDA were analysed by real-time PCR and were found to be ubiquitously upregulated by type I IFN. In some cases expression was more highly upregulated in T and B cells than in DCs. Results are plotted as fold change in expression in cells cultured in IFN-I versus medium alone, after normalising test mRNA levels to 28S RNAlevels.

stimulation. Our original aim was to identify DC-specific genes, however enhanced expression in T and B cells does not necessarily rule out an important role in regulating DC-specific functions.

Members of the Schlafen (Slfn) gene family have been shown to be differentially expressed during the course of T cell development (Schwarz *et al.*, 1998). Slfn4 expression decreases in T cells during progression from the triple negative ($CD3^-$, $CD4^-$, $CD8^-$) to the single positive (either $CD8^+$ or $CD4^+$) stage, yet increases following activation. None of the Slfn genes had previously been shown to be expressed in DCs or induced by IFN-I . For these reasons we decided to investigate expression of Slfn4 and Slfn5 identified in the six hour RDA and the $CD8^+$ DC RDA respectively. Slfn4 was more highly upregulated in DCs treated for two hours with IFN-I than in T cells treated for the same time and was down-regulated in B cells (Figure 3.9). Slfn5 expression was markedly different, its expression increasing by almost 20-fold in T cells treated with IFN-I.

In addition to investigating the effect of IFN-I on the expression of these genes, we made two other comparisons. First, we compared expression in DCs, T cells and B cells cultured for two hours in medium alone. As shown in figure 3.10, 9130002C22Rik was more highly expressed in T cells and B cells than in DCs. Slfn4 and AI448571 were also expressed at higher levels in T and B cells than in DCs, although the differences were not as large. By contrast Slfn5 expression was lower in T cells than DCs and Mx1 intron expression was decreased in both T and B cells compared to DCs.

Second, we compared expression of these genes in DCs that had been cultured for two hours or six hours in medium alone. Since splenic DCs mature spontaneously when placed in culture, we wanted to know whether this process, like IFN-I-induced maturation, was

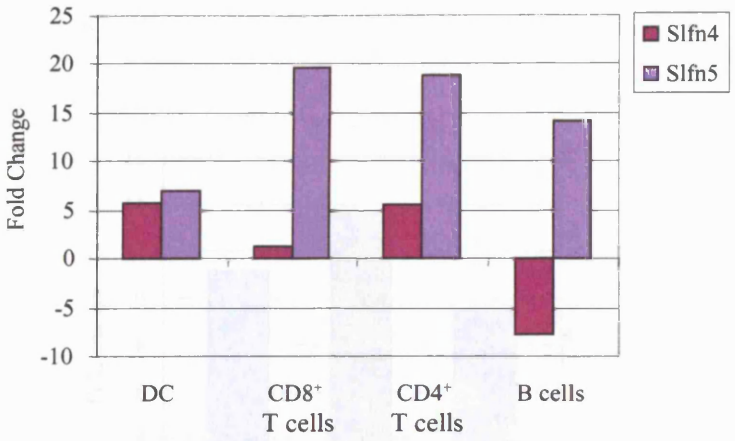


Figure 3.9: Comparison of Slfn4 and Slfn5 expression in IFN-treated DCs, T cells and B cells. Real-time PCR was carried out for RNA extracted from DCs, T and B cells cultured in medium alone or with 2 x10^4 U IFN-I. Slfn4 expression was increased the most in DCs treated for 2 hours with IFN-I but decreased in B cells. Slfn5 had extremely elevated expression in T cells treated with IFN. Results are plotted as fold change in expression in IFN-treated cells versus that in the same cells cultured for the same time in medium alone.

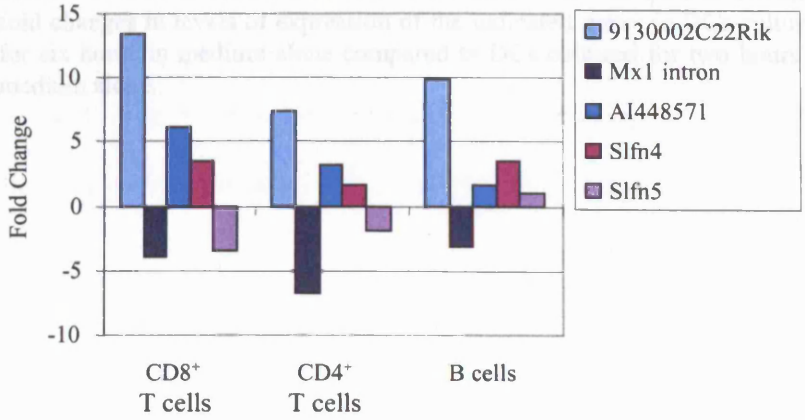


Figure 3.10: Levels of expression of the genes identified in RDAs varies between cell types. Real-time PCR was used to compare expression of the indicated sequences using RNA from DCs cultured in medium alone for two hours as the reference sample and RNA from T cells and B cells also cultured for two hours in medium alone as the test samples.

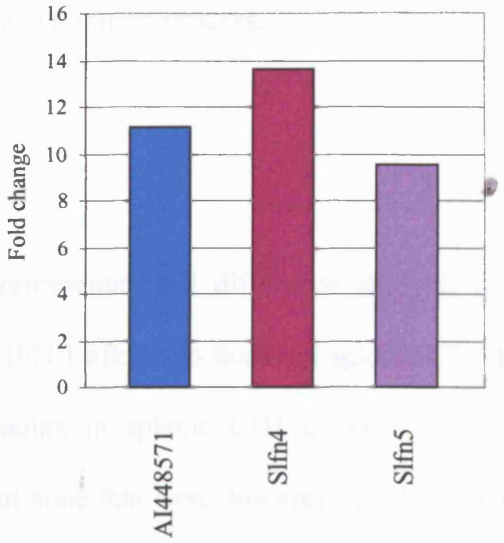


Figure 3.11: Expression of AI448571, Slfn4 and Slfn5 are induced by culture of DCs in medium alone. Real time PCR was used to calculate fold changes in levels of expression of the indicated genes in DCs cultured for six hours in medium alone compared to DCs cultured for two hours in medium alone.

associated with upregulation of these genes. In fact, AI448571, Slfn4 and Slfn5 were all markedly upregulated in DCs between two and six hours of culture (17-24 fold, Figure 3.11). This indicates that expression of these genes is being upregulated as the DCs mature, and that IFN-I is accelerating the maturation process.

3.3 Discussion

We have carried out representational difference analysis to investigate differential gene expression induced by IFN-I after two hours in splenic $CD11c^+$ DCs, $CD8^-$ and $CD8^+$ DC subsets and after six hours in splenic $CD11c^+$ DCs. We detected genes which were upregulated by IFN-I but none that were downregulated. The majority of genes identified in these experiments fell into two broad categories. Firstly, we found genes which had previously been characterized as IFN-induced, such as Pml, Gbp2 and the Ifit gene family. Secondly, we identified genes and which had not been previously shown to be induced by IFN; these genes had little functional characterization such as Slfn4 or were expressed sequences representing putative or novel genes with no assigned function.

Identification of genes whose expression is known to be induced by IFN-I confirmed that we were identifying true difference products. One of these genes, interferon response factor 7 (Irf7) which we detected at six hours of IFN-I stimulation, belongs to a family of transcription factors which regulate the cellular response to extracellular pathogens (Taniguchi *et al.*, 2001). Irf7 was first identified as a protein which bound to the promoter of an Epstein Barr gene and was linked to controlling the latency of this virus (Zhang *et al.*, 1997). It is expressed predominantly in lymphoid tissues as four different isoforms (Zhang *et al.*, 1997; Au *et al.*, 1998) and is induced by IFN-α, viral infection and LPS (Au *et al.*, 1998). IRF7 is a key mediator in the expression of type I IFNs themselves. Translocation

of IRF7 to the nucleus is dependent on virally-induced phosphorylation, preventing inappropriate IFN-I production. Therefore it is not surprising that we did not detect induction of the IFN-I genes themselves.

Pml was identified as the gene whose disruption in humans leads to acute promyelocytic leukemia (Goddard *et al.*, 1991) and has since been implicated in multiple cellular functions: cell growth, apoptosis, regulation of transcription and viral inhibition (Zhong *et al.*, 2000), (Ruggero *et al.*, 2000). Pml expression is regulated by signals of cellular stress such as viral infection and heat shock. It is upregulated by both type I and type II IFNs and could therefore mediate anti-viral and pro-apoptotic effects of IFN. In support of this, TNF-related apoptosis inducing ligand (TRAIL) has recently been identified as a downstream transcriptional target of Pml (Crowder *et al.*, 2004). The multiple functions of Pml seem inextricably linked with multiprotein complexes known as nuclear bodies (NBs) with which Pml is associated. Pml is necessary for the formation and integrity of NBs to which a wide variety of other proteins are also recruited. These include the apoptotic protein Daxx and other IFN-induced proteins such as the anti-viral protein Mx1. However, one isoform of Pml which is not associated with NBs has recently been shown to be a modulator of TGF-β signaling (Lin *et al.*, 2004).

Gbp2 has a role in growth regulation (Gorbacheva *et al.*, 2002), and belongs to a family of guanylate binding proteins (Gbps) which were first identified as some of the most abundantly expressed proteins produced after IFN stimulation (Boehm *et al.*, 1998), and have demonstrated anti-viral activity (Anderson *et al.*, 1999). However, Gbps are more strongly up-regulated by IFN γ than by IFN-I (Cheng *et al.*, 1986).

The relative abundance of Gbps and Pml would likely contribute to their frequent cloning in these experiments. Another factor could be that they are preferentially amplified: the Pml gene is large, (4.3kb) and contains numerous *Dpn*II restriction sites which would yield up to five different amplifiable fragments in an RDA, depending on the Pml isoform.

Two of the novel sequences, after carrying out a BLAST search of the Ensemble database, were found to be located within the intronic sequence of two genes, Z-DNA binding protein (Zbp) and Mx1. It is likely that these transcripts were isolated before splicing out of the introns had occurred. Zbp (also known as DLM-1) was originally identified as a gene upregulated in response to the presence of tumours and is upregulated in macrophages after four hours stimulation by IFN-γ (Fu *et al.*, 1999). Mx1 is a well known anti-viral whose expression is controlled by IFN-I (Staeheli *et al.*, 1986a).

Three further novel genes were upregulated in IFN-I treated DCs. Firstly, 9130002C22Rik, a full length cDNA clone which encodes a protein sharing 92% amino acid identity with a recently described protein, very large inducible GTPase-1 (VLIG-1). VLIG-1 is a member of a new family of IFN-inducible GTPases (Klamp *et al.*, 2003). Second we identified a sequence known as AI448571 whose function remains unknown. The final novel gene was known at the time as "similar to hypothetical protein FLJ34922", located within 100kb of the original Slfn gene family on chromosome 11. This gene has recently been redesignated Slfn5 and is a member of a new subgroup of the Slfn family (Geserick *et al.*, 2004). Members of this subgroup have a unique C terminus containing motifs present in RNA helicases. Another member of the Slfn gene family, Slfn4, was upregulated in DCs treated for six hours with IFN-I. Slfn genes are differentially regulated in thymocyte development and have been implicated in cell growth control (Schwarz *et al.*, 1998). Expression of many of the Slfn genes is induced by IFN-γ and LPS in bone-marrow derived macrophages,

although Slfn4 was only observed to be elevated by LPS treatment (Geserick *et al.*, 2004). Notably, induction of Slfn genes by LPS was dependent on IFN-I signaling. In addition to being induced during LPS or IFN-γ induced activation, transcription of Slfn genes was upregulated during differentiation of the myeloid cell line, M1, into macrophage-like cells. We found that expression of Slfn4 and Slfn5 were highly elevated in DCs cultured for six hours compared to DCs cultured for two hours. Since DCs mature when isolated and placed in culture, it is possible that these Slfn genes could also be involved in regulating DC maturation.

We tested the expression patterns of these genes, including the Mx1 intronic sequence, by real-time PCR and found that each of the novel genes were upregulated in DCs, T and B cells by IFN-I. With the exception of the Mx1 intronic sequence, they were expressed at higher levels in untreated T and B cells than in untreated DCs. Therefore it appears that these genes do not confer a cell-specific function for DCs and are more likely to serve a more general function such as anti-viral activity.

RelB, a component of the transcription factor NF-κB, was upregulated in DCs treated with IFN-I for six hours. NF-κB is plays a key role in both the innate and adaptive immune responses. By mediating signal transduction from TLRs NF-κB initiates production of inflammatory cytokines and prevents apoptosis (Bonizzi *et al.*, 2004). In human monocyte-derived DCs, CD40 ligand-induced maturation and APC function is dependent on NF-κB (O'Sullivan *et al.*, 2002). Since IFN-I is known to induce CD40 expression by murine DCs (Montoya *et al.*, 2002), it could in this way amplify the CD40 ligation signal to further enhance DC maturation. RelB also plays an important role in DC differentiation/maturation, since RelB knockout mice are deficient in $CD8^-$ DCs (Wu *et al.*, 1998).

Several of the sequences which were isolated belonged to genes which have no known function. In an attempt to predict something about the possible function of these genes we used a bioinformatics approach. Where translated sequences were available, we carried out InterPro and protein BLAST searches which, in some cases, identified domains which hinted at their function. The translated sequence of the RIKEN cDNA 9530019H02 gene contained a conserved domain belonging to the Ras GTPase superfamily and was most closely related to the Rab subfamily of proteins. Rab proteins are involved in many aspects intracellular vesicle transport, including different stages of endocytosis, exocytosis and vesicle movement between the ER and Golgi (Novick *et al.*, 1997). The Rab subfamily of proteins is large, containing at least 40 members in mammals, likely reflecting the complexity of vesicle transport systems and the need for different systems in different cell types. Indeed expression of some Rab GTPases is restricted to certain cell types as they carry out specific functions only required in those cell types (Stenmark *et al.*, 2001). The ML (MD-2 related lipid recognition) domain identified in the epididimal secretory protein (also known as Niemann-Pick type C2) is involved in innate immunity (Inohara *et al.*, 2002). This domain is present in MD-2 and MD-1 which act as co-factors for TLR4 and RP105 LPS recognition respectively (Shimazu *et al.*, 1999; Miyake *et al.*, 2000). The KIAA0431 protein contains zinc-finger binding domains which are a common feature of DNA-binding proteins and indicate a function as a transcription factor.

The number of genes identified by RDA was limited and had not provided us with any promising candidates for conferring an enhanced ability to stimulate secondary immune responses. The technique of cDNA RDA does have its limitations and may not be appropriate when a large number of differences are expected. Since certain products will be

preferentially enriched, many differentially expressed genes could be missed. Although cDNA RDA is very sensitive and can detect absolute differences between genes that are expressed at very low levels it may not detect small (3-5 fold) differences in transcript abundance (Hubank *et al.*, 1999). We did not detect a change in expression in co-stimulatory molecules CD80 or CD86, whose expression at the cell surface is known to be induced by type I IFNs. In addition, we did not identify any chemokines or cytokines known to be induced by IFN in these experiments, for instance CXCL9, CXCL10 and IL-15. Using the technique of RDA, genes which may be expressed at high levels but which do not increase more than three-fold after IFN treatment may not be detected. In order to gain a more global picture of changes in gene expression after IFN treatment, we decided to use the approach of microarray analysis.

Chapter 4 :

Identification of genes regulated by IFN-I in DCs by microarray analysis

4.1 Introduction

In recent years, microarrays have been developed as a powerful tool for examining gene expression, allowing simultaneous analysis of the expression of thousands of transcripts from a biological sample. The completion of genome sequencing projects has meant that arrays covering entire genomes can be manufactured, providing a means for truly global gene expression analysis. A great deal of valuable data can be obtained through microarray experiments, although the enormous volume of data generated means that analysis is difficult and time consuming.

Two kinds of array are widely used, cDNA and oligonucleotide arrays, which are manufactured and analysed in different ways. cDNA arrays are produced by spotting PCR products representing specific genes onto a glass or membrane matrix and fixed by ultraviolet irradiation (Duggan *et al.*, 1999). Our experiments were carried out on Affymetrix oligonucleotide arrays, which are made by building the oligonucleotides onto the array step by step using a combination of photolithography and combinatorial chemistry (Pease *et al.*, 1994). A solid support covered with photoprotected hydroxyls is illuminated through a mask which removes the photolabile protecting groups so that a nucleoside phosphoramidite can be coupled to the hydroxyl group. Cycles of illumination and coupling are repeated, generating a specific set of oligonucleotide probes at known locations on the array. This method eliminates the possibility of misidentification of an array feature which may occur when dealing with large libraries of cDNA clones.

Affymetrix arrays use several different oligonucleotides to probe expression of the same gene transcript (known as a probe set), thereby reducing any cross-hybridising effects. Additionally, each oligonucleotide has a mismatched partner which serves as a control for cross-hybridisation and is used for background subtraction. The use of multiple short (25-mer) oligonuceotides on the Affymetrix arrays may provide the greatest discrimination between related sequences but at the expense of poorer hybridisation efficiency (Hardiman, 2004). Another difference between the two systems is that whereas samples for comparison by Affymetrix arrays are hybridised to separate arrays, samples for comparison on cDNA arrays are hybridised to the same array and are distinguished using a two-colour dye system. Hybridisation to replicate arrays can be an advantage since the control sample can be compared to limitless test samples.

In the last few years several groups have utilised microarrays for the exploration of DC function. An early focus of these studies was to identify genes regulated during differentiation and maturation of human $CD14^+$ monocytes into immature and mature DCs (Lapteva *et al.*, 2001; Le Naour *et al.*, 2001). The validity of this approach was confirmed by the identification of genes known to be regulated during differentiation and maturation such as genes encoding MHC class II and co-stimulatory molecules and the chemokine receptor CCR7. In addition, genes not previously associated with DC maturation were identified, including genes encoding cell adhesion molecules and secreted factors.

The response of DCs to different activation stimuli, including CD40L (Tureci *et al.*, 2003), pathogens and their components, has also been investigated using microarray technology. Distinct subsets of genes were shown to be regulated in human monocyte-derived DCs in response to *Escherichia coli*, *Candida albicans* and influenza virus (Huang *et al.*, 2001a).

Similarly, Grannuci *et al.*, have examined the gene expression profile in the mouse DC line D1 when driven to maturation by TNF-α or LPS (Granucci *et al.*, 2001b) and over a time course of activation by Gram-negative bacteria (Granucci *et al.*, 2001a). These studies have revealed a wide range of genes important in the different stages of DC maturation, from loss of phagocytic capability to the efficient stimulation of T and B cells. In addition to giving an overall view of the relationship between gene regulation and kinetics of DC maturation, these studies have provided fresh insights into DC function. For example IL-2, an essential mediator of T cell function, was found to be upregulated in DCs by Gram negative bacteria. Further *in vitro* analysis showed that DC-derived IL-2 was necessary for maximal T cell proliferation in a mixed lymphocyte reaction assay (Granucci *et al.*, 2001a). Also using the mouse cell line D1, Trottein et al. found that schistosoma mansoni larvae and their eggs elicited markedly different transcriptional responses in DCs, with the eggs preferentially inducing inflammatory cytokines, chemokines and IFN response genes (Trottein *et al.*, 2004). The difference in gene expression profiles was in part due to the autocrine action of IFN-β which was produced by egg-stimulated DCs, and could help to explain the different immune responses induced by them.

Oligonucleotide arrays have also been used successfully to determine differential gene expression between mouse splenic DC subsets (Edwards *et al.*, 2003a), which showed that the $CD4^-$ and double negative subsets were more similar to each other in gene expression profiles than the $CD8^+$ subset. Finally, gene expression analysis has been used to examine the effects of uptake of tumour-lysates on murine bone marrow-derived DCs (BMDCs) function (Grolleau *et al.*, 2003).

Microarray analysis has also been used to identify type-I IFN regulated genes. In a human fibrosarcoma cell line, 94 and 268 genes increased in expression by more than two-fold after

IFN-α2a and IFN-β stimulation respectively (Der *et al.*, 1998). Since different IFN-α subtypes and IFN-β exert themselves in distinct biological ways despite binding to the same receptor, data of this type can help to explain the phenomenon. Genes most highly up-regulated by the type I IFNs included well known interferon-stimulated genes such as MxA, MxB, 2-5A synthetase and STAT1. In addition, novel interferon-stimulated genes were identified, including genes involved in the apoptotic effect of IFNs.

We wished to obtain a comprehensive profile of genes regulated by IFN-α4 in DCs. To this end, we have used oligonucleotide arrays to identify genes differentially expressed in murine splenic and BMDCs treated with IFN-α4. Several hundred IFN-regulated genes were identified, including known IFN-stimulated genes and genes which we had previously identified by RDA. Analysis of the data from splenic DCs revealed several genes with a potential influence on DC function, which were chosen for further study. Real-time PCR was used to confirm IFN-induced expression of these genes in DCs and to determine their pattern of expression in other cell types of the immune system. In addition we confirmed the regulation of cytokines and chemokines at the protein level by ELISA.

4.2 Results

4.2.1 Gene expression in splenic DCs treated with IFN-I

4.2.1.1 Experimental overview

$CD11c^+$ DCs were initially isolated by FACS sorting as before. However, we subsequently found that we could consistently isolate DCs of high purity (>98% $CD11c^+$) by positive selection on consecutive MACS columns (Figure 4.1). Since we could recover more than double the number of DCs using this method than by FACS sorting, we used MACS columns to purify DCs for the remaining experiments. RNA was isolated from DCs which had been cultured either alone or with 2×10^4 units of IFN-α4/ml for two or six at hours 37^0C. As an indication of the quality of the experiment we assessed the regulation of Mx1 by real-time PCR. In one case Mx1 was not expressed at higher levels in the DCs treated with IFN-α4, and therefore these samples were discarded. The cRNA was hybridised to the Affymetrix murine genome U74Av2 array, which contains approximately 6000 functionally characterised genes and 6000 EST clusters. An example of a scanned array is given in figure 4.2. The experiments were carried out in triplicate and analyzed using Genespring software.

4.2.1.2 Normalisation and filtering criteria

Normalisation of data was carried out using Genespring 7 software. Differences in the overall signal intensities between arrays was corrected for by normalisation to the median value: the signal intensity for each probe set is divided by the median of the signal intensity for all the probe sets on the array. This step corrects for any differences between the arrays which are due to assay variability, such as sample concentration, hybridisation or staining efficiencies. The second normalisation step in Genespring is a per gene normalisation,

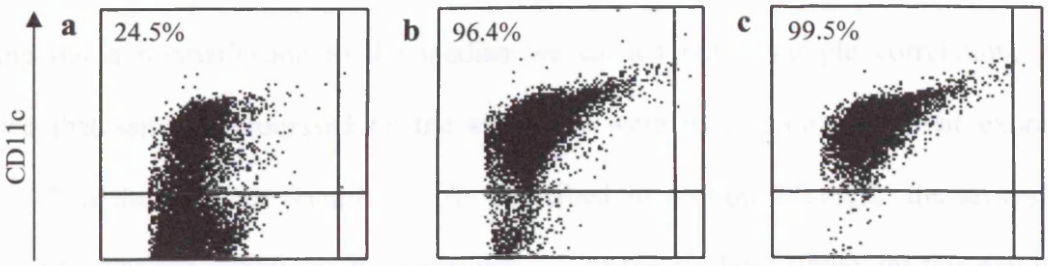


Figure 4.1 Purity of splenic DCs isolated by magnetic bead sorting
Splenic DCs were digested by collagenase/DNase followed by enrichment of low density cells on a Nycoprep density gradient (a). Cells were then stained with CD11c-FITC then with anti-FITC microbeads and positively selected on a MACS column (b). To isolate cells of high purity the cells were then isolated on a second MACS column (c).

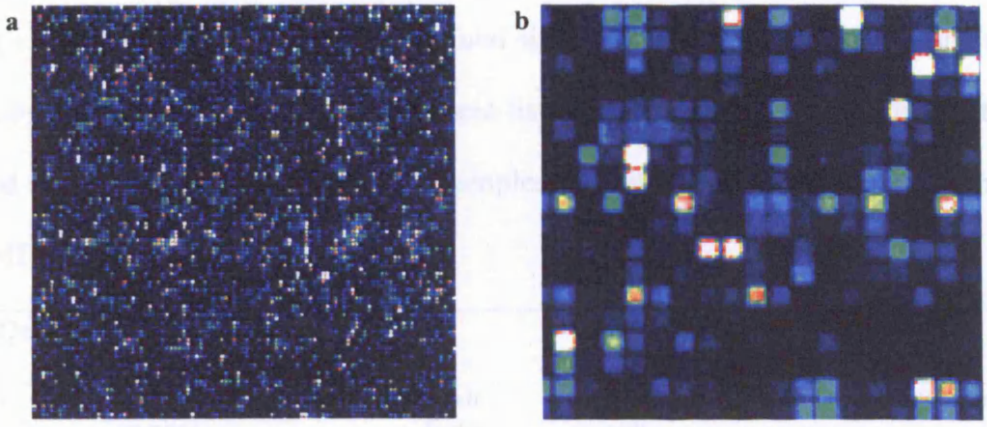


Figure 4.2 Section of a scanned Affymetrix array
Biotin-labeled cRNA samples were hybridised to Affymetrix arrays and stained with streptavidin-PE. The arrays were scanned on a GeneChip scanner 2000. a) an enlarged section and b) a further enlarged section of a scanned murine genome 430 2.0 array.

which is used to determine the level of transcript on one array relative to another. This normalisation can be done either by comparing the signal value to the median value for the probe sets or by normalising to the corresponding probe set in a specified sample. After carrying out a normalisation to the median we carried out a sample correlation, which revealed that samples processed on the same day were more similar in their expression profiles than the replicate samples. As described in section 2.2.6.2.2, the arrays were reviewed for various quality control measures. At the six hour timepoint this day to day sample variation can be seen in the number of genes whose expression has been detected as present (Table 4.1). For these reasons, the per gene normalisation was carried out between specific samples. This means that the measurements for the IFN-treated samples were normalised to the measurements from the control samples isolated on the same day. Following normalisations, the data was filtered so as to only consider those genes which were being expressed in at least two out of a total six samples, and which were changing in expression by at least 1.5 fold. The filtered gene list was then subjected to a Welch's t-test, a t-test used when the variances between the samples are not assumed to be equal, with a p-value cut-off of 0.05.

Table 4.1 Quality control measurements

Sample Name	Replicate number	Q (Noise)	Scale factor	Genes present	GAPDH	B-actin
Untreated 2h	1	2.59	0.828	43.0%	2.04	1.97
	2	1.84	0.653	51.5%	1.92	1.51
	3	2.75	0.576	49.5%	1.83	1.27
IFN-treated 2h	1	1.97	0.891	45.1%	1.73	1.72
	2	1.76	0.548	53.8%	1.9	1.48
	3	2.43	0.555	51.4%	2.4	1.45
Untreated 6h	4	2.27	0.922	41.5%	1.88	1.55
	5	1.69	3.349	29.2%	2.82	2.83
	6	1.78	4.327	27.1%	3.7	2.67
IFN-treated 6h	4	2.1	1.038	37.0%	2	1.62
	5	1.59	7.38	22.2%	3.08	2.69
	6	1.98	7.464	22.1%	4.18	3.64

4.2.1.3 Analysis of differentially expressed genes

The expression of 201 genes changed significantly by at least 1.5 fold after 2h IFN stimulation and 270 genes after 6h , of which 48 were common to both (Figure 4.3). Many of the genes identified in these experiments were known IFN-induced genes which we had also previously identified by RDA, for example Ifit2 and Ifit3, Pml and Gbp2, confirming that the results were reliable. By RDA we had identified a thymidylate kinase family member known as thymidylate kinase family, LPS-inducible member (Tyki). This gene showed the highest fold-increase in expression of any of the genes, and was increased by more than 100-fold in the DC IFN sample compared to the untreated control at two hours of culture. This high fold change is in part due to the extremely low level of expression in the untreated control sample, which was barely detectable. Thymidylate kinase is an essential enzyme in the synthesis of dTTP, but it is not clear what role thymidylate kinase family members may play in immunity. We identified genes involved in a wide range of functions; anti-viral (Mx1), antigen presentation (Tap1), signaling (Stat1), transcription factors (Irf7), chemokines (CXCL9), cytokines (IL-15), and their receptors (CCR5, IL-6R). Many of the genes identified were of unknown function.

Genes which were detected as differentially expressed after two hours (2h) but not after six hours (6h) (Table 4.2) included CCR7, which is essential for the migration of maturing DCs to the draining lymph node. The signal values from the 6h data show that CCR7 was being expressed at high levels by both untreated and IFN-treated DCs at this timepoint, and therefore the fold difference between them was lower. It is possible that the expression of CCR7 mRNA by the IFN-DC had reached a plateau by six hours. This was true for a number of genes, including Ifi204 and Tap1, which were clearly still being maintained at elevated levels by IFN at 6h but the fold increase compared to the untreated samples was less

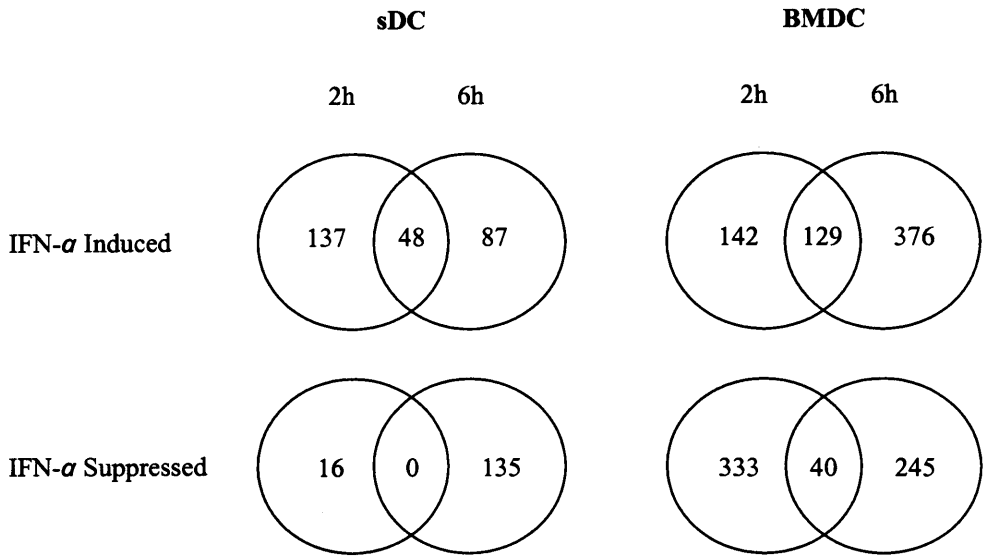


Figure 4.3 Number of genes whose level of expression changed by at least 1.5 fold after IFN-α4 treatment Samples from CD11c$^+$ sDCs or BMDCs treated *in vitro* in the presence of 2 x 10^4 units IFN-α4 or in media alone were hybridised to Affymetrix murine U74Av2 or 430 2.0 arrays. Data generated from BMDCs is presented here for completeness and is discussed in section 4.2.2. Data was analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05.

Gene	Description	Fold Change	2h Signal 0	2h Signal IFN	6h Signal 0	6h Signal IFN	Affymetrix Accession	Genbank Accession
EST	EST AA793972	14.1	11	124	23	53	97162_at	AI451676
Mx2	Myxovirus resistance 2	12.5	39	492	401	860	102699_at	J03368
Ifi204	interferon activated gene 204	8.3	8	67	182	187	98466_r_at	M31419
AI447904	expressed sequence AI447904	8.0	34	276	366	450	103615_at	AA727023
Il15	interleukin 15	7.6	8	68	86	152	161037_at	U14332
Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	6.9	34	237	125	152	97322_at	AI835093
Trim30	tripartite motif protein 30	6.3	52	332	266	294	98030_at	J03776
Slfn1	schlafen 1	6.0	20	123	300	465	102264_at	AF099972
AI481105	expressed sequence AI481105	5.5	107	598	456	735	102965_at	AW121646
LOC236219	expressed sequence LOC236219	5.4	30	162	72	104	102254_f_at	AA289585
Pttg1	pituitary tumor-transforming 1	5.3	8	46	21	28	101026_at	AF071209
Gbp3	guanylate binding protein 3	5.2	90	532	739	1556	103202_at	AW047476
Tor3a	torsin family 3, member A	5.2	49	258	200	248	96533_at	AI508931
Ifi204	interferon activated gene 204	5.0	209	1055	1900	3067	98465_f_at	M31419
Gpr33	G protein-coupled receptor 33	4.9	53	261	111	197	94184_at	AF045766
Trim21	tripartite motif protein 21	4.9	105	541	356	744	92942_at	AA138192
Pml	promyelocytic leukemia	4.4	96	408	442	676	99015_at	U33626
Tpst1	protein-tyrosine sulfotransferase 1	3.9	68	275	197	227	103032_at	AF038008
Ifi35	interferon-induced protein 35	3.8	204	775	712	866	100013_at	AW121732
Pnpt1	polynbonucleotide nucleotidyltransferase 1	3.7	47	182	123	144	99194_at	AW124271
EST	Mus musculus transcribed sequences	3.7	16	55	89	144	98597_at	AW048912
Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	3.7	221	756	624	615	102104_f_at	AI504305
Sap30	sin3 associated polypeptide	3.7	65	246	56	62	160068_at	AF075136
Fndc3a	fibronectin type III domain containing 3a	3.6	89	324	256	427	104419_at	AI132380
Ifi204	interferon activated gene 204	3.5	148	503	584	842	92251_f_at	AA960657
2310005P05Rik	RIKEN cDNA 2310005P05 gene	3.5	6	21	7	11	160916_at	AI838150
Usp25	Ubiquitin specific protease 25	3.4	15	49	21	28	95858_at	C76988
Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	3.2	48	166	41	64	103518_at	X15592
6720480F16Rik	RIKEN cDNA 6720480F16 gene	3.2	12	35	10	23	103768_at	AW209561
Tnfsf10 (TRAIL)	tumor necrosis factor (ligand) superfamily, member 10	3.1	4	12	20	22	94112_at	U37522
EST	Mus musculus transcribed sequences	3.1	15	46	29	41	96579_at	AA267568
Adar	adenosine deaminase, RNA-specific	3.0	80	246	186	180	96188_at	AF052506
Snx2	sorting nexin 2	3.0	182	536	345	419	96331_at	AI842754
Stat1	signal transducer and activator of transcription 1	2.9	98	283	583	557	101465_at	U06924
Ogfr	opioid growth factor receptor	2.8	266	752	555	636	160668_at	AI838195
Trim21	tripartite motif protein 21	2.8	36	103	126	168	102678_at	L27990
Irf7	interferon regulatory factor 7	2.7	60	165	328	506	162202_f_at	AV373853
Fln29-pending	FLN29 gene product	2.7	463	1307	1160	1570	103254_at	AW049897
D1Ertd622e	DNA segment, Chr 1, ERATO Doi 622, expressed	2.7	102	277	187	232	160597_at	AW047450
etnk1	ethanolamine kinase 1	2.7	159	424	319	354	160393_at	AI853226
Isgf3g	interferon dependent positive acting transcription factor 3 gamr	2.6	301	810	495	490	103634_at	U51992
Rnf34	ring finger protein 34	2.5	60	153	247	248	101481_at	AI852555
Dck	deoxycytidine kinase	2.5	403	975	440	608	98071_f_at	X77731
Adar	adenosine deaminase, RNA-specific	2.5	26	65	86	106	102741_at	AW046250
1810054D07Rik	RIKEN cDNA 1810054D07 gene	2.4	5	12	3	5	93462_at	AA168418
Zfp90	zinc finger protein 90	2.4	21	47	28	34	92934_at	X79828
Dck	deoxycytidine kinase	2.3	17	39	31	33	98072_r_at	X77731
Max	Max protein	2.3	177	420	201	267	99095_at	M63903
Nmi	N-myc (and STAT) interactor	2.3	46	107	171	254	101424_at	AF019249
Myd88	myeloid differentiation primary response gene 88	2.3	135	315	356	510	102430_at	X51397
9130427A09Rik	RIKEN cDNA 9130427A09 gene	2.3	68	155	128	246	93484_at	AW061306
Csprs	HSR; Mouse HSR mRNA, clone pMmHSRc-[1,3,3E,10 and 10l	2.3	41	94	112	124	101846_r_at	M55219
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	2.2	236	530	865	1189	94881_at	AW048937
Rasa4	RAS p21 protein activator 4	2.2	699	1544	1239	1737	160965_at	AA163960
Dtnb	dystrobrevin, beta	2.2	22	47	15	16	102210_at	AJ003007
Anxa1	annexin A1	2.1	24	50	42	69	93037_i_at	M69260
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)	2.1	165	342	323	361	98067_at	U09507
Nedd9	neural precursor cell expressed, developmentally down-regulat	2.1	50	102	33	56	101469_at	AF009366
Map2k1	mitogen activated protein kinase kinase 1	2.0	158	325	375	388	92585_at	L02526
Tom70a	translocase of outer mitochondrial membrane 70 homolog A (yeast)	2.0	140	283	254	384	95432_f_at	AI844034
Ddx24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	2.0	151	297	118	125	99096_at	U46690
D530037H12Rik	RIKEN cDNA D530037H12 gene	2.0	17	33	23	33	92653_at	AI482432
Stard3	START domain containing 3	2.0	173	348	365	398	95607_at	X82457
Ccr5	chemokine (C-C motif) receptor 5	2.0	22	44	45	64	102718_at	AF022990
BC004022	cDNA sequence BC004022	2.0	169	336	171	207	103930_at	AI413179
Fnbp4	formin binding protein 4	2.0	42	84	89	71	101422_at	AW121377
Mov10	Moloney leukemia virus 10	2.0	212	414	178	163	103025_at	X52574
Casp1	caspase 1	2.0	123	244	363	413	102064_at	L28095

Signal intensity

0-10
10-100
100-250
250-500
500-1000
1000-2000
2000-3000
3000-4000
4000-5000
>5000

Table 4.2 (continued over page) IFN-induced genes in splenic DCs after 2h but not 6h of culture. Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine U74Av2 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key.

Gene	Description	Fold Change	2h Signal 0	2h Signal IFN	6h Signal 0	6h Signal IFN	Affymetrix Accession	Genbank Accession
Insl3	insulin-like 3	1.9	10	18	38	24	101873_at	X95603
Gnb4	guanine nucleotide binding protein, beta 4	1.9	36	69	146	186	93949_at	M63658
Ppicap	peptidylprolyl isomerase C-associated protein	1.9	148	287	599	769	97507_at	X67809
6330442E10Rik	RIKEN cDNA 6330442E10 gene	1.9	79	156	72	108	103424_at	AI844839
Arhh	ras homolog gene family, member H	1.9	49	96	61	89	103231_at	AA739233
Sh3bp2	SH3-domain binding protein 2	1.9	33	64	23	66	92975_at	L14543
4632413K17Rik	RIKEN cDNA 4632413K17 gene	1.9	18	34	20	15	102784_at	AI843949
Rfc3	replication factor C (activator 1) 3	1.9	32	62	79	94	160072_at	AV026570
Tnfrsf1a	tumor necrosis factor receptor superfamily, member 1a	1.9	216	411	146	243	92793_at	X57796
Csprs	component of Sp100-rs	1.9	28	52	52	61	101845_s_at	M55219
Snx2	sorting nexin 2	1.8	125	229	48	95	96333_g_at	AW259199
Emp1; TMP	M.musculus emp-1 gene.	1.8	168	312	422	458	97426_at	X98471
Mbd2	methyl-CpG binding domain protein 2	1.8	74	135	163	209	160833_at	AF072243
Ccng2	cyclin G2	1.8	147	267	324	502	98478_at	U95826
5031425D22Rik	RIKEN cDNA 5031425D22 gene	1.8	51	93	13	11	103221_at	AA940352
Ythdf1	YTH domain family 1	1.8	195	345	92	105	96949_at	AI853542
Atp6v0a2	ATPase, H+ transporting, lysosomal V0 subunit a isoform 2	1.8	150	272	195	212	103681_at	AW049274
Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	1.8	1048	1877	1671	2136	103035_at	U60020
Anxa1	annexin A1	1.8	47	98	108	165	161703_f_at	AV003419
Gyg1	glycogenin 1	1.8	141	250	167	328	100597_at	AW049730
Zfp50	zinc finger protein 50	1.8	19	34	22	11	161031_at	AI842128
Slit1	slit homolog 1 (Drosophila)	1.8	241	429	237	291	160307_at	AW121695
Minpp1	multiple inositol polyphosphate histidine phosphatase 1	1.8	42	74	24	19	99640_at	AW045481
Cr2	complement receptor 2	1.8		10	14	10	102289_r_at	M29281
Tcf4	transcription factor 4	1.7		16			160483_at	U16322
D10Wsu159e	Mus musculus transcribed sequences	1.7	169	327	333	401	100748_at	AA409010
D5Bwg0860e	DNA segment, Chr 5, Brigham & Women's Genetics 0860 exp	1.7		14		13	161062_r_at	AW049573
Hnrpa1	alternative splicing modulator; RNA-binding protein; Mus musc	1.7	62	106	52	65	100371_at	U65316
Klrk1	killer cell lectin-like receptor subfamily K, member 1	1.7	58	55	165	246	93678_s_at	AF054819
Nrp1	neuropilin 1	1.7	11	16	28	28	92773_at	AF079528
Scarb2	scavenger receptor class B, member 2	1.7	224	382	282	310	101389_at	AB008553
Sdcbp	syndecan binding protein	1.7	131	221	100	139	93017_at	AF077527
Nono	non-POU-domain-containing, octamer binding protein	1.7	39	66	84	84	93831_at	AI316087
Vrk1	vaccinia related kinase 1	1.7	71	119	54	56	97393_at	AF080253
Atp6v0a2	ATPase, H+ transporting, lysosomal V0 subunit a isoform 2	1.7	82	139	103	108	160960_at	AA881202
Evi2	ecotropic viral integration site 2	1.7	1077	1806	355	597	98026_g_at	M34896
Trim25	tripartite motif protein 25	1.7	544	897	1021	1197	100475_at	D63902
Golga3	golgi autoantigen, golgin subfamily a, 3	1.7	34	58	69	85	93951_at	AB029537
CD47	CD47 antigen	1.7	270	453	381	337	95020_at	AI848868
Hk2	hexokinase II	1.6	222	366	205	265	94375_at	Y11666
Usp38	ubiquitin specific protease 38	1.6	64	107	56	62	160605_s_at	AI157260
Bcl6	B-cell leukemia/lymphoma 6	1.6	422	691	426	377	103015_at	U41465
Cybb	cytochrome b-245, beta polypeptide	1.6	107	176	103	173	100300_at	U43384
Nfil3	nuclear factor, interleukin 3, regulated	1.6	22	36		25	102955_at	U83148
B130024L21Rik	RIKEN cDNA B130024L21 gene	1.6	213	355	230	297	161723_at	AV264147
5830406C15Rik	RIKEN cDNA 5830406C15 gene	1.6	10	17	11		103223_at	AW120767
Klrk1	killer cell lectin-like receptor subfamily K, member 1	1.6	64	106	185	247	93679_at	AF030313
Scotin	scotin gene	1.6	820	1352	1951	2532	95102_at	AW123754
Plscr1	phospholipid scramblase 1	1.6	231	371	349	483	102839_at	D78354
Pon3	paraoxonase 3	1.6		12	14	23	93940_at	L76193
Evl	Ena-vasodilator stimulated phosphoprotein	1.6	66	106	41	66	161808_f_at	AV371846
2010012F05Rik	RIKEN cDNA 2010012F05 gene	1.6	1087	1726	1651	1922	103555_at	AI843520
Stag2	stromal antigen 2	1.6	77	121	61	61	104595_at	AI846890
HRP12	heat-responsive protein 12	1.6		11		10	96048_at	U50631
Sugt1	SGT1, suppressor of G2 allele of SKP1	1.5	119	184	331	346	93366_r_at	AI838149
Cd47	CD47 antigen	1.5	793	1234	1163	1216	103611_at	AB012693
Ankrd17	ankyrin repeat domain 17	1.5	29	42	14		104683_at	AW120783
Adamdec1	ADAM-like, decysin 1	1.5	113	173	131	121	98976_at	AJ242912
Wars; WRS	tryptophanyl-tRNA synthetase	1.5	53	81	71	100	98606_s_at	X69656
Phxr1	per-hexamer repeat gene 1	1.5	70	107	65	61	101705_at	X12809
Elf1	E74-like factor 1	1.5	35	54	51	56	160721_at	U19617
CD200	CD200 antigen	1.5	40	61	220	233	101851_at	AF029215
Auh	AU RNA binding protein/enoyl-coenzyme A hydratase	1.5	66	93	113	124	96650_at	AI837724
Crebbp	CREB binding protein	1.5	109	167	90	76	95886_g_at	AA177826
Usp22	ubiquitin specific protease 22	1.5	27	41	12		97960_at	AW125800
D730040F13Rik	RIKEN cDNA D730040F13 gene	1.5	20	30	36	35	104661_at	AF031164
Rasd1	RAS, dexamethasone-induced 1	1.5	20	30	24	31	99032_at	AF009246
Snx2	sorting nexin 2	1.5	11	17	21	18	96332_at	AW259199
Ccr7	chemokine (C-C motif) receptor 7	1.5	1026	1524	4185	4556	104443_at	L31580

Signal intensity
0-10
10-100
100-250
250-500
500-1000
1000-2000
2000-3000
3000-4000
4000-5000
>5000

Table 4.2 (continued) IFN-induced genes in splenic DCs after 2h but not 6h of culture

than 1.5 due to high levels of expression in the untreated sample. In contrast, the TNF receptor (p55 subunit) mRNA which was induced by IFN-α4 at 2h, was lower at six hours in both untreated and IFN-treated samples. At 2h, the majority of differentially expressed genes represented those that were induced by IFN-α4, with only 16 genes being suppressed (Table 4.3). Most notably, IL-6 receptor expression was lower in IFN-DC. IL-6 is thought to maintain DCs in an immature state, and has been shown to inhibit LPS-induced maturation of BMDCs (Park *et al.*, 2004b). Therefore, by down-regulating the IL-6 receptor DCs could escape this suppression, enabling their maturation.

