Plasticity in gene expression programmes of Dendritic Cells responding to ~~pathogens and derived components~~ ANTIGENIC

Antonia Pui-Yue Kwan

Submitted to the University of London for the degree of Doctor of Philosophy

January 2005

Virus Genomics and Bioinformatics Group
Department of Immunology and Molecular Pathology
Division of Infection and Immunity
Windeyer Institute of Medical Sciences
University College London

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

Abstract

Dendritic cells (DCs) are professional antigen presenting cells whose function is to initiate and shape an appropriate adaptive immune response. This requires an ability to distinguish differences between whole pathogens, in order to orchestrate effective downstream immunological outcomes. However, cellular re-programming of DC functions during these events are not well understood.

A paradigm of dendritic cell biology is that DCs have two modes of function that relate to their differentiation states. An immature DC functions as an immune sentinel, to monitor and interrogate its surroundings for pathogens. Encounter with such stimuli results in a process termed "maturation", where DCs acquire the properties of effective antigen presenting cells. However, this process of differentiation is complex. In this thesis, gene expression profiling of DCs exposed to pathogen components has revealed three distinct phases of maturation, with statistically significant expression of subsets of genes characterising these phases. Transcriptional regulation of the signalling pathways involving p38 and ERK MAP kinases important to DC function were identified. Specific inhibitors of p38 and ERK confirmed their differential role in DC maturation, with p38 activity being necessary for the initiation of DC maturation, whilst ERK activity persists to maintain DC survival.

Concurrent with the core maturation process is the DCs' ability to differentially respond to pathogens. Gene expression analysis of DCs exposed to whole viruses supports the model of DC plasticity to different pathogenic stimuli. Using exploratory cluster analysis and a novel vector algebra method, core and pathogen-specific gene expression programmes were identified. The programmes involving the differential regulation of cytokines were confirmed at the transcript level and at the protein level.

Together these data show that DCs mature to effective antigen presenting cells via an orchestrated pattern of at least three gene expression programmes. Superimposed on this core maturation response are pathogen-specific transcriptional programmes. Therefore, we conclude that DCs can translate different pathogenic stimuli into core DC maturation and pathogen-specific responses that together shape an appropriate adaptive immune response.

Acknowledgements

The completion of this thesis has depended on the efforts of numerous people apart from myself and for this I thank them. First and foremost thanks to my supervisor Paul Kellam for his enthusiasm, encouragement, dedication and constant support. Also thanks to my second supervisor Benny Chain for his advice and generous enthusiasm. Many thanks also to Mary Collins and Chris Boshoff for their invaluable advice. Thanks to Robin Weiss who has made the Wohl Virion Centre such an enjoyable place to work. I am very grateful to the University of London's Triangle Trust and Universities UK Overseas Research Student Award for funding me throughout this PhD.

Many thanks also to Tom Freeman and Debbie Williams at the Human Genome Mapping Project Resource Centre, University of Cambridge, for providing the cDNA microarrays. And thanks to our collaborators Wendy Barclay at the University of Reading, Toby Tuthill, Dave Rowlands and Eric Blair at the University of Leeds, for providing the purified virus preparations and for their invaluable advice and enthusiasm.

And warmest thanks to members of the Virus Genomics and Bioinformatics Group past and present for making the lab such a wonderful place to work: Catherine Gale, Rob Gifford, Eva Gramoustianou, Natasha Hidvegi, Ria Holzerlandt, Stephane Hue, Richard Jenner, Cheryl Li, Richard Myers, Jane Rasaiyaah, Edward Tsao, and Sam Wilson. Evenings at the pub, long hours and weekends will never be the same again. And many thanks to members of the Chain/Katz lab: Gabriele Pollara, Matthew Handley, Paul Kaye, and Cheryl Chiang, for tolerating my constant presence in their tissue culture lab.

Thanks also to Gordon Stewart concerning all matters MB PhD, and David Katz particularly for all his help and tireless support with issues regarding funding.

Lastly, I would like to thank my parents and my brother for tolerating my long absences from home, and for their constant encouragement and support.

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Abbreviations

ACTB	Actin, beta
AMP	Antimicrobial peptide (Dm)
ANOVA	Analysis of variance
AOAH	Acyloxyacyl hydrolase
APC	Antigen presenting cell
APOBEC	Apolipoprotein B mRNA editing enzyme catalytic polypeptide
ARHGAP	Rho GTPase activating protein
ATP	Adenosine triphosphate
AV	Annexin V
BAK1	Bcl2-antagonist/killer 1
Bcl2	B cell CLL/lymphoma 2
BIRC	Baculoviral IAP repeat-containing
bp	base pairs
CASP	caspase
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CD40L	CD40 ligand
cDNA	complementary DNA
CFLAR	CASP8 and FADD-like apoptosis regulator
CMV	Cytomegalovirus
cos	cosine
cov	covariance
CR	Complement receptor
CRADD	CASP2 and RIPK1 domain containing adaptor with death domain
cRNA	complementary RNA
CTL	Cytotoxic T lymphocyte
CTS	Cathepsin
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
Cy	cyanine
DAVID	Database for Annotation, Visualisation, and Integrated Discovery
DAXX	Death-associated protein 6
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM3 grabbing non-integrin
DD	Death domain
DEDD	Death effector domain-containing protein
DEPC	Diethyl pyrocarbonate
Dm	Drosophila melanogaster
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate mix
dsRNA	double-stranded RNA
DV	Dengue virus
eIF	Eukaryotic translation initiation factor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorter
FADD	Fas (TNFRSF6)-associated via death domain
FCS	Foetal calf serum
FDR	False Discovery Rate
FITC	fluorescein isothiocyanate
FLN29	FLN29 gene product

FOSL1	fos-like antigen 1
FPR	False positive rate
GADD	Growth arrest and DNA-damage-inducible
GALT	Gut-associated lymphoid tissue
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GO	Gene Ontology
gp	glycoprotein
HBSS	Hanks' Balanced Salt Solution
HCV	Hepatitis C virus
HDAC	Histone deacetylase
HGMP	Human Genome Mapping Project
HIV-1	Human immunodeficiency virus-1
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HRV	Human Rhinovirus
HSP	Heat shock protein
HSV	Herpes simplex virus
IAP	Inhibitor of apoptosis
ICAM	Intercellular adhesion molecule
IER	Immediate early response
IFIT	Interferon-induced protein with tetratricopeptide repeats
IFITM	Interferon induced transmembrane protein
IFN	Interferon
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IKB	Nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor
IL-1	Interleukin-1
Imd	Immune deficiency (Dm)
IRAK	Interleukin-1-receptor associated kinase
IRES	Internal ribosomal entry site
IRF	Interferon regulatory factor
IS	Immunological synapse
ISG	Interferon-stimulated genes
ISGF	Interferon-stimulated gene factor
ISRE	Interferon-stimulated response element
ITG	Integrin
ITGBP	Integrin binding protein
JAK	Janus kinase
JNK	c-jun kinase
kb	kilobase
KEGG	Kyoto Encyclopedia of Genes and Genomes
KSHV	Kaposi's sarcoma-associated herpesvirus
LBP	LPS-binding protein
LCP	Lymphocyte cytosolic protein
LDL-R	Low-density lipoprotein receptor
LFA-1	Lymphocyte function-associated antigen type I (integrin αL)
log	logarithm
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTA	Lipotechoic acid
Mal	MyD88-adapter-like
MALT	Mucosa-associated lymphoid tissue
MAP2K	Mitogen-activated protein kinase kinase
MAP3K	Mitogen-activated protein kinase kinase kinase
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein 1
MDC	Myeloid dendritic cell

MDCK	Madin-Darby canine kidney
MDS	Multi-dimensional scaling
MEF	Mouse embryonic fibroblast
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
MMTV	Mouse mammary tumour virus
moDC	monocyte-derived dendritic cell
MOI	Multiplicity of infection
mRNA	messenger RNA
MT	Metallothionein
MV	Measles virus
MWU	Mann-Whitney U test
MyD88	Myeloid differentiation primary response gene 88
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAPA	NSF attachment protein alpha
NCBI	National Center for Biotechnology Information
NES	Nuclear export signal
NF-κB	Nuclear factor of kappa light polypeptide gene enhancer in B cells
NK	Natural killer
NLS	Nuclear localisation sequence
NP	nucleoprotein
NRP	Neuropilin
NSF	N-ethylmaleimide-sensitive factor
OAS1	2',5'-oligoadenylate synthetase 1
PAK1	p21/Cdc42/Rac1-activated kinase 1
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDC	Plasmacytoid dendritic cell
PFAM	Protein Families Database of alignments and hidden Markov models (HMMs)
pfu	plaque forming units
PG	Peptidoglycan
PGRP	Peptidoglycan recognition protein (Dm)
PI	Propidium iodide
PKR	dsRNA-activated protein kinase R
PMT	Photomultiplier tube
poly(I:C)	polyinosinic-polycytidylic acid
PRKA	Protein kinase A
PRR	Pattern recognition receptor
PSMD10	Proteasome 26S subunit 10
PSME2	Proteasome activator subunit 2
RAC1	Ras-related C3 botulinum toxin substrate 1
RANTES	Regulated upon activation, normally T-expressed and secreted
RHD	Rel homology domain
RIPK	Receptor (TNFRSF)-interacting serine-threonine kinase
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	ribonucleoprotein complex
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RSV	Respiratory syncytial virus
RT-PCR	Reverse transcription polymerase chain reaction
RV	Rhinovirus
SAM	Significance Analysis of Microarrays

SARM	SAM and ARM containing protein
SD	Standard deviation
SE	Spring embedding
SEMA	Semaphorin
SERPINA1	Serine (or cysteine) proteinase inhibitor, clade A, member 1
SMAC	Supramolecular activation cluster
SNR	Signal to noise ratio
SOM	Self-organising map
SOS1	Son of sevenless homolog 1
SRF	Serum response factor
ssRNA	single-stranded RNA
STAT	Signal transduction and activator of transcription
TAB	TAK-1-binding protein
TAK-1	Transforming growth factor b-activated kinase-1
TANK	TRAF family member-associated NF-κB activator
TAP1	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)
TBK	TRAF family member-associated NF-κB activator (TANK) binding kinase
TESS	Transcription element search software
TGFβ	Transforming growth factor b
TH	T helper cell
TICAM-1	TIR domain-containing adaptor molecule-1
TICAM-2	TIR domain-containing adaptor molecule-2
TIMP	Tissue inhibitor of metalloproteinases
TIR	Toll/IL-1 receptor
TIRAP	TIR domain-containing adapter protein
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNFα	Tumour necrosis factor alpha
TNFAIP	Tumour necrosis factor alpha induced protein
TNFR	Tumour necrosis factor receptor-associated factor
TNFRSF	Tumour necrosis factor receptor superfamily
TNFSF	Tumour necrosis factor (ligand) superfamily
TOR1B	Torsin family 1, member B
TRADD	TNFRSF1A-associated via death domain
TRAF	Tumour necrosis factor receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRANSFAC	The Transcription Factor Database
TRIF	TIR domain-containing adapter inducing IFN-b
TRIKA	TRAF6-regulated IKK activator
UBE	Ubiquitin conjugating enzyme
UV	Ultra-violet
VAK	Virus-associated kinase
VEGF	Vascular endothelial growth factor
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
WASP	Wiskott-Aldrich syndrome protein
wnt	wingless-type MMTV integration site family

Chapter 1

Introduction

The vertebrate immune response to microbial pathogens relies on both innate and adaptive components. The innate response is immediate, providing a first line defence against many common microorganisms. Effector cells of the innate immune response comprise of neutrophils, basophils, eosinophils, monocytes, macrophages and dendritic cells that coordinate additional host responses by synthesising a wide range of inflammatory modulators. Adaptive immune components, comprising of T and B lymphocytes, have evolved a more antigen-specific means of defense that also provides increased level of protection from a subsequent re-infection with the same pathogen. The cells of the innate system play a crucial part in the initiation and subsequent direction of the adaptive immune response. Moreover, because there is a delay of 4 to7 days before the initial adaptive immune response takes effect, the innate response is crucial for controlling infections during this period.

The key role of dendritic cells (DCs) is to function as a bridge between the innate and adaptive immune response. Dendritic cells are an integral part of the innate immune system. As immune sentinels, dendritic cells survey their surroundings for potential pathogenic and inflammatory stimuli. Upon receipt of the right stimulation, DCs undergo a complex process of "maturation", with the end result of presenting antigen to T cells to initiate and shape the downstream adaptive immune response. Their role at the interface of innate and adaptive immunity make studies of dendritic cells crucial to the understanding of how immune responses to a variety of pathogenic stimuli are generated.

1.1 Innate immunity

Only vertebrates have developed a highly specific immune response that is based on the selection of somatically recombined B and T cell receptors. Other animals and plants lack this adaptive immune system, and instead rely on a relatively sophisticated system of innate immune defences. Even vertebrates rely totally on this innate immune response for the week it takes to develop a specific adaptive immune response. Similar to the adaptive immune response, a primary challenge to the innate immune system is how to discriminate the large numbers of potential pathogens from self cells and proteins, in order to mount an effective response.

The challenge of recognition of a large number of potential microorganisms is met by the use of pattern recognition receptors (PRRs) that recognise surface determinants conserved among microbes but absent in the host. Such patterns include lipopolysaccharides (LPS), peptidoglycans (PGs), mannans and other proteins and lipids specific to pathogens. PRRs activate the innate immune response by multiple and complex signalling cascades that ultimately regulate the transcription of target genes encoding effector molecules. The molecular signatures of pathogens have been termed "pathogen-associated molecular patterns" (PAMPs).

1.1.1 Pattern recognition receptors

Much of what is now known about innate immunity has come from studies in *Drosophila* (Brennan and Anderson, 2004). *Drosophila* is devoid of an adaptive immune system and relies on innate immune reactions for its defence. Genetic and molecular approaches have shown that *Drosophila* is a powerful model system to study innate immunity, as innate immunity seems to be remarkably conserved from flies to mammals (Hoffmann and Reichhart, 2002).

Drosophila innate immune responses comprise a humoral and cellular response that is distinct from the vertebrate adaptive immune system. *Drosophila* cellular defences consist essentially of phagocytosis, and the humoral response consists of the synthesis of antimicrobial peptides (AMPs). *Drosophila* produces nine distinct antimicrobial peptides with activity directed against various fungi, Gram-positive and Gram-negative bacteria (Brennan and Anderson, 2004). The promoters of the genes encoding these

peptides contain sequence motifs relating to mammalian NF-κB response elements (Hetru et al., 2003). Induction of antimicrobial peptides are a result of activation of two immune signalling pathways, namely Toll and immune deficiency (Imd). *Drosophila* Toll was originally identified as a transmembrane receptor required for the establishment of the dorsal-ventral axis in the developing embryo (Anderson et al., 1985). The Toll pathway is activated mainly by Gram-positive bacteria and fungi. In contrast, the Imd pathway modulates immune responses to Gram-negative bacteria. However, most AMP genes can be regulated by either pathway, depending on the type of infection. The selective activation of Toll and Imd by different classes of pathogens does however lead to specific AMP gene expression programmes (De Gregorio et al., 2002). The components of the Drosophila Toll and Imd pathways are shown in Figure 1.1.

The signalling pathway of *Drosophila* Toll show remarkable similarity to the mammalian interleukin-1 (IL-1) pathway, which leads to activation of NF-κB, a transcription factor responsible for many aspects of the inflammatory and immune responses (Lemaitre et al., 1996). The cytoplasmic domains of *Drosophila* Toll and IL-1 receptor are highly conserved and are referred to as the Toll/IL-1 receptor (TIR) domain. This TIR domain in *Drosophila* Toll interacts with adaptor proteins DmMyD88, Tube and Pelle, that have a death domain (DD) region (Horng and Medzhitov, 2001; Letsou et al., 1991; Shelton and Wasserman, 1993; Tauszig-Delamasure et al., 2002). Fly mutants of each adaptor proteins do not mount a normal protective antifungal response when challenged (Lemaitre et al., 1996; Tauszig-Delamasure et al., 2002). Upon activation, the Toll receptor-adaptor complex signals to dissociate Cactus, the ankyrin-repeat inhibitor protein, from the Rel protein by phosphorylation. There are three Rel proteins in *Drosophila*: Dorsal (Nusslein-Volhard et al., 1980; Steward, 1987), Dorsal-related immunity factor (DIF) (Ip et al., 1993), and Relish (Dushay et al., 1996). Toll is activated by the polypeptide Spätzle, which is cleaved to its active form as the end result of a proteolytic cascade (LeMosy et al., 1999). Therefore, in *Drosophila*, rather than a direct recognition of microbial structures by Toll, fungal and Gram-positive infectious agents probably trigger a proteolytic cascade that leads to the processing of Spätzle, which in turn activates Toll. In support of this, mutations of a gene encoding a peptidoglycan-recognition protein (PGRP) PGRP-SA and a serine protease *Persephone* result in compromised anti-Gram-positive

bacteria and antifungal responses (Michel et al., 2001). This also indicates the existence of distinct extracellular pathways for activation of Toll by Gram-positive bacteria and fungi, involving PGRP-SA and Persephone, respectively. Most likely molecules upstream of Persephone detects fungal infection, and a serine protease downstream of PGRP-SA triggers the proteolytic cascade to activate Spätzle.

The Imd pathway governs defense reactions against Gram-negative bacteria. As for the Toll pathway, the immune induction of the relevant AMPs relies on a member of the Rel family, Relish. Relish is not inhibited by Cactus, but carries its own inhibitory sequences in the form of several carboxy-terminally located ankyrin repeat domains (Dushay et al., 1996). The mechanism of proteolytic cleavage of Relish is not known. Another peptidoglycan recognition protein, PGRP-LC, has been shown to mediate signals from bacterial LPS, and has been demonstrated to be crucial for activating the Imd/Relish pathway (Choe et al., 2002).

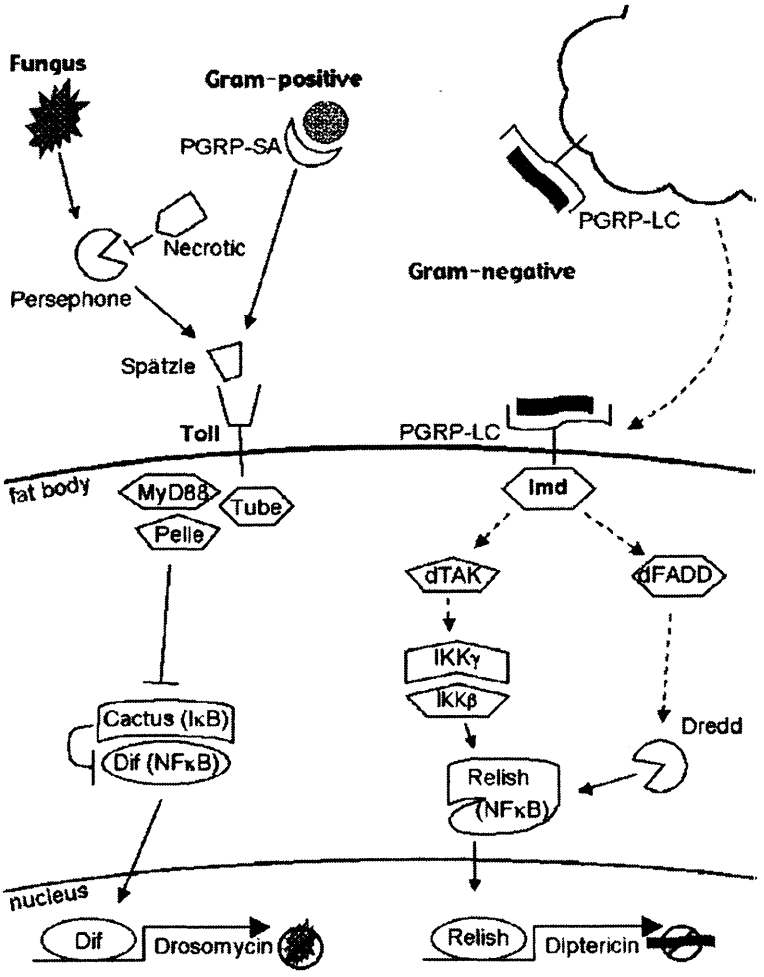


Figure 1.1 Drosophila Toll and Imd pathways of innate immunity

Fungus and Gram-positive bacteria are recognised by Persephone and PGRP-SA respectively, which activate Spätzle. Spätzle is the natural ligand for Drosophila (Dm)Toll, and the activation of DmToll leads to downstream signalling events resulting in the activation and nuclear translocation of Dif. Gram-negative bacteria are recognised by PGRP-LC, which activates the Imd pathway. Similar downstream signalling events leads to the activation and nuclear translocation of a Relish, another homolog of human NF-κB. The activation of Dif and Relish leads to the transcription of genes encoding antimicrobial peptides (AMPs) that are specific for the type of incoming pathogen (fungus, Gram-positive or Gram-negative bacteria). Drosophila with mutant Toll or Imd have impaired antifungal and antibacterial responses.
(Figure adapted from Brennan and Anderson, 2004)

1.1.2 Toll-like receptor family

A year after the discovery of the role of the *Drosophila* Toll in host defence, a mammalian homologue of *Drosophila* Toll was identified (Medzhitov et al., 1997). Toll-like receptors (TLR) are a family of pattern recognition receptors that have important functions in innate immune defence. There are now currently 11 members in the TLR family in humans (Figure 1.2A). TLRs function as "taste receptors" to detect signatures of pathogens, and have evolved to recognise a range of microbial proteins, lipids, and nucleic acids (Table 1.1).

TLR family members are characterised structurally by the presence of leucine-rich repeat (LRR) domains in their amino-terminal extracellular domain, and a TIR domain in their carboxy-terminal intracellular domain. TLR4 was the first mammalian TLR identified, and ectopic overexpression was shown to cause induction of genes for several inflammatory cytokines and costimulatory molecules (Medzhitov et al., 1997).

So far, the TLR members 1-10 have been divided into five subfamilies based on their amino acid sequence and genomic structure (Takeda et al., 2003) (Figure 1.2B). The TLR2 subfamily is composed of TLR1, TLR2, TLR6, and TLR10; the TLR9 subfamily is composed of TLR7, TLR8, and TLR9. TLR3, TLR4, and TLR5, form their own subfamilies.

TLRs are differentially expressed within the cell. TLR4, TLR5, and members of the TLR2 subfamily are expressed on the cell surface; TLR3 and members of the TLR9 subfamily are expressed intracellularly in the endosomal-lysosomal compartments (Ahmad-Nejad et al., 2002; Latz et al., 2004; Nishiya and DeFranco, 2004). It is tempting to speculate that cell surface-expressed TLRs rapidly sense bacterial infections by recognising extracellular bacterial cell wall or virus envelope constituents. Analogous to this, TLR3 and members of the TLR9 subfamily that are expressed in endosome compartments most likely recognise nucleic acids and other components of intracellular bacteria and viruses when these are present in phagosome-endosome compartments. Together, this may help optimise recognition of ligands, through the distribution of receptors to where their ligands are most likely encountered.

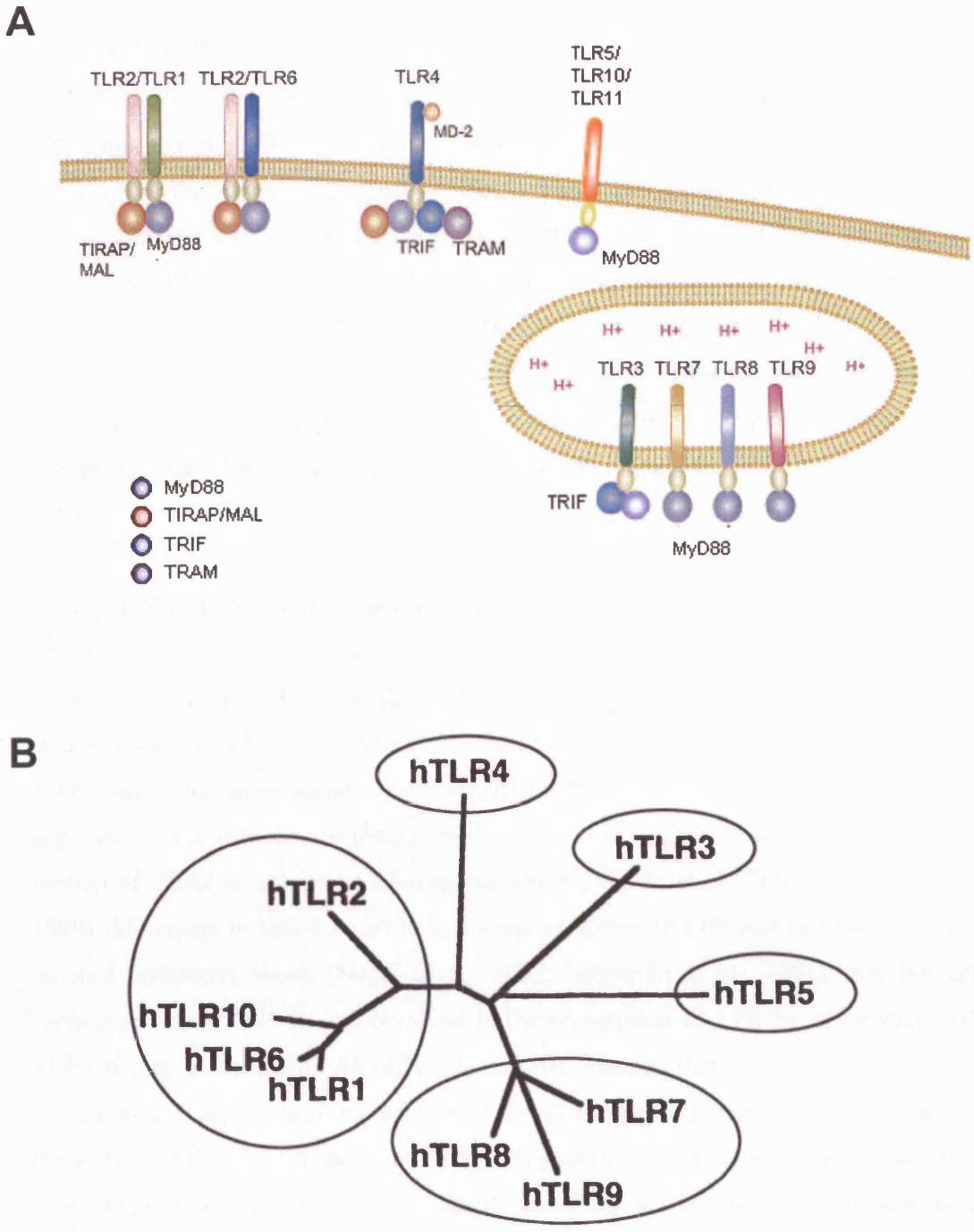


Figure 1.2 Human Toll-like receptor family

A Differential cellular localisation of the members of the human Toll-like receptor family. Cell surface TLRs include TLRs 1, 2, and 6, which may heterodimerise and recognise a broad range of bacterial lipoproteins; TLR4 which specifically recognises a wide range of molecular patterns on pathogens; TLR5 which recognises flagellin of Gram-negative bacteria; TLR11 which recognises forms of uropathogenic bacteria; and TLR10 whose natural ligand has not yet been discovered. TLRs 3, 7, 8, 9 are involved in recognising nucleic acids from viruses and bacteria, and are localised intracellularly in lysosomes. (Figure adapted from Boehme and Compton, 2004)
B Phylogenetic tree of the first 10 members of the human toll-like receptor family. The phylogenetic tree was derived from alignment of the amino acid sequences for human TLRs. (Figure adapted from Takeda, 2003)

Toll-like receptor 4

Toll-like receptor 4 (TLR4) was the first human TLR to be discovered in 1997 (Medzhitov et al., 1997). Ectopic overexpression of this receptor caused the induction of the genes for several inflammatory cytokines and costimulatory molecules, including interleukin (IL)-1, CXCL-8 (formerly known as IL-8), IL-6, and CD80. In 1998, two groups identified the gene responsible for the hyporesponsiveness to bacterial endotoxin in two mouse strains, C3H/HeJ and C57BL10/ScCr (Poltorak et al., 1998; Qureshi et al., 1999). Mutation in the *tlr4* gene was responsible for hyporesponsiveness to LPS, and TLR4-deficient mice generated by gene targeting are also hyporesponsive to LPS, confirming that TLR4 is an essential receptor for the recognition of LPS (Hoshino et al., 1999).

Recognition of LPS requires other molecules in addition to TLR4. LPS is recognised by CD14 expressed preferentially on the surface of monocytes, macrophages and neutrophils (da Silva Correia et al., 2001; Moreno et al., 2004). The binding of LPS may also be potentiated by LPS-binding protein (LBP) present in the serum (Hailman et al., 1994). MD-2 has been found to be essential for the transport of TLR4 from the Golgi apparatus to the cell surface (Nagai et al., 2002), and associates with the extracellular portion of TLR4 to enhance LPS-responsiveness (Akashi et al., 2000; Shimazu et al., 1999). Mutations in MD-2 result in hyporesponsiveness to LPS and resistance to LPS-induced endotoxin shock (Nagai et al., 2002; Schromm et al., 2001). Another cell surface protein RP105 is also involved in the recognition of LPS by associating with TLR4 (Ogata et al., 2000). RP105 contains LRR domains that is structurally related to those found in the extracellular portion of the TLRs, and is preferentially expressed on the surface of B cells (Miyake et al., 1994). Together, this suggests that the extracellular components that lead to TLR4-mediated signalling in human is as complex as in Drosophila.

In addition to LPS, TLR4 also recognises glycoproteins present in virus envelopes. Viral ligands that trigger TLR4 include the fusion protein of Respiratory Syncytial virus (RSV) (Kurt-Jones et al., 2000), envelope proteins of Mouse Mammary Tumour virus (MMTV) (Burzyn et al., 2004), and the core protein of hepatitis C virus (HCV) (Duesberg et al., 2002).

TLR4 also recognises endogenous ligands, particularly heat shock proteins (HSPs). The functions of HSPs include helping proteins achieve their native conformation, to reach their correct cellular destination, and resist protein denaturation due to cellular stressors. In normal conditions, HSPs are abundant and intracellular. Tissue damage and cell death results in the release of HSPs into the extracellular milieu, which are recognised by macrophages, dendritic cells, neutrophils and monocytes through HSP receptors, including TLR4. A wide variety of stress conditions such as heat shock, ultraviolet (UV) radiation, and infections induce the increased synthesis of heat shock proteins, and their detection by innate immune cells are a potent signal for the presence of danger, stress and infectious agents. Recent evidence also point to HSPs as potent activators of pro-inflammatory and innate immune responses (Tsan and Gao, 2004). HSP60, HSP70 and HSP90 are recognised by TLR4, alone or in combination with TLR2 (Ohashi et al., 2000; Triantafilou and Triantafilou, 2004; Vabulas et al., 2001). Triggering of TLRs singly or in combination may contribute to additional specificity of the downstream immune response.

Toll-like receptor 2

Toll-like receptor 2 (TLR2) recognises a variety of microbial components (Table 1.1), ranging from components of Gram positive and Gram negative bacteria, mycoplasma, and parasites. Analysis of TLR2-deficient mice showed that TLR2 is critical to the recognition of peptidoglycan and lipoproteins (Schwandner et al., 1999). Accordingly, TLR2-deficient mice showed higher susceptibility to infection by *Staphylococcus aureus* than wild-type mice (Takeuchi et al., 2000). Furthermore, TLR2 recognises several atypical types of LPS from *Leptospira interrogans* and *Porphyromonas gingivalis*, in contrast to TLR4 which recognises lipopolysaccharides from enterobacteria such as *Escherichia coli* and *Salmonella* (Erridge et al., 2004; Tapping et al., 2000). The properties of atypical LPS differ structurally from enterobacteria LPS by the number of acyl chains in the lipid A component (Netea et al., 2002).

It has been shown that TLR2 is recruited to macrophage phagosomes after the internalisation of yeast mannan, and this triggers the downstream TLR2 signalling pathway resulting in the production of pro-inflammatory cytokines including TNFα (Underhill et al., 1999). This demonstrates that TLR2 recruitment to phagosomes is a functional link between macrophage phagocytosis and the production of pro-

inflammatory cytokines. This may also indicate a potential cooperation between other non-TLR pattern recognition receptors that recognise and internalise pathogens, and TLRs that are recruited to phagosomes and trigger cytokine production. This further increases the likelihood that specific targeting of TLRs to defined locations facilitates the recognition of specific ligands.

TLR2 cooperates with TLR1 and TLR6. TLR1 and TLR6 are very closely related in their amino acid sequence, showing 69.3% overall identity and over 90% identity in the TIR domains (Takeuchi et al., 1999). Functional association of TLR2 with TLR1 and TLR6 confers discrimination among different microbial components, allowing differentiation between triacylated bacterial and diacylated mycoplasmal lipopeptides. TLR2 and TLR6 functionally cooperate to recognise diacylated lipopeptides (Ozinsky et al., 2000; Takeuchi et al., 2001), while TLR2 and TLR1 cooperate to recognise triacylated lipopeptides (Takeuchi et al., 2002; Wyllie et al., 2000). TLR1 may also be responsible for recognising subtle differences among lipid moieties of lipopeptides. The ability of TLR2 to functionally associate with at least TLR1 and TLR6 allows finer discrimination between pathogen components than perhaps allowed by single TLRs.

Toll-like receptor 3

The ligand for Toll-like receptor 3 (TLR3) was identified in 2001 to be double-stranded RNA (dsRNA), a molecular pattern associated with viral infection, as it is produced by many virus families at some point during their replication cycle (Alexopoulou et al., 2001). Using a synthetic analogue to dsRNA, polyinosinic-polycytidylic acid (poly(I:C)), *TLR3*$^{-/-}$ mice were shown to have impaired responses to poly(I:C) in terms of IL-6 and TNFα production, and impaired upregulation of CD69, CD80 and CD86 in B cells. Responses to poly(I:C) in *MyD88*$^{-/-}$ mice were also impaired in terms of dendritic cell production of IL-12 and nitric oxide (NO_2) production from macrophages, demonstrating the involvement of MyD88 downstream of TLR3 signalling in cytokine responses.

There are however other cellular receptors for dsRNA, including dsRNA-dependent protein kinase (PKR) (Proud, 1995). However, PKR-deficient mice still showed some responses to dsRNA, demonstrating the presence for additional cellular receptors for this viral motif (Yang et al., 1995). Because so many viruses synthesise dsRNA or

RNAs with extensive secondary structures during their replicative cycle, dsRNA is an important viral motif for the host to recognise. Accordingly, recognition of dsRNA by TLR3 also results in the activation of interferon regulatory factor (IRF)-3, a transcription factor that can activate a number of interferon-stimulated genes (ISGs), including interferon-α and interferon-β (IFNα/β) in the absence of prior secreted interferon (Servant et al., 2002). Therefore, in addition to the core response induced by all TLRs through the adaptor protein MyD88, the antiviral action of TLR3, through the activation of IRF-3, is mediated through another adaptor protein TRIF/TICAM-1, as discussed in Section 1.1.3.

The endogenous ligand for TLR3 has been shown to be messenger RNA (mRNA), presumably through the presence of dsRNA in dynamic secondary structures (Kariko et al., 2004). Similar to HSPs, the presence of extracellular mRNA may be the result of cellular necrosis, and act as a signal for surrounding tissue damage that may trigger an immune response in innate immune cells that detect mRNA through TLR3.

In contrast to TLR4 and TLR2, studies have shown an intracellular localisation of TLR3 in dendritic cells (Matsumoto et al., 2003). This is in contrast to the cell surface expression of TLR3 in fibroblasts (Matsumoto et al., 2002). Exogenous dsRNA still stimulated IL-12 and IFNα/β production in DCs, demonstrating that TLR3 is triggered after dsRNA is internalised. This confers cell-type specificity in the induction of antiviral responses via TLR3, where cells with a high capacity for endocytosis may be more likely to detect viral nucleic acids intracellularly, as opposed to stromal cells like fibroblasts that do not have a capacity to phagocytose, and rely upon detection of dsRNA extracellularly.

Toll-like receptor 9

Bacterial DNA is distinguished from vertebrate DNA by its high frequency of unmethylated CpG motifs (Bestor, 1990; Hendrich and Tweedie, 2003). These structural differences allow bacterial DNA to be distinguished from self DNA (Heeg et al., 1998). The discovery that unmethylated bacterial CpG-DNA motifs have immunostimulatory properties (Krieg et al., 1995), and the recognition that bacterial DNA induces the nuclear translocation of NF-κB with the subsequent production of pro-inflammatory cytokines TNFα and IL-6 (Sparwasser et al., 1997), alluded to the

role of bacterial CpG DNA as a potential molecular pattern recognised by TLRs. Subsequently, it was discovered that Toll-like receptor 9 (TLR9) is essential for the recognition of bacterial DNA (Bauer et al., 2001).

In addition to bacterial CpG DNA, TLR9 also recognises herpes simplex virus-1 (HSV-1) and HSV-2, demonstrated in murine and human plasmacytoid DCs respectively (Hochrein et al., 2004; Krug et al., 2004; Lund et al., 2003). Lund *et al* also demonstrated that purified HSV-2 genomic DNA can trigger the release of IFNα, which is inhibited by inhibitory CpG oligonucleotide in a dose-dependent manner. The production of IFNα required the adaptor protein MyD88. TLR9 has also been shown to recognise malaria schizonts (Pichyangkul et al., 2004).

Comparison between TLR signalling activated by LPS and CpG-DNA revealed different intracellular localisation of TLR4 and TLR9. Similar to TLR3, TLR9 is also expressed intracellularly, and upon stimulation with CpG-DNA, TLR9 with its ligand and MyD88 both co-localise to lysosomal compartments (Ahmad-Nejad et al., 2002). This recruitment of MyD88 to endosomal compartments is also seen for TLR7 and TLR8 with their respective ligands (Heil et al., 2003). The requirement for endocytosis for TLR9 triggering is demonstrated by the failure of TLR9-mediated signal transduction in the presence of inhibitors of endocytosis (Ahmad-Nejad et al., 2002; Lund et al., 2003). This is in contrast to the independence of LPS internalisation and triggering of TLR4 (Ahmad-Nejad et al., 2002), showing that signalling by TLRs is also specific to intracellular location. Accordingly, expression of intracellular TLRs such as TLR3, and members of the TLR9 family, are targeted to locations within the cell to optimise their detection of viral and bacterial nucleic acids. The contribution of such TLRs to the antiviral response is further detailed in Section 1.2.3.2.

Table 1.1 Ligands for human Toll-like receptors

Activating ligands for members of the human Toll-like receptor family, subdivided into classes of pathogens from which the components are derived. Endogenous (host) proteins can also act to trigger TLRs. Such pathogen components trigger TLRs to raise the alarm for activating the immune system.

	Bacterial components	*Viral components*	*Fungal / parasitic components*	*Endogenous factors*
TLR1	Tri-acyl lipopeptides (mycobacteria)			
TLR2	Lipoproteins (Gram- bacteria, mycoplasma, spirochetes) PTG, LTA (Gram+ bacteria) Porins (Neisseria) Lipoarabinomannan (mycoplasma) Atypical LPS (Leptospira, porphyromonas) H. pylori	HSV-1 Measles virus (Haemagluttinin)	Glycoinositolphospholipids (Trypanosoma cruzi) Glycolipids (Treponema maltophilum) Zymosan (fungi)	
TLR3		dsRNA, MCMV		mRNA
TLR4	Lipopolysaccharide (Gram- bacteria)	RSV (Fusion protein), MMTV (Envelope protein)	Cryptococcus neoformans	HSP
TLR5	Flagellin (Gram- bacteria)			
TLR6	Di-acyl lipopeptides (mycobacteria) LTA		Zymosan (fungi)	
TLR7		ssRNA		
TLR8		ssRNA		
TLR9	CpG DNA, H. pylori	HSV-1, HSV-2, MCMV	Plasmodium falciparum schizonts	
TLR10		*Not determined*		
TLR11	Not determined (uropathogenic bacteria)			

1.1.3 TLR-mediated signalling pathways

Both TLR and IL-1 receptor (IL-1R) families activate similar signalling cascades upon stimulation, due to their similar cytoplasmic domains. Neither Toll-like receptors nor the IL-1 receptor possess intrinsic kinase activity, and rely on recruitment of adaptor proteins to mediate downstream signalling events. There are currently 5 members of the TLR adaptor family, all of which possess a TIR domain at the carboxy-terminal (McGettrick and O'Neill, 2004). MyD88 was the first adaptor protein identified to mediate signalling events downstream of IL-1R and TLR4 (Medzhitov et al., 1998; Muzio et al., 1997; Wesche et al., 1997). Stimulation of *MyD88*$^{-/-}$ mice with LPS showed incomplete abolition of downstream TLR4-induced responses including activation of NF-κB and MAP kinases, suggesting that other adaptors probably existed (Kawai et al., 1999). Mal/TIRAP (MyD88-adapter-like or TIR domain-containing adapter protein) was the second adaptor protein discovered (Fitzgerald et al., 2001; Horng et al., 2001), and has subsequently been found to function with MyD88 downstream of TLR2 and TLR4 (Horng et al., 2002; Oshiumi et al., 2003a; Yamamoto et al., 2002a). Other TIR-containing TLR adapters include TRIF (TIR domain-containing adaptor inducing IFN-β), also known as TICAM-1 (TIR domain-containing adaptor molecule-1), which functions downstream of TLR3 and TLR4 (Oshiumi et al., 2003a; Hoebe et al., 2003a; Yamamoto et al., 2002b); TRAM (TRIF-related adaptor molecule), also known as TICAM-2 (TIR domain-containing adaptor molecule-2), which functions downstream of TLR4 as a bridging adaptor to TRIF/TICAM-1 (Fitzgerald et al., 2003b; Oshiumi et al., 2003b; Yamamoto et al., 2003b); and SARM (SAM and ARM containing protein), of which a *C. elegans* ortholog has been found to contribute to innate immunity (Couillault et al., 2004). The existence of different adaptors allows recruitment by TLRs, singly or in combination, to activate distinct signalling pathways. MyD88 probably functions as a universal adaptor for TLRs, with the possible exception of TLR3 (Jiang et al., 2003), whereas the usage for Mal, TRIF/TICAM-1 and TRAM/TICAM-2 are more limited. The role of SARM in human TLR signalling is yet to be demonstrated.

The variety of TLR adaptor proteins further suggest that differential adaptor utilisation by TLRs may mediate receptor-specific patterns of gene expression. The signalling pathways of TLR4 and TLR3 demonstrating MyD88-dependent and MyD88-independent signalling are discussed below.

MyD88-dependent signalling pathway

The adaptor protein MyD88 has a TIR domain in its C-terminal portion and a death domain (DD) in its N-terminal portion (Muzio et al., 1997). Downstream of MyD88, members of the IRAK (IL-1 receptor associated kinase) family are recruited. IRAK-4 is crucial to the induction of innate immunity (Suzuki et al., 2002), and activates IRAK-1. Upon activation, IRAK-1 becomes hyperphosphorylated and interacts with TRAF6 (tumour necrosis factor (TNF) receptor-associated factor 6). TRAF6 is a member of the TNF receptor-associated factor (TRAF) family of proteins, which have been characterised as adaptor molecules that mediate signals downstream of the TNF receptor superfamily, leading to the activation of NF-κB and MAP (mitogen-activated protein) kinases (Wajant et al., 2001). TRAF6 is the only member of the TRAF family to participate in IL-1 receptor and TLR signalling (Cao et al., 1996; Zhang et al., 1999), suggesting that TRAF6 may be a central point where signals induced by the TLR and TNF receptor families converge.

TRAF6 complexes with a number of different proteins, two immediate factors called TRIKA1 (TRAF6-regulated IKK activator 1) and TRIKA2. TRIKA1 is a dimeric ubiquitin-conjugating enzyme complex composed of Ubc13 and Uev1A. This Ubc complex together with TRAF6 catalyses the activation of IKK (Deng et al., 2000), which go on to phosphorylate the inhibitory proteins IκBs, resulting in the activation of NF-κB (Siebenlist et al., 1994). The IKKs also phosphorylate and regulate p105 (NFKB1) and p100 (NFKB2) leading to their processing to p50 and p52 respectively (Siebenlist et al., 1994). TRIKA2 is composed of TAK-1 (transforming growth factor β (TGFβ)-activated kinase-1, also known as mitogen-activated protein kinase kinase kinase 7), TAB1 (TAK1-binding protein 1) and TAB2 (Ninomiya-Tsuji et al., 1999; Takaesu et al., 2000). The two complexes associated with TRAF6 can activate both NF-κB and MKK6 (mitogen-activated protein kinase kinase 6), the upstream kinase of p38 and JNK (Jiang et al., 2003; Wang et al., 2001). TAK-1, together with TRAF6, therefore acts as a branch point that activates both the NF-κB pathway and the MAPK pathway (Wang et al., 2001) (Figure 1.3).

MyD88-independent signalling pathway

TLR4 and TLR3 are unique among the members of the TLR family in that these two receptors can signal independently of MyD88, and still result in the activation and nuclear translocation of NF-κB (Yamamoto et al., 2003a). The MyD88-independent signalling pathway is mediated through the adaptor protein TRIF (also known as TICAM-1) (Hoebe et al., 2003a; Oshiumi et al., 2003a; Yamamoto et al., 2002b). TRIF is recruited directly by TLR3, and indirectly by TLR4 via a bridging adaptor TRAM (also known as TICAM-2) (Fitzgerald et al., 2003b; Oshiumi et al., 2003b; Yamamoto et al., 2003b) (Figure 1.3).

TRIF associates with TRAF6, which then binds to TBK-1 (TRAF family member-associated NF-κB activator (TANK) binding kinase 1) and IKKε (Fitzgerald et al., 2003a). Both TBK1 and IKKε are distantly related to IKKα and IKKβ, and activate the antiviral response through activation of NF-κB and IRF-3 (Fitzgerald et al., 2003a; Fitzgerald et al., 2003b; McWhirter et al., 2004; Sato et al., 2003; Sharma et al., 2003; Takeuchi et al., 2004), the coordinate activation of NF-κB and IRF-3 being necessary for the induction of IFNβ (Panne et al., 2004). It has also been demonstrated that poly(I:C)-induced activation of TLR3 results in TRIF binding to TRAF6, and downstream events similar to that described for MyD88-dependent signalling, involving TAK-1, TAB1 and TAB2 also lead to the activation of NF-κB and MAP kinases (Jiang et al., 2003).

Though TLR3 and TLR4 signal through common MyD88-dependent and MyD88-independent signalling pathways, gene expression studies (Doyle et al., 2002; Doyle et al., 2003) and investigations of other activation parameters (Hoebe et al., 2003b; Wietek et al., 2003) have indicated a diverging gene expression program mediated by the two TLRs. The existence of known TLR3- and TLR4-specific signalling pathways might also suggest the existence of similar diversity in signalling pathways of other TLRs.

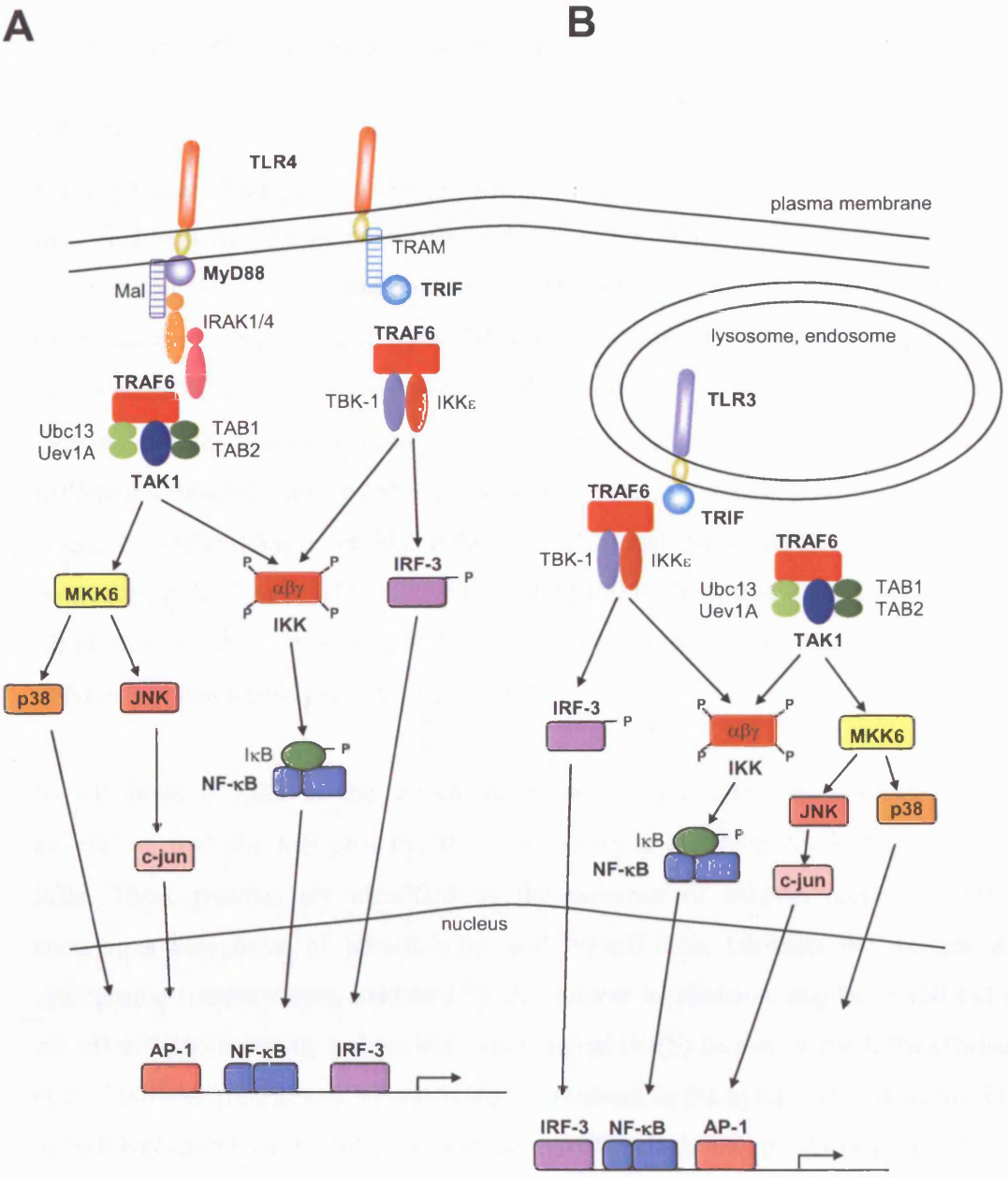


Figure 1.3 MyD88-dependent and MyD88-independent signalling pathways

The suggested intracellular signalling pathways downstream of TLR4 on the cell surface (A) and TLR3 in intracellular compartments (B). TLR4 utilises both a MyD88-dependent and MyD88-independent signalling pathway. The MyD88-independent signalling pathway is mediated by the adaptor protein TRIF, recruited directly by TLR3, and indirectly by TLR4 via a bridging adaptor (TRAM). TRAF6 seems to be activated downstream of both MyD88 and TRIF, and the proteins associated with TRAF6 may differ depending on the activating adaptor protein. Transcription factors IRF-3 and NF-κB are activated downstream of TLR3/4 activation, and JNK can activate c-jun to form the transcription factor AP-1. p38 also enters the nucleus and may have a role in histone modification that is required for genes whose promoter regions are masked by surrounding histones. p38 may also mediate a phagocytic gene program (Blander and Medzhitov, 2004; Doyle et al., 2004). The activation of the combination of transcription factors leads to the characteristic gene expression program of immune cells characterised by cytokine and interferon production and cell-specific changes downstream of NF-κB activation.

1.1.4 Transcription factors activated in TLR signalling pathways

NF-κB

Nuclear factor of kappa light polypeptide gene enhancer in B cells (NF-κB) was first identified as a nuclear factor necessary for the transcription of immunoglobulin (Ig) light chains in B cells (Sen and Baltimore, 1986). Subsequently NF-κB was shown to be expressed in a variety of cell types. NF-κB proteins are evolutionarily conserved. In *Drosophila*, NF-κB members include Dorsal, Dorsal-related immunity factor and Relish (Section 1.1.1). Mammalian NF-κB members include: RelA (p65), RelB, c-Rel, p105/p50 (NF-κB1), and p100/p52 (NF-κB2), related through their Rel homology domains (RHD) (Ghosh et al., 1998). NF-κB1 and NF-κB2 are synthethised as precursors, p105 is cleaved to release p50, and p100 is cleaved to release p52. The NF-κB proteins are dimeric *in vivo*, and p65/p50 heterodimers are the most abundant form of NF-κB in many cells (Siebenlist et al., 1994).

NF-κB proteins exist in the cytoplasm in an inactive form, as a result of their association with the IκB proteins, the most common of which are IκBα, IκBβ, and IκBε. These proteins are identified by the presence of ankyrin repeats. There is continuous movement of NF-κB-IκBα and NF-κB-IκBε between the nuclear and cytoplasmic compartments, mediated by the nuclear localisation sequences (NLSs) on NF-κB p50/p65 subunits, and nuclear export signal (NES) located in the IκBα (Birbach et al., 2002). Complexes of NF-κB-IκBβ are retained in the cytoplasm due to masking of both NLS in NF-κB by IκBβ, in contrast to IκBα which only masks one NLS (Malek et al., 2001).

The activation of NF-κB is the most well-characterised outcome of TLR signalling. This results in the production of a range of inflammatory cytokines and acute phase proteins such as IL-1, TNFα, IL-6, CXCL8, IL-12p40, ICAM1 (Baeuerle and Henkel, 1994). The activation of NF-κB in dendritic cells also has important functional consequences affecting DC activation (Section 1.2.3.1).

AP-1

The activating protein-1 (AP-1) family of transcription factors consists of homodimers and heterodimers of the Jun and Fos family (Karin, 1995). The activity of AP-1 is upregulated through phosphorylation by the MAP kinases JNK (c-Jun NH_2-terminal kinase, MAPK8) and ERK (extracellular signal-regulated kinase, MAPK3) (Karin, 1995). MAP kinase pathways involving ERK and JNK are activated by a range of stimuli such as growth factors and cytokines, bacterial and viral infections (Chu et al., 1999). LPS and peptidoglycan has been shown to enhance the transcriptional activity of AP-1 (Mackman et al., 1991), and viral infection and dsRNA activate AP-1 through the induction of JNK (Chu et al., 1999). Differential activation of TLRs has been shown to correspond to variation in downstream activities of the ERK pathway (Agrawal et al., 2003). Activation of the MAP kinase pathway downstream of TLRs may result from the downstream effects of TAK-1, though such a link has not yet been reported.

IRF-3

Interferon regulatory factors (IRFs) constitute a family of transcription factors that mediate activation of interferons and interferon-related response genes (Taniguchi et al., 2001). There are nine members of the IRF family. IRFs are differentially induced by viral infections (Bose and Banerjee, 2003), and are activated by downstream signalling pathways of cytokine receptors including interferons (Taniguchi et al., 2001). IRF-3 can also be activated downstream of TLR3 and TLR4 signalling (Doyle et al., 2002), and it has been recently shown that IRF-7 is also activated downstream of TLR-9 and MyD88 (Honda et al., 2004). IRF-3 and IRF-7 are also activated directly as a result of viral infections, but their activation are temporally segregated. IRF-3 is activated upon viral entry (Hiscott et al., 1999), resulting in the activation of the IFNβ and IFNα1 genes. Feedback signalling from the interferon receptor results in the activation of interferon-stimulated genes (ISGs) including IRF-7, which results in the transcription of the full range IFNs, thereby amplifying the antiviral response. Virus infection can activate IRF-3 via numerous signalling pathways, and kinases that phosphorylate IRF-3 include a virus-associated kinase (VAK) and the dsRNA-activated protein kinase R (PKR) (Servant et al., 2002).

The coordinate activation and combinatorial regulation of multiple transcription factors, and the integration of their downstream effects, results in a fine-tuned transcriptional control of gene expression. With respect to the immune response, clusters of genes regulated in cell type- or pathogen-specific manners are likely to represent specific gene expression programmes that are regulated by unique combinations of such core transcription factors.

1.1.5 Expression and distribution of TLRs

Expression of TLRs vary between cell types, ranging from the ubiquitous (TLR1) to more limited expression of the other TLRs (Hornung et al., 2002; Muzio et al., 2000). Most innate immune cells express TLRs, including myeloid and plasmacytoid dendritic cells, macrophages, monocytes (Armstrong et al., 2004), eosinophils (Nagase et al., 2003), neutrophils (Hayashi et al., 2003), and natural killer (NK) cells (Becker et al., 2003; Schmidt et al., 2004). TLRs are especially important in antigen presenting cells, including DCs, macrophages and B cells, where active phagocytosis contributes to increased recognition of PAMPs. TLR activation also results in DC maturation (Section 1.2.3.1) Consequently, such cells express the widest range of TLRs. Dendritic cells further differentiate their expression of TLRs, depending on subset and stage of maturation. Myeloid DCs (MDCs) and plasmacytoid DCs (PDCs) express a broadly complementary panel of TLRs: MDCs express TLR2, TLR3, TLR4, TLR5, TLR6, TLR8; PDCs express TLR7, TLR9 (Jarrossay et al., 2001; Kadowaki et al., 2001; Krug et al., 2001). However, the data regarding exclusive expression of TLRs by particular subsets is conflicting. For example, TLR7 has also been shown to be expressed on myeloid DCs (Ito et al., 2002; Krug et al., 2001). The differential expression of TLRs according to DC subsets allows a theoretical "division of labour" where the two subsets of DCs are able to recognise different antigenic ligands. TLR3 is the only TLR that is transcriptionally upregulated upon DC maturation (Matsumoto et al., 2003), possibly through an interferon-dependent pathway (Doyle et al., 2003; Heinz et al., 2003; Tanabe et al., 2003). Transcript levels of other TLRs (TLR1, TLR2, TLR3, TLR7) have also been shown to be induced in macrophages in response to viral infections, in an IFN-dependent manner (Miettinen et al., 2001).

In addition to the expression of TLRs on cells of the innate immune system, TLRs are also expressed on cells resident at barrier sites to infection such as epithelial cells,

endothelial cells, fibroblasts and keratinocytes (Takeda et al., 2003). The location of these stromal cells also function to contribute to inflammatory responses by recognising pathogens (Sato and Iwasaki, 2004; Zarember and Godowski, 2002).

1.1.6 Regulation of adaptive immunity by TLRs

The presence of TLRs on a wide variety of cell types means that activation of TLRs can mediate a range of effector functions that influence adaptive immunity. The specificity conferred by TLRs in terms of responses to specific microorganisms forms the basis for driving a pathogen-specific immune response. Upon triggering of TLRs, downstream events result in a complex and coordinated change in DCs that allow acquisition of a "mature" phenotype, which make DCs effective antigen presenting cells that interact with T cells and to initiate and shape the downstream adaptive immune response (Section 1.2.3.1).

In addition to the "maturation" effects on dendritic cells, TLR triggering also leads to the secretion of a variety of pro-inflammatory cytokines by stromal cells that express TLRs, innate immune cells, and DCs (Andonegui et al., 2003; Farina et al., 2004; Gewirtz et al., 2001; Kagnoff and Eckmann, 1997; Mazzoni and Segal, 2004; Schmitz et al., 2004; Supajatura et al., 2001). The secretion of pro-inflammatory cytokines has a wide range of effects, and cytokines as intercellular messengers are important in modulating the immune response (Holloway et al., 2002). The activation of MyD88 downstream of TLR signalling resulting in the production of pro-inflammatory cytokines by dendritic cells has also been shown to be essential for naïve CD4 T cell activation (Pasare and Medzhitov, 2004). Studies have shown that although PAMPs that stimulate different TLRs induce similar changes of surface phenotype of DCs, they often induce distinct patterns of cytokines which may be pathogen-specific (Pulendran et al., 2001; Qi et al., 2003). Triggering of different TLRs, singly or in combination, may lead to subtly different programs of activation, including differential range of effector cytokines secreted by cells.

1.2 Dendritic cells

1.2.1 Dendritic cell biology

Dendritic cells (DCs) are specialised antigen presenting cells in the immune system. These cells constitute the innate immune system, and interface with adaptive immunity in their immune response. The nature of the infectious stimuli influences how DCs respond and mature. Encounter with pathogens in the periphery provides activation signals which are transduced via TLRs and the relevant downstream signalling pathways. DCs then carry this information into lymph nodes where they prime antigen-specific T cells and induce a polarised response specific for the pathogen. The antigen-specific nature of the adaptive immune response is therefore reliant on the ability of DCs to distinguish between pathogens and affect appropriate outcomes.

1.2.2 Dendritic cell subsets

The existence of numerous DC subsets strongly suggest their importance and specialised functions as antigen presenting cells. DCs are responsible for the induction of central and peripheral tolerance, as well as for priming effector and memory T cells to induce the adaptive immune response to infection. These apparently contradictory roles could be attributed to either distinct DC lineages endowed with unique T cell stimulatory capacity, or to a single DC type, which is instructed by environmental stimuli to perform different functions (McLellan and Kampgen, 2000).

DCs arise from myeloid and lymphoid progenitors in the bone marrow, which differentiate from the pluripotent haematopoietic stem cells. Different DC subsets carry different lineage markers that distinguish their origin. Myeloid DCs (MDCs) share a $CD34^+$ precursor with monocytes and macrophages, and are $CD1a^{high}$, $CD11b^+$, $CD11c^+$, and $CD14^-$. DCs generated *in vitro* by culturing $CD14^+$ monocytes in granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) (Sallusto and Lanzavecchia, 1994) are the classical *in vitro* monocyte-derived DC (moDC) which stimulate effector and memory responses in T cells.

Thymic DCs are involved in clonal deletion of self-reactive T cells (Ardavin et al., 1993). Lymphoid progenitors were found to differentiate into thymic DCs, as well as T, B and natural killer (NK) cells, giving rise to a lymphoid origin of DCs to distinguish

them from the classical myeloid DCs (Ardavin et al., 1993). As thymic DCs are involved in the negative selection of T cells in the thymus and the induction of tolerance, these cells will not be further explored here.

Plasmacytoid cells were discovered more recently in 1958, their name attributed to their morphological similarities to plasma cells. Because these cells did not possess markers of plasma cells, and were found in T cell-zones in lymph nodes, they were first termed plasmacytoid T cells. Plasmacytoid T cells can differentiate into dendritic cells, and appear to be the main natural interferon-producing cells (NIPC) in the blood (Grouard et al., 1997; Siegal et al., 1999). The origin of plasmacytoid DCs (PDCs) has not yet been resolved, with arguments for both a myeloid and lymphoid origin (Olweus et al., 1997; Shigematsu et al., 2004; Spits et al., 2000). PDCs are $CD4^+$, $CD123\alpha^+$ (IL-3 receptor), $CD11b^-$ and $CD11c^-$. Immature plasmacytoid DCs can secrete large amounts of IFNα at the early stages of viral infection and can be induced to mature in response to viruses (Yonezawa et al., 2003) and ligands of TLR7 and 9, which are expressed on PDCs (Kadowaki et al., 2001). Recently, it has been shown that PDCs, upon injection with dsRNA, together with the resulting secreted type I interferons, can differentiate into MDCs (Zuniga et al., 2004). Developmental plasticity is also suggested by recent evidence showing that PDCs can differentate from both common myeloid and common lymphoid progenitors (Shigematsu et al., 2004). The distinction and relationship between DC subsets are constantly re-evaluated, and are probably not as straightforward as was once thought.

Functional differences between dendritic cell subsets mainly involve innate functions. Myeloid and plasmacytoid DCs express a complementary range of TLRs (Section 1.1.5), and the pattern of cytokine production is also different. Myeloid DCs secrete large quantities of IL-12 upon ligation of TLRs, whereas plasmacytoid DCs secrete large quantities of IFNα (Ito et al., 2002). However, upon CD40 ligation, both myeloid and plasmacytoid DCs secrete IL-12 (Krug et al., 2001). Similarly, dsRNA introduced into the cytosol of myeloid DCs are also induced to secrete high amounts of IFNα (Diebold et al., 2003). Myeloid and plasmacytoid DCs are also plastic in their induction of adaptive immune responses, having been shown to be able to polarise both T_H1 and T_H2 responses (Boonstra et al., 2003; de Heer et al., 2004; Farkas et al., 2004;

Kapsenberg, 2003). This indicates that the ability to initiate differential adaptive immune responses is a property of both myeloid and plasmacytoid DCs.

1.2.2.1 Distribution of dendritic cells

Dendritic cells are widely distributed, and the localisation of these cells reflects their function *in vivo*, at primary barrier sites and areas of maximum antigen encounter such as epidermis in the skin (Langerhans cells and dermal DCs), mucosa lining the gut (gut-associated lymphoid tissue GALT), respiratory, and urogenital tracts (together known as mucosa-associated lymphoid tissue MALT). At these peripheral locations, DCs form a contiguous network of cells extending their surface projections between resident epithelial cells. The unifying feature of DCs is their dendritic morphology, contributing to their ability to effectively sample their surroundings for antigens.

Different subsets of DCs have also been shown to localise to specific tissues and compartments, and show distinct migration patterns (Penna et al., 2002). Myeloid DCs comprise tissue resident DCs in the skin, epithelial surfaces, liver, lung, mucosa, as well as circulating DCs in the blood. In the absence of maturation stimuli, these immature DCs constitutively migrate at a low rate to draining lymph nodes, and do not induce effector responses (Kurts et al., 1997). In inflammatory conditions and upon infection, resident DCs respond to infection and migrate to lymph nodes to stimulate T cells. Monocytes from the blood are also recruited to the site of infection and differentiate into DCs under the influence of the local cytokine environment (Randolph et al., 1999). The constant flux of migration of maturing DCs leaving inflamed tissues and recruitment of monocytes into inflamed tissues are regulated at the level of chemokine receptor expression and chemokine production (Sallusto et al., 2000). In contrast, plasmacytoid DCs are found in the blood and localise to secondary lymphoid organs (thymus, bone marrow, spleen, tonsils, and lymph nodes) (Cella et al., 1999a). The difference in distribution and migration patterns may be attributable to differential chemokine production and chemokine receptor usage in myeloid DCs and plasmacytoid DCs (Penna et al., 2002; Sallusto et al., 1999), where myeloid DCs are recruited to sites of inflammation and infection, and plasmacytoid DCs recruited to secondary lymphoid tissues, by virtue of their expression of particular chemokine receptors.

Signals from surrounding tissues are also interpreted by DCs to promote different types of T cell responses (Vieira et al., 2000). DCs from bronchial and intestinal mucosa skew responses toward T_{helper} (T_H) 2 (Alpan et al., 2001; Stumbles et al., 1998), whereas those isolated from spleen promote T_H1 responses (Everson et al., 1996). DCs in the respiratory tract and intestinal mucosa are exposed to a plethora of inhaled allergens and commensal microflora respectively, where an immune response activated by DCs may be inappropriate and pathogenic. Therefore, the location in which DCs reside may preferentially induce a T_H1/T_H2 response. However, the demonstration that DCs in the lung can still mount T_H1-type responses show that DC subset and location do not define the type of responses as either T_H1 or T_H2 (Dahl et al., 2004; Stumbles et al., 1998). The context in which DCs are activated are important in influencing DC responses to pathogens, in order to effectively tailor appropriate pathogen-specific immune responses for the local environment.

1.2.3 Dendritic cell function in immune response

Dendritic cells are absolutely required for the initiation of T cell immunity. Because antigen-specific T cells are rare, about 1 in 10^4 to 10^6, and infected cells displaying specific antigen-MHC complexes do not often express co-stimulatory molecules necessary to drive clonal expansion of antigen-specific T cells, there is a need to focus immune-initiating stimuli to appropriate T cells. Dendritic cells provide the means of such a focus. This function of DCs as professional antigen presenting cells is one that is acquired through the final differentiation process from immature DCs to mature DCs. This "maturation" process is triggered by infectious agents and inflammatory products, and is central to the function of DCs.

1.2.3.1 Dendritic cell maturation

Immature dendritic cells are resident in tissues and blood. Immature DCs have a very high capacity for endocytosis and antigen uptake, effectively sampling their surroundings for infectious agents. Antigen uptake is achieved by phagocytosis (Inaba et al., 1993; Reis e Sousa et al., 1993), pinocytosis (Sallusto et al., 1995), and receptor-mediated endocytosis (Jiang et al., 1995; Stahl and Ezekowitz, 1998; Tan et al., 1997). In addition to expressing pattern recognition receptors to mediate antigen uptake, immature DCs also express a wide range of activatory Toll-like receptors which allow

the recognition of a range of infectious agents. Immature DCs respond quickly and vigorously to many microbial and inflammatory stimuli via TLRs and cytokine receptors. Interestingly, DCs express high levels of all NF-κB proteins (Granelli-Piperno et al., 1995), not just activities. This may help to explain how DCs react so quickly and vigorously to many stimuli that signal through NF-κB, for example TLRs and receptors of the tumour necrosis factor receptor family (TNFRs).

Triggering of TLRs and cytokine receptors results in the activation of a complex "maturation" program in DCs that involves migration out of peripheral tissues into draining lymph nodes, phenotypic changes, and secretion of cytokines (Banchereau and Steinman, 1998). DCs downregulate endocytic receptor expression and endocytosis itself (Garrett et al., 2000), and upregulate molecules that are required to interact with T cells. These include intercellular adhesion molecule (ICAM)-1 (also known as CD54) to attach to integrins expressed on T cell, CD40, which interacts with CD40L on T cells to increase DC survival and enhance DC-T cell interactions, upregulation of co-stimulatory molecules CD80 and CD86, secretion of chemokines such as CXCL8, CCL3 and CCL4 (Caux et al., 1994), and IL-12 production (Cella et al., 1996; Krug et al., 2001; Reis e Sousa et al., 1997). Mature DCs are very potent at stimulating T cells: low levels of antigen and small numbers of DCs can induce a strong T cell responses (Bhardwaj et al., 1993).

DCs provide three signals that are necessary to activate naïve $CD4^+$ T_{helper} cells. Stimulatory signal 1 results from the ligation of T cell receptors (TCR) by pathogen-derived peptides presented with MHC class II complexes. This determines the antigen-specificity of the response. Stimulatory signal 2 is in the form of co-stimulation for the antigen-specific T cell. Without co-stimulation, antigen-specific T_H cells are rendered anergic, which may lead to tolerance (Harding et al., 1992; Tan et al., 1993). DCs also secrete polarised cytokines, IL-12 or IL-4, as signal 3 to polarise the downstream T cell response to T_H1- or T_H2- directed immunity (Kalinski et al., 1999; Kapsenberg, 2003). Such cytokine-induced polarising conditions have been shown to activate key transcription factors that instruct T_H1 and T_H2 differentiation (Lu et al., 2004). Polarisation of T cell responses also depend on the activation state of DCs, determined by the strength of the antigen signal (Eisenbarth et al., 2002; Oh and Eichelberger, 2000; Ruedl et al., 2000) as well as the length of time after the DCs are exposed to the

antigen (Langenkamp et al., 2000). The cytokines secreted by DCs, and the activation state of DCs, are both strongly dependent on the environment in which they were stimulated, and the nature of the pathogens.

However, in addition to the role of cytokines and DC activation states in polarising a T cell response, a recent study has attributed Notch ligands Delta and Jagged expressed on DCs to be responsible for affecting a T_H1 and T_H2 response in $CD4^+$ T_{helper} cells respectively (Amsen et al., 2004). Jagged was expressed on DCs upon exposure to T_H2-predisposing stimuli, and corresponding Notch activation on T cells resulted in a T_H2 polarised response. Delta expression on DCs correlated with T_H1 polarisation, though the induction of T_H1 responses does not absolutely require the Notch pathway. This shows that T cell polarisation is more complex than initially suggested, involving specific ligand-receptor interactions between DCs and T cells in addition to polarising cytokines in the surrounding environment, and suggests an additional dimension that should be considered in investigating plasticity of DCs in response to pathogenic stimuli.

Adaptive immunity is therefore influenced by numerous factors including pathogen-derived signals and cellular environmental signals that effectively polarise DCs to modulate downstream adaptive immune responses that are tailored for the particular pathogen in a particular context of host infection.

1.2.3.2 Dendritic cell response to infection

The location of DCs at immune barrier sites render them particularly susceptible to infections with pathogens. Many pathogens have targeted DCs in order to modulate the host immune response to ensure the survival and dissemination of the pathogen. Bacteria, viruses, fungi and parasites have all been shown to interact with DCs, with varying outcomes. Microbes can interfere at several stages of DC-induced immunity. These include the generation of DCs from precursor cells, DC survival and maturation, antigen processing and presentation, T cell activation and priming. Conversely, the responses regulated by DCs to pathogens, through recognition by differential TLRs and PRRs, are also specifically tailored in order to affect appropriate immune responses to the pathogen in question. The interplay between pathogen-driven immune dysfunction

and pathogen-specific immune responses are clearly illustrated in pathogen interactions with DCs.

Bacteria

Phagocytes of the innate immune system and the humoral and cellular responses of adaptive immunity are the main effectors to combat bacterial infections. Macrophages and immature DCs as phagocytic cells are important for initiating the adaptive immune response, as they can degrade bacterial peptides and present these peptides on MHC class I and class II molecules for T cell recognition. Accordingly, bacteria target the two antigen processing pathways to subvert antigen presentation and avoid T cell recognition. This is achieved by preventing antigen processing by inhibiting phagosome maturation (Clemens and Horwitz, 1995; Sturgill-Koszycki et al., 1994), inhibition of MHC class II surface expression (Noss et al., 2001), and impairing MHC class I synthesis and expression (Kirveskari et al., 1999). However, the expression of TLRs that recognise various bacterial components, including lipoproteins, lipopolysaccharide, and bacterial CpG DNA, together with scavenger receptors and mannose receptors, allow macrophages and DCs to rapidly and efficiently uptake and activate in response to bacteria. Macrophages and dendritic cells probably have complementary roles in the immune response to bacterial infections. Macrophages are highly phagocytic, though do not have the migratory capacity of DCs. Therefore, macrophages are probably important in presenting bacterial components to effector T cells at infected tissue sites, producing and eliciting cytokines, and controlling bacterial replication (Harding et al., 2003). Conversely, DCs are critical at initiating the adaptive immune response to bacterial infections by presenting bacterial peptides to antigen-specific T cells in the lymph nodes.

Intracellular bacteria such as *Mycobacterium leprae* have been shown to infect DCs (Hanekom et al., 2003; Hashimoto et al., 2002; Henderson et al., 1997). The two forms of leprosy, tuberculoid and lepromatous, are characterised by T_H1- and T_H2-type responses respectively (Modlin, 1994). *M. leprae* activates TLR1 and TLR2, which were found to be more highly expressed in lepromatous lesions of tuberculoid leprosy compared to lesions in lepromatous leprosy (Krutzik et al., 2003). The clinical spectrum of disease corresponds to immune responses to the pathogen, strongly suggesting the involvement of dendritic cells in polarising the immune response.

Fungus

Fungi generally display either of two growth modes, yeast-like or filamentous (hyphae) forms. Dimorphic fungi can alternate between a yeast phase and a hyphal phase, depending on environmental stimuli (Wendland, 2001). Virulence is associated with fungi dimorphism (van Burik and Magee, 2001), and examples of dimorphic fungi are *Candida albicans*, *Saccharomyces cerevisiae*, and *Aspergillus fumigatus*. DCs are unique in that they can phagocytose both yeast and hyphae forms of *C. albicans* and *A. fumigatus*, and these dimorphic forms engage distinct receptors on DCs. This results in differential downstream effects in terms of T cell activation and cytokine secretion (Buentke and Scheynius, 2003). In the case of *Candida*, the yeast form, recognised by the mannose receptor, results in DCs activating T_H1-driven immunity, characterised by secretion of IL-12. The hyphae form of *Candida* is recognised by complement receptor 3 (CR3) and FcγR, and internalisation mediated by the two receptors results in DCs activating T_H2-driven immunity, characterised by secretion of IL-4 (d'Ostiani et al., 2000; Montagnoli et al., 2002). The conidia and hyphae forms of *Aspergillus fumigatus* induce similarly disparate T_H1 and T_H2 responses in murine DCs respectively, as measured by their cytokine production (Bozza et al., 2002). The engagement of distinct receptors by dimorphic forms of fungi may explain the link between fungi morphology and virulence, as differential engagement of receptors results in differential immune outcomes (Romani et al., 2004).

Parasites

Parasites have also been shown to modulate immune responses by targeting DCs (McKee et al., 2004; Sher et al., 2003). Many factors affect DC responses to parasites, including the stage of parasite life cycle (Prina et al., 2004). *Toxoplasma gondii* preferentially invades immature DCs without activating these cells, and renders the immature DCs resistant to subsequent activation by TLR ligands or CD40L (McKee et al., 2004). A non-activatory infection of DCs by *Leishmania amazonensis* also results in impaired DC function (Prina et al., 2004).

The diseases caused by *Leishmania* infection present as a clinical spectrum that correlates with the level of immune response to the pathogen. Resistant and susceptible mouse strains (C57BL/6 and BALB/c mice respectively) mount differential T_{helper}

responses that correlate with disease outcome. Localised self-limiting infections in C57BL/6 mice are critically dependent on development of a T_H1 response; non-healing lesions in BALB/c mice are characterised by a T_H2 response. DCs have been shown to be infected with amastigote forms of *L. major* (Moll et al., 1993), which are released from macrophages and other lysed host cells. This corresponds to delayed DC activation and initiation of cellular immunity, prolonging the initial phase of parasite growth, resulting in a robust infection with high parasite loads. Gene expression studies have shown that responses of DCs and macrophages infected with *Leishmania* include downregulation of IFNγ-induced genes (Chaussabel et al., 2003). As the production of IFNγ from T_H1 cells is crucial to activate macrophages to kill parasites, the lack of activation signals received by *L. major*-infected macrophages contributes to the delay in induction of the immune response. In contrast, *L. major*-infected DCs retain the ability to secrete IL-12 (von Stebut et al., 1998), which contributes to the initiation of protective T_H1-dominated immunity.

Virus

Interactions between viruses and dendritic cells have been widely investigated. DCs recognise and allow virus entry by various mechanisms: the expression of specific receptors and co-receptors on the cell surface that mediate DC activation and maturation as well as internalisation of the virus, and the endocytic capacity of DCs depending on the stage of DC maturation. Similar to bacteria, fungi and parasites, the clinical disease manifested by viral infection is dependent upon the general immune status of the host, as well as virus-specific factors.

Detection of viruses and the surrounding microenvironment

The first step in innate antiviral immunity is the recognition of viral components via pattern recognition receptors, and detection of tissue injury in the microenviroment. Many TLRs recognise viral components (Table 1.1). TLR2 has been shown to recognise herpes simplex virus (HSV)-1 (Kurt-Jones et al., 2004) and the haemagglutinin (HA) protein of measles virus (MV) (Bieback et al., 2002). TLR3 recognises dsRNA (Alexopoulou et al., 2001), a motif found in viruses as a replication intermediate and as secondary structures in the viral genome. TLR4 has been shown to recognise the fusion (F) protein of respiratory syncytial virus (RSV) (Kurt-Jones et al., 2000) and murine mammary tumour virus (MMTV) (Burzyn et al., 2004). TLR7 and TLR8 have both

been shown to recognise single-stranded (ss)RNA (Diebold et al., 2004; Heil et al., 2004). TLR7 also recognises Influenza genomic RNA (Diebold et al., 2004). TLR9 recognises HSV-1 and HSV-2 viral DNA, independently of viral replication (Hochrein et al., 2004; Krug et al., 2004; Lund et al., 2003). As viral nucleic acids are generally not "seen" by the cell until internalisation, it has been hypothesised that TLRs 3, 7, 8 and 9 localise in endosomal compartments in the cell and recognise viral ligands at the point of endosomal degradation of viral particles. This intracellular localisation has been shown for TLR3 (Matsumoto et al., 2003) and TLR9 (Ahmad-Nejad et al., 2002; Latz et al., 2004; Takeshita et al., 2001). Novel TLR chimera studies have also implicated the intracellular location of TLR7 and TLR8 (Nishiya and DeFranco, 2004). In contrast, TLRs that detect viral glycoproteins (TLR2 and TLR4) are expressed on the cell surface.

The antiviral response is the hallmark of viral infection, characterised by the production of type I interferons. Accordingly, TLRs that recognise viral ligands are able to induce such an antiviral response. TLR3 and TLR4 utilise a MyD88-independent signalling pathway to mediate antiviral effects in DCs (Section 1.1.3) via the adaptor TRIF/TICAM-1 and the subsequent activation of IRF-3. Activation of IRF-3 activates IFNβ and other ISGs. Signalling downstream of members of the TLR9 family are strictly MyD88-dependent (Wagner, 2004). TLR9 induces IFNβ through MyD88 (Hoshino et al., 2002), and the activation of TLR9 by HSV-2 in plasmacytoid DCs results in the production of IFNα that is both MyD88- and IRF7-dependent (Kawai et al., 2004; Lund et al., 2003). TLR7 recognises ssRNA, and ssRNA viruses including Influenza and vesicular stomatitis virus (VSV) results in IFNα production in murine PDCs (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004). Such TLRs that recognise viruses, especially intracellular TLRs that recognise foreign nucleic acids, are likely to synergistically trigger transcription factors to activate the innate antiviral response via the production of type I interferons and other host inflammatory mediators (Triantafilou et al., 2005).

In addition to TLRs that trigger DC activation and maturation, other pattern recognition receptors expressed on DCs may contribute to receptor-mediated endocytosis and viral entry. These PRRs include carbohydrate-binding C-lectin transmembrane proteins that function as receptors that recognise carbohydrate moieties on pathogens. DC-SIGN

(DC-specific ICAM-3 grabbing non-integrin, CD209), langerin (CD207), mannose receptor (MR, CD206), and human DEC-205 (CD205) are expressed on DCs. The expression of these receptors vary according to DC maturation status (Kato et al., 2000), and generally function to mediate endocytosis. The mannose receptor expressed on DCs has been shown to be responsible for non-specific recognition of enveloped DNA and RNA viruses and the subsequent stimulation of IFNα production (Milone and Fitzgerald-Bocarsly, 1998).

DC-SIGN also recognises viral envelopes of human immunodeficiency virus (HIV)-1 (Geijtenbeek et al., 2000), dengue virus (DV) (Tassaneetrithep et al., 2003), hepatitis C virus (HCV) (Lozach et al., 2003; Pohlmann et al., 2003), Ebola virus (Alvarez et al., 2002), and cytomegalovirus (CMV) (Halary et al., 2002). This suggests that DC-SIGN is a pathogen receptor with broad specificities. The use of DC-SIGN may be a broad pathogen strategy to utilise non-activating carbohydrate receptors expressed on dendritic cells to escape immunity (Geijtenbeek and van Kooyk, 2003; van Kooyk et al., 2004; van Kooyk and Geijtenbeek, 2003). The uptake of HIV-1 by DC-SIGN does not result in viral entry (Geijtenbeek et al., 2000), and HCV seems to utilise this uptake mechanism as a way of avoiding lysosomal degradation (Ludwig et al., 2004). This may be a strategy of the virus to take advantage of the highly mobile DC to facilitate viral dissemination.

Effector components of innate immunity initiated by DCs

The primary antiviral innate response is the secretion of type I interferons (IFNα/β). Interferons (IFNs) were originally discovered as antiviral proteins that inhibit virus replication. The ability to produce and respond to interferons is not restricted to specialised immune cells. Stromal cells like epithelial cells and fibroblasts can also produce and respond to interferon by inducing an antiviral state (Stark et al., 1998). In addition to their antiviral properties, IFNs are involved in many other physiological processes including cell growth and proliferation, cell death, activation of the immune response including NK cells and macrophages, enhancement of MHC class I expression, T_H1 polarisation, and differentiation of DC (Stark et al., 1998). The IFN signalling pathway has been extensively studied. On binding of IFNs to their cognate receptors, the JAK-STAT (Janus kinases and Signal transducers and activators of transcription) signal transduction pathway is triggered, culminating in the transcription

of interferon-stimulated genes (ISGs) that mediate the functions of interferons. The proteins encoded by ISGs include many antiviral effectors: the dsRNA-activated protein kinase R (PKR), which inhibits viral protein synthesis via phosphorylation of the eukaryotic translation initiation factor (eIF)2α; the 2',5'-oligoadenylate synthetase (OAS1), which activates RNase L to degrade viral RNA; and the Mx GTPases (Mx1 and Mx2), which block viral transport inside the cell (Haller and Kochs, 2002; Pavlovic et al., 1993; Stark et al., 1998). Other ISGs include ISG56 (which inhibits protein translation via eukaryotic translation initiation factor eIF3), and the P200 family of interferon-inducible proteins which impair cell proliferation through cellular transcription factors such as NF-κB, YY1, p53, MyoD1, E2F, and c-myc (Asefa et al., 2004).

Plasmacytoid DCs are the most powerful type I interferon-producing cells, and are sometimes called natural interferon-producing cells (NIPCs). However, viral infection also induces interferon production from myeloid DCs (Diebold et al., 2003). Infection of MDC with a variety of viruses including herpes simplex virus (HSV)-1 (Pollara et al., 2004), measles virus (MV) (Tanabe et al., 2003) and Influenza (Coccia et al., 2004) leads to low levels of IFNα/β production, and Influenza virus infection results in MxA expression in infected DCs, conferring resistance to cytopathic effects of the virus (Cella et al., 1999b). The production of IFNα/β has very powerful effects both in the infected cells and neighbouring uninfected cells, due to the autocrine-paracrine effects that IFNs exert. Viruses have numerous mechanisms of inhibiting the IFN innate defence at different levels (Basler and Garcia-Sastre, 2002; Garcia-Sastre, 2004).

In addition to interferons, the cytokine TNFα also mediates antiviral effects (Guidotti and Chisari, 2001). TNFα, in addition to its role as a pro-inflammatory cytokine, has numerous additional immunomodulatory functions, including promoting an antiviral state in cells (Bose et al., 2003), inducing DC differentiation (Morrison et al., 2003; Santiago-Schwarz et al., 1993; Santiago-Schwarz et al., 1998), and triggering apoptosis via signalling through TNF receptors (Gupta, 2001). TNFα binds to TNF receptors 1 and 2 expressed on the cell surface, and downstream signalling results in the activation of NF-κB. This results in DC differentiation and maturation, as well as the transcription of NF-κB-responsive antiviral genes, for example IFNβ. Additionally, TNFα can also

recruit and activate effector cells such as macrophages, NK cells and T cells to participate in the antiviral response.

Recruitment of effector cells such as neutrophils, monocytes, macrophages and NK cells to the site of infection requires the production of other cytokines to mediate chemotaxis, such as CXCL8, CXCL10, CCL3, CCL4, and CCL5 (Moser et al., 2004). These effector cells are important for the lysis of virally-infected cells (mediated by NK cells), and promote an inflammatory response to raise the alarm for the immune system. The production of cytokines are among the earliest immune mediators produced upon virus infections, and their role to coordinate cellular activation, proliferation, differentiation and chemotaxis help to orchestrate the induction and maintenance of the innate and adaptive antiviral responses.

The importance of the cytokine and chemokine system in modulating antiviral immunity is also supported by the numerous mechanisms viruses (mostly large DNA viruses such as poxviruses and herpesviruses) have evolved to evade the immune system by encoding homologues of cytokines, chemokines and their receptors (Alcami, 2003). Many virus-encoded mechanisms block the effector functions of cytokines, such as the antiviral state induced by IFNs or apoptosis triggered by TNFα, as well as TNFα or IL-1/TLR signalling. Poxviruses encode four soluble viral TNF receptors (Hu et al., 1994; Loparev et al., 1998; Saraiva and Alcami, 2001; Smith et al., 1991a; Smith et al., 1996), likely to block the activity of TNFα in different tissues. Vaccinia virus encodes a soluble IFNα/β-binding protein that binds to the cell surface to prevent type I interferons from binding to cellular IFN receptors and inducing an antiviral state in surrounding uninfected cells (Colamonici et al., 1995).

Adaptive antiviral immune response

As mentioned previously, DCs are essential to the initiation of the adaptive immune response. This involves the activation of distinctive effector cells, including B cells, $CD4^+$ helper T cells, $CD8^+$ cytotoxic T cells, which are capable of specific recognition of microbial components. Viruses can modulate the adaptive immune response by interfering with dendritic cell function in a variety of ways: to suppress the maturation and migration capacity of DCs; expression of viral gene products that directly alter the immune response, for example cytokines and cytokine receptor homologues;

interference with antigen presentation; and skew T cell responses through altered cytokine production. Interfering with dendritic cell function is a strategic target for viruses, with the aim to subvert the host antiviral response. Some of the mechanisms used by viruses to escape adaptive immunity through their interactions with dendritic cells are described below.

DC activation and maturation

Influenza virus productively infects both myeloid and plasmacytoid DCs, and results in DC activation, including upregulation of co-stimulatory molecules, increased T cell stimulatory capacity, and secretion of polarising cytokines (Cella et al., 2000; Cella et al., 1999b; Fonteneau et al., 2003; Oh and Eichelberger, 2000). In contrast, Measles virus also replicates in myeloid DCs (Fugier-Vivier et al., 1997; Servet-Delprat et al., 2000) and induces their maturation. However, MV-infected DCs fail to stimulate T cells, and even induce T cell apoptosis. This occurs through the expression of viral glycoproteins on the DC surface which negatively signals to T cells, and secretion of TNFSF10 (formerly known as TNFα related apoptosis-inducing ligand or TRAIL) to mediate cytotoxicity (Dubois et al., 2001; Fugier-Vivier et al., 1997; Hahm et al., 2004; Vidalain et al., 2000). The contrast between Influenza and Measles virus effects on DCs may contribute and relate to the clinical disease manifested in the host, where Influenza infection is a relatively acute infection that is resolved in a matter of weeks, whereas Measles virus can cause lymphopaenia and severe immunosuppression for several weeks (Wright and Webster, 2001; Griffin, 2001).

Antigen presentation

Clinical isolates of human cytomegalovirus (CMV) have been shown to productively infect monocyte-derived DCs (Grigoleit et al., 2002; Moutaftsi et al., 2002). CMV-infected DCs compared to uninfected DCs have decreased surface expression of MHC class I and class II molecules, CD40, CD80, and consequently impaired immunostimulatory capacity. CMV glycoproteins also specifically target MHC class I-dependent antigen presentation (Rehm et al., 2002), to inhibit the surface expression of processed viral antigens associated with MHC class I molecules, and escape $CD8^+$ T cell cytotoxicity.