48 genes showed elevated expression in IFN-treated versus untreated DCs after both 2h and 6h of culture (Table 4.4). Many genes associated with DC function were induced by IFN at both timepoints, including IL-6, CD40 and the chemokines CXCL9 and CXCL10. A non-classical MHC class I gene from the TL locus was identified (H2-T10), which is recognised by the γδ-T cell receptor. Ly6a, a haemopoietic stem cell marker, was expressed at very high levels after 6h of IFN-treatment. Ly6a expression is associated with lymphocyte activation (Malek *et al.*, 1986), and has been shown to be dramatically induced in T cells by IFN-I and to a lesser extent IFN-γ (Dumont *et al.*, 1986). Ly6a is expressed constitutively on splenic DCs (Izon *et al.*, 1994). Despite intense investigation, the function of Ly6a is still unclear, although it may be involved in cell signaling (Malek *et al.*, 1986; Rock *et al.*, 1986) and cell adhesion (Bamezai *et al.*, 1995). Several other familiar genes are present in the data set, including Tyki and Pml. Another highly expressed gene is Vig1 (viral hemorrhagic septicemia virus (VHSV) induced gene 1), which we had previously identified as IFN-induced by RDA. In mice, DCs have been identified as the main cell population which induce Vig1 expression in response to vesicular stomatitis virus (VSV) infection and pseudorabies virus (PrV) by both IFN-independent and IFN-I-dependent pathways,

Gene	Description	Fold Change	2h Signal 0	2h Signal IFN	6h Signal 0	6h Signal IFN	Affymetrix Accession	Genbank Accession
Gla; Ags	galactosidase, alpha	3.1	42	13	45	56	102341_at	L46651
Cggbp1	CGG triplet repeat binding protein 1	2.3				15	95574_f_at	AI641995
Sap18	Sin3-associated polypeptide 18	2.2	15		57	66	161303_at	AV054196
Nfkbiz	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, zeta	1.9	16		14	15	161903_f_at	AV374591
Galnt4	polypeptide N-acetylgalactosaminyltransferase 4	1.9	14		24	24	161016_at	AA673574
St6gal1	beta galactoside alpha 2,6 sialyltransferase 1	1.8	28	15	25	53	94431_at	D16106
Il6ra	interleukin 6 receptor, alpha	1.8	162	91	68	99	104268_at	X51975
EST	Mouse DNA sequence from clone CT7-BM573K1 on chromosome 17	1.7	62	37	202	192	102169_at	AL078630
1810029F08Rik	RIKEN cDNA 1810029F08 gene	1.7	35	21	57	75	161888_r_at	AV371196
Apoa2	apolipoprotein A-II	1.6	14		48	59	99648_at	X62772
Sec63	SEC63-like (S. cerevisiae)	1.6	31	20	54	98	99350_at	C76102
2310020H20Rik	RIKEN cDNA 2310020H20 gene	1.6	31	20		39	95645_at	AI837440
A430096B05Rik	RIKEN cDNA A430096B05 gene	1.6	23	15	56	43	162286_r_at	AV373294
Elk1	ELK1, member of ETS oncogene family	1.5	41	27	91	97	96593_at	X87257
Orc1l	origin recognition complex, subunit 1-like (S.cereviaiae)	1.5	14		19	31	92458_at	AJ003133
Dri1	dead ringer homolog 1 (Drosophila)	1.5	20	13	37	35	103496_at	U60335

Table 4.3 IFN-suppressed genes in splenic DCs after 2h but not 6h of culture.
Legend as in table 4.4 below

Gene	Description	2h Fold Change	2h Signal 0	2h Signal IFN	6h Fold Change	6h Signal 0	6h Signal IFN	Affymetrix Accession	Genbank Accession
TDKl	thymidylate kinase homologue	124.2		662	4.7	248	1194	103066_at	L32973
Ifit2	interferon-induced protein with tetratricopeptide repeats 2	32.0	52	1669	2.5	1905	4675	103639_at	U43085
Ifit3; Ifi49	interferon-induced protein with tetratricopeptide repeats 3	26.0	37	1003	2.2	1161	2530	93956_at	U43086
Ifit1	interferon-induced protein with tetratricopeptide repeats 1	23.9	42	869	1.8	1404	2490	100981_at	U43084
Cxcl10	chemokine (C-X-C motif) ligand 10	20.6	85	1827	3.8	600	2266	93858_at	M33266
Ifi47	interferon gamma inducible protein	16.5	55	896	1.9	1027	2027	104750_at	M63630
Usp18	ubiquitin specific protease 18	13.8	108	1531	2.5	1670	4151	95024_at	AW047653
Tgtp	T-cell specific GTPase	12.8	155	1899	2.0	2111	4230	102906_at	L38444
Mx1	myxovirus (influenza virus) resistance 1	12.1	147	1746	2.2	1516	3378	98417_at	M21038
Ifi1	interferon inducible protein 1	11.5	44	497	1.6	340	560	97409_at	U19119
Ifi203	interferon activated gene 203	10.9	48	507	2.0	149	297	93321_at	AF022371
Dnr12	Diabetic nephropathy-related gene 1	10.0	29	315	2.7	260	722	100880_at	AA816121
Igtp	interferon gamma induced GTPase	9.7	41	425	2.5	343	866	160933_at	U53219
Vig1	viral hemorrhagic septicemia virus(VHSV) induced gene 1	9.2	248	2199	2.9	1515	4384	104177_at	AA204579
Igtp	interferon gamma induced GTPase	9.2	75	668	2.0	538	1068	98410_at	AJ007972
IL-6	interleukin 6	8.4	50	466	3.7	102	416	102218_at	X54542
Isg20	cDNA for interferon stimulated protein (20kDa)	8.0	85	689	2.8	604	1659	103432_at	AW122677
Irf7	interferon regulatory factor 7	6.2	105	664	2.3	985	2240	104669_at	U73037
Isg15	cDNA for interferon stimulated protein (15kDa)	5.9	78	433	2.1	447	933	161511_f_at	AV152244
Pnp	purine-nucleoside phosphorylase	4.8	237	1227	2.6	1396	3659	93290_at	U35374
Gbp2	guanylate nucleotide binding protein 2	4.4	71	353	3.0	764	2336	104597_at	AJ007970
Ifih1	Interferon-induced with helicase C domain 1	4.3	177	760	1.7	660	1101	103446_at	AA959954
G1p2	interferon, alpha-inducible protein	4.1	381	1569	2.0	2595	5214	98822_at	X56602
Daxx	Fas-death domain associated protein Daxx	4.0	114	467	1.9	584	1149	96125_at	AF110520
Mad	Max dimerization protein	4.0	23	88	3.4	57	192	94688_at	X83106
Socs1	suppressor of cytokine signaling 1	3.7	70	275	3.1	61	250	92832_at	U88325
Ifi205	interferon activated gene 205	3.4	891	2990	1.7	3805	6521	94224_s_at	M74123
EST	EST	3.2	138	477	1.8	480	878	160799_at	AW060549
CD40	tumor necrosis factor receptor superfamily, member 5	3.2	189	612	1.6	468	756	92962_at	M83312
Slfn2	schlafen 2	3.0	296	895	1.6	783	1271	92472_f_at	AF099973
Fgl2	precursor; Mouse fibrinogen-like protein gene, exon 2.	2.9	297	867	2.0	712	1438	97949_at	M16238
Cxcl9	chemokine (C-X-C motif) ligand 9	2.8	619	1755	2.8	1073	2960	101436_at	M34815
Samhd1	SAM domain and HD domain, 1	2.8	455	1329	1.9	2187	4223	103080_at	U15635
Slfn2	schlafen 2	2.7	676	1876	1.7	1782	3100	92471_i_at	AF099973
Ifrg15	IFN-α responsive gene	2.7	189	495	1.5	557	864	93997_at	AI853475
March5	Membrane-associated ring finger (C3HC4) 5	2.5	394	1003	1.6	905	1429	99604_at	AI848713
Cited2	Cbp/p300-interacting transactivator 2	2.5	213	525	2.9	169	494	101973_at	Y15163
Ccr5	C-C chemokine receptor 5	2.5	113	287	2.8	100	280	161968_f_at	AV370035
Xdh	xanthine dehydrogenase	2.4	16	37	4.0	27	110	97950_at	X75129
Socs3	suppressor of cytokine signaling 3	2.3	75	188	2.6	59	158	92232_at	U88328
Irf1	interferon regulatory factor 1	2.3	443	974	1.9	843	1573	102401_at	M21065
Ly6a	lymphocyte antigen 6 complex, locus A	2.1	396	855	2.5	2892	7318	93078_at	X04653
H2-T10	histocompatibility 2, T region locus 10	1.9	314	626	1.5	995	1514	93865_s_at	M35244
Adfp	adipose differentiation related protein	1.7	206	369	2.0	374	757	98589_at	M93275
Evi2	ecotropic viral integration site 2	1.6	812	1337	1.7	323	544	98025_at	M34896
Bzrp	benzodiazepine receptor, peripheral	1.6	424	695	1.5	312	553	93042_at	D21207
Ccnd1	cyclin D1	1.6	528	879	1.8	1804	2732	94232_at	AI849928
Ifitm3	IFN-induced transmembrane protein 3	1.5	979	1493	1.5	2443	3709	160253_at	AW125390

Table 4.4 IFN-induced genes in splenic DCs after both 2h and 6h culture.
Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine U74Av2 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

respectively (Boudinot *et al.*, 2000). The human homologue, cig5, is also induced by IFN-I and has been shown to inhibit the replication of human cytomegalovirus (HCMV) (Chin *et al.*, 2001).

In contrast to the 2h data, there were more IFN-suppressed genes than IFN-induced genes at 6h. The genes induced by IFN at 6h but not 2h are listed in table 4.5. These included Ly86 (also known as MD-1) which is found associated with, and positively regulates the cell surface expression of a B cell surface molecule which promotes cell proliferation, RP105, (Miyake *et al.*, 1994; Miyake *et al.*, 1998). RP105/Ly86 shares structural similarity to TLR4/Ly96 and has an important role in the immune response to LPS (Ogata *et al.*, 2000). Ly86 is necessary for maximum proliferation of B cells in response to LPS (Nagai *et al.*, 2002). RP105/Ly86 expression was originally thought to be restricted to B cells. However it has recently been shown to be expressed on macrophages and DCs (Divanovic *et al.*, 2005). In addition it was shown that RP105/Ly86 can interact directly with TLR4/MD-2 and acted to suppress TLR4 signaling in a transfected cell line. TLR4-induced secretion of TNF, IL-12p70 and IL-6 by DCs was increased in RP105-deficient mice, indicating that RP105 acts as a negative regulator of TLR4 responses. Therefore this could represent an example of how type I IFNs could dampen an inflammatory response.

Interestingly, IL-6 signal transducer expression was suppressed at 6h by IFN-α4 (Table 4.6), which represents a second possible mechanism for avoidance of IL-6 regulation, along with the downregulation of IL-6 receptor expression seen after 2h.

Gene Name	Gene description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Ifng	interferon gamma	48.9		56		11	99334_at	K00083
Iigp-pending	interferon inducible GTPase 1	11.1	44	478	18	137	96764_at	AJ007971
Slfn4	schlafen4 (Slfn4)	9.8	123	1217	18	94	92315_at	AF099977
Iigp-pending	interferon inducible GTPase 1	8.6	58	502	15	100	103963_f_at	AA914345
Fcgr1	Fc receptor, IgG, high affinity I	5.5	47	212	33	66	102879_s_at	M31314
ART3	ADP-ribosyltransferase 3	5.1	11	47			98924_at	Y08027
Il10ra	interleukin 10 receptor, alpha	3.3	69	182	153	337	96525_at	L12120
Ccl4	chemokine (C-C motif) ligand 4	3.0	105	303	487	798	94146_at	X62502
Inpp5b	inositol polyphosphate-5-phosphatase B	3.0	402	1191	102	173	94398_s_at	AF040094
Ptgs2	prostaglandin-endoperoxide synthase 2	2.9	33	91	338	405	104647_at	M88242
Cnn3	calponin 3, acidic	2.6	44	115	51	58	160150_f_at	AW125626
Inpp5b	inositol polyphosphate-5-phosphatase B	2.6	415	1065	142	239	94399_at	AI843172
Lgals9	lectin, galactose binding, soluble 9	2.5	341	846	116	317	103335_at	U55060
Il1rn	interleukin 1 receptor antagonist	2.5	564	1444	316	427	93871_at	L32838
Atf3	activating transcription factor 3	2.4	609	1489	1440	2314	104155_f_at	U19118
Ch25h	cholesterol 25-hydroxylase	2.4	32	79	13	20	104509_at	AF059213
Gzmb	granzyme B	2.3	78	191	39	75	102877_at	M12302
C3	complement component 3	2.3	93	222	99	121	93497_at	K02782
Perp	PERP, TP53 apoptosis effector	2.3	21	43	12	13	97825_at	AI854029
Dnase1l3	deoxyribonuclease 1-like 3	2.2	38	60	92	97	94107_at	AF047355
Pttg1	pituitary tumor-transforming 1	2.2	388	859	122	323	101027_s_at	AF069051
Anxa1	annexin A1	2.2	275	613	204	399	93038_f_at	M69260
Gjb2	gap junction membrane channel protein beta 2	2.1	68	152	65	111	98423_at	M81445
Socs3	suppressor of cytokine signalling-3 (SOCS-3)	2.1	113	237	97	192	162206_f_at	AV374868
Caml	calcium modulating ligand	2.1	68	134	72	73	104529_at	U21960
Enam	enamelin	2.1	27	55	14	10	94623_at	U82698
Atf3	activating transcription factor 3	2.0	307	634	344	552	104156_r_at	U19118
Cpne4	cytokine inducible SH2-containing protein	2.0	147	304	88	113	100022_at	D89613
Phc2	polyhomeotic-like 2 (Drosophila)	2.0	122	245	255	464	92867_at	AF060076
Irf6	interferon regulatory factor 6	2.0	42	84	18	19	92440_at	U73029
Serpina3g	spi2 proteinase inhibitor	2.0	1275	2511	727	1448	102860_at	M64085
Sct	secretin	2.0	703	1388	146	211	92755_f_at	X73580
4930422J18Rik	RIKEN cDNA 4930422J18 gene	2.0	58	114	54	76	161013_f_at	AI596360
Lag3	lymphocyte-activation gene 3	1.9	193	372	132	192	98392_at	X98113
Icsbp1	interferon consensus sequence binding protein 1	1.9	636	1224	995	1232	98002_at	M32489
Lgals9	lectin, galactose binding, soluble 9	1.9	68	130		26	161301_f_at	AV049898
Mthfr	5,10-methylenetetrahydrofolate reductase	1.9	288	546	121	386	102360_at	AW214225
Hck	hemopoietic cell kinase	1.9	586	1122	773	1095	93483_at	J03023
Ndr1	N-myc downstream regulated 1	1.9	404	777	461	532	160464_s_at	U60593
Carhsp1	calcium regulated heat stable protein 1	1.9	187	311	73	95	96884_at	AI847631
Ccl5	chemokine (C-C motif) ligand 5	1.8	4313	7897	2005	3055	98406_at	AF065947
Pstpip1	proline-serine-threonine phosphatase-interacting protein 1	1.8	189	341	109	205	103946_at	U87814
Slc30a1	solute carrier family 30 (zinc transporter), member 1	1.8	12	23	21	23	93938_at	U17132
Dnmt2	DNA methyltransferase 2	1.8	22	39	26	25	93470_at	AF012129
5430432M24Rik	RIKEN cDNA 5430432M24 gene	1.8	72	126	64	73	103343_at	AI845815
1110014K08Rik	RIKEN cDNA 1110014K08 gene	1.8	447	788	479	760	98910_at	AI846118
Flnb	filamin, beta	1.8	351	611	315	463	95637_at	AI838592
Trpm1	transient receptor potential cation channel, subfamily M, member 1	1.8	73	128	81	92	102251_at	AF047714
Ly6e	Thymic shared antigen-1 (TSA-1) gene	1.8	8681	12075	3734	4338	101487_f_at	U47737
Rnf4	ring finger protein 4	1.7	616	1076	859	1252	93782_at	AI844517
Nedd8	neural precursor cell expressed, developmentally down-regulated gene 8	1.7	40	72	15	16	162180_r_at	AV367714
Fpr-rs1	formyl peptide receptor, related sequence 1	1.7	22	38			95770_s_at	AF071179
Camk4	calcium/calmodulin-dependent protein kinase IV	1.7	25	45	10	11	104455_at	J03057
E330014L15Rik	RIKEN cDNA E330014L15 gene	1.7	408	705	271	463	103517_at	AA822898
Prg	proteoglycan, secretory granule	1.7	388	657	941	1186	94085_at	M34803
2310004I24Rik	RIKEN cDNA 2310004I24 gene	1.7	165	279	113	205	104334_at	AW060314
Arf6	ADP-ribosylation factor 6	1.7	36	61	44	47	100462_at	D87903
Prdx1	peroxiredoxin 1	1.7	6360	10827	1497	2097	97758_at	AB023564
Casp4	caspase 4, apoptosis-related cysteine protease	1.7	554	923	100	288	102905_at	Y13089
Olfr89	olfactory receptor 89	1.7	23	40		10	102583_at	AJ132195
Kcnma1	potassium large conductance calcium-activated channel, Ma1	1.7	33	57	16	14	97759_at	U09383
Atp6v1d	ATPase, H+ transporting, V1 subunit D	1.7	664	1121	539	744	96951_at	AI839795
Ly86	lymphocyte antigen 86	1.6	593	968	559	871	94425_at	AB007599
Gdap10	ganglioside-induced differentiation-associated-protein 10	1.6	170	282	357	609	94192_at	Y17860
Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	1.6	109	181	105	188	96912_s_at	X15591
Gch	GTP cyclohydrolase 1	1.6	121	196	279	425	102313_at	L09737
Ppp1r15b	protein phosphatase 1, regulatory (inhibitor) subunit 15b	1.6	174	284	275	422	95609_at	AA869927
Plac8	placenta-specific 8	1.6	2925	4733	2403	2783	98092_at	AA790307
Akr1a4	aldo-keto reductase family 1, member A4 (aldehyde reductase)	1.6	3105	4970	1640	2250	96888_at	AI839814
Tgoln1	trans-golgi network protein	1.6	78	124	160	179	99144_s_at	D50031
Sct	secretin	1.6	617	981	210	198	92756_r_at	X73580
2410002M20Rik	RIKEN cDNA 2410002M20	1.6	109	173	119	178	104713_at	AA863717
Hat1	histidine aminotransferase 1	1.6	585	936	740	1112	97895_f_at	AW125218
IL2R	Mouse interleukin 2 receptor	1.6	56	87			101917_at	M26271
1200016E24Rik	RIKEN cDNA 1200016E24 gene	1.6	4988	7757	3758	4368	99849_at	C85523
4930565N07Rik	RIKEN cDNA 4930565N07 gene	1.6	797	1212	431	570	93779_at	AA261092
Ig Vkappa-PCG-4	M.musculus Ig Vkappa-PCG-4 gene.	1.5	72	111	54	53	96975_at	X82691
Krt1-2	keratin complex 1, acidic, gene 2	1.5	82	126	68	72	97767_at	X75649
Ung	uracil-DNA glycosylase	1.5	79	122	61	81	102792_at	U55040
Npc2	Niemann Pick type C2	1.5	2252	3471	1024	1377	160344_at	AB021289
pyp	pyrophosphatase	1.5	603	927	194	416	160314_at	AI839803
Btg3	B-cell translocation gene 3	1.5	97	147	176	182	96146_at	D83745
Psmb9	proteosome subunit, beta type 9	1.5	1124	1699	707	949	93085_at	D44456
Csk	c-src tyrosine kinase	1.5	114	172	188	225	94046_at	U05247
Atp1b3	ATPase, Na+/K+ transporting, beta 3 polypeptide	1.5	833	1256	1565	1912	99579_at	U58761
Tap2	transporter 2, ATP-binding cassette, sub-family B (MDR/TAP)	1.5	250	379	209	295	102873_at	U60091
Rnh1	ribonuclease/angiogenin inhibitor 1	1.5	1252	1904	524	753	100621_at	AI848825

Table 4.5 IFN-induced genes in splenic DCs after 6h but not 2h of culture Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine U74Av2 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

Gene Name	Gene description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Ccl22	chemokine (C-C motif) ligand 22	2.9	1500	519	365	237	102310_at	AF052505
Slc39a7	solute carrier family 39 (zinc transporter), member 7	2.7	75	28	200	184	103371_at	AF100956
Rad50	RAD50 homolog	2.7	132	50	77	77	100459_at	U66887
Gus	beta-glucuronidase	2.6	111	43	527	475	97538_at	M19279
Spic	Spi-C transcription factor	2.6	631	245	209	193	103454_at	AA182189
Igsf4	immunoglobulin superfamily, member 4	2.6	642	250	935	1190	93604_f_at	AF061260
Erh	enhancer of rudimentary homolog (Drosophila)	2.5	350	139	632	621	94040_at	D73368
BC005662	cDNA sequence BC005662	2.4	177	74	116	121	160897_at	AW060889
Kctd12	potassium channel tetramerisation domain containing 12	2.3	187	81	558	661	104735_at	AI842065
Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase	2.3	51	22	111	128	94818_at	AW047223
Cd1d1	CD1d1 antigen	2.3	60	26	216	264	103422_at	M63695
Ugt1a1	UDP-glucuronosyltransferase 1 family, member 1	2.2	652	291	299	264	99580_s_at	U16818
1810003N24Rik	RIKEN cDNA 1810003N24 gene	2.2	116	54	235	214	97237_at	AI837771
Fdps	farnesyl diphosphate synthetase	2.2	292	136	149	185	99098_at	AW045533
Sf3a1	splicing factor 3a, subunit 1	2.1	94	44	126	109	96027_at	AW120546
Apbb1ip	amyloid beta (A4) precursor protein-binding, B1 interacting protein	2.1	310	146	736	844	102710_at	AF020313
Bub1	budding uninhibited by benzimidazoles 1 homolog	2.1	30	14	93	98	104097_at	AF002823
U2af1	U2 small nuclear ribonucleoprotein auxiliary factor 1	2.1	89	43	435	283	97486_at	AA693246
Taf9	TAF9 RNA polymerase II, TBP-associated factor	2.1	213	103	257	249	103074_f_at	AI842969
Gsr	glutathione reductase 1	2.0	408	199	103	102	160646_at	AI851983
Fdps	farnesyl diphosphate synthetase	2.0	225	111	135	144	160424_f_at	AI846851
Il1b	interleukin 1 beta	2.0	2386	1174	1461	1229	103486_at	M15131
Tcte1l	t-complex-associated-testis-expressed 1-like	2.0	24	12			95056_r_at	AW122747
Sqle	squalene epoxidase	2.0	214	107	118	111	94322_at	D42048
2310046H11Rik	RIKEN cDNA 2310046H11 gene	2.0	103	52	208	224	161342_r_at	AV136028
rnf6	ring finger protein (C3H2C3 type) 6	2.0	36	19	45	48	104049_at	AW047134
psmd1	proteasome 26S subunit, non-ATPase, 1	2.0	658	333	268	304	95483_at	AW123318
Ivns1abp	influenza virus NS1A binding protein	2.0	182	93	312	324	97302_at	AI854285
A430106J12Rik	RIKEN cDNA A430106J12 gene	1.9	84	44	58	62	95288_i_at	AA189811
Sca10	spinocerebellar ataxia 10 homolog	1.9	337	175	375	406	99127_at	X61506
Gsto1	glutathione S-transferase omega 1	1.9	294	154	377	380	97819_at	AI843119
Adh5	alcohol dehydrogenase 5 (class III), chi polypeptide	1.9	247	133	330	381	98625_s_at	M84147
Cat	catalase	1.9	137	74	83	96	160479_at	M29394
Cxcr4	chemokine (C-X-C motif) receptor 4	1.9	166	90	407	299	102794_at	Z80112
Ptma	prothymosin alpha	1.8	1017	553	1128	2245	100718_at	X56135
6330406L22Rik	RIKEN cDNA 6330406L22 gene	1.8	620	337	535	490	100511_at	AI154249
psarl	presenilin associated, rhomboid-like	1.8	139	76	189	186	96641_at	AW060674
Cct8	chaperonin subunit 8 (theta)	1.8	164	90	245	267	160102_at	Z37164
0610011I04Rik	RIKEN cDNA 0610011I04 gene	1.8	265	146	395	441	96605_at	AI787183
Il6st	interleukin 6 signal transducer	1.8	130	71	162	171	94345_at	AI843709
Klrd1	killer cell lectin-like receptor, subfamily D, member 1	1.8	495	275	473	478	93677_at	AF030311
Fnbp1	Formin binding protein 1	1.8	243	135	273	268	103638_at	AW046460
Kpna2	karyopherin (importin) alpha 2	1.8	68	37	84	83	92790_at	D55720
Cdkap1	CDK2 (cyclin-dependent kinase 2)-associated protein 1	1.8	339	189	251	264	98532_at	AF011644
1500010B24Rik	RIKEN cDNA 1500010B24 gene	1.8	264	147	212	232	93358_at	AI836451
1300006C06Rik	RIKEN cDNA1300006C06 gene	1.8	210	116	202	212	97401_at	AW124244
B230365D05Rik	RIKEN cDNA B230365D05 gene	1.8	231	130	181	182	104315_at	AI846773
Anxa3	annexin A3	1.8	553	315	299	341	101393_at	AJ001633
Ntan1	N-terminal Asn amidase	1.7	51	29	52	56	96666_at	U57692
Atp6ap1	ATPase, H+ transporting, lysosomal accessory protein 1	1.7	214	123	312	331	94043_at	AB031290
Cops6	COP9 (constitutive photomorphogenic) homolog, subunit 6	1.7	144	83	218	214	99106_at	AF071315
Atf1	activating transcription factor 1	1.7	138	80	207	236	100984_at	M63725
Dock2	dedicator of cyto-kinesis 2	1.7	181	106	166	171	103462_at	AW122239
2510049I19Rik	RIKEN cDNA 2510049I19 gene	1.7	255	148	408	392	97866_at	AI842858
Cdc34	cell division cycle 34 homolog	1.7	155	91	107	98	94048_at	AW120683
Lip1	lysosomal acid lipase 1	1.7	275	163	157	115	102123_at	Z31689
Rpn1	similar to ribophorin I	1.7	40	24	82	97	161400_f_at	AV276050
Galt	galactose-1-phosphate uridyl transferase	1.7	56	33	53	65	104616_g_at	M96265
1200015A19Rik	RIKEN cDNA 1200015A19 gene	1.7	219	131	181	178	95737_at	AW120814
Stip1	stress-induced phosphoprotein 1	1.7	80	48	131	139	100081_at	U27830
BC032204	cDNA sequence BC032204	1.7	323	194	366	394	160815_at	AA709534
Set	SET translocation	1.7	327	197	422	350	98550_at	AI854006
1810044O22Rik	RIKEN cDNA 1810044O22 gene	1.7	69	41	72	92	96878_at	AW048566
Tm7sf1	Mus musculus transmembrane 7 superfamily member 1	1.7	1422	857	928	778	103017_at	AI060729
Hcfc1	host cell factor C1	1.7	206	125	171	158	100900_at	AW209053
Jtb	jumping translocation breakpoint	1.7	416	251	1034	979	102790_at	AB016490
Lta4h	leukotriene A4 hydrolase	1.7	603	365	741	775	100540_at	M63848
Bcat2	branched chain aminotransferase 2, mitochondrial	1.7	73	44	55	57	100443_at	AF031467

Table 4.6 (continued over page) IFN-suppressed genes in splenic DCs after 6h but not 2h of culture. Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine U74Av2 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

Gene Name	Gene description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Tpm1	tropomyosin 1, alpha	1.6	526	319	251	220	160532_at	M22479
S100a4	S100 calcium binding protein A4	1.6	637	388	645	607	99051_at	M36579
Eif3s10	eukaryotic translation initiation factor 3, subunit 10 (theta)	1.6	91	55	171	183	94250_at	U14172
3230402J05Rik	RIKEN cDNA 3230402J05 gene	1.6	42	26	39	26	93203_f_at	AW107463
Fto	fatso	1.6	140	86	97	99	98460_at	AJ237917
Matr3	matrin 3	1.6	545	333	1137	1147	96012_f_at	AI835367
Cops3	COP9 (constitutive photomorphogenic) homolog, subunit 3	1.6	61	38	120	138	99113_at	AF071313
1110031E24Rik	RIKEN cDNA 1110031E24 gene	1.6	48	30	114	116	98057_at	AW121162
Cct5	chaperonin subunit 5 (epsilon)	1.6	578	355	891	942	93295_at	Z31555
Rbbp4	retinoblastoma binding protein 4	1.6	282	174	372	372	92647_at	U35141
1300010A20Rik	RIKEN cDNA 1300010A20 gene	1.6	180	111	152	167	104398_at	AI846222
3300001G02Rik	RIKEN cDNA 3300001G02 gene	1.6	223	138	162	170	97412_at	AI843157
Adprtl2	ADP-ribosyltransferase (NAD+; poly(ADP-ribose) polymerase)-like 2	1.6	137	85	203	230	100903_at	AJ007780
2310066N05Rik	RIKEN cDNA 2310066N05 gene	1.6	90	56	85	86	97802_at	AI850195
2700038L12Rik	RIKEN cDNA 2700038L12 gene	1.6	189	118	349	339	160293_at	AI839817
Nde1	nuclear distribution gene E homolog 1	1.6	89	55	83	81	94910_at	AW120739
Adss2	adenylosuccinate synthetase 2, non muscle	1.6	216	135	230	221	99038_at	L24554
G3bp	Ras-GTPase-activating protein SH3-domain binding protein	1.6	406	253	840	755	103642_at	AB001927
Wdr13	WD repeat domain 13	1.6	57	36	61	63	94374_at	AI850378
Mtch2	mitochondrial carrier homolog 2	1.6	185	116	239	317	160262_at	AI839901
2410195B05Rik	RIKEN cDNA 2410195B05 gene	1.6	163	103	118	93	96677_at	AI849566
Srebf1	sterol regulatory element binding factor 1	1.6	308	194	234	218	93264_at	AI843895
Ext1	exostoses (multiple) 1	1.6	309	195	131	148	102811_at	X96639
Emp3	epithelial membrane protein 3	1.6	1373	866	1868	1789	93593_f_at	U87948
0610009D07Rik	RIKEN cDNA 0610009D07 gene	1.6	359	226	334	353	95714_at	AI226264
1810009M01Rik	RIKEN cDNA 1810009M01 gene	1.6	617	390	672	751	97885_at	AB031386
2310047I15Rik	RIKEN cDNA 2310047I15	1.6	274	173	348	372	97448_at	AI845165
txnl2	thioredoxin-like 2	1.6	433	275	641	749	102103_f_at	AI482454
Apg5l	autophagy 5-like	1.6	84	54	45	55	94225_at	AI844679
H2afz	H2A histone family, member Z	1.6	1152	734	1485	1579	101954_at	U70494
0610043B10Rik	RIKEN cDNA 0610043B10 gene	1.6	308	198	164	152	96615_at	AI840137
Vcp	valosin containing protein	1.6	933	597	638	690	100710_at	Z14044
Cks2	CDC28 protein kinase regulatory subunit 2	1.6	135	87	181	196	97527_at	AA681998
Gclc	glutamate-cysteine ligase, catalytic subunit	1.6	825	529	231	222	99649_at	U85414
1810012E07Rik	RIKEN cDNA 1810012E07 gene	1.6	180	116	303	221	99655_at	AB025405
Uqcrc2	RIKubiquinol cytochrome c reductase core protein 2	1.6	472	304	341	378	102000_f_at	AI842835
Ncb5or	NADPH cytochrome B5 oxidoreductase	1.5	299	194	364	420	97922_at	AW045597
selenoprotein	selenoprotein	1.5	189	122	139	136	96249_at	AW122105
D6Wsu116e	EST D6Wsu116e	1.5	325	211	281	312	98880_at	AW124955
Mt2	metallothionien II; Mouse metallothionein II (MT-II) gene.	1.5	486	304	96	90	101561_at	K02236
Cct7	chaperonin subunit 7 (eta)	1.5	311	202	660	765	160562_at	Z31399
Rnu22	RNA, U22 small nucleolar	1.5	132	86	296	277	97838_at	AA684508
Stk18	serine/threonine kinase 18	1.5	61	40	132	132	98996_at	L29480
1110001M20Rik	RIKEN cDNA 1110001M20 gene	1.5	107	70	139	193	95477_at	AW125185
pdap1	PDGFA associated protein 1	1.5	171	112	171	173	104080_at	AW212479
Macf1	microtubule-actin crosslinking factor 1	1.5	134	88	263	317	98402_at	AI843799
8030499H02Rik	RIKEN cDNA 8030499H02 gene	1.5	98	64	136	141	103889_at	AI834833
Capzb	capping protein (actin filament) muscle Z-line, beta	1.5	516	338	938	1137	95142_s_at	U10407
Psma1	proteasome subunit, alpha type 1	1.5	176	115	179	216	96892_at	AI836804
C530002L11Rik	RIKEN cDNA C530002L11 gene	1.5	271	177	353	429	96710_at	AI854262
Dlgh1	discs, large homolog 1	1.5	234	154	162	177	160849_at	U93309
Tial1	Tial1 cytotoxic granule-associated RNA binding protein-like 1	1.5	153	101	233	237	99947_at	U55861
G6pdx	glucose-6-phosphate dehydrogenase X-linked	1.5	323	212	88	96	94966_at	Z11911
Acly	ATP citrate lyase	1.5	240	158	351	383	160207_at	AW121639
Sucla2	succinate-Coenzyme A ligase, ADP-forming, beta subunit	1.5	125	82	126	129	93501_f_at	AF058955
C79802	expressed sequence C79802	1.5	72	47	69	89	95659_at	AF041476
Dstn	destrin	1.5	696	459	426	479	94492_at	AB025406
Oat	ornithine aminotransferase	1.5	204	134	279	283	92848_at	X64837
Gdpd1	glycerophosphodiester phosphodiesterase domain containing 1	1.5	123	81	181	212	95470_at	AI846025
Ccndbp1	cyclin D-type binding-protein 1	1.5	235	155	258	247	101483_at	AI850862
Cd63	Cd63 antigen	1.5	486	309	401	431	160493_at	D16432
Tcf7	transcription factor 7, T-cell specific	1.5	677	450	393	431	97994_at	AI019193
Ddx54	DEAD (Asp-Glu-Ala-Asp) box polypeptide 54	1.5	100	67	168	174	95433_at	AW049776
1110033G07Rik	RIKEN cDNA 1110033G07 gene	1.5	34	23	55	58	97407_at	AI848025
Pygb	brain glycogen phosphorylase	1.5	64	43	36	40	97489_at	AI846739
Tebp-pending	telomerase binding protein, p23	1.5	153	103	340	332	94259_at	AB024935
Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, g1	1.5	540	366	664	785	92798_at	AA870675

Table 4.6 (continued) IFN-suppressed genes in splenic DCs after 6h but not 2h of culture

4.2.1.4 Identification and analysis of genes with potential function in DC mediated immune responses

Our original aim was to identify IFN-I regulated genes which enhanced the ability of DCs to initiate and sustain an effective adaptive immune response. We hoped to find genes encoding cell surface molecules involved in either recognition and uptake of antigen, cell-cell interactions or cell migration. We were also interested in genes encoding secretory factors which could affect the function and/or migration of other cells, thereby influencing the type of response elicited.

We identified five genes of unknown function containing domains which suggested a role in immune function (Table 4.7). Several of the genes contained transmembrane domains indicating cell surface localization, in addition to domains common to other proteins involved in the immune response such as a domain found in tumour necrosis family (TNF) receptors and CD40. The gene Ppicap encodes a protein which contains the scavenger receptor cysteine-rich domain (Chicheportiche *et al.*, 1994), a highly conserved domain found in diverse secreted and cell surface proteins. These domains are thought to mediate protein-protein interactions and ligand binding (Freeman *et al.*, 1990). Two of the genes identified, designated Ms4a4c and Ms4a6b, belong to the membrane-spanning 4A (Ms4a) family. This is a large family with members including CD20 and FcεRIβ, all of which have conserved transmembrane domains and are thought to be components of oligomeric cell surface complexes (Liang *et al.*, 2001). Members of this family are expressed in different tissues and cell types, and the predicted extracellular domains share little sequence homology which suggests they serve multiple diverse roles. Ms4a4c and Ms4a6b are expressed at very high levels in the lymphoid organs and at low levels in non-lymphoid organs (Liang *et al.*, 2001). In addition, Ms4a4c has been shown to be differentially expressed in the DC subsets

Gene Name	Symbol	Affymetrix Accession	Protein domains	Interpro	Proteins containing domain/ Domain function
G-protein coupled receptor33	Gpr33	94184_at	Rhodopsin-like GPCR family	IPR000276	Chemokine receptors
IMAGE 4168084		102965_at	TNFR/CD27/30/40/9 5 cysteine-rich region	IPR001368	TNF receptors, CD40, CD27, CD30, 4-1BB, FAS, OX40
Membrane-spanning 4 domains 4a4c	Ms4a4c	98373_at	Transmembrane domains		
			CD20 domain	IPR007237	CD20, IgE Fc receptor β subunit
Membrane-spanning 4 domains 4a6b	Ms4a6b	97322_at	Transmembrane domains		
			CD20 domain	IPR007237	CD20, IgE Fc receptor β subunit
Peptidylprolyl isomerase C-associated protein	Ppicap	97507_at	Scavenger receptor domain	IPR001190	Macrophage scavenger receptor-1, CD5, CD6, Mac-2, MARCO
			BTB/POZ domain	IPR000210	Dimerisation

Table 4.7 Genes induced by IFN-α4 in sDCs with potential function in DC-mediated immune responses

(Edwards *et al.*, 2003a). One of the genes chosen for further study was G-protein coupled receptor 33 (Gpr33), a putative seven transmembrane protein sharing highest homology with chemoattractant receptors (Marchese *et al.*, 1998).

We analysed expression of these genes by real-time PCR in DCs, T and B cells treated *in vitro* with IFN-α4. The results confirmed the microarray data; all genes were expressed at higher levels in IFN-treated DCs after both 2h and 6h of culture (Figure 4.4). The level of expression for all of the genes was higher in untreated DCs cultured for 6h compared to 2h (Figure 4.4b). The majority of the genes were also elevated in T and B cells treated with IFN-α4 compared with untreated controls, in some cases to a greater extent than in DCs. For example, Ms4a4c mRNA levels were more than 15-fold higher in $CD4^+$ T cells and Ppicap mRNA was around 10-fold higher in both T and B cells. We also compared the level of gene expression between untreated DCs and non-DCs cultured for 2h. Ms4a6b was expressed predominantly in T cells, while Ms4a4c was expressed at higher levels in B cells and at lower levels in $CD4^+$ T cells compared to DCs.