DC interaction with T cells

Varicella zoster virus (VZV) and human immunodeficiency virus-1 (HIV-1) also utilise the function of DCs as T cell stimulators as a means of transport to facilitate their dissemination to their target cells. Varicella zoster virus establishes a productive infection in both immature and mature myeloid DCs (Abendroth et al., 2001; Morrow et al., 2003), though its effects on plasmacytoid DCs are unknown. Experiments have shown that VZV-infected DCs showed no significant decrease in cell viability, and no significant phenotypic maturation. Importantly, VZV-infected DCs cocultured with T cells resulted in infection of T cells, and infectious virus was recovered from T cells. This suggests that VZV is able to utilise the infection of DCs as a means of transport to infect target T cells in the lymph nodes. Similarly, HIV-1, shown to infect both myeloid and plasmacytoid DCs (Canque et al., 1996; Fong et al., 2002; Patterson et al., 2001) has also been proposed to utilise DCs to transmit infectious virus to target T cells (Tsunetsugu-Yokota et al., 1995). DC-SIGN is a carbohydrate-binding C-lectin transmembrane protein expressed on DCs that interacts with ICAM3 expressed on T cells to stabilise DC-T cell interactions (Geijtenbeek et al., 2000), as well as functioning as a pattern recognition receptor that recognises carbohydrate moieties on pathogens. DC-SIGN binds to HIV-1 glycoprotein gp120, resulting in internalisation into low pH non-lysosomal compartments. This internalised virus retains infectivity, and when DCs are in contact with T cells, the virus infects target T cells *in trans* (Geijtenbeek et al., 2000; Kwon et al., 2002). HIV-1 infection of T cells contributes to AIDS disease progression, and illustrates the virus' strategy to hijack DCs as a means of transport and virus dissemination.

A DC-centric view of immunity provides a means of understanding the way in which immune responses to pathogens are initiated and shaped, and a helpful viewpoint from which to focus immunotherapeutic strategies such as the design of vaccines and tumour therapies. The concerted orchestration by the dendritic cell involves capturing and processing antigens, resulting in effector and antigen presenting functions that propagate the innate response and initiate the adaptive immune response to pathogens. Consequently, pathogens have evolved to subvert DC function in numerous ways, and the dynamic interplay between pathogen and dendritic cell is manifested in the host immune response to infection.

1.3 RNA viruses

1.3.1 Replication of Influenza virus

Influenza is a member of the *Orthomyxoviridae* family of viruses. These are enveloped viruses with a segmented single-stranded negative sense RNA genome. Influenza A viruses are divided into subtypes depending on the envelope glycoproteins haemagluttinin (HA) and neuraminidase (NA). Species specificity is largely determined by the amino acid sequence of HA. Influenza uses carbohydrates as their host cell recognition molecules, and entry into host cells is initiated by the binding of HA spikes to *N*-acetyl-neuraminic acid (sialic acid) residues on cell surface glycoproteins and glycolipids (Skehel and Wiley, 2000). This binding is followed by endocytosis via clathrin-coated pits into endocytotic vesicles and finally endosomes. Fusion of the endosome with an acidic lysosome lowers the pH of the vesicle to around 5, triggering cleavage of HA into HA_1 and HA_2. This activates the membrane fusion function of HA_2. Fusion of the virus envelope with the endosome membrane allows the 8 segments of the Influenza genome to be released directly into the cytoplasm. Specific nuclear targeting sequences in NP result in translocation of the nucleocapsid into the nucleus.

Influenza is unique among the RNA viruses in that replication occurs in the cell nucleus. The Influenza genome consists of 8 segments (7 segments in Influenza C). Each segment is associated with nucleoprotein (NP) to form RNP (RNA and nucleoprotein) associated with 3 polymerase polypeptides, PB1, PB2 and PA (Portela and Digard, 2002). The three viral polymerase proteins are encoded on segments 1, 2, and 3, and together form an enzyme complex that functions in both transcription and replication.

The segments of RNA are wrapped around the NP proteins, together with the 3 polymerase polypeptides. This nucleocapsid then identifies patches of cell membrane that contain viral envelope glycoproteins, associated with matrix protein on the inner surface. Interactions of nucleocapsid proteins with matrix proteins initiates the formation of the bud, where the nucleocapsid becomes enwrapped in membrane to form a vesicle that is then "pinched off" from the membrane to release the enveloped virions (Whittaker, 2001).

1.3.2 Immune response to Influenza infection

Influenza is an acute respiratory infection with high morbidity and significant mortality, primarily because of its complications in very young and very old persons (Renegar, 1992). Symptoms include sudden onset of malaise and fever, followed by upper and lower respiratory manifestations as well as myalgia and headache. In adults, the fever and other systemic features usually last 3 days, whereas respiratory symptoms may persist for 1 to 2 weeks.

Influenza virus replicates throughout the respiratory tract in epithelial cells and resident macrophages and dendritic cells. Virus is recoverable from upper and lower respiratory tracts of infected individuals. The virus is released from the apical surface of the cell, which may serve to limit the infection locally and prevent systemic dissemination. However this also concentrates the virus in the lumen of the respiratory tract and facilitates dissemination to the next susceptible host (Blau and Compans, 1996). The extremely short incubation period between infection and clinical illness implies that innate immunity is important.

Studies in experimentally infected human volunteers have correlated the formation of symptoms to cytokine production, especially IL-6, IFNα, TNFα, and CXCL8 (Hayden et al., 1998; Van Reeth, 2000). Whereas IL-6 and IFNα correlated with acute symptoms and viral titres, TNFα responses peaked when viral shedding and symptoms were subsiding, and CXCL8 levels correlated with lower respiratory tract symptoms which occurred later in the disease process.

Both humoral and cellular immunity are important in the clearance of Influenza. Antibodies to Influenza HA, NA, NP, and M proteins are produced, and the level of serum antibody to HA and NA correlate with resistance to illness (Couch, 2003). Both $CD4^+$ and $CD8^+$ T cells contribute to clearance of Influenza, the former providing help to the latter to lyse virally infected cells. $CD8^+$ T cells are activated via the presentation of viral antigens by professional APCs.

1.3.3 Influenza interactions with DCs

Influenza has been shown to be capable of establishing productive infection in both myeloid and plasmacytoid DCs (Bhardwaj et al., 1994; Cella et al., 2000; Cella et al., 1999b). The interaction of Influenza with DCs is necessary for efficient anti-Influenza T cell responses (Bhardwaj et al., 1994; Oh and Eichelberger, 2000), and a T_H1-mediated immune responses is necessary for recovery from Influenza virus infection in murine experimental models (Lopez et al., 2002). Influenza infection of immature MDCs results in DC activation, upregulation of surface expression of maturation markers, and increased T cell stimulatory capacity (Cella et al., 1999b). The production of IFNα and IL-12 by Influenza-infected DCs also suggests the ability to drive polarised T_H1 responses (Cella et al., 2000; Cella et al., 1999b). However, the dose of Influenza exposed to DCs may also affect to the polarity of the T cell response induced by the infected DCs (Oh and Eichelberger, 2000).

DCs residing in the respiratory tract are believed to play a central role in the induction of adaptive immune responses to pulmonary infections. Intranasal inoculation of mice with Influenza show that DCs and macrophages recovered from the lungs and mediastinal lymph nodes of infected mice are indeed antigen-positive and are able to stimulate virus-specific cytotoxic T cells (Hamilton-Easton and Eichelberger, 1995). However the migration of respiratory DCs to regional lymph nodes has been shown to be limited to the early phase of pulmonary infection; after 24 hours the DCs are refractory to migration following further stimulation (Legge and Braciale, 2003). This highlights the importance of timing of antigen exposure in considering the generation of the downstream adaptive immune response.

While it is agreed that DCs exposed to inactivated Influenza are also able to elicit CTL responses by presenting viral antigens with MHC class I molecules (Bender et al., 1995; Saurwein-Teissl et al., 1998), there is contention as to whether virus replication is necessary to drive a T_H1-type immune response (Lopez et al., 2001; Lopez et al., 2002; Saurwein-Teissl et al., 1998). However, comparisons between effects elicited from DCs exposed to live and inactivated virus is limited to measurement of cytokine production, which may not fully capture the range of DC responses.

1.3.4 Replication of Rhinovirus

Rhinoviruses (RV) are members of the *Picornaviridae* family of viruses. These are non-enveloped viruses with a single-stranded RNA genome of positive polarity. Other members include foot and mouth disease virus, poliovirus, coxsackie virus, and hepatitis A virus. There are over 100 serotypes of Rhinovirus; depending on the entry receptor utilised, Rhinoviruses are classified as major (approximately 90 serotypes) or minor (approximately 10 serotypes) group viruses that utilise the intercellular adhesion molecule-1 (ICAM1 or CD54) or low-density lipoprotein receptor (LDL-R) respectively (Greve et al., 1989; Hofer et al., 1994; Staunton et al., 1989). Rhinoviruses replicate in the nasopharynx, are extremely acid-labile, and are important agents of the common cold.

Interaction of Rhinovirus capsid with its entry receptor is followed by receptor-mediated endocytosis via clathrin coated pits. Uncoating is triggered by acidification of endosomes, which results in conformation change in the capsid. Fusion of the capsid with the vesicle membrane leads to release of the viral genome into the cytoplasm (Racaniello, 2001).

Rhinoviruses replicate in the cytoplasm, and the process starts with translation of the positive strand RNA genome. The 5' end of the viral genomes contain long untranslated regions (leaders), which contain conserved sequences to direct cellular translational machinery to an internal site, termed the internal ribosomal entry site. Ribosomes and other cellular proteins are then recruited, including the C-terminal fragment of the cleaved eukaryotic translation initiation ofactor (eIF) 4G, permitting attachment of ribosomal subunits to the viral genomic RNA molecule. RV proteins are synthesized by the translation of a single, long, open reading frame on the viral (+)sense RNA genome, followed by cleavage of the polyprotein by virus-encoded proteases $2A^{pro}$ and $3C^{pro}$ to release individual proteins. This strategy allows the synthesis of multiple protein products from a single RNA genome (Racaniello, 2001).

Synthesis of viral RNAs begins as soon as the first polyproteins have undergone their nascent and early maturation cleavages to provide the enzymes and other factors necessary for the process. The viral RNA-dependent RNA polymerase 3D ($3D^{pol}$) allows the initiation of complementary (-)sense strand (cRNA) synthesis. This

replicative intermediate serves as the template for multiple (+)sense strands, some of which are translated, and some of which form genomic viral RNA (vRNA). Viral RNA is packaged into preformed capsids, and infectious viruses are released upon cell lysis.

1.3.5 Immune response to Rhinovirus

Rhinoviruses are the most common upper respiratory pathogens, inducing the majority of common colds worldwide. The incubation period to onset of virus shedding into nasal secretions is 1 to 4 days (Douglas et al., 1966). Local symptoms include sneezing, nasal obstruction and discharge, sore throat and cough, while systemic symptoms such as headache and malaise also feature. Illness is maximal for 2 to 3 days; then the symptoms progressively improve.

The primary site of infection is the epithelial surface of the nasal mucosa. Similarly to Influenza, symptoms of Rhinovirus-induced colds are attributed to chemical mediators, including IL-1β, IL-6, CXCL8 and TNFα detected at elevated levels in nasal secretions of infected persons (Gern et al., 1996a; Terajima et al., 1997; Zhu et al., 1997; Zhu et al., 1996). However, the relative importance of different cytokines and effector cells are yet to be determined.

Though the lower respiratory tract is less susceptible to Rhinovirus infection than the nasopharynx, Rhinoviruses can also infect the lower respiratory tract, and this leads to the development of lower respiratory symptoms. This is particularly important for patients in vulnerable age groups, patients with chronic lung conditions, and immunocompromised patients.

1.4 DNA microarrays

1.4.1 Introduction to genomics

Genomics is defined as the comprehensive study of whole sets of genes, gene products, and their interactions. The completion of the draft human genome sequence in 2001 (Lander et al., 2001; Venter et al., 2001), in addition to completed genome sequences of over 150 bacterial genomes and 1500 viral genomes (Entrez gene, September 2004), has made the opportunity for the study of gene expression on a genomic scale of both host and pathogens possible. The interaction between host and pathogens is the question that drives research in infectious diseases and immunology, and exploring this on a global genome scale offers unparalleled insights into the interactions of the host and pathogen.

DNA microarrays are an ideal tool for exploring the genome in a way that is both systematic and comprehensive. Studying the regulation of gene expression at the level of transcript abundance is the rationale behind microarray experiments. This is founded on the basis that there exists a tight connection between the function of a gene product and its expression pattern. Gene promoters that control expression of genes are constantly responding to changing inputs of information about the identity, internal state and surrounding environment of the cell. Therefore the complement of genes that are expressed dictates cellular phenotype and function that are specific to a particular state. As we learn to infer biological consequences of specific features of gene expression patterns, microarrays allow a comprehensive and dynamic view of the studied system.

1.4.2 Description of DNA microarrays

The basis behind all microarrays is the precise positioning of DNA fragments (probes) at high density on a solid support so that they can act as molecular detectors. In practice, microarrays vary from the solid support used (such as glass or filters), the surface modifications with various substrates, the type of DNA fragments on the array (such as cDNA, oligonucleotides, or genomic fragments), whether the gene products are pre-synthesised and deposited or synthesised *in situ*, to the machinery used to place the fragments on the array (such as ink jet printing, spotting, mask or micro-mirror based *in situ* synthesis). Currently, combinations of these variables are used to generate three main types of microarrays: filter arrays, spotted glass slide arrays, and *in situ* synthesised oligonucleotide arrays (Affymetrix GeneChips).

Expression analysis using glass slide microarrays is typically done by the competitive hybridisation of two targets (typically known as test and reference), each labelled with a specific fluorescent dye (cyanine dyes Cy™5 and Cy™3) (Schena et al., 1995; Shalon et al., 1996). Because levels of gene expression are relative, the use of a universal reference allows comparative analysis between multiple test samples. A large quantity of reference RNA must be made at the outset. Where several batches are required, pairwise comparisons should be made to control for and reduce batch-to-batch variation. Universal references are also available from commercial suppliers, including Stratagene and Clontech. The degree of batch-to-batch variation from commercially supplied reference is not clear. Affymetrix array users may use a reference-like pool of spiked RNAs and have developed algorithms to facilitate comparisons across groups of arrays, similar to the method for spotted arrays reference comparisons, and for determination of absolute concentrations of cellular RNA species.

In order to measure gene expression, RNA (total RNA or messenger RNA) needs to be labelled. This is usually achieved by the incorporating labelled nucleotides into cDNA reverse-transcribed from RNA. For two-colour microarrays the fluorophores are usually Cy3 and Cy5. Nucleic acid from the two samples competitively hybridise to complementary probes on the array. The output of such array image analysis is a ratio between the Cy3 and Cy5 signal, a measure of the relative amounts of each sequence between the two samples. The use of a Cy3-labelled reference also serves as an internal control for hybridisation conditions of each array (Figure 1.4).

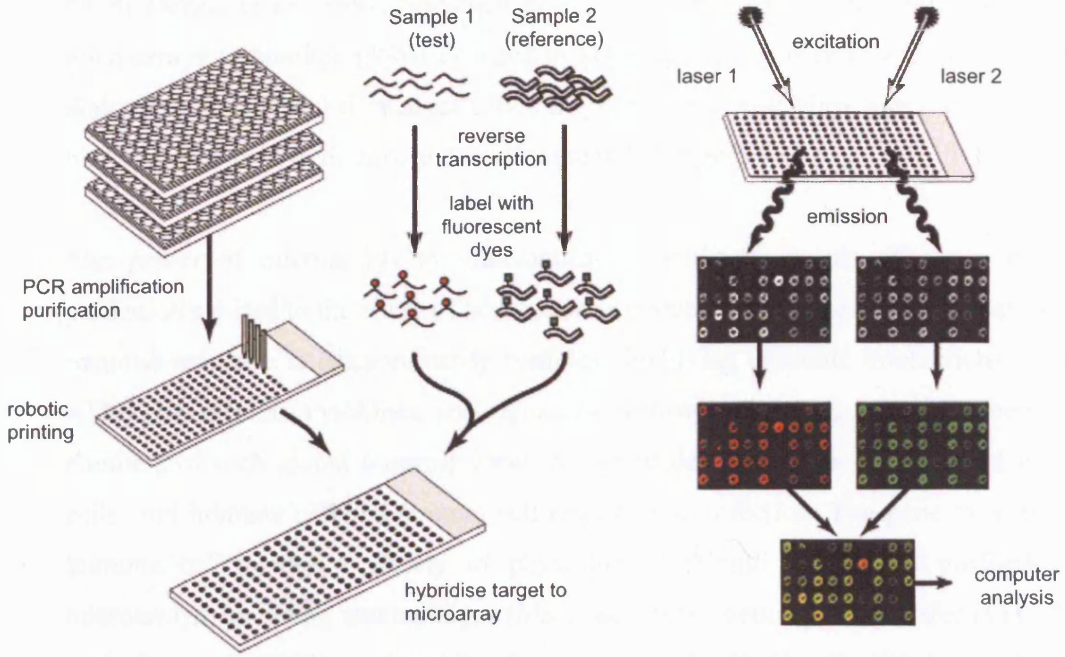


Figure 1.4 Two-colour microarray experiment protocol

The sequences of genes of interest are obtained and amplified. Following the printing of desired PCR amplification products onto glass slides, biological samples are reverse transcribed and labelled with a fluorophore, commonly Cy5. A standardised reference mixture is similarly reverse transcribed and labelled with Cy3. The two samples (test and reference) are mixed together and are allowed to competitively hybridise onto the glass array. After an overnight incubation, the arrays are washed and scanned. This involves exciting the microarray with two lasers that emit wavelengths corresponding the excitation energy of the two fluorophores. The corresponding signals emitted by the fluorescently-labelled hybridised spots are detected by photomultiplier tubes. The resulting image showing ratios of signal intensities detected at the two excitation wavelengths are analysed by various software, and the data extracted for further computer analysis. (Figure adapted from Duggan et al., 1999)

1.4.3 Using DNA arrays to study gene expression

Microarrays have been applied to address wide-ranging biological problems, from the purpose of diagnostics in the clinical setting (DeRisi et al., 1996; Golub et al., 1999; Rhodes and Chinnaiyan, 2004), to understanding the cell cycle in yeast (Chu et al., 1998; DeRisi et al., 1997; Spellman et al., 1998; Wodicka et al., 1997). The use of microarrays to monitor global changes in gene expression is now widespread, and its ability to monitor global changes offers a systems-level overview into the responses of biological systems to disease and environmental changes.

The power of microarrays to systematically profile thousands of genes makes it particularly suited to the study of host immune system and its response to infection. The immune response is extraordinarily complex, involving dynamic interactions between wide array of cells, cytokines, and signalling pathways. To date, there have been large numbers of such global transcriptional studies of developmental pathways of immune cells, and immune cell and stromal cell responses to infection. The gene expression of immune cells under a variety of physiological stimuli have been profiled using microarrays, including macrophages (Ma et al., 2003), neutrophils (Fessler et al., 2002; Malcolm et al., 2003), eosinophils (Temple et al., 2001), B cells (Ollila and Vihinen, 2003), T cells (Geserick et al., 2004; Huang et al., 2004), plasma cells (Tarte et al., 2003; Underhill et al., 2003), and dendritic cells (Dietz et al., 2000; Ju et al., 2003; Le Naour et al., 2001; Tureci et al., 2003). These studies have proved very useful in shedding light on the global transcriptional changes that occur in the development and evolution of a cell's activation, differentiation, and response to physiological stimulation in the course of an immune response. Additionally, understanding of normal function of cells is crucial to the understanding of pathology, for example in the case of B cell tumours. B cell tumours can arise from cells in any stage of B cell development and differentiation, and the resulting tumour phenotype mirrors that of the corresponding B cell differentiation stage from which the tumour arose. Accordingly, understanding B cell development allows the accurate classification of B cell tumours, and has effects on directing suitable therapy (Jenner et al., 2003).

There are also numerous studies profiling responses to various stimuli of non-immune cells such as endothelial cells (Ahn et al., 2003; Wang et al., 2004; Warke et al., 2003), epithelial cells (Bohn et al., 2004; Zhang et al., 2003b), fibroblasts (Blader et al., 2001),

liver cells (Aizaki et al., 2002; Smith et al., 2003) and cells of the central nervous system (Galey et al., 2003; Roep, 2003). In addition, the use of microarrays in disease classification, especially in oncology, has revealed important biological markers, and affects clinical management of disease (Guo, 2003; van 't Veer et al., 2002).

The dynamic interaction between host and pathogen has also been studied using microarrays, and *in vitro* studies have included monitoring expression changes in macrophages (Nau et al., 2002; Rodriguez et al., 2004), dendritic cells (Granucci et al., 2001a; Huang et al., 2001; Izmailova et al., 2003), neutrophils (Fessler et al., 2002), peripheral blood mononuclear cells (PBMCs) (Boldrick et al., 2002; Feezor et al., 2003), T cells (Corbeil et al., 2001; Jones and Arvin, 2003), and B cells (Jenner et al., 2003; Kanamori et al., 2004), in response to various bacteria, viruses, parasites, and their components.

The importance of these studies in furthering our understanding of the immune response to pathogens are further detailed in Section 1.4.5.

1.4.4 Microarray data analysis

There are numerous methods for extracting biological information for the results of microarray experiments. As the technology is well-established, analysis techniques have also flourished, and numerous publications describing the analysis pipeline of microarrays are readily available (Knudsen, 2002; Causton et al., 2003). This section therefore focuses on analysis methods suitable for biological problems addressed with microarrays, and the clustering analysis and visualisation beyond basic data analysis (Quackenbush, 2002).

1.4.4.1 Binary comparisons

The simplest type of microarray experiment is a binary system, where the comparison between two samples identifies differentially expressed genes. Such experiments may address the effects of a particular drug (Wilson et al., 1999), or comparisons between normal and cancerous tissue (DeRisi, 1996). Replicates should be performed on such data to confirm results (Lee et al., 2000; Yang and Speed, 2002). Biological replication is essential in experimental design because it allows an estimate of variability. The

ability to assess such variability allows identification of biologically reproducible changes in gene expression levels

As transcriptional profiling has grown in popularity, statistical methods for interpreting the data have proliferated. Variants of common statistical tests generally involve two parts: calculating a test statistic and determining the significance of the observed statistic. A standard statistical test for detecting significant change between repeated measurements of a variable in two groups is the *t*-test (Kerr et al., 2000); this can be generalised to multiple groups via the analysis of variance (ANOVA) F statistic (Churchill, 2004; Park et al., 2003). Variations on the *t*-test statistic (often called "*t*-like tests") for microarray analysis are abundant (Golub et al., 1999; Tusher et al., 2001). However, the *t*-test assumes the data to be normally distributed. As gene elements are not individual entities, and the correlation between genes significant and complex, non-parametric tests are also used. Rank-based non-parametric statistics include both traditional statistical methods (Zhan et al., 2002) and ones especially designed for microarray data (Ben-Dor et al., 1999; Park et al., 2003; Troyanskaya et al., 2002). However, when testing thousands of genes, correction for multiple testing is important. The standard Bonferroni correction (multiplying the uncorrected *p*-value by the number of genes tested) is overly restrictive. This is because corrections are based on the assumption that each gene represents an independent test. A way of estimating the distribution of the data is from randomising the class labels of the data. Such permutation tests, generally carried out by repeated scrambling the samples' class labels and computing the *t* statistics for all genes in the scrambled data, best captures the unknown structure of the data (Tusher et al., 2001).

1.4.4.2 Time-series analyses

Analysis methods for microarray experiments that focus on differential gene expression over a time-series have received relatively little attention. A lack of robust statistical methods tailored to such problems has meant that most analyses generally applied for binary comparisons are also used for time-series analyses. In particular, analysis of variance (ANOVA) has been applied to time-series analysis. This requires replicate samples at each timepoint, and binary comparisons are used to find differentially expressed genes between timepoints. This approach does not rely on any assumptions

about the spacing between timepoints, but does not take into account known temporal relationships of gene expression over time.

Another way of approaching time-series analysis is by assuming that there is some relationship between the timepoints, for example a linear relationship or cyclical phenomena. Mathematical models can then be applied (linear modelling or Fourier transformations respectively) as a statistical analysis tool (Spellman et al., 1998).

Experimental designs that follow biological processes over time offer much insight to such processes. Applying current binary analysis methods that do not take into account the temporal relationship between samples means that such initial knowledge is lost. However, if the temporal element is considered, then assumptions need to be made about the gene expression pattern in order to apply mathematical models for statistical tests. If there are no prior knowledge or expectations on the biological response, clustering remains a way to discover temporal relationships.

1.4.4.3 Pattern discovery

The term "clustering" applies to a wide variety of unsupervised methods for organising multivariate data into groups with roughly similar patterns. Clustering has many applications in expression data analysis. Clues to unknown gene function may be inferred from clusters of genes similarly expressed across many samples. Clustering samples over expression levels of multiple genes has been proposed as a way of defining new disease subclasses (Alizadeh et al., 2000; Golub et al., 1999). Cluster analysis may be used primarily for data reduction and visualisation, or it may be used to generalise or predict the categorisation of new samples (Jenner et al., 2003).

The most frequently used clustering method is hierarchical clustering. In this method, all data points start in their own clusters, and the two clusters most closely related by some similarity metric (usually the Pearson correlation coefficient) are merged. The process of merging the two closest clusters is repeated until a single cluster remains. This arranges the data into a tree structure that can be broken into the desired number of clusters by cutting across the tree at a particular height. Tree structures are easily viewed and understood, and the hierarchical clusters provide potentially useful information about the relationships between clusters.

Partition or centroid algorithms require the number of clusters (k) to be specified, and start with k data points that may be chosen randomly or deliberately. These k points are then used as centre points of an initial set of clusters. The algorithm then partitions the samples into the k clusters, the centres of each cluster readjusted for the new clusters' centre points, and this is iterated until all the samples are assigned to the number of specified clusters. The k-means method is one such approach. A variation of this method that allows samples to influence the location of neighbouring clusters is known as the self-organising map or Kohonen map (Kohonen et al., 1996; Tamayo et al., 1999). Such maps are particularly valuable for describing the relationships between clusters.

There are many other clustering methods, such as clustering based on a pre-defined data model. However, the optimal clustering method is still dependent on the data and the goals of the microarray experiments. Clustering enables pattern discovery and a reduction in dimensionality that makes the data easier to understand.

Visualisation and understanding of microarray data can also be facilitated by reducing dimensionality in the data. Principal component analysis (Landgrebe et al., 2002; Raychaudhuri et al., 2000), multi-dimensional scaling (Bittner et al., 2000; Khan et al., 1998), and single value decomposition (Alter et al., 2000; Holter et al., 2000) are related techniques that facilitate visualisation and understanding by reducing the dimensionality of the data. Each of these approaches projects the data into a new space that retains a large amount of the original data's variation. These techniques rely on the idea that most of the data's variation can be explained by a smaller number of transformed variables. When this is true, a two- or three-dimensional representation of highly multi-dimensional data may offer invaluable insight. However, much information can be lost, and noise that contributes to variability will also be captured.

1.4.4.4 Domain knowledge

Beyond cluster analysis, data mining including function prediction and promoter prediction together to infer regulatory pathways and networks further enable a systems-view of microarray gene expression data. Genes with unknown function that appear in the same cluster as genes with known function may be inferred to have similar function.

This is because genes in the same cluster share similar transcription response to different conditions, and this is likely to be caused by some commonality in function or role. Comparison of array results with information in the literature and in public expression databases facilitate function prediction. This approach relies heavily on existing functional annotation which is often incomplete. Similarly, genes that are co-expressed may be co-regulated, and share common transcription factor binding sites in their promoter. This can be explored by transcription factor databases (Transcription Element Search Software TESS: http://www.cbil.upenn.edu/tess, Schug and Overton, 1997) that hold sequences of transcription factor binding sites for known transcription factors. Inferring regulatory networks and complex gene relationships rely on time-series data and steady-state data of gene knockouts. Bayesian networks applied to published data have rediscovered known relationships, propose revisions or contradictions of others, and suggest novel interactions (Friedman et al., 2000; Kim et al., 2003a; Savoie et al., 2003; Tamada et al., 2003). In general, models that incorporate existing constraints from other data sources seem to produce hypotheses that agree better with existing biological knowledge than do models learned from expression data alone (Hartemink et al., 2002).

1.4.5 Gene expression profiling in immunology

To date, there are over 100 publications involving microarrays as applied to infectious agents causing pathogenesis, and over 50 publications involving microarrays as applied to host immune response to infections. From the perspective of pathogen studies, microarrays have been applied to the understanding genetic determinants that contribute to pathogenicity, such as microbial toxins and virulence factors (Bohn et al., 2004; Geiss et al., 2003; Lorenz, 2002; Raman et al., 2004; Schoolnik, 2002; Yang et al., 2002). In terms of host studies, microarray investigations into cells of the innate and adaptive immune system have yielded a vast amount of information as to how these cells are activated in response to pathogens and physiological stimuli (Alizadeh et al., 2000; Diehn et al., 2002; Ehrt et al., 2001; Huang et al., 2001; Staudt and Brown, 2000). However, it is the information and knowledge gleaned from host-pathogen interaction studies that provide the key to understanding the immune response to infection, the infectious disease process. This has allowed us to understand not only how the host "sees" microbes, but to dissect the level at which this discrimination occurs.

1.4.5.1 Stereotyped responses

As the function of innate immunity is to provide an immediate host defence response to incoming pathogens, components of innate immunity regulate responses which are largely similar regardless of the pathogen encountered, such as the acute inflammatory response mounted in response to infection. So far, gene expression array experiments have been carried out in monocytes, macrophages, dendritic cells and neutrophils, in response to various pathogens and physiological stimuli (Section 1.4.3). Stereotyped responses are generally responsible for pro-inflammatory "alarm" signals that marshal components of innate defence, including pleiotropic acute inflammatory cytokines. Such responses are usually also mediated in response to whole pathogens, inactivated or killed pathogens as well as pathogen components (Boldrick et al., 2002; Huang et al., 2001; Malcolm et al., 2003; Nau et al., 2002; Subrahmanyam et al., 2001). Such microarray results are in agreement known biology of innate immune cells that express patterm recognition receptors to recognise diverse pathogens via conserved pathogen-associated molecular patterns. It will be important to identify the central regulators of this common immune response, and the ways in which microbes attempt to thwart this process.

In a seminal study, by comparing the gene expression responses of dendritic cells to a bacterium (*Escherichia coli*), a virus (Influenza A) and a fungus (*Candida albicans*), Huang *et al* found a core of 166 genes that were commonly regulated by each organism in dendritic cells (Huang et al., 2001). The inclusion of multiple timepoints allowed the dynamics of expression of these genes to be elucidated, indicating how microarray studies can show the sequence of events and pathways involved in immune responses. Similarly, time-series analysis of dendritic cell responses to single pathogens or pathogen components have also revealed cascades of transcription programming that allow insight into dynamics of gene expression regulated by dendritic cells (Granucci et al., 2001a; Matsunaga et al., 2002; Tureci et al., 2003). Peripheral blood mononuclear cells (PBMCs) and macrophages exposed to different bacteria also regulate stereotyped responses (Boldrick et al., 2002; Nau et al., 2002), including immune activation genes that have both local and systemic effects. Stereotyped responses are generally attributed to activation of toll-like receptors that signal to activate common transcription factors like NF-κB.

1.4.5.2 Differentiating between infectious agents

In addition to stereotyped responses, dendritic cells and macrophages have also been shown to demonstrate pathogen-specific responses (Boldrick et al., 2002; Chaussabel et al., 2003; Huang et al., 2001; Nau et al., 2002). Pathogen-specificity is important for DCs to coordinate the downstream adaptive immune response, and effector functions of macrophages should be similarly tailored towards particular pathogens. Huang *et al* showed that DCs are able to regulate unique transcriptional programs in response to *E. coli* and Influenza. Responses to bacteria showed preferential induction of innate immune response genes such as neutrophil chemoattractant cytokines and inflammatory genes; in contrast, responses to virus showed preferential induction of antiviral genes including interferons. Macrophages can similarly differentiate different strains of bacteria (Nau et al., 2002), where *Mycobacterium tuberculosis* infection in macrophages results in a compromised production of IL-12 compared to infection with *E. coli*. Pathogen-specificity at the innate immune cell level is important in modulating differential downstream adaptive immune responses that are tailored to incoming pathogens.

1.4.5.3 Infection-related experimental issues

There are important experimental issues to consider with experiments that aim to determine pathogen-specificity in the responses of various cell types. For example, the study of Huang *et al* determined DC responses to virus by the host genes regulated in response to Influenza. However, influenza virus establishes a productive infection in DCs (Bhardwaj et al., 1994), and has numerous host immunomodulatory properties in its encoded genes (Geiss et al., 2001; Geiss et al., 2002). Similarly, a "specific" response of impaired IL-12 production as a result of interaction between macrophages and *M. tuberculosis* (Nau et al., 2002) is possibly an active process mediated by the pathogen to downmodulate the immune response. Therefore, the question addressed by such microarray experiments, to discover differential pathogen-specific host responses, need to distinguish between gene expression profiles of host infected-states and gene expression profiles of the host responding to recognition of different pathogens. To address the ability of the host to discriminate between pathogens, responses to inactivated pathogens or defined pathogen components should be explored in parallel to control for possible confounding effects of active replication and pathogen-modulated responses downstream of infection.

The temporal aspect of host response to pathogens is also an important consideration in determining downstream immune responses. The sequence of host responses is central to understanding the development and evolution of the immune response as well as immunopathogenesis. Experiments examining host gene expression responses to infection that have been published to date include measurements at only one or two timepoints (Chaussabel et al., 2003; Detweiler et al., 2001; Dietz et al., 2000; Messmer et al., 2003). This leaves open the possibility that a phase shift in the kinetics of the responses to two stimuli will be interpreted as a significant difference in gene expression profiles. Extended timecourse experiments are therefore critical in fully evaluating the regulation of gene expression in response to pathogen stimuli. Similarly, the "dose" effect of pathogen input varies the "potency" of gene expression changes (Boldrick et al., 2002; Eisenbarth et al., 2002; Oh and Eichelberger, 2000; Ruedl et al., 2000), and this should be appropriately controlled for.

1.5 Aims of this thesis

Discovering the determinants and subsequent outcome of dendritic cell responses to pathogens and their derived components are crucial for the understanding of the generation of host immune responses to pathogens, pathogen strategies to escape and downmodulate the immune response, and the interplay which leads to the eventual outcome of an infection. It may also provide information about potential intervention therapies that may be applied to benefit the host in the face of pathogenic infections.

The aim of this thesis is to harness the power of DNA arrays to globally profile dendritic cell responses to a number of pathogens and derived components, in order to understand how dendritic cells transcriptionally regulate responses that affect their role as antigen presenting cells. The questions that are addressed include:

1. What are the transcriptional responses of dendritic cells to established maturation stimuli, LPS and dsRNA?
2. What are the gene expression changes that occur during the dendritic cell "maturation" response?
3. What are the responses of DCs to different purified preparations of live and inactivated RNA viruses (Influenza and Rhinovirus)?
4. What are the gene expression changes in DCs responding to such RNA viruses, and are there core and virus-specific responses?
5. What are the functional consequences of transcriptional regulation of DC responses to such pathogens and pathogen components?

The results of attempts to answer these questions, together with their interpretation, are the subjects of Chapters 3 to 5. Chapter 3 deals with the array results of dendritic cell responses to LPS and dsRNA, which further characterises the DC maturation response. Chapter 4 contains results of DC responses to Influenza and Rhinovirus measured by phenotype changes and DC viability, as well as initial array analyses of DC transcriptional responses to the two viruses. Chapter 5 further explores the theme of transcriptional plasticity of DCs in response to viruses, and investigates the functional consequences of such transcriptional plasticity.

Chapter 2

Materials and Methods

2.1 Buffers and solutions

Table 2.1. Constituents of buffers and solutions.

Deoxynucleotide triphosphate mix (dNTPs)	100 mM of deoxyadenosine triphosphate (dATP), deoxythymidine triphosphate (dTTP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP)
20x Saline sodium citrate (SSC)	3 M sodium chloride, 0.3 M sodium citrate, pH 7.0
Tris-acetate-EDTA (TAE)	40 mM Tris pH 7.8, 20 mM sodium acetate, 1 mM ethylenediaminetetraacetic acid (EDTA)
Tris-EDTA (TE)	10 mM Tris pH 7.4, 1 mM EDTA
Hanks' Balanced Salt Solution (HBSS)	138 mM sodium chloride, 5 mM potassium chloride, 4 mM sodium bicarbonate, 1 mM calcium chloride, 0.5 mM magnesium chloride, 0.4 mM potassium phosphate (monobasic), 0.3 mM sodium phosphate (dibasic), pH 7.4
Saline-sodium phosphate-EDTA buffer (SSPE)	2.98 M sodium chloride, 0.02 M EDTA, 0.2 M phosphate buffer, pH 7.4,
Phosphate buffered saline (PBS)	137 mM sodium chloride, 2 mM potassium chloride, 10 mM sodium hydrogen phosphate (dibasic), 2 mM potassium hydrogen phosphate (dibasic), pH 7.4

2.2 Dendritic cells

2.2.1 Culturing dendritic cells

Culturing dendritic cells involves a 8-day protocol, starting from day 0 with the collection of blood. The source of dendritic cells was either peripheral venous blood from healthy volunteers, or buffy coats from the National Blood Service (NBS). Buffy coats are the leukocyte-enriched fractions separated in the processing of whole blood donations from voluntary donors for the National Health Service. The buffy coats received from the NBS are deemed unsuitable for platelet production due to an extended bleed time of 15 minutes. Both buffy coat and peripheral blood preparations are diluted in Hanks' Balanced Salt Solution (HBSS) (Gibco Invitrogen, UK) (in a ratio of blood to HBSS of 2:1 for whole blood from healthy volunteers, and 1:6 for buffy coats), and carefully layered on 17.5 ml Ficoll Lymphoprep (Nycomed, Norway). This is centrifuged at 800g for 30 minutes to allow separation of plasma, leukocytes and

erythrocytes. The leukocytes form an interface, and this layer is harvested, spun down (250g, 10 minutes), and washed twice with HBSS by repeat centrifugation and resuspension. The cells are then plated into 6-well plates (Becton Dickinson Falcon, UK) in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco Invitrogen, UK) supplemented with 10% foetal calf serum (FCS), 100 μg/ml streptomycin, and 100 U/ml penicillin (hereafter referred to as complete medium), and incubated at 37°C, 5% CO_2 for 2 hours which allows the monocytes to adhere to the plate. In the case of buffy coats, plating is preceded by a red-cell lysis stage, which involves incubation of the harvested cells with 10 ml Red blood cell lysing buffer (Sigma-Aldrich, UK) for 10 to 15 minutes to lyse remaining erythrocytes. Lysed red cells are removed by repeat washing with HBSS, and the cells are then plated in complete medium. After 2 hours at 37°C, 5% CO_2, non-adherent cells are removed by gentle pipetting, and the remaining adherent cells are cultured in complete medium with human recombinant GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) (both gifts from Schering-Plough Research Institute), at 37°C, 5% CO_2.

On day 4, the cells are collected and layered onto Lymphoprep, and again centrifuged at 800g for 30 minutes. The layer of cells was collected, washed, and counted with a haemocytometer. Dendritic cells were distinguished from small contaminating T cells, B cells, and NK cells. The number of small cells counted determined the amount of mouse anti-CD2 antibody (mouse mAb MAS 593, IgG_{2b}; Harlan SeraLabs, UK), anti-CD3 antibody (supernatant mouse mAb UCHT1, IgG_1; gift from P.C.L. Beverley, Edward Jenner Institute for Vaccine Research, Compton, UK) and anti-CD19 antibody (supernatant mouse mAb BU12, IgG_1; gift from D. Hardie, Birmingham University, UK) that is incubated with the cells. The cells are incubated with anti-CD2, anti-CD3 and anti-CD19 antibodies at 4°C for 30 minutes in 2 ml of complete medium. After 2 washes in cold HBSS, the cells are incubated with sheep anti-mouse IgG-coated immunomagnetic beads (Dynal, Merseyside, UK, 10μl per 10^6 small cells counted) at 4°C for 45 minutes on a rotating mixer, which removes the T, B and NK cells that have bound the mouse antibodies. The beads are then removed, the remaining cells counted, and re-plated in complete medium with GM-CSF and IL-4 at a density of 5×10^5 DCs/ml. The resulting DCs were >90% pure as judged by flow cytometry (Chapter 3, Figure 3.1A).

On day 7 the immature DCs are ready to use. To induce maturation with LPS (Salmonella Minnesota strain) or polyinosinic-polycytidylic acid (poly(I:C), both from Sigma-Aldrich, UK), these are added directly into the culture medium, at concentrations of 100 ng/ml for LPS and 25 μg/ml for poly(I:C). Control mock-stimulated DCs are treated with the equivalent volume of complete medium.

2.2.2 Infecting dendritic cells

Day 7 immature DCs were collected, centrifuged, and counted. Depending on the required multiplicity of infection (MOI), the appropriate number of cells were again centrifuged, the supernatant removed, and infected with virus in a small volume (approximately 100μl). This virus-DC mixture was incubated at 37°C, 5% CO_2 for 1 hour. After an hour, HBSS was added to the cells, and this was centrifuged and resuspended, and repeated, to ensure removal of unbound virus. The cells were then replated in complete medium with GM-CSF and IL-4 for the required amount of time at 37°C, 5% CO_2, before RNA harvesting or phenotyping by flow cytometry.

2.2.3 Virus preparations

Sucrose-gradient purified Influenza virus (strain A/PR8/34, H1N1) and Rhinovirus (major group RV16) were gifts from collaborators at the University of Reading (W. Barclay) and University of Leeds (R. Rowlands and T. Tuthill) respectively. The virus preparations were stored in aliquots of 50 μl or 100 μl at -70°C until required. The titre of the viruses were at $1\text{-}3\times10^9$ plaque forming units (pfu)/ml, as determined on Madin-Darby canine kidney (MDCK) or HeLa (Ohio strain) cells for Influenza and Rhinovirus respectively.

2.2.4 Inactivation of viruses

Virus aliquots were thawed on ice, and the appropriate volume was transferred to a 0.5 ml eppendorf, and exposed directly to short wave UV-light (R-52 Grid Lamp, UVP, Cambridge, UK) on ice for 2 minutes. This was then used as inactivated virus, and incubated with DCs as in Section 2.2.2.

2.2.5 Monitoring extracellular phenotype by flow cytometry

Stimulated or virus-exposed DCs were collected after the appropriate length of time, and monitored for surface expression of a number of maturation markers, including MHC class II (HLA-DR, supernatant mouse mAb L243, IgG_{2a}; gift from P. C. L. Beverley), MHC class I (HLA-ABC, W6/32; Serotec, Oxford, UK), CD86 (supernatant mouse mAb BU63, IgG_1; gift from D. Hardie), and ICAM-1 (mouse mAb HA58, IgG_1, eBioscience, UK). DC surface expression of CD1a (supernatant mouse mAb NA1/34, IgG_{2a}; gift from A. McMichael, John Radcliffe Hospital, Oxford, UK) and CD14 (supernatant mouse mAb HB246, IgG_{2b}; gift from P. C. L. Beverley) was also determined. DCs were first incubated in HBSS containing 0.1% sodium azide and 10% rabbit serum (blocking solution) to block non-specific binding, at 10^5 cells per 100 µl, at 4°C for 15 minutes. The cells were then incubated with 50 µl of the appropriate primary antibody at 4°C for 30 minutes. Cells were washed twice in blocking solution, and then incubated with 50 µl of rabbit anti-mouse IgG conjugated to fluorescein isothiocyanate (FITC, DakoCytomation, UK, diluted 1:20) at 4°C for 30 minutes in the dark. Cells were then washed twice in HBSS containing 0.1% sodium azide (wash solution), then resuspended in 50 µl wash solution and fixed in 100 µl 3.7% formaldehyde. Cells were then analysed by flow cytometry within 2 days using a FACScan with Cellquest software (Becton Dickinson, UK).

2.2.6 Monitoring intracellular protein production by flow cytometry

Intracellular staining was used to monitor levels of intracellular protein in DCs, including cathepsin E (mouse mAb CE1.1, IgM), Influenza nucleoprotein (NP, mouse mAb AA5H, IgG_{2a}; ImmunologicalsDirect, Oxfordshire, UK), and whole Rhinovirus particles (guinea pig HRV16 antiserum, American Type Culture Collection, UK).

DCs were collected and spun down, and fixed in 400 µl of 4% paraformaldehyde at 4°C for 10 minutes. Cells were washed twice in HBSS containing 2% FCS and 0.1% sodium azide (wash solution), and then permeabilised in 100 µl 0.1% Triton-X100 (Sigma-Aldrich, UK) at 4°C for 10 minutes. The cells were again washed twice in wash solution, and then blocked in HBSS containing 10% goat serum and 0.1% sodium azide (blocking solution), at 3×10^5 cells per 100 µl, at 4°C for 10 minutes. The cells were then incubated with 50 µl of the appropriate primary antibody at 4°C for 45 minutes, washed

twice in blocking solution, then incubated with 100 µl of the appropriate secondary antibody for 45 minutes at 4°C in the dark. Cells were then washed twice in wash solution, and analysed by flow cytometry immediately.