Expression of Gpr33 was interesting since the level of mRNA was not elevated by IFN-α4 in any cell type other than DCs. In addition, as well as being higher in DCs after 6h of culture, expression was lower in untreated T cells. Although expression in B cells was similar to that in DCs, it was expressed at a lower level after IFN-α4 treatment.

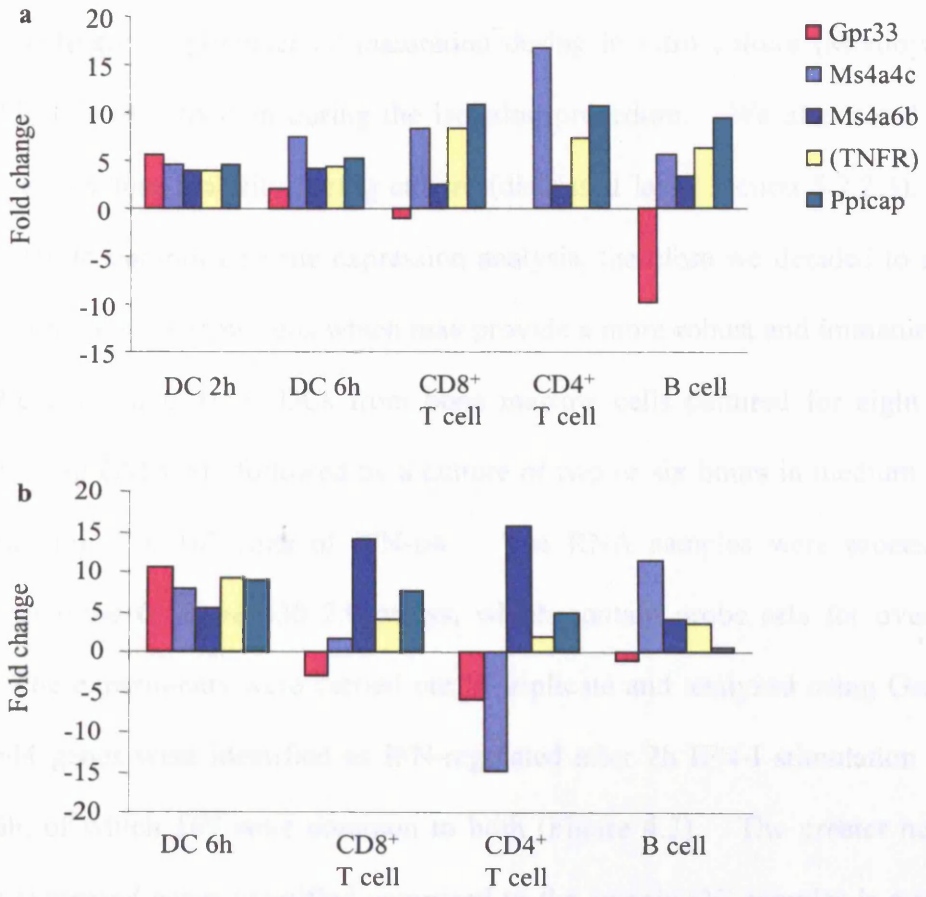


Figure 4.4 Levels of expression of the genes of interest identified by microarray varies between cell types. Real-time PCR was carried out for RNA extracted from DCs, T and B cells cultured in medium alone or with 2 x10^4 U IFN-I. $CD4^+$ and $CD8^+$ T cells and B cells were isolated from the lymph nodes by Dynabead depletion followed by FACS sorting. Cell purities were >99%. **a)** Results are plotted as fold change in expression in IFN-treated versus untreated DC. **b)** Results are plotted as fold change in expression of untreated cells compared to the untreated DC at 2h.

4.2.2 Microarray analysis of BMDCs treated with IFN-I

4.2.2.1 Experimental overview

Splenic DCs undergo a 'spontaneous' maturation during in vitro culture (Montoya *et al.*, 2002), possibly due to activation during the isolation procedure. We also noted that the splenic DCs rapidly lose viability during culture (discussed later, section 5.2.2.3). These factors are likely to complicate gene expression analysis, therefore we decided to compare DCs derived from bone marrow cells which may provide a more robust and immature source of DCs. We isolated $CD11c^+$ DCs from bone marrow cells cultured for eight days in medium containing GM-CSF, followed by a culture of two or six hours in medium alone or medium containing 2 x 10^4 units of IFN-α4. The RNA samples were processed and hybridized to Murine Genome 430 2.0 arrays, which contain probe sets for over 39,000 transcripts. The experiments were carried out in triplicate and analyzed using Genespring software. 644 genes were identified as IFN-regulated after 2h IFN-I stimulation and 790 genes after 6h, of which 169 were common to both (Figure 4.3). The greater number of differentially expressed genes identified compared to the splenic DC samples is a reflection of the greater number of transcripts present on the arrays.

4.2.2.2 Analysis of differentially expressed genes

Less than one third of the genes differentially expressed at the 2h timepoint only were upregulated by IFN-I (142 out of 475). These are listed in table 4.8; for simplicity, transcripts representing ESTs for which limited annotations were available are not shown. As expected, IFN-induced genes Ifit1 and Ifit2 were identified. These still appear to be elevated by IFN-I at 6h but did not pass the fold change cut-off point of 1.5. One gene whose expression we had not previously detected as being IFN-induced was Pnp (purine-nucleoside phosphorylase) which was highly expressed at both the 2h and 6h timepoints. In

Gene	Description	Fold Change	2h Signal 0	2h Signal IFN	6h Signal 0	6h Signal IFN	Affymetrix Accession	Genbank Accession
Ifit2	interferon-induced protein with tetratricopeptide repeats 2	19.1	214	3509	3195	4346	1418293_at	NM_008332
Tyki	thymidylate kinase family LPS-inducible member	14.2	126	1832	724	2153	1450484_a_at	AK004595
Oas1b	2'-5' oligoadenylate synthetase 1B	13.7	10	139	67	106	1425119_at	BC012877
Acac	acetyl-Coenzyme A carboxylase	8.1		47	11	22	1444556_at	BB223018
Ifit1	interferon-induced protein with tetratricopeptide repeats 1	7.6	476	3251	2238	3308	1450783_at	NM_008331
Socs1	suppressor of cytokine signaling 1	7.3	29	222	56	164	1450446_a_at	AB000710
Slc6a14	solute carrier family 6 member 14	4.4					1420504_at	AF320226
Nt5c3	5'-nucleotidase, cytosolic III	4.2		20			1431306_at	BG518810
Vig1-pending	viral hemorrhagic septicemia virus(VHSV) induced gene 1	4.2	1327	4975	3632	4815	1436058_at	BB132493
Panx3	pannexin 3	4.1		10	11		1456073_s_at	AV220875
Pttg1	pituitary tumor-transforming 1	3.9	33	131	54	126	1419620_at	NM_013917
Cyp2f2	cytochrome P450, family 2, subfamily f, polypeptide 2	3.8		16			1448792_a_at	NM_007817
Stat1	signal transducer and activator of transcription 1	3.5	130	430	418	662	1440481_at	BB229853
Slfn3	schlafen 3	3.5	16	55	55	117	1450322_s_at	NM_011409
Timeless	timeless homolog (Drosophila)	3.3	50	158	148	292	1417586_at	NM_011589
Cited2	Cbp/p300-interacting transactivator	3.2	655	1968	1160	2108	1452207_at	Y15163
Socs1	suppressor of cytokine signaling 1	3.1	50	156	33	25	1440047_at	AV237386
Znfn1a2	zinc finger protein, subfamily 1A, 2 (Helios)	3.0	24	73	57	65	1456956_at	BB291816
Pml	promyelocytic leukemia gene	3.0	35	108	26	106	1456103_at	BB667149
Klrk1	killer cell lectin-like receptor subfamily K, member 1	3.0	46	135	59	144	1450495_a_at	AF039026
Rslcan1	regulator of sex-limitation candidate 1	2.8	115	317	177	296	1438004_at	BB324894
Nudt11	nudix (nucleoside diphosphate linked moiety X)-type motif 11	2.8		14			1435061_at	AI853080
Trim34	tripartite motif protein 34	2.8	70	193	145	190	1421550_a_at	NM_030684
Xdh	xanthine dehydrogenase	2.7	422	1070	699	884	1424607_a_at	BC020078
Hsh2	hematopoietic SH2 protein	2.6	23	60	38	61	1442130_at	BG091940
Trex1	three prime repair exonuclease 1	2.5	353	848	817	1156	1450672_a_at	NM_011637
Aldh1b1	aldehyde dehydrogenase 1 family, member B1	2.5	141	364	322	651	1451260_at	BC020001
Mthfr	5,10-methylenetetrahydrofolate reductase	2.4	224	530	324	709	1434087_at	BG069750
Prkr	protein kinase, interferon-inducible double stranded RNA dependent	2.3	77	177	126	166	1422005_at	M93567
Trim25	tripartite motif protein 25	2.2	285	638	538	782	1426415_a_at	AI746456
Trim25	tripartite motif protein 25	2.1	880	1778	2253	2519	1419879_s_at	AA960166
Bace2	beta-site APP-cleaving enzyme 2	2.1					1435581_at	BE947462
Pfdn5	prefoldin 5	2.1					1422297_at	NM_027044
Kdr	kinase insert domain protein receptor	2.1	79	159	134	282	1449379_at	NM_010612
Galk2	galactokinase 2	2.1		11			1438642_at	AV278176
Zfp354b	zinc finger protein 354B	2.0		13			1421379_at	AF184112
Foxp2	forkhead box P2	1.9	14	26	19	17	1438231_at	BB376288
Eya3	eyes absent 3 homolog (Drosophila)	1.9	20	38	38	53	1439590_at	AV273072
Gpr120	G protein-coupled receptor 120	1.9	134	237	117	167	1439489_at	AV025152
Milt2h	homolog of human MLLT2 unidentified gene	1.8	67	120	211	310	1425640_at	AF074266
Lrat	lecithin-retinol acyltransferase	1.8	10	18		11	1421345_at	NM_023624
Rs1h	retinoschisis 1 homolog (human)	1.8	26	44	22	29	1421085_at	NM_011302
Vcam1	vascular cell adhesion molecule 1	1.8	34	61	26	35	1451314_a_at	L08431
Pttg1	pituitary tumor-transforming 1	1.8	1372	2353	2181	3298	1438390_s_at	AV105428
Tm4sf10	transmembrane 4 superfamily member 10	1.7		16	11		1449885_at	NM_138751
Gpr44	G protein-coupled receptor 44	1.7	74	126	39	60	1441578_at	BB142123
Pla2g2a	phospholipase A2, group IIA	1.7	16	27	20	23	1450128_at	NM_011108
Tln	talin	1.7	17	28	19	15	1422367_at	NM_019485
Mcf2	mcf.2 transforming sequence	1.7	10	16	11		1419021_at	NM_133197
Prlpc3	prolactin-like protein C 3	1.7	13	23	19	15	1424387_at	AF466150
Zc3hav1	zinc finger CCCH type, antiviral 1	1.7	173	291	200	296	1428378_at	AK004770
Pnp	purine-nucleoside phosphorylase	1.6	2178	3358	2815	3831	1416530_a_at	BC003788
Napb	N-ethylmaleimide sensitive fusion protein attachment protein beta	1.6	17	26	17		1452444_at	AU067119
Zc3hav1	zinc finger CCCH type, antiviral 1	1.6	33	51	38	63	1431909_at	AK013726
Wdr20	WD repeat domain 20	1.6	32	49	26	31	1430004_s_at	AK015014
Rfxank	regulatory factor X-associated ankyrin-containing protein	1.6	24	37	19	20	1425670_at	BC010971
Epb4.1l1	erythrocyte protein band 4.1-like 1	1.5	53	83	53	54	1434575_at	BB794965
Cul4a	cullin 4A	1.5	70	108	43	65	1426060_at	BC007159
Evl	Ena-vasodilator stimulated phosphoprotein	1.5	71	109	61	70	1440885_at	AW546029
Cetn1	centrin 1	1.5	39	61	40	49	1453881_x_at	AK015808
Fgs	forssman glycolipid synthetase	1.5	41	62	33	42	1426148_at	AF292399
Tuba-rs1	tubulin alpha, related sequence 1	1.5	31	47	23	28	1427832_at	M19413

Table 4.8 IFN-induced genes in BMDCs after 2h but not 6h of culture. Samples from $CD11c^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine U74Av2 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

humans, a deficiency in Pnp is associated with cellular imunodeficiency, where patients have reduced T cell numbers and function (Markert, 1991). Mice carrying Pnp mutations have abnormal thymocyte development and splenic leukocytes from these mice have reduced numbers of T cells with impaired responses to IL-2 (Snyder *et al.*, 1997).

Many of the genes suppressed by IFN-I at two hours were expressed at low levels, and their downregulation may not have a significant impact on biological function (Table 4.9). One gene whose expression was greatly reduced is Egr2 (early growth response 2), encoding a zinc-finger transcription factor which has been shown to regulate FasL expression (Mittelstadt *et al.*, 1999). Recently, it has been reported that Egr2 is necessary for full induction of T-cell anergy (Harris *et al.*, 2004) and is involved in promoting TCR-induced negative regulation of T cell activation (Safford *et al.*, 2005). A role for Egr2 in dendritic cell function has not been investigated.

169 genes were detected as differentially expressed at both the 2h and 6h timepoints. Where enhanced expression was seen at the 2h timepoint (129 genes, table 4.10), expression was also increased at 6h. Similarly, genes suppressed at 2h were also suppressed at 6h (40 genes, table 4.11): no genes were enhanced at one timepoint and suppressed at the other. Many of the upregulated genes are commonly associated with DC function, including the chemokines CXCL9, CXCL10 and CXCL11, which all act via the CXCR3 receptor that is expressed mainly on activated Th1 cells (Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998b). The relatively low expression of CXCL11 mRNA compared to CXCL10 may reflect its potent chemotactic ability, since it induces optimal chemotaxis at concentrations 10 fold lower than does CXCL10 (Meyer *et al.*, 2001). Induction of CXCL11 had not been detected in splenic DCs, and is represented on the U74Av arrays used in those experiments. In support of previous work (Mattei *et al.*, 2001), we saw upregulation of IL-15 and the IL-15 receptor α

Gene	Description	Fold Change	2h Signal 0	2h Signal IFN	6h Signal 0	6h Signal IFN	Affymetrix Accession	Genbank Accession
H13	histocompatibility 13	10.8	33		10		1438456_at	BB327773
Mapk7	mitogen-activated protein kinase 7	7.4	54		85	24	1418060_a_at	NM_011841
Mtss1	metastasis suppressor 1	6.2	28		20	24	1446284_at	BB157298
Slc6a13	solute carrier family 6 (neurotransmitter transporter, GABA), member 13	4.7	29		39	11	1424338_at	BC023117
Man2b1	mannosidase 2, alpha B1	4.7	91	20	74	55	1436781_at	BE634501
Brd3	bromodomain containing 3	3.8	43	11	29	18	1436960_at	BB753423
Egr2	early growth response 2	3.6	673	186	183	121	1427682_a_at	X06746
Ppp2r5a	protein phosphatase 2, regulatory subunit B (B56), alpha isoform	3.4	19				1438914_at	AW741731
Cxcr4	chemokine (C-X-C motif) receptor 4	3.2	535	167	680	475	1448710_at	D87747
Herpud1	homocysteine-inducible, ER stress-inducible, ubiquitin-like domain 1	2.6	745	283	559	377	1435626_a_at	AI835088
Sh3d3	SH3 domain protein 3	2.6	37	14	35	33	1446023_at	AV024397
Adora2b	adenosine A2b receptor	2.5	235	92	50	46	1450214_at	NM_007413
Slitl2	Slit-like 2 (Drosophila)	2.5	150	60	95	77	1455812_x_at	BB530515
Ddit3	DNA-damage inducible transcript 3	2.5	37	15	30	31	1443897_at	BB200603
Angptl4	angiopoietin-like 4	2.5	71	29	52	25	1417130_s_at	NM_020581
Pdgfb	platelet derived growth factor, B polypeptide	2.4	114	47	58	39	1450413_at	NM_011057
Rpl12	ribosomal protein L12	2.4	86	36	83	40	1435655_at	BG807990
Sesn2	sestrin 2	2.4	64	27	55	44	1425139_at	BC005672
Egr2	early growth response 2	2.4	1266	533	483	329	1427683_at	X06746
Catna1	catenin alpha 1	2.4	31	13	28	32	1436631_at	BG066669
Idb3	inhibitor of DNA binding 3	2.4	112	47	69	56	1416630_at	NM_008321
Mtss1	metastasis suppressor 1	2.3	84	36	79	65	1440847_at	BB326749
Adrb2	adrenergic receptor, beta 2	2.3	96	42	32	36	1437302_at	AV083350
Map4k4	mitogen-activated protein kinase kinase kinase kinase 4	2.3	91	40	51	46	1440609_at	BB113015
Grtp1	GH regulated TBC protein 1	2.3	19		14	19	1439150_x_at	BB480256
Gpi1	glucose phosphate isomerase 1	2.3	77	34	96	82	1456909_at	BF017016
Matr3	matrin 3	2.3	59	26	39	26	1436796_at	BB237072
Trappc5	trafficking protein particle complex 5	2.2	58	26	37	18	1448999_at	AV226526
Hspa8	heat shock protein 8	2.2	88	41	139	142	1431182_at	AK004608
Jundm2	Jun dimerization protein 2	2.2	117	54	116	116	1450350_a_at	NM_030887
Ier2	immediate early response 2	2.1	289	136	114	89	1416442_at	NM_010499
Pgf	placental growth factor	2.1	158	75	111	93	1418471_at	NM_008827
Pim3	proviral integration site 3	2.1	706	335	656	563	1437100_x_at	BB206220
Lox	lysyl oxidase	2.1	84	40	121	85	1416121_at	NM_010728
Chic2	cysteine-rich hydrophobic domain 2	2.1	23	11	12	15	1444018_at	BB794854
Zfp146	zinc finger protein 146	2.1	71	34	47	28	1422135_at	NM_011980
Phospho1	phosphatase, orphan 1	2.1	79	38	35	27	1457063_at	AI447357
Per1	period homolog 1 (Drosophila)	2.1	163	79	203	200	1449851_at	AF022992
Spic	Spi-C transcription factor (Spi-1/PU.1 related)	2.1	81	39	47	37	1418555_x_at	NM_011461
Gna11	guanine nucleotide binding protein, alpha 11	2.1	84	41	60	58	1449144_at	NM_010301
Ptger4	prostaglandin E receptor 4 (subtype EP4)	2.1	557	271	293	238	1424208_at	BC011193
DXImx41e	DNA segment, Chr X, Immunex 41, expressed	2.0	23	11	24	33	1458023_at	BG070379
Gas5	growth arrest specific 5	2.0	186	93	141	120	1436222_at	AW547050
Myo9b	myosin IXb	2.0	35	17	25	21	1438533_at	BM121122
C130032F08	hypothetical protein C130032F08	2.0	110	56	90	74	1434043_a_at	AV286809
Il4i1	interleukin 4 induced 1	2.0	633	320	462	330	1415926_at	NM_053074
Plu1	putative DNA/chromatin binding motif 1	2.0	78	40	154	103	1427143_at	BC019446
Axot	axotrophin	2.0	79	40	100	85	1440966_at	BB248730
Fosl2	fos-like antigen 2	1.9	386	198	147	110	1437247_at	BM245170
Fgd3	FYVE, RhoGEF and PH domain containing 3	1.9	97	50	62	52	1433398_at	AK018025
Cdkl2	cyclin-dependent kinase-like 2 (CDC2-related kinase)	1.9	54	28	43	37	1457625_s_at	BE979937
Zfp275	Zinc finger protein 275	1.9	362	188	235	175	1418397_at	BC019962
Nolc1	nucleolar and coiled-body phosphoprotein 1	1.9	67	35	94	89	1450087_a_at	NM_053086
Pld2	phospholipase D2	1.9	145	77	111	98	1417237_at	NM_008876
Flrt3	fibronectin leucine rich transmembrane protein 3	1.9	791	417	322	232	1453102_at	BE945486
Trim13	tripartite motif protein 13	1.9	287	153	198	160	1417888_at	NM_023233
Gas5	growth arrest specific 5	1.9	162	86	103	73	1424843_a_at	BC004622
Ndst3	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 3	1.9	15		13	16	1443277_at	BB392250
Flrt3	fibronectin leucine rich transmembrane protein 3	1.9	576	310	218	157	1429310_at	AK017456
Dido1	death inducer-obliterator 1	1.9	53	28	18	23	1437182_at	BM224349
Mt1	metallothionein 1	1.8	170	93	235	198	1451612_at	BC027262
Brd8	bromodomain containing 8	1.8	47	26	38	32	1429526_at	AK014475
Kif1b	kinesin family member 1B	1.8	36	20	25	15	1451642_at	BE199508
Siat4a	sialyltransferase 4A (beta-galactoside alpha-2,3-sialytransferase)	1.8	70	39	70	59	1441216_at	BB024025
Atp10d	ATPase, Class V, type 10D	1.8	38	21	27	22	1436544_at	BB016769
Cblb	Casitas B-lineage lymphoma b	1.8	101	56	99	82	1443086_at	BB534113
Erdr1	erythroid differentiation regulator 1	1.8	46	26	20	28	1452566_at	BC021831
E2f3	E2F transcription factor 3	1.8	141	80	62	41	1434564_at	BQ176318
Gsn	gelsolin	1.8	95	54	96	94	1439687_at	BM207947
Rb1	retinoblastoma 1	1.8	830	471	1531	1123	1428615_at	AK008952
Hbxap	hypothetical protein Hbxap	1.8	13		10		1442207_at	BB283202
Arf2	ADP-ribosylation factor 2	1.8	44	25	34	24	1438661_a_at	AV023312
Ptger2	prostaglandin E receptor 2 (subtype EP2)	1.8	157	89	138	98	1449310_at	BC005440
Tm7sf1	transmembrane 7 superfamily member 1	1.8	665	380	430	304	1423164_at	BB325447
Zfp68	Zinc finger protein 68	1.8	17	10	12		1459979_x_at	AV232843
Zfp36l2	zinc finger protein 36, C3H type-like 2	1.7	1845	1057	2559	2757	1437626_at	BB031791

Table 4.9 (continued over page) IFN-suppressed genes in BMDCs after 2h but not 6h of culture. Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine U74Av2 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

Gene	Description	Fold Change	2h Signal 0	2h Signal IFN	6h Signal 0	6h Signal IFN	Affymetrix Accession	Genbank Accession
Zfp397	zinc finger protein 397	1.7	46	27	22	18	1435664_at	AV342828
Rpl30	ribosomal protein L30	1.7	43	25	32	21	1438076_at	BE447059
Pknox1	Pbx/knotted 1 homeobox	1.7	53	31	32	20	1450172_at	AA409923
MGC57096	hypothetical protein MGC57096	1.7	18	11	14	11	1457157_at	BB247624
Trim2	tripartite motif protein 2	1.7	36	21	20	18	1417028_a_at	BB667078
Fus	fusion, derived from t(12;16) malignant liposarcoma (human)	1.7	82	48	54	52	1455831_at	BE985138
Fbxo32	F-box only protein 32	1.7	64	38	63	59	1448747_at	AF441120
Es10	esterase 10	1.7	157	92	115	77	1447724_x_at	BB324282
Map4k4	mitogen-activated protein kinase kinase kinase kinase 4	1.7	102	60	102	75	1448050_s_at	BF450398
Sca2	spinocerebellar ataxia 2 homolog (human)	1.7	302	178	109	85	1433455_at	AW536901
Cxcl7	chemokine (C-X-C motif) ligand 7	1.7	52	31	79	59	1418480_at	NM_023785
Nedd8	neural precursor cell expressed, developmentally down-regulated 8	1.7	38	22	28	16	1429180_at	AK017821
Mapk8	mitogen activated protein kinase 8	1.7	37	22	25	24	1437045_at	AV345871
Erdr1	erythroid differentiation regulator 1	1.7	425	252	216	140	1439200_x_at	BE686792
Rnpc1	RNA-binding region (RNP1, RRM) containing 1	1.7	49	29	31	25	1421265_a_at	NM_019547
Pou2f1	POU domain, class 2, transcription factor 1	1.7	83	49	68	67	1427695_a_at	X56230
Ube2i	ubiquitin-conjugating enzyme E2I	1.7	88	53	74	62	1429545_at	BI080812
G2a	G protein-coupled receptor G2A	1.7	85	50	52	36	1421755_at	NM_019925
Skp2	S-phase kinase-associated protein 2 (p45)	1.7	59	35	39	31	1437033_a_at	BB784099
Mtr3-pending	Mtr3 (mRNA transport regulator 3)-homolog (yeast)	1.7	79	47	37	19	1435544_at	BB446614
Serpinh1	serine (or cysteine) proteinase inhibitor, clade H, member 1	1.7			10		1450843_a_at	BI220012
Arf2	ADP-ribosylation factor 2	1.7	104	62	77	69	1416459_at	NM_007477
Dnaja3	DnaJ (Hsp40) homolog, subfamily A, member 3	1.7	108	64	83	66	1427894_at	AK012169
Irf4	interferon regulatory factor 4	1.7	590	356	271	207	1421173_at	NM_013674
Trim27	tripartite motif protein 27	1.7	256	154	132	83	1456375_x_at	BB290427
Btg2	B-cell translocation gene 2, anti-proliferative	1.7	190	115	207	221	1416250_at	BG965405
Nsd1	nuclear receptor-binding SET-domain protein 1	1.7	21	13	11	10	1457464_at	BG070926
Aldh2	aldehyde dehydrogenase 2, mitochondrial	1.7	68	41	62	42	1437410_at	BB513627
Syt13	synaptotagmin 13	1.6	38	23	29	30	1434470_at	BB244585
Ubtf	upstream binding transcription factor, RNA polymerase I	1.6	38	23	19	11	1460304_a_at	BB832806
Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase	1.6	161	98	105	82	1425517_s_at	BI657000
Epha4	Eph receptor A4	1.6	22	14	22	12	1421929_at	BB706548
Sfpq	splicing factor proline/glutamine rich	1.6	36	22	26	20	1436898_at	BI738328
Zfp472	zinc finger protein 472	1.6	84	52	60	58	1425058_at	BC023090
Lsm10	U7 snRNP-specific Sm-like protein LSM10	1.6	216	133	201	152	1417515_at	NM_138721
Picalm	phosphatidylinositol binding clathrin assembly protein	1.6	78	48	107	116	1455773_at	BG797225
Rps17	ribosomal protein S17	1.6	72	45	39	27	1438502_x_at	AA030209
Cdc37l	cell division cycle 37 homolog (S. cerevisiae)-like	1.6	147	91	109	89	1433574_at	BE824561
Zfp292	zinc finger protein 292	1.6	159	98	219	196	1436308_at	BB315561
Ulk1	Unc-51 like kinase 1 (C. elegans)	1.6	209	130	222	159	1448370_at	AF072370
Facl2	fatty acid Coenzyme A ligase, long chain 2	1.6	573	357	591	531	1450643_s_at	BI413218
Masp2	mannan-binding lectin serine protease 2	1.6	83	52	36	31	1436318_at	AV338665
Spata13	spermatogenesis associated 13	1.6	132	82	52	50	1437865_at	AW546433
Dusp9	dual specificity phosphatase 9	1.6	19	12	21	17	1454737_at	AV295798
Tgfbr1	transforming growth factor, beta receptor I	1.6	1982	1247	1082	668	1420895_at	BM248342
Glb1	galactosidase, beta 1	1.6	42	26	25	18	1435795_at	BE956926
Wee1	wee 1 homolog (S. pombe)	1.6	196	124	237	281	1416773_at	BC006852
Perq1	PERQ amino acid rich, with GYF domain 1	1.6	141	89	131	102	1423460_at	BI688428
Gstm1	glutathione S-transferase, mu 1	1.6	144	91	105	103	1425626_at	J03952
Epm2aip1	EPM2A (laforin) interacting protein 1	1.6	116	73	85	73	1434105_at	AV276770
Cdca4	cell division cycle associated 4	1.6	359	226	141	190	1456240_x_at	BB329505
B3gat1	beta-1,3-glucuronyltransferase 1 (glucuronosyltransferase P)	1.6	24	15	13	16	1454821_at	BB424673
Solh	small optic lobes homolog (Drosophila)	1.6	648	413	721	543	1427785_x_at	BC022685
Pde4b	phosphodiesterase 4B, cAMP specific	1.6	168	107	141	102	1422473_at	AF326555
Il17r	interleukin 17 receptor	1.6	187	119	82	88	1420904_at	NM_008359
Slc38a2	solute carrier family 38, member 2	1.6	128	82	112	99	1429593_at	BE854973
Cxcl1	chemokine (C-X-C motif) ligand 1	1.6	965	616	359	341	1457644_s_at	BB554288
Spred2	sprouty protein with EVH-1 domain 2, related sequence	1.6	80	52	37	29	1436892_at	BB133520
Htf9c	Hpall tiny fragments locus 9c	1.6	106	69	70	61	1448115_at	NM_008307
Gfi1b	growth factor independent 1B	1.6	49	31	30	29	1420399_at	NM_008114
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	1.5	666	431	277	207	1456005_a_at	BB667581
Gspt1	G1 to phase transition 1	1.5	48	32	27	30	1446550_at	BB162021
Cbx2	chromobox homolog 2 (Drosophila Pc class)	1.5	101	65	79	66	1434116_at	BI693188
Zfp36l1	zinc finger protein 36, C3H type-like 1	1.5	136	88	136	137	1422528_a_at	BB036959
Adora2b	adenosine A2b receptor	1.5	102	66	32	36	1434772_at	BB795854
Pigq	phosphatidylinositol glycan, class Q	1.5	107	70	51	49	1426055_a_at	AF030178
Tardbp	TAR DNA binding protein	1.5	372	242	213	143	1455655_a_at	BM935796
Cdk5r	cyclin-dependent kinase 5, regulatory subunit (p35)	1.5	183	119	68	80	1433451_at	BB177836
Sirt7	sirtuin 7	1.5	200	131	74	49	1434054_at	BB342453
Nr4a1	nuclear receptor subfamily 4, group A, member 1	1.5	88	57	95	96	1416505_at	NM_010444
Zipro1	zinc finger proliferation 1	1.5	72	48	54	57	1416370_at	NM_011757
Trim27	tripartite motif protein 27	1.5	204	134	145	102	1448101_s_at	NM_009054
Fbs1	fibrosin 1	1.5	168	111	130	174	1451645_at	BB162362
Spg20	spastic paraplegia 20	1.5	84	56	83	87	1451520_at	BB040507
Bcl2l11	BCL2-like 11 (apoptosis facilitator)	1.5	224	150	127	85	1456006_at	BB667581
Imap38	immunity-associated protein	1.5	17	12	52	53	1449988_at	NM_008376

Table 4.9 (continued) IFN-suppressed genes in BMDCs after 2h but not 6h of culture

Gene	Description	2h Fold Change	2h Signal 0	2h Signal IFN	6h Fold Change	6h Signal 0	6h Signal IFN	Affymetrix Accession
Ifit1	interferon-induced protein with tetratricopeptide repeats 1	95.8	43	2670	3.7	999	3394	1435529_at
Cxcl9	chemokine (C-X-C motif) ligand 9	78.1	7	514	46.4	17	731	1418652_at
Tyki	thymidylate kinase family LPS-inducible member	58.5	11	553	5.5	220	1201	1422095_a_at
Mx2	myxovirus (influenza virus) resistance 2	50.9	11	464	2.1	279	580	1419676_at
Ms4a4c	membrane-spanning 4-domains, subfamily A, member 4C	30.6	12	245	11.9	67	795	1420671_x_at
Igp-pending	interferon-inducible GTPase	30.2	10	266	8.0	87	723	1419042_at
Ifit3	interferon-induced protein with tetratricopeptide repeats 3	29.4	69	1954	2.7	1213	3160	1449025_at
Isg20	interferon-stimulated protein	26.6	19	360	5.6	170	920	1419569_a_at
Tgtp	T-cell specific GTPase	24.2	77	1837	4.7	481	2359	1449009_at
Samhd1	SAM domain and HD domain, 1	19.6	10	174	1.8	88	165	1444064_at
Slfn1	schlafen 1	18.1	45	393	2.9	208	594	1418612_at
Cxcl11	chemokine (C-X-C motif) ligand 11	14.7	36	373	5.7	114	633	1419697_at
Ms4a4c	membrane-spanning 4-domains, subfamily A, member 4C	12.5	17	206	9.6	81	524	1450291_s_at
Iigp	interferon inducible GTPase 1	11.2	47	530	5.7	224	1257	1419043_a_at
Mx1	myxovirus (influenza virus) resistance 1	10.6	233	2462	2.1	1377	2892	1451905_a_at
Igtp	interferon gamma induced GTPase	10.6	82	858	2.4	382	875	1417793_at
Serpina3g	serine (or cysteine) proteinase inhibitor, clade A, member 3G	9.7	99	958	12.0	166	1972	1424923_at
Usp18	ubiquitin specific protease 18	9.7	296	2520	2.1	1411	2983	1418191_at
Vig1	viral hemorrhagic septicemia virus(VHSV) induced gene 1	9.6	372	3284	1.5	2920	4476	1421008_at
Zbp1	Z-DNA binding protein 1	8.5	22	135	4.4	51	223	1419604_at
Ifi47	interferon gamma inducible protein	7.9	271	1981	1.7	1468	2412	1417292_at
Ifi1	interferon inducible protein 1	7.4	222	1620	2.2	747	1599	1418825_at
Cxcl9	chemokine (C-X-C motif) ligand 9	6.6	68	105	7.6	14	104	1456907_at
Igtp	interferon gamma induced GTPase	6.4	130	826	2.8	467	1273	1417141_at
Vig1	viral hemorrhagic septicemia virus(VHSV) induced gene 1	5.8	732	3912	1.5	2940	4398	1421009_at
Il15	interleukin 15	5.0	61	401	3.1	333	973	1418219_at
Ifi203	interferon activated gene 203	4.8	269	1141	3.0	500	1525	1451567_a_at
Oasl1	2'-5' oligoadenylate synthetase-like 1	4.7	310	1353	1.8	1370	2425	1424339_at
Trim21	tripartite motif protein 21	4.5	190	851	1.9	387	744	1448940_at
Il15ra	interleukin 15 receptor, alpha chain	4.1	59	240	2.3	173	401	1448681_at
Slfn5	schlafen 5	3.8	64	239	2.1	185	383	1458458_at
Trim21	tripartite motif protein 21	3.8	66	252	2.1	121	254	1418077_at
Oas2	2'-5' oligoadenylate synthetase 2	3.7	70	232	2.7	142	386	1425065_at
Lhx2	LIM homeobox protein 2	3.5	12	44	11.7	8	93	1418317_at
Slfn5	schlafen 5	3.3	124	420	1.8	242	439	1442640_at
CD40	tumor necrosis factor receptor superfamily, member 5	3.3	166	523	3.2	307	952	1460415_a_at
Pml	promyelocytic leukemia	3.1	75	225	2.4	145	342	1448757_at
Trim30	tripartite motif protein 30	3.1	553	1569	2.1	1113	2239	1417961_a_at
Slfn4	schlafen 4	3.1	82	216	2.4	125	300	1427102_at
Mad	Max dimerization protein	3.1	173	509	2.0	598	1170	1455104_at
Stat2	signal transducer and activator of transcription 2	3.1	79	251	1.8	221	398	1421911_at
Daxx	Fas death domain-associated protein	3.0	351	1038	2.0	841	1697	1419026_at
Ly6e	lymphocyte antigen 6 complex, locus E	2.9	59	169	1.9	118	235	1439773_at
Ms4a6b	membrane-spanning 4-domains, subfamily A, member 6B	2.8	57	157	2.6	90	238	1418826_at
Cxcl10	chemokine (C-X-C motif) ligand 10	2.8	1448	3856	1.6	2274	3674	1418930_at
Mad	Max dimerization protein	2.8	318	857	1.9	594	1126	1434830_at
Il15ra	interleukin 15 receptor, alpha chain	2.8	38	107	2.6	72	180	1422397_a_at
Plec1	plectin 1	2.7	154	403	1.9	383	706	1452178_at
Bagl-pending	B aggressive lymphoma	2.7	330	869	1.6	598	954	1416897_at
Card4	caspase recruitment domain 4	2.5	163	401	2.9	193	555	1454733_at
Nat2	N-acetyltransferase 2 (arylamine N-acetyltransferase)	2.5	35	88	2.8	51	139	1449981_a_at
Gbp2	guanylate nucleotide binding protein 2	2.4	894	2130	2.1	1676	3434	1418240_at
Prkr	protein kinase, interferon-inducible double stranded RNA dependent	2.3	82	195	2.2	78	174	1422006_at
Centa2	centaurin, alpha 2	2.2	53	120	2.3	63	144	1425639_at
Tap1	transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	2.2	369	810	1.8	783	1439	1416016_at
Rpl41	ribosomal protein L41	2.2	42	90	0.6	76	42	1454639_x_at
Ms4a6c	membrane-spanning 4-domains, subfamily A, member 6C	2.1	76	165	2.5	103	242	1450234_at
Cpo	coproporphyrinogen oxidase	2.1	25	53	2.5	22	56	1431296_at
Oas1g	2'-5' oligoadenylate synthetase 1G	2.0	720	1350	1.8	1032	1861	1424775_at
Dtr	diphtheria toxin receptor	1.9	75	144	2.3	84	193	1418350_at
Pols	polymerase (DNA directed) sigma	1.9	100	187	2.0	146	286	1438003_at
Tor3a	torsin family 3, member A	1.8	82	145	1.6	97	151	1450454_at
Ifrg15-pending	interferon alpha responsive gene	1.8	554	965	2.0	480	951	1458364_s_at
Lck	lymphocyte protein tyrosine kinase	1.7	43	74	1.9	40	77	1425396_a_at
Tlr8	toll-like receptor 8	1.7	47	80	1.9	39	75	1450267_at
Ctla2a	cytotoxic T lymphocyte-associated protein 2 alpha	1.6	27	44	3.2	30	91	1452352_at
Casp2	caspase 2	1.6	174	276	1.9	266	518	1448165_at
Plac8	placenta-specific 8	1.6	46	72	1.9	78	151	1451335_at

Table 4.10 IFN-induced genes in BMDCs after both 2h and 6h culture.
Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine U74Av2 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2..