2.2.7 Monitoring apoptosis by Annexin V staining

The Becton Dickinson Pharmingen Annexin V-FITC Apoptosis detection kit (BD Biosciences, UK) was used to monitor DC viability following virus infections and other stimuli. Following culture with the appropriate stimulus, DCs were collected, washed and centrifuged, and resuspended in 100 µl 1× Annexin V Binding Buffer at 1×10^6 cells/ml. The cells were incubated with 5 µl of AnnexinV-FITC and 5 µl of Propidium Iodide for 15 minutes in the dark at room temperature, after which 400 µl 1× Annexin V Binding Buffer was added to the samples, and the samples were analysed by flow cytometry immediately.

2.2.8 Harvesting dendritic cells for arrays

Cultured DCs were harvested at the appropriate timepoints after incubation with LPS or poly(I:C), or infection with virus. For time 0, the stimulus was added to the media, then the medium was immediately removed with a pipette, and TRIZOL® reagent (Invitrogen, UK) added directly to the plate containing adherent DCs. The medium containing non-adherent DCs was centrifuged, the supernatant removed and frozen at -20°C, and the TRIZOL® from the plate was used to resuspend the cell pellet, and this was then frozen at -70°C. This was the protocol for harvesting subsequent timepoints, where the supernatants were stored at -20°C and the TRIZOL® aliquot was used to lyse both adherent and non-adherent DCs.

2.3 Cell culture

Table 2.2 Cell lines used in as reference RNA in this study

Cell line	*Origin*	*Culture conditions*
HeLa	Cervical epithelium	DMEM[†], 10% FCS
HuH7	Hepatoma	DMEM, 10% FCS
Ramos	B cell	RPMI[‡], 10% FCS
MRC5	Embryonic fibroblast	DMEM, 10% FCS
BMVEC	Breast microvascular endothelium	EGM-2-MV, 5% FBS (with BulletKit[§])
SSCEM	T cell	RPMI, 10% FCS
U937	Monocytes	RPMI, 10% FCS

[†] Dulbecco's Modified Eagle's Medium (Gibco Invitrogen, UK)
[‡] Roswell Park Memorial Institute 1640 medium (Gibco Invitrogen, UK)
[§] Microvascular Endothelial Growth Medium-2 (Clonetics, BioWhittaker, Cambrex, USA)

2.3.1 Thawing cells

Cells removed from liquid nitrogen were thawed rapidly at 37°C. Cells were added to 20 ml of the appropriate medium, Dulbecco's Modified Eagle's Medium (DMEM) or Roswell Park Memorial Institute (RPMI)-1640 medium (both from Gibco Invitrogen, UK) with 10% foetal calf serum (Helena Biosciences, UK), 100 U/ml penicillin and 100 µg/ml streptomycin (both from Gibco Invitrogen, UK). The cells were then pelleted at 325g for 5 minutes, resuspended in 10 ml media and counted with a haemocytometer. The cells were centrifuged for a further 5 minutes and resuspended at 5×10^6 cells/ml. The medium was replaced after 24 hours.

Breast microvascular endothelial cells (BMVEC) (gift from C Boshoff, University College London, UK) were maintained in EGM-2-MV (Clonetics, Biowhittaker, Cambrex, USA) with supplements provided as single-use aliquots in the Bullet-Kit (including 5% foetal bovine serum (FBS), human recombinant epidermal growth factor, fibroblast growth factor, vascular endothelial growth factor, insulin-like growth factor, vitamin C, hydrocortisone, gentamicin, amphotericin-B).

2.3.2 Passaging cells

Cells were cultured in growth medium with 10% FCS (or 5% FBS for BMVECs) and antibiotics penicillin and streptomycin (or gentamicin and amphotericin-B for BMVECs) in 5% CO_2 at 37°C for the initial 1-2 weeks after thawing. Cells were split 1:2 to 1:5, depending on cell density and rate of growth, approximately twice a week.

2.3.3 Mycoplasma testing

After thawing, cells were grown for 1 week in the same growth medium that did not contain antibiotics, and the culture medium was harvested for mycoplasma testing at the Institute of Cancer Research. The cell lines in Table 2.2 used for making the microarray common reference were verified as mycoplasma negative by Hoechst (DNA) stain.

2.3.4 Freezing cells

Cells were centrifuged at 325g for 5 minutes and resuspended at 10^7 cells/ml in cold medium (DMEM or RPMI-1640) with 20% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. An equal volume of media containing 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin and 20% dimethyl sulphoxide (DMSO, Sigma-Aldrich, UK) was added. Cells were aliquoted into cryovials (Nunc, USA) and gradually cooled to -70°C in an isopropanol-containing cryo-container (Nalgene, USA) before being transferred to liquid nitrogen after 24 hours.

2.3.5 Harvesting cells for reference RNA

Cells were gradually bulked up over 2 to 3 weeks (depending on rate of growth), gradually seeding into larger flasks. When sufficient numbers of cells were cultured, the culture medium was replaced with fresh medium 24 hours before harvesting. For adherent cells, the culture medium was removed, and TRIZOL® directly added to the flask, 1 ml TRIZOL® per 10 cm^2. The cell lysate was passed several times through a pipette, and aliquoted into cryovials and frozen at -70°C. For non-adherent cells, the culture medium with cells was removed, centrifuged, and TRIZOL® was added directly to the cell pellet, at 1 ml per 5-10×10^6 cells.

2.4 Dendritic cell microarrays

Human cDNA microarrays were supplied by the Human Genome Mapping Project Resource Centre (HGMP-RC) Microarray Programme. The majority of the clones printed on these arrays are from the HuGen cDNA set, the HGMP-RC's minimally redundant human gene set, as well as PCR inserts from an "Angiogenesis set" prepared by the Reproductive Molecular Research Group, Department of Pathology, University of Cambridge. Throughout the period from 2002 to 2004, the content and layout of these human arrays have changed from array version 1 to array version 2, to further incorporate clones from the Mammalian Gene collection. Accordingly, 35 out of 45 microarray experiments that are included in this thesis were hybridised on version 1 microarrays, and the remaining 10 on version 2 microarrays (Table 2.3, Figure 3.4).

Table 2.3 Gene elements and microarray experiments hybridised to different microarrays

	Human cDNA array version 1	*Human cDNA array version 2*
Gene elements	9,216	11,520
Experiments	Control, LPS, poly(I:C) (24)†	LPS (3)
	Influenza (6)	Inactivated Influenza (1)
	Inactivated Influenza (5)	Rhinovirus (6)

†number of arrays hybridised with RNA from DCs treated with the indicated stimuli

2.4.1 Total RNA purification

Frozen TRIZOL lysates (sections 2.2.8 and 2.3.5) were thawed at 37°C and centrifuged at 12,000g for 10 minutes at 4°C to remove insoluble material. The supernatant was transferred to a new eppendorf tube and left at room temperature for 5 minutes. Chloroform was then added, 200 µl per ml of TRIZOL, and the solution shaken by hand for 15 seconds. After incubation at room temperature for 2-3 minutes, the aqueous layer was separated from the organic layer by centrifuging at 12,000g for 15 minutes at 4°C. The top aqueous layer was transferred to a new tube and 500µl (per ml TRIZOL) of chloroform was added to repeat the chloroform extraction. After incubation at room temperature for 2-3 minutes and centrifuging at 12,000g for 15 minutes at 4°C, the aqueous layer was transferred to a new tube. Isopropanol was added (500 µl per ml TRIZOL) to the aqueous layer to precipitate RNA. The solution was vortexed and incubated at room temperature for 10 minutes before the RNA was pelleted by centrifugation at 12,000g for 15 minutes at 4°C. The supernatant was discarded and the pellet washed with 1 ml 75% ethanol and centrifuged at 7,500g for 5 minutes at 4°C.

The supernatant was removed once more and the pellet dried at room temperature. The RNA was then resuspended in 100µl diethyl pyrocarbonate (DEPC)-treated distilled water. RNA was quantified by UV-spectrophotometry, measuring UV absorbance at 260 nm. An absorbance of 1 unit/cm was taken to be equivalent to 40 µg/ml RNA. The purity of RNA was measured by the ratio of the absorbencies at 260 nm and 280 nm. Contaminating DNA was removed from RNA by treatment with DNase I (Promega, UK) according to the following protocol:

Starting RNA	*50µg*	*100µg*	*150µg*	*200µg*
RNA	100	100	100	100
DNase buffer	15	20	30	40
DNase I (1U/µl)	5	10	15	20
DEPC water	30	70	155	240
Total	150µl	200µl	300µl	400µl

This was incubated at 37°C for 1 hour. The reaction was stopped by adding $1/10^{th}$ volume of terminator mix (0.1M EDTA pH 8, 1 mg/ml glycogen). The RNA was re-extracted by adding an equal volume of phenol:chloroform:isoamylalcohol (25:24:1, Sigma-Aldrich, UK). The mixture was vortexed for 15 seconds, centrifuged at 14,000g for 10 minutes, and the top aqueous layer transferred to a new tube. This was then repeated with phenol:chloroform:isoamylalcohol, and once with chloroform to remove any remaining phenol. RNA was precipitated at -20°C for 2 hours with $1/5^{th}$ volume ammonium acetate (8M) and 2.5 volumes of 95% ethanol. RNA was pelleted by centrifugation at 14,000g for 30 minutes at 4°C, the pellet washed with 200 µl 80% ethanol, and centrifuged at 14,000g for 10 minutes. The ethanol was removed, and the RNA pellet was air-dried on the bench at room temperature. RNA was resuspended 100 µl DEPC-treated distilled water, quantified and quality-assessed using the Agilent Bioanalyzer (Agilent, UK) RNA 6000 Nano assay protocol for total RNA, and stored at -70°C.

2.4.2 mRNA purification

mRNA was purified from total RNA using Oligotex (Qiagen, UK), which separates nucleic acids containing poly-dA sequences through hybridisation to oligo-dT bound to microscopic polystyrene-latex particles. Before purification, the Oligotex suspension and OBB buffer (binding buffer) was warmed to 37°C, vortexed and then returned to room temperature, and buffer OEB (elution buffer) heated to 70°C. Total RNA was

made up to 250 µl in DEPC-treated water and mixed with 250 µl buffer OBB and 15 µl Oligotex suspension. This was incubated at 70°C for 3 minutes to denature the RNA, and the Oligotex and RNA allowed to anneal at room temperature for 20 minutes. Subsequently, the Oligotex was pelleted by centrifugation at 14,000g for 2 minutes and the supernatant discarded. The pellet was resuspended in 400 µl buffer OW2 (wash buffer), mixed and applied to a Qiagen spin column. This was centrifuged at 14,000g for 1 minute, the Oligotex resuspended in an additional 400 µl buffer OW2 and centrifuged again. The bound mRNA was eluted by resuspending the Oligotex in 100 µl of buffer OEB (70°C) and centrifuging through the column at 14,000g for 1 minute. This was repeated to give a final volume of 200 µl mRNA in elution buffer. The mRNA was quantified and quality-assessed by using the Agilent Bioanalyzer (Agilent, UK) RNA 6000 Nano assay protocol for messeger RNA, and stored at -70°C.

2.4.3 mRNA labelling

mRNA was concentrated to approximately 100 ng/µl by centrifugation through a Microcon YM-30 column (Millipore, UK) at 14,000g for 8 minutes. The mRNA was recovered by inverting the column and centrifuging at 1000g for 3 minutes. Reference RNA was made by mixing mRNA from the different cell lines in the following proportions: HeLa 25%, U937 25%, SSCEM 15%, Ramos 15%, HuH7 10%, MRC5 5%, BMVEC 5% (Table 3.2). The RNA was labelled using the CyScribe First-Strand cDNA Labelling Kit (Amersham Pharmacia Biotech, UK). The initial annealing reaction mixtures included the concentrated mRNA from sample or reference, 1 µl oligo(dT) primer, 1 µl random nonamers, and DEPC-treated water up to a final volume of 11 µl. This was mixed and heated at 70°C for 5 minutes, and then the mixture cooled at room temperature for 10 minutes. The RNA was then reverse transcribed, incorporating Cy5 (sample) and Cy3 (reference) fluorophores, to make differentially labelled cDNA.

RNA, oligo(dT), random nonamers	11µl
5× CyScript buffer	4µl
0.1M DTT	2µl
dCTP nucleotide mix	1µl
Cy3 or Cy5 dCTP (1mM)	1µl
CyScript reverse transcriptase (100U/µl)	1µl

This was mixed, vortexed and the incubated at 42°C for 90 minutes. The remaining mRNA was then denatured with 2.5 µl 0.5M EDTA (pH 8.0) and 10 µl 0.1M NaOH at 70°C for 10 minutes, then neutralised with 10 µl 0.1M HCl. Cot-1 DNA (3µl, Invitrogen, UK) was added to suppress hybridisation of repetitive DNA, and the final volume made up to 300 µl with Tris-EDTA (TE, pH 8.0).

Unincorporated nucleotides were removed and the labelled-cDNA concentrated by serial centrifugations through a Microcon YM-30 column (Millipore, UK) at 14,000g for 5 minutes. At each stage the filtrate was retained, the Microcon column filled with 300 µl TE and centrifuged again. This was repeated two times, and the concentrated cDNA was recovered by inverting the Microcon column and centrifuging at 1,000g for 3 minutes. The desired volume was 13 µl or below. The initial 20 µl of dilute cDNA, 20 µl of the subsequent filtrates, and 1/25th of the final concentrated cDNA was run on a 1% agarose gel (with no ethidium bromide) at 50V for 2 hours. Cy-dye incorporation and removal of unincorporated Cy-dyes was verified by scanning the gel with a fluorimeter (Storm 860, Molecular Dynamics, UK). The scanner emits light at 635nm to detect Cy5 incorporation, and at 450nm to detect Cy3 incorporation.

2.4.4 Hybridisation

The hybridisation mix was made as follows:

20× saline sodium phosphate EDTA (SSPE, Sigma, UK)	20µl
0.5M EDTA	1.1µl
poly-dA (8µg/µl, Amersham Biosciences, UK)	2µl
yeast tRNA (4µg/µl, Sigma, UK)	2µl
Cy5-labelled sample cDNA	
Cy3-labelled reference cDNA	
TE (pH 8.0)	up to 45 µl
10% SDS (added last)	1µl

The cDNA was denatured at 98°C for 2 minutes, then incubated at 37°C for 20 minutes. After incubation, 1µl 100× Denhardt's solution (Sigma-Aldrich, UK) was added to the hybridisation mix, and any insoluble material removed by centrifugation at 14,000g for 15 minutes. Meanwhile, the HGMP glass array was placed inside the hybridisation cassette (Ambion (Europe), UK) and warmed to 65°C for around 15 minutes. The mixed cDNA probe was pipetted onto the glass array, and an ethanol-washed glass

coverslip (22×64mm, no.0 thickness, Chance-Propper, UK) was placed on top of the glass microarray in the chamber. The hybridisation cassette was humidified by addition of 150 µl of 4× SSPE, the lid was screwed on, and the whole cassette was transferred to a 65°C waterbath for 16-18 hours overnight.

After the overnight hybridisation, the array was the removed and placed in 2× SSPE at 50°C until the coverslip became detached from the array, and the array was subsequently washed in 2× SSPE for 2 minutes, 1× SSPE for 2 minutes, and 0.1× SSPE for 3 minutes. The microarray was rapidly dried by centrifugation at 200g for 2 minutes. The array was then scanned with a GenePix 4000B microarray scanner (Axon Instruments, US).

2.4.5 Scanning and data extraction

The microarrays were scanned with a GenePix 4000B microarray scanner, controlled by GenePix Pro 3.0 software (Axon Instruments, USA), at 10 µm resolution. Cy3 and Cy5 were simultaneously excited at 532 nm and 635 nm respectively and the resultant emitted light detected with two photomultiplier tubes (PMTs). The voltages across the PMTs were adjusted so that the signals from the array elements were balanced. The PMTs were generally set around 650V to maximise the signal to background ratio. The GenePix software combines the data from the two channels to create a single composite image. Arrays found to have hybridised unevenly or with high background were repeated.

A GAL file (GenePix Array List file) was fitted over the array image using a spot-finding software algorithm. Array elements from which no signal could be detected were flagged as not found by the GenePix software. All 9,216 (Human cDNA array version 1) or 11,520 (Human cDNA array version 2) elements on each array were checked by eye and the template altered if necessary. Array elements spoiled by debris, scratches or with a high local background were flagged as bad. Data were extracted from the image by the software using the adjusted template. Signal intensity is measured as the average number of bits, ranging from 0 to 65,535 units, for spot pixels in the image file. Expression ratios were calculated by the software as the median of the

ratios between the local background-subtracted Cy3 and Cy5 signals, on a pixel-by-pixel basis.

The data were exported to HGMP_Analyser, a spreadsheet created in Microsoft Excel, and the median of ratios filtered using Boolean operators to remove flagged array elements and elements for which the signal to background ratio (termed signal to noise ratio SNR) was below the averaged SNR of designated negative genes in each array in the Cy3 and Cy5 channels (Table 3.3). The expression ratios for the gene elements that pass the SNR filter were then log-transformed (to base 2), and exported for use in Cluster.

2.5 Microarray data analysis

2.5.1 Cluster analysis

The $\log_2$ expression ratios from different microarray experiments were imported into Cluster software (Eisen et al., 1998). The first operation in Cluster allows removal of genes that do not have an expression ratio above the negative SNR cut-off determined on a per-array basis. For each set of arrays used for Cluster analysis, only the genes that passed the negative SNR filter for all the arrays in the set were used for further analysis (filtered genes on 100% presence). Genes and arrays were then normalised by median centring across genes and arrays. The genes were then ordered with a 1-dimensional self-organising map (SOM) algorithm. The number of nodes was set to the square root of the number of gene elements to the nearest whole number (e.g. $\sqrt{4320} = 65.6 = 66$ nodes). This ordering was used to control the orientation of nodes generated by hierarchical clustering, to take into account relationships between neighbouring clusters that is incorporated in the SOM algorithm. Both arrays and genes were clustered by average-linkage hierarchical clustering using the uncentred Pearson correlation as the similarity metric (the algorithm by which the similarity of the expression patterns are judged). Hierarchical clustering does not take into account temporal relationship between array samples. The results were visualised with the software Treeview (Eisen et al., 1998).

Treeview is the complement software to Cluster that allows visualisation of clustered data. The data matrix table of expression ratios are represented graphically by a colour

that is defined by the user: black for $\log_2$ expression ratios of 0, increasingly positive $\log_2$ expression ratios with reds of increasing intensity, and increasingly negative $\log_2$ expression ratios with greens of increasing intensity. A representation of the array sample and gene dendogram are appended to the coloured table indicates the nature of the relationships between the samples and the genes in the table.

2.5.2 Significance Analysis of Microarrays (SAM)

Significance Analysis of Microarrays, a program run as an Microsoft Excel add-in, allows binary comparisons of microarray data, a gene-specific *t*-test that gives significantly regulated genes, and includes a permutation algorithm that allows estimation of error, termed the false discovery rate (FDR) (Tusher et al., 2001). From Cluster, genes that passed the 100% presence filter (i.e. genes that pass SNR filter across all arrays analysed) were separated into corresponding technical repeats to give two expression ratios per gene. This effectively doubles the number of arrays analysed, as each array that formerly contained gene duplicates are separated into two separate technical repeat arrays (Table 3.4). These normalised $\log_2$ expression ratios across different array experiments (mock-stimulated control, LPS and poly(I:C), 27 arrays), with the corresponding Genbank Accession numbers, were used as input data for SAM.

SAM was used to compare genes that were regulated by DCs in response to LPS and poly(I:C) stimulus compared to control mock-stimulated DCs. The comparisons are shown in Table 3.4. The estimation of false discovery rate (FDR) is determined by a user-defined Delta value, which also determines a threshold for identifying significantly regulated genes (Section 3.2.7.1). Delta values for each binary comparison were chosen to maintain a consistent FDR of approximately 5% (i.e. 5 genes in every 100 genes found to be significantly regulated may be falsely identified). Lists of significantly up- and down-regulated genes were assembled for each binary comparison (numbers of genes shown in Table 3.5), which is correlated to a graphical output in Excel, which plots the observed (input) data values of $\log_2$ expression ratios (y-axis) versus the expected (permuted) values (x-axis) (Figure 3.11).

2.5.3 Mann-Whitney U (Wilcoxon-rank) test

MWU was used as a non-parametric statistical method to determine genes significantly regulated by DCs in response to LPS and dsRNA. The same input data for SAM was used for Mann-Whitney U (MWU) analysis in Microsoft Excel (Table 3.4). The expression ratio for each gene element was converted into ranks relative to that gene element across the arrays in the binary comparison. A U-value for each array element was calculated by:

$$U_i = n_{i,1}n_{i,2} + ((n_{i,1}(n_{i,1}+1))/2) - R_1$$

Where:

$n_{i,1}$ = the number of non-filtered expression ratios for the i^{th} array element in the LPS- or poly(I:C)-stimulated DC samples (6)

$n_{i,2}$ = the number of non-filtered expression ratios for the i^{th} array element in the control mock-stimulated DC samples (6)

R_1 = sum of ranks for the i^{th} array element in the LPS- or poly(I:C)-stimulated DC samples

The U values were converted to the standard normal variable for the i^{th} array element (Z_i) with the equation:

$$Z_i = \frac{U_i - ((n_{i,1}n_{i,2})/2)}{\sqrt{(n_{i,1}n_{i,2}(n_{i,1}+n_{i,2}+1)/12)}}$$

The probability associated with each Z-value was calculated in Excel (ZTEST), and the level of significance (chosen to be $p<0.01$) determined the number of genes found to be significantly regulated by DCs in response to LPS or poly(I:C) compared to control mock-stimulated DCs (Table 3.6).

2.6 Transcript confirmation of microarray data

Table 2.4 Primers used for transcript confirmation

Gene	Sequence	Product size	Reference
TNFα	F: ATGAGCACTGAAAGCATGAT R: TGACTGCCTGGGCCAGAGGG	232 bp	—
CCL2	F: TGTGCCTGCTGCTCATAG R: GAATCCTGAACCCACTTCTG	235 bp	(Beck et al., 1999)
CD40	F: GTCAGTGCTGTTCTTTGTGC R: AAGATGATGGGGATCACCAC	502 bp	Jenner RG
NF-κB	F: ATTGAAGTGATCCAGGCAGC R: GCAGCTGGCAAAGCTTAGTA	529 bp	Jenner RG
CD86	F: GTATTTTGGCAGGACCAGGA R: GCCGCTTCTTCTTCTTCCAT	664 bp	(Denfeld et al., 1995)
CXCL12	F: ATGAACGCCAAGGTCGTGG R: TCTGAAGGGCACAGTTTGG	175 bp	—
α-tubulin	F: ATGCGTGAGTGCATCTCCA R: GGCATAGTTATTGGCAGCA	298 bp	Jenner RG

2.6.1 RT-PCR

RT-PCR confirmation was performed for the genes shown in Table 2.4, in DCs stimulated with LPS and poly(I:C). Total RNA was purified by the methods detailed in Section 2.4.1 and contaminating DNA removed by treatment with DNase I (Promega, UK). The integrity and quantity of RNA was assessed by the Agilent Bioanalyzer RNA Nano 6000 Assay. The Stratagene StrataScript™ First-Strand Synthesis System (Stratagene, USA) was used for cDNA synthesis. 3 μl of oligo-dT primers (0.1 μg/μl) were annealed to 5 μg of total RNA in 41 μl final volume by heating at 65°C for 5 minutes and cooled slowly at room temperature for 10 minutes. cDNA was synthesised in the following reaction:

RNA and primers	41μl
10× first-strand buffer	5μl
RNase Block Ribonuclease Inhibitor (40U/μl)	1μl
dNTPs (100 mM)	2μl
StrataScript™ reverse transcriptase (50U/μl)	1μl

The reaction was mixed gently and incubated at 42°C for 1 hour, and then terminated at 95°C for 5 minutes. The cDNA was cooled on ice for subsequent use in PCR amplification immediately, or stored at -20°C until needed.

cDNA was amplified for transcripts shown in Table 2.4. The PCR conditions for the primers are shown in Table 2.5. The PCR reactions were carried out in 200 µl thin-walled tubes using Bioline BioTaq DNA polymerase (Bioline, UK):

cDNA	2µl
Primers	2 + 2µl
10× NH_4 buffer	5µl
$MgCl_2$ (50 mM)	1.5µl
dNTPs (10 mM)	1µl
DNA polymerase	0.25µl
Distilled water	36.25µl

PCR products were resolved on a 1.5% agarose gel containing 0.2 µg/ml ethidium bromide. Size of the PCR products were verified by GeneRuler™ DNA ladders 1 kb (to resolve 250 bp to 10 kb) and 100 bp (to resolve 80 bp to 1000 bp) (MBI Fermentas, UK).

Table 2.5 PCR conditions for PCR amplification

Gene	*PCR conditions*
TNFα, α-tubulin	1. 95°C 2 minutes 2. 2. 95°C 30 seconds 3. 57°C 30 seconds 4. 72°C 1 minute 5. repeat steps 2-4 for 35 cycles 6. 72°C 2 minutes
CCL2	1. 94°C 2 minutes 2. 94°C 1 minute 3. 55°C 1 minute 4. 72°C 1 minute 5. repeat steps 2-4 for 35 cycles 6. 72°C 5 minutes
CD40, NF-κB	1. 94°C 2 minutes 2. 94°C 30 seconds 3. 58°C 30 seconds 4. 72°C 1 minute 5. repeat steps 2-4 for 40 cycles 6. 72°C 5 minutes
CD86	1. 94°C 2 minutes 2. 94°C 1 minute 3. 55°C 1 minute 4. 72°C 1 minute 5. repeat steps 2-4 for 40 cycles 6. 72°C 5 minutes
CXCL12	1. 95°C 2 minutes 2. 95°C 30 seconds 3. 55°C 30 seconds 4. 72°C 1 minute 5. repeat steps 2-4 for 40 cycles 6. 72°C 2 minutes

2.6.2 Real-time RT-PCR

Real-time RT-PCR confirmation was performed for TNFα in DCs stimulated with different virus treatments. Total RNA was purified by the methods detailed in Section 2.4.1 and contaminating DNA removed by treatment with DNase I. The integrity and quantity of RNA was assessed by the Agilent Bioanalyzer RNA Nano 6000 Assay. 1 μl of oligo-dT primers (0.5 μg/μl) were annealed to 100 ng of total RNA in 12 μl final volume by heating at 70°C for 10 minutes and cooled slowly at room temperature for 10 minutes. cDNA was synthesised in the following reaction:

RNA and oligo-dT primers	12μl
5× first-strand buffer	4μl
DTT (0.1M)	2μl
dNTPs (100 mM)	1μl
Promega AMV reverse transcriptase (9U/μl)	1μl

The reaction was mixed gently and incubated at 42°C for 50 minutes, and then terminated at 70°C for 15 minutes. The cDNA was cooled on ice for subsequent use in PCR amplification immediately, or stored at -20°C until needed.

Specific TaqMan® primers and FAM™ dye-labelled probe for TNFα were purchased from Applied Biosystems (Assays-on-Demand™ Gene Expression product number Hs00174128). The PCR reaction for TaqMan® quantification were as follows:

cDNA diluted in RNase free water	22.5μl
20× TNFα assay mix (primers and probe)	2.5μl
2× TaqMan® Universal Master Mix buffer	25μl

The reactions were prepared in a 96-well optical reaction plate (ABgene, Surrey, UK) and run on the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, USA). The PCR thermal cycling conditions were:

1. 50°C for 2 minutes
2. 95°C for 10 minutes
3. 95°C for 15 seconds
4. 60°C for 1 minute
5. repeat steps 3 and 4 for 40 cycles.

Quantification of TNFα transcripts in virus-exposed DC timecourse samples were calculated based on relative changes to samples at time zero using the comparative C_T (cycle threshold) method (User Bulletin #2, Applied Biosystems). This method was adapted so that C_T values of virus-exposed DC samples were first normalised to C_T values of control mock-stimulated DC samples at corresponding timepoints, and then these values were divided by the respective time zero virus-exposed DC samples to derive fold changes of TNFα transcript relative to time zero.

2.7 Protein quantification of cytokine secretion

2.7.1 TNFα sandwich immunoassay

Transcript levels of TNFα were further investigated by measuring bioactive TNFα in culture supernatants from DCs stimulated with LPS, poly(I:C), and exposed to different virus treatments. Sandwich enzyme-linked immunosorbent assay (ELISA) for TNFα was performed with a commercial kit from eBioscience, and using Maxisorp Flat-bottom 96-well plates (Nunc, UK). Phosphate buffered saline (pH 7.0) with 0.05% Tween 20 (Sigma-Aldrich, UK) was used for all washes, and the experiment was performed according to the manufacturer's protocol. Biotin-conjugated anti-human antibody was detected by avidin-horseradish peroxidase (HRP) linked detection antibody, and this bound conjugate was allowed to react to tetramethylbenzidine (TMB) substrate solution. After the reaction was stopped by addition of sulphuric acid (2N H_2SO_4), absorbances were read at 450 nm on a microplate reader (Dynex Technologies, UK). TNFα concentrations were calculated by interpolation from a standard curve and all determinations were performed in triplicate.

2.7.2 FAST®Quant MicroSpot ELISA

A novel cytokine array platform from Schleicher & Schuell BioScience, Germany, is a high throughput system for multiplex cytokine quantification. The FAST®Quant Human II system allows the simultaneous detection of 10 different cytokines: IL-8 (CXCL8), GM-CSF, IL-10, IL-1β, IL-12p70, IL-2, MCP-1 (CCL2), IL-4, RANTES (CCL5), and IL-6. The 10 cytokines are spotted in triplicate, along with 6 "dummy" controls, in nitrocellulose, making up 36 spots per reaction pad. Each assay consists of 4 slides containing 16 reaction pads each, making up a total of 64 reaction pads. A cytokine

cocktail mixture of a fixed concentration of the 10 cytokines is provided with the assay, and diluted to form a titration curve with 8 dilutions. This allows the assay of a further 56 individual samples, enabling the simultaneous measurement of the levels of 10 different cytokines in triplicate. DC culture supernatants stimulated with LPS, poly(I:C), and exposed to different virus treatments were assayed for the presence of the 10 cytokines.

Biotinylated anti-cytokine antibodies linked to streptavidin-Cy5 (Amersham Bioscience, UK) enabled cytokine quantification by fluorescence intensity. The slides were scanned on the GenePix 4000B microarray scanner at 10 μm resolution. Images from the 4 slides were "stitched" together using ArrayVision™ FAST® version 8.0 software (Imaging Research Inc). A spot-finding algorithm was applied, and a "protocol editor" file with details of assayed cytokines and titration curve generated standard curves for the 10 cytokines which correlated fluorescence intensities to cytokine concentrations. The data of fluorescence intensities and converted cytokine concentration values were exported to Microsoft Excel, and further analysed to adjust for saturated fluorescence intensities by extrapolating from the standard titration curves.

2.7.3 Type I interferon sandwich immunoassay

To assess the contribution of secreted type I IFNs in the induction of the common antiviral responses in DCs, bioactive IFNα and IFNβ in culture supernatants from DCs exposed to different virus treatments were investigated. Sandwich ELISA for IFNα and IFNβ were performed with two commercial kits from PBL Biomedical Laboratories. The human IFNα ELISA kit is specific for all 13 subspecies of IFNα (IFN-αA, α2, αA/D, αB2, αC, αD, αG, αH, αI, αJ, αK, α4b, and αWA). All the reagents were supplied with the kit, and the experiment was performed according to the manufacturer's protocol. Absorbances were read at 450 nm on a microplate reader (Dynex Technologies). IFNα and IFNβ concentrations were calculated by interpolation from standard curves and all determinations were done in triplicate.

Chapter 3

Dendritic cell activation and differentiation

Introduction

The activation and differentiation of dendritic cells, commonly referred to as the dendritic cell "maturation program", was profiled using DNA microarrays. Current investigations into dendritic cell maturation are assessed by a number of biological endpoints which are measured at the proposed end of the maturation, 24 or 48 hours after dendritic cell stimulation. Such outcomes include upregulation of the cell surface expression of antigen presenting molecules, co-stimulatory molecules, adhesion molecules and chemokine receptors, morphological changes in the acquirement of dendritic processes, loss of phagocytic activity, the increased capacity to stimulate T cells, and the secretion of inflammatory and chemotactic cytokines, including polarising cytokines to mediate a polarised T cell response. We propose that measurement of these outcomes at the end of the maturation process belie its complexity. Transcriptional profiling using DNA microarrays of dendritic cells throughout this maturation process will allow more detailed understanding of dendritic cell activation and differentiation, thereby revealing in greater detail the intricacies of dendritic cell function.

Dendritic cell maturation was investigated using characteristic maturation-inducing agents, lipopolysaccharide (LPS) and polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of double-stranded (ds)RNA. These are ligands for two members of the toll-like receptor (TLR) family, TLR4 and TLR3 respectively, both of which share similar downstream signalling components. The time-dependent nature of dendritic cell maturation indicate the importance of monitoring transcriptional changes over time, rather than simply at the end of maturation. This has revealed distinct stages of dendritic cell maturation, defined by transcriptional profiles, which correspond to dendritic cell phenotype as they progress through activation and differentiation.

Results

3.1 Growing and characterising dendritic cells

Dendritic cells (DCs) were cultured from monocytes isolated from peripheral blood of healthy donors, or buffy coats obtained from the National Blood Service. Immature DCs were differentiated from monocytes in the presence of GM-CSF and IL-4 (Section 2.2.1). The phenotype of *in vitro*-derived immature DCs are similar to those of myeloid DCs, which are $CD1a^{high}$ and $CD14^{-}$, $HLA\text{-}DR^{+}$, $CD86^{low}$ (Figure 3.1A, B). Upon stimulation with maturation-inducing agents such as LPS and poly(I:C), immature DCs undergo phenotypic changes resulting in upregulation of HLA-DR and CD86 (Figure 3.1B). Titration of LPS and poly(I:C) was carried out to determine the optimal dose for HLA-DR and CD86 upregulation (Figure 3.1C), without compromising the viability of the DCs (assessed by the size and geometry of cells by flow cytometry, data not shown). The antigen doses, 100 ng/ml for LPS, 25 μg/ml for poly(I:C), resulted in similar upregulation of HLA-DR and CD86 as determined by flow cytometry (Figure 3.1B, C, D).

3.2 Gene expression analysis of DC maturation

3.2.1 Sample preparation

Immature dendritic cells differentiated from buffy coat monocytes cultured in GM-CSF and IL-4 were used for all array experiments. LPS and poly(I:C) were added on day 7 to immature DCs to induce maturation (Section 2.2.1). Alternatively, an equivalent volume of culture medium was added as a control for mock-stimulated DCs. Cells were harvested over a 24-hour timecourse. Each timecourse consisted of cells from the same buffy coat preparation. Biological replicates at 0, 5, and 18 hours post-stimulus were performed to confirm reproducibility of the arrays and to control for inherent biological variability between different donor sources of these buffy coats (Table 3.1). Total RNA was extracted, DNase treated, and quality- and quantity-assessed by Agilent Bioanalyzer RNA 6000 Nano Assay (Figure 3.2A, B). From total RNA, mRNA was purified (Figure 3.2C), reverse transcribed to cDNA and labelled with Cy5. This was hybridised to arrays together with equivalent amounts of Cy3-labelled reference cDNA.

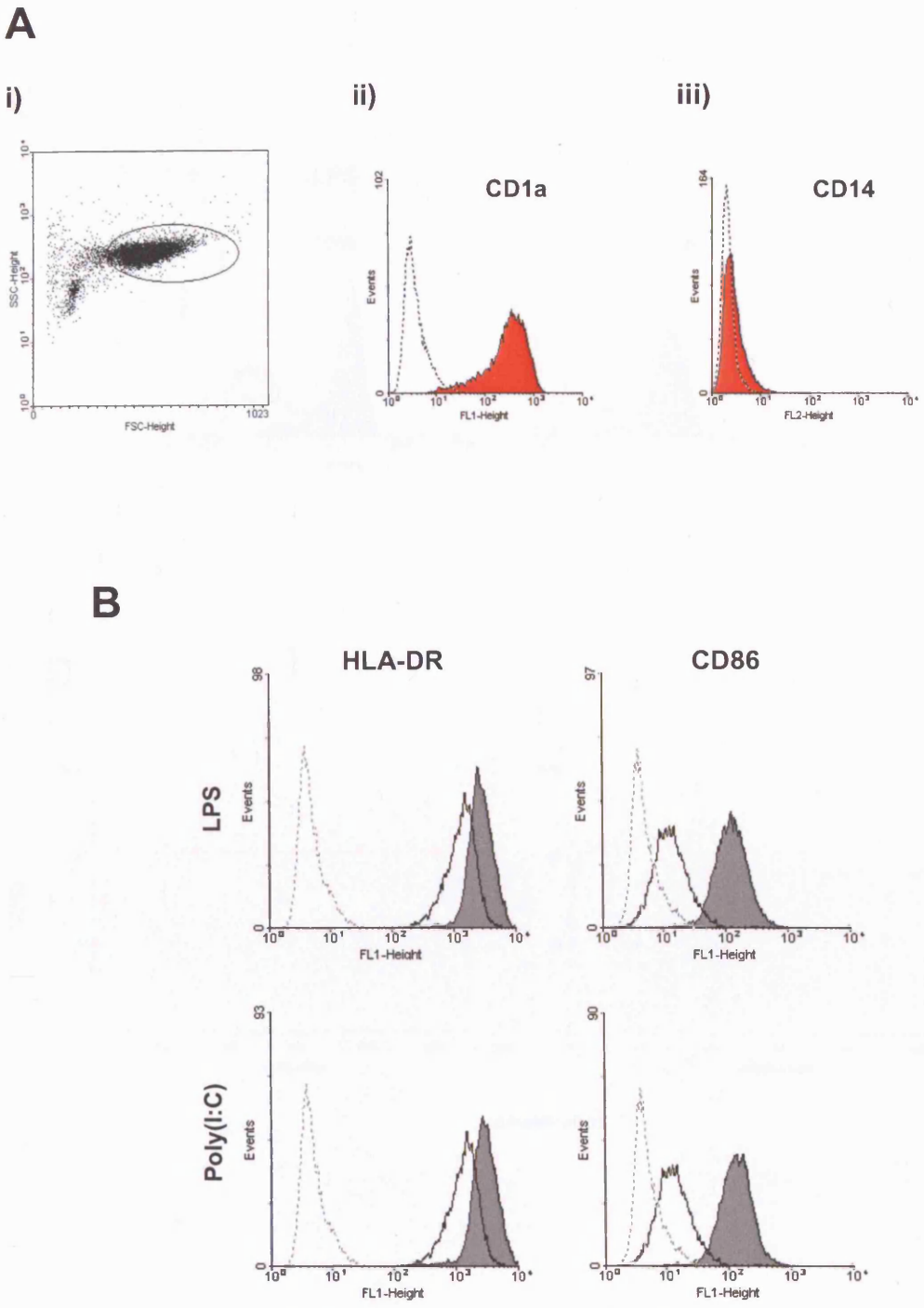


Figure 3.1 Immunophenotype of immature and mature dendritic cells

A (i) Immature DCs are gated by forward and side scatter (>90% of cells within gated population), and are (ii) $CD1a^{high}$ (red histogram) and (iii) $CD14^{-}$ (red histogram).
B Mature DCs, 24 hours after stimulation with LPS (100ng/ml) or poly(I:C) (25μg/ml) upregulate surface expression of HLA-DR and C86 (grey histograms), compared to mock-stimulated control DCs (black unfilled histogram).
(isotype controls shown by dotted histogram for all plots)

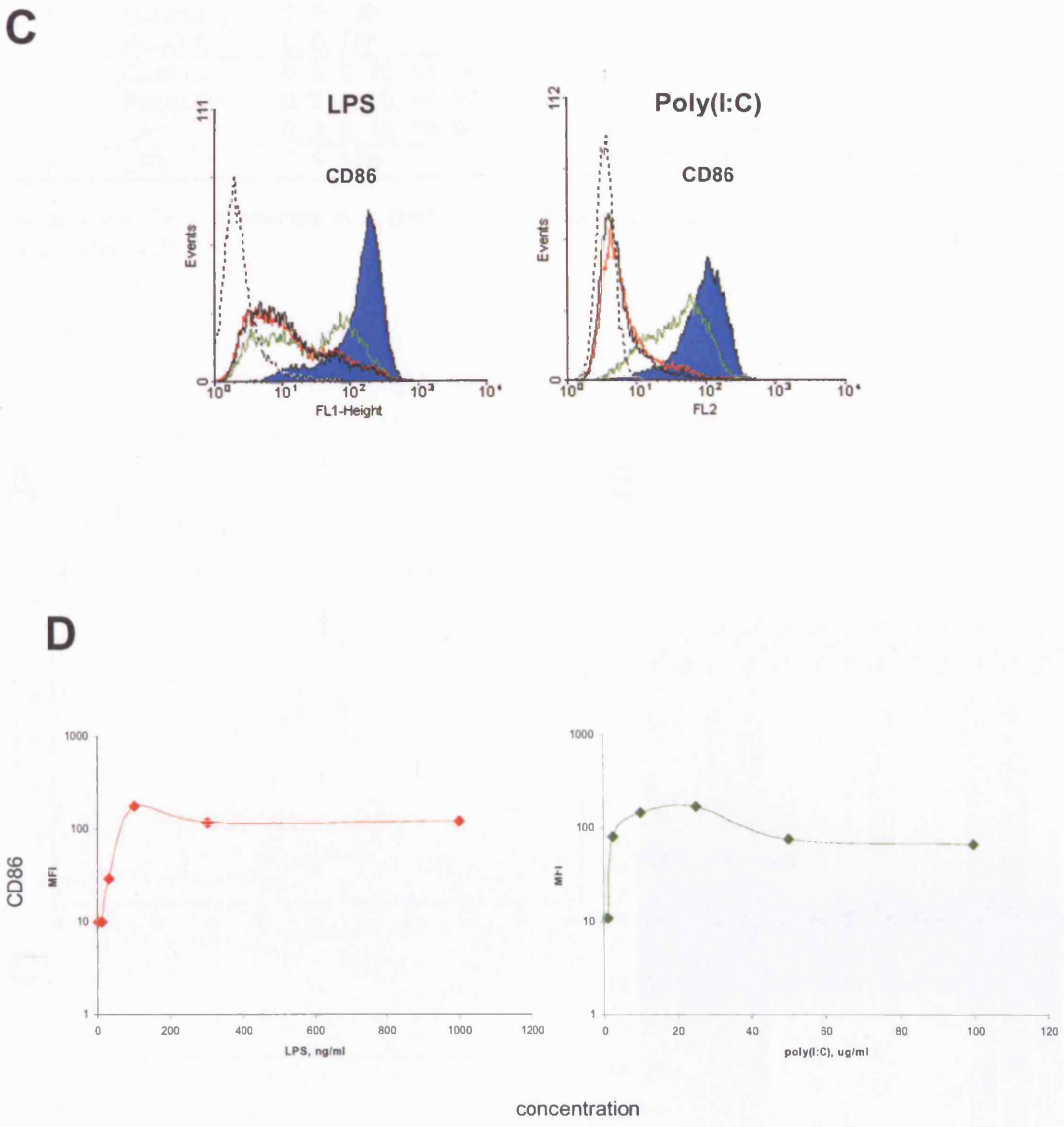


Figure 3.1 Immunophenotype of immature and mature dendritic cells (continued)

C Escalating amounts of LPS (3ng/ml, black histogram; 10ng/ml, red histogram; 30 ng/ml, green histogram; 100 ng/ml, blue filled histogram), and poly(I:C) (2.5 μg/ml, black histogram; 5 μg/ml, red histogram; 10μg/ml, green histogram; 25μg/ml, blue filled histogram) lead to different degrees of CD86 upregulation.
D Titration of amounts of LPS and poly(I:C) show peak CD86 surface expression at 100ng/ml for LPS, and 25μg/ml for poly(I:C).
(isotype controls shown by dotted histogram for all plots)

Table 3.1 Source of cells for DC maturation timecourse arrays

Buffy coat	*Stimulus*	*Timepoints*	*HGMP array*	*Reference batch*[†]
1	Control	0, 5, 18h	Human cDNA array version 1	1
	Poly(I:C)	0, 5, 18h	Human cDNA array version 1	
2	Control	0, 2, 5, 10, 18, 24h	Human cDNA array version 1	2
	Poly(I:C)	0, 2, 5, 10, 18, 24h	Human cDNA array version 1	
	LPS	0, 2, 5, 10, 18, 24h	Human cDNA array version 1	
3	LPS	0, 5, 18h	Human cDNA array version 2	3

[†]Reference RNA consisted of a mixture of mRNAs extracted from different human cell lines (see Table 3.2)

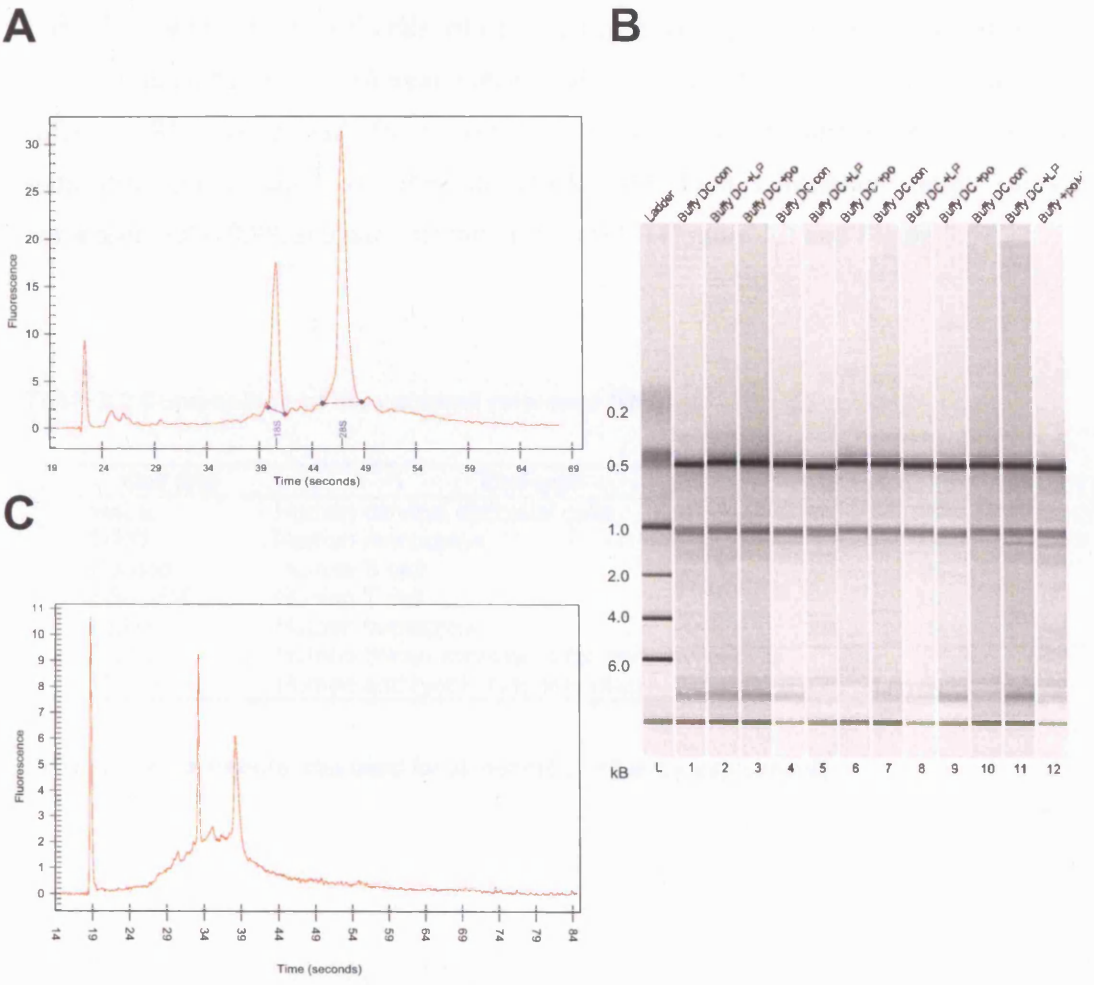


Figure 3.2 Dendritic cell RNA as measured by Agilent Bioanalyzer RNA 6000 NanoAssay

A total RNA, showing 18S and 28S ribosomal peaks, and no contaminating DNA
B "virtual" electrophoretic gel of total RNA with bands corresponding to ribosomal RNA
C messenger RNA purified from total RNA.
Quantification of samples is achieved by comparison with a known concentration of RNA ladder (lane L), which contains distinct sized fragments resulting in 6 peaks, seen in the gel in **B**.

3.2.2 Creating a common reference for gene expression profiling

A common reference RNA approach was used for the two-colour microarrays as previous studies have shown that this method allows multi-comparative analyses across different samples (Introduction Section 1.4.2). The microarrays used for this study contain a broad range of human cDNA probes for different cell types and gene functions. It is therefore important that a reference RNA is representative of this spectrum, in order that the number of detectable expression ratios are maximised for each sample. The reference RNA was designed to cover a range of different cell types to broaden the range of transcripts represented. The relative composition of the reference RNA mixture used is shown in Table 3.2.

Large batches (5×10^7 to 10^8 cells) of each cell type were grown to minimise differences in RNA abundances in different batches of reference RNA. In total, 3 batches of reference RNA were made from these cells. Comparison of sample arrays hybridised with different batches of reference RNA gave high correlation ratios (median correlation ratio 0.89, standard deviation (SD) 0.11) (Figure 3.3 and Figure 3.5).

Table 3.2 Composition of the common reference RNA[†]

Cell line	*Cell type*	*Composition (%)*
HeLa	Human cervical epithelial cells	25
U937	Human monocytes	25
Ramos	Human B cell	15
SS-CEM	Human T cell	15
HuH7	Human hepatoma	10
BMVEC	Human breast microvascular endothelial cells	5
MRC5	Human embryonic lung fibroblast	5

[†] This reference mixture was used for all dendritic cell array experiments

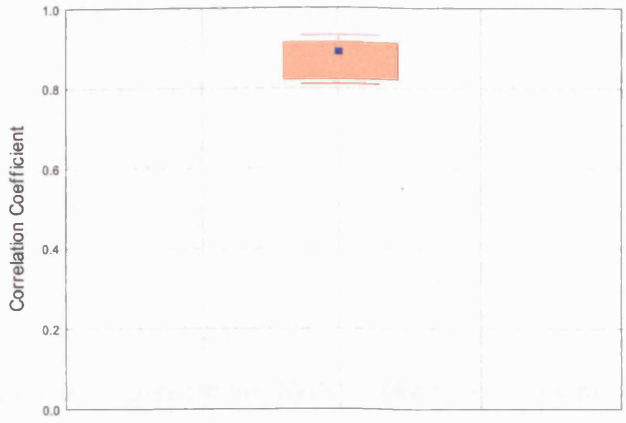


Figure 3.3 Correlation coefficients of biological repeat arrays

Biological replicates of dendritic cell experiments (mock-stimulated, LPS- and poly(I:C)-stimulated DC timecourses at t=0, t=5, t=18, from different buffy coat preparations (as indicated in Table 3.1) hybridised with 3 different batches of reference RNA were compared to assess the correlation between batches of reference RNA. The median correlation coefficient was 0.89 (blue square), standard deviation (SD) 0.11. The box indicates the 25th and 75th quartiles, the whiskers the range of correlation coefficients.

3.2.3 Filtering noise from data

Before array data from multiple samples can be compared, high variance and low intensity spot data need to be removed. Each element on the arrays is quantified by two intensity values at 635 nm (Cy5 emission - sample) and 532 nm (Cy3 emission - reference) as detected by two PMTs. The feature intensity measurements for each spot have corresponding background intensity measurements. The signal-to-noise ratio (SNR) is derived from the quotient of feature intensity divided by background intensity for the two wavelengths. The microarrays contain a set of control genes that were used for data filtering (Table 3.3). These control genes are replicated within each block of the arrays, resulting in 24 replicates of control genes. These serve as a robust internal control for each array hybridisation (Figure 3.4). Negative controls and yeast gene probes were selected as they gave consistently low or background intensity values for both wavelengths. The SNRs of these negative controls were averaged over 24 repeats spotted on the array to give an averaged value for filtering the remaining elements on the array. The gene elements with either Cy5 or Cy3 SNR greater than the filter values were interpreted as having a positive signal above background, and were used for further analysis. Each array was filtered independently, so the genes selected for further analysis from each array were only dependent on experimental variation that manifested in that particular array hybridisation experiment.

Table 3.3 Control genes present on HGMP cDNA arrays version 1 and version 2

Control genes - cDNA array version 1		*Control genes – cDNA array version 2*	
Positive control 1	Human	Positive control	Total human genomic DNA
Positive control 2	Human		
Housekeeping gene 1		Housekeeping gene 1	Actin gamma 1
Housekeeping gene 2		Housekeeping gene 2	Glyceraldehyde-3-phosphate dehydrogenase
Housekeeping gene 3		Housekeeping gene 3	Ubiquinol-cytochrome c reductase core protein II
Housekeeping gene 4		Housekeeping gene 4	Casein kinase II beta polypeptide
Housekeeping gene 5		Housekeeping gene 5	EST highly similar to NY-REN-37 antigen
Housekeeping gene 6		Housekeeping gene 6	Human hydroxymethyl glutaryl-CoA lyase
Housekeeping gene 7		Housekeeping gene 7	Neuroblastoma RAS viral oncogene homolog
Housekeeping gene 8		Housekeeping gene 8	Eukaryotic translation initiation factor 4A, isoform 1
Housekeeping gene 9		Housekeeping gene 9	Ubiquinol-cytochrome c reductase core protein II
Housekeeping gene 10		Housekeeping gene 10	Enoyl Coenzyme A hydrolase, short chain 1
Housekeeping gene 11			
Dynamic range control 1	relative abundance 3.3% - Yeast	Dynamic range control 1	Yeast Intergenic Region from Chrom XI
Dynamic range control 2	relative abundance 1% - Yeast	Dynamic range control 2	Yeast Intergenic Region from Chrom XI
Dynamic range control 3	relative abundance 0.1% - Yeast	Dynamic range control 3	Yeast Intergenic Region from Chrom VII
Dynamic range control 4	relative abundance 0.033% - Yeast	Dynamic range control 4	Yeast Intergenic Region from Chrom VII
Dynamic range control 5	relative abundance 0.01% - Yeast	Dynamic range control 5	Yeast Intergenic Region from Chrom XII
Dynamic range control 6	relative abundance 0.0033% - Yeast	Dynamic range control 6	Yeast Intergenic Region from Chrom XII
Negative control 1	Arabidopsis thaliana protein G1p	Negative control 1	Arabidopsis thaliana protein G1p
Negative control 2		Negative control 2	Poly-dA oligonucleotide
Negative control 3	50%DMSO	Negative control 3	Spotting buffer
Negative control 4	Bacterial	Negative control 4	Bacillus subtilis gene
Negative control 5	Bacterial	Negative control 5	Bacillus subtilis gene
Ratio control 1	1:3 – Yeast	Ratio control 1	Yeast Intergenic Region from Chrom XII
Ratio control 2	3:1 – Yeast	Ratio control 2	Yeast Intergenic Region from Chrom XII
Ratio control 3	1:10 – Yeast	Ratio control 3	Yeast Intergenic Region from Chrom XII
Ratio control 4	10:1 – Yeast	Ratio control 4	Yeast Intergenic Region from Chrom XIII

The genes in red are the ones used to determine negative filter values for Cy5 and Cy3 SNRs. All the control genes, except the positive controls, were spotted on every subgrid of the HGMP cDNA version 1 array, and on every other subgrid of the HGMP cDNA version 2 array (Figure 3.4), resulting in 24 copies of each negative control gene. The average Cy5 and Cy3 SNR for the negative control genes (9 for HGMP cDNA array version 1, 15 for HGMP cDNA array version 2) were averaged, and used as a filter SNR for the remaining gene elements on the arrays.

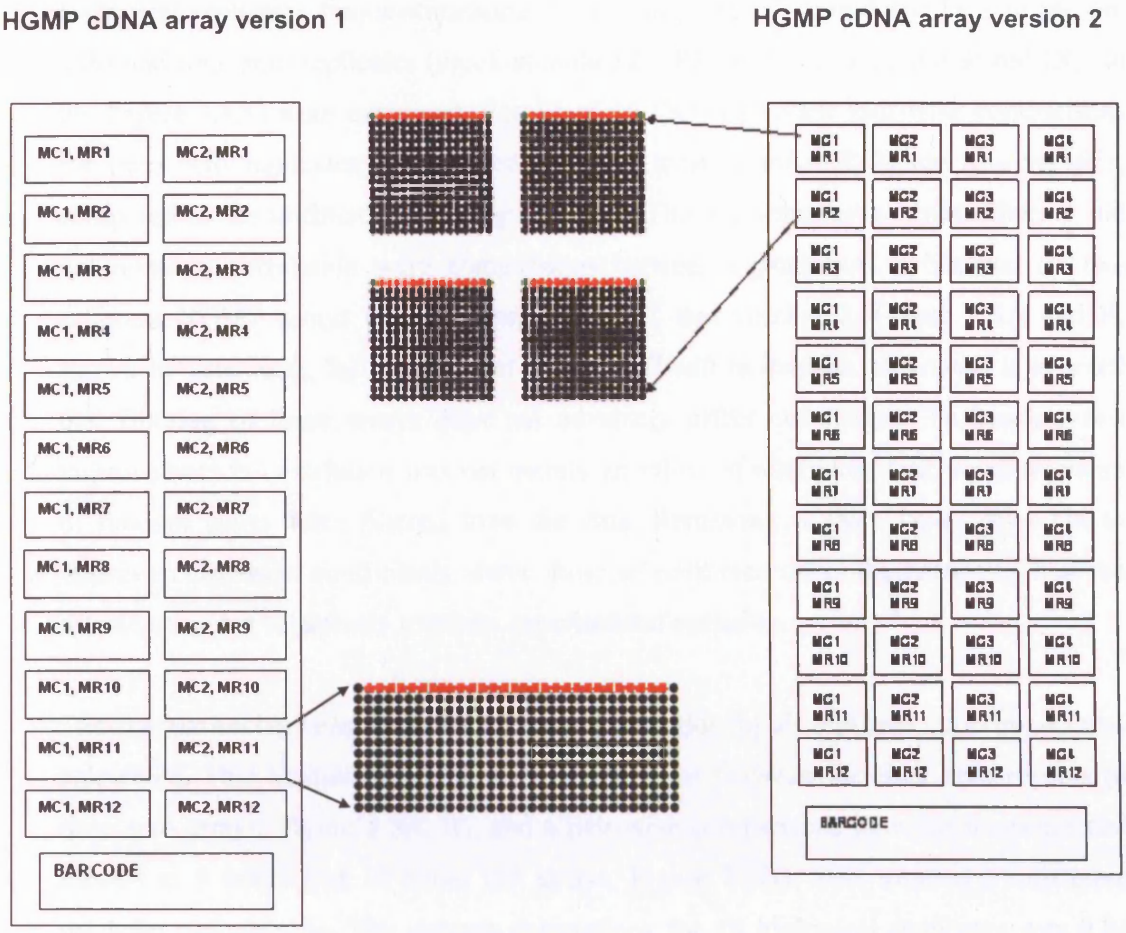


Figure 3.4 Layout of HGMP cDNA version 1 and 2 array

The HGMP cDNA version 1 arrays were used for dendritic cell timecourse experiments from buffy coat preparations 1 and 2 (Table 3.1). The layout of the array is such that gene elements spotted in metacolumn (MC) 2 are the same as the gene elements spotted in MC1. The control genes (shown in red) were spotted on the top row of every subgrid, resulting in 24 control gene data points, to serve as a monitor of hybridisation conditions across the array.
The HGMP cDNA version 2 arrays were used for dendritic cell timecourse experiments from buffy coat preparations 3, and array experiments in Chapter 4. Here, gene elements in MC3 and MC4 are the same as the gene elements in MC1 and MC2 respectively. The range of control genes (in red) were spotted on the top row over two subgrids, giving 24 data points for all the negative control genes.

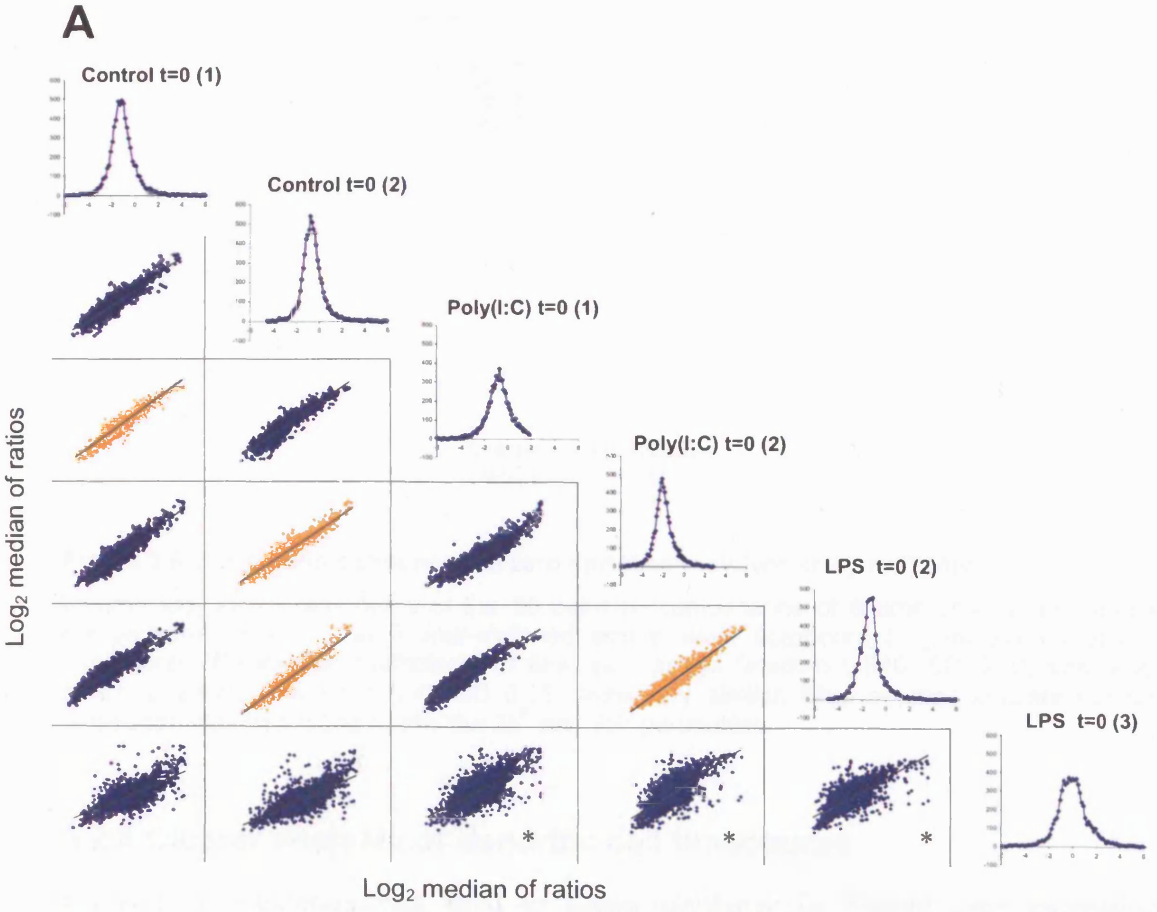
The signal to background filtering was applied on a per array basis to the entire dataset. On average, filtering removed 15% of the array elements (1383 gene elements, SD=10.4, number of arrays (n) = 45). To confirm that the filtering criteria actually reduce experimental variation, the effect on the correlation between the time-matched biological replicates (mock-stimulated, LPS- and poly(I:C)-stimulated DCs at 0h, 5h, 18h) and time zero replicates (mock-stimulated, LPS- and poly(I:C)-stimulated DCs at 0h, Figure 3.5A) were examined. For 12 of 15 (80%) possible pair-wise comparisons for time zero replicates, the filtered data are more correlated, hence less variable, compared to the unfiltered data (Figure 3.5B). The 3 comparisons where filtering did not improve correlation were comparisons between experiments hybridised on two different HGMP arrays (cDNA array version 1 and version 2, Figure 3.5A and B, shown by asterixes), for reasons that remain difficult to explain. However, it is noted that filtering of these arrays does not adversely affect correlation. To confirm this improvement in correlation was not merely an effect of removing data, equal numbers of random genes were filtered from the data. Removing random genes does not to improve correlation coefficients above those of unfiltered data, demonstrating that the filtering process selectively removes experimental variation.

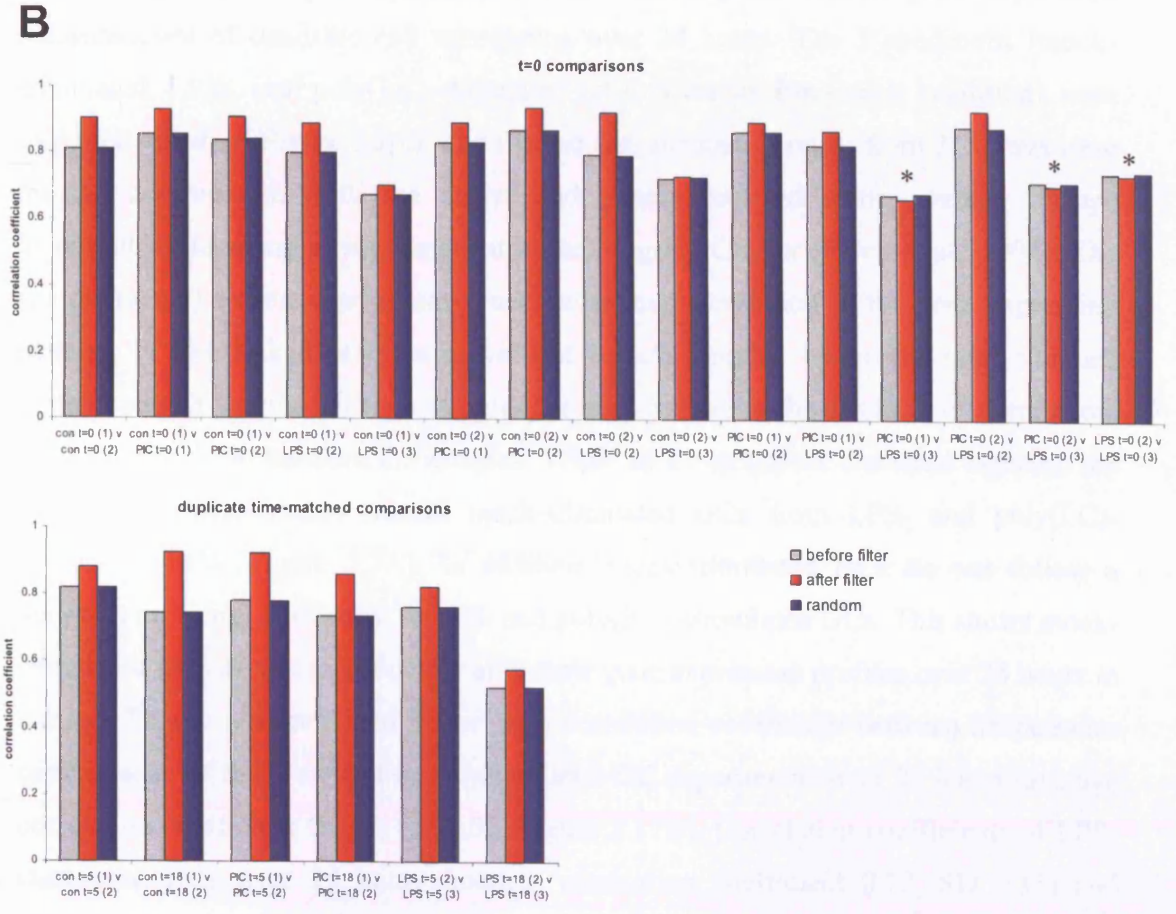
The correlation between filtered $\log_2$ expression ratios for all replicate experiments was calculated. This included 15 pair-wise comparisons between the time zero repeats (6 time zero arrays, Figure 3.5A, B), and 6 pair-wise comparisons between time-matched repeats at 5 hours and 18 hours (12 arrays, Figure 3.5B). This totalled 21 different pairwise comparisons. The average correlations for all biological replicates was 0.84 (n=21 different pairwise comparisons, from 18 biological replicate arrays) (Figure 3.6).

The consistency of the high correlation coefficients between biological replicates show that DCs derived from different buffy coat preparations are cross-comparable, both as immature mock-stimulated DCs (correlation between array experiments at time zero), and in their responses to LPS and poly(I:C) (correlation between time-matched array experiments at t=5 and t=18). The filtering method used is effective at removing experimental variation that arises from hybridisation conditions and inefficient Cy-dye labelling, and is effective at removing noisy data by improving correlation coefficients between arrays of experimental replicates. This forms a good basis for further array analysis.

Figure 3.5 Filtering microarray data by signal to noise ratio (next page)

A Histograms showing distribution of $\log_2$ median of ratios for the six time zero arrays, and the corresponding scatter plots showing correlation between the arrays. The orange scatter plots show correlation between dendritic cells derived from the same buffy coat preparations, which tend to be higher. LPS t=0 (3) is hybridised on the HGMP cDNA version 2 array, resulting in lower correlation when compared to other time zero arrays. The asterixes show the three pairwise comparisons where filtering did not improve correlation (Section 3.2.3).
B Correlation coefficients before and after filtering for all 21 pairwise comparisons, 15 comparisons between time zero arrays and 6 comparisons between time-matched arrays. Comparison of the correlation coefficients after filtering to random removal of the same number of genes shows that 18 out of 21 total correlation coefficients improved by selective filtering based on Cy5 and Cy3 SNR of negative genes. **The 3 comparisons that did not improve with selective filtering (asterixed) were arrays hybridised on version 2 arrays (Section 3.2.3).**


A
Control t=0 (1)
Control t=0 (2)
Poly(I:C) t=0 (1)
Poly(I:C) t=0 (2)
LPS t=0 (2)
LPS t=0 (3)
Log2 median of ratios
Log2 median of ratios


B
t=0 comparisons
correlation coefficient
duplicate time-matched comparisons
correlation coefficient
before filter
after filter
random

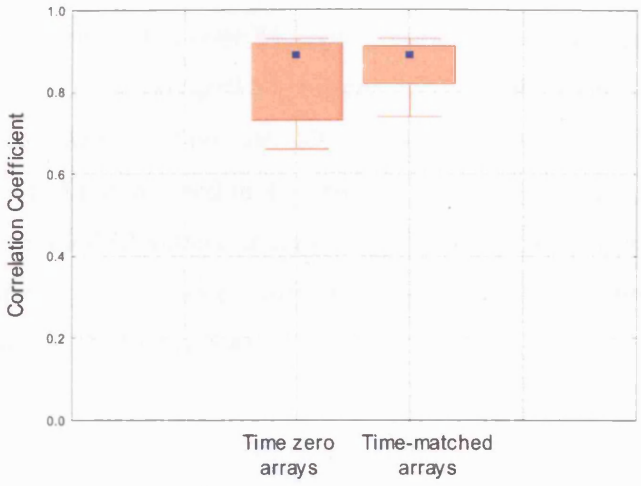


Figure 3.6 Correlation between time zero and time-matched array replicates

Filtered $\log_2$ expression ratios of the 15 pairwise comparisons of 6 time zero arrays, and 9 comparisons between the 9 time-matched arrays, were compared to generate correlation coefficients. Correlation coefficients of time zero arrays (median 0.840, SD 0.10) and time-matched arrays (median 0.836, SD 0.11) were very similar. Blue squares indicate median correlation coefficient, box marks the 25^{th} and 75^{th} percentiles.

3.2.4 Cluster analysis of dendritic cell timecourse

Hierarchical clustering was used to assess similarity in filtered gene expression measurements of dendritic cell stimulation over 24 hours. The 3 conditions (mock-stimulated, LPS-, and poly(I:C)-stimulated DCs, 9 arrays from each condition) were clustered together (Figure 3.7A). The filtered $\log_2$ median of ratios from 27 arrays were median centred and both the arrays and genes clustered using average linkage hierarchical clustering as implemented in the program Cluster (Eisen et al., 1998). The sample (array) cluster dendogram generated relates correlation in the gene expression patterns of two linked samples to vertical branch lengths, where the total summed vertical branch lengths between samples are proportional to the distance, or correlation, in gene expression between the samples. When all 27 arrays are clustered together, the dendogram differentiates control mock-stimulated DCs from LPS- and poly(I:C)-stimulated DCs (Figure 3.7A). In addition, mock-stimulated DCs do not follow a temporal ordering, in contrast to LPS- and poly(I:C)-stimulated DCs. This shows mock-stimulated DCs do not significantly alter their gene expression profiles over 24 hours in culture. This is also reflected in the high correlation coefficient between 36 pairwise comparisons of the 9 control mock-stimulated DC experiments over 24 hours (average correlation coefficient 0.915, SD 0.02, Figure 3.17B). Correlation coefficients of LPS-stimulated DCs over 24 hours (median correlation coefficient 0.72, SD 0.15) and

poly(I:C)-stimulated DCs over 24 hours (median correlation coefficient 0.81, SD 0.08) were significantly lower ($p<10^{-8}$, Figure 3.7B). Both LPS-stimulated and poly(I:C)-stimulated DC arrays at time zero also cluster with the control mock-stimulated arrays (Figure 3.7A). As mentioned in Section 3.2.3, the slight discrepancy of the experiments hybridised on HGMP version 2 arrays appear as outliers within the temporal clusters, but are noted to still cluster within the correct temporal ordering (labelled as LPS3 arrays, Figure 3.7A, Figure 3.8).

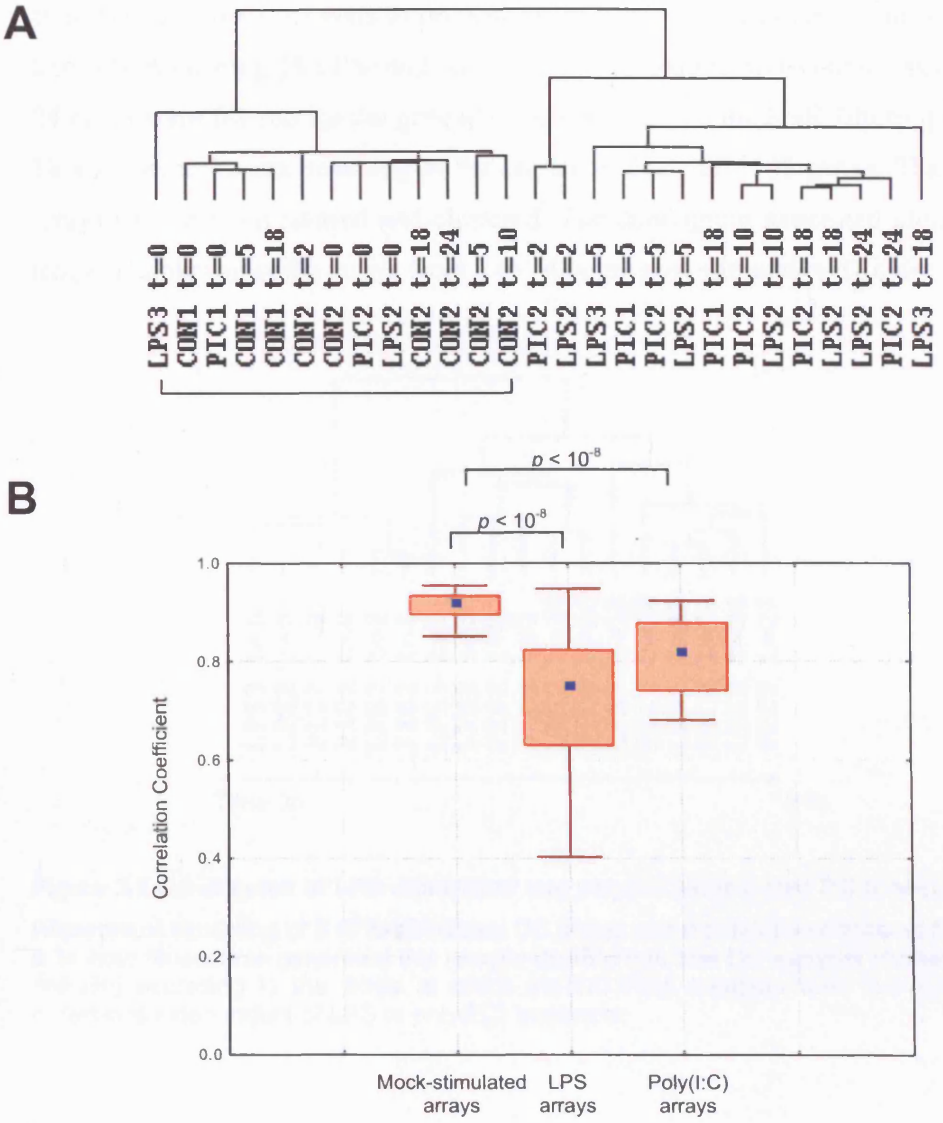


Figure 3.7 Relationship between DC timecourse arrays

A Dendogram of DC timecourse arrays 27 arrays from the 3 conditions were clustered together. Control mock-stimulated DC arrays cluster together with LPS and poly(I:C)-stimulated t=0 arrays, and LPS- and poly(I:C)-stimulated arrays cluster separately, following a temporal order.

B Correlation between control mock-stimulated, LPS, and poly(I:C) arrays
Log_2 gene expression ratios of mock-stimulated, LPS- and poly(I:C)-stimulated arrays were compared, 36 pairwise comparisons for 9 arrays for each condition. Blue squares indicate median correlation coefficient, box shows 25th and 75th percentiles. Correlation between control mock-stimulated DC arrays is significantly higher than correlation between LPS- and poly(I:C)-stimulated DC arrays over 24 hours ($p<10^{-8}$, calculated by the standard Student's paired *t*-test).

Because of the high correlation coefficient between mock-stimulated DC timecourse arrays, these experiments can be treated as biological replicates. They were excluded from further cluster analysis to prevent disproportionate skewing of the sample cluster tree. The remaining 18 LPS- and poly(I:C)-stimulated DC timecourse experiments over 24 hours were filtered for the gene elements that passed the SNR filtering criteria in all 18 array experiments, resulting in the cluster analysis of 5108 genes. These genes and arrays were median centred and clustered. The dendogram generated clearly reflects a temporal ordering in the arrays from 0 to 24 hours post stimulation (Figure 3.8).

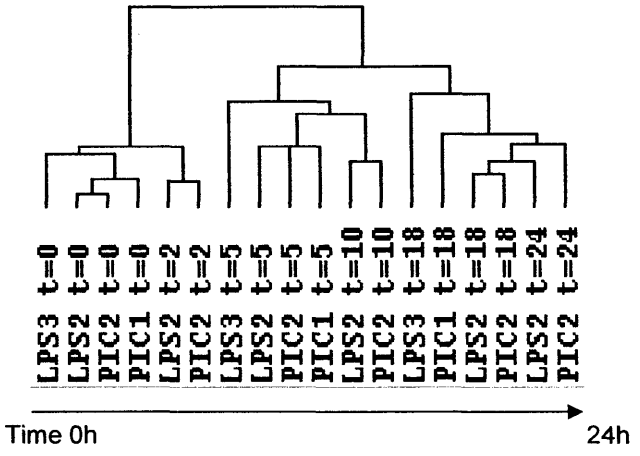


Figure 3.8 Dendogram of LPS-stimulated and poly(I:C)-stimulated DC timecourses

Hierarchical clustering of 9 LPS-stimulated DC arrays and 9 poly(I:C)-stimulated DC arrays over a 24-hour timecourse generated this sample dendogram. The DC samples cluster in a temporal ordering according to the times at which the DC RNA samples were harvested, and the ordering is independent of LPS or poly(I:C) treatment.

3.2.5 Temporal ordering of transcriptional changes during DC "maturation"

The dendogram reveals that transcriptional responses of dendritic cells to LPS and poly(I:C) are very similar, with greater time-dependent relationships than diversity generated by LPS- and poly(I:C)-dependent maturation responses. Both TLR4 and TLR3 share common intracellular signalling pathways that involve the adaptor proteins MyD88 and TRIF, resulting in the activation of similar transcription factors, which help to explain the common response. This also confirms the equivalence of the two stimuli in terms of comparable dosage effecting similar levels of transcriptional responses in DCs (Figure 3.1B).

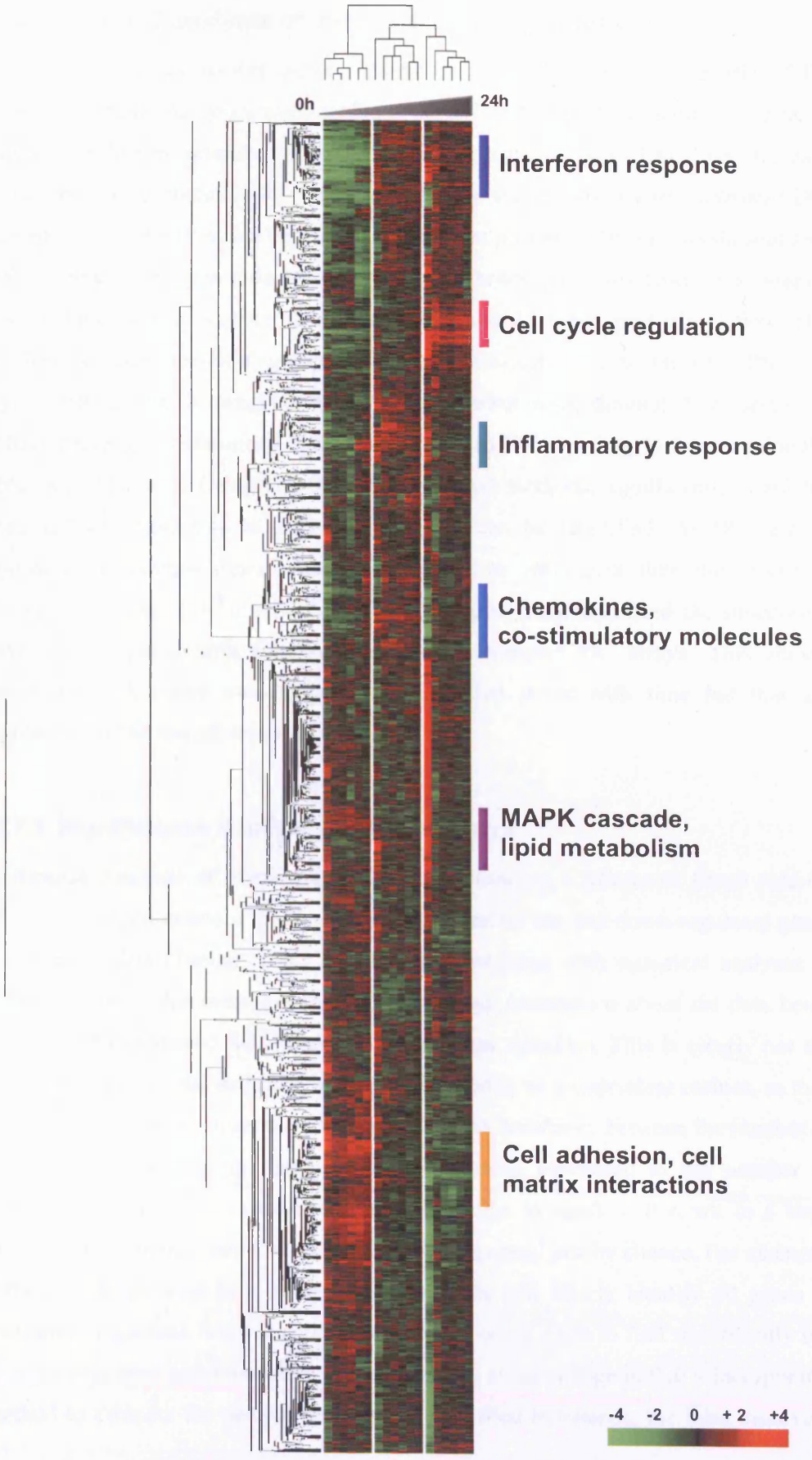
The branch structure of the tree suggests there may be distinct stages in the DC maturation process: the first cluster groups together samples between 0 and 2 hours, showing that gene expression patterns of DCs responding to LPS and poly(I:C) are more similar at early timepoints than at later timepoints; the next cluster groups together samples between 5 and 10 hours post stimulation, corresponding to an intermediate "transitional" stage of DC maturation; the last cluster groups together samples between 18 and 24 hours post stimulation, reflecting similarity in the gene expression profiles of fully mature DCs. The ordering of the samples therefore reflects the time-dependent "maturation" response of dendritic cells, and allows the identification of defined stages of the DC maturation response.

3.2.6 Distinct transcriptional signatures delineates stages of DC maturation

The program Treeview (Eisen et al., 1998) was used to visualise the expression pattern of the filtered set of 5108 genes expressed across the 18 LPS- and poly(I:C)-stimulated dendritic cell arrays (Figure 3.9). This analysis shows how the genes vary across the timecourse during the process of DC maturation. Genes cluster together by similar function and/or similar expression across the arrays (termed gene expression signatures), a number of which can be identified in this dataset. The kinetics of expression of gene groups have important functional implications for dendritic cell responses to antigens.

Figure 3.9 Hierarchical clustering LPS- and poly(I:C)-stimulated DC arrays (next page)

Genes in the 18 LPS- and poly(I:C)-stimulated DC arrays were filtered for genes which passed the SNR filter in all 18 arrays, and the resulting 5108 genes and 18 arrays were clustered together with hierarchical clustering. The dendrogram on the left represents the relationship between genes in terms of their expression pattern. Gene expression is shown as a pseudo-coloured representation of $\log_2$ expression ratio with red being above and green below the row/column median level of expression (set to 0) as shown by the scale. The sample dendogram is shown enlarged in Figure 3.8. The coloured bars to the right mark the position of gene function clusters (gene expression signatures), which vary in expression across the dataset.


0h
24h
Interferon response
Cell cycle regulation
Inflammatory response
Chemokines,
co-stimulatory molecules
MAPK cascade,
lipid metabolism
Cell adhesion, cell
matrix interactions
-4 -2 0 +2 +4

3.2.7 Statistical analysis of transcriptional signatures

In order to more specifically define subsets of genes that are characteristic of DC maturation stages, the genes were analysed according to the three maturation phases, evident from the tree structure of the DC timecourse arrays (Figure 3.8). From this data, DC maturation can therefore be classified into three stages, where ***early activated*** DCs combine gene expression data between 0 and 2 hours post-stimulation, ***transitional*** DCs combine gene expression data between 5 and 10 hours post-stimulation, and ***mature*** DCs combine gene expression data between 18 and 24 hours post-stimulation. This classification and temporal grouping allows statistical comparison of LPS- and poly(I:C)-stimulated DC arrays to time-matched control mock-stimulated DC arrays, as we have previously determined that mock-stimulated DCs are largely transcriptionally stable over 24 hours. Using two statistical analysis methods, significantly regulated genes in DCs responding to LPS and poly(I:C) can be identified. As DCs are so responsive to external stimuli, and even culture *in vitro* over time can result in phenotypic changes, it is important that LPS- and poly(I:C)-stimulated DC timecourse arrays are compared with time-matched mock-stimulated DC arrays. This should control for background transcriptional changes that occur with time but that are independent of the stimuli received.

3.2.7.1 Significance Analysis of Microarrays

Significance Analysis of Microarrays (SAM) functions as a Microsoft Excel Add-in, and was developed as a tool for discovering significantly up- and down-regulated genes in microarray data (Tusher et al., 2001). A general issue with statistical analyses of microarray data is that most statistical tests make an assumption about the data being normally distributed, and that genes are independent variables. This is clearly not the case for microarray data, as genes cannot be assumed to be independent entities, as they can co-vary and can be co-regulated. In addition, the imbalance between the number of genes that are variable (in magnitude of thousands), compared to the number of observations in terms of experimental samples (often in tens), will result in a large number of genes being identified as significantly regulated just by chance. For example, a 99% confidence level in a dataset of 5000 genes will falsely identify 50 genes as significantly regulated. SAM functions as a gene-specific *t*-test to find significantly up- and down-regulated genes between samples, but has an advantage in that it incorporates a method to estimate the percentage of genes identified by chance, the false discovery

rate (FDR). The FDR is generated by balanced permutations of the measurements blind to the way the data is classified (equivalent to the *null* hypothesis). Within SAM, a threshold (termed Delta) can be defined that determines the number of genes identified as significantly up- or down-regulated. Delta is linked to the FDR. The higher the threshold for discovery of significant genes, the smaller set of genes identified as significantly regulated, but consequently, the lower the FDR, i.e. a smaller number of genes that are likely to be called significant based on chance. This relationship between the number of significant genes discovered, the threshold (Delta) and the FDR is shown in Figure 3.10, for one of the comparisons between poly(I:C)-stimulated DCs and mock-stimulated DCs at a time interval.