		2h			6h			
		Fold	Signal		Fold	Signal	Affymetrix	Genbank
Gene	Description	Change	0	IFN	Change	0	IFN	Accession	Accession
Herpud1	endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	2.9	1017	350	1.6	759	483	1448185_at	NM_022331
Pdgfb	platelet derived growth factor, B polypeptide	2.8	532	190	1.7	239	137	1450414_at	BC023427
Aps	adaptor protein with pleckstrin homology and src	2.3	132	57	1.8	72	39	1450718_at	NM_018825
Gpr68	G protein-coupled receptor 68	2.3	472	206	1.7	456	266	1455000_at	BB538372
Ubl4	ubiquitin-like 4	2.3	106	46	1.6	83	54	1424539_at	BI650739
Cnk	cytokine inducible kinase	2.1	137	64	1.8	99	55	1434496_at	BM947855
Stk10	serine/threonine kinase 10	2.0	364	185	1.7	257	148	1417751_at	NM_009288
Slc39a14	solute carrier family 39 (zinc transporter), member 14	1.8	249	136	2.0	177	90	1427035_at	BB399837
Cebpa	CCAAT/enhancer binding protein (C/EBP), alpha	1.8	617	343	1.5	438	285	1418982_at	BC011118
Eif2ak3	eukaryotic translation initiation factor 2 alpha kinase 3	1.8	187	104	1.5	183	119	1449278_at	NM_010121
Rit1	Ras-like without CAAX 1	1.7	82	47	1.7	63	36	1428710_at	AK018785
Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	1.7	141	82	2.2	181	81	1447363_s_at	AU045529
Rab40c	Rab40c, member RAS oncogene family	1.7	231	137	1.6	290	183	1424331_at	BC027008
Ptpre	protein tyrosine phosphatase, receptor type, E	1.6	1013	622	1.8	552	307	1418539_a_at	NM_011212
Rnu22	RNA, U22 small nucleolar	1.6	361	224	1.9	388	206	1433674_a_at	BQ177137
Madh6	MAD homolog 6 (Drosophila)	1.6	78	49	1.6	126	77	1422771_at	AF010133
Slco4a1	solute carrier organic anion transporter family, member 4a1	1.6	216	137	1.6	116	73	1455803_at	AV375182
Ptpre	protein tyrosine phosphatase, receptor type, E	1.5	1540	1008	1.7	600	359	1418540_a_at	U35368
Bet1l	blocked early in transport 1 homolog (S. cerevisiae)-like	1.5	108	71	1.7	85	51	1430549_at	AV252862
Txnrd3	thioredoxin reductase 3	1.5	83	54	1.8	63	35	1449623_at	AI196535

Table 4.11 IFN-suppressed genes in BMDCs after both 2h and 6h culture.
Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine 430 2.0 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

Gene	Description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Dnase1l3	deoxyribonuclease 1-like 3	25.3		62		15	1421057_at	BC012671
Ms4a4b	membrane-spanning 4-domains, subfamily A, member 4B	11.6		31		20	1423467_at	BB199001
Pou3f1	POU domain, class 3, transcription factor 1	5.4	11	54		15	1460038_at	BG065255
Tlr3	toll-like receptor 3	5.1	69	334	42	156	1422782_s_at	NM_126166
Cxcl11	chemokine (C-X-C motif) ligand 11	4.7		44		39	1419698_at	NM_019494
Il6	interleukin 6	4.2	96	428	315	1067	1450297_at	NM_031168
Tnfsf10	tumor necrosis factor (ligand) superfamily, member 10	4.1	41	156		33	1420412_at	NM_009425
Cspg2	chondroitin sulfate proteoglycan 2	4.0	90	346	66	137	1427256_at	BI692925
Cspg2	chondroitin sulfate proteoglycan 2	3.8	121	449	89	167	1421694_a_at	NM_019389
Ch25h	cholesterol 25-hydroxylase	3.7	254	854	585	1245	1449227_at	NM_009890
Ifi203	interferon activated gene 203	3.6	28	103	24	101	1425008_a_at	L14559
cathepsin C	cathepsin C	3.3	287	932	297	577	1437939_s_at	BM237633
Tnfrsf5	tumor necrosis factor receptor superfamily, member 5	3.3	236	697	189	461	1449473_s_at	NM_011611
Fgl2	fibrinogen-like protein 2	3.1	355	1114	150	456	1421854_at	NM_008013
Tnfrsf5	tumor necrosis factor receptor superfamily, member 5	3.1	250	729	195	511	1439221_s_at	BB220422
Gnb4	guanine nucleotide binding protein, beta 4	2.9	167	470	162	214	1419470_at	BI713933
Ifi203	interferon activated gene 203	2.8	405	1120	266	1037	1448775_at	NM_008328
Hmgn3	high mobility group nucleosomal binding domain 3	2.7	45	114	54	71	1434875_a_at	AV018952
Mov10	Moloney leukemia virus 10	2.6	55	148	37	85	1416380_at	NM_008619
Mef2c	myocyte enhancer factor 2C	2.6	10	26	20	21	1421028_a_at	AI595932
Gnb4	guanine nucleotide binding protein, beta 4	2.6	68	178	80	96	1419469_at	NM_013531
Slco3a1	solute carrier organic anion transporter family, member 3a1	2.6	41	69	13	15	1418030_at	BB337160
Rpl35a	ribosomal protein L35a	2.6	89	219	70	169	1436032_at	BB201888
Fcgr1	Fc receptor, IgG, high affinity I	2.6	76	194	117	214	1436625_at	BB075261
Fcgr1	Fc receptor, IgG, high affinity I	2.5	303	755	355	561	1417876_at	AF143181
Bbx	bobby sox homolog (Drosophila)	2.5	18	46	20	10	1422741_a_at	AV255109
Tmem2	transmembrane protein 2	2.5	94	230	85	135	1451458_at	BC019745
Dnr12	diabetic nephropathy-related gene 1	2.4	743	1766	653	1255	1434380_at	BM241271
Dmtf1	cyclin D binding myb-like transcription factor 1	2.4					1439376_x_at	BB357590
Oas3	2'-5' oligoadenylate synthetase 3	2.4	263	592	209	403	1425374_at	AB067534
Pou3f1	POU domain, class 3, transcription factor 1	2.4	30	70	17	43	1422068_at	NM_011141
Pdgfrl	platelet-derived growth factor receptor-like	2.4	48	113	47	56	1454824_s_at	BB699957
Zbp1	Z-DNA binding protein 1	2.3	254	589	161	322	1429947_a_at	AK008179
Gpr86	G protein-coupled receptor 86	2.3	57	133	158	262	1428700_at	AK008013
Spred1	sprouty protein with EVH-1 domain 1, related sequence	2.3	59	136	113	163	1428777_at	BG060646
Dtr	diphtheria toxin receptor	2.3	60	141	69	114	1418349_at	NM_010415
Abcb1a	ATP-binding cassette, sub-family B (MDR/TAP), member 1A	2.3	27	63	17	33	1419759_at	BB819674
Mad	Max dimerization protein	2.3	56	134		13	1422002_at	L38926
Oasl2	2'-5' oligoadenylate synthetase-like 2	2.3	829	1891	286	920	1453196_a_at	BQ033138
Cd86	CD86 antigen	2.3	365	886	331	372	1449858_at	NM_019388
Hmgn3	high mobility group nucleosomal binding domain 3	2.2	27	60	27	50	1431777_a_at	AK002970
Fgf23	fibroblast growth factor 23	2.2	15	33	29	27	1416124_at	BB840359
Sgcb	sarcoglycan, beta (dystrophin-associated glycoprotein)	2.2	65	141	39	43	1419667_at	NM_011890
Itpr1	inositol 1,4,5-triphosphate receptor 1	2.2	56	121	106	77	1460203_at	NM_010585
Pttg1	pituitary tumor-transforming 1	2.2	338	744	243	365	1424105_a_at	AF069051
Nmi	N-myc (and STAT) interactor	2.2	281	621	211	290	1425719_a_at	BC002019
Pdgfrl	platelet-derived growth factor receptor-like	2.2	22	47	21	25	1436501_at	AW554551
Rps24	ribosomal protein S24	2.2	39	80	36	42	1455195_at	BM119287
Sgcb	sarcoglycan, beta (dystrophin-associated glycoprotein)	2.2	66	140	88	103	1419668_at	AK014381
Ly6a	lymphocyte antigen 6 complex, locus A	2.2	766	1666	527	722	1417185_at	BC002070
Slfn5	schlafen 5	2.1	234	508	90	375	1456288_at	BB134615
Fnbp2	formin binding protein 2	2.1	34	70	32	32	1427967_at	BE653295
Zfp318	zinc finger protein 318	2.1	11	22	16	12	1425347_a_at	BB168536
Tgfb1i4	transforming growth factor beta 1 induced transcript 4	2.1	64	136	57	65	1454758_a_at	AU016382
Znfn1a2	zinc finger protein, subfamily 1A, 2 (Helios)	2.1	197	396	163	195	1437542_at	BB319935
Casp3	caspase 3, apoptosis related cysteine protease	2.1	58	121	69	58	1426165_a_at	D86352
Hrasls3	HRAS like suppressor 3	2.1	146	294	119	133	1451611_at	BC024581
H2-Bf	histocompatibility 2, complement component factor B	2.1	376	717	339	414	1417314_at	NM_008198
Aim1	absent in melanoma 1	2.1	134	272	158	178	1426942_at	BM233292
Mertk	c-mer proto-oncogene tyrosine kinase	2.1	345	706	357	534	1422869_at	NM_008587
Il12b	interleukin 12b	2.1	105	212	120	135	1449497_at	AF128214
Ripk2	receptor (TNFRSF)-interacting serine-threonine kinase 2	2.1	302	619	305	506	1450173_at	NM_138952
Lap3	leucine aminopeptidase 3	2.1	738	1526	605	825	1450860_at	AK010384
Zc3hdc1	zinc finger CCCH type domain containing 1	2.1	804	1646	459	998	1426774_at	BM227980
Pnp	purine-nucleoside phosphorylase	2.1	1661	3409	953	1841	1453299_a_at	AK008143
Spred1	sprouty protein with EVH-1 domain 1, related sequence	2.1	59	122	127	140	1452911_at	AK017680
Xm1	5'-3' exoribonuclease 1	2.0	43	88	47	48	1450308_a_at	NM_011916
C3ar1	complement component 3a receptor 1	2.0	252	517	421	597	1442082_at	BB333624
Il18rap	interleukin 18 receptor accessory protein	2.0	27	55	39	39	1421291_at	NM_010553
Crsp9	cofactor required for Sp1 transcriptional activation, subunit 9	2.0	33	68	19	31	1430419_at	AK009549
Hhex	hematopoietically expressed homeobox	2.0	145	291	137	261	1423319_at	AK014111
G1p2	interferon, alpha-inducible protein	2.0	1381	2754	495	1207	1431591_s_at	AK019325
Apobec3	apolipoprotein B editing complex 3	2.0	322	646	197	317	1417470_at	NM_030255
Tpst1	protein-tyrosine sulfotransferase 1	2.0	266	525	104	251	1421733_a_at	NM_013837
Apaf1	apoptotic protease activating factor 1	2.0	150	291	205	223	1452870_at	AK018076

Table 4.12 (continued over page) IFN-induced genes in BMDCs after 6h but not 2h of culture. Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine 430 2.0 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

Gene	Description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Ogfr	opioid growth factor receptor	2.0	122	243	94	142	1422512_a_at	AW476433
Katna1	katanin p60 (ATPase-containing) subunit A1	2.0	242	475	215	232	1450949_at	AK012319
Cyp27a1	cytochrome P450, family 27, subfamily a, polypeptide 1	2.0	138	269	104	133	1417590_at	NM_024264
Fabp3	fatty acid binding protein 3, muscle and heart	2.0	155	308	108	134	1416023_at	NM_010174
Tgfb1i4	transforming growth factor beta 1 induced transcript 4	2.0	377	741	358	393	1454971_x_at	BB357514
Snn	stannin	2.0	150	295	161	254	1452789_at	AK012171
Mgat4a	mannoside acetylglucosaminyltransferase 4, isoenzyme A	2.0	91	178	96	110	1444347_at	BG070161
Lrp8	low density lipoprotein receptor-related protein 8	2.0	175	341	171	193	1440882_at	BB750940
Pcaf	p300/CBP-associated factor	2.0	198	378	251	288	1434037_s_at	AV094898
Ptgs2	prostaglandin-endoperoxide synthase 2	2.0	123	237	125	72	1417263_at	M94967
Ptpn2	protein tyrosine phosphatase, non-receptor type 2	2.0	69	28	15	15	1425198_at	BG076152
Znf212	Zinc finger protein 212	1.9	91	177	98	129	1451292_at	BC006693
Bckdhb	branched chain ketoacid dehydrogenase E1, beta polypeptide	1.9	56	106	53	64	1427153_at	AW047304
Cited2	Cbp/p300-interacting transactivator 2	1.9	297	561	145	426	1421267_a_at	NM_010828
Itpr1	inositol 1,4,5-triphosphate receptor 1	1.9	59	115	91	80	1417279_at	NM_008789
Stat1	signal transducer and activator of transcription 1	1.9	505	957	249	583	1420915_at	BM239586
Bcl9	B-cell CLL/lymphoma 9	1.9	[illegible]	[illegible]	55	66	1451574_at	BC019641
Stat1	signal transducer and activator of transcription 1	1.9	732	1376	351	792	1450034_at	AW214029
Pvrl4	poliovirus receptor-related 4	1.9	42	79	60	55	1428701_at	AK004821
Il18bp	interleukin 18 binding protein	1.9	110	210	84	120	1450424_a_at	AF110803
Tcf4	transcription factor 4	1.9	212	403	110	189	1416724_x_at	AI639846
Slamf8	SLAM family member 8	1.9	187	353	213	264	1425294_at	BC024587
Spred1	sprouty protein with EVH-1 domain 1, related sequence	1.8	41	75	76	79	1423162_s_at	BQ044290
Tgfb1i4	transforming growth factor beta 1 induced transcript 4	1.8	296	567	278	312	1425742_a_at	AF201285
Trim26	tripartite motif protein 26	1.8	162	293	143	183	1424929_a_at	AF230395
Daam1	dishevelled associated activator of morphogenesis 1	1.8	222	403	193	221	1455244_at	BB794633
Schip1	schwannomin interacting protein 1	1.8	44	81	66	78	1423025_a_at	NM_013928
Bbx	bobby sox homolog (Drosophila)	1.8	64	114	60	59	1425835_a_at	AF454944
Tbc1d1	TBC1 domain family, member 1	1.8	17	30	14	37	1447016_at	BB258507
Cklfsf6	chemokine-like factor super family 6	1.8	617	1106	735	771	1451114_at	BC027248
Ccnd2	cyclin D2	1.8	372	657	547	611	1430127_a_at	AK007904
Samhd1	SAM domain and HD domain, 1	1.8	1869	3359	1595	2417	1418131_at	NM_018851
Sema4c	semaphorin 4C	1.8	47	85	51	58	1433920_at	BQ032843
Rap2c	RAP2C, member of RAS oncogene family	1.8	350	626	332	426	1460430_at	AK008416
Fcrl3	Fc receptor-like 3	1.8	174	309	164	240	1425225_at	BC027310
Phip	pleckstrin homology domain interacting protein	1.8	88	157	48	66	1438535_at	BB523030
Gpr108	G protein-coupled receptor 108	1.8	231	414	357	375	1426078_a_at	AF376726
Sp100	nuclear antigen Sp100	1.8	303	540	175	279	1451821_a_at	U83636
Stx11	syntaxin 11	1.8	213	374	192	276	1453228_at	AK017897
Hdac1	histone deacetylase 1	1.8	532	938	655	742	1448246_at	NM_008228
Il13ra1	interleukin 13 receptor, alpha 1	1.8	273	483	190	211	1451775_s_at	S80963
Casp4	caspase 4, apoptosis-related cysteine protease	1.8	648	1131	516	788	1449591_at	NM_007609
Azi2	5-azacytidine induced gene 2	1.8	329	571	322	439	1431822_a_at	AK004992
Xdh	xanthine dehydrogenase	1.8	182	321	221	237	1451006_at	AV286265
Tmem2	transmembrane protein 2	1.8	103	184	84	138	1424711_at	BB667229
Usp12	ubiquitin specific protease 12	1.8	229	396	296	397	1434483_at	AW539189
Gtpat12	gene trap PAT 12	1.8	71	126	54	81	1434306_at	BF319015
Plcl2	phospholipase C-like 2	1.8	392	680	363	394	1426450_at	BM207017
Lrp8	low density lipoprotein receptor-related protein 8	1.8	36	67	39	38	1421459_a_at	NM_053073
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1	1.8	12	21	21	27	1439947_at	C87524
Stat2	signal transducer and activator of transcription 2	1.7	379	659	122	390	1450403_at	AF088862
Dr1	down-regulator of transcription 1	1.7	106	185	123	157	1416019_at	NM_026106
P2ry12	purinergic receptor P2Y, G-protein coupled 12	1.7	53	91	56	71	1431724_a_at	AK013804
Gmppb	GDP-mannose pyrophosphorylase B	1.7	184	327	142	225	1439030_at	BI410722
Kitl	kit ligand	1.7	11	18	[illegible]	14	1415854_at	BC011322
Tmpo	thymopoietin	1.7	212	367	176	180	1428976_at	AK017463
Tcf4	transcription factor 4	1.7	162	275	88	139	1424089_a_at	U16321
Upp1	uridine phosphorylase 1	1.7	434	746	380	473	1448562_at	NM_009477
syntaxin 11	Mus musculus transcribed sequences	1.7	63	92	73	84	1457780_at	BB767243
Fgf23	fibroblast growth factor 23	1.7	707	1186	998	1173	1434745_at	BQ175880
Bfar	bifunctional apoptosis regulator	1.7	370	624	278	359	1426490_at	AK013874
Tor3a	torsin family 3, member A	1.7	501	857	266	583	1428660_s_at	AK009693
Phip	pleckstrin homology domain interacting protein	1.7	58	109	37	78	1443522_s_at	BM221262
Arhh	ras homolog gene family, member H	1.7	119	200	108	163	1429319_at	BM243660
Zfp313	zinc finger protein 313	1.7	209	360	132	192	1422959_s_at	NM_030743
Csrp1	cysteine and glycine-rich protein 1	1.7	237	403	273	230	1425810_a_at	AF092921
Stat1	signal transducer and activator of transcription 1	1.7	1091	1847	440	830	1450033_a_at	NM_009283
Etohd2	ethanol decreased 2	1.7	540	897	516	612	1433910_at	BM208041
Usp12	ubiquitin specific protease 12	1.7	30	51	42	56	1427478_at	BI558267
Mitc1	MAD homolog 4 interacting transcription coactivator 1	1.7	215	367	220	206	1433606_at	BG069232
Atp11b	ATPase, Class VI, type 11B	1.7	502	850	473	595	1451388_a_at	BC026741
Slamf7	SLAM family member 7	1.7	442	738	336	429	1453472_a_at	AK016183
C3ar1	complement component 3a receptor 1	1.7	540	899	368	631	1419482_at	BC003728
Ccl2	chemokine (C-C motif) ligand 2	1.7	469	791	1156	1558	1420380_at	AF065933

Table 4.12 (continued) IFN-induced genes in BMDCs after 6h but not 2h of culture

Gene	Description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Tnk1	tyrosine kinase, non-receptor, 1	1.7	19	32	38	36	1425635_at	AF307746
Zfpm1	zinc finger protein, multitype 1	1.7	117	196	80	99	1451046_at	AA014267
Cd86	CD86 antigen	1.7	169	278	194	188	1420404_at	BC013807
Crkl	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	1.7	35	58	29	33	1421953_at	NM_007764
Mllt2h	homolog of human MLLT2 unidentified gene	1.7	32	53	41	40	1425641_at	BB586268
Lyn	Yamaguchi sarcoma viral (v-yes-1) oncogene homolog	1.7	378	634	491	486	1425598_a_at	M64608
Icsbp1	interferon consensus sequence binding protein 1	1.7	618	1006	586	833	1416714_at	BC005450
Fgf23	fibroblast growth factor 23	1.6	290	485	356	448	1448229_s_at	BM118679
Timeless	timeless homolog (Drosophila)	1.6	48	77	30	36	1417587_at	BM230269
Ogfr	opioid growth factor receptor	1.6	569	922	416	691	1422511_a_at	NM_031373
Fnbp2	formin binding protein 2	1.6	94	156	160	160	1434406_at	BQ174400
Tlk2	tousled-like kinase 2	1.6	259	425	145	232	1431827_a_at	AK014829
Homer1	homer homolog 1 (Drosophila)	1.6	152	247	130	176	1439662_at	BM124609
Tor3a	torsin family 3, member A	1.6	286	463	195	452	1421998_at	AV290846
Prpf4	PRP4 pre-mRNA processing factor 4 homolog (yeast)	1.6	92	149	84	108	1429724_at	BG918505
Homer1	homer homolog 1 (Drosophila)	1.6	231	365	204	248	1437363_at	BQ043238
Ankrd17	ankyrin repeat, domain 17	1.6	474	768	440	471	1436775_a_at	AW557826
Mppe1	metallophosphoesterase 1	1.6	233	378	235	275	1440880_at	BI648107
Brd2	bromodomain containing 2	1.6	480	783	480	473	1437210_a_at	AI506310
Ifi205	interferon activated gene 205	1.6	1919	3106	1258	2710	1452231_x_at	M74124
Mpv17	Mpv17 transgene, kidney disease mutant	1.6	20	33	41	44	1441076_at	BM939297
Tmod3	tropomodulin 3	1.6	317	508	263	284	1423089_at	AK017725
Tbc1d1	TBC1 domain family, member 1	1.6	32	52	46	43	1419446_at	NM_019636
Il12rb2	interleukin 12 receptor, beta 2	1.6	114	184	132	161	1421623_at	NM_008354
Tmpo	thymopoietin	1.6	477	765	421	443	1452036_a_at	AA153892
Glipr2	GLI pathogenesis-related 2	1.6	178	289	193	186	1428492_at	AK017557
Cds1	CDP-diacylglycerol synthase 1	1.6	58	93	56	58	1456114_at	BI152841
Adar	adenosine deaminase, RNA-specific	1.6	324	521	120	227	1425405_a_at	AF291876
Il13ra1	interleukin 13 receptor, alpha 1	1.6	193	311	200	192	1427165_at	AA879826
Cds1	CDP-diacylglycerol synthase 1	1.6	56	94	71	72	1428680_at	AK014670
Map4k5	mitogen-activated protein kinase kinase kinase kinase 5	1.6	86	139	85	128	1438702_at	BG075699
Rbl1	retinoblastoma-like 1 (p107)	1.6	197	309	209	229	1424156_at	U27177
Casp8	caspase 8	1.6	606	959	639	742	1424552_at	BC006737
Lgals9	lectin, galactose binding, soluble 9	1.6	1579	2510	1412	1749	1421217_a_at	NM_010708
Bfar	bifunctional apoptosis regulator	1.6	190	299	155	202	1426488_at	AV235863
Hcph	hemopoietic cell phosphatase	1.6	648	1024	597	556	1460188_at	NM_013545
Nkd1	naked cuticle 1 homolog (Drosophila)	1.6	16	24	24	29	1440446_at	BM021767
Rfc3	replication factor C (activator 1) 3	1.6	67	106	62	64	1432538_a_at	AK013095
Insl6	insulin-like 6	1.6	453	714	291	352	1418346_at	NM_013754
Icsbp1	interferon consensus sequence binding protein 1	1.6	444	681	230	356	1448452_at	BG069095
Nr3c1	nuclear receptor subfamily 3, group C, member 1	1.6	56	88	48	59	1421866_at	NM_008173
Cpne3	copine III	1.6	168	259	179	157	1428514_at	AK017651
Rab19	RAB19, member RAS oncogene family	1.6	203	317	121	178	1430148_at	BM241400
H2-T10	histocompatibility 2, T region locus 10	1.6	1243	1940	654	1037	1449875_s_at	NM_010395
Pdk3	pyruvate dehydrogenase kinase, isoenzyme 3	1.6	445	706	284	323	1426410_at	AV086243
Eva	epithelial V-like antigen	1.6	26	42	24	29	1416237_at	NM_007962
Kif24	kinesin family member 24	1.6	19	30	14	23	1431937_at	AK016916
Fgf23	fibroblast growth factor 23	1.6	67	105	93	108	1416123_at	BM118679
Pfkfb3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3	1.5	389	593	165	143	1416432_at	NM_133232
Crlf3	cytokine receptor-like factor 3	1.5	489	754	380	435	1460338_a_at	BB161253
Ddx24	DEAD (Asp-Glu-Ala-Asp) box polypeptide 24	1.5	215	331	308	284	1415713_a_at	U46690
Ccl3	chemokine (C-C motif) ligand 3	1.5	878	1398	1822	2321	1419561_at	NM_011337
Rin2	Ras and Rab interactor 2	1.5	238	363	256	334	1426368_at	AK014548
Mgat1	mannoside acetylglucosaminyltransferase 1	1.5	378	581	311	415	1423609_a_at	BB205495
Skil	SKI-like	1.5	69	106	101	78	1422054_a_at	U36203
Whsc1l1	Wolf-Hirschhorn syndrome candidate 1-like 1 homolog (human)	1.5	48	73	48	56	1459907_a_at	BI658695
Slc25a12	solute carrier family 25 (mitochondrial carrier, Aralar), member 12	1.5	391	583	385	401	1428440_at	AK019150
Pcsk7	proprotein convertase subtilisin/kexin type 7	1.5	170	256	184	189	1417180_at	NM_008794
Atm	ataxia telangiectasia mutated homolog (human)	1.5	61	88	65	68	1421205_at	NM_007499
Stx3	syntaxin 3	1.5	197	299	284	284	1434559_at	AW536415
Bpgm	2,3-bisphosphoglycerate mutase	1.5	84	88	88	98	1415865_s_at	BC004589
Crkl	v-crk sarcoma virus CT10 oncogene homolog (avian)-like	1.5	214	331	190	220	1436950_at	AW541802
Tceb3	transcription elongation factor B (SIII), polypeptide 3	1.5	226	343	275	296	1434117_at	AV264059
Fsrg3	female sterile homeotic-related gene 3	1.5	32	49	55	56	1450178_at	NM_054054
Golga3	golgi autoantigen, golgin subfamily a, 3	1.5	141	212	103	107	1419159_at	D78270
T2bp	Traf2 binding protein	1.5	384	583	338	522	1426501_a_at	BB277065
Atp8a1	ATPase, aminophospholipid transporter (APLT), class I, type 8A,1	1.5	71	110	90	80	1423597_at	BB303874
Fgfr4	fibroblast growth factor receptor 4	1.5	30	46	39	51	1427846_x_at	AF127140
Slc7a2	solute carrier family 7 , member 2	1.5	79	121	81	73	1450703_at	BF533509
Cyp1b1	cytochrome P450, family 1, subfamily b, polypeptide 1	1.5	71	98	93	101	1416612_at	NM_009994
Mina	myc induced nuclear antigen	1.5	162	241	221	232	1451042_a_at	AK013451
Edg2	endothelial differentiation, lysophosphatidic acid GPCR, 2	1.5	45	68	49	53	1448606_at	U70622
Psmb9	proteosome (prosome, macropain) subunit, beta type 9	1.5	762	1160	479	689	1450696_at	NM_013585
Bbx	bobby sox homolog (Drosophila)	1.5	165	248	93	96	1430820_a_at	BC024449

Table 4.12 (continued) IFN-induced genes in BMDCs after 6h but not 2h of culture

Gene	Description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Maff	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein F (avian)	2.4	221	92	401	186	1418936_at	BC022952
Rragd	Ras-related GTP binding D	2.3	95	42	78	67	1434909_at	BF462770
Galnt9	polypeptide N-acetylgalactosaminyltransferase 9	2.3	156	68	229	163	1434055_at	BB048942
Ing3	inhibitor of growth family, member 3	2.2	62	28	65	48	1422806_x_at	BB020556
Txnrd3	thioredoxin reductase 3	2.2	60	26	55	42	1423869_s_at	AF349659
Tcerg1	transcription elongation regulator 1 (CA150)	2.1	40	19	41	24	1450100_a_at	AW046403
H2afx	H2A histone family, member X	2.1	358	171	477	318	1416746_at	NM_010436
Fasn	fatty acid synthase	2.1	60	29	292	182	1423828_at	AF127033
Cyhr1	cysteine and histidine rich 1	2.0	37	18	68	46	1451153_a_at	BC025112
Epm2aip1	EPM2A (laforin) interacting protein 1	2.0	36	17	63	38	1434106_at	BB175879
Tgfbr1	transforming growth factor, beta receptor I	2.0	76	39	148	63	1420894_at	D25540
Sprr1	small proline rich-like 7	2.0	16	8	11	16	1420332_x_at	NM_027137
Smt3h1	SMT3 (supressor of mif two, 3) homolog 1 (S. cerevisiae)	2.0	41	21	37	20	1417033_at	AF296657
Eli	elongation factor RNA polymerase II	2.0	156	80	144	71	1460643_at	BC024894
Klhl2	kelch-like 2, Mayven (Drosophila)	2.0	33	17	63	64	1426978_at	AW682368
Zdhhc7	zinc finger, DHHC domain containing 7	1.9	15	8	38	38	1438876_at	BB667355
Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	1.9	211	110	173	125	1416961_at	NM_009773
Cerk	ceramide kinase	1.9	187	98	297	178	1434034_at	BI905090
Ampd3	AMP deaminase 3	1.9	210	110	447	216	1422573_at	D85596
Il6ra	interleukin 6 receptor, alpha	1.9	59	31	90	79	1452416_at	X53802
Mbp	myelin basic protein	1.9	87	45	67	61	1425263_a_at	L07508
Tfap4	transcription factor AP-4 (activating enhancer-binding protein 4)	1.9	24	13	18	16	1418167_at	NM_031182
Cdk4	cyclin-dependent kinase 4	1.9	218	117	229	192	1422440_at	NM_009870
Trim37	tripartite motif protein 37	1.9	94	51	95	78	1436393_a_at	BG065227
Hist2h2bb	histone 2, H2bb	1.8	39	21	46	31	1447854_s_at	AV127319
Cnr2	cannabinoid receptor 2 (macrophage)	1.8	78	43	90	49	1450476_at	NM_009924
Dapk1	death associated protein kinase 1	1.8	311	170	291	222	1427358_a_at	BC026671
Ube3a	ubiquitin protein ligase E3A	1.8	22	12	16	16	1445727_at	BB186188
Fcmd	Fukuyama type congenital muscular dystrophy homolog (human)	1.8	57	32	59	30	1435801_at	AV365405
Ssa2	Sjogren syndrome antigen A2	1.8	1	1	1	1	1436533_at	AV232359
Cd97	CD97 antigen	1.8	395	215	356	240	1418394_a_at	NM_011925
Poln	polymerase (DNA directed), eta (RAD 30 related)	1.8	22	12	33	19	1420988_at	AB037184
Gclc	glutamate-cysteine ligase, catalytic subunit	1.8	219	123	187	124	1424296_at	BC019374
Arhf	ras homolog gene family, member f (in filopodia)	1.8	15	8	14	11	1441510_at	BB157359
C76746	expressed sequence C76746	1.8	285	162	307	194	1428391_at	AK004767
Sca7	spinocerebellar ataxia 7 homolog (human)	1.8	42	24	41	45	1442186_at	BE945939
Zfp99	putative zinc finger protein 99 sequence	1.8	63	36	32	30	1452610_at	AK009842
Gle1l	GLE1 RNA export mediator-like (yeast	1.8	24	13	18	23	1440719_at	AV251087
Eno2	enolase 2, gamma neuronal	1.8	39	22	27	20	1418829_a_at	NM_013509
Tex292	testis expressed gene 292	1.8	165	94	240	173	1423884_at	BC027399
Skp2	S-phase kinase-associated protein 2 (p45)	1.8	30	17	23	23	1460247_a_at	NM_013787
Rora	RAR-related orphan receptor alpha	1.8	165	94	137	82	1420583_a_at	NM_013646
Ahr	aryl-hydrocarbon receptor	1.8	704	402	1181	891	1422631_at	NM_013464
Fbxo5	F-box only protein 5	1.8	71	40	98	62	1429499_at	AK011820
Tcfe2a	transcription factor E2a	1.7	71	41	89	64	1426297_at	AF352579
Ptov1	prostate tumor over expressed gene 1	1.7	66	38	34	46	1416945_at	NM_133949
Srd5a2l	steroid 5 alpha-reductase 2-like	1.7	127	73	196	199	1439241_x_at	BB825787
Akap8	A kinase (PRKA) anchor protein 8	1.7	129	74	182	132	1450983_at	BG069776
Plec1	plectin 1	1.7	24	14	32	21	1437554_at	BM232239
Gys3	glycogen synthase 3, brain	1.7	226	130	124	94	1416737_at	NM_008195
Mtss1	metastasis suppressor 1	1.7	670	390	1056	807	1434036_at	AV024771
Tardbp	TAR DNA binding protein	1.7	186	108	305	202	1423723_s_at	BC012873
Spata13	spermatogenesis associated 13	1.7	38	22	68	49	1434958_at	BG075163
GTP binding protein 3	GTP binding protein 3	1.7	46	27	33	18	1450980_at	BM207274
Sox7	SRY-box containing gene 7	1.7	41	24	36	25	1416564_at	NM_011446
Lrrc5	leucine-rich repeat-containing 5	1.7	298	175	329	202	1433506_at	BB315861
Fnbp1	formin binding protein 1	1.7	62	36	36	21	1451806_at	BC003867
Cdadc1	cytidine and dCMP deaminase domain containing 1	1.7	44	26	59	27	1439105_at	BE200391
Acvrl1	activin A receptor, type II-like 1	1.7	106	62	111	96	1435825_at	BG969012
Fgfr1op	Fgfr1 oncogene partner	1.7	176	104	219	115	1428919_at	BB667817
Orm1	orosomucoid 1	1.7	79	47	51	34	1451054_at	BE628912
Cdr2	cerebellar degeneration-related 2	1.7	51	30	57	38	1417430_at	NM_007672
Rad23a	RAD23a homolog	1.7	124	74	108	92	1417619_at	NM_026320
Pcm1	pericentriolar material 1	1.7	17	10	31	22	1418525_at	BG088656
Ormdl3	ORM1-like 3 (S. cerevisiae)	1.7	109	65	85	82	1419450_at	NM_025661
Galnt6	polypeptide N-acetylgalactosaminyltransferase 6	1.7	599	356	621	392	1434399_at	AV231866

Table 4.13 (continued over page) IFN-suppressed genes in BMDCs after 6h but not 2h of culture. Samples from CD11c$^+$ DCs cultured in vitro in the presence of 2 x 10^4 U IFN-α4 (IFN) or in media alone (0) were hybridised to Affymetrix murine 430 2.0 arrays. Data were generated from the mean of three experiments carried out on independently isolated DCs. Data were analysed in Genespring 7.2 and changes of more than 1.5 fold were determined to be significant by Welch's approximate t-test where p-values were less than or equal to 0.05. Normalised signal intensities were colour coded to indicate the level of expression, as shown in the key for table 4.2.

Gene	Description	Fold Change	6h Signal 0	6h Signal IFN	2h Signal 0	2h Signal IFN	Affymetrix Accession	Genbank Accession
Mki67ip	Mki67 (FHA domain) interacting nucleolar phosphoprotein	1.7	145	97	248	176	1424001_at	AY030275
Rps6ka2	ribosomal protein S6 kinase, polypeptide 2	1.7	50	35	25	17	1441311_at	BG063083
Lpin1	lipin 1	1.7	309	186	353	229	1426516_a_at	AK014526
Ercc1	excision repair cross-complementing rodent repair deficiency, group 1	1.7	62	37	78	56	1430138_at	AW987309
Osbpl3	oxysterol-binding protein-like 3	1.7	394	238	420	288	1428484_at	AK004768
Rnu22	RNA, U22 small nucleolar	1.6	205	124	217	135	1433675_at	BQ177137
Tnrc15	trinucleotide repeat containing 15	1.6	86	58	103	68	1451397_at	BC027137
Creb1	cAMP response element binding protein (Creb1)	1.6				15	1421583_at	AF448508
Sfrs2	splicing factor, arginine/serine-rich 2 (SC-35)	1.6	218	133	266	164	1452439_s_at	AF250135
Gas7	growth arrest specific 7	1.6	135	82	86	75	1417859_at	NM_008088
Ms4a1	membrane-spanning 4-domains, subfamily A, member 1	1.6					1450912_at	BB236617
Msh6	mutS homolog 6 (E. coli)	1.6	42	26	49	33	1416915_at	U42190
Syncrip	synaptotagmin binding, cytoplasmic RNA interacting protein	1.6	96	59	161	92	1450743_s_at	BG920261
Golgb1	golgi autoantigen, golgin subfamily b, macrogolgin 1	1.6	231	142	188	113	1435032_at	BM213299
Sh3glb1	SH3-domain GRB2-like B1 (endophilin)	1.6	62	38	64	67	1434916_at	AV377318
Hoxa1	homeo box A1	1.6	21	13	18	17	1420565_at	NM_010449
Pitpnb	phosphotidylinositol transfer protein, beta	1.6	91	56	96	49	1420307_a_at	BG975479
Uchrp	ubiquintin c-terminal hydrolase related polypeptide	1.6	63	39	112	121	1448230_at	NM_009462
Pdcd4	programmed cell death 4	1.6	112	69	98	69	1456393_at	AI642124
Dusp19	dual specificity phosphatase 19	1.6	37	23	55	39	1426447_at	AK009187
Slc6a12	solute carrier family 6 (neurotransmitter transporter, betaine/GABA),12	1.6	165	102	167	89	1449382_at	NM_133661
Nudt6	nudix (nucleoside diphosphate linked moiety X)-type motif 6	1.6	22	13	25	20	1424510_at	BC027267
Jtv1	JTV1 gene	1.6	192	120	228	162	1424151_at	BC026972
Mrps18b	mitochondrial ribosomal protein S18B	1.6	142	88	187	137	1451164_a_at	BC021752
Map4k4	mitogen-activated protein kinase kinase kinase kinase 4	1.6	235	146	189	170	1434184_s_at	BQ175905
Dnajb10	DnaJ (Hsp40) homolog, subfamily B, member 10	1.6	74	47	73	39	1448657_a_at	NM_020266
Lpin1	lipin 1	1.6	199	125	277	166	1418288_at	NM_015763
Kpnb1	karyopherin (importin) beta 1	1.6	201	126	251	176	1416925_at	NM_008379
Siva	Cd27 binding protein (Hindu God of destruction)	1.6	88	54	91	64	1418377_a_at	NM_013929
Rai14	retinoic acid induced 14	1.6	192	121	217	167	1417400_at	AF274866
Abce1	ATP-binding cassette, sub-family E (OABP), member 1	1.6	418	264	592	445	1416015_s_at	NM_015751
Erdr1	erythroid differentiation regulator 1	1.6	501	317	684	600	1427820_at	BC021831
Pdxk	pyridoxal (pyridoxine, vitamin B6) kinase	1.6	57	36	23	29	1427930_at	BG063905
Syncrip	synaptotagmin binding, cytoplasmic RNA interacting protein	1.6	153	98	234	188	1422769_at	BG920261
Map4k4	mitogen-activated protein kinase kinase kinase kinase 4	1.6	88	56	75	57	1422615_at	NM_008696
Me2	malic enzyme 2, NAD(+)-dependent, mitochondrial	1.6	163	104	135	93	1426572_at	BF730769
Hs1bp3	HS1 binding protein 3	1.6	107	68	106	109	1433843_at	AW541327
Ibsp	integrin binding sialoprotein	1.6	19	12	15	16	1417485_at	L20232
Solh	small optic lobes homolog (Drosophila)	1.6	776	499	662	399	1434416_a_at	BB022975
zinc finger protein s11-6	zinc finger protein s11-6	1.6	62	40	106	64	1434355_at	BB159201
Tbrg4	transforming growth factor beta regulated gene 4	1.6	53	34	92	75	1448796_s_at	BB768873
Rassf3	Ras association (RalGDS/AF-6) domain family 3	1.6	292	188	214	177	1448546_at	BC011511
Mtss1	metastasis suppressor 1	1.6	344	222	529	338	1424826_s_at	AA250031
Solh	small optic lobes homolog (Drosophila)	1.5	322	209	287	172	1434417_at	BB022975
Ccnf	cyclin F	1.5	93	60	136	86	1422513_at	NM_007634
Acpp	acid phosphatase, prostate	1.5	577	375	510	372	1441975_at	BB008092
Sec14l1	SEC14-like 1 (S. cerevisiae)	1.5	82	54	67	53	1451908_a_at	BC006766
Thrap3	thyroid hormone receptor associated protein 3	1.5	59	38	67	54	1452125_at	BG075035
additional sex combs like	additional sex combs like 1	1.5	137	89	203	143	1435077_at	BE956516
Il4i1	interleukin 4 induced 1	1.5	266	173	348	216	1438917_x_at	AW240611
Atf4	activating transcription factor 4	1.5	840	548	1210	737	1448135_at	M94067
Arhgef16	Rho guanine nucleotide exchange factor (GEF) 16	1.5	188	123	265	215	1438178_x_at	BB368056
integrin beta 3	integrin beta 3	1.5	98	63	65	65	1455257_at	AV352983
Acpp	acid phosphatase, prostate	1.5	340	223	207	143	1453943_a_at	AK020693
Agpat4	1-acylglycerol-3-phosphate O-acyltransferase 1	1.5	574	378	533	376	1436640_x_at	BE994529
Tef	thyrotroph embryonic factor	1.5	283	186	250	195	1424175_at	BC017689
Rassf3	Ras association (RalGDS/AF-6) domain family 3	1.5	35	24	36	25	1417015_at	NM_138956
Dgkd	diacylglycerol kinase, delta	1.5	142	94	159	113	1433564_at	BB422487
Ccnb1	cyclin B1	1.5	151	99	219	164	1418076_at	X58708
Mafg	v-maf musculoaponeurotic fibrosarcoma oncogene family, protein G (avian)	1.5	314	207	418	273	1448916_at	BC002092
Unc119	unc-119 homolog (C. elegans)	1.5	312	206	351	273	1418123_at	BC001990
Cdca3	cell division cycle associated 3	1.5	217	145	241	180	1452040_a_at	BI081061
Zrf2	zuotin related factor 2	1.5	272	181	313	240	1417657_s_at	NM_009584
Txnl	thioredoxin-like	1.5	22	15	22	25	1437906_x_at	AV106191
Hps4	Hermansky-Pudlak syndrome 4 homolog (human)	1.5	147	99	181	121	1448629_at	NM_138646
Itga10	integrin, alpha 10	1.5	24	16	29	30	1440235_at	BB151146

Table 4.13 (continued) IFN-suppressed genes in BMDCs after 6h but not 2h of culture

chain. Three members of the membrane-spanning 4-domain family of proteins, two of which whose expression we had investigated by real-time PCR in splenic DCs, were also induced by IFN-α in BMDCs.

A large number of genes (376) were induced by IFN-I at the 6h timepoint but not the 2h timepoint (Table 4.12). This was the only condition in which IL-12p40 mRNA was detected as IFN-induced, and was expressed at relatively low levels. Similarly this is the only condition where CD86 was significantly upregulated by IFN-α4. In addition to detecting dramatic enhancement of CCL2 (MCP-1) and CCL3 (MIP-1α), known be induced during DC maturation (Sallusto *et al.*, 1999b), we detected a gene named chemokine-like factor super family 6 and a putative cytokine receptor named cytokine receptor-like factor 3 which may merit further investigation. Genes which were suppressed at 6h but not at 2h are shown in table 4.13.

It should be noted that, several of the genes listed were also upregulated at the 2h timepoint, for example Pnp which was discussed earlier. This is because differential expression has been detected by different probe sets. Therefore they have not been grouped together in the list containing genes differentially regulated at both 2h and 6h.

4.2.3 Comparison of gene expression in splenic DCs and BMDCs

4.2.3.1 Merging and clustering of splenic DC and BMDC data

Since we had used different versions of microarrays to analyse gene expression in splenic DCs (sDCs) and BMDCs, it was impossible to do a direct comparison using probe set identification numbers. Therefore, in order to make comparisons between the two data sets it was necessary to make an independent analysis. The data was normalised in Genespring as described previously. Following this, the entire data sets were exported and the sDC and

BMDC data sets merged using data comparison files provided by the array manufacturer (Affymetrix). We only used the data present in the "best match" comparison files, which are matched based on a stringent set of parameters that includes the percentage identity between the representative sequences and the amount of overlap between the target sequences chosen for probe selection. Where probe sets existed in the 430 2.0 array but did not match to probe sets in the U74Av2 array, or vice versa, the data was retained for separate analysis.

The data were then filtered on the following criteria: the change in expression for a gene must be greater 1.5 fold in at least one of the conditions, and have an associated p-value of less than 0.05 in the paired t-test. In addition, the gene must be detected as present (i.e. being expressed) in all three replicates in the condition displaying higher expression. Following this, the data were visualised using the TIGR multiple-experiment viewer (TMEV)(Saeed *et al.*, 2003). The data were clustered using a hierarchical method, which was done purely for visualisation purposes and was not intended to infer functional relationships within groups of genes.

More than 1000 genes were differentially expressed in at least one of the conditions tested (figure 4.5a). Of these, more than half of those found in the BMDCs (430 2.0 array) had no corresponding probe set for the sDC data (U74Av2 array), which was expected. However 437 genes whose expression changed more than 1.5 fold were represented on both arrays (Figure 4.5b). Of these, 17 genes were enhanced by IFN-α in all four conditions, which included the usual suspects: CXCL9, CXCL10, Tyki, Mx1 and known IFN-induced genes. 48 genes were enhanced in at least three of the conditions. However, many of the induced genes were detected in only one of the conditions. Of the suppressed genes, only one was common to both sDCs and BMDCs, which was CXCR4.