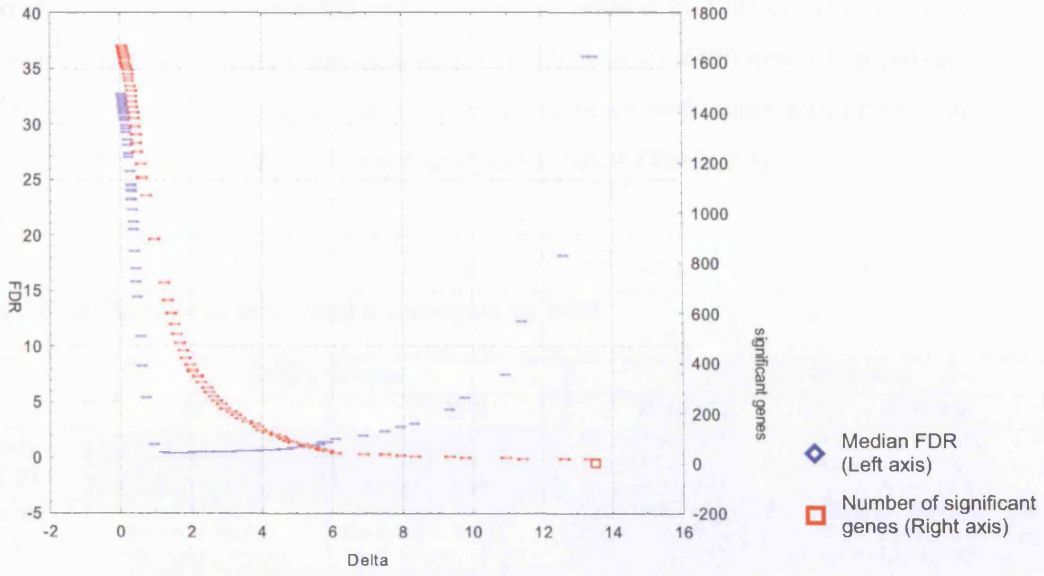


Figure 3.10 Relationship between Delta, FDR and significant genes in SAM

For a range of delta values, the effects on FDR (blue diamonds, left axis) and the number of significant genes called (red squares, right axis) are shown. As Delta increases, the number of significant genes called decreases, approximately exponentially. As Delta increases, FDR decreases to a minimum, after which increasing Delta results in an increasing FDR.

For analysis of significantly up- and down-regulated genes in LPS- and poly(I:C)-stimulated DCs compared to control mock-stimulated DCs at the defined stages of maturation, a FDR of 5% was chosen. This means 1 in 20 genes identified by SAM as differentially regulated between two conditions will be a false discovery. This seemed an acceptable compromise between the number of significant genes found and the number of genes that were found by chance (Figure 3.10 and Table 3.5). The relationship between Delta, FDR and number of significant genes called for each binary

comparison is unique to the comparison in question, therefore having a standard FDR for each binary test allowed comparison between the sets of significant genes that were generated for different comparisons. The comparisons analysed by SAM are shown in Table 3.4.

SAM requires a minimum of four gene measurements in each group to generate the balanced permutations for calculating FDR. To facilitate this analysis we partitioned each array into two, based on the duplicate gene spots, to give two measurements per gene. Partitioning the data in this way results in six measurements per gene within each group. Because of this strategy, only the genes where both duplicates on the arrays passed the SNR filter in all 27 arrays were analysed in SAM. The array data across the 27 arrays (Figure 3.7A) were filtered and median centred in Cluster. This normalised data was used as the initial input data in SAM. There were 4320 genes that passed the SNR filter across all 27 arrays, and of these, 1762 genes with duplicates on each array (termed "technical" repeats A, B) were analysed in SAM (Table 3.4).

Table 3.4 Partitioning of array data for analysis by SAM

	LPS v Control		Poly(I:C) v Control	
	LPS	Control	Poly(I:C)	Control
Early 0h, 2h	LPS2 0h(A), 0h(B) LPS3 0h(A), 0h(B) LPS2 2h(A), 2h(B)	Con1 0h(A), 0h(B) Con2 0h(A), 0h(B) Con2 2h(A), 2h(B)	PIC1 0h(A), 0h(B) PIC2 0h(A), 0h(B) PIC2 2h(A), 2h(B)	Con1 0h(A), 0h(B) Con2 0h(A), 0h(B) Con2 2h(A), 2h(B)
Transitional 5h, 10h	LPS2 5h(A), 5h(B) LPS3 5h(A), 5h(B) LPS2 10h(A), 10h(B)	Con1 5h(A), 5h(B) Con2 5h(A), 5h(B) Con2 10h(A), 10h(B)	PIC1 5h(A), 5h(B) PIC2 5h(A), 5h(B) PIC2 10h(A), 10h(B)	Con1 5h(A), 5h(B) Con2 5h(A), 5h(B) Con2 10h(A), 10h(B)
Mature 18h, 24h	LPS2 18h(A), 18h(B) LPS3 18h(A), 18h(B) LPS2 24h(A), 24h(B)	Con1 18h(A), 18h(B) Con2 18h(A), 18h(B) Con2 24h(A), 24h(B)	PIC1 18h(A), 18h(B) PIC2 18h(A), 18h(B) PIC2 24h(A), 24h(B)	Con1 18h(A), 18h(B) Con2 18h(A), 18h(B) Con2 24h(A), 24h(B)

The data from 27 arrays were partitioned to analyse significantly regulated genes by DCs responding to LPS- and poly(I:C)-stimulation at three maturation stages. Technical repeats of duplicate genes on each array (A, B) were used to increase the number measurements in the groups in each comparison.

Six comparisons were made, to find genes significantly differentially regulated by DCs responding to LPS and poly(I:C) compared to control mock-stimulated DCs at the three maturation stages (Table 3.4). SAM generates a graph plot of *expected* gene expression values (x-axis) from balanced random permutations of the data, against *observed* gene expression values (y-axis) from the input data (Figure 3.11). The plot demonstrates that genes that are differentially and significantly regulated between the compared groups lie

outside of the line of identity ($y=x$, solid blue lines in Figure 3.11) (i.e. different to gene expression values that are generated by random permutations of data groups). The tramlines on the plot serve as the user-defined threshold that determines whether or not the genes are significantly regulated. Genes which lie outside the tramlines are assigned as significantly up- or down-regulated between the data groups, genes that lie inside the tram lines are not significantly regulated between the data groups. The width of the tramlines is determined by the Delta value. This therefore affects the number of significant genes that are found, as illustrated in Figure 3.10. The wider the tramlines, the larger the Delta value, the smaller number of significant genes found, and consequently the smaller the FDR. Each of the 6 comparisons have adjusted Delta values to correspond to a FDR of approximately 5%. The numbers of significantly regulated genes in the comparison groups are shown in Table 3.5.

Table 3.5 Significantly up- and down-regulated genes as identified by SAM

	UP	DOWN	Total	FDR
	LPS v Control			
Early	127	217	334 (18)	5.05%
Transitional	464	534	998 (50)	4.96%
Mature	482	523	1005 (51)	5.09%
	Poly(I:C) v Control			
Early	50	67	117 (6)	4.82%
Transitional	478	568	1046 (53)	5.06%
Mature	459	593	1052 (52)	4.95%

The number of genes found to be significantly up- or down-regulated in the comparisons of LPS- and poly(I:C)-stimulated DCs compared to mock-stimulated DCs. The numbers in parentheses indicate the number of genes out of the total found to be significantly regulated that may be identified by chance (as corresponding to the FDR).

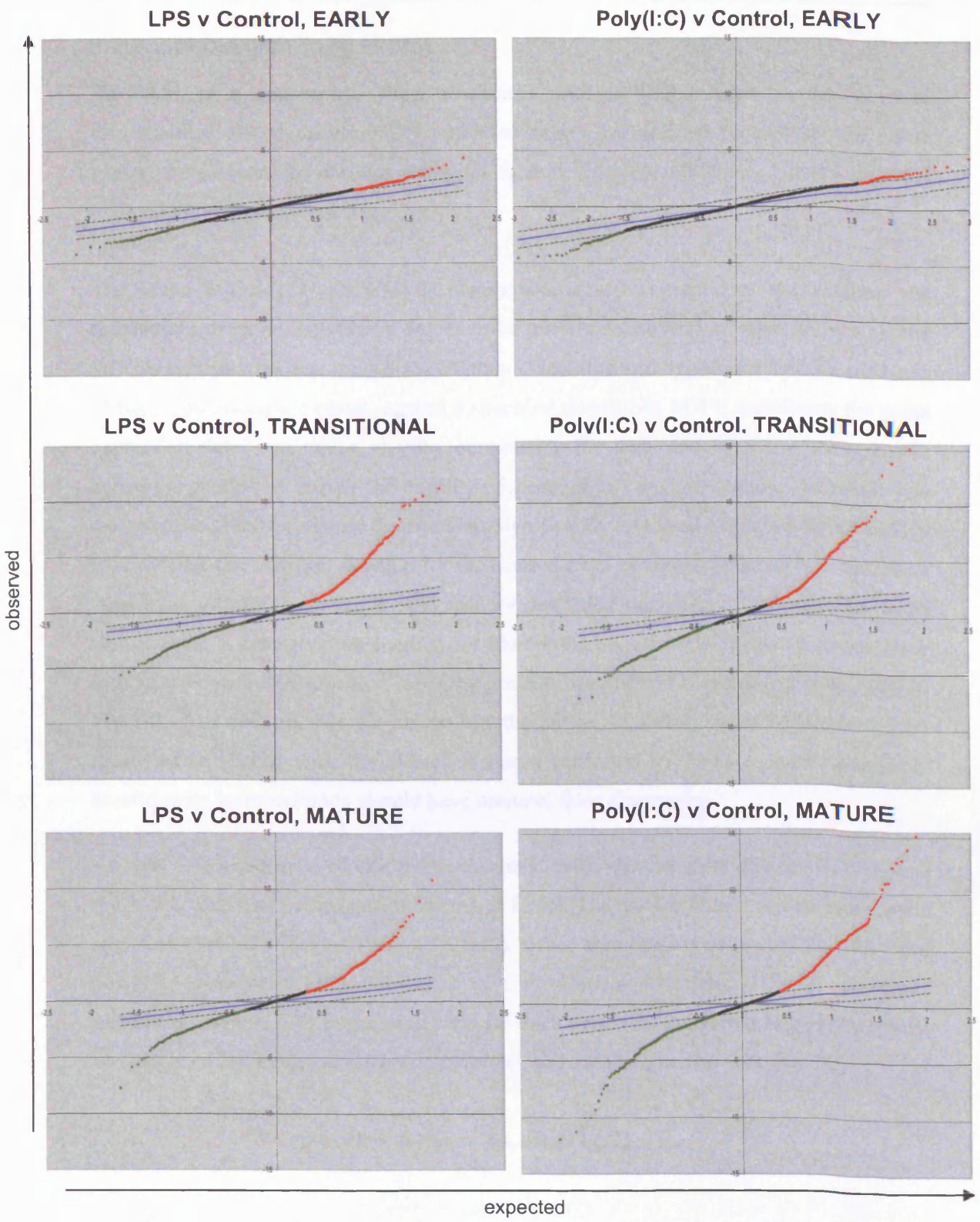


Figure 3.11 SAM plots of comparisons between stimulated and mock-stimulated DCs

Six comparisons were made between LPS- and poly(I:C)-stimulated DCs and control mock-stimulated DCs at three different maturation stages. Genes that are found to be significantly up-regulated in stimulated DCs are shown in red, genes significantly down-regulated are shown in green. The blue lines are $y=x$ for each plot, the dotted black lines are the tramlines determined by Delta, which corresponds to the width of the tramlines.

3.2.7.2 Mann-Whitney U test

As SAM is a parametric *t*-test associated with a FDR related to the random misclassification of differentially regulated genes, we decided to analyse the same binary comparisons by another statistical method. The Mann-Whitney U test was used as an additional statistical test.

The Mann-Whitney U (MWU) is a non-parametric test that does not assume the distribution of gene expression values to be a normal distribution. Also known as the Wilcoxon rank sum test, the Mann-Whitney U tests the *null* hypothesis that the medians of two populations are equal, against a specified alternative. MWU transforms the gene expression data into ranks, thereby normalising the data, and compares these ranks across the groups by testing the equality of means across the two groups. The result is a *z*-score related to the normal distribution. Significantly regulated genes can be identified by choosing the relevant *p*-value for the desired level of significance. MWU has been used as a standard non-parametric test for analysing microarray data, and has been shown to be a conservative method for identifying significantly regulated genes (low true positive rate TPR), with a low false positive rate (FPR) (Troyanskaya et al., 2002). We therefore reason that by comparing the subset of differentially regulated genes identified by SAM with the subset of genes generated by MWU, genes commonly identified by both methods should have minimal false discoveries.

As with SAM, normalised filtered data from Cluster was the input data for MWU, and the MWU test was performed in Microsoft Excel. The same 6 binary comparisons were analysed (Table 3.5) by MWU to identify genes significantly regulated in LPS- and poly(I:C)-stimulated DCs compared to control mock-stimulated DCs at the three maturation stages. The expression ratio for each gene was converted into ranks across the arrays undergoing the binary comparison, and the U-value was calculated by:

$$U_i = n_{i,1}n_{i,2} + ((n_{i,1}(n_{i,1}+1))/2) - R_1$$

Where:

$n_{i,1}$ = the number of gene expression ratios for the i^{th} array element in the LPS- or poly(I:C)-stimulated DC samples (6)

$n_{i,2}$ = the number of gene expression ratios for the i^{th} array element in the control mock-stimulated DC samples (6)

R_i = sum of ranks for the i^{th} array element in the LPS- or poly(I:C)-stimulated DC samples.

The U values were converted to the standard normal variable for the i^{th} array element (Z_i) with the equation:

$$Z_i = \frac{U_i - ((n_{i,1}n_{i,2})/2)}{\sqrt{(n_{i,1}n_{i,2}(n_{i,1}+n_{i,2}+1)/12)}}$$

The probability associated with each *z*-value was calculated in Excel (ZTEST).

The number of genes identified by MWU, at the significance level of $p<0.01$ were very comparable to the numbers computed by SAM (Table 3.6 and Table 3.5).

Table 3.6 Significantly up- and down-regulated genes identified by Mann-Whitney U test

	LPS v Control				*Poly(I:C) v Control*			
	UP	DOWN	Total	*p*	UP	DOWN	Total	*p*
Early	104	148	252	0.01	50	53	103	0.01
Transitional	353	361	714	0.01	351	424	775	0.01
Late	342	384	726	0.01	379	396	775	0.01

Using Microsoft Access, genes that were commonly identified by the two statistical tests SAM (FDR 5%) and MWU (*p*-value <0.01) were used for further analysis (Table 3.7 and Figure 3.12). These genes, commonly identified by both statistical methods, should be reliable markers for genes regulated by DCs in response to LPS- and poly(I:C)-stimulation. MWU consistently identifies a smaller set of significantly regulated genes compared to SAM; this is probably due to the difference in the acceptable level of error of the two methods; a FDR of 5% approximates to a 95% confidence interval, whereas a *p*-value of 0.01 approximates to a 99% confidence interval. If the FDR used in SAM is lowered to 1%, the number of genes identified by SAM as significantly regulated by LPS- or poly(I:C)-stimulated DCs compared to mock-stimulated DCs should be even more comparable to the number of genes identified by MWU as significantly regulated between stimulated and mock-stimulated DCs.

Table 3.7 Significantly regulated genes commonly identified by SAM and MWU

		LPS v Control			*Poly(I:C) v Control*		
		SAM†	MWU‡	Common	SAM†	MWU‡	Common
Early	UP	127	104	82	50	50	38
	DOWN	217	148	138	67	53	43
Transitional	UP	464	353	334	478	351	343
	DOWN	534	361	353	568	424	419
Late	UP	482	342	323	459	379	369
	DOWN	523	384	374	593	396	391

† Number of genes significantly up- and down-regulated at 5% FDR
‡ Number of genes significantly up- and down-regulated at p <0.01

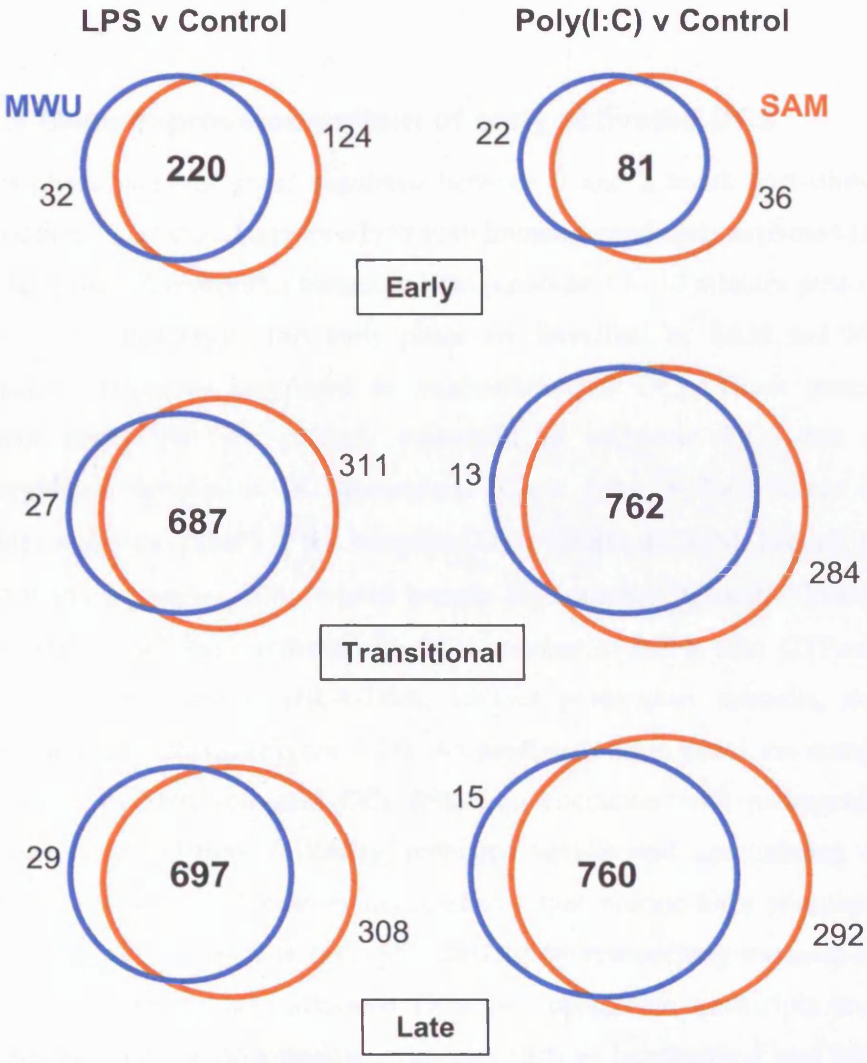


Figure 3.12 Comparisons of significantly regulated genes found by SAM and MWU

Genes identified by SAM and Mann-Whitney U in the 6 comparisons of LPS-stimulated and poly(I:C)-stimulated DCs compared to mock-stimulated DCs. Significant genes identified by MWU are shown in blue, genes identified by SAM are shown in orange, common genes shown in bold (overlap in Venn diagram).

3.2.8 Common genes regulated upon DC maturation

Using Microsoft Access, the genes commonly regulated by DCs responding to both LPS and poly(I:C) were identified (Figure 3.13A). These were defined as the core set of genes significantly regulated in DCs in response to LPS and poly(I:C) over the 24 hour maturation process. These core genes were regulated over different maturation stages (Figure 3.13B), and included genes that were differentially expressed at each stage as well as genes regulated across more than one of the three phases of DC maturation. For example, the differential expression of 295 genes in LPS- and poly(I:C)-stimulated DCs compared to mock-stimulated DCs is commonly observed in both transitional and mature stage DCs (Figure 3.13B).

3.2.8.1 Gene expression pattern of early activated DCs

As this phase includes genes regulated between 0 and 2 hours post-stimulation, the transcriptional signature corresponds to both immature and early activated DCs (Figure 3.14). In reality 0h represents transcript changes about 5 to 10 minutes post-stimulation, and the genes that typify this early phase are identified by SAM and MWU to be significantly regulated compared to mock-stimulated DCs. These genes therefore represent genes that are strongly expressed in immature DCs that are slowly downregulated throughout DC maturation. These genes include tissue inhibitor of metalloproteinases (TIMP) 2, the integrins (ITG) ITGβ2, ITGαM, ITGα2, ITGβ1- and ITGβ4-binding proteins, actin related protein 2/3 complex, Wiskott-Aldrich syndrome protein, p21/Cdc42/Rac1-activated kinase 1, partner of Rac1, Rho GTPase activating protein 1, MHC class II (HLA-DM), various proteasome subunits, the mannose receptor, and E-cadherin (Figure 3.14). As predicted, these genes are compatible with the function of early-activated DCs following encounter with pathogens, including cytoskeleton remodelling following pathogen uptake and upregulating the antigen processing machinery. Recent evidence shows that murine DCs stimulated by TLR ligands transiently upregulate endocytic capacity by remodelling the actin cytoskeleton (West et al., 2004). Early activated DCs also upregulate transcripts encoding pro-inflammatory and chemo-attractive cytokines such as interleukin-1 and interleukin-16, and genes encoding cAMP signalling such as protein kinase A (PRKA), PRKA anchor protein, cAMP responsive element binding protein-like 1. However, the early-activated DC transcriptional signature seems more stimulus-dependent compared to at other

maturation stages (Figure 3.13A), perhaps reflecting inherent differences between LPS and dsRNA that are initially perceived by the DC. These differences are much less evident at later timepoints.

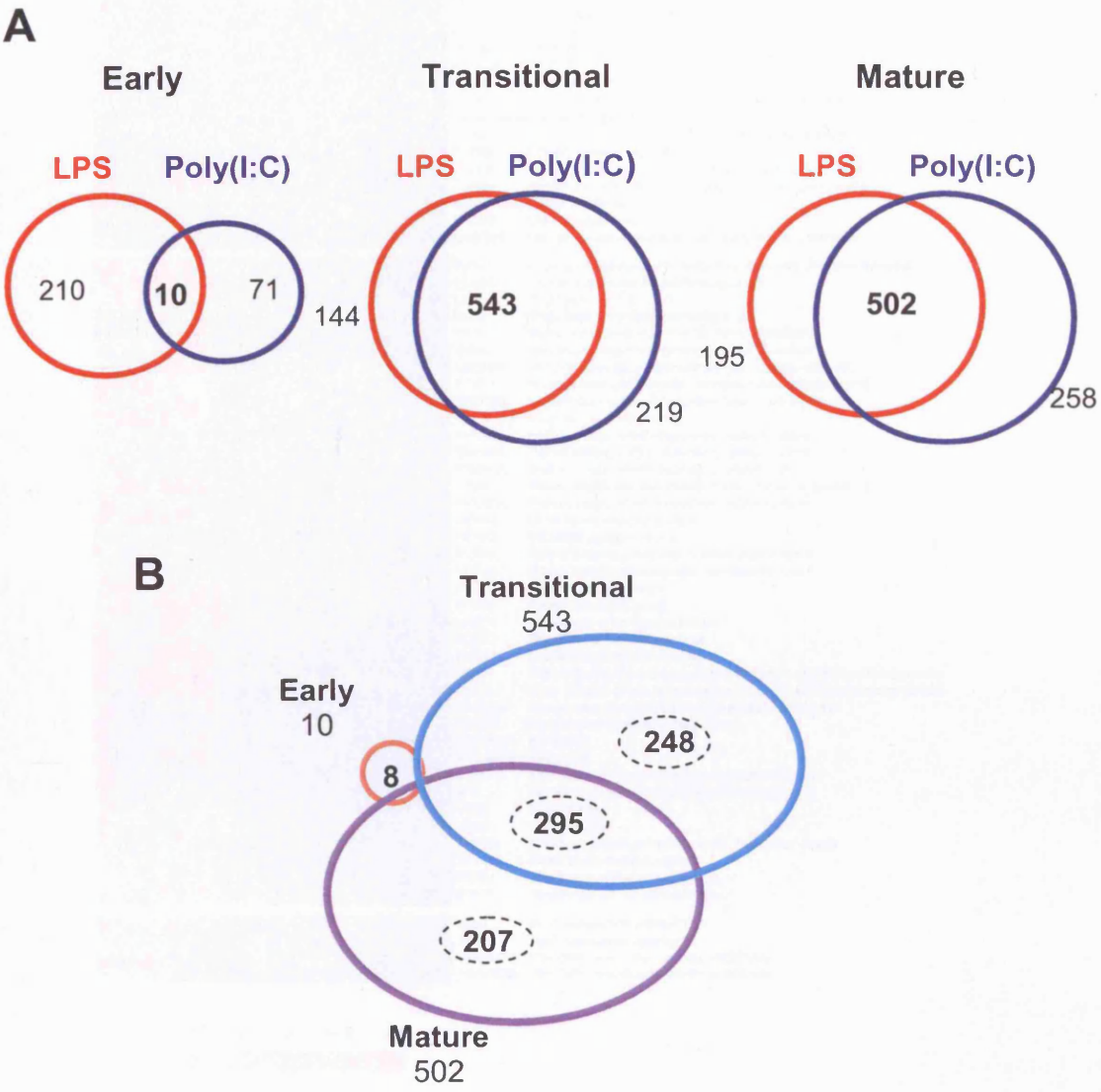


Figure 3.13 Genes commonly regulated by DCs in response to LPS and poly(I:C)

A Genes identified as significantly regulated by LPS-stimulated DCs (red) and poly(I:C)-stimulated DCs (blue) compared to control mock-stimulated DCs at the different maturation stages were analysed, and genes that were commonly regulated by DCs responding to both LPS and poly(I:C) are shown as the intersection of Venn diagrams.
B Commonly regulated genes were regulated at specific maturation stages. For example, out of 543 genes commonly regulated by DCs responding to LPS and poly(I:C) at the transitional stage, 248 genes were specific to this transitional stage, whereas 295 genes were also regulated by DCs responding to LPS and poly(I:C) at the mature stage.

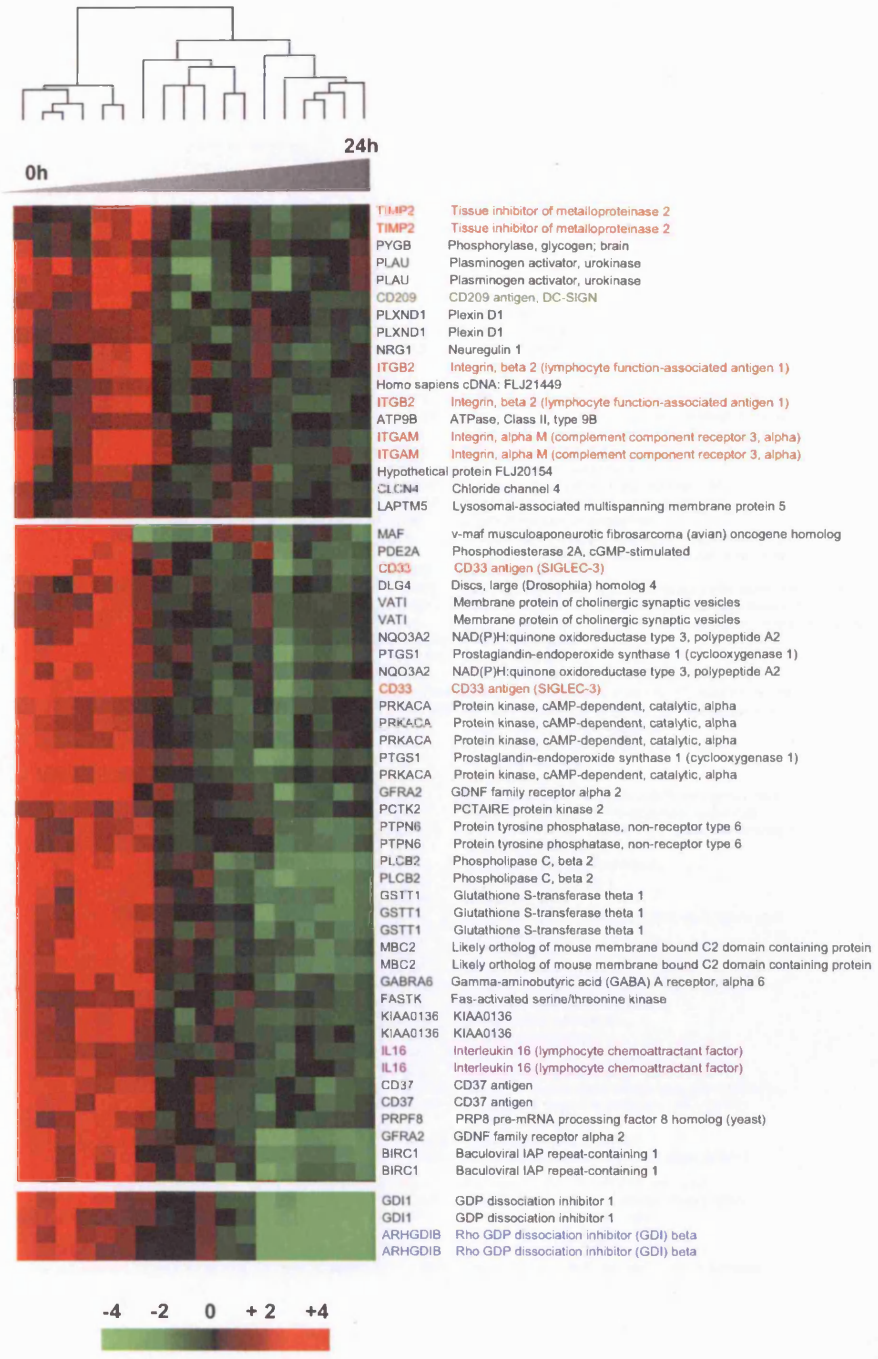


Figure 3.14 Transcriptional signature of early activated DCs

This gene expression signature of early activated DCs is enriched for genes involved in actin/cytoskeleton remodelling (genes in blue), cell-cell adhesion (genes in red), and antigen uptake and processing (genes in green). Inflammatory genes are also present in this profile (in purple). These genes are most strongly induced in early activated DCs.

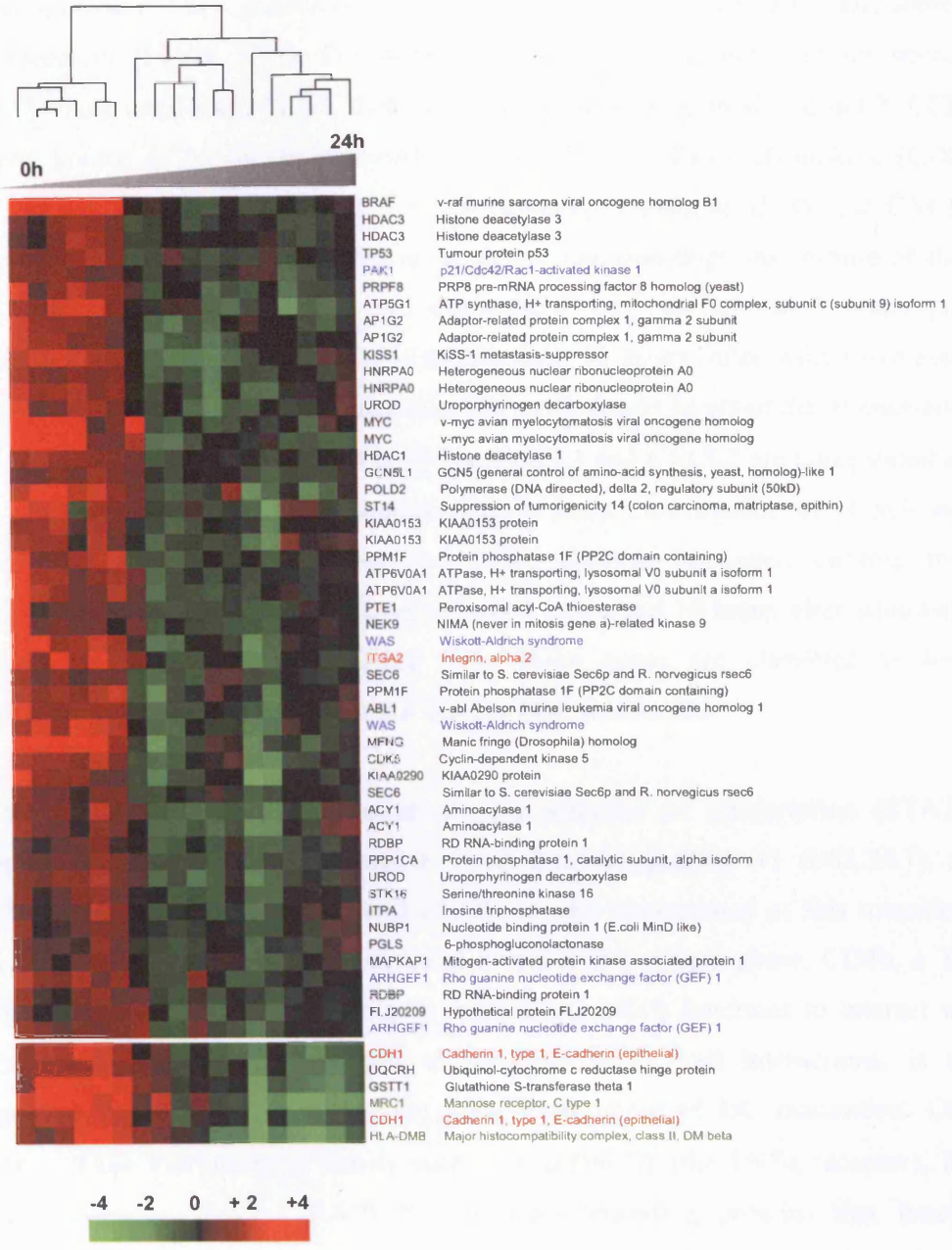


Figure 3.14 Transcriptional signature of early activated DCs (continued)

3.2.8.2 Gene expression in transitional DCs

Genes regulated by transitional phase DCs encode transcription factors as well as a broader spectrum of upregulated pro-inflammatory cytokines, chemokines and some of their receptors (Figure 3.15). Pro-inflammatory cytokines include tumour necrosis factor (TNFα), interleukin (IL)-1, IL-6, IL-15, chemokine (C-C motif) ligand 2 (CCL2, formerly known as monocyte chemoattractant protein (MCP)-1), chemokine (C-X-C motif) ligand 1 (CXCL1), CXCL2, CXCL8 (formerly known as IL-8), and CXCL9. Receptors for IL-1 and IL-2 are also upregulated. The transcriptional profile of these acute pro-inflammatory cytokines are upregulated very strongly at 5 hours post-stimulation, and this upregulation is sustained until 10 hours, after which expression returns to basal levels by 18 hours post-stimulation. It should be noted that transcripts of some pro-inflammatory cytokines like TNFα, CXCL1 and CXCL2 are upregulated at 2 hours after LPS- and poly(I:C)-stimulation (Figure 3.15). This is reflective of their rapid induction kinetics following TLR4 and TLR3 ligation. However, because these transcripts are significantly upregulated at both 5 hours and 10 hours after stimulation compared to control mock-stimulated DCs, these genes are classified as being significantly regulated at the transitional phase of DC maturation.

The transcription factor signal transducer and activator of transcription (STAT)4, apoptosis-regulatory genes like caspase 4, bcl-2-related protein A1 (BCL2A1), and BH3-interacting domain death agonist (BID) are also upregulated at this transitional phase, and transcript levels return to basal levels at the mature phase. CD40, a TNF family receptor expressed on DCs upon maturation, which functions to interact with CD40 ligand expressed on T cells to facilitate DC-T cell interactions, is also upregulated in this manner during the transitional phase of DC maturation. Other members of the TNF receptor family such as TNFRSF1A (the TNFα receptor), TNF receptor-associated factor (TRAF) 2, and genes encoding proteins that function downstream of TNFα signalling including CRADD (CASP2 and RIPK1 domain containing adaptor with death domain) are also regulated with similar kinetics. Reverse-transcription (RT) polymerase chain reaction (PCR) analysis confirmed the induction of TNFα, CCL2 and CD40 transcript at this transitional phase (see Section 3.2.8.3, Figure 3.17B).

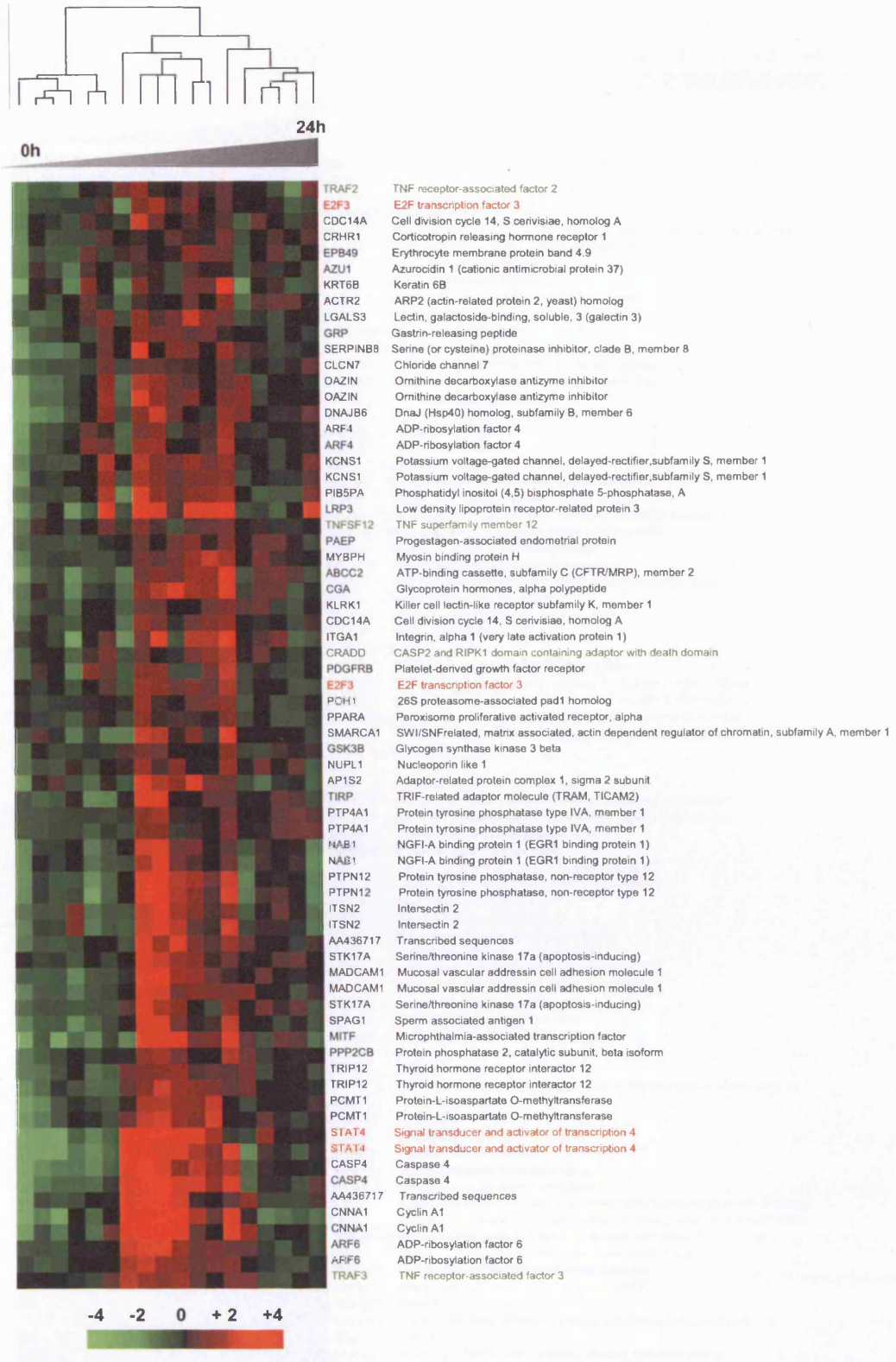


Figure 3.15 Transcriptional signature of transitional DCs

This gene expression signature is enriched for genes encoding pro-inflammatory cytokines (genes in purple), transcription factors (genes in red), and genes involved in TNFα signalling (genes in green). There are also anti-apoptotic and cell-signalling proteins encoded by genes within this cluster. This gene expression profile characterises transitional DCs.

Figure 3.15 Transcriptional signature of transitional DCs (continued)

From the Venn diagram in Figure 3.13B, over half of the genes significantly regulated by DCs responding to LPS and poly(I:C) at the transitional phase are also significantly regulated at the mature phase of DC maturation. The gene expression profile of this sustained response is shown in Figure 3.16. This response includes additional genes encoding proteins downstream of TNFα signalling: TRAF family member-associated NF-κB activator (TANK), TAK1-binding protein 2, TRADD (TNFRSF1A-associated via death domain), TNFα-induced protein 3 (TNFAIP3, a transcription factor formerly known as A20), TNFAIP2, TNFAIP6, TNFRSF10C (formerly known as TRAIL receptor 3, a decoy receptor without a death domain), and TNFSF13B (formerly known as B cell activating factor BAFF). Some of these genes have apoptosis regulating functions, including the pro-apoptotic TRADD, caspase 3, caspase 10 and DPF2, a transcription factor also known as requiem. Anti-apoptotic genes upregulated in the transitional and mature phase of DC maturation include CFLAR (CASP8 and FADD-like apoptosis regulator, also known as c-FLIP), baculoviral inhibitor of apoptosis (IAP) repeat-containing 2 (BIRC2, also known as cIAP1), BIRC3 (cIAP2), death-associated protein 6 (DAXX), and immediate early response 3 (IER3). BIRC2 inhibits apoptosis by binding to TRAF1 and TRAF2, IER3 functions to protect against TNFα and Fas-induced apoptosis, and DAXX is a transcriptional repressor that interacts with CFLAR and suppresses JNK activation (Kim et al., 2003b) and pro-apoptotic gene expression (Chen and Chen, 2003). NF-κB subunits p50 and p65, as well as NF-κB inhibitors IκBα, are similarly upregulated in the transitional and mature phase.

Additional upregulated genes including transcription factors and immune-function genes are activated downstream of cytokine signalling. These include STATs (STAT1, STAT3, STAT5A), interferon-responsive genes IFIT5 (interferon-induced protein with tetratricopeptide repeats 5), 2',5'-oligoadenylate synthetase (OAS1), IFITM1 and IFITM2, Mx2, interferon-stimulated protein 15kDa (ISG15), immuno-proteasome subunits, interferon regulatory factor (IRF)2, and co-stimulatory molecules CD86 and CD83, markers of DC maturation. Overall, this suggests that the induction and sustained upregulation of these genes over the transitional and mature phase function downstream of secreted cytokines that function in an autocrine manner to target the activation of transcription factors, immune-response and pro-survival genes in DCs, and are involved in mediating DC differentiation.

Figure 3.16 Transcriptional signature in transitional and mature phase DCs

Genes shown are characteristically upregulated over the transitional and mature phase of DC activation and differentiation. Such genes include transcription factors (genes shown in red), immune-response genes (shown in blue), genes regulating apoptosis (shown in purple), and additional genes activated downstream of TNFα and other cytokine signalling (shown in green).