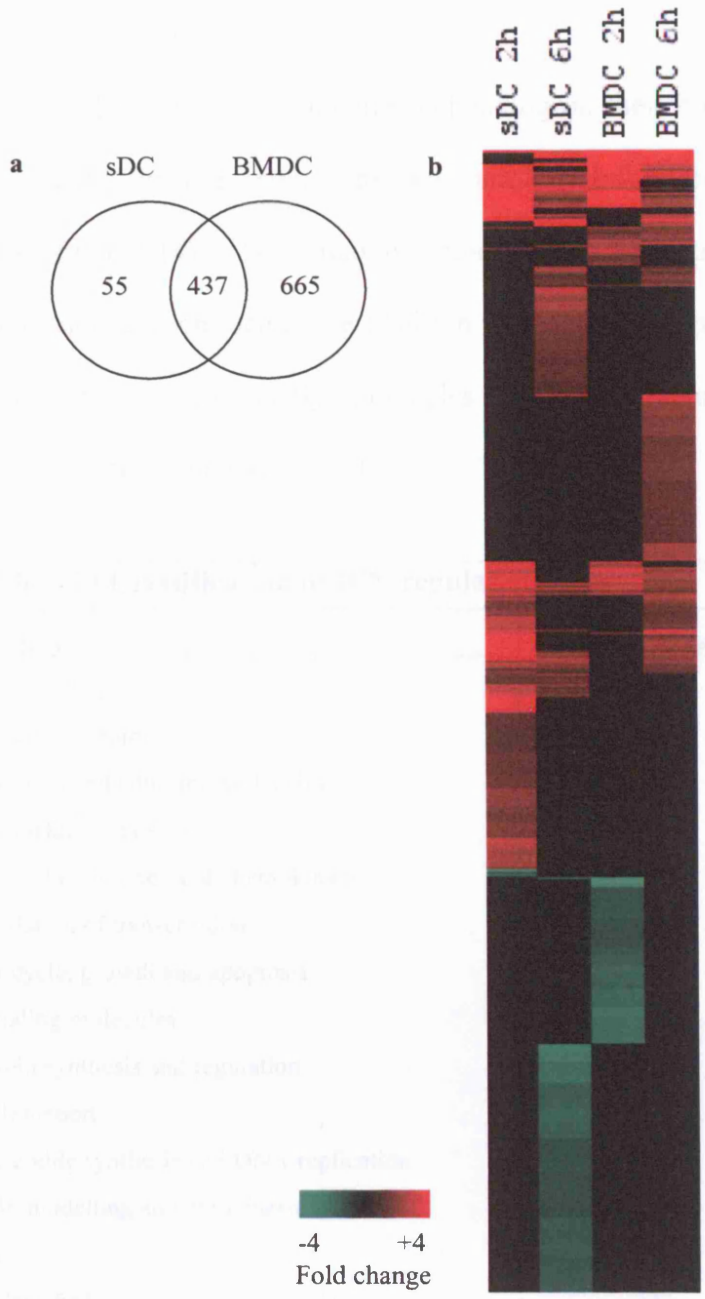


Figure 4.5 Comparison of genes regulated by IFN-α4 in sDCs and BMDCs. Data were normalised in Genespring 7.2, and were generated from the mean of three experiments carried out on independently isolated DCs. sDC and BMDC data sets were exported and merged and genes which did not change in expression by at least 1.5 fold with an associated t-test p-values of less than or equal to 0.05 in at least one condition were filtered out. a) Comparison of probe sets (where IFN-regulation was detected in at least one condition) from sDC and BMDC data sets. b) Data was clustered using TMEV. Change in expression level is indicated by green colouring (suppressed) or red colouring (induced).

4.2.3.2 Functional classification

The genes were classified into various categories depending on their biological function or the biological or molecular function inferred by gene ontology information available on the Affymetrix database (Table 4.14). There may be some overlap between the categories, for instance many of the transcription factors are likely to play roles in regulation of cell cycle and apoptosis, such as Pml. Additionally, molecules involved in signal transduction may also act as transcription factors, such as STAT1.

Table 4.14 Classification of IFN-regulated genes

Category	Number of genes
Classical ISGs	41
Cytokine receptors	10
Migration and adhesion molecules	23
Cell surface expressed	77
Secreted (cytokines and chemokines)	21
Regulation of transcription	106
Cell cycle, growth and apoptosis	69
Signaling molecules	75
Protein synthesis and regulation	66
Ion transport	33
Nucleotide synthesis and DNA replication	40
Actin modelling and cytokinesis	15
ESTs	279
Unclassified	332

4.2.3.2.1 Classical ISGs

As would be expected, many known IFN-induced genes were identified in both the sDCs and the BMDCs, and included several anti-viral genes (Figure 4.5c). In general, these genes were most strongly induced at the 2h timepoint, but it should be noted that the transcription level of these genes in the 6h untreated controls was high. It is possible that these genes are being induced by endogenous IFN-I produced by the DCs. A group of genes was induced at

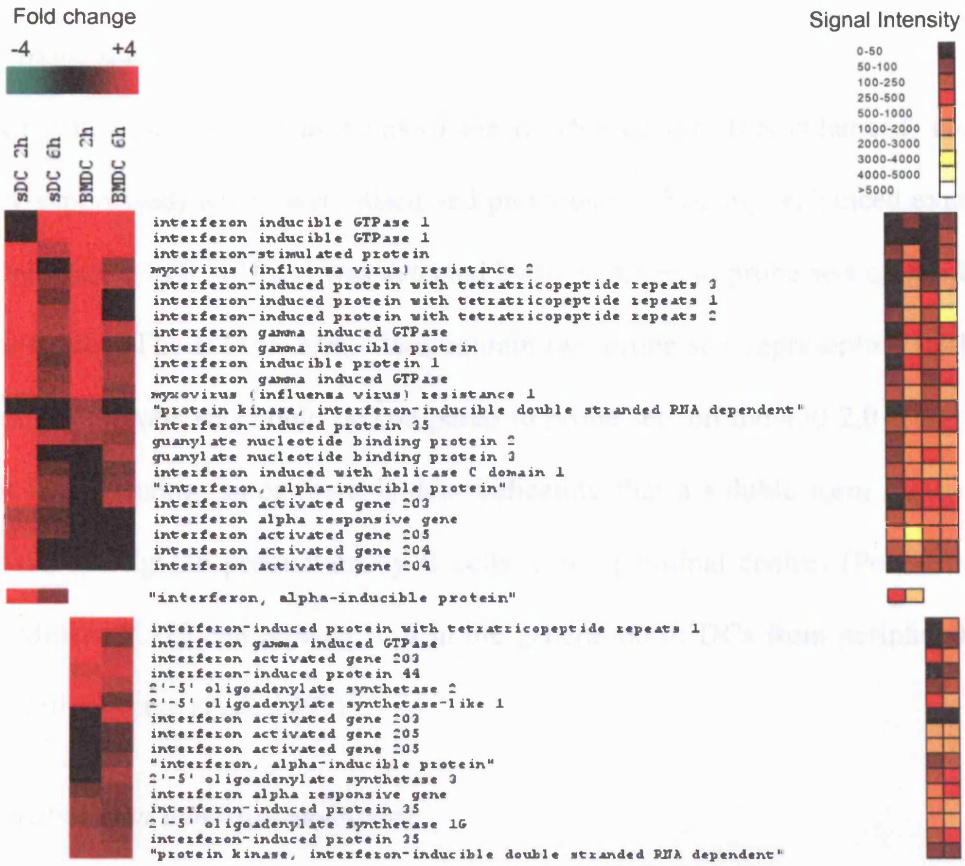


Figure 4.5c Comparison of expression of classical ISGs in sDCs and BMDCs. Data were normalised in Genespring 7.2, and were generated from the mean of three experiments carried out on independently isolated DCs. sDC and BMDC data sets were exported and merged, and filtered to include only genes whose expression changed by at least 1.5 fold ($p=<0.05$) in at least one condition. Data was clustered using TMEV. Change in expression level is indicated by green colouring (suppressed) or red colouring (induced). Signal intensities for the control samples were colour coded to indicate the level of expression, as shown in the key.

2h in sDCs but not BMDCs. Again, mRNA expression was high in the BMDC untreated controls where enhanced gene expression was not detected.

4.2.3.2.2 Cytokine receptors

This group of genes includes the α-chains of the IL-15 receptor (IFN-enhanced) and IL-6 receptor (IFN-suppressed) which were discussed previously. Notably, enhanced expression of the IL-13 receptor α1 (IL-13Rα1) was detected by three different probe sets on the 430 2.0 array (Figure 4.5d). The U74Av array does contain two probe sets representing IL-13Rα1, but neither of these probe sets could be compared to probe sets on the 430 2.0 array. This enhancement is interesting, since there is data indicating that a soluble form of IL-13Rα1 promotes IgG2a and IgG2b production by B cells in the germinal centres (Poudrier *et al.*, 1999). In addition, IL-13 can replace IL-4 in the generation of DCs from peripheral blood mononuclear cells (Morse *et al.*, 1999).

4.2.3.2.3 Migration and adhesion molecules

In this group of genes whose function is associated with cell migration and adhesion, none were enhanced in both splenic and BMDC data sets (Figure 4.5e). In sDCs there was enhanced expression of both CCR5 and CCR7 at 2h of IFN-stimulation. Upregulation of CCR7 is expected, since maturing DCs need to express this receptor in order to migrate to the LN. CCR5 upregulation was unexpected however, since it has previously been shown that after LPS-maturation of human DCs, CCR5 mRNA levels remained stable whilst cell surface expression was lost (Sallusto *et al.*, 1998c). Also unexpected was the downregulation of CXCR4, which is again in contrast to results shown in human LPS-matured DCs which strongly upregulate CXCR4 mRNA (Sallusto *et al.*, 1998c). However, the upregulation of CXCR4 mRNA was not reflected in expression on the cell surface, which was not detectable in mature DCs.

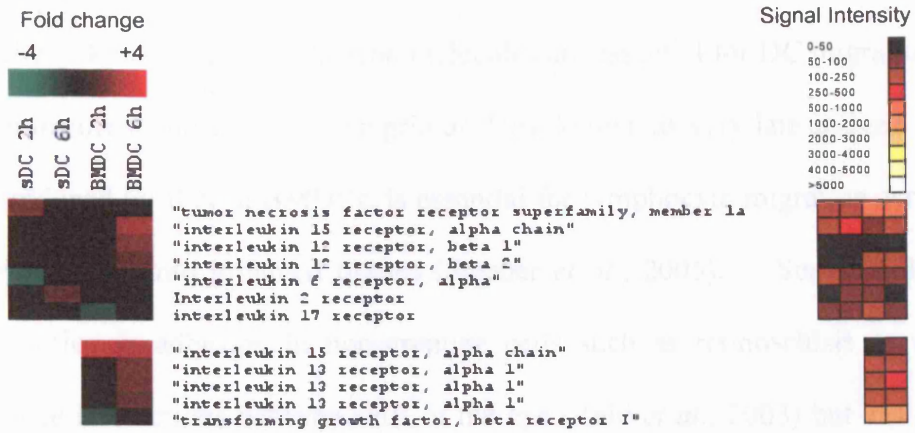


Figure 4.5d Comparison of IFN-α4 regulated expression of cytokine receptor genes in sDCs and BMDCs Legend as in figure 4.2.3.1c

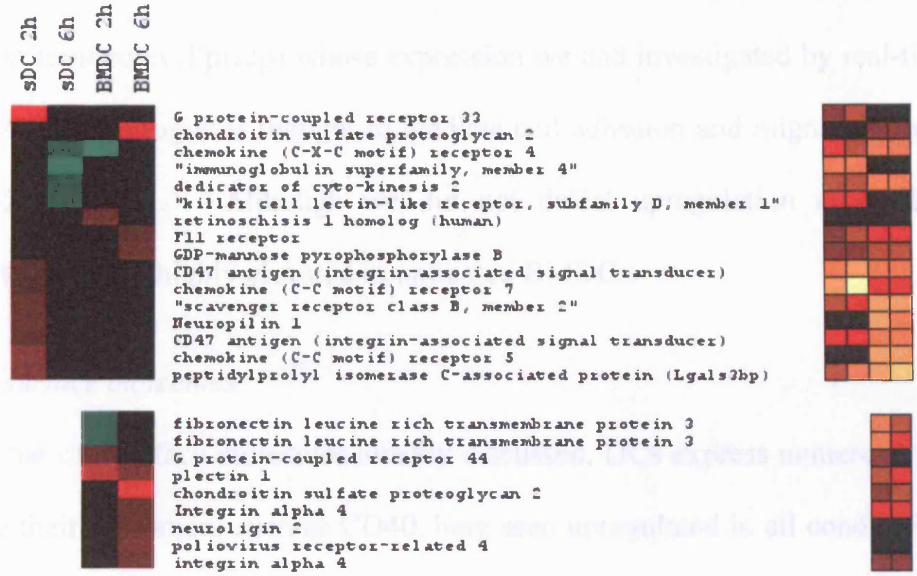


Figure 4.5e Comparison of IFN-α4 regulated expression of genes involved in cell adhesion and migration in sDCs and BMDCs Legend as in figure 4.2.3.1c

In addition to chemokine receptors, adhesion molecules are essential for DC migration. One cell adhesion molecule identified here, Integrin α4 (also known as very late antigen 4, VLA-4), which was induced by IFN in BMDCs, is essential for lymphocyte migration across high endothelial venules and into inflamed tissues (Steeber *et al.*, 2005). Some of the genes identified have roles in adhesion in non-immune cells such as retinoschisis 1, which is thought to mediate interactions between cells of the eye (Reid *et al.*, 2003) but has not been implicated in DC function.

Another gene upregulated by IFN in sDCs but not BMDCs included peptidylprolyl isomerase C-associated protein/lectin (Ppicap) whose expression we had investigated by real-time PCR and whose human homologue is thought to mediate cell adhesion and migration through its ligand MAC-2/galectin-3. Although we did not detect upregulation in BMDCs, the expression of this gene is highly elevated in untreated BMDCs.

4.2.3.2.3 Cell surface molecules

In addition to the cell surface molecules already discussed, DCs express numerous receptors which mediate their activation, such as CD40, here seen upregulated in all conditions except after 2h in BMDCs (Figure 4.5f). Several TLRs were enhanced by IFN-I in BMDCs: TLR3, TLR7, and TLR8. TLR3 recognises dsRNA, whilst TLR7 and TLR8 recognise viral ssRNA (Takeda *et al.*, 2005). Therefore, this may represent a mechanism which allows DCs to respond correctly to the pathogen, since the presence of type-I IFNs indicate viral infection. None of the probe sets for these TLRs are directly comparable to probe sets on the U74Av array; indeed only TLR3 is represented at all on these arrays.

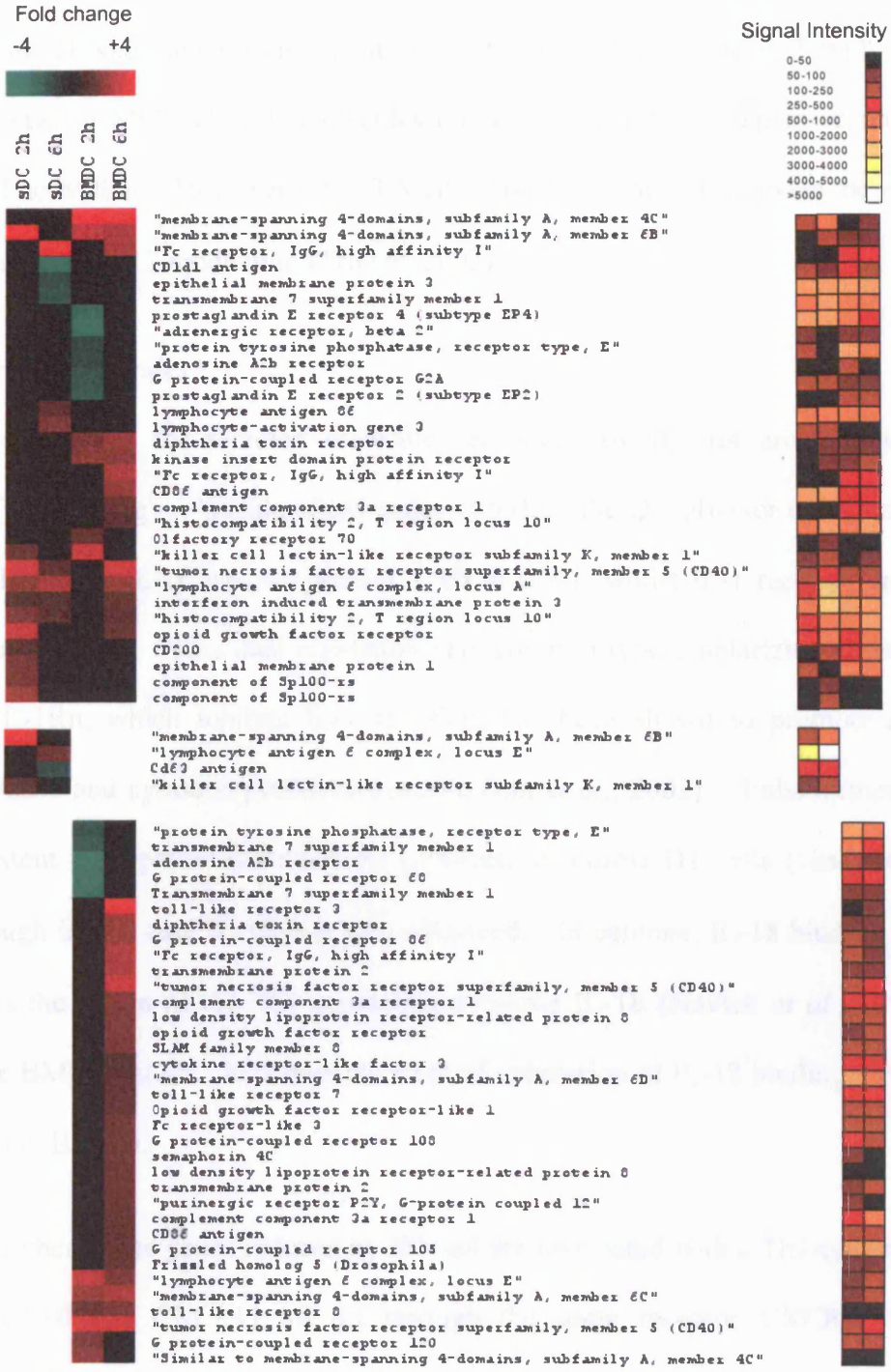


Figure 4.5f Comparison of IFN-α4 regulated expression of genes encoding cell surface molecules in sDCs and BMDCs Legend as in figure 4.2.3.1c

Another cell surface molecule, lymphocyte activation gene 3 (LAG-3) which was induced only in splenic DCs at the 6h timepoint, is more commonly associated with T cells. Interactions between MHC class II molecules on APCs and LAG-3 expressed on T cells inhibits T cell activation. However LAG-3-MHC class II interactions activate the APC and stimulate IFN-γ and IL-12 production (Triebel, 2003).

4.2.3.2.4 Secreted molecules

The majority of genes for secreted molecules enhanced by IFN-α4 are cytokines and chemokines (Figure 4.5g). We identified genes encoding the DC effector cytokines IL-15, IL-6 and IL-12p40. IL-1β was suppressed at 6h in sDCs, whilst IL-1 receptor antagonist (IL-1Rn) was enhanced. This dual regulation may confer a type-1 polarizing signal on the DCs, since IL-1Rn, which inhibits IL-1 function, has been shown to promote a type-1 antibody response and cytokine production profile (Lin *et al.*, 2002). Enhancement of IL-1Rn is consistent with previous studies on LPS-treated murine D1 cells (Granucci *et al.*, 2001b), although in this case IL-1β was also enhanced. In contrast, IL-18 binding protein , which inhibits the action of the Th1 promoting cytokine IL-18 (Novick *et al.*, 1999), was induced in the BMDCs at 6h. However the level of expression of IL-18 binding protein was low compared to IL-1Rn.

In general, the chemokine genes induced by IFN-α4 are associated with a Th1-type response. CXCL9, CXCL10 and CXCL11 all act through the same receptor CXCR3, which is primarily expressed by activated Th1 cells (Bonecchi *et al.*, 1998; Sallusto *et al.*, 1998b). CCL4 and CCL5, enhanced in sDCs at 6h, are also thought to have Th1 inducing properties, although CCL2, which was induced in BMDCs at 6h, promotes Th2 effector cell development (Luther *et al.*, 2001). sDCs also strongly suppressed CCL22 expression in response to IFN-α at the 6h timepoint. This chemokine directs the migration of Th2 cells via

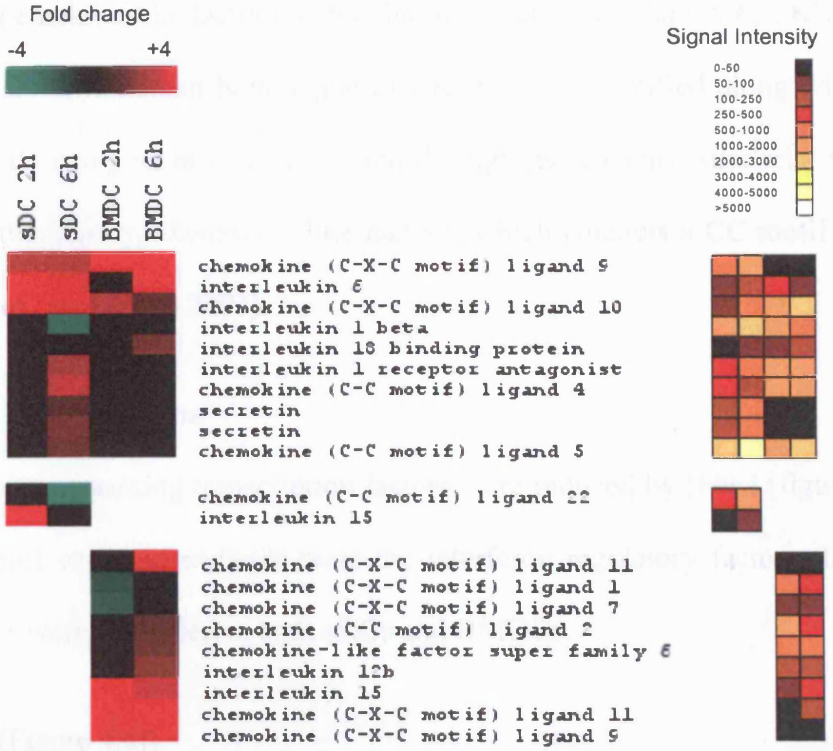


Figure 4.5g Comparison of IFN-α4 regulated expression of genes encoding secreted molecules in sDCs and BMDCs Legend as in figure 4.2.3.1c

CCR4 (Zlotnik *et al.*, 2000). Both CCL1 and CCL7 were suppressed by BMDCs at 6h. CCL1 and CCL7 are agonists for CCR8 and CCR3 respectively, which are also expressed on Th2 cells (Sallusto *et al.*, 1998a; Colantonio *et al.*, 2002).

A gene encoding a chemokine-like factor (chemokine-like factor superfamily 6, CKLF6) was identified in BMDCs. The human homologue of CKLF6 was identified along with other members of this family using an *in silico* approach through its sequence similarity with the original member of this family, chemokine-like factor1, which contains a CC motif and has chemotactic properties (Han *et al.*, 2003).

4.2.3.2.5 Regulation of transcription

A large number of genes encoding transcription factors were induced by IFN-I (figure 4.5h). Among these, the most easily identifiable were the interferon regulatory factors (IRFs), of which IRF7 and IRF1 were identified in both sDCs and BMDCs.

4.2.3.2.6 Signaling (Figure 4.5i)

Few genes involved in cell signaling were commonly regulated in sDCs and BMDCs. One of these, STAT1 (signal transducer and activator of transcription 1) mediates signaling from the IFN-I receptor which involves its dimerisation with STAT2 (induced in BMDCs). Members of the SOCS (suppressor of cytokine signaling) family of proteins, were also induced by IFN-I. Whereas SOCS-1 was induced in both DC types, SOCS-3 upregulation was only detected in sDCs. Both SOCS-1 and SOCS-3 are known to be induced by many types of stimuli including interleukins, IFN-α and IFN-γ in addition to TLR ligands LPS and CpG DNA (Fujimoto *et al.*, 2003). SOCS-1 and -3 regulate complex negative-feedback mechanisms since they can inhibit multiple cytokine receptor signal transduction pathways.

Figure 4.5h Comparison of IFN-α4 regulated expression of genes involved in transcription regulation in sDCs and BMDCs Legend as in figure 4.2.3.1c

Figure 4.5i Comparison of IFN-α4 regulated expression of genes encoding signaling molecules in sDCs and BMDCs Legend as in figure 4.2.3.1c

4.2.3.2.7 Cell growth/cell cycle/apoptosis

Many genes with putative roles in either cell cycle, differentiation or cell death were differentially expressed (figure 4.5j). A number of genes were regulated similarly in sDCs and BMDCs. These included Fas death domain associated protein (Daxx), a signaling molecule associated with the Fas receptor (Yang *et al.*, 1997). IFN-α enhances the expression and nuclear translocation of Daxx where it associates with nuclear bodies and induces cell cycle arrest and apoptosis (Gongora *et al.*, 2001). As previously discussed, Pml, whose expression was also induced by IFN is an essential component of nuclear bodies and also mediates apoptosis.

Lectin, galactose binding, soluble 9/ Galectin-9 (Lgals9/Gal-9) was induced after 6h in both types of DC. Lgals9 functions as a proapoptotic factor for many cell types including thymocytes and activated T cells (Wada *et al.*, 1997; Kashio *et al.*, 2003). In addition Lgals9 acts as a chemotactic factor for eosinophils (Hirashima, 1999).

4.2.3.2.8 Protein synthesis and regulation

This group includes genes with a range of functions in the regulation of proteins including their translation, such as ribosomal proteins, folding (chaperonin subunits) and their transport, for example sorting nexin 2 (figure 4.5k). Several genes in this group are involved in protein ubiquitination or encode proteases which act on ubiquitinated proteins. A known IFN- stimulated gene, ubiquitin specific protease 18 (Usp18), which was induced by IFN at both timepoints and DC type, regulates signaling through the JAK-STAT pathway. This gene was also identified by RDA. It acts by unconjugating the IFN-stimulated protein ISG15, a ubiquitin-like protein, from other intracellular proteins. Since cells lacking Usp18 are hypersensitive to type I IFN, it is thought to act as a negative regulator of IFN-signaling (Malakhova *et al.*, 2003).

Figure 4.5j Comparison of IFN-α4 regulated expression of genes encoding molecules involved in cell cycle, growth and apoptosis in sDCs and BMDCs Legend as in figure 4.2.3.1c

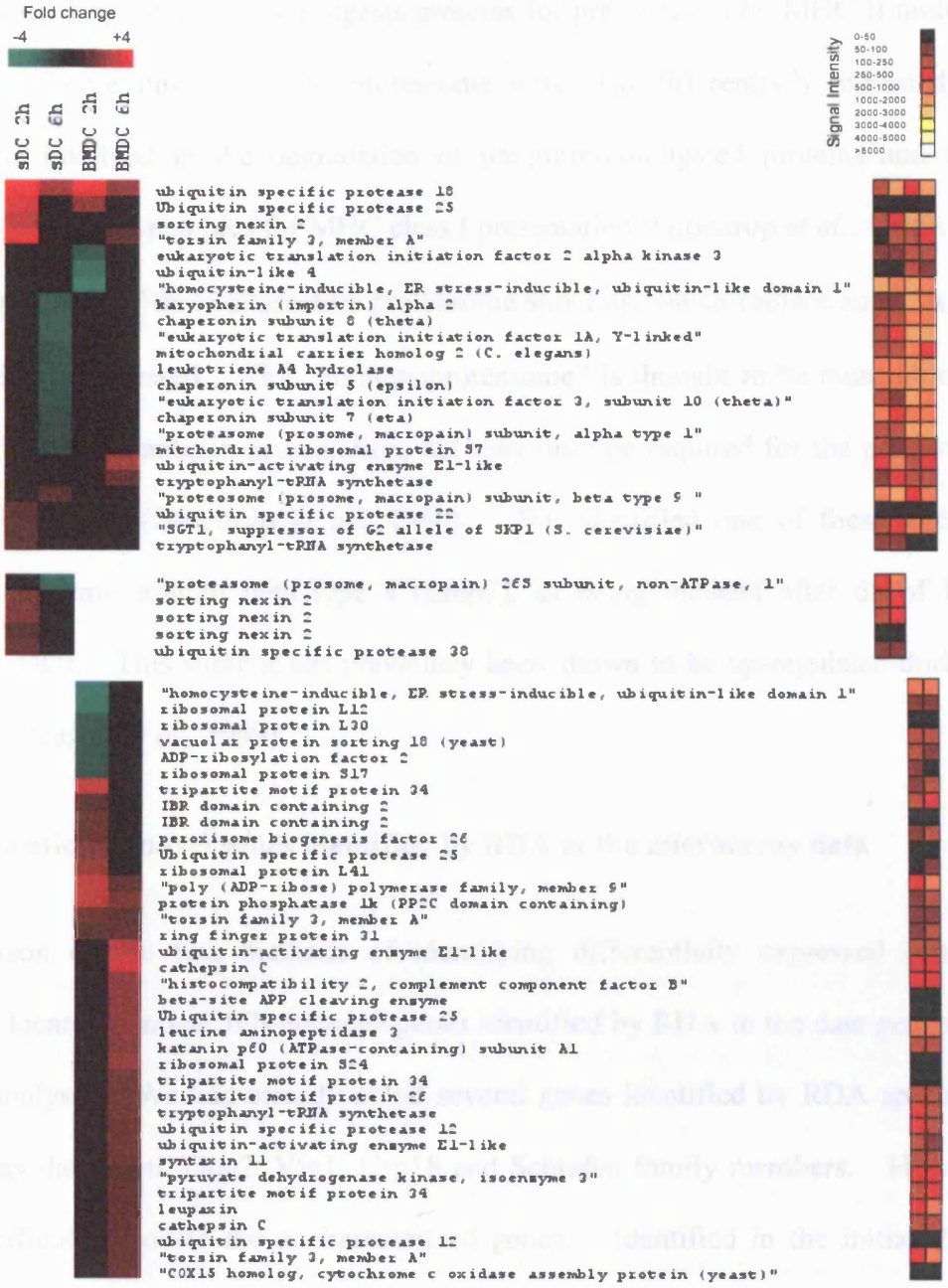


Figure 4.5k Comparison of IFN-*α*4 regulated expression of genes encoding molecules involved in protein regulation in sDCs and BMDCs
Legend as in figure 4.2.3.1c

We also identified a protease which digests proteins for presentation by MHC II molecules, cathepsin C. Three subunits of the proteasome were also differentially regulated. The proteosome is involved in the degradation of ubiquitin-conjugated proteins and is also important in generating peptides for MHC class I presentation (Groettrup *et al.*, 1996). IFN-γ induces expression of three alternative proteasome subunits, which replace subunits which are constitutively expressed. This "immunoproteasome" is thought to be more efficient at supplying peptides for antigen presentation, but may only be required for the processing of certain viral antigens (Groettrup *et al.*, 1996). We identified one of these alternative subunits, proteasome subunit beta type 9 (Lmp2), as being induced after 6h of IFN-α4 treatment in sDCs. This subunit has previously been shown to be up-regulated during DC maturation (Macagno *et al.*, 1999).

4.2.4 Identification of novel genes identified by RDA in the microarray data

For comparison of the two methods of identifying differentially expressed genes, we attempted to locate the novel IFN-induced genes identified by RDA in the data generated by microarray analysis. We had already noted several genes identified by RDA appearing in the microarray data: Pml, Gbp2, Vig1, Usp18 and Schlafen family members. However, it was more difficult to locate the uncharacterised genes. Identified in the initial RDA on $CD11c^+$ DCs, the gene with NCBI accession NM_175026 was enhanced 8-fold in sDCs at 2h in the microarray data and was also enhanced in BMDCs at both 2h and 6h. Another gene of unknown function identified by RDA in DCs at 6h of IFN-I stimulation, known as epididimal secretory protein (Table 3.4), was of interest due to a domain present in proteins which bind TLRs. Reflecting the RDA, this gene's expression was not found to be enhanced by IFN-I in sDCs after 2h, but was enhanced 1.5 fold at 6h (Table 4.5, NCBI Accession AB021289, this gene is also known as Niemann Pick type C2). Other genes identified by RDA were not

detected in the microarray analysis due to either failure to pass statistical criteria or because they were not represented on the U74Av2 arrays. Although all the genes were represented on the Murine 430 2.0 arrays, these data were from BMDCs and did not appear to correspond to data from the RDA which had been derived from sDCs. Again, this highlights the apparent difference in the responses of sDCs and BMDCs to IFN-I.

4.2.5 Analysis of cytokines released by in vitro cultured DCs

Expression of mRNA for several cytokines was differentially regulated by IFN-α4 (Figure 4.6). To test whether regulation of these genes at the mRNA level was reflected at the level of protein expression we measured the levels of these cytokines produced by *in vitro* cultured DCs. Supernatants from splenic DCs or BMDCs cultured for 2h – 24h in media alone or containing 2 x10^4 units/ml IFN-α4, were assayed for the presence of cytokines by ELISA.

In general, regulation of cytokines secreted by DCs in response to IFN-α4 correlated with the mRNA expression data. Overall, the levels of cytokines produced by the BMDCs were higher than from splenic DCs (Figure 4.6). This could be explained by the cell size; since BMDCs are larger than splenic DCs they may have a higher capacity for cytokine production. However, this is unlikely to account for the large difference seen for some cytokines (CXCL10), or for the two cases where expression could not be detected in splenic DCs (CCL2 and CCL12).

Lower levels of IL-1β mRNA were detected after 6h IFN treatment of splenic DCs compared to untreated controls. At the same time, expression of IL-1 receptor agonist (IL-1Rn) mRNA was upregulated. We could not detect any change in the level of secreted IL-1β, although its function may be blocked by increased levels of IL-1Rn. Due to reagent unavailability we could not test the levels of IL-1Rn by ELISA. IL-6 expression was

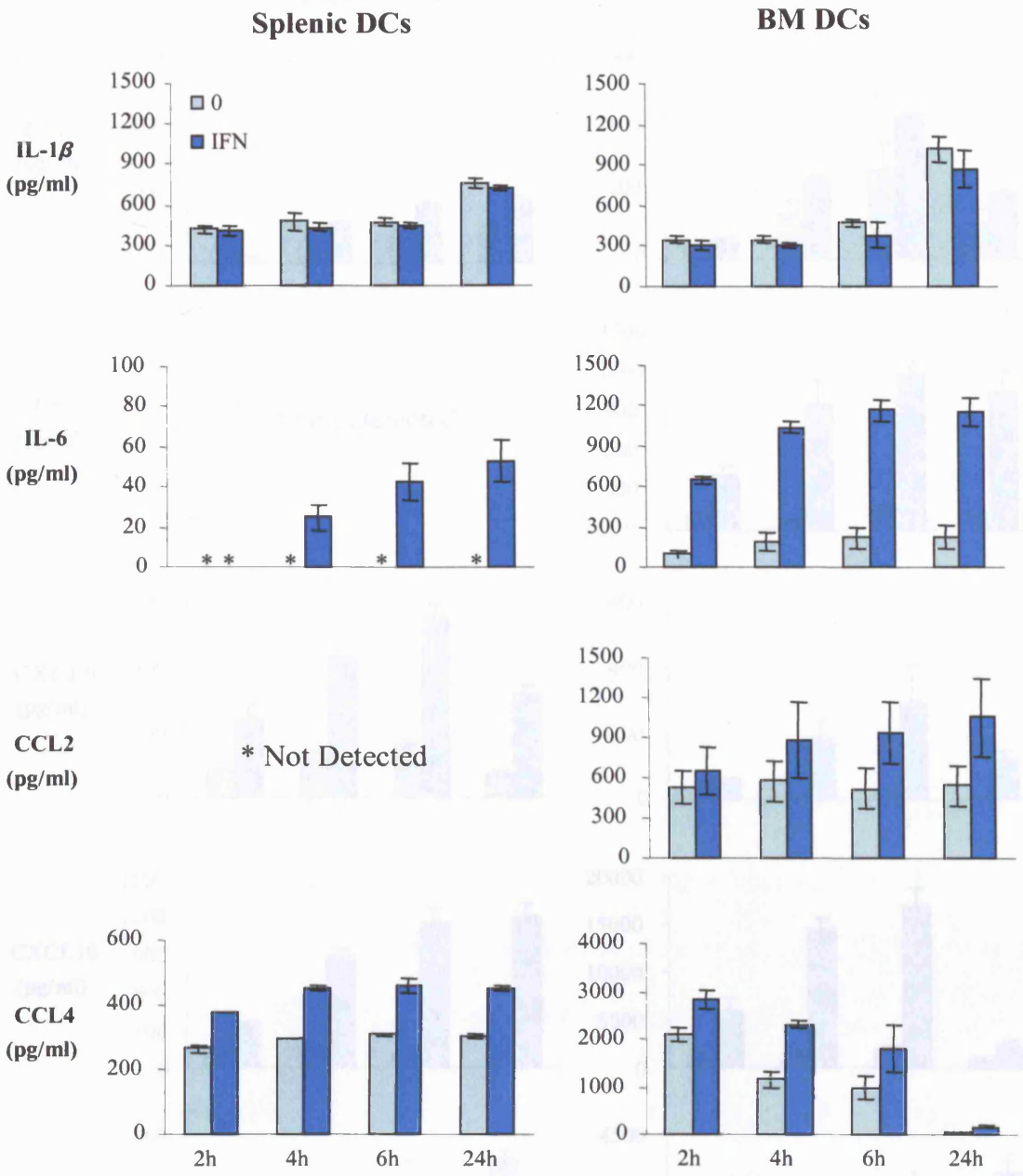


Figure 4.6 (continued over page) Secretion of cytokines by DCs
Splenic DCs (>90% CD11c$^+$) or BMDCs (> 93% CD11c$^+$) were cultured in media alone (□ 0) or with 2 x 10^4 units/ml IFN-α4 (■ IFN). Splenic DCs were cultured at 3.5 x10^6 cells/ml, and BMDCs 4 x 10^6/ml. At the indicated time points, aliquots of the cultures were removed and cytokines in the supernatants detected by ELISA. Values are the mean of two experiments, error bars indicating the standard error. * Indicates that cytokines were not detected.

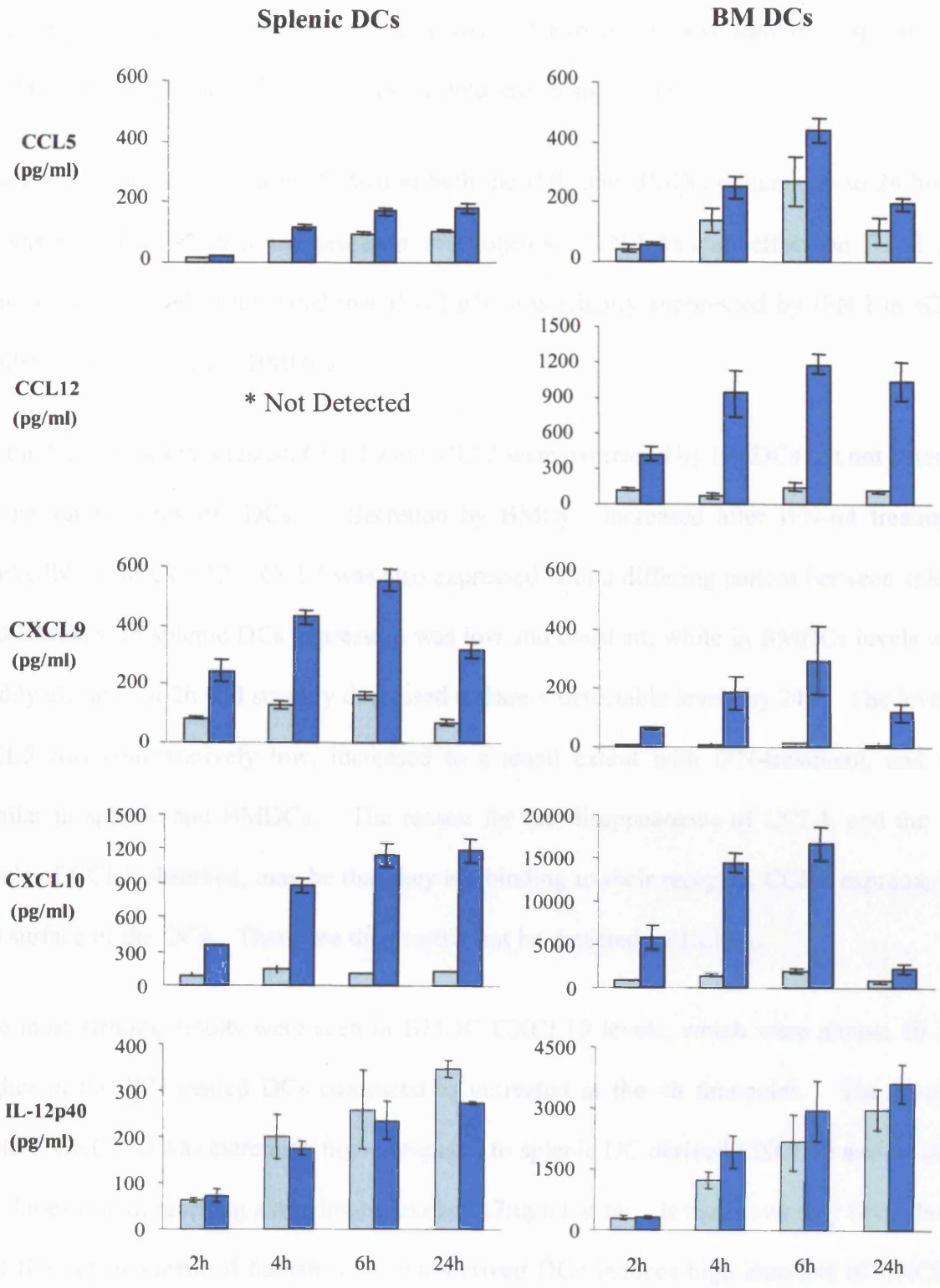


Figure 4.6 (continued) Secretion of cytokines by DCs

markedly enhanced by IFN-α4 and the pattern of expression was similar in splenic and BMDCs. However, levels were at least 20-fold less in splenic DCs.