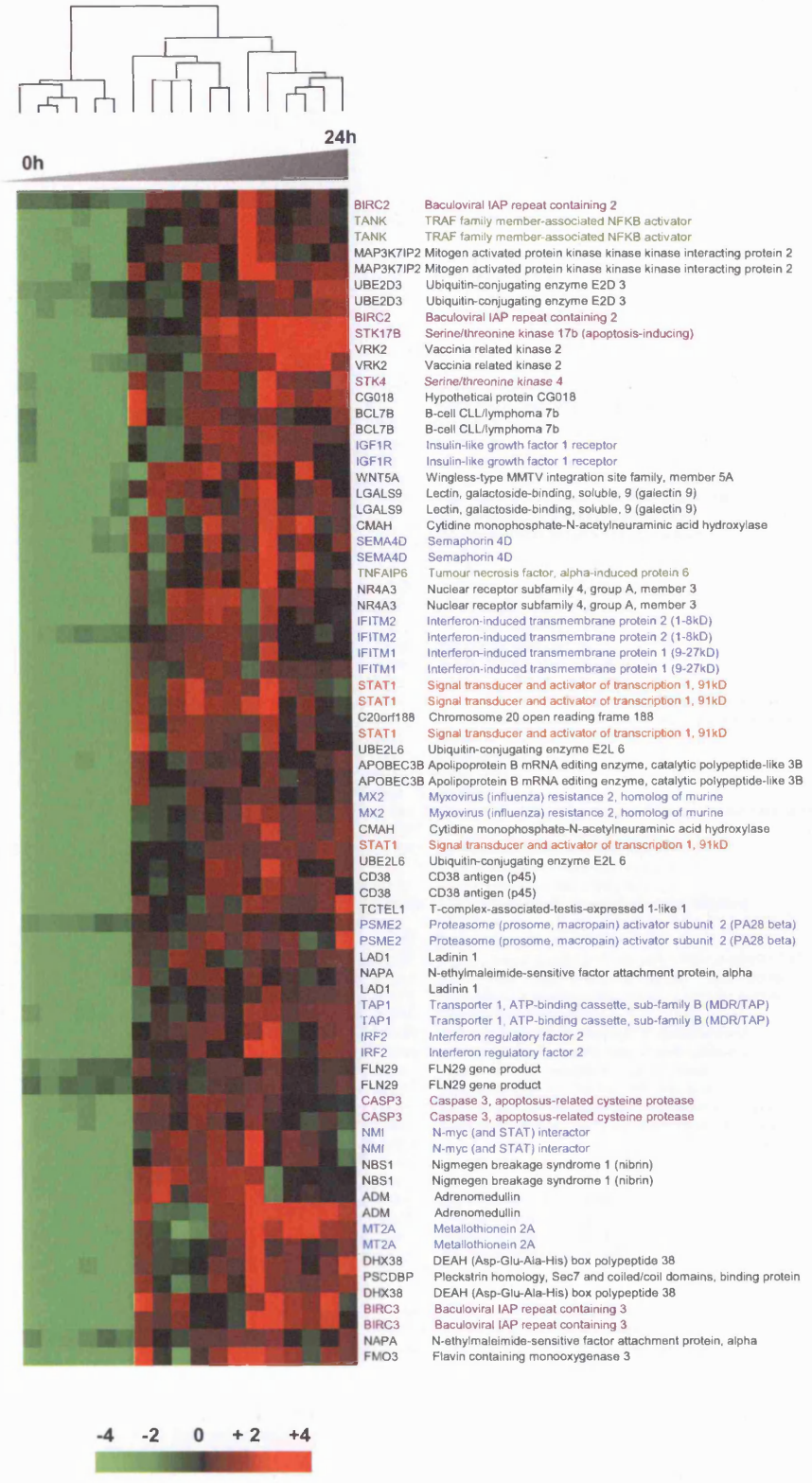


Figure 3.16 Transcriptional signature in transitional and mature phase DCs (continued)

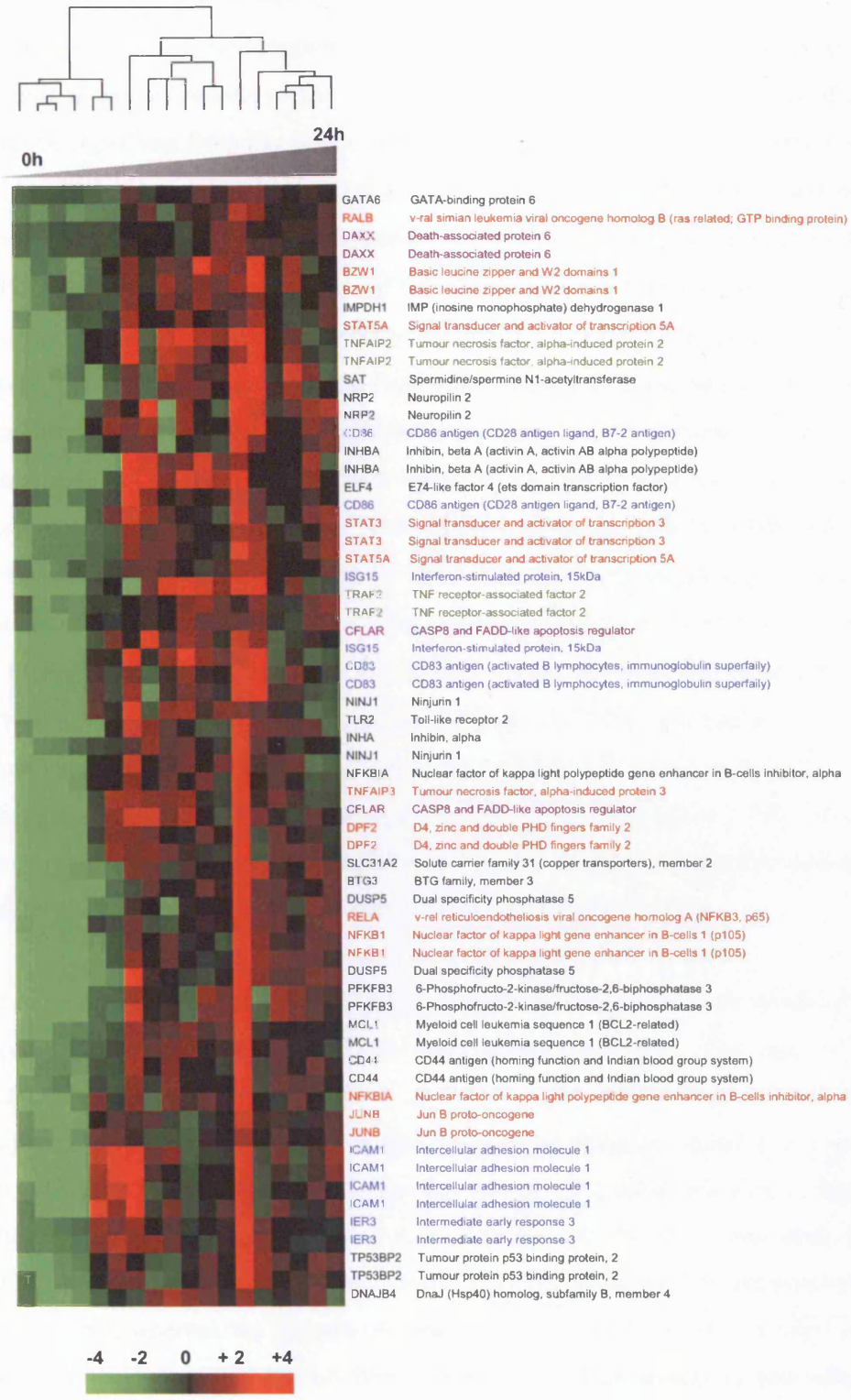


Figure 3.16 Transcriptional signature in transitional and mature phase DCs (continued)

3.2.8.3 Cytokine production by DCs

The upregulation of transcription factors, immune-response and pro-survival genes at the transitional and mature phase of DC maturation are likely to be a result of autocrine feedback signalling from the initial burst of cytokine and chemokine production (Figure 3.17A). TNFα transcript is induced as early as two hours post- LPS- and poly(I:C)-stimulation (Figure 3.17A, B). Upregulation of genes encoding proteins associated with TNFα signalling and transcription factors downstream of TNFα signalling support this hypothesis. This suggests that TNFα produced by DCs in response to LPS- and poly(I:C)-stimulation functions in an autocrine manner to contribute to DC maturation, in addition to its general pro-inflammatory role in the context of infection and inflammation (Chomarat et al., 2003; Ritter et al., 2003; Van Lieshout et al., 2004). When measured by enzyme-linked immunoassay from DC culture supernatants, a very marked burst of TNFα production by DCs was observed, beginning as early as two hours post-stimulus (Figure 3.18A). This correlates with both the microarray-based and RT-PCR transcript confirmation (Figure 3.17), both showing the induction of TNFα mRNA by 2 hours post-stimulation. In addition to TNFα, production of other pro-inflammatory cytokines (IL-6) and chemokines (CXCL8, CCL2 and CCL5) was also investigated using the FAST®Quant cytokine array system (Section 2.7.2). This showed a very rapid production of these cytokines by DCs at early timepoints, which reached peak levels between 2 and 5 hours post-stimulation (Figure 3.18A).

The rate of production of these cytokines appears to vary, with peak levels of cytokine detected between 2 hours (CXCL8) and 10 hours (CCL5). The rate of cytokine production of mock-stimulated DCs and LPS- and poly(I:C)-stimulated DCs was compared, approximately derived as the levels of cytokine produced over a given time period (Figure 3.18B). This shows that the rate of cytokine production is significantly higher in stimulated DCs, confirming at the protein level the microarray data. However, CXCL8 protein levels reach the maximum detection limit by 2 hours post-stimulation (6000 pg/ml), whereas the microarray data show CXCL8 transcript induced at 2 hours post-stimulus. Functional studies have shown CXCL8 responses to pro-inflammatory mediators are rapid (Roebuck, 1999). CXCL8 is regulated primarily at the level of gene transcription, and its promoter sequence contains binding sites for the inducible transcription factors NF-kB, AP-1, NF-IL6 (further explored in Section 5.4.2). This suggests that sampling at an earlier timepoint before 2 hours will show induction of

CXCL8 transcript, leading to CXCL8 protein detection at 2 hours. Microarray data suggests there is further transcriptional upregulation of CXCL8 between 2 and 5 hours after LPS- and poly(I:C)-stimulation.

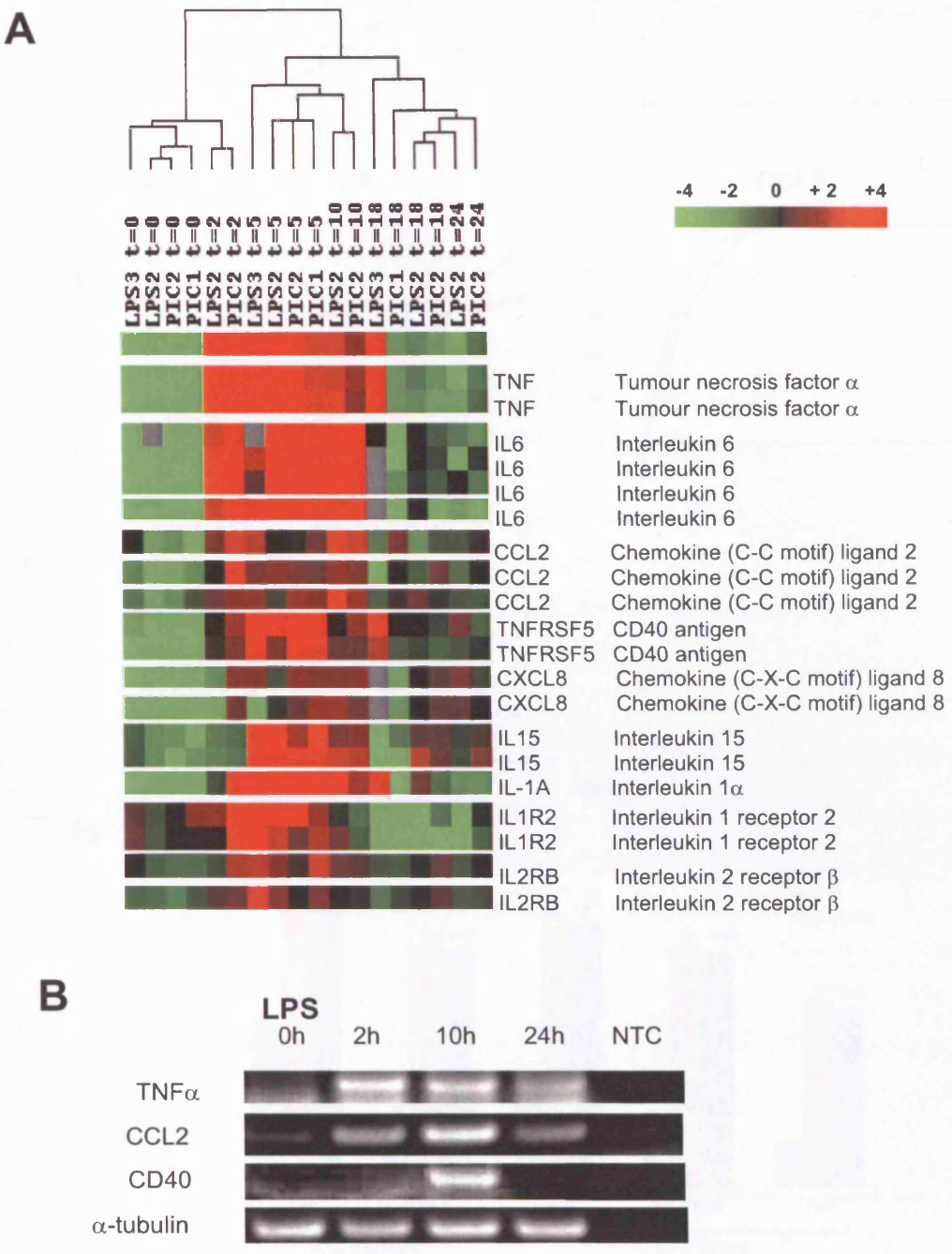


Figure 3.17 Transcript levels of pro-inflammatory cytokines in stimulated DCs

A Microarray gene expression detail of the upregulation of pro-inflammatory cytokines and chemokines expressed in LPS- and poly(I:C)-stimulated DCs
B RT-PCR confirmation for TNFα, CCL2, and CD40, in DCs stimulated with LPS. DC RNA was harvested at the specified timepoints. NTC is no cDNA template control, α-tubulin is the non-changing housekeeping gene.

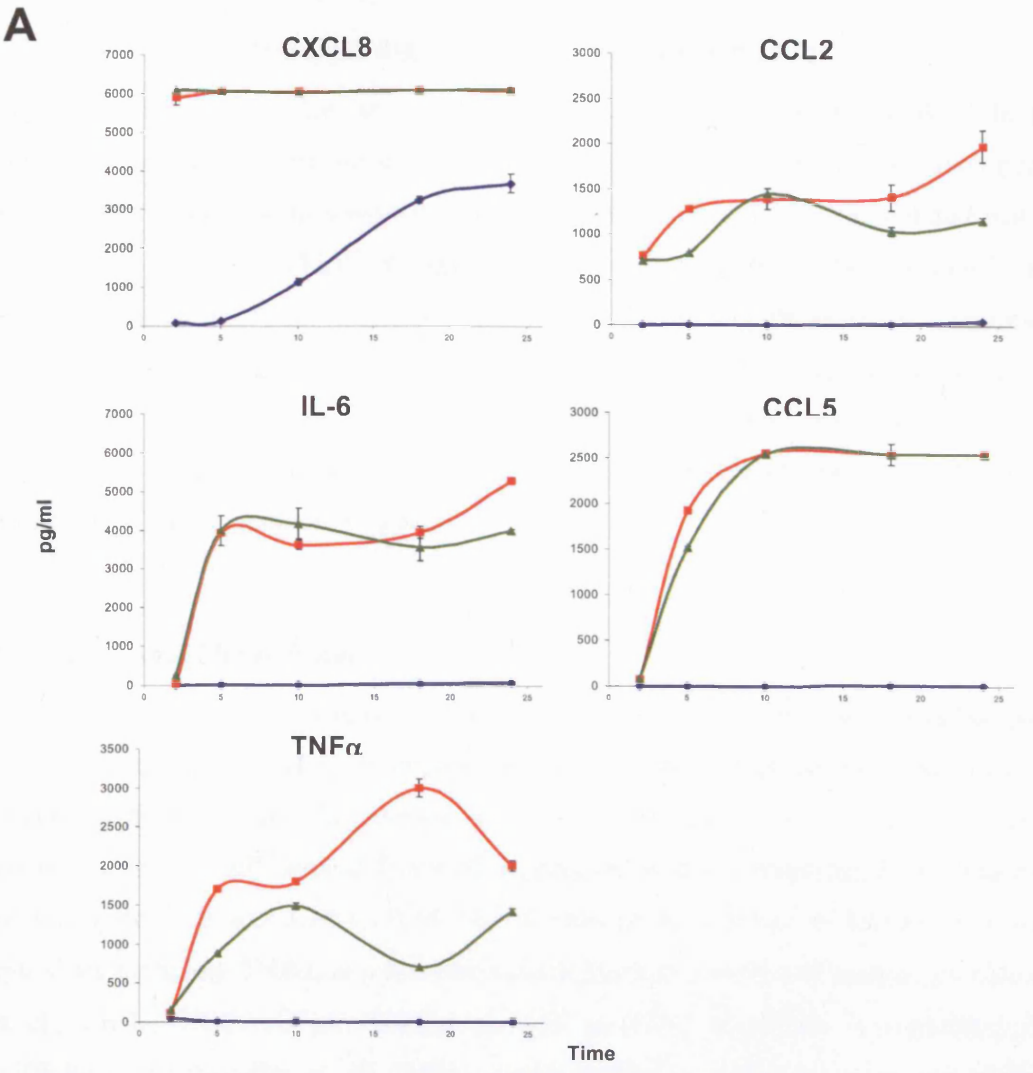


Figure 3.18 Protein levels of pro-inflammatory cytokines in stimulated DCs

A Levels of CXCL8, CCL2, IL-6, CCL5 and TNFα produced in DCs stimulated with medium (blue ♦), LPS (red ■) and poly(I:C) (green ▲), measured from DC culture supernatant. Error bars represent SD.
B Rate of cytokine production, measured over the hours post stimulation as specified. Bars with asterix (*) indicate significant difference compared to time-matched mock-stimulated DCs, at $p<0.01$. Two asterixes (**) indicate significance at $p<0.001$.

3.2.8.4 Gene expression signature in late mature DCs

Genes regulated at the late phase of DC maturation are generally involved in the inducible immune and antigen presenting functions of DCs (Figure 3.19). Such genes are well-represented in the gene expression signature shared by transitional and mature phase DCs (Section 3.2.8.2), such as upregulation of transcription factors STATs and NF-κB downstream of initial cytokine production. In this late phase of DC maturation, further chemokines, chemokine receptors, cell motility and cell adhesion molecules are upregulated, which mostly likely function to facilitate and maintain DC-T cell interactions, in agreement with the antigen presenting role of DCs stimulating T cells to initiate the adaptive immune response.

Cytokines and Chemokines

Cytokines are clearly important in modulating immune cell function. Cytokine gene expression at this late stage of DC maturation gives an indication as to the intended target for these signals. Chemokine (C-X-C motif) ligand 12 (CXCL12, formerly known as stromal cell-derived factor 1) is upregulated at the transcript level in mature DCs (Figure 3.19 and 3.20A). CXCL12 is induced by a range of pro-inflammatory cytokines including TNFα, and is a chemoattractant for T cells and monocytes (Blades et al., 2002a; Blades et al., 2002b), as well as being important in regulating DC trafficking (Vanbervliet et al., 2003). Upregulation of CXCL12 transcript (Figure 3.19A), followed by its secretion, may function to form a gradient of CXCL12 which attracts T cells to facilitate T cells and DCs making contact. The receptor for CXCL12 is CXCR4 (Bleul et al., 1996), which is also upregulated on DCs at this late phase, correlating with an increased CXCR4 surface expression (Figure 3.20B). CXCR4 is not expressed on immature DCs, but is upregulated upon DC maturation (Lin et al., 1998a; Sallusto et al., 1998) and infection of DCs by HIV-1 (Kawamura et al., 2001). The upregulation of CXCL12 and its receptor CXCR4 on mature DCs suggests a further autocrine role for CXCL12, in addition to its T cell chemoattractant properties.

The TGFβ receptor is also upregulated at this late phase of DC maturation. The upregulation of TGFβ receptor follows that of TGFβ upregulation during the transitional phase of DC maturation. This phase shift in time suggests the earlier upregulation of TGFβ transcript leads to production of functional TGFβ, and this may

be linked to the upregulation of its receptor. Alternatively, the delayed upregulation of TGFβ receptor allows an orchestrated and appropriate TGFβ effect. The autocrine effects of TGFβ include inhibitory and regulatory effects on dendritic cells (Geissmann et al., 1999; Sato et al., 2000a; Strobl and Knapp, 1999) and T cells (Campbell et al., 2001; Chen et al., 2001; Schramm et al., 2003; Wahl et al., 2004). The extensive transcriptional modulation of cytokines and their receptors highlight the functional importance of their effects on DCs as well as their effects on polarising T cell responses.

Immunological synapse

In order for DCs to present antigen in the form of the MHC-peptide complex to T cells, an immunological synapse must be formed, which consists of central and peripheral supramolecular activation clusters (c-SMAC and p-SMAC). The central clusters involve the signalling molecules which include the ligand-specific T cell receptor and the relevant MHC molecule, and CD28 on T cells receiving co-stimulation in the form of CD80 or CD86 from DCs. The peripheral cluster consists of adhesion molecules (integrins from T cells interacting with ICAMs from DCs) (Monks et al., 1998). Formation of this immunological synapse (IS) involves extensive microtubule and cytoskeleton reorganisation (Das et al., 2002; Dustin and Cooper, 2000; Krummel and Davis, 2002; Wulfing and Davis, 1998).

Histone deacetylase HDAC6 is upregulated in mature phase DCs, and plays a role in linking the tubulin cytoskeleton with formation of the immunological synapse (Hubbert et al., 2002; Zhang et al., 2003c). Synaptogyrin and synaptopodin, similarly upregulated at this stage, may also be involved as structural scaffolds in IS formation (Figure 3.19). NAPA (N-ethylmaleimide-sensitive factor (NSF) attachment protein alpha, formerly known as SNAPα) was upregulated in the transitional and late phase of DC maturation, and is also involved in membrane fusion and possibly as part of IS formation (Das et al., 2004; Sollner et al., 1993).

The small GTP-binding protein Rac1 is also upregulated strongly at this mature phase (Figure 3.19). Upon maturation, DCs reorganise their actin cytoskeleton and project dendrites which increase the potential surface area of contact to capture antigen-specific T cells (Granucci et al., 2003a; Mempel et al., 2004; Miller et al., 2004). Small GTPases

of the Rho family regulate the actin cytoskeleton (Burridge and Wennerberg, 2004) and subquently control aspects of the immune response that involves cytoskeleton remodelling. It has been recently shown that Rac1 and Rac2 expression by mature DCs are required for T cell priming, formation of dendrites, and the the polarised short-range movement toward T cells (Benvenuti et al., 2004).

Neuropilin 2 (NRP2), upregulated at transitional and late stages of DC maturation, may also participate in DC-T cell interactions (Figure 3.20C). Neuropilin 1 (NRP1) and NRP2 receptors were initially described as receptors for axon guidance factors of the class III semaphorin subfamily (He and Tessier-Lavigne, 1997). Subsequently, it was shown that in endothelial cells NRP1 and NRP2 function as receptors for some forms of vascular endothelial growth factors (VEGF), and promote angiogenesis and cell migration (Soker et al., 1998). NRP1 is expressed on immature monocytes-derived DCs (Tordjman et al., 2002) and plasmacytoid DCs (Dzionek et al., 2002), and has been shown to be essential for the initiation of primary immune response, involved in DC-T cell adhesion and clustering (Tordjman et al., 2002). From the array results, NRP1 transcript is present in immature DCs, and this is downregulated as the DCs progress through maturation. Conversely, NRP2 is upregulated as DCs mature (Figure 3.19C). This suggests that the expression of neuropilins may be important in mediating DC-T cell interactions, and their roles in axonal guidance in the nervous system as well as angiogenesis and cell migration in endothelial cells may point to new possibilities in their roles in the immune system.

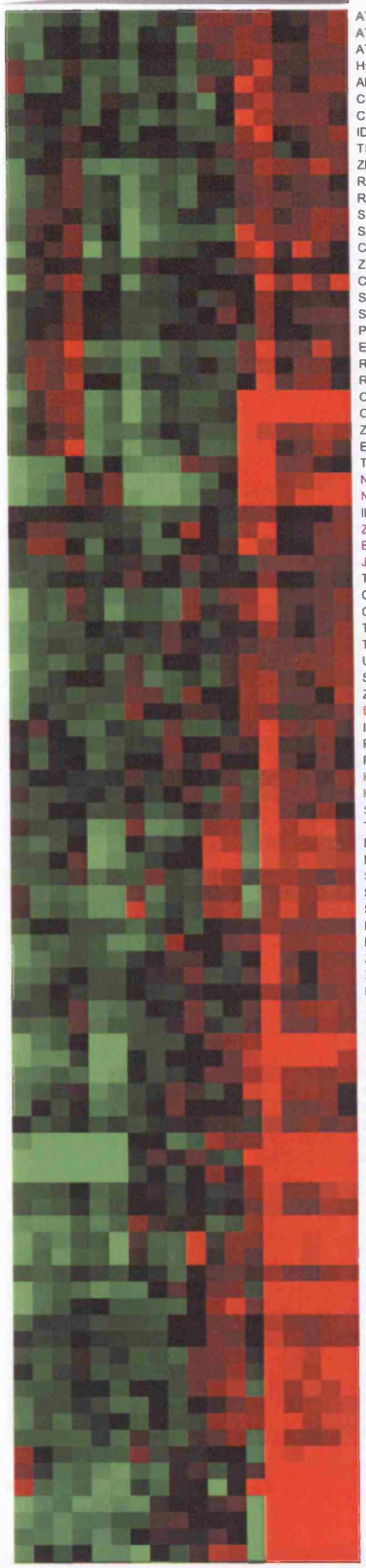
NRP1 and NRP2 have varying affinities for different members of the semaphorin family, and this may confer specificity in their actions. Dendritic cells, T cells and B cells express semaphorins that are involved in activation of these cells during immune responses. Semaphorin 4D (SEMA4D or CD100) has been shown to be required for T cell activation, and DCs also upregulate SEMA4D at the late stage of maturation (Figure 3.20C). SEMA4D binding to its receptor CD72 on B cells results in enhanced B cell responses to antigen and CD40 ligation (Kumanogoh and Kikutani, 2001). The coordinated upregulation of NRP1 and NRP2 as well as their ligands, VEGF and semaphorins, may function to regulate dendritic cell migration and T cell activation at the immunological synapse that involves cell adhesion functions mediated by neuropilins and their ligands.

Apoptosis regulation

As dendritic cells mature, they approach the end of their lifespan and die by apoptosis. Some apoptosis-regulating genes that function downstream of TNFα signalling upregulated at the transitional and mature phase were discussed in Section 3.2.8.2. Additional apoptosis regulatory genes upregulated at this mature phase include growth arrest and DNA-damage-inducible (GADD) genes (GADD45), death effector domain-containing protein (DEDD), caspase 3, cullin 1, and TRAF5. Mature DCs need to carefully regulate apoptosis to balance effecting their own survival to perform their antigen presentation function, and to appropriately apoptose to prevent excessive immune stimulation. For example, GADD45β, a NF-κB-responsive gene, is upregulated during the transitional and late phase of DC maturation. GADD45β functions by suppressing the JNK cascade (Papa et al., 2004), which results in apoptosis in DCs.

Figure 3.19 Transcriptional signature of late mature DCs (next page)

This gene expression signature is enriched for genes involved in apoptosis regulation (genes shown in red), DC migration (genes shown in blue), cell-cell interaction (genes shown in green), and additional transcription factors (genes shown in purple). These genes are most strongly induced in late mature DCs.



ATP6J ATPase, H+ transporting, lysosomal (vacuolar proton pump), member J
ATP6E ATPase, H+ transporting, lysosomal (vacuolar proton pump), 31kD
ATP6E ATPase, H+ transporting, lysosomal (vacuolar proton pump), 31kD
Hypothetical protein LOC157567
ABCC5 ATP-binding cassette, subfamily C (CFTR/MRP), member 5
CLCN6 Chloride channel 6
CLCN6 Chloride channel 6
IDS Iduronate 2-sulfatase (Hunter syndrome)
TSG101 Tumour susceptibility gene 101
ZNF22 Zinc finger protein 22 (KOX 15)
RAD23B RAD23 homolog B (S. cerevisiae)
RAD23B RAD23 homolog B (S. cerevisiae)
SIAH1 Seven in absentia homolog 1 (Drosophila)
SIAH1 Seven in absentia homolog 1 (Drosophila)
CREG Cellular repressor of E1A-stimulated genes
ZNF384 Zinc finger protein 384
CREG Cellular repressor of E1A-stimulated genes
SNX1 Sorting nexin 1
SNX1 Sorting nexin 1
PIGC Phosphatidylinositol glycan, class C
EST, putative cAMP-dependent protein kinase type II, beta regulatory chain
RBPSUH Recombining binding protein suppressor of hairless (Drosophila)
RBPSUH Recombining binding protein suppressor of hairless (Drosophila)
CCNG2 Cyclin G2
CCNG2 Cyclin G2
ZNF22 Zinc finger protein 22 (KOX 15)
EST, putative cAMP-dependent protein kinase type II, beta regulatory chain
TIAM1 T-cell lymphoma invasion and metastasis 1
NFIL3 Nuclear factor, interleukin 3 regulated
NFIL3 Nuclear factor, interleukin 3 regulated
ILF1 Interleukin enhancer binding factor 1
ZNF142 Zinc finger protein 142
BCL6 B-cell CLL/lymphoma 6 (zinc finger protein 51)
JUND Jun D proto-oncogene
TSG101 Tumour susceptibility gene 101
CST7 Cystatin F (leukocystatin)
CST7 Cystatin F (leukocystatin)
TGIF TGFB-induced factor (TALE family homeobox)
TGIF TGFB-induced factor (TALE family homeobox)
USP11 Ubiquitin specific protease 11
SEPW1 Selenoprotein W, 1
ZYG ZYG homolog
DEDD Death effector domain-containing
IGFBP3 Insulin-like growth factor binding protein 3
PPP4C Protein phosphatase 4 (formerly X), catalytic subunit
PPP4C Protein phosphatase 4 (formerly X), catalytic subunit
HDAC6 Histone deacetylase 6
HDAC6 Histone deacetylase 6
SPK Symplekin; Huntingtin interacting protein 1
TRAF5 TNF receptor-associated factor 5
MNDA Myeloid cell nuclear differentiation antigen
MNDA Myeloid cell nuclear differentiation antigen
SEMA3C Semaphorin 3C
SLC20A1 Solute carrier family 20 (phosphate transporter), member 1
SLC20A1 Solute carrier family 20 (phosphate transporter), member 1
LRP8 Low density lipoprotein receptor-related protein 8, apolipoprotein E receptor
EST, putative histone H2B
XPNPEP1 X-prolyl aminopeptidase 1, soluble
XPNPEP1 X-prolyl aminopeptidase 1, soluble
DDX9 DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 9
Hypothetical protein 669
Hypothetical protein 669
CASP3 Caspase 3, apoptosis-related cysteine protease
CASP3 Caspase 3, apoptosis-related cysteine protease
CUL1 Cullin 1
MGEA5 Meningioma expressed antigen 5 (hyaluronidase)
KPNB2 Karyopherin (importin) beta 2
HDAC6 Histone deacetylase 6
CCL19 Chemokine (C-C motif) ligand 19
CHST2 Carbohydrate (chondroitin 6/keratan) sulfotransferase 2
CHST2 Carbohydrate (chondroitin 6/keratan) sulfotransferase 2
DBI Diazepam binding inhibitor (GABA receptor modulator, acyl Coenzyme A binding protein)
DBI Diazepam binding inhibitor (GABA receptor modulator, acyl Coenzyme A binding protein)
TGFBR3 Transforming growth factor, beta receptor III
NK4 Natural killer cell transcript 4
NK4 Natural killer cell transcript 4
RAC1 Ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1)
Hypothetical protein MGC4276 similar to CG8198
H2AFL H2A histone family, member L
SMARCD1 SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1
SMARCD1 SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 1
CAPN1 Calpain 1 (mu/l) large subunit
CAPN1 Calpain 1 (mu/l) large subunit
NLVCF Nuclear localisation signal deleted in velocardiofacial syndrome
EST, putative histone H2B
KIAA0053 KIAA0053 gene product
NLVCF Nuclear localisation signal deleted in velocardiofacial syndrome
KLF5 Kruppel-like factor 5 (intestinal)
EXTL2 Exostoses (multiple)-like 2
EXTL2 Exostoses (multiple)-like 2
Hypothetical protein MGC4276 similar to CG8198
EST
TGFBR3 Transforming growth factor, beta receptor III
ALDH1 Aldehyde dehydrogenase 1, soluble

Figure 3.19 Detail of the transcriptional signature in mature phase DCs (continued)

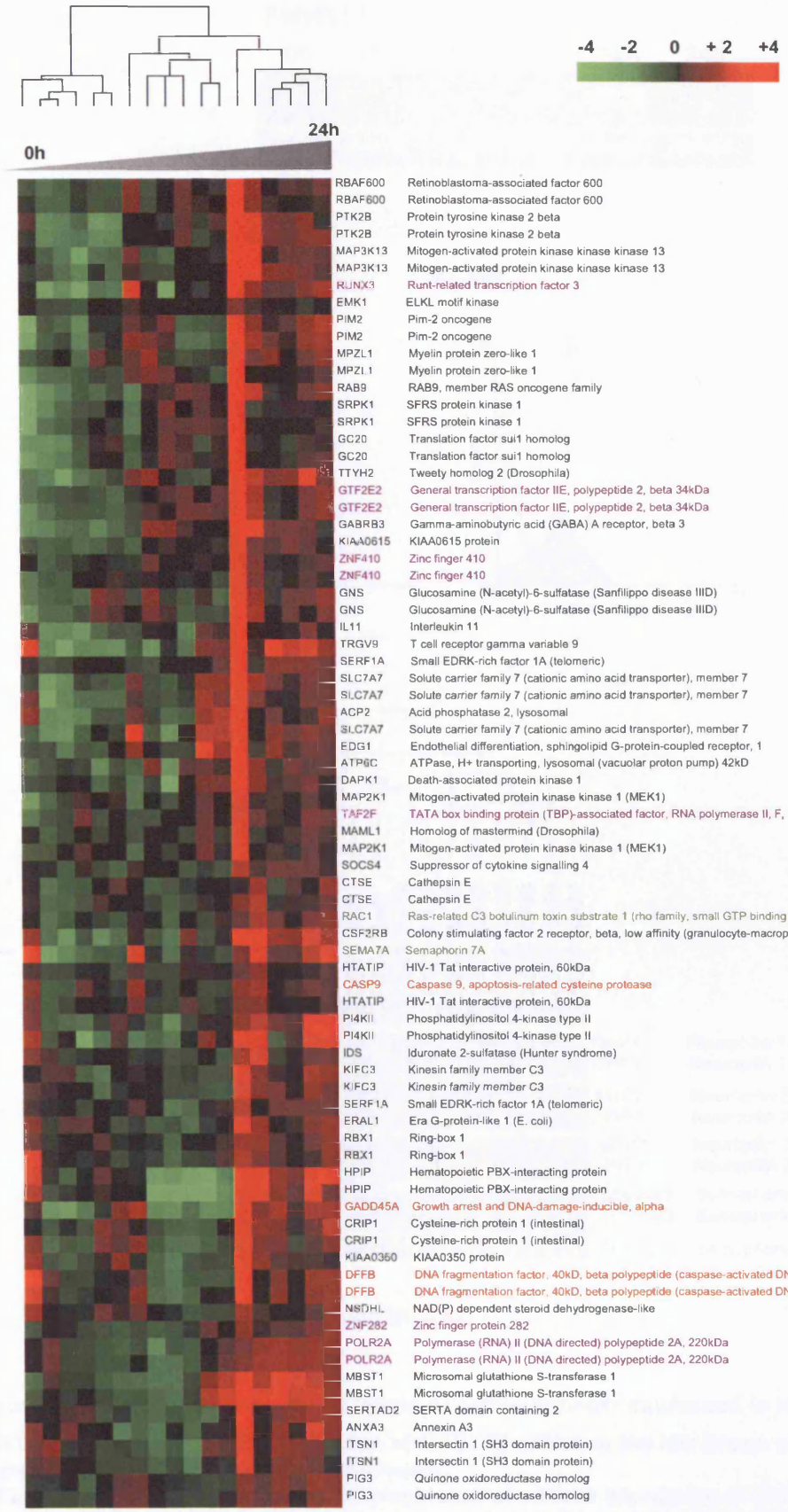


Figure 3.19 Detail of the transcriptional signature in mature phase DCs (continued)

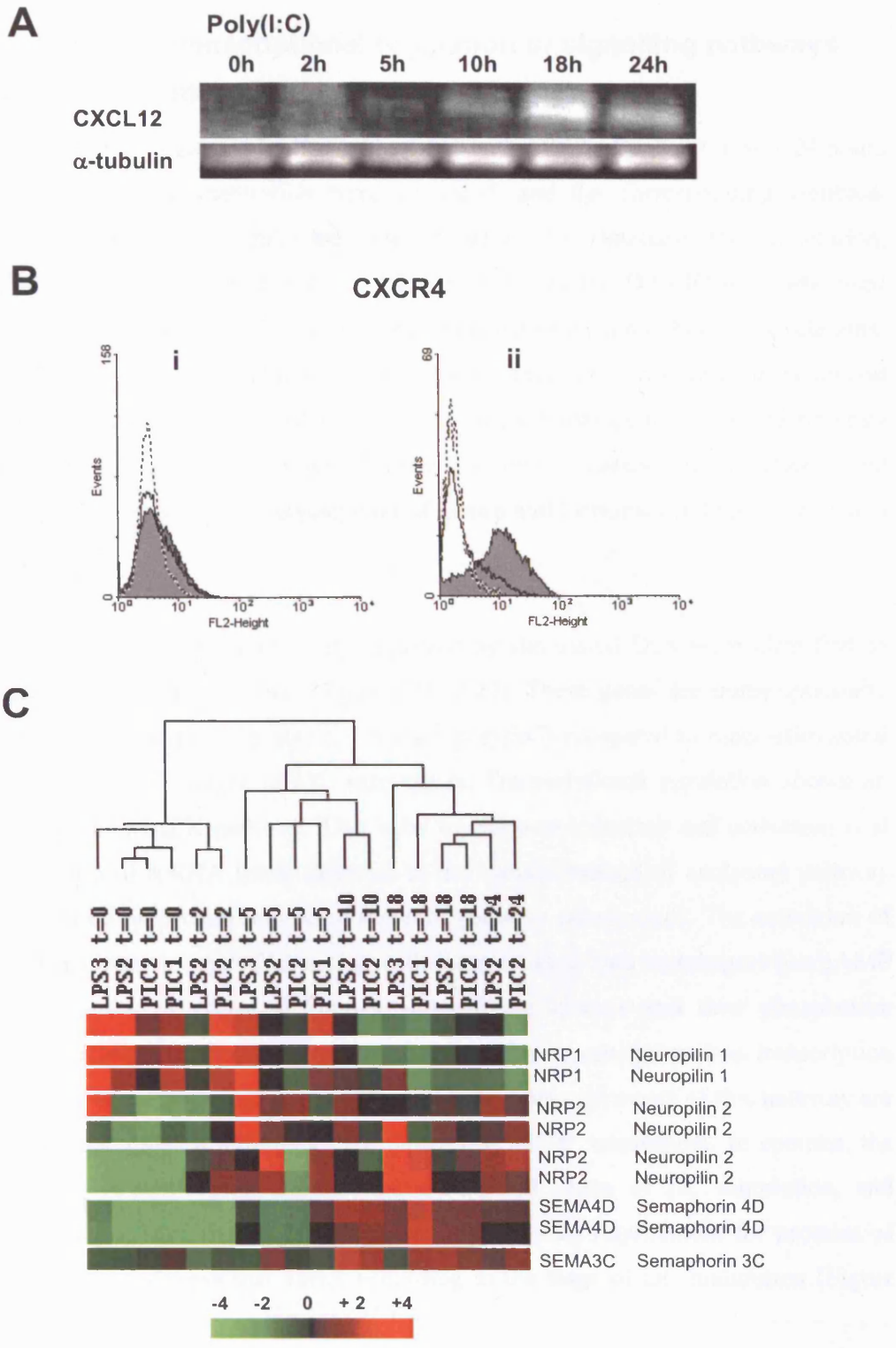


Figure 3.20 Transcript and protein levels of genes strongly expressed in mature DCs

A RT-PCR confirmation of upregulation of CXCL12 mRNA at the late phase of DC maturation, following a timecourse of poly(I:C) stimulation.
B Flow cytometric analysis showing upregulation of surface expression of CXCR4 on DCs. (i) CXCR4 at 5 hours after poly(I:C)-stimulation, (ii) CXCR4 at 24 hours post-stimulation. Grey filled histogram – CXCR4, black histogram – mock-stimulated DCs, dotted histogram – isotype control.
C Array-based transcript levels of neuropilin 1, neuropilin 2, and their ligands the semaphorins.

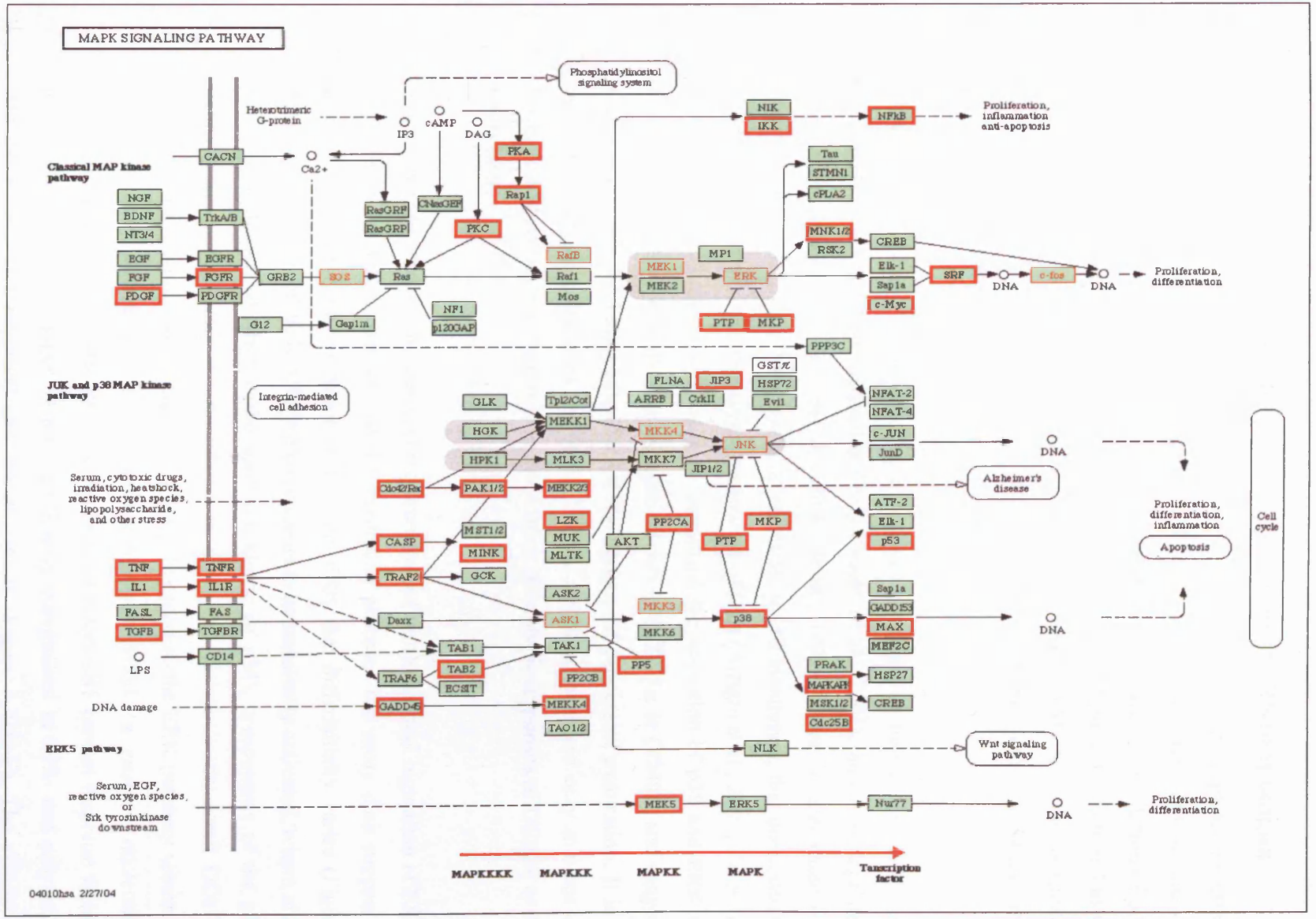
3.2.9 Differential transcriptional regulation of signalling pathways during DC maturation

Genes coordinately regulated by DCs stimulated with LPS and poly(I:C) over 24 hours of DC activation and maturation were compiled, and the corresponding Genbank Accession numbers were uploaded into DAVID, the Database for Annotation, Visualisation, and Integrated Discovery (Dennis et al., 2003). DAVID is a web-based client/server application that allows mapping of gene accession numbers via a relational database to detailed functional annotation. DAVID draws upon resources from several public databases, including LocusLink at the NCBI (National Center for Biotechnology Information), Gene Ontology (GO), Protein Families Database of alignments and HMMs (PFAM), and Kyoto Encyclopedia of Genes and Genomes (KEGG) charts and pathways.

Approximately 50 genes significantly regulated by stimulated DCs were identified as involved in the MAPK pathway (Figure 3.21, 3.22). These genes are transcriptionally up- or down-regulated in response to LPS and poly(I:C) compared to mock-stimulated controls, at different stages of DC maturation. Transcriptional regulation shows an activation of the MAPK pathway. This view of pathway induction and activation is at the transcriptional mRNA level, different to the classic method of analysing pathway activation based on protein phosphorylation of pathway components. The activation of the MAPK pathway by cytokines and growth factors and second messengers like cAMP lead to activation of upstream kinases, downstream kinases and their phosphatase inhibitors. Focusing on the end kinases and their effector proteins such as transcription factors, the ERK pathway and the corresponding upstream elements of this pathway are consistently upregulated throughout the timecourse of DC maturation. In contrast, the p38 pathway is only upregulated at the transitional phase of DC maturation, and downregulated at later stages. This suggests a differential requirement for proteins of the two MAPK pathways that varies according to the stage of DC maturation (Figure 3.22).

Figure 3.21 Mitogen-activated protein kinase signal transduction pathway

Genes identified as significantly regulated by DCs in response to LPS and poly(I:C) were submitted to DAVID, and a number of genes encoded proteins that were components of the MAPK signalling pathway. This pathway was visualised by KEGG charts. The component proteins in red are encoded by genes which were significantly regulated on the transcript level by DCs in response to LPS and poly(I:C) at different stages of maturation.