IL-12 p40 levels steadily accumulated in both the sDC and BMDC cultures up to 24 hours. It was not clear whether the presence of exogenous IFN-I had an effect on IL-12 p40 expression, although it appeared that IL-12 p40 was slightly suppressed by IFN-I in sDCs, whilst being enhanced in BMDCs.

Of the CC-chemokines tested, CCL2 and CCL12 were expressed by BMDCs but not detected in the supernatants of sDCs. Secretion by BMDCs increased after IFN-α4 treatment, markedly so for CCL12. CCL4 was also expressed with a differing pattern between splenic and BMDCs: in splenic DCs expression was low and constant, while in BMDCs levels were highly elevated at 2h and steadily decreased to barely detectable levels by 24h. The level of CCL5 was comparatively low, increased to a small extent with IFN-treatment, and was similar in splenic and BMDCs. The reason for the disappearance of CCL4, and the low levels of CCL5 observed, may be that they are binding to their receptor, CCR5 expressed on the surface of the DCs. Therefore they would not be detected by ELISA.

The most striking results were seen in BMDC CXCL10 levels, which were almost 10-fold higher in the IFN-treated DCs compared to untreated at the 4h timepoint. The level of BMDC CXCL10 was extremely high compared to splenic DC derived CXCL10 and all other cytokines tested, reaching a maximum level of 17ng/ml at 6h. It has previously been shown that IFN-α2 treatment of human monocyte-derived DCs induces high amounts of CXCL10 production (Padovan *et al.*, 2002; Lande *et al.*, 2003). After 24h of culture, the level of CXCL10 in BMDCs cultures was severely demised, while in splenic DC cultures the level of

CXCL10 was maintained. CXCL9 expression by BMDCs was a fraction of CXCL10, reaching a maximum of 300pg/ml in 6h IFN-treated samples.

4.3 Discussion

We have used microarrays to identify genes whose expression in DCs is regulated by IFN-α4. In addition to previously reported IFN-induced and DC maturation-induced genes, we have identified novel genes with potential roles in DC function. IFN-induced upregulation of several of the genes, which encode putative cell surface molecules, was verified by quantitative PCR. Of particular interest was a gene encoding a chemokine receptor-like molecule, Gpr33, which was induced by IFN-α4 in DCs but not T or B cells, and is the subject of chapter 5.

Previous studies using microarrays to analyse gene expression in DCs activated by maturation stimuli have used either *in vitro*-derived human DCs or the mouse DC cell line D1. The maturation stimuli used in these studies were either pathogens or their components, which act via TLRs, and whose actions are at least partly dependent on type-I IFNs. We have investigated the expression of IFN-α4 induced genes in *ex-vivo* splenic DCs and DCs generated *in vitro* from bone marrow cells (BMDCs). The use of sDCs allows investigation on "real" DCs rather than *in vitro* derived DCs which are unlikely to behave in exactly the same manner as DCs would *in vivo*. However, sDCs are activated by mechanical manipulation, and do not survive prolonged periods in culture, thereby complicating their analysis. For these reasons we also chose to investigate BMDCs which are more robust *in vitro*. These DCs are likely to represent DCs developing from a myeloid lineage, since they are differentiated in the presence of GM-CSF and may differ from DCs differentiated *in vivo* which do not require GM-CSF for their development (Ni *et al.*, 2001; Gilliet *et al.*, 2002).

However these cells display the characteristics of DCs in their expression of cell surface markers, including high levels of MHC II, and their ability to induce proliferation in the MLR (Inaba *et al.*, 1992; Lutz *et al.*, 1999). The BMDCs can be further matured by TNF-α or LPS (Inaba *et al.*, 1992; Lutz *et al.*, 1999) and type I IFNs contribute to their full maturation (Montoya *et al.*, 2002).

Analysis of microarray data was performed in two different ways, and was initially carried out using Genespring software. A total of 471 and 1265 IFN-regulated genes were identified in sDCs and BMDCs respectively. This large discrepancy in the number of differentially regulated genes is most likely due to the number of transcripts present on the arrays used. The fold change cut-off point we chose for identification of differentially expressed genes was 1.5 fold. Commonly, a fold change cut-off of 2 is used in gene expression studies of this type. However we believed that this may exclude many interesting genes which are expressed at high levels in the control samples. In these cases the fold change may be low, but the absolute level of transcript may have increased a significant amount. An example of this is CCR7, essential for migration of maturing DCs to the lymph nodes and known to be induced by IFN-I in DCs (Parlato *et al.*, 2001). We found relatively high levels of CCR7 mRNA in untreated sDCs, which increased by 1.5 fold after IFN-α4 treatment.

We carried out expression analysis of genes from sDCs on U74Av2 arrays (representing 12,000 transcripts) and from BMDCs on 430 2.0 arrays (representing 39,000 transcripts). We chose to use the 430 2.0 arrays for analysis of the BMDCs samples since they provided comprehensive coverage of the mouse genome on single arrays but had not been available when carrying out the analysis on sDCs samples. However, since the probe sets for these arrays were designed on updated sequence information from the Unigene database they cannot be directly compared to probe sets present on the U74Av2 arrays. We attempted to

overcome this problem using Affymetrix conversion files which allowed us to compare at least part of the information from the two data sets. The majority of probe sets on the U74Av2 array which represented genes differentially expressed in sDCs were comparable to probe sets on the 430 2.0 arrays (437 out of 492). However it was apparent that the majority of differentially expressed genes were not commonly regulated in both sDCs and BMDCs. Strikingly, only one IFN-suppressed gene (CXCR4) was detected in both sDC and BMDC data sets. Those genes which were consistently induced in both sDCs and BMDCs (87 in total) included CXCL9 and CXCL10, in addition to many known IFN-stimulated genes.

In an attempt to obtain an overall picture of how IFN-induced gene expression could affect DC function, we grouped the differentially expressed genes into functional categories. Since control of DC migration is important for vaccination strategies, we were particularly interested in this group of genes. DOCK2 (Dedicator of cytokinesis 2) is a scaffold protein necessary for efficient migration of lymphocytes in response to chemokines. However, its suppression in sDCs may not affect their migration since migration defects in DOCK2 deficient mice appear to be lymphocyte specific (Reif *et al.*, 2002). More surprising perhaps, is the enhancement of the F11 receptor seen in BMDCs at 6h. F11 receptor has been shown to be expressed on the surface of BMDCs but did not increase after maturation with TNF-α. Furthermore, F11 receptor deficient mice displayed enhanced trafficking of DCs from the skin to the lymph nodes (Cera *et al.*, 2004), therefore we would not expect this receptor to be induced during DC maturation.

Peptidylprolyl isomerase C-associated protein (Ppicap, also known as cyclophilin C associated protein and galactosidase binding, soluble, 3 binding protein, Lgals3bp) a gene upregulated in sDCs after 2h IFN-I stimulation, has a potential role in cell adhesion and migration. Ppicap was identified on the basis of its ability to bind the peptidylprolyl

isomarase cyclophilin C (Friedman *et al.*, 1993), and was independently isolated as a cell surface antigen present on mouse macrophages (Chicheportiche *et al.*, 1994), but was subsequently shown to be a secreted protein (Trahey *et al.*, 1999). Ppicap was proposed to be the mouse homologue of human MAC-2 binding protein (hMAC-2BP) (Chicheportiche *et al.*, 1994), which has been shown to mediate cell adhesion through interactions with its ligand, MAC-2/Galectin-3 (Inohara *et al.*, 1996). MAC-2 /Galectin-3, is a secreted molecule which modulates various aspects of immunity, acting as a chemoattractant to monocytes and macrophages, and mediating DC interactions with naïve lymphocytes, thereby facilitating antigen presentation (Rabinovich *et al.*, 2002). However, Ppicap and hMAC-2BP appear to be functionally distinct since Ppicap does not bind MAC-2 and hMAC-2BP does not bind cyclophilin C (Jalkanen *et al.*, 2001). Alternative to a role in adhesion or migration, Ppicap appears to modulate host responses to endotoxins by regulating levels of proinflammatory cytokines (Trahey *et al.*, 1999).

DCs are known to rapidly produce inflammatory chemokines in response to inflammatory stimuli (Sallusto *et al.*, 1999b). This is likely to serve two functions: recruitment of monocytes and immature DCs to the site of inflammation and downregulation of cognate receptors on maturing DCs permitting their migration away from the inflamed tissue. CCL4 (MIP-1β) is an inflammatory chemokine which is expressed by most mature haematopoietic cells, including LPS-stimulated monoctyes and activated T cells, B cells and NK cells, and is important in the recruitment of T cells, B cells and immature DCs to the site of infection (Menten *et al.*, 2002). Additionally, CCL4 acts as a chemoattractant for NK cells and increases their cytolytic ability (Menten *et al.*, 2002). CCL4 signals through CCR5 which in humans is expressed on memory but not naïve T cells, and on Th1 but not Th2 cells (Loetscher *et al.*, 1998). In human dendritic cells CCL4 has been shown to be induced by

LPS, TNF-α and CD40L (Sallusto *et al.*, 1999b), with expression peaking at between six and twelve hours of stimulation. We saw an increase in CCL4 levels after IFN-α4 treatment in both sDCs and BMDCs. Concentrations of CCL4 in the supernatants from sDCs were sustained up to 24h, whereas levels in the BMDCs cultures peaked at 2h and steadily declined thereafter. Perhaps this difference could be explained by differing cell surface CCR5 expression, that is if BMDCs express higher levels of CCR5 they may internalise CCL4 more rapidly. As previously mentioned, production of CCL4 and CCL5 are thought to be important in mediating downregulation of CCR5 from the cell surface, which is rapidly internalised in maturing DCs (Sallusto *et al.*, 1999b).

CCL5 like CCL4 is an agonist for CCR5, in addition to CCR1, CCR3 and CCR4. CCL5 mediates the trafficking of T cells, monocytes, basophils, eosinophils, NK cells and DCs. At high concentrations CCL5 can induce T-cell proliferation or apoptosis, and the release of pro-inflammatory cytokines such as IL-2, IL-5, IFN-γ and CCL4 (Appay *et al.*, 2001). Again, we saw steady levels of CCL5 in cultures of sDCs but in BMDCs supernatants levels had begun to fall by 24h. This is in contrast to results in LPS-treated monocyte derived DCs where levels of CCL5 continued to rise at 24h (Sallusto *et al.*, 1999b). Notably, though, LPS-treatment is also known to down-regulate CCR5 on monocyte-derived DCs (Sallusto *et al.*, 1998c).

We also detected a downregulation of CXCR1 expression after IFN-α4 treatment, which is consistent with the phenotype of mature DCs (Sallusto *et al.*, 1998c). In accordance with recent observations (Lande *et al.*, 2003) we saw upregulation of CXCL9 and CXCL10 in IFN-α4 treated DCs. In addition to their importance in mediating migration of T cells, these chemokines also function as mediators of T cell responses, and have been shown to enhance

T cell proliferation and IFN-γ production (Whiting *et al.*, 2004). However, in disagreement with previous reports we also saw IFN-suppression of CXCR4 (Sallusto *et al.*, 1998c).

Type-I IFNs act on all cell types to induce the expression of anti-viral factors. Therefore a large number of the genes identified in this study represent genes involved in anti-viral defense and are not specifically involved in DC function. A database aimed at listing and classifying all IFN-stimulated genes has been constructed and includes data from a human fibrosarcoma cell line (de Veer *et al.*, 2001). Many of the genes identified in our experiments are present in this IFN-stimulated gene database, which may indicate a DC non-specific role for these genes in immunity. However genes such as IL-15, an important mediator of DC function, are also listed in this database, therefore we cannot rule out the importance of genes simply due to their upregulation by IFN-I in unrelated systems.

Chapter 5 : Characterisation of Gpr33

5.1 Introduction

Essential to DC function is their ability to migrate in response to chemotactic factors. Immature DCs express receptors for inflammatory chemokines such as CXCR1, CCR1, CCR2 and CCR5, as well as receptors for bacterial and complement chemoattractants (Sallusto *et al.*, 2000). This enables rapid movement of DCs to sites of infection for uptake of antigen. At the site of infection, DCs are exposed to maturation stimuli, triggering a reprogramming of DC function. This reprogramming includes a switch in chemokine receptor expression enabling the DCs to respond to a different set of chemokines which direct their migration to the lymph nodes.

Chemokine receptors belong to a large family of cell surface receptors, the G-protein-coupled receptors (GPCRs). GPCRs are integral membrane proteins containing seven transmembrane domains which send signals to the cell through trimeric G proteins (Baldwin, 1994). The human formyl-peptide receptor (FPR), and its variants FPR-like 1 and FPR-like 2 (FPRL1 and FPRL2) also belong to the GPCR family, and like chemokine receptors, mediate cell migration (Le *et al.*, 2002). Since these receptors were originally thought to bind solely to N-formyl peptides, which are derived from bacterial and mitochondrial proteins, it followed that these receptors could be involved in directing cells towards sites of bacterial infection or tissue damage. It is now known that a wide variety of non-formylated peptides are also recognised by the FPRs (Le *et al.*, 2002). These peptides are both pathogen-derived (HIV) and host-derived, such as an annexin 1 peptide. Commonly, the host-derived peptides are associated with disease; for instance, one agonist for FPRL1 is a

prion peptide. In the mouse, at least six formyl peptide receptor genes exist, of which three encode proteins orthologous to either FPR or FPRL1 and bind a similar variety of ligands to their human counterparts.

G-protein coupled receptor 33 (Gpr33) is an orphan GPCR which shares highest amino acid identity to another orphan GPCR, Gpr32, chemokine-like receptor 1 (CMKLR1), and the formyl-peptide receptors (Marchese *et al.*, 1998). Human FPR is expressed in immature myeloid DCs but is downregulated on DC maturation (Yang *et al.*, 2000), and FPRL1 is downregulated even as DC precursors differentiate into immature DCs (Yang *et al.*, 2001). FPRL1 expression is maintained in macrophages which implies an involvement in innate host defense rather than adaptive responses. However FPRL2 expression is maintained on DCs after maturation, suggesting a role in trafficking of both immature DCs to the site of infection, and mature DCs to the lymphoid organs for antigen presentation to T cells (Yang *et al.*, 2002). We have shown that Gpr33 mRNA is expressed at higher levels in DCs treated with IFN-I than in untreated DCs, which suggests that it may be involved in regulating the movement of mature DCs.

To gain an insight into the function of Gpr33, we used real-time PCR to analyse expression in DC subsets and other immune cells. In addition, we attempted to generate monoclonal and polyclonal antibodies against Gpr33 that would allow us to study expression at the protein level and investigate how this is regulated by stimulation with IFN-I. At the same time we produced Gpr33 transfected cell lines for the initial purpose of testing the antibodies and secondly, for screening possible ligands of this receptor.

5.2 Results

5.2.1 Analysis of Gpr33 expression by real-time PCR

5.2.1.1 Expression of Gpr33 in subsets of DCs treated with IFN-I

DC subsets reside in distinct anatomical locations and differ in their responses to chemokines (Pulendran *et al.*, 1997; Iwasaki *et al.*, 2000). Therefore we investigated whether Gpr33 was differentially expressed in subsets of DCs. We isolated the $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^-CD8^-$ subsets (these will be referred to as $CD11c^{high}$ since pDCs express intermediate levels of CD11c) and pDCs from the spleen by depleting lymphocytes and granulocytes followed by MACS sorting. The DCs were treated *in vitro* for two hours with IFN-α4. The results of real-time PCR analysis showed that only the $CD4^+$ $CD8^-$ subset showed a small increase (1.8 fold) in Gpr33 expression after IFN-I treatment (data not shown). We reasoned that, since isolation of the subsets was lengthy and required many manipulations, that the DCs were being activated and maturing rapidly during the two hour incubation period, even without IFN-I stimulus. Therefore to avoid culturing the DCs we decided to treat the DCs with IFN-α4 *in vivo*. We also altered the isolation method to sort $CD11c^{high}$ subsets by FACS to avoid excessive cell manipulations. Mice were injected intravenously (i.v) with IFN-α4 and sacrificed after 30 minutes. The $CD11c^{high}$ DCs and pDCs were isolated from the spleen by MACS, the three $CD11c^{high}$ subsets were sorted on expression of CD4 and CD8 by FACS.

First, we compared expression of Gpr33 between control and IFN-treated DCs. Upregulation of Gpr33 in $CD11c^{high}$ DC subsets treated with IFN-α4 varied between five and twenty fold (Figure 5.1a). The data did not indicate that any one subset preferentially upregulated Gpr33 expression, as the mean fold changes for each, which ranged from eight to

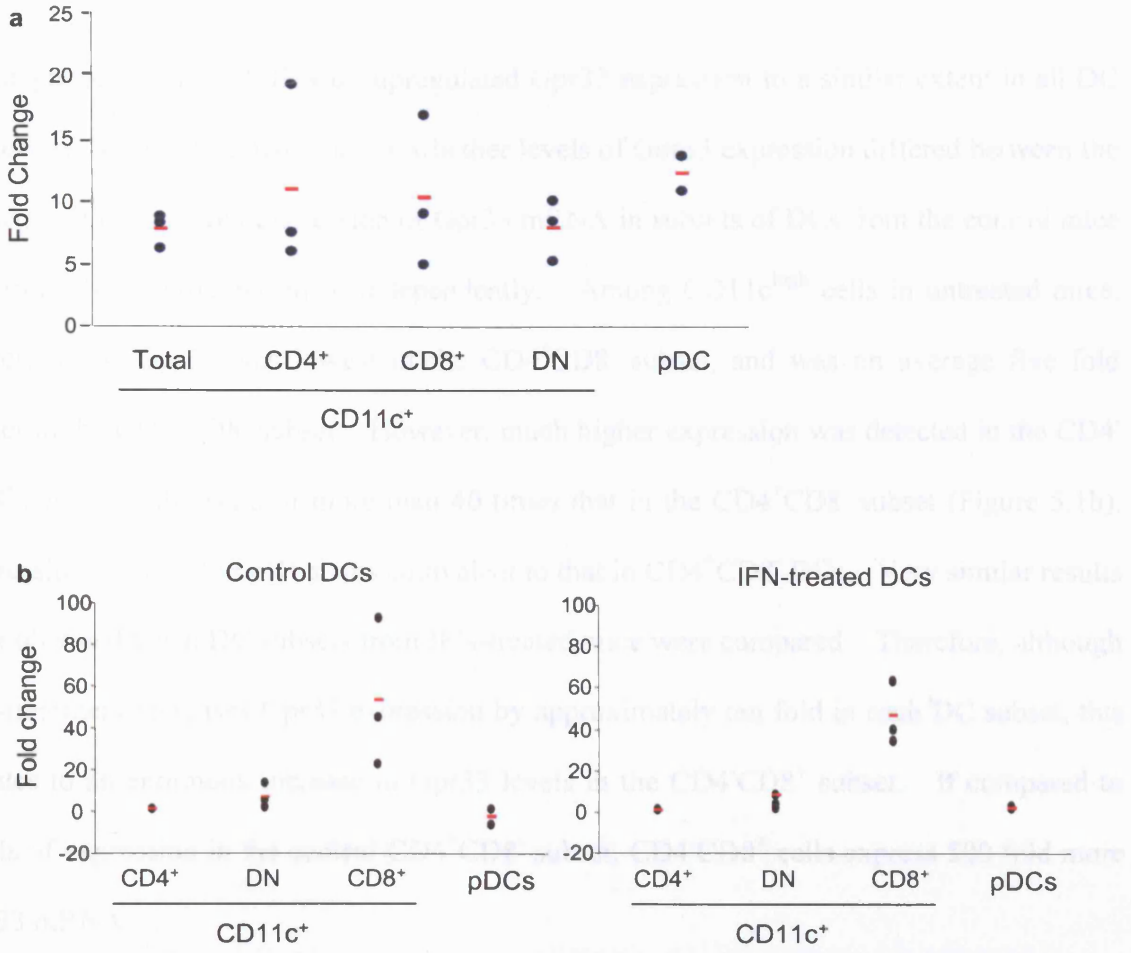


Figure 5.1 Expression of Gpr33 in subsets of DCs treated with IFN-α4
Mice were injected i.v. with 1 x 10^5 units IFN-α4 or PBS, 30 minutes later mice were sacrificed and the DCs isolated from the spleen. $CD11c^+$ DCs and pDCs were isolated by MACS, DC subsets were sorted by FACS. DN represents the double negative $CD4^-CD8^-$ subset. Cell purities were >90%. RNA was isolated and expression of Gpr33 analysed by real-time PCR. **a)** Results are plotted as fold change in expression in cells from IFN-treated mice versus control. **b)** Results are plotted as fold change in expression compared to the $CD11c^+CD4^+CD8^-$ subset (therefore values for $CD4^+CD8^-$ DCs are always equal to 1 ie no change in expression). Data is from three independent experiments, except pDCs, for which only two experiments were carried out.

eleven fold, were not significantly different. Expression of Gpr33 changed comparably in pDCs, with an average increase of 12 fold in IFN-treated compared to control pDCs.

Although treatment with IFN-α4 upregulated Gpr33 expression to a similar extent in all DC subsets, we were interested to know whether levels of Gpr33 expression differed between the subsets. We compared expression of Gpr33 mRNA in subsets of DCs from the control mice and from the IFN-treated mice independently. Among $CD11c^{high}$ cells in untreated mice, expression of Gpr33 was lowest in the $CD4^+CD8^-$ subset, and was on average five fold higher in the $CD4^-CD8^-$ subset. However, much higher expression was detected in the $CD4^-CD8^+$ subset, with levels of more than 40 times that in the $CD4^+CD8^-$ subset (Figure 5.1b). Expression of Gpr33 in pDCs was equivalent to that in $CD4^+CD8^-$ DCs. Very similar results were obtained when DC subsets from IFN-treated mice were compared. Therefore, although IFN-treatment increases Gpr33 expression by approximately ten fold in each DC subset, this equates to an enormous increase in Gpr33 levels in the $CD4^-CD8^+$ subset. If compared to levels of expression in the control $CD4^+CD8^-$ subset, $CD4^-CD8^+$ cells express 500 fold more Gpr33 mRNA.

5.2.1.2 Expression of Gpr33 over a time course of in vitro DC culture

Expression of Gpr33 was measured by real-time PCR in splenic $CD11c^+$ DCs which had been cultured with or without 2 x 10^4 U/ml IFN-I for two, four of six hours. Consistent with the results of microarray analysis, the results showed higher expression of Gpr33 mRNA in IFN-treated DCs at the two hour time point, but not at the later time points (Figure 5.2). Expression of Gpr33 in the untreated DCs was stable over time, whereas expression in the IFN-treated DCs was downregulated after four hours, and returned to the level of expression in the untreated DCs by six hours of culture.

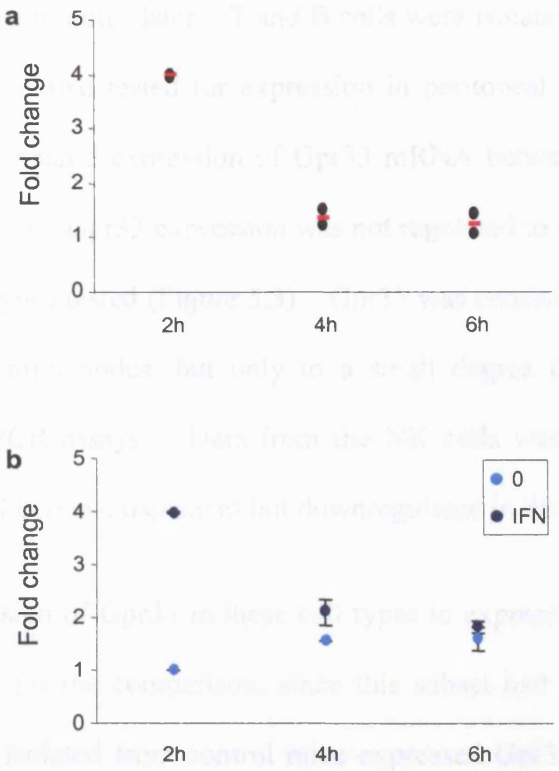


Figure 5.2 Expression of Gpr33 over a timecourse of IFN-treatment in splenic DCs
$CD11c^+$ DCs isolated from spleens by MACS were cultured in vitro in media alone (0) or with 2 x 10^4 U IFN-α4/ml (IFN) for the indicated times. Cell purities were >85%. RNA was isolated and expression of Gpr33 analysed by real-time PCR. **a)** Results are plotted as fold change in expression in IFN-treated versus untreated DCs. **b)** Results are plotted as fold change in expression compared to the untreated DCs at 2h (therefore value for untreated DCs at 2h equals 1 ie no change in expression). Data is from two independent experiments.

5.2.1.3 Expression of Gpr33 in lymphocytes and macrophages treated in vivo with IFN-I

Next, we tested whether expression of Gpr33 was restricted to DCs, and whether expression in other cell types could also be upregulated by IFN-α4. Mice were injected i.v. with IFN-α4 or PBS and sacrificed 30 minutes later. T and B cells were isolated from both the spleen and the lymph nodes. We also tested for expression in peritoneal macrophages and NK cells. Again, we first compared expression of Gpr33 mRNA between cells isolated from IFN-treated and control mice. Gpr33 expression was not regulated to a significant degree by IFN-α4 in any of the cell types tested (Figure 5.3). Gpr33 was consistently upregulated in B cells isolated from the lymph nodes, but only to a small degree (2 fold), which is not meaningful in real time PCR assays. Data from the NK cells was ambiguous as Gpr33 appeared to be upregulated in one experiment but downregulated in the other.

We then compared expression of Gpr33 in these cell types to expression in DCs. We used the $CD4^+CD8^-$ DC subset for the comparison, since this subset had the lowest expression. None of the lymphocytes isolated from control mice expressed Gpr33 at different levels to the $CD4^+CD8^-$ DC subset, except in macrophages, where expression of Gpr33 was around five fold lower (Figure 5.4). When cells from IFN-treated mice were compared, a relative decrease in Gpr33 expression was observed, reflecting the failure of non-DCs to upregulate expression. This was especially true in macrophages where expression was around 40 fold lower than that in $CD4^+CD8^-$ DCs. Expression in B cells was four to six fold lower and in $CD8^+$ T cells eight fold lower. Expression was more variable in $CD4^+$ T cells but on average was between six and ten fold less than that in $CD4^+CD8^-$ DCs.

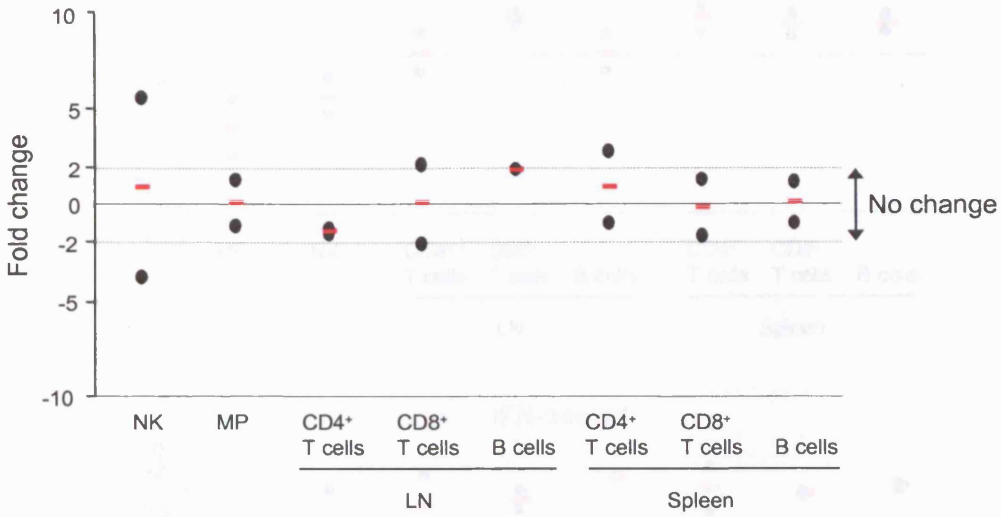


Figure 5.3 Regulation of Gpr33 by IFN-I in cells of the immune system
Mice were injected i.v. with 1 x 10^5 units IFN-α4 or PBS, and sacrificed 30 minutes later. $CD4^+$ and $CD8^+$ T cells and B cells were isolated from both the spleen and the lymph nodes (LN) by Dynabead depletion followed by MACS. Natural killer cells (NK) were isolated from the spleen by MACS followed by FACS sorting. Peritoneal macrophages (MP) were sorted by FACS. Cell purities were >90%. RNA was isolated and expression of Gpr33 analysed by real-time PCR. Results from two independent experiments are plotted as fold change in expression in cells from IFN-treated mice versus control.

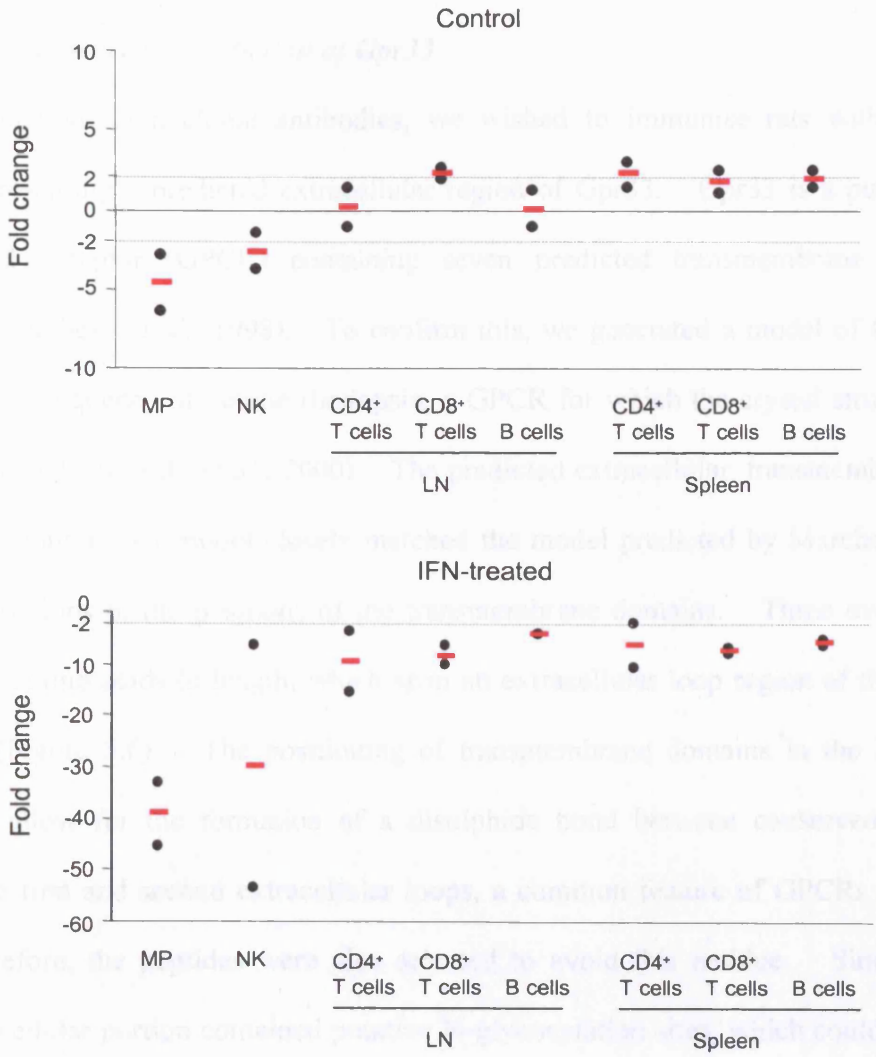


Figure 5.4 Expression of Gpr33 in cells of the immune system compared to expression in DCs Mice were injected i.v. with 1 x 10^5 units IFN-α4 or PBS, and sacrificed 30 minutes later. $CD4^+$ and $CD8^+$ T cells and B cells were isolated from both the spleen and the lymph nodes (LN) by Dynabead depletion followed by MACS. Natural killer cells (NK) were isolated from the spleen by MACS followed by FACS. Peritoneal macrophages (MP) were sorted by FACS. Cell purities were >90%. RNA was isolated and expression of Gpr33 analysed by real-time PCR. Results are plotted as fold change in expression in cells as indicated versus $CD4^+CD8^-$ DCs. Data is from two independent experiments.

5.2.2 Generation of monoclonal Abs against Gpr33

5.2.2.1 Secondary structure prediction of Gpr33

In order to generate monoclonal antibodies, we wished to immunise rats with peptide sequences representing a predicted extracellular region of Gpr33. Gpr33 is a putative G-protein-coupled receptor (GPCR) containing seven predicted transmembrane domains (Figure 5.5) (Marchese *et al.*, 1998). To confirm this, we generated a model of Gpr33 by aligning it to the sequence of bovine rhodopsin, a GPCR for which the crystal structure has been determined (Palczewski *et al.*, 2000). The predicted extracellular, transmembrane and intracellular regions in our model closely matched the model predicted by Marchese *et al.*, with slight variations in the positions of the transmembrane domains. Three overlapping peptides of 15 amino acids in length, which span an extracellular loop region of the protein were chosen (Figure 5.6). The positioning of transmembrane domains in the Marchese model would allow for the formation of a disulphide bond between conserved cysteine residues in the first and second extracellular loops, a common feature of GPCRs (Murphy, 1994). Therefore, the peptides were also selected to avoid this residue. Since the N-terminal extracellular portion contained putative N-glycosylation sites, which could interfere with antibody recognition, we chose not to immunise with peptides with this amino acid sequence.

5.2.2.2 Immunisations and screening of hybridomas

A combination of the three overlapping peptides (named A1, A2 and A3), synthesized as multiple antigenic peptides, was used to immunize rats either intraperitoneally (i.p.) or subcutaneously (s.c.). After 15 days the rat serum was tested for production of peptide specific antibodies. The rat injected i.p. produced the best response against the A1 peptide and a weaker response to the A2 peptide. We did not detect any antibodies specific for the

Figure 5.5 Alignment of the deduced amino acid sequence of Gpr32 with related GPCRs, including Gpr33
Adapted from Marchese et al., 1998. Amino acids identical with the receptor encoded by Gpr32 are boxed and shaded. Potentially, a di-sulphide bond exists between cysteine residues marked *. The region used for immunisation for generation of monoclonal is marked by the red line. The conserved acidic-arginine-aromatic triplet is marked by the blue line.

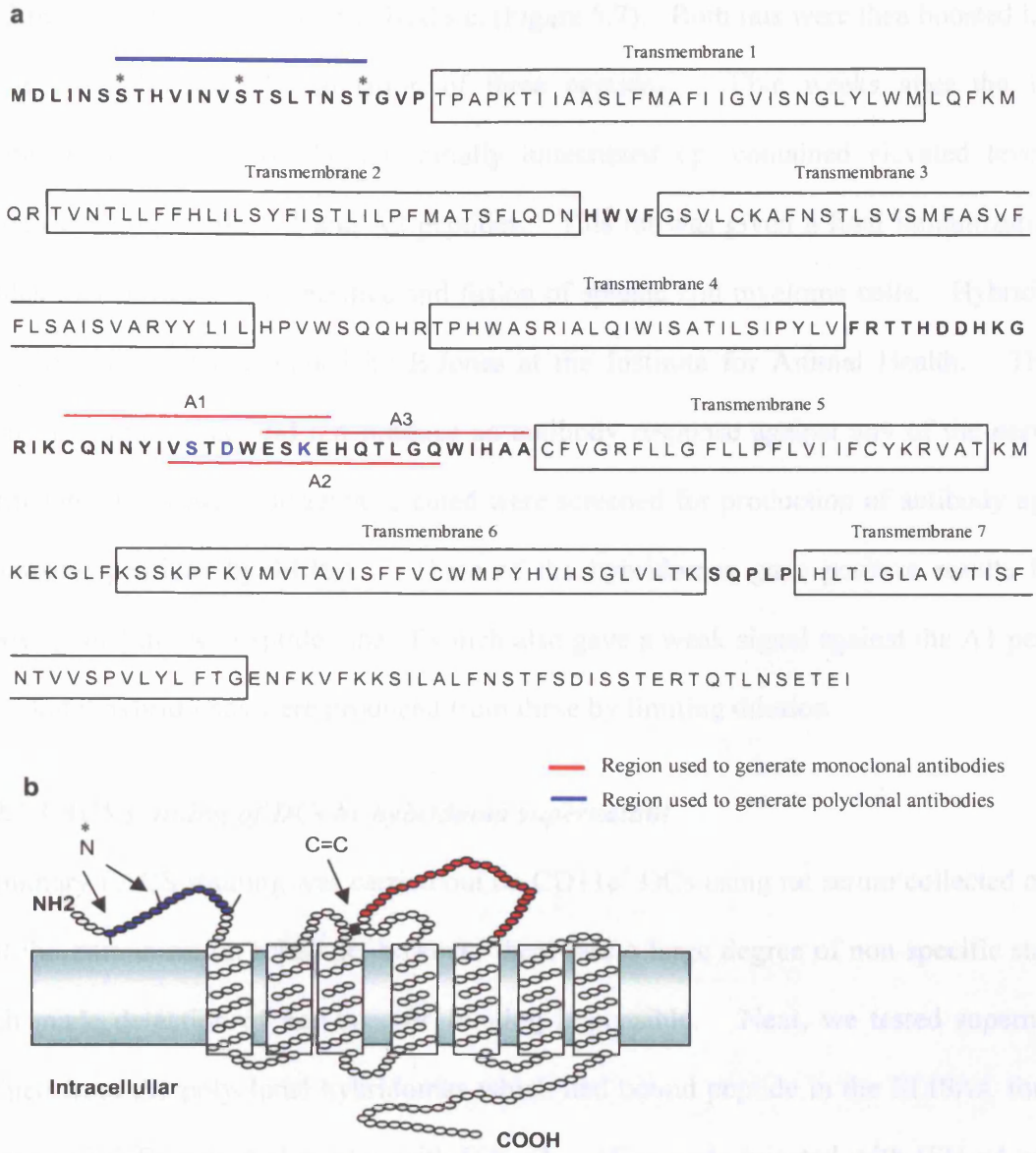


Figure 5.6 Design of peptides for generation of anti-Gpr33 antibodies
a, Three overlapping peptides (A1, A2 and A3) spanning an extracellular loop between transmembrane domains four and five were chosen for the immunisation of rats. Residues in blue are those which differ in the rat homologue (XP_234113). Additionally, polyclonal antibodies were raised against the N-terminal region. Residues in bold type are extracellular. Asparagine residues marked * are putative glycosylation sites. **b,** Schematic representation of Gpr33 structure, showing the putative extracellular loop against which we attempted to raise monoclonal antibodies (residues marked in red), potential N-linked glycosylation sites and di-sulphide bond (C=C).

Gpr33 peptides from the rat immunized s.c. (Figure 5.7). Both rats were then boosted i.p. on day 18 with the same combination of three peptides. Five weeks after the initial immunization, the sera of the rat initially immunized i.p. contained elevated levels of antibody specific for the A1 and A2 peptides. This rat was given a final immunization of peptides four days prior to sacrifice and fusion of splenic and myeloma cells. Hybridomas were generated and maintained by B.Jones at the Institute for Animal Health. The rat initially immunized s.c. did not produce an antibody response against any of the peptides. Supernatants from the hybridomas created were screened for production of antibody against immunising peptides by ELISA. Three of the hybridomas gave positive results in the ELISA against the A2 peptide, one of which also gave a weak signal against the A1 peptide. Monoclonal hybridomas were produced from these by limiting dilution.

5.2.2.3 FACS staining of DCs by hybridoma supernatant

Preliminary FACS staining was carried out on $CD11c^+$ DCs using rat serum collected on day 35 of the immunization schedule, however there was a large degree of non-specific staining which made detection of any specific binding impossible. Next, we tested supernatants obtained from the polyclonal hybridomas which had bound peptide in the ELISAs, for their ability to bind DCs treated *in vivo* with IFN-α4. Mice were injected with IFN-α4 or PBS alone and 12 hours later $CD11c^+$ DCs were isolated. After IFN-treatment, the hybridoma clone LF7 appeared to stain a small subset of DCs (Figure 5.8).

Since we identified Gpr33 in DCs treated *in vitro*, it was possible that expression of this molecule was more efficiently induced *in vitro*. Therefore we isolated DCs and treated them *in vitro* with IFN-α4 for three, four or six hours. We noted that during the incubation a large proportion of the cells were dying; the number of cells in the live cell gate was reduced from

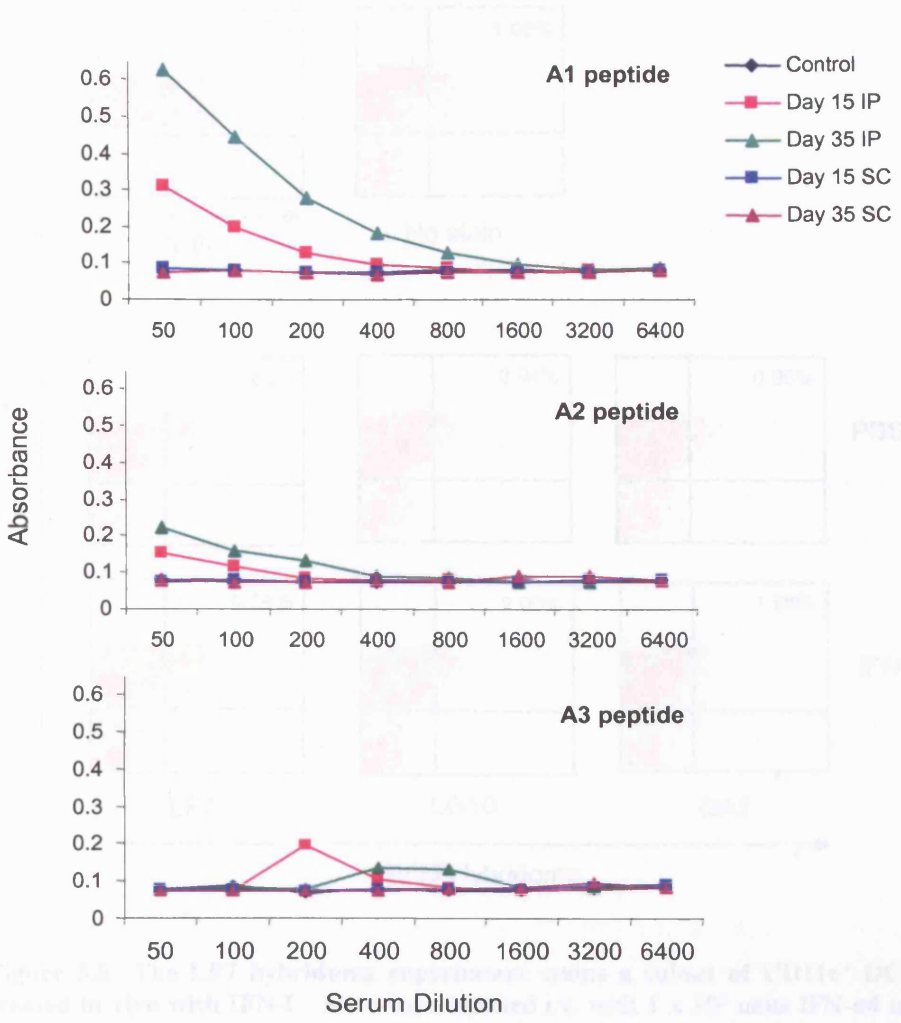


Figure 5.7 Sera from the rat immunised intraperitoneally contained antibodies against the A1 and A2 Gpr33 peptides Serum taken from rats initially immunised subcutaneously (SC) or intraperitoneally (IP), and thereafter immunised IP were tested for their ability to bind the three immunising peptides, A1, A2 and A3 by ELISA.

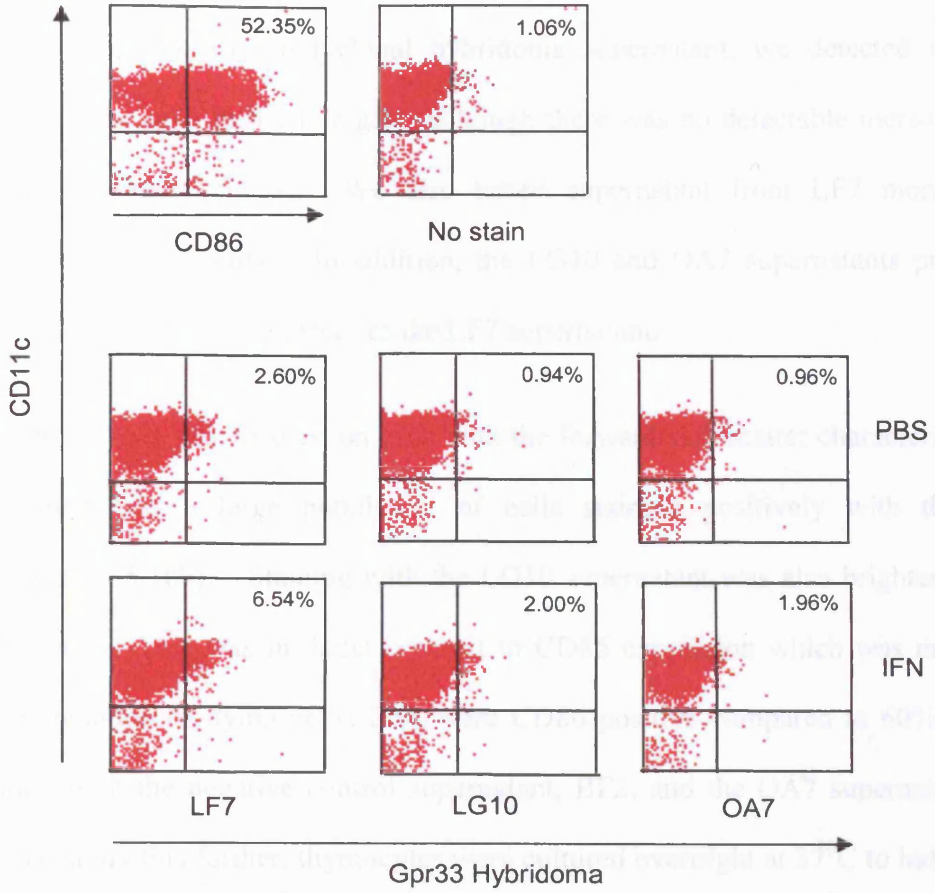
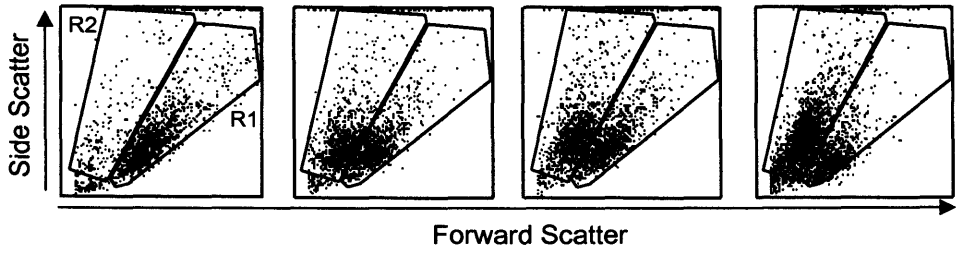


Figure 5.8 The LF7 hybridoma supernatant stains a subset of CD11c$^+$ DCs treated in vivo with IFN-I Mice were injected i.v. with 1 x 10^5 units IFN-α4 or PBS alone. 12 hours later CD11c$^+$ DCs were isolated from the spleen by MACS sorting. Cells were stained using either a rat anti-CD86 antibody or the Gpr33 hybridoma supernatants, followed by an anti-rat PE conjugated antibody.

70% when cells were kept on ice to 25% or less after six hours of incubation at 37^0C (Figure 5.9). Again, using the LF7 polyclonal hybridoma supernatant, we detected a small proportion of positive cells in the live gate, although there was no detectable increase after IFN-α4 treatment (Figure 5.10a). We also tested supernatant from LF7 monoclonal hybridomas with similar results. In addition, the LG10 and OA7 supernatants produced some staining but not to the same extent as the LF7 supernatant.

However, when analysis was focused on cells with the forward/side scatter characteristics of dying cells, there was a large population of cells staining positively with the LF7 supernatants (Figure 5.10b). Staining with the LG10 supernatant was also brighter in this population of cells. This was in direct contrast to CD86 expression which was markedly lower in the population of dying cells: 20% were CD86 positive compared to 60% of live DCs. Staining with the negative control supernatant, BF2, and the OA7 supernatant was unchanged. To study this further, thymocytes were cultured overnight at 37^0C to induce cell death. The results showed that the antibody from the LF7 hybridoma stained a population of dead or dying cells (Figure 5.11).

Since BMDCs survive for longer periods in culture, we also attempted to stain BMDCs using the LF7 hybridoma supernatant. BMDCs were cultured for 4 or 24 hours alone or in the presence of IFN-α4. A small population of cells was stained by the LF7 hybridoma supernatant after four hours of culture but this was not increased by IFN-α4 treatment or blocked by addition of Gpr33 peptides (Figure 5.12). Again, the staining was much more pronounced in dead/dying cells. The same was true after 24 hours where the majority of LF7 stained cells were dying and the staining was not affected by the addition of the Gpr33 peptides. Therefore, it appeared that the interaction between the monoclonal LF7 antibody and dead cells was a non-specific one.



	ICE	3h	4h	6h
R1	70.11%	43.70%	41.29%	23.60%
R2	13.37%	48.28%	52.30%	68.69%

Figure 5.9 DCs cultured at 37^{0}C lose viability over time
CD11c$^+$ DCs were isolated, and either stored on ice or cultured at 37^{0}C for the indicated times in media alone or in the presence of 2 x 10^4 units IFN-α4/ml. The region R1 contains live cells, cells in the region R2 are dead/dying. These cells were untreated, but a similar effect was seen with IFN-treated cells.

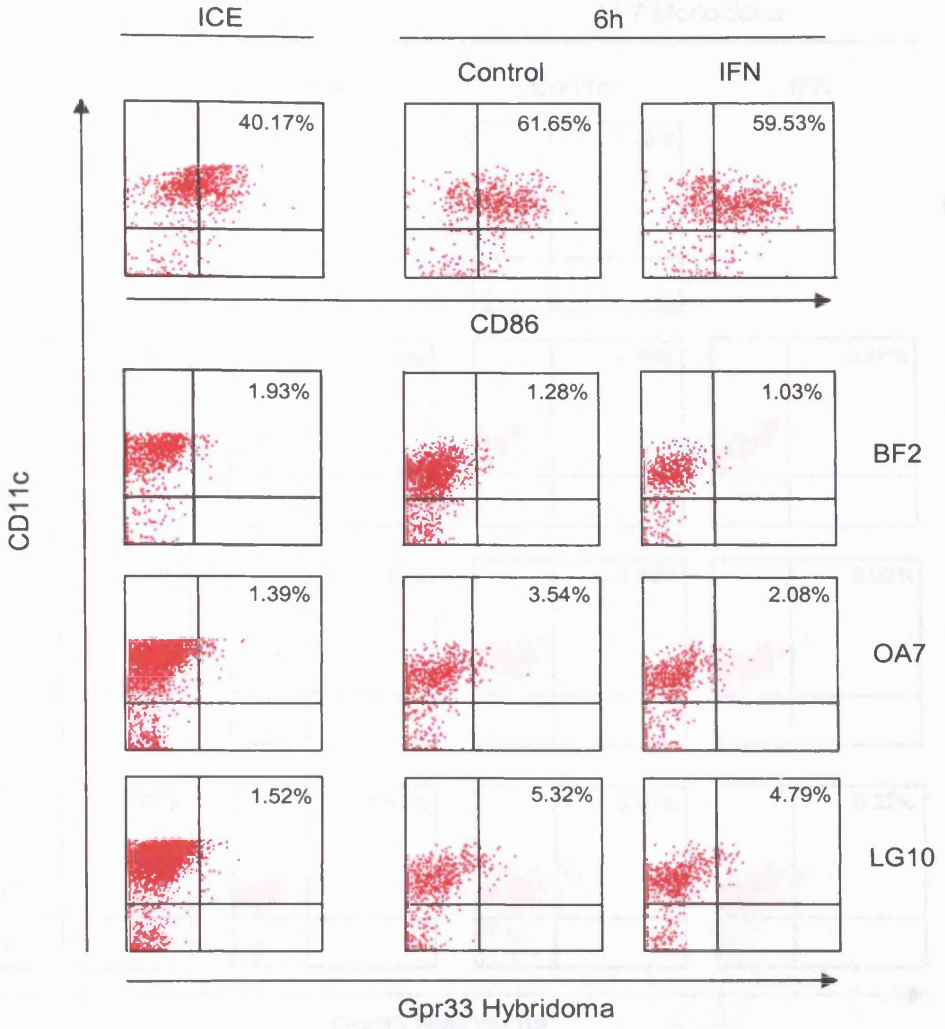


Figure 5.10a (continued over page) Staining of in vitro-treated splenic DCs by the Gpr33 hybridoma supernatants $CD11c^+$ DCs were isolated from the spleen by MACS sorting and either stored on ice or cultured at 37^0C for the indicated times in media alone or in the presence of 2 x 10^5 units IFN-α4/ml. Cells were stained using either a rat anti-CD86 antibody or the Gpr33 hybridoma supernatants, followed by an anti-rat PE conjugated antibody. BF2 is a Gpr33 hybridoma which tested negative by ELISA. Cells were gated on the live gate R1 (Figure 5.9).

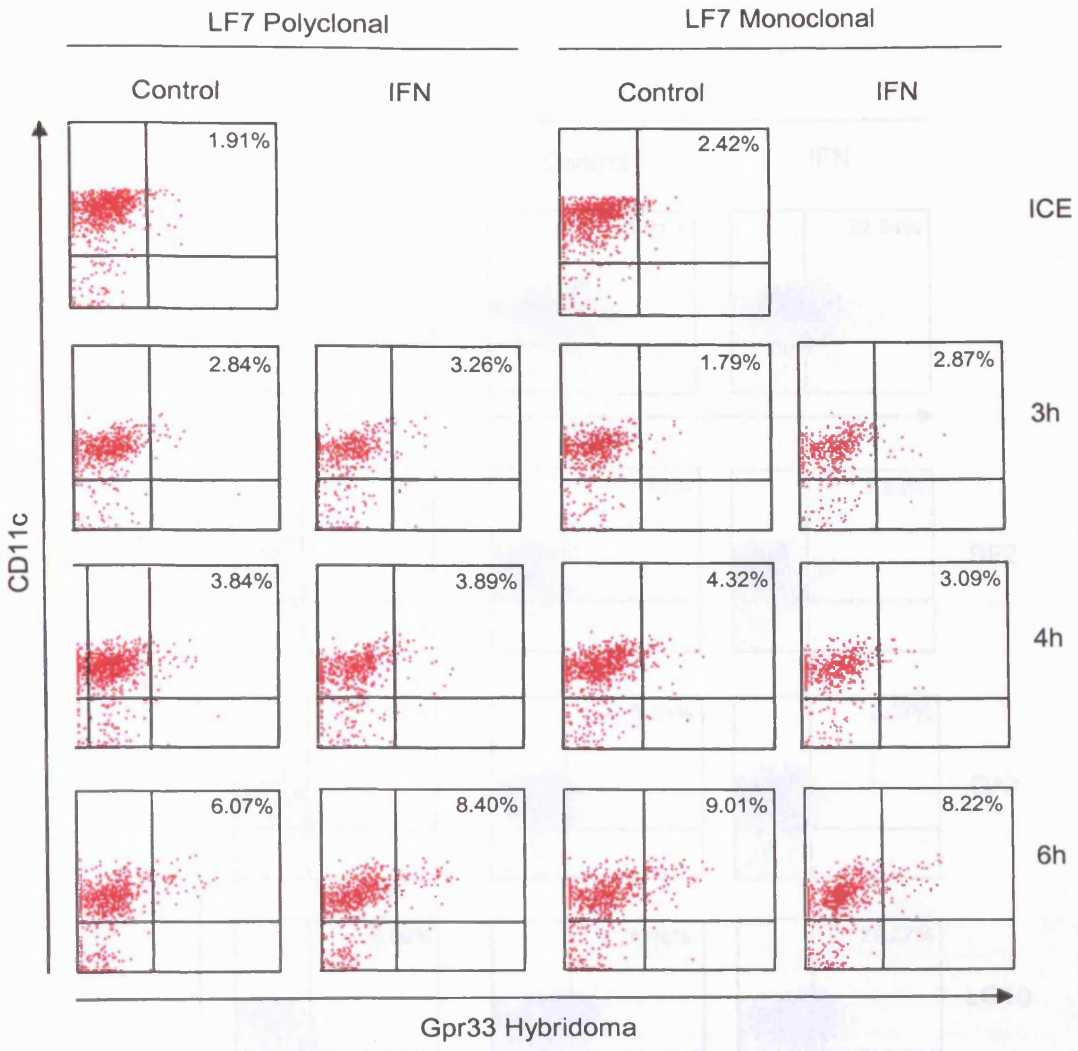


Figure 5.10a (continued) Staining of in vitro-treated splenic DCs by the Gpr33 hybridoma supernatants

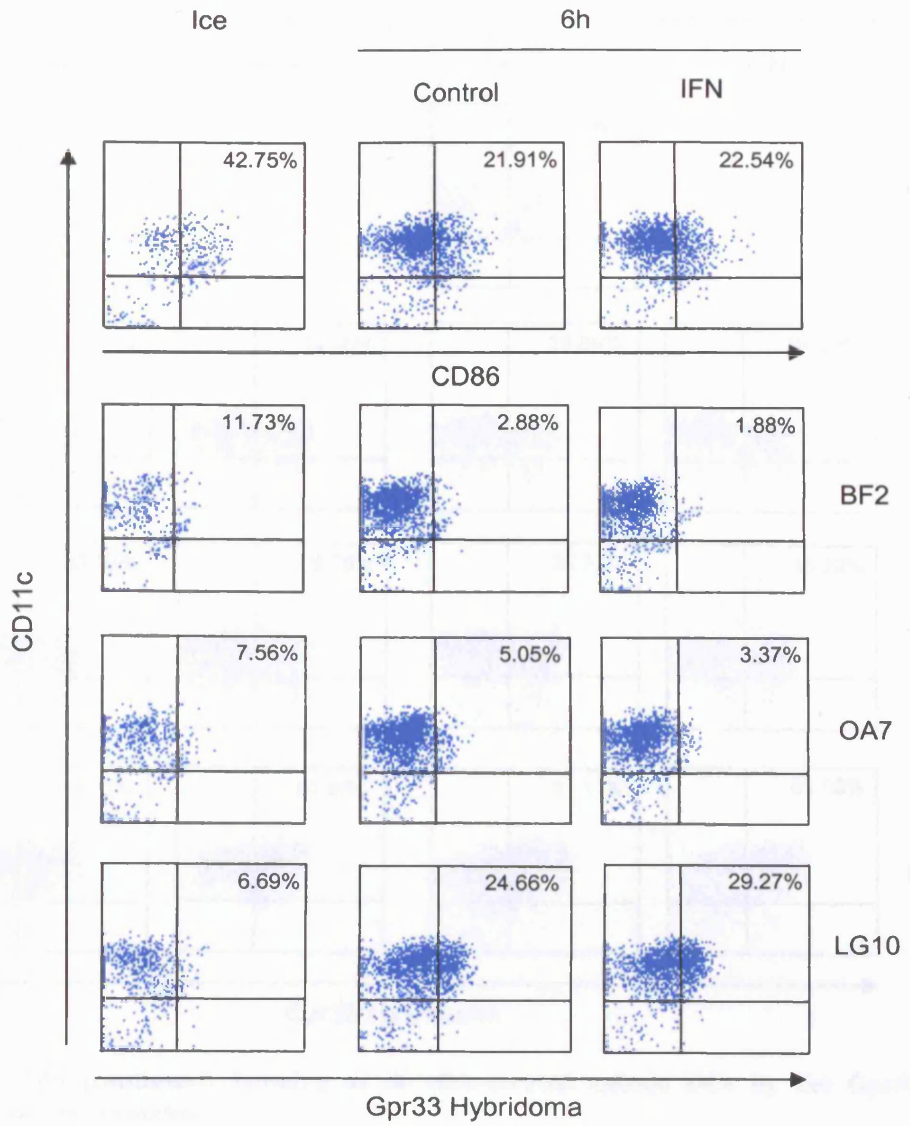


Figure 5.10b (continued over page) Staining of in vitro-treated splenic DCs by the Gpr33 hybridoma supernatants $CD11c^+$ DCs were isolated from the spleen by MACS sorting and either stored on ice or cultured at 37^0C for the indicated times in media alone or in the presence of 2×10^5 units IFN-α4/ml. Cells were stained using either a rat anti-CD86 antibody or the Gpr33 hybridoma supernatants, followed by an anti-rat PE conjugated antibody. BF2 is a Gpr33 hybridoma which tested negative by ELISA. Cells were gated on the region R2 (figure 5.9), cells with forward/side scatter characteristics of dying cells.

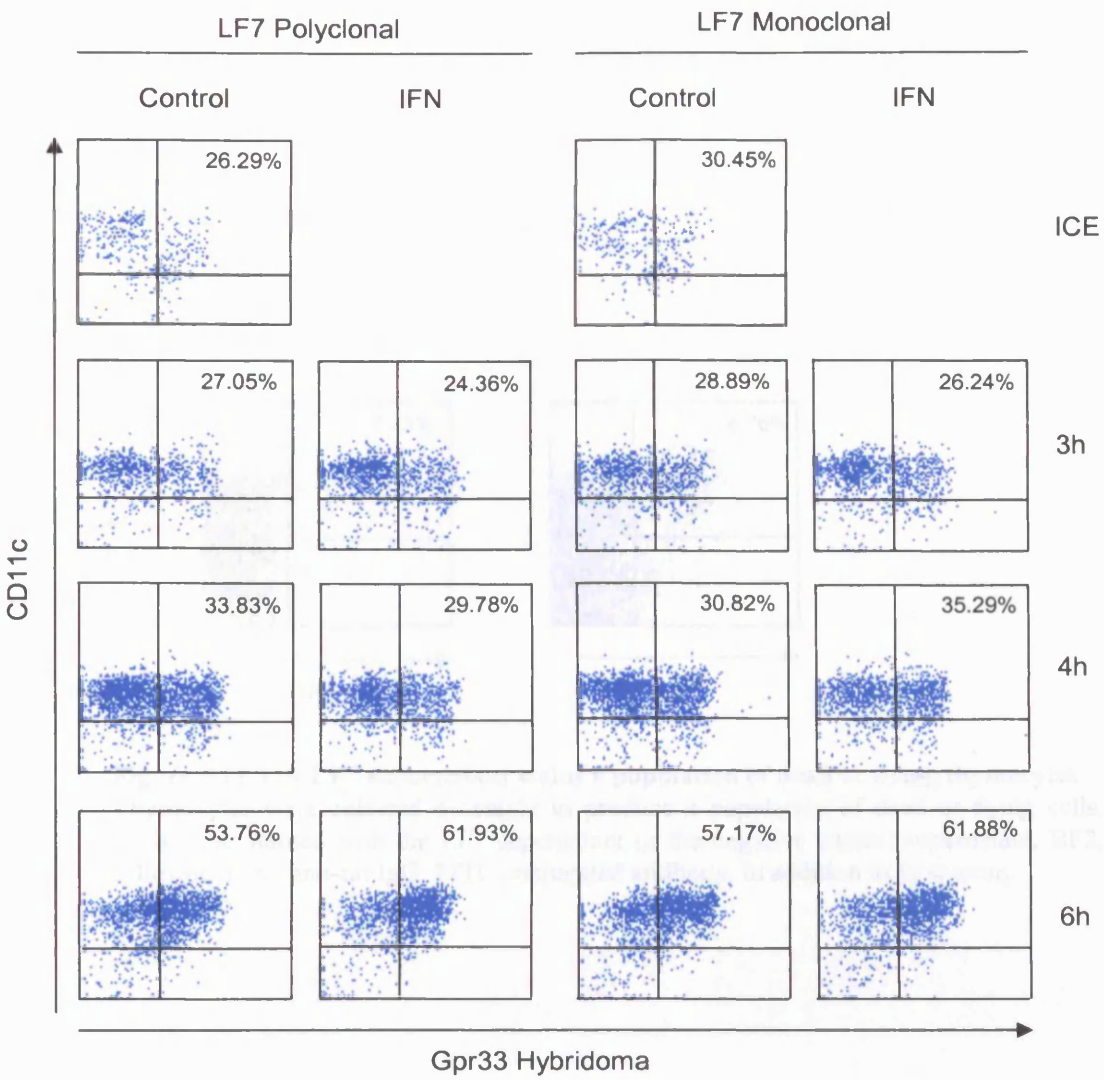


Figure 5.10b (continued) Staining of in vitro-treated splenic DCs by the Gpr33 hybridoma supernatants

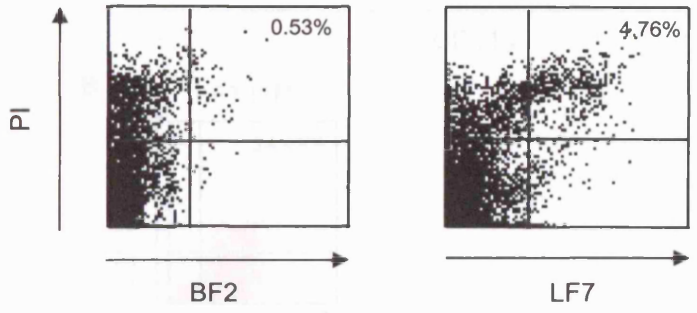


Figure 5.11 The LF7 supernatant stains a population of dead or dying thymocytes
Thymocytes were cultured overnight to produce a population of dead or dying cells. Cells were stained with the LF7 supernatant or the negative control supernatant, BF2, followed by an anti-rat IgG, FITC conjugated antibody, in addition to PI staining.

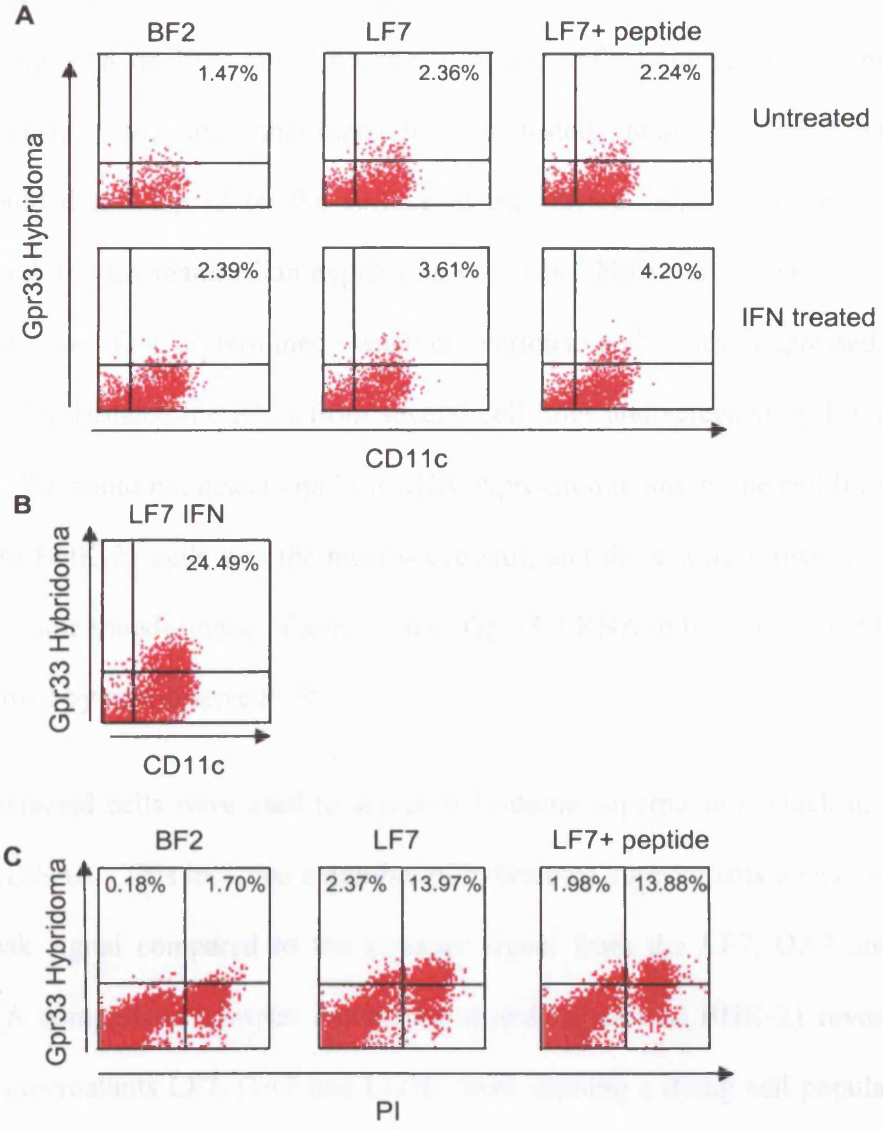


Figure 5.12 LF7 hybridoma supernatant binds non-specifically to dying BMDCs BMDCs were cultured *in vitro* with or without 2 x 10^4 U IFN-α4 for 4 hours **(A + B)** or 24 hours **(C).** BMDCs were harvested and stained for expression of CD11c and Gpr33. Where indicated, we attempted to block binding of the LF7 supernatant by addition of the Gpr33 peptides A1, A2 and A3 (each at 20μg/ml). **A,** Live cell gate, **B,** Dead cell gate and **C** BMDCs harvested at 24 hours were additionally stained with PI to detect dead cells.

5.2.2.4 Screening of hybridoma supernatants using a Gpr33 transfected cell line

Since any staining seen on DCs could be due to non-specific binding, or binding of the antibody to cell surface molecules other than Gpr33, we tested whether the Gpr33 hybridoma supernatants could detect Gpr33 on the surface of transfected cells. Full-length Gpr33 cDNA was cloned into the mammalian expression vector pcDNA6. To choose a target cell for transfection, we first determined whether various cell lines expressed Gpr33 endogenously. We isolated the RNA from several cell lines and screened by RT-PCR for Gpr33 mRNA. We could not detect Gpr33 mRNA expression in any of the cell lines tested. Transfection into BHK-21 cells was the most successful, and these were grown in selective media to obtain stable transfectants. Expression of Gpr33 mRNA in the transfected BHK-21 cells was confirmed by quantitative PCR.

The Gpr33 transfected cells were used to screen hybridoma supernatants which had bound peptide in the ELISAs. This included a number of hybridoma supernatants which had given a relatively weak signal compared to the stronger signal from the LF7, OA7 and LG10 supernatants. A comparison between untransfected and transfected BHK-21 revealed that the hybridoma supernatants LF7, OA7 and LG10, were staining a dying cell population (PI positive) in both cases (Figure 5.13). No specific staining of viable cells was detected.

Since we used linear peptides to immunise the rats, it was also possible that the monoclonal antibodies were not recognizing the protein in its native form. To test this, we ran Gpr33 transfected cell lysates on a denaturing SDS-PAGE gel and probed the blot with the Gpr33 monoclonal antibody. No bands were detected (data not shown).

Taken together, these data indicated that the antibodies raised by immunization with Gpr33 peptides did not bind to Gpr33 protein. However, although we tested for Gpr33 mRNA

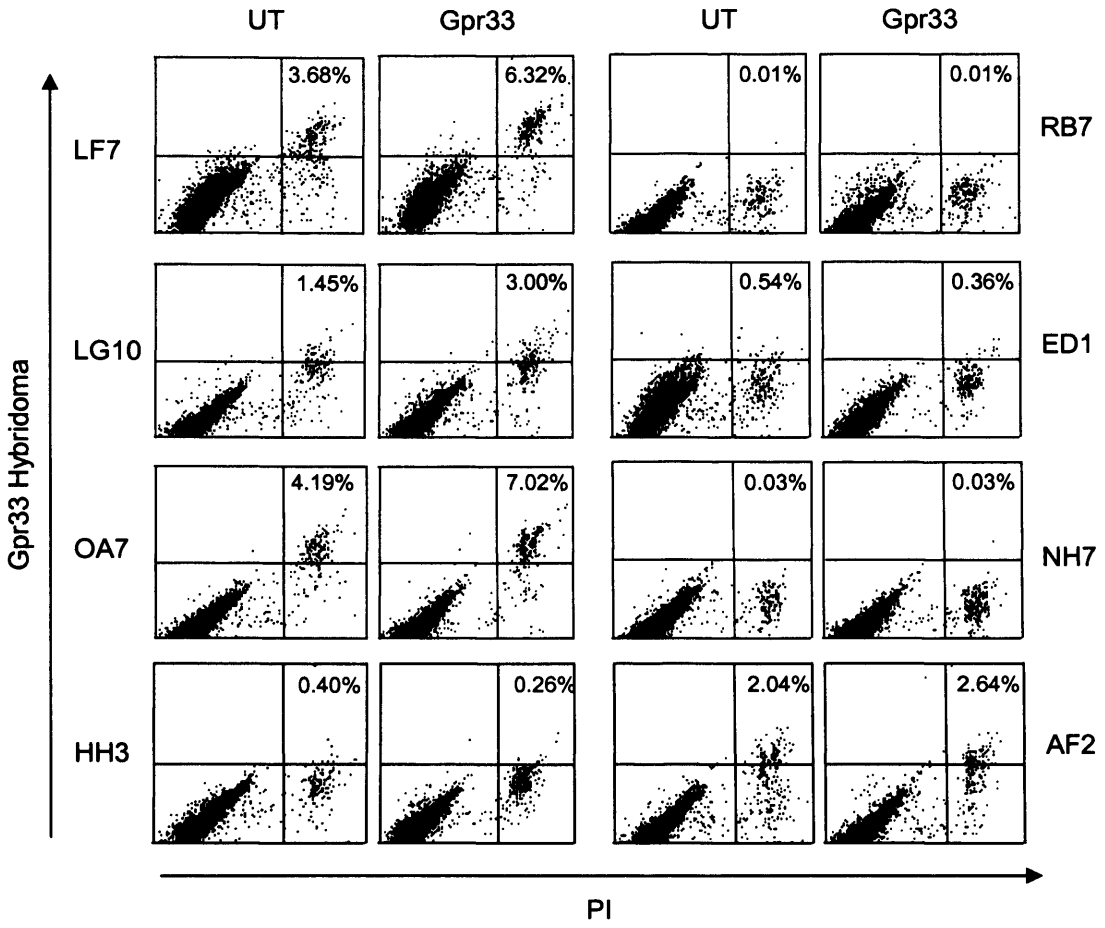


Figure 5.13 Screening of Gpr33 hybridoma supernatants by FACS staining of transfected cells (Figure continued over page) Supernatants from hybridomas were used to stain BHK-21 cells which had been stably transfected with Gpr33 (Gpr33) and compared with staining of untransfected cells (UT).

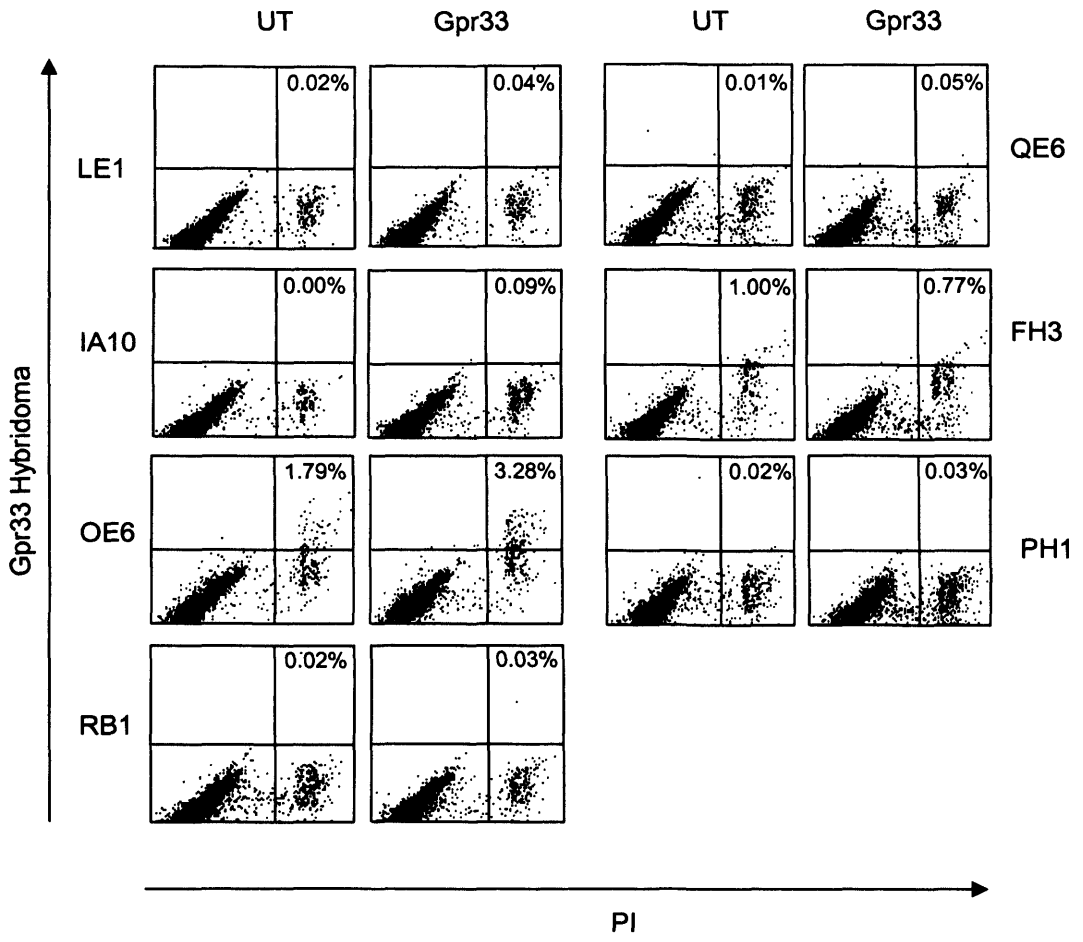


Figure 5.13 (continued) Screening of Gpr33 hybridoma supernatants by FACS staining of transfected cells

expression in the transfected BHK-21 cells, it was impossible to know whether the mRNA was being translated into protein, or whether the Gpr33 protein was being correctly trafficked to the cell surface.

5.2.3 Generation of a FLAG-Gpr33 fusion protein

5.2.3.1 Cloning and expression of a FLAG-Gpr33 fusion protein

To generate a model in which we could verify Gpr33 expression at the cell surface independently of the specificity of our anti-Gpr33 monoclonal antibodies, we cloned the Gpr33 cDNA into the expression vector pFLAG-CMV4, which contains an N-terminal FLAG marker. Transfected cells expressing the FLAG -Gpr33 fusion protein could then be detected using an anti-FLAG antibody. As a positive control, murine CCR7 was cloned in parallel into the same vector. The murine pre-B cell line Baf/3 was transfected and grown in selective media to obtain stable transfectants. Expression of Gpr33 was confirmed by real-time PCR. Gpr33 mRNA was detected in untransfected cells, but was expressed at levels 600 fold higher in transfected cells. CCR7 was also expressed endogenously, but unfortunately, we could detect no difference in expression in the transfected cells.

5.2.3.2 FACS analysis of FLAG-Gpr33 transfected cells

We could not detect cell surface expression of the FLAG-Gpr33 fusion protein in stably transfected Baf/3 cells using the anti-FLAG M2 antibody (Figure 5.14). A small population of cells stained positively with the anti-FLAG M2 antibody but this was true even for the untransfected cells. This suggests that the M2 antibody is cross-reacting with a protein expressed by the Baf/3 cells.

Additionally, intracellular staining was carried out to test whether the fusion protein was being expressed in the cytoplasm. In this case there was a dramatic shift in staining in both

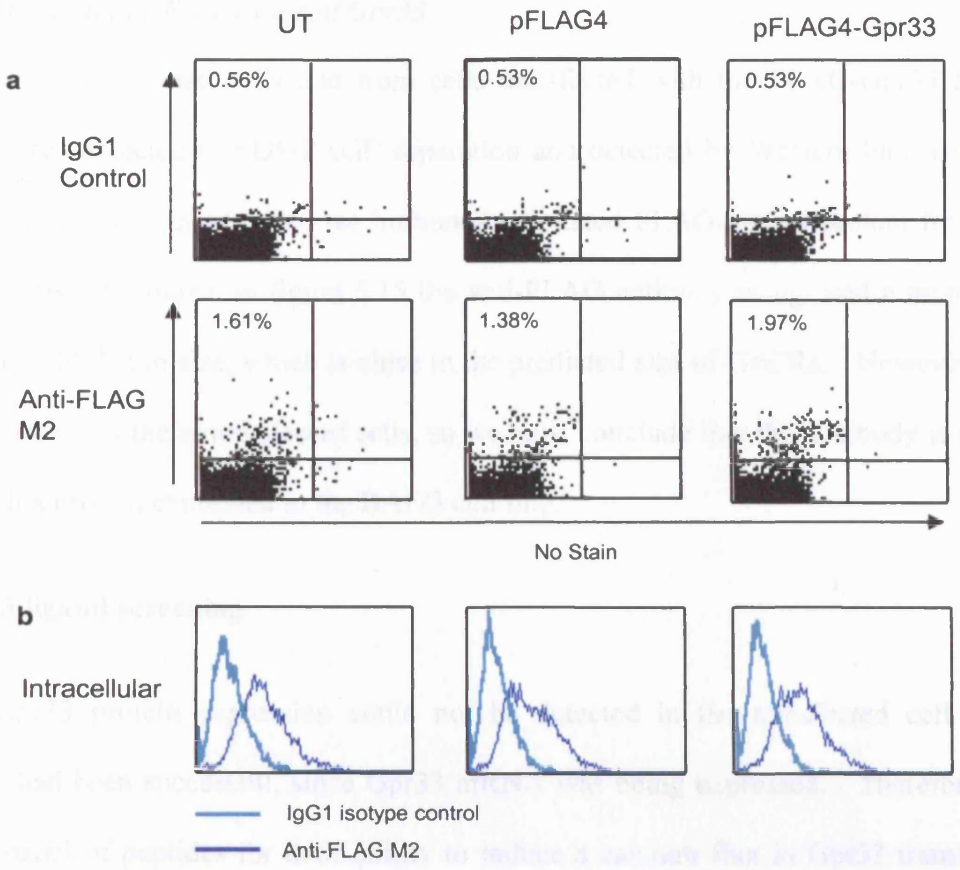


Figure 5.14 Staining of FLAG-Gpr33 transfectants by the anti-FLAG M2 antibody Baf/3 cells which were either untransfected (UT), transfected by the pFLAG-CMV-4 vector alone (pFLAG4), or with the pFLAG-CMV-4-Gpr33 construct (pFLAG4-Gpr33) were stained with the M2 anti-FLAG antibody **a)** Cell surface staining **b)** Intracellular staining.

the untransfected and Gpr33 transfected cells, again this strongly indicates that the M2 antibody is cross-reacting with an unrelated protein.

5.2.3.3 Western blot of FLAG-tagged Gpr33

Lysates from untransfected cells and from cells transfected with the FLAG-Gpr33 fusion construct, were subjected to SDS-PAGE separation and detected by Western blot using an anti-FLAG antibody. In addition, we immunoprecipitated FLAG-tagged protein from the transfected cells. As shown in figure 5.15 the anti-FLAG antibody recognised a protein of approximately 40kDa in size, which is close to the predicted size of GPCRs. However, this protein also exists in the untransfected cells, so we must conclude that this antibody is cross-reacting with a protein expressed in the BAF/3 cell line.

5.2.4 Gpr33 ligand screening

Although Gpr33 protein expression could not be detected in the transfected cell lines, transfection had been successful, since Gpr33 mRNA was being expressed. Therefore, we screened a panel of peptides for their ability to induce a calcium flux in Gpr33 transfected cells. The peptides and proteins chosen for screening were known agonists for receptors with the highest homology to Gpr33 (Marchese *et al.*, 1998)(Table 5.1). The majority of the agonists were identified as ligands for human receptors, but at least two, amyloid β protein and temporin A (TA), have been shown to act on mouse FPR2 (mFPR2), the homologue for human FPRL1. We detected a calcium flux in cells treated with the MMK-1 peptide. However, this flux was apparent in both untransfected and transfected cells (Figure 5.16). No calcium flux was detected for any of the other peptides tested. Therefore we were unable to identify a ligand for Gpr33 amongst the panel of peptides screened.

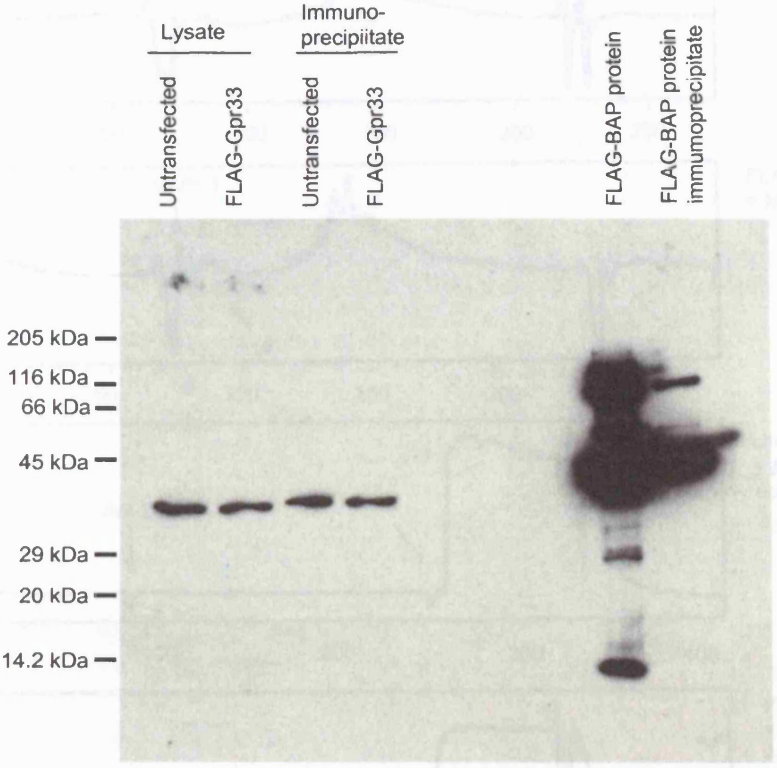


Figure 5.15 Immunoblot of FLAG-Gpr33 transfected cells
Proteins from Baf/3 cells, untransfected or tranfected with the FLAG-Gpr33 contruct were detected on a Western blot using the anti-FLAG M2 antibody. The samples were either cell lysates or proteins immunoprecipitated from the lysates using anti-FLAG M2 antibody. The FLAG-BAP protein is a control with a size of 49 kDa.

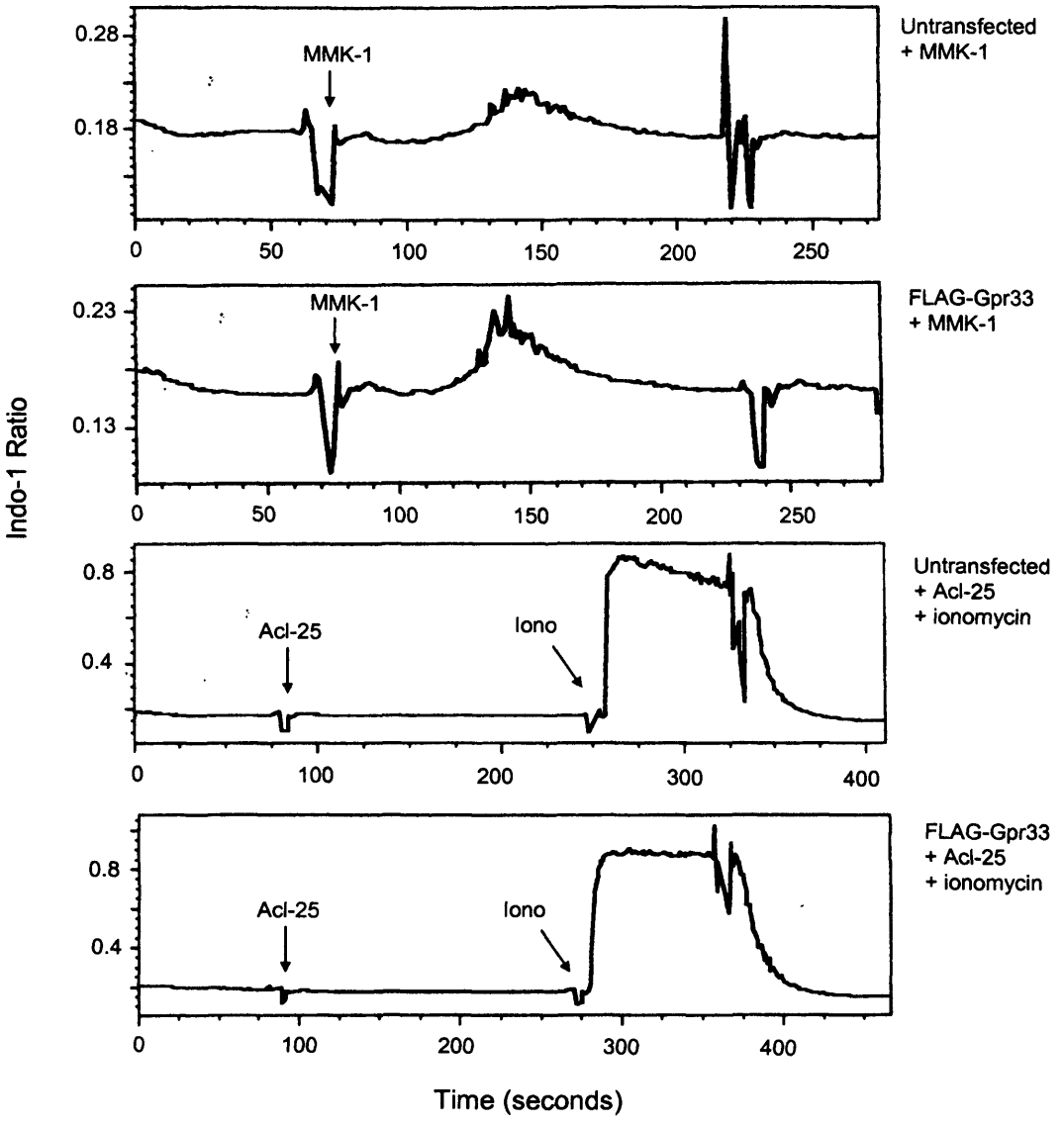


Figure 5.16 Gpr33 ligand screening Gpr33 transfected Baf/3 cells loaded with Indo-1 dye were stimulated with peptides or proteins and the calcium flux detected by the ratio of flourescence at 330 nm to 398 nm. Ionomycin (Iono), which is known to induce a clacium flux in all cell types, was used a positive control.

Table 5.1 Peptides and proteins screened in calcium flux assay

Peptide	Sequence	Receptor	Reference
A5	SLLWLTCRPWEAM	FPRL1	(Klein *et al.*, 1998)
Annexin 1 (Acl-25)	Ac-AMVSEFLKQAWFIEN EEQEYVQTVK	FPR, FPRL1, FPRL2	(Ernst *et al.*, 2004)
HIV-derived F	EGSDTITLPCRIKQFINMWQE	FPRL1	(Deng *et al.*, 1999)
HIV-derived T20	Ac-YTSLIHSLIEESQNQQEKN EQELLELDKWASLWNWF-NH2	FPR	(Su *et al.*, 1999)
HIV-derived V3	RIHIGPGRAFYTTKN	FPRL1	(Shen *et al.*, 2000)
Humanin	MAPRGFSCLLLLTSEIDLPVKRRA	FPRL1	(Ying *et al.*, 2004)
I4G10-C	FLPIIASLLGKLL-NH2	FPRL1	(Chen *et al.*, 2004)
I4S10-C	FLPIIASLLSKLL-NH2	FPRL1	(Chen *et al.*, 2004)
MMK-1	LESIFRSLLFRVM	FPRL1	(Klein *et al.*, 1998)
Rana-6	FISAIASMLGKFL-NH2	FPRL1	(Chen *et al.*, 2004)
T1P	FLPIVGKLLSGLL-NH2	Unknown	(Chen *et al.*, 2004)
TA	FLPLIGRVLSGIL-NH2	FPRL1, mFPR2	(Chen *et al.*, 2004)
W	WKYMVm	FPR, FPRL1, FPRL2	(Le *et al.*, 1999) (Christophe *et al.*, 2001)
Amyloid β protein	DAEFGHDSGFEVRHQKLVFFAED VGSNKGAIIGLMVGGVVIA	FPRL1, mFPR2	(Tiffany *et al.*, 2001)
Prion protein (106-126)	KTNMKHMAGAAAAGAVVGGLG	FPRL1	(Le *et al.*, 2001c)
Chemerin (human)	NCBI: Y14838	CMKLR1	(Meder *et al.*, 2003; Wittamer *et al.*, 2003)
Chemerin (mouse)	NCBI: NP_082128	CMKLR1	(Meder *et al.*, 2003; Wittamer *et al.*, 2003)
fMLF	Formyl-MLF	FPR	(Le *et al.*, 2001a)

5.2.5 Generation of polyclonal antibodies against Gpr33

The peptide sequences which we used to immunise mice for production of monoclonal antibodies spanned a predicted extracellular loop of Gpr33. However, it is impossible to predict the conformation of this region, and the native protein could be folded in such a way that this region is not accessible to an antibody or is not recognised by an antibody raised against linear peptides. Therefore, we decided to raise antibodies against another site in Gpr33, the N-terminal region (Figure 5.6). Although this portion of the molecule contains potential N-glycosylation sites, it might adopt a more linear structure *in vivo*. Polyclonal antibodies were raised against this region by conjugating the N-terminal 15 amino acids to a carrier protein and immunising rabbits.

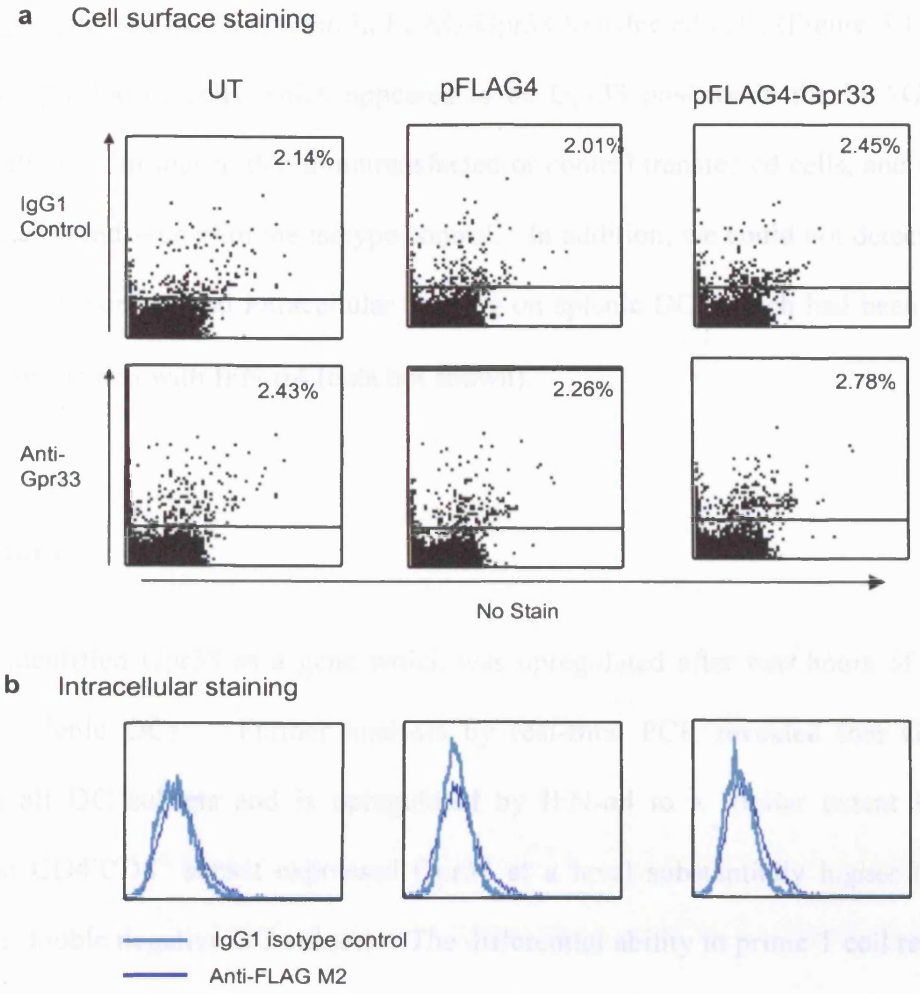


Figure 5.17 Staining of FLAG-Gpr33 transfectants with polyclonal anti-Gpr33
Baf/3 cells which were either untransfected (UT), transfected by the pFLAG-CMV-4 vector alone (pFLAG4), or with the pFLAG-CMV-4-Gpr33 construct (pFLAG4-Gpr33) were stained with the rabbit polyclonal anti-Gpr33 antibody **a)** Cell surface staining **b)** Intracellular staining.

5.2.5.1 FACS staining with the polyclonal anti-Gpr33 antibody

Using the polyclonal anti-Gpr33 antibody, we attempted to detect both cell surface and intracellularly expressed Gpr33 protein in FLAG-Gpr33 transfected cells (Figure 5.17). As shown, the proportion of cells which appeared to be Gpr33 positive in the FLAG-Gpr33 transfected cells was similar to that in untransfected or control transfected cells, and was not above the background seen with the isotype control. In addition, we could not detect Gpr33 expression by cell surface and intracellular staining on splenic DCs which had been treated either *in vivo* or *in vitro* with IFN-α4 (data not shown).

5.3 Discussion

We initially identified Gpr33 as a gene which was upregulated after two hours of IFN-α4 treatment in splenic DCs. Further analysis by real-time PCR revealed that Gpr33 is expressed in all DC subsets and is upregulated by IFN-α4 to a similar extent in each. However, the $CD4^-CD8^+$ subset expressed Gpr33 at a level substantially higher than the $CD4^+CD8^-$ or double negative DC subsets. The differential ability to prime T cell responses observed by $CD8^+$ and $CD8^-$ DC subsets may depend to some extent, on their tissue distribution and migratory properties. Indeed, DC subsets are distributed in distinct anatomical locations in both the spleen (Pulendran *et al.*, 1997) and the Peyer's patch (Iwasaki *et al.*, 2000). In the spleen, $CD8^+$ DCs are concentrated in the T cell rich PALS, whereas $CD8^-$ DCs are found in the marginal zone (Pulendran *et al.*, 1997). Similarly, in the Peyer's patch, $CD8^-$ DCs are positioned for antigen capture in the subepithelial dome, with the $CD8^+$ DCs residing in the T-cell rich interfollicular region. This difference in location could be attributed to expression of CCR6 by $CD8^-$ DCs and their ability to migrate towards CCL20/MIP-3α (Iwasaki *et al.*, 2000).

$CD8^+$ and $CD8^-$ DCs subsets also appear to have different capacities for migration *in vivo*. In experiments where DCs were injected into the footpad of mice, only $CD8^-$ DCs were detectable in the draining lymph node after 22-24 hours (Ruedl *et al.*, 1999; Smith *et al.*, 1999). Drake et al. (Drake *et al.*, 2001), showed that although both subsets could migrate to the draining LN after s.c. injection, it was the $CD8^-$ subset that preferentially did so. In a more recent study, only $CD8^+$ DCs which had been matured were seen to traffic to the draining LN, but again with lower efficiency than $CD8^-$ DCs (Colvin *et al.*, 2004b). This apparent difference in LN homing ability could be attributed to a lower capacity to migrate in response to CCL19 and CCL21 seen *in vitro*, even though no difference in chemokine receptor expression between the subsets could be detected . The route of administration is also important, since both mature DC subsets localised in the T cell areas of the spleen after i.v. injection. The impaired ability of $CD8^+$ DCs to reach the LN after s.c injection may be due to a lack of expression of adhesion molecules which would prevent crossing of endothelial cells into lymphatic vessels. In mice, $CD8^-$ DCs express higher levels of the adhesion molecule CD11b. However, this does not appear to mediate *in vitro* transmigration of epithelial cells in response to CCR7 ligands (Colvin *et al.*, 2004a). Alternatively, expression of as yet unidentified chemokine receptors could represent a mechanism for retention of $CD8^+$ DCs in the peripheral tissues. Gpr33 represents a candidate which could confer the different migratory properties of $CD8^+$ DCs.

Gpr33 shares 35% amino acid identity with chemokine-like receptor 1 (CMKLR1, formerly Chem23, Dez) (Marchese *et al.*, 1998), which is expressed by pDCs in human blood (Zabel *et al.*, 2005). CMKLR1 mediates migration to a proteolytically regulated chemoattractant, chemerin, activated during blood coagulation, suggesting a role for CMKLR1 in attracting

pDCs to sites of tissue damage. In contrast to CMKLR1 expression, Gpr33 expression was much lower on pDCs than on the $CD8^+$ DCs. In addition, treatment of Gpr33 transfected cells with chemerin did not induce a calcium flux, and so Gpr33 and CMKLR1 appear to have different ligand specificities. Therefore the function of the two receptors may not be closely related.

To gain further insight into the function of Gpr33, we investigated mRNA expression in other cells of the immune system. Expression of Gpr33 mRNA in lymphocytes was comparable to the untreated $CD4^+CD8^-$ DCs, with no detectable regulation by IFN-α4. Expression of Gpr33 was lower in macrophages than in $CD4^+CD8^-$ DCs which suggests that the role of Gpr33 is not to mediate migration of phagocytic cells to sites of infection, as is thought to be the case for formyl peptide receptors (Le *et al.*, 2002). However it may be of interest to test for expression in neutrophils, which are also known to respond to N-formylated peptides (Schiffmann *et al.*, 1975).

To characterise cell surface expression of Gpr33, we attempted to raise antibodies against it. First, we generated rat monoclonal antibodies against the 2^{nd} putative extracellular loop by immunisation with three overlapping peptides. Although we isolated hybridomas which produced antibodies against the immunising peptides, they did not appear to detect native protein expressed on a transfected cell line or on IFN-α4-treated DCs. There are problems inherent with this approach. Firstly, the amino acid sequence of rat Gpr33 is similar in sequence to mouse Gpr33, with only three amino acids differing over the 26 amino acid sequence chosen for the immunisations (Figure 5.6). The immunising peptides A1 and A2 contained all three of these amino acid changes, whereas the A3 peptide only contained one amino acid difference, which could explain why the rats did not mount a response to this peptide. Secondly, since the immunisations were with linear peptides, and our initial

screening method selected antibodies specific for the linear peptides, we may have isolated hybridomas which were not specific for the native conformation of this extracellular loop of Gpr33. Unfortunately, since the isotype of the three monoclonal antibodies generated was IgM, they would be of relatively low affinity and may have worked in the ELISA due to the presence of multiple antigens which would bind the pentameric structure more efficiently. It is likely that these antibodies would not recognise cell surface-expressed Gpr33 as efficiently. To test whether the antibodies could detect Gpr33 in a denatured form, we carried out Western blotting of transfected cell lines. Since this was unsuccessful, it was impossible to determine whether the antibodies were not specific for Gpr33 or whether Gpr33 mRNA in the transfected cells was not being translated. A lack of correlation between mRNA expression and cell surface expression of chemokine receptors has previously been reported (Sallusto *et al.*, 1998c).

We tried to overcome these problems in two ways, firstly, we attempted to clone Gpr33 in frame with an expression tag in order to verify expression on the surface of transfected cells. We could not detect cell surface or intracellular expression by FACS or by Western blot, which suggested that the Gpr33 mRNA was not being translated. However, if the N-terminus of Gpr33 is glycosylated this could interfere with binding to the FLAG tag. In addition, we have cloned Gpr33 with the initiating methionine still present. Since the FLAG tag is short, the start codon is only 40 base pairs from the Gpr33 start codon. In this case the ribosome may have a preference to initiate translation at the second methionine which would prevent translation of the FLAG-Gpr33 fusion protein.

Secondly, we had antibodies raised against the N-terminal region of Gpr33. We initially chose to avoid this region due to the presence of three potential N-glycosylation sites, which would interfere with antibody binding. However, antibodies against chemokine receptors

are commonly raised against this region, and antibodies against CMKLR1 were generated in this way (Zabel *et al.*, 2005). It must be noted though, that unlike Gpr33, the N-terminal region of CMKLR1 does not contain any N-glycosylation sites.

The final stage of the characterisation of Gpr33 was to determine the ligand. To do this we screened a panel of ligands for their ability to induce a calcium flux in Gpr33 transfected cells. We chose a panel of ligands which were already known to be agonists for CMKRL1 and the formyl peptide receptors. However, it is difficult to predict the nature of the ligand when the closest relatives share such low homology (35%). Indeed, neither chemerin, the CMKRL1 ligand, or any of the formyl peptide receptor ligands induced a calcium flux in Gpr33 transfected cells, although again, we have to accept the possibility that Gpr33 protein was not expressed in the transfected cells.

Gpr33 contains conserved residues which indicate that it is a functional GPCR. A conserved acidic-arginine-aromatic triplet present at the N-terminal end of the second cytoplasmic loop has been suggested to interact with G-proteins (Attwood *et al.*, 1991). Although Gpr33 does not contain the initial acidic residue, the arginine and aromatic tyrosine are present (Figure 5.5). In addition, the C-terminal region contains several threonine and serine residues, which are potential phosphorylation sites in desensitised receptors (Murphy, 1994). However, without the identification of a functional ligand, Gpr33 remains an orphan GPCR.

Chapter 6: Final Discussion

DCs have been described as 'nature's adjuvant', since during an infection they facilitate the proliferation of naive antigen-specific T cells. This unique property makes them prime candidates for the development of new vaccination strategies. In order to manipulate DCs so that they can be used to develop effective vaccines, a detailed knowledge of their biology is required. One aspect of DC biology which requires particular attention is their migration, both to sites of infection for efficient uptake of antigen, and to the secondary lymphoid tissues where they can interact with T cells. Secondly, it is vital to determine how stimuli from pathogens and the host affect DC maturation and how they influence the type of response elicited by the DCs. In addition, a knowledge of the molecular mechanisms by which DCs exert their immunogenic function may aid the development of efficient vaccines.

Early studies in the mouse showed that antigen- or gene-loaded DCs could induce immunity *in vivo* (Inaba *et al.*, 1990; Sornasse *et al.*, 1992) and that DCs loaded with tumour antigens could induce anti-tumour immunity (Gilboa *et al.*, 1998). *Ex vivo* generated, antigen-loaded DCs have been used as vaccines in patients with cancer (Davis *et al.*, 2003) and chronic HIV infection (Lu *et al.*, 2004). However, antigen-loaded immature DCs can induce tolerance (Fu *et al.*, 1996) and the antigen presenting DC subset can also influence the outcome of the immune response (Maldonado-Lopez *et al.*, 1999; Pulendran *et al.*, 1999). Therefore, these factors must be considered in DC-based vaccine design.

Currently, DNA vaccines which directly manipulate DCs *in vivo* are being developed. These vaccines work by using a combination of plasmids which encode proteins to both recruit and activate DCs, along with the plasmid encoding the specific antigen. For example, in one study co-vaccination with plasmids encoding CCL3 (MIP-1α) and fms-like

tyrosine kinase 3 (Flt3L) enhanced IFN-γ production by T cells specific for the antigen encoded by a co-injected plasmid (Sumida *et al.*, 2004). In this case CCL3 acts by recruiting immature DCs expressing the receptor CCR5, to the site of vaccination. Once there, they are activated by Flt3L, which also expands DC numbers. Therefore, this increases the number of DCs taking up antigen and the likelihood that these DCs will mature and migrate to the LN.

DC maturation is a complex process, which has evolved to facilitate 1) efficient processing and presentation of antigens which have been captured at sites of infection, 2) transport of the antigens to sites where they can be recognised by antigen-specific naive T cells and 3) delivery of antigen together with signals which instruct T cells on the type of response to be elicited (Banchereau *et al.*, 2000). DC maturation must therefore be tightly regulated to avoid the initiation of inappropriate immune responses. DC maturation can be triggered directly by pathogen components, or indirectly by 'danger signals' such as inflammatory cytokines (Matzinger, 2002; Medzhitov *et al.*, 2002). Indeed the presence of different cytokines during maturation of DCs *in vitro* influences how they function in the adaptive immune response. For example DCs differentiated in the presence of IL-4 are less efficient at inducing CTL priming than DCs differentiated in the presence of IL-15 (Mohamadzadeh *et al.*, 2001). Therefore careful consideration must be taken in how DCs to be used for vaccination are generated *in vitro*.

DCs matured in the presence of IFN-α strongly promote a Th1 response (Santini *et al.*, 2000), which is vital in anti-viral and anti-tumour immunity. IFN-α has been used in combination with poly(I:C) and a type-1 polarising cytokine cocktail (TNF-α/IL-1β/IFN-γ) to generate mature type-1-polarised DCs in a serum-free medium (Mailliard *et al.*, 2004). In addition to exhibiting the phenotypic and migrational characteristics of mature DCs, these

DCs had the ability to produce IL-12 after CD40L stimulation, making them superior inducers of antigen-specific CTLs. To identify the mechanisms by which IFN-I is able to enhance the maturation and function of DCs, we have investigated genes regulated by IFN-I in splenic and BM-derived DCs.

6.1 Identification of genes induced in DCs by IFN-I using RDA

By using the technique of RDA we hoped to rapidly and economically identify IFN-induced genes associated with DC function, since this technique requires only basic laboratory equipment and relatively inexpensive molecular biology reagents. This initial approach proved valuable, since in addition to identifying several known IFN-induced genes we also succeeded in identifying transcribed products representing novel ISGs.

Initially, RDA was used to identify genes regulated by IFN-I after a two hour culture of total splenic DCs or DCs separated into the $CD11b^+$ ($CD8^-$) and $CD11b^-$ ($CD8^+$) DC subsets. To verify that the novel genes or sequences identified were induced by IFN-I we either probed a southern blot or carried out quantitative PCR on independently generated samples. Although we found novel sequences or genes which were exclusively upregulated by IFN-I in either DC subset we did not exclude the possibility that this was due to day to day experimental variation. This is possible, since none of the novel sequences detected in the RDA on sorted DC subsets were detected in the RDA on total DCs, and conversely the novel sequences identified in total DCs were not detected in either DC subset. Therefore it is not clear whether these differences truly reflect differences between the subsets or experimental variation. This could be determined by carrying out Q-PCR using RNA isolated from sorted DC subsets.

In addition we carried out an RDA to identify genes differentially regulated after six hours of culture in the presence of IFN-I. This RDA yielded promising results since in addition to identification of an MHC class II gene, which is consistent with a mature DC phenotype, we identified a number of genes which had not previously been shown to be IFN-induced such as the p101 regulatory subunit of P13-kinase. Several genes with unknown function were also identified at the six hour timepoint, and we extended the characterisation of these genes by searching for functional domains in their amino acid sequence. The genes were found to contain domains which are present in proteins involved in nucleic acid binding, intracellular protein transport and pathogen recognition. Although these proteins did not enter the original remit of this study they may be of interest in DC biology. In particular, the Niemann-Pick type C2 (Npc2) protein, which we also detected by microarray analysis after six hours IFN-I treatment may merit further investigation. Npc2 contains the ML (MD-2-related lipid recognition) domain found in proteins which act as cofactors for cell surface recognition of LPS (Inohara *et al.*, 2002). A second ML domain containing protein, known as MD-1 or Ly86, was also found to be induced by IFN-I in splenic DC after six hours of culture using microarray analysis. However, a specific function cannot be inferred simply by the presence of an ML domain since these domains may also be involved in host lipid recognition and metabolism (Inohara *et al.*, 2002). Another gene identified in this experiment encodes lymphocyte cytosolic protein (LPL), also known as plastin-2, which plays an important role in control of bacterial infections (Chen *et al.*, 2003). LPL-deficient mice cannot generate an adhesion-dependent respiratory burst in response to integrin ligands. LPL is an actin crosslinking protein, however it does not control integrin-dependent phagocytosis, adhesion or migration. Instead it is thought that actin remodelling by LPL provides a scaffold needed for the cells to assemble the integrin-initiated signaling pathway (Chen *et al.*, 2003).

As expected, a number of genes identified were known ISGs involved in the anti-viral response and apoptosis, such as Gbp2 and Pml. In fact, the pleiotropic nature of IFN-I may impede this type of experiment, since it means that the proportion of identified genes involved specifically in regulating DC function is likely to be low. One way to differentiate between ubiquitously expressed ISGs and those whose expression is restricted to DCs is to cross-check with public databases of known ISGs such as that of de Veer et al (de Veer *et al.*, 2001). However the data available in this resource is somewhat limited. Therefore quantitative PCR to detect expression of genes in other cells of the immune system was used to determine the expression of unknown ISGs, several of which were also highly upregulated by IFN-I in T and B cells. It may also be useful to test for expression of these genes in non-immune cells.

Although the RDA experiments had been successful, the data was restricted to only a subset of genes induced by IFN-I. This was in part due to the preferential amplification of certain genes, particularly at the two hour timepoint where many of the cloned difference products represented the genes Gbp2 and Pml. This could possibly have been resolved by competing these genes out by spiking the driver samples with the Gbp2 and Pml sequences. Indeed, where a large number of differences are expected between two samples, iterative RDA can be carried out, where the products of an initial RDA are amplified and added to the driver in a subsequent experiment. This prevents the amplification of previously identified products and allows the amplification of a different set of difference products (Frazer *et al.*, 1997).

6.2 DC activation

Our aim was to identify genes regulated directly by IFN-I signaling in DCs. This necessitates their culture *in vitro*, rather than *in vivo* treatment where the IFN-I could act

indirectly through other cell types which in turn could interact with DCs to influence their function. Even *in vitro* however, cytokines produced by the DCs, such as IL-15, can act in an autocrine fashion to further induce their maturation (Mattei *et al.*, 2001). A further complication of using an *in vitro* culture system is that isolation and culture of murine DCs is known to induce their maturation. For example, DCs isolated from the spleen are known to be activated by adhesion to plastic (Ossevoort *et al.*, 1992) and DCs from the adherent or low density fraction of the spleen have been shown to strongly upregulate CD86 expression after overnight culture (Inaba *et al.*, 1994). This upregulation was not due to contaminating LPS, since DCs from the LPS-nonresponder strain C3H/HeJ also had elevated levels of CD86 after overnight culture. In addition, Langerhans cells cultured *in vitro* mature into DCs with potent immunostimulatory capacity (Schuler *et al.*, 1985). GM-CSF, which is produced by many cells including the keratinocytes of the skin (Witmer-Pack *et al.*, 1987), and IL-1 have been shown to mediate Langerhans cell maturation (Witmer-Pack *et al.*, 1987; Heufler *et al.*, 1988). It is possible that during tissue dissociation these cytokines are released in sufficient quantities to activate the DCs. Alternatively, it has been suggested that inhibition of DC maturation could occur *in vivo*, and removing them from this environment may trigger maturation (Reis e Sousa, 2004).

The mechanical manipulations involved in DC isolation may trigger DC maturation. Dissociation of BM-derived DCs and transfer to fresh plates for overnight culture has been shown to result in maturation equivalent to that induced by LPS (Gallucci *et al.*, 1999). In addition, recent experiments showed that induction of a calcium flux in one DC, triggered by only the touch of a micropipette tip, was transmitted in a matter of seconds via connecting structures known as tunneling nanotubules to surrounding DCs (Watkins *et al.*, 2005). The transmitted calcium flux was enough to induce veil extension in DCs similar to that seen with

direct LPS stimulation. This highlights the sensitivity of DCs to mechanical stimulation and the concept that DC maturation can be triggered by disrupting interactions with the surrounding cells *in situ*. This would complicate the analysis of gene expression, since if the DCs are maturing in culture medium alone this may lessen the differences seen in IFN-treated DCs.

The 'spontaneous' maturation of splenic DCs during culture has been demonstrated (Montoya *et al.*, 2002). In this case the DC maturation was shown to be partially dependent on the autocrine action of IFN-I, since addition of anti-IFN-I inhibited up-regulation of CD40, CD86 and MHC class I. Indeed, this could explain the upregulation of several of the novel ISGs identified by RDA during culture in the absence of exogenous IFN-I. Production of IFN-I by the DCs also potentially reduces the number of IFN-induced genes identified. However, although both splenic and BM-derived DCs produce IFN-I, these levels are extremely low (8-16 units/10^6 DCs) (Montoya *et al.*, 2002) compared to the levels in our culture conditions where exogenous IFN-I was added (4000 units/10^6 DCs).

6.3 Global analysis of gene expression in IFN-I stimulated DCs

Using microarray analysis we identified more than one thousand genes and expressed sequences whose expression was upregulated by IFN-I in either splenic or BM-derived DCs. These genes fall into one of several groups. Firstly we found known IFN-induced genes whose function is unlikely to be specifically linked with the function of DCs. Of these, some have well defined roles. For example, Mx1 is a prototypical anti-viral gene, whilst the function of others, like the Ifit genes is less clear. Second we found genes which have previously been shown to be induced by IFN-I in DCs such as IL-15, CXCL9 and CXCL10, and play important roles in DC biology. These types of genes act as useful markers to

indicate that the experiments are working correctly. In fact, before carrying out microarray analysis we tested for Mx1 upregulation in IFN-I treated samples by quantitative PCR.

The number of ESTs identified in these experiments is extremely high, with just under 25% of all differentially regulated sequences being ESTs. These sequences represent a large number of genes with the potential for shaping the immune response directed by DCs. However when little or no functional characterisation is available for these sequences it is challenging to determine which, if any encode proteins important for mature DC function. One way would be to use a circumstantial approach, that is, to test whether a certain gene's expression pattern coincides with that of other genes known to be involved in a specific process. This clustering technique is widely used when dealing with microarray data, but is only feasible where multiple timepoints and/or stimuli have been tested. In addition, this type of study may be more amenable to a population of cells which have been generated in culture and therefore are more homogenous in their stage of maturation (Granucci *et al.*, 2001b). Also, since many aspects of DC maturation are likely to be occurring simultaneously, it may not be possible to link genes to particular processes simply by examining the timing of their expression.

Another approach for determining the function of ESTs is to carry out systematic searches using the advanced bioinformatics tools now publicly available on the world-wide web. In this respect, the Netaffx web resource (Affymetrix) is extremely useful since many of the ESTs have been annotated with information such as the conserved protein domains present and known homology with other proteins. However, attempting to extract functional information on ESTs in this manner is extremely time consuming and may not always yield meaningful results. As more information becomes available on databases such as Netaffx, the analysis of microarray data will be greatly facilitated.

As discussed, the wide reaching effects of IFN-I may hinder the identification of genes associated with DC function. Ideally, parallel experiments to identify IFN-I induced genes in cells unrelated to the immune system could have been carried out. The IFN-regulated genes identified could then easily be subtracted from the data from DCs to generate a list of DC-specific IFN-I induced genes.

When analysing the expression of mRNA it must be taken into consideration that changes in mRNA levels may not necessarily 'translate' into a significant change in the level of protein sufficient for a functional consequence. For example, expression of mRNA for certain chemokine receptors, as has been shown for CCR1, CCR2, CCR5 and the IL-8 receptor, does not always correlate with cell surface expression or a functional response (Sozzani *et al.*, 1997; Sallusto *et al.*, 1998c). Therefore it is important to verify protein levels by other means such as ELISA, which we have used to quantitate levels of several cytokines and chemokines identified. Related to this issue is the matter of determining the fold change in mRNA levels that are considered to be significant. Genes whose expression increases by a high fold change may initially appear interesting but might not always be the most significant, for example if they are expressed at very low levels. Similarly the abundance of a transcript cannot generally be considered as a factor in determining a gene's importance since certain proteins, for example secreted proteins, may be required in much larger quantities than others, such as cell surface receptors. Finally, a small increase in the level of mRNA may result in the expression of protein such that a certain threshold is reached, allowing a functional response. Similarly a small change in protein may affect the ratio of subunits which make up for example, a cytokine, which could allow a functional form to predominate.

In addition to known DC ISGs, we also identified novel IFN-I regulated genes which could play important roles in DC function. These included genes with known function, such as the IL-6 receptor and the IL-6 receptor signal transducer, which were both suppressed by IFN-I. The large number of genes identified encoded proteins with diverse molecular functions from transcription to cell adhesion, and which control many cell processes such as cell cycle and cell migration. Although it may be difficult to extrapolate how each of these genes may influence DC function, the data help to provide an overall picture of the properties IFN-I treatment confers on DCs. For example, we detected upregulation of several TLRs in BMDCs after IFN-I treatment; in particular, TLRs which are known to bind viral components were identified. Therefore, stimulation with IFN-I appears to promote the expression of molecules for detection of viruses which may be necessary for their full maturation or development into Th1 promoting DCs. IFN-I also increased expression of MyD88 in splenic DCs, which mediates signal transduction from all TLRs. As would be expected, the cytokine and chemokine production by IFN-I treated DCs would strongly promote a Th1 profile.

In addition, these studies provide a wealth of data for future studies and several genes identified may merit further investigation. One of these, chemokine-like factor super family 6 was induced after six hours of IFN-I treatment in BMDCs. It would be interesting to test whether this protein has chemotactic activity towards T or B cells.

6.4 Gpr33

From the microarray data, we chose to investigate a gene which we believed could be involved in the migration of murine DCs, Gpr33, that was upregulated after two hours of IFN-I stimulation in splenic DCs. In addition to acting as a chemoattractant receptor, it is

also possible that Gpr33 acts as a pattern recognition receptor (PRR). Formyl peptide receptors (FPRs), of which FPRL1, FPR1 and FPRL2 share 31-33% amino acid identity with Gpr33, are known to bind molecules derived from pathogens, as well as molecules from damaged host cells and abnormal self proteins which are associated with disease (Le *et al.*, 2002). Binding of FPRs activates phagocytes, increasing cell migration, phagocytosis and release of proinflammatory cytokines (Le *et al.*, 2001a). Therefore these receptors appear to act as PRRs which alert cells to the presence of danger.

DCs are known to express FPRs and migrate in response to various of their agonists (Sozzani *et al.*, 1995), however whether they also mediate DC activation signals is unclear. This is perhaps not unlikely, since CCR5, also a GPCR involved in cell migration can send maturation signals to DCs. One ligand for CCR5, CCL5 has been shown to induce DC activation, as measured by the release of inflammatory chemokines after stimulation (Fischer *et al.*, 2001). In addition, ligation of CCR5 has been shown to mediate IL-12 production by DCs after stimulation with the Toxoplasma gondii extract, STag (Aliberti *et al.*, 2000). Therefore this chemokine receptor has the dual function of recruiting DCs to sites of infection and inducing their activation. It is possible that activation of DCs may occur through other GPCRs such as Gpr33.

Importantly however, FPR signaling is known to be responsible for the heterologous de-sensitization of various chemokine receptors including CCR5. Activation of FPRL1 by the HIV-1 gp120 F peptide or the synthetic peptide WKYMVm results in downregulation of CCR5 and CXCR4 (Deng *et al.*, 1999; Le *et al.*, 2001b; Li *et al.*, 2001). LXA_4, a ligand for FPRL1 which is induced by STag, also down-modulates CCR5, and results in "DC paralysis" where DCs can no longer produce IL-12 on re-stimulation by STag (Aliberti *et al.*, 2002). In fact there is considerable evidence demonstrating the suppression of IL-12 production by

FPR signaling (Braun *et al.*, 2001), which could prevent excessive and potentially damaging IL-12 production. An inability to produce IL-12 during culture with $CD4^+$ T cells has been shown to be associated with final maturation of DCs even though these DCs induced T cell IFN-γ production (Kalinski *et al.*, 1999b). Although sharing homology with FPRs, it is likely that Gpr33 binds distinct ligands and may be linked to different signaling pathways which could potentially produce the opposite outcome, such as suppression of IL-10 production. Finally, heterologous desensitization of CCR5 may be important for migration of DCs out of infected tissue, since downregulation of CCR5 has been shown during DC maturation (Sallusto *et al.*, 1998c).

Although the gene encoding Gpr33 in humans is a pseudogene, since it contains a stop codon at nucleotide position 418 of the open reading frame, there may be a functional homologue present in humans which carries out a similar function. If Gpr33 does recognise pathogen components, the loss of Gpr33 in humans may be explained by differences in evolutionary pressure exerted by different organisms which invade mice and humans. This is seen in the FPR family, since three FPR genes exist in the mouse for which there are no human counterparts suggesting the gene cluster has undergone differential expansion and therefore functional divergence of the encoded receptors (Le *et al.*, 2001a). Similarly, in the TLR family, TLR11 is functional in the mouse whereas the presence of a stop codon prevents TLR11 production in humans (Zhang *et al.*, 2004). Conversely, TLR10 is functional in humans but not mice (Takeda *et al.*, 2005). There could be two reasons for this, firstly it could be that the two TLRs have redundant functions and were therefore disposable. Alternatively they may have specificity for a pathogen component that is no longer relevant to the organism, in which case mutations preventing its function would not be disadvantageous.

We have demonstrated the potential of microarray analysis for identifying genes relevant to DC biology and therefore their application in DC-based vaccination. However we have also highlighted difficulties in the characterisation of interesting genes. The approach we took was to screen differentially expressed genes by identifying conserved protein domains or homology to functionally characterised proteins. This provides an indication as to the novel protein's function, which is essential for the design of experiments for their characterisation. The discovery of an IFN-I induced GPCR related to chemoattractant receptors provided a promising candidate for several reasons. It should be expressed at the cell surface and therefore antibodies against it could be raised. Although the ligand binding site for Gpr33 is not known, it was also possible that the antibodies raised would block the ligand binding site and therefore be useful in functional assays. Finally, the nature of the protein also made it possible for us to carry out ligand screening, which if successful could have been tested in migration assays. Future studies could be directed at secreted molecules which could be produced and tested for their function on other immune cells or on DCs themselves.

In conclusion, we have generated a large volume of data regarding the regulation of gene expression by IFN-I in DCs and have demonstrated the possibility of utilising this data to relate gene expression to the behaviour of DCs. Overall, the microarray data provides information on the processes triggered during activation of DCs by IFN-I. Translating expression data into functional properties, and identification of genes crucial for immunity induced by DCs remains a challenge for the future.

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