MAPK SIGNALING PATHWAY
Phosphatidylinositol signaling system
Heterotrimeric G-protein
IP3
cAMP
DAG
PKA
Rap1
RasGRF
CNrasGEF
PKC
RapB
Raf1
Mos
Ras
SOS
GRB2
Gab1m
NF1
p120GAP
G12
CACN
Ca2+
Classical MAP kinase pathway
NGF
BDNF
NT3/4
TrkA/B
EGF
FGF
PDGF
EGFR
FGFR
PDGFR
MEK1
MEK2
MP1
ERK
PTP
MKP
MNK1/2
RSK2
CREB
Elk-1
Sap1a
c-Myc
SRF
c-fos
DNA
Proliferation, differentiation
NIK
IKK
NFkB
Proliferation, inflammation anti-apoptosis
Tau
STMN1
cPLA2
PPP3C
GSTπ
HSP72
Evi1
FLNA
JIP3
ARRB
CrkII
NFAT-2
NFAT-4
c-JUN
JunD
JNK
JUK and p38 MAP kinase pathway
Integrin-mediated cell adhesion
GLK
HGK
HPK1
Tpl2/Cot
MEKK1
MLK3
MKK4
MKK7
JIP1/2
Alzheimer's disease
ATF-2
Elk-1
p53
Proliferation, differentiation, inflammation
Apoptosis
Cell cycle
Serum, cytotoxic drugs, irradiation, heatshock, reactive oxygen species, lipopolysaccharide, and other stress
Cdc42/Rac
PAK1/2
MEKK2/3
LZK
MUK
MLTK
MST1/2
MINK
GCK
CASP
TRAF2
TNF
IL1
TNFR
IL1R
FAS
TGFBR
CD14
FASL
TGFB
LPS
Daxx
ASK2
ASK1
TAK1
TAB1
TAB2
ECSIT
TRAF6
GADD45
MEKK4
TAO1/2
DNA damage
AKT
PP2CA
PP2CB
PP5
PTP
MKP
MKK3
MKK6
p38
Sap1a
GADD153
MAX
MEF2C
PRAK
MAPKAPK
MSK1/2
Cdc25B
HSP27
CREB
NLK
Wnt signaling pathway
ERK pathway
Serum, EGF, reactive oxygen species, or Src tyrosinkinase downstream
MEK5
ERK5
Nur77
MAPKKKK
MAPKKK
MAPKK
MAPK
Transcription factor
04010hsa 2/27/04

3.2.10 MAPK inhibitors affect DC maturation at different stages

The differential requirement for MAPK pathway components can be explored by using specific pharmacological inhibitors to p38 and ERK. The pyridinyl imidazole SB203580 (hereafter referred to as SB) is selective in its binding and inhibition of p38 activity, not its phosphorylation (Tong et al., 1997). PD98059 (hereafter referred to as PD) prevents the activation of MEK1 (systematic name MAP2K1), the upstream activating kinase of ERK1/ERK2, and therefore prevents the activation and phosophorylation of ERK1/ERK2 (Alessi et al., 1995).

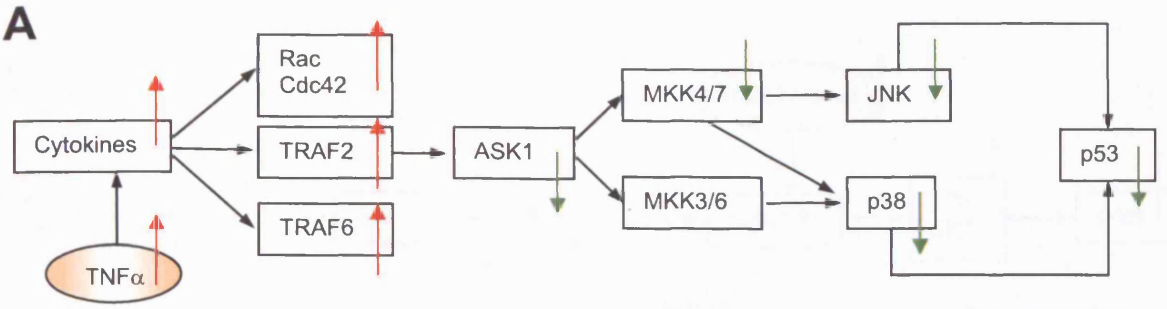
ERK has been shown to function as a negative regulator in human dendritic cell phenotypic and functional maturation (Puig-Kroger et al., 2001), and also regulates survival in mouse dendritic cells (Rescigno et al., 1998). There is also extensive literature documenting the effects of p38 inhibition on DC maturation, that demonstrate p38 to be necessary in the phenotypic maturation of DCs (Arrighi et al., 2001; Yu et al., 2004). However, these studies focus on measuring the activation of p38 and ERK in DCs within minutes or hours after stimulation with LPS, TNFα or CD40L, and suggest a continuous role for both p38 and ERK throughout the process of maturation. It is a common theme to extrapolate the initial MAPK activities measured only minutes or hours post-stimulation to their role in modulating phenotypic responses of DCs 24 or 48 hours later.

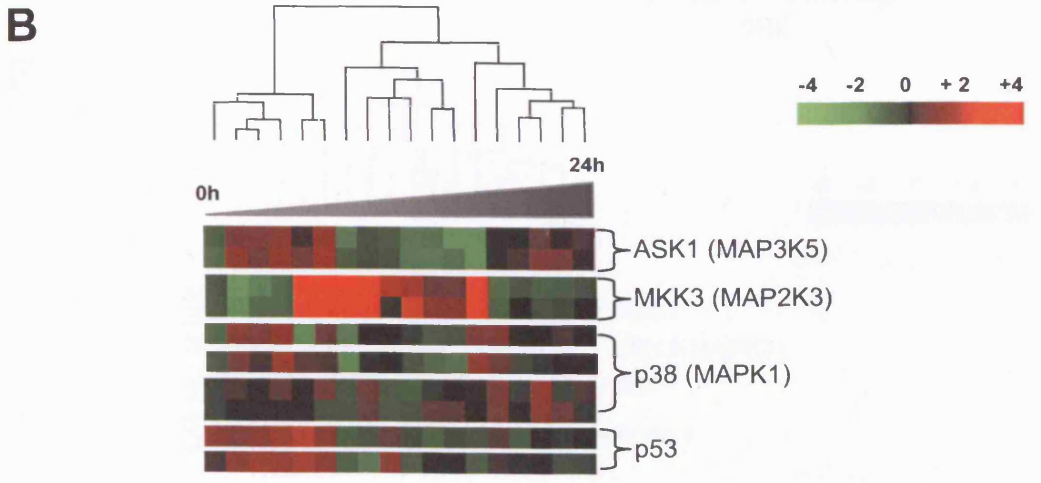
There is no literature on the temporal requirement and transcriptional regulation of ERK and p38 in the DC activation and differentiation process. The array data suggest a temporal transcriptional regulation of p38 and ERK that differentially varies (Figure 3.22). The p38 arm of the MAPK pathway seems to be transiently activated, where after initial upregulation of MKK3 (the upstream kinase of p38), components of the p38 pathway are significantly downregulated in LPS- and poly(I:C)-stimulated DCs at transitional or mature stages (Figure 3.22A-C). In contrast, the ERK pathway seems to be consistently activated. The upstream component SOS1 (a guanine nucleotide exchange factor that activates Ras), downstream effectors SRF (serum response factor) and FOSL1 (fos-like antigen 1) are significantly upregulated in LPS- and poly(I:C)-stimulated DCs at transitional and mature phases (Figure 3.22D-F). The effects of inhibiting the two MAPK components p38 and ERK at two different timepoints on DC phenotype, function, and viability was therefore investigated.

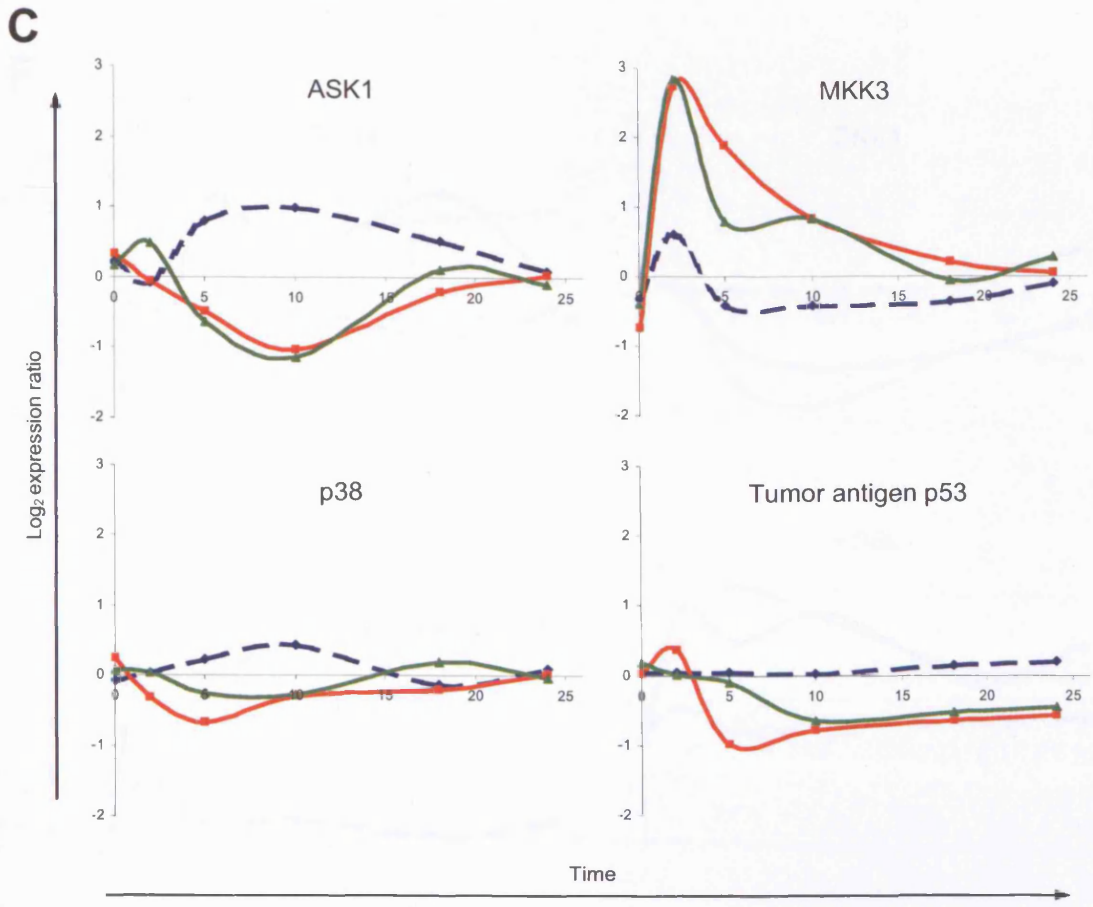
Figure 3.22 Transcriptional regulation of MAPK pathways (next 2 pages)

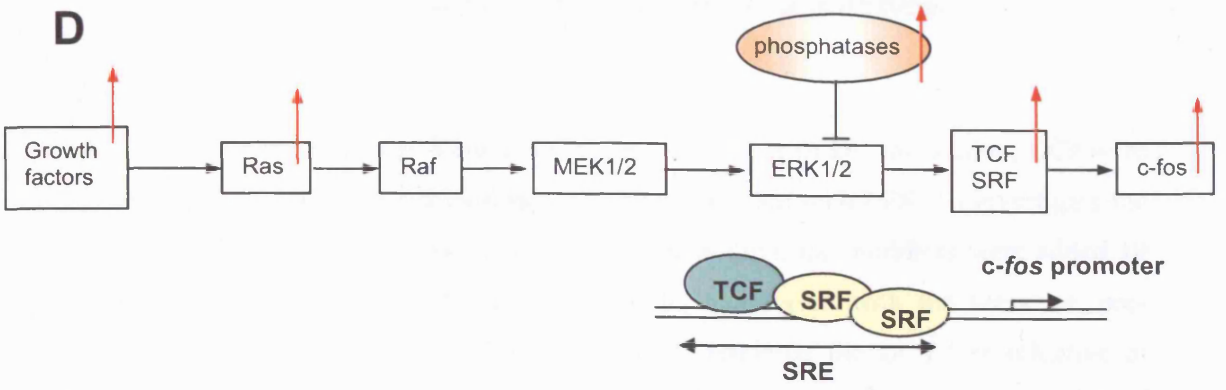
A Components of p38/JNK pathway significantly up- and down-regulated (shown by red and green arrows respectively) in the core DC activation and differentiation response to LPS and poly(I:C);
B Representation of normalised log_2 expression ratios of components of the p38 pathway, as visualised in Treeview;
C Log_2 expression ratios of the same components (shown in **B**) of the p38 pathway plotted over time, in mock-stimulated DCs (broken blue lines), LPS-stimulated DCs (red lines), and poly(I:C)-stimulated DCs (green lines).

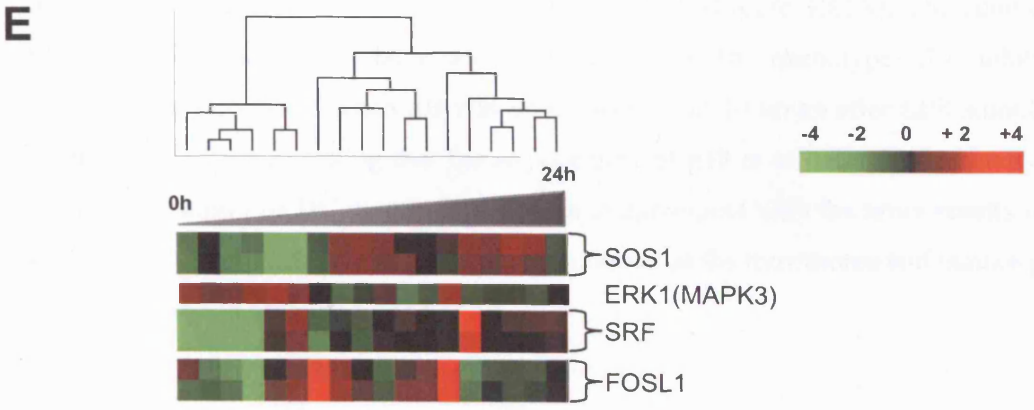
D Components of ERK pathway significantly up- and down-regulated (shown by red and green arrows respectively) in the core DC activation and differentiation response to LPS and poly(I:C);
E Representation of normalised log_2 expression ratios of components of the ERK pathway, as visualised in Treeview;
F Log_2 expression ratios of the same components (shown in **E**) of the ERK pathway plotted over time, in mock-stimulated DCs (broken blue lines), LPS-stimulated DCs (red lines), and poly(I:C)-stimulated DCs (green lines).

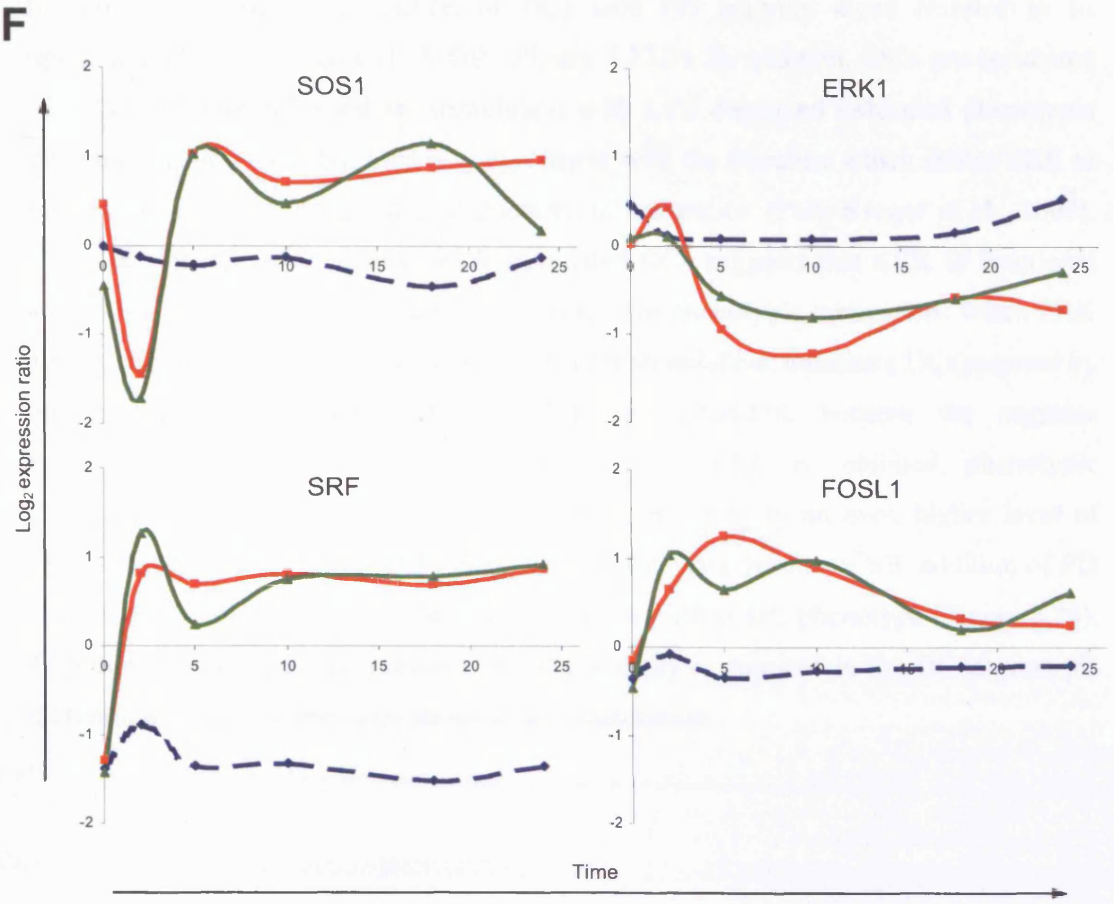

A
Cytokines
TNFα
Rac
Cdc42
TRAF2
TRAF6
ASK1
MKK4/7
MKK3/6
JNK
p38
p53


B
-4 -2 0 +2 +4
0h
24h
ASK1 (MAP3K5)
MKK3 (MAP2K3)
p38 (MAPK1)
p53


C
ASK1
MKK3
p38
Tumor antigen p53
Log2 expression ratio
Time


D
phosphatases
Growth factors
Ras
Raf
MEK1/2
ERK1/2
TCF SRF
c-fos
c-fos promoter
TCF
SRF
SRF
SRE


E
-4 -2 0 +2 +4
24h
0h
SOS1
ERK1(MAPK3)
SRF
FOSL1


F
SOS1
ERK1
SRF
FOSL1
Log2 expression ratio
Time

3.2.10.1 Role of p38 and ERK in affecting DC phenotype

p38 pathway

To investigate the role of p38 and ERK at the early phase of DC maturation, DCs were pre-incubated with SB or PD inhibitor prior to stimulation with LPS. To investigate the role of p38 and ERK at the late phase of DC maturation, the inhibitors were added 10 hours after DCs were stimulated with LPS. In accordance with the literature, pre-incubation of DCs with SB (inhibition of p38) rendered the DCs less effective at upregulating CD86 and HLA-DR in response to LPS (Figure 3.23A). The addition of SB inhibitor alone did not have noticeable effects on DC phenotype. This inhibitory effect was not observed when SB was added to DCs at 10 hours after LPS stimulation (Figure 3.24), demonstrating that the requirement of p38 in affecting DC phenotype is at the early stages of DC maturation. This is in agreement with the array results which show a downregulation of p38 pathway components at the transitional and mature phase of DC maturation (Figure 3.22).

ERK pathway

In contrast to SB, pre-incubation of DCs with PD inhibitor alone resulted in an upregulation of CD86 and HLA-DR (Figure 3.23B). In addition, DCs pre-incubated with PD inhibitor followed by stimulation with LPS displayed enhanced phenotypic maturation (Figure 3.23B). This is in agreement with the literature which shows ERK to function as a negative regulator of phenotypic maturation (Puig-Kroger et al., 2001). The effect of PD on immature mock-stimulated DCs suggests that ERK is functional and active in immature DCs acting as a "brake" for phenotypic maturation. When ERK activity is inhibited by PD in the absence of LPS stimulation, immature DCs respond by upregulating surface expression of CD86 and HLA-DR, because the negative maturation control is released. In addition, when ERK is inhibited, phenotypic maturation may be further potentiated by LPS, resulting in an even higher level of surface expression of CD86 and HLA-DR. Similar to the results of SB, addition of PD to DCs at 10 hours after LPS stimulation does not affect DC phenotype (Figure 3.24). Together, this suggests suppression of ERK activity is required in the DC phenotypic maturation process at the early stages of DC maturation.

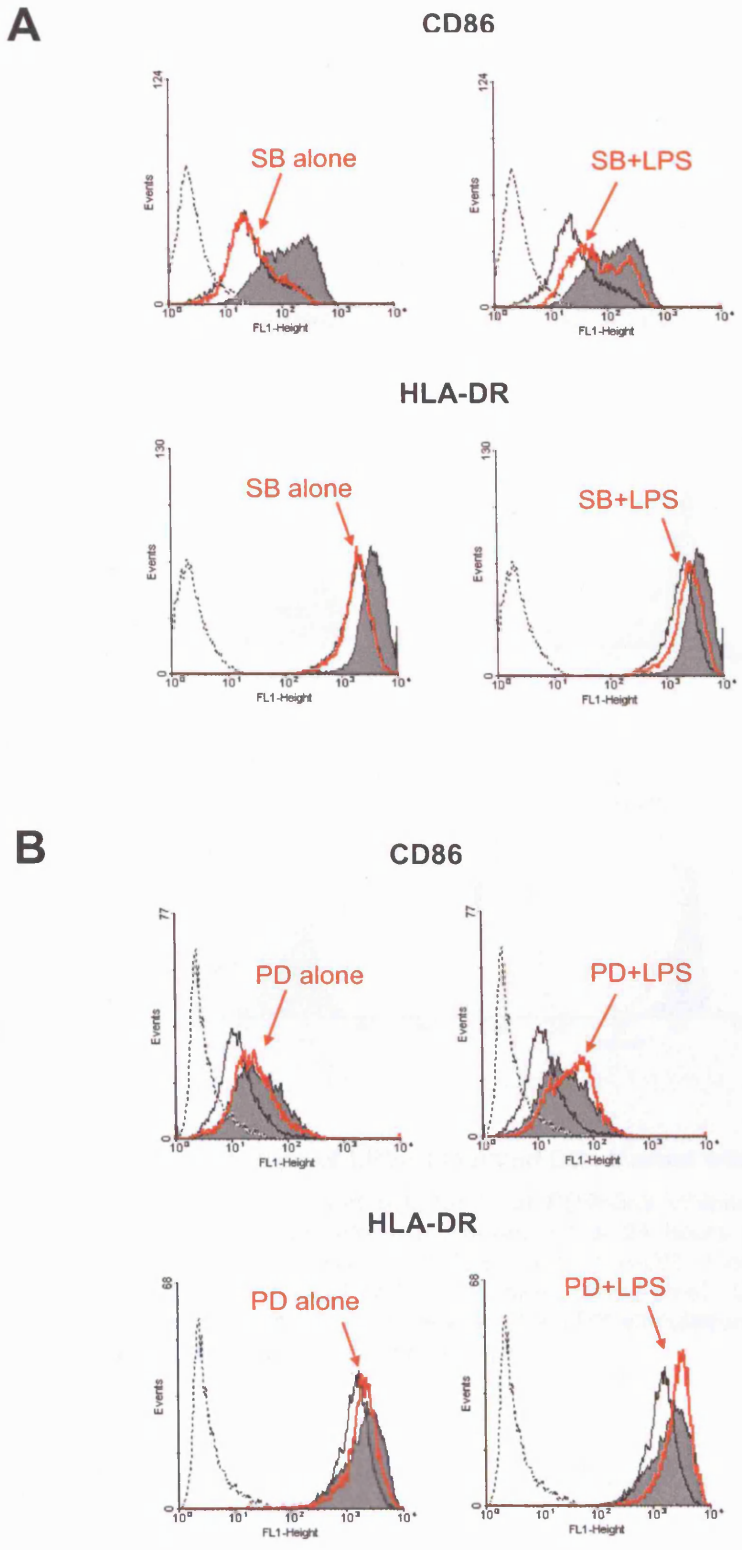


Figure 3.23 Immunophenotype of DCs pre-incubated with SB or PD inhibitor

DCs were stimulated with LPS following pre-treatment with p38 or ERK inhibitors. Surface expression of CD86 and HLA-DR of mock-stimulated DCs (black unfilled histogram), DCs stimulated with LPS (grey filled histogram), and treatment with (**A**) SB203580 (red histogram) with or without LPS stimulation, and (**B**) PD98059 (red histogram) with or without LPS stimulation, were measured by flow cytometry at 24 hours post-LPS-stimulation. Dotted histograms show the relevant isotype controls.

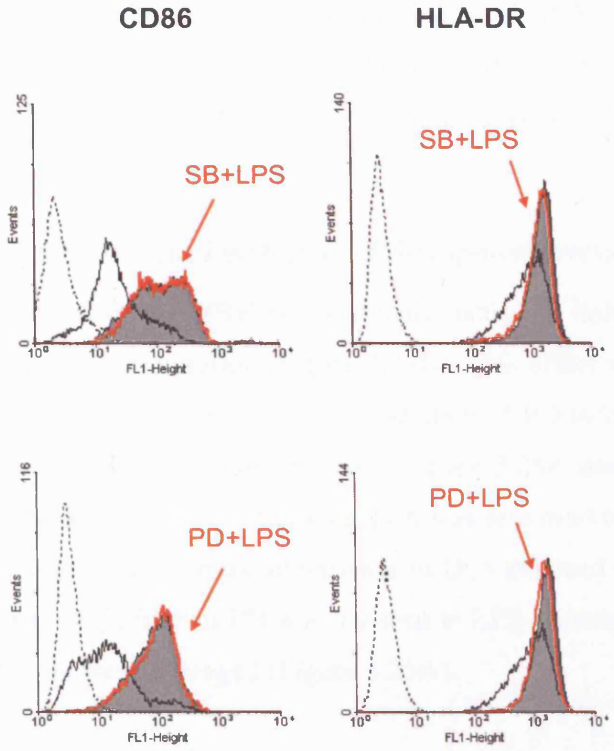


Figure 3.24 Immunophenotype of LPS-stimulated DCs treated with SB and PD inhibitors

DCs were stimulated with LPS, and SB203580 or PD98059 inhibitors were added 10 hours after LPS-stimulation. Flow cytometry was carried out at 24 hours post-LPS-stimulation, and cells analysed for surface expression of CD86 and HLA-DR. Mock-stimulated DCs (black unfilled histogram), LPS-stimulated DCs (grey filled histograms), DCs treated with SB (top panel) or PD inhibitor (bottom panel) 10 hours after LPS-stimulation (red histograms). Dotted histograms show the relevant isotype controls.

3.2.10.2 Donor variability and differential responses to MAPK inhibition

Typical of experiments involving human dendritic cells, there are inherent donor variability in responses to fixed concentrations of various stimuli such as LPS and poly(I:C). The same variable effect is observed for DC responses to fixed concentrations of PD inhibitor, evident in the levels of HLA-DR and CD86 surface expression. This suggests that sensitivity of DCs to SB or PD inhibitor reflects donor variability, possibly reflecting differences in the basal activities of p38 and ERK.

The role of ERK and its relationship to LPS-responsiveness

Data presented here show that ERK is functionally active in immature DCs to prevent inappropriate phenotypic maturation (Figure 3.23B). This effect was consistently found among DCs from different donors, where the addition of PD to immature DCs resulted in enhancement of CD86 surface expression (Figure 3.25A and B, p-value = 0.011). The effect of PD on HLA-DR surface expression was less marked, though still showed a general trend of increased surface expression in DCs exposed to PD (Figure 3.25B). However, the enhancing effect of PD was not seen in LPS-stimulated DCs, when results from different donors were averaged (Figure 3.25A).

Further analysis of the effects of PD inhibitor in individual donors showed a negative correlation between a donor's DCs' responsiveness to LPS (as determined by CD86 upregulation in response to LPS) and the enhancing phenotypic effect of PD in mock-stimulated DCs (Figure 3.26). The stronger response a donor's cells have to LPS stimulation, the less CD86 upregulation occurs when PD is added to immature DCs (e.g. donor 4 in Figure 3.26). Conversely, where there is a weaker response to LPS as measured by CD86 upregulation, there is a commensurate phenotype enhancing effect of PD (e.g. donor 1 in Figure 3.26). This implies that donors whose DCs have a potential to strongly respond to LPS (indicated by level of CD86 upregulation in response to LPS) may have correspondingly higher basal levels of ERK. Therefore, a fixed dose of PD may be suboptimal and not be sufficient at inhibiting ERK activity fully in the corresponding immature DCs. It is important to note that this individual variation was not seen when the results were averaged (Figure 3.25A), highlighting the importance of examining individual variation as well as averaged trends.

This data suggests that individual donor DCs have different levels of ERK activity that is correlated to their LPS-responsiveness. It is therefore likely that a titration of PD inhibitor per donor will identify levels where PD is able to inhibit all ERK activity, shown by the greatest phenotypic enhancement of CD86 surface expression indicating the release of the negative maturation control of the ERK pathway.

The role of p38 and its relationship to LPS-responsiveness

In contrast to the subtle effects of ERK inhibition that vary with individuals, results of p38 inhibition showed consistent impaired phenotypic maturation in SB-treated DCs responding to LPS (Figure 3.27). There does not seem to be a relationship between the inhibition of p38 and LPS responsiveness as measured by CD86 surface expression in individual donor DCs.

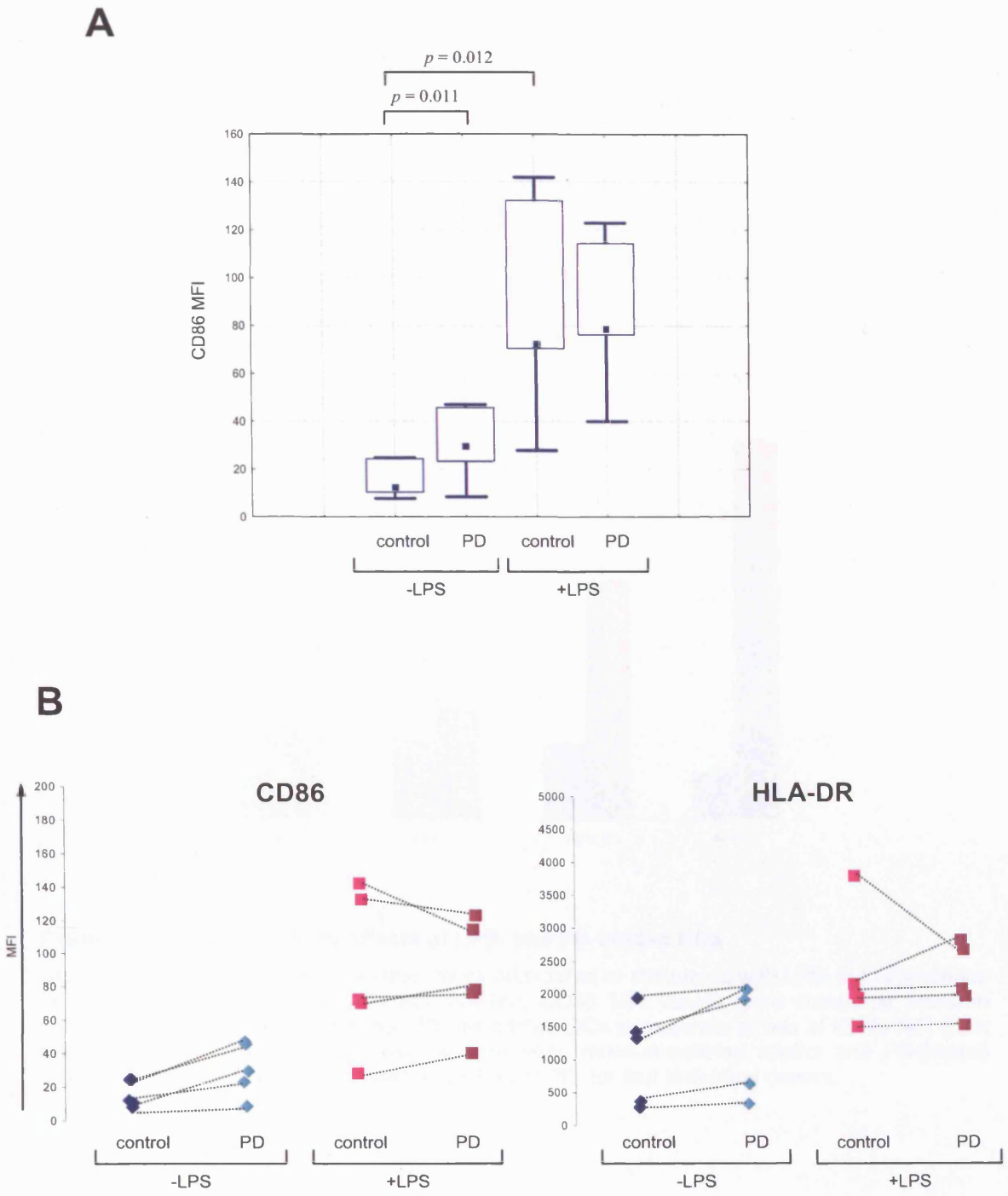


Figure 3.25 Donor variability effects on phenotype of PD-treated DCs

A DCs from individual donors were either mock-stimulated (-LPS) or stimulated with LPS (+LPS), with or without pre-treatment with PD inhibitor. Averaged CD86 MFI values from flow cytometric analysis of 5 donors, visualised as box-and-whisker plots, were compared between the groups. *P*-values indicated are generated by the Student's *t*-test for comparisons between control mock-stimulated and control LPS-stimulated DCs, and between mock-stimulated control and mock-stimulated PD-treated DCs.
B The CD86 MFI values plotted for each individual donor to visualise donor-specific responses to PD-treatment in mock-stimulated (-LPS) and LPS-stimulated (+LPS) DCs.

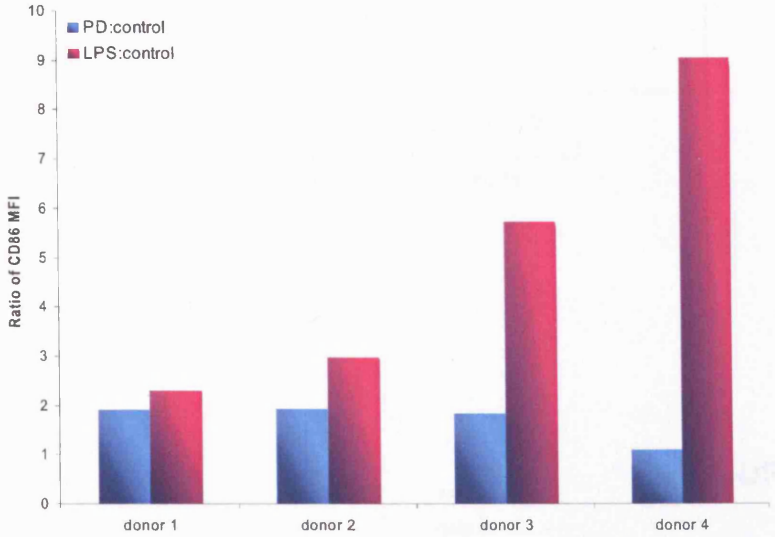


Figure 3.26 Donor variability effects of LPS- and PD-treated DCs

DCs from individual donors were either mock-stimulated or stimulated with LPS, in the presence or absence of pre-treatment with PD inhibitor. CD86 MFI values were compared between control mock-stimulated and control LPS-stimulated DCs to generate a ratio of CD86 MFI (pink bars) indicating LPS-responsiveness, and between mock-stimulated control and PD-treated DCs (blue bars), indicating the enhancing effect of PD, for four individual donors.

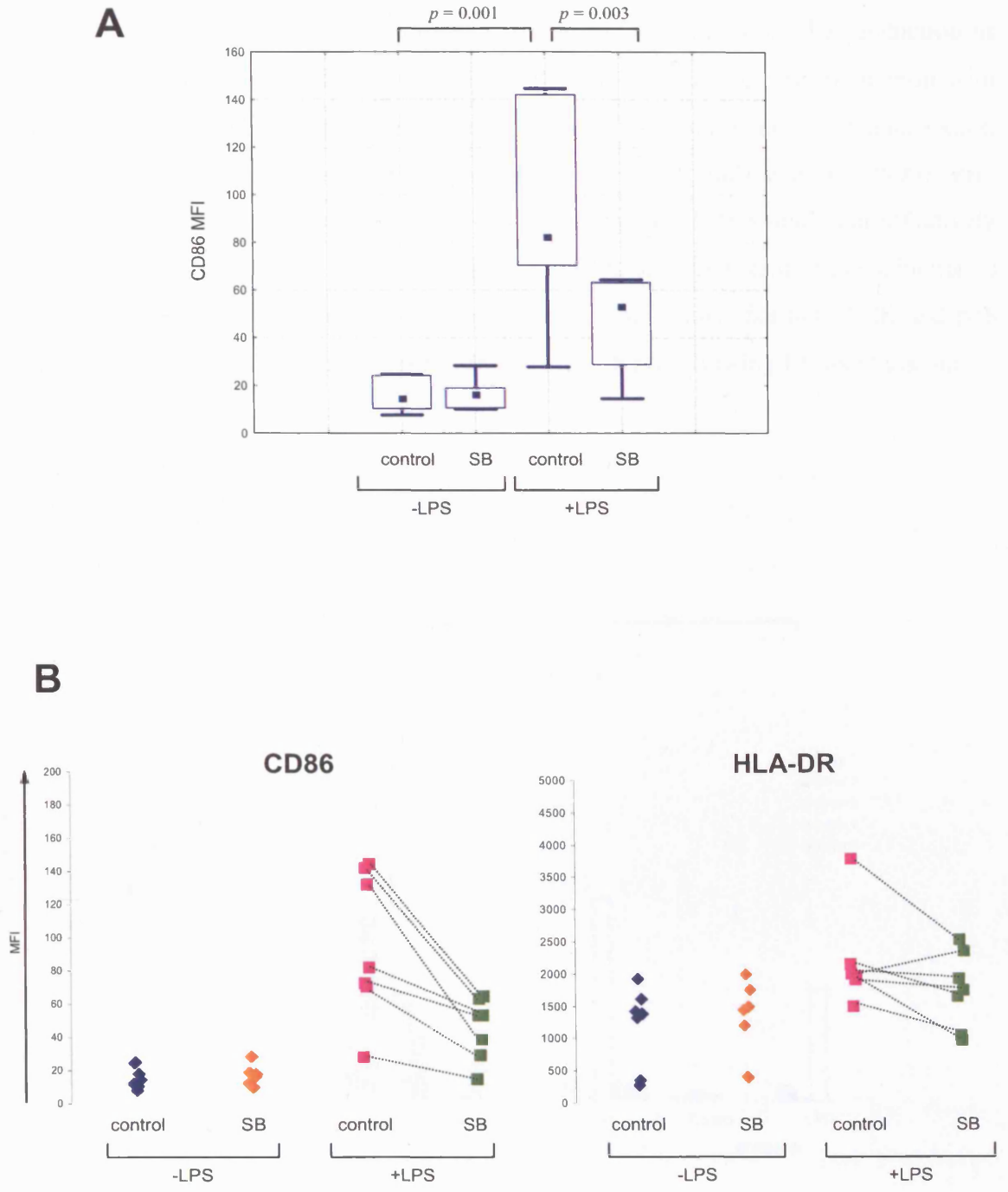


Figure 3.27 Donor variability effects on phenotype of SB-treated DCs

A DCs from individual donors were either mock-stimulated (-LPS) or stimulated with LPS (+LPS), with or without pre-treatment with SB inhibitor. Averaged CD86 MFI values from flow cytometric analysis of 7 donors, visualised as box-and-whisker plots, were compared between the groups. *P*-values indicated are generated by the Student's *t*-test for comparisons between control mock-stimulated and control LPS-stimulated DCs, and between LPS-stimulated control and LPS-stimulated SB-treated DCs.
B The CD86 MFI values plotted for each individual donor to visualise donor-specific responses to SB-treatment in LPS-stimulated control and LPS-stimulated SB-treated DCs.

3.2.10.3 Role of ERK and p38 in TNFα production

To investigate further the role of ERK and p38 in DC maturation, the production of TNFα in DCs pre-incubated with SB and PD inhibitor followed by stimulation with LPS was investigated. TNFα was chosen because of its rapid induction characteristics, and its clear role in DC maturation (Lyakh et al., 2000; Nelson et al., 1999). Pre-treatment of DCs with both SB and PD inhibitor before LPS-stimulation effectively reduced TNFα production in DCs responding to LPS, and the extent of the reduction is dependent on the individual donors (Figure 3.28). This shows that both ERK and p38 are required in maturing DCs for the production of TNFα following LPS-stimulation.

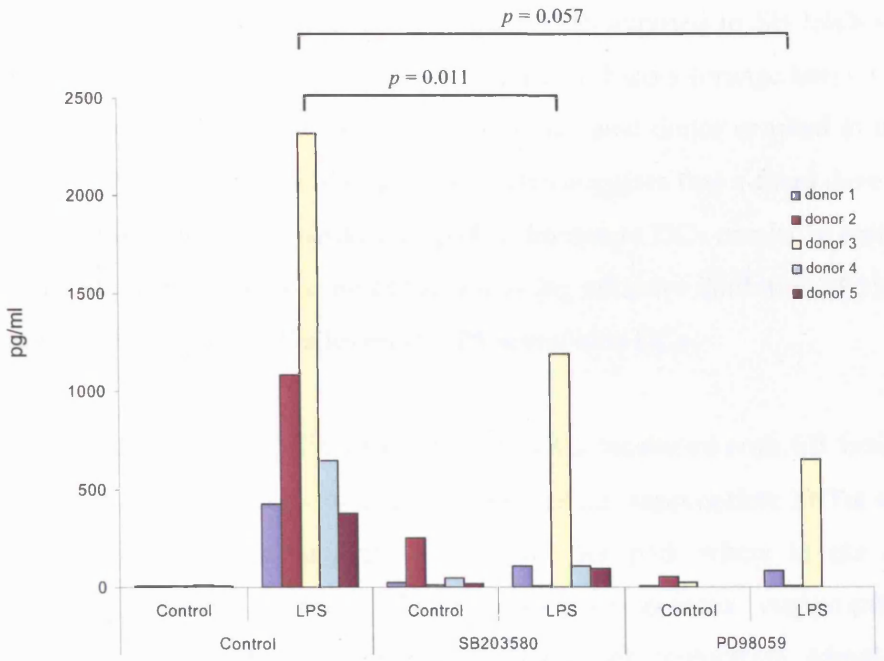


Figure 3.28 Effects of SB and PD inhibitors on TNFα production in DCs

DCs were either untreated (control) or pre-treated with SB (SB203580) or PD (PD98059). DCs were then mock-stimulated (control) or stimulated with LPS (LPS) for 24 hours in culture. DC culture supernatants were harvested after 24 hours, and levels of TNFα were measured by a sandwich immunoassay for TNFα (Section 2.7.1). Results show 5 donor samples for pre-treatment with SB, 3 donor samples for pre-treatment with PD. Averaged levels of TNFα production were compared between LPS-stimulated control DCs and inhibitor-treated DCs to determine level of significance by the Student's paired *t*-test.

The role of p38 in TNFα secretion by DCs

DCs pre-incubated with SB inhibitor alone consistently produced a small but detectable amount of TNFα, compared to untreated control DCs (Figure 3.28). This also seemed to be donor-dependent, but comparison between mock-stimulated SB-treated DCs and control DCs with averaged levels of TNFα production across donors masked this effect at the individual donor level. This enhancement of TNFα production by SB treatment was investigated. In mock-stimulated DCs, pre-treatment with SB slightly increased the level of TNFα production. This increase in the level of TNFα production is correlated to the reduction in levels of TNFα production as a result of SB pre-treatment in LPS-stimulated DCs (Figure 3.29). This can be visualised on a plot of the ratio of TNFα secretion between SB treated and control DCs at the immature (mock-stimulated) and mature (LPS-stimulated) stage, where an enhancement in TNFα secretion is seen as a ratio greater than 1, and a reduction in TNFα secretion is seen as a ratio less than 1. For example, immature DCs from donor 2 (Figure 3.29) exposed to SB inhibitor produce the highest levels of TNFα compared to the other 4 donors (orange bars). Conversely, SB pre-treatment in LPS-stimulated DCs from the same donor resulted in the greatest reduction of TNFα production (purple bars). This suggests that a fixed dose of SB that effectively inhibits the basal activity of p38 in immature DCs results in optimal TNFα production, and that the same dose of SB producing effective inhibition of p38 results in the greatest reduction in TNFα levels in LPS-stimulated DCs.

This enhancing effect of TNFα production by DCs incubated with SB inhibitor alone suggests the role of basal activity of p38 in preventing inappropriate TNFα secretion by immature DCs. This data suggests a dual role for p38, where in the absence of appropriate maturation stimuli, p38 functions to prevent inappropriate TNFα production. However, in the presence of appropriate maturation stimuli, p38 acts synergistically or functions downstream of TLR signalling to result in optimal and appropriate TNFα production by DCs. Therefore, the higher the basal p38 activity in donor DCs, the less enhancement by TNFα production is seen when DCs are pre-treated with suboptimal dose of SB inhibitor. Similarly, this amount of SB inhibitor is also less effective at inhibiting p38 to reduce levels of TNFα production in the same DCs stimulated with LPS. Individual donor DCs also appear to have different levels of p38 activity that is correlated to their LPS-responsiveness related to TNFα production.

It is therefore likely that a titration of SB inhibitor per donor will identify levels where SB is able to inhibit all p38 activity and thereby release the negative control of TNFα production by the p38 pathway in immature DCs.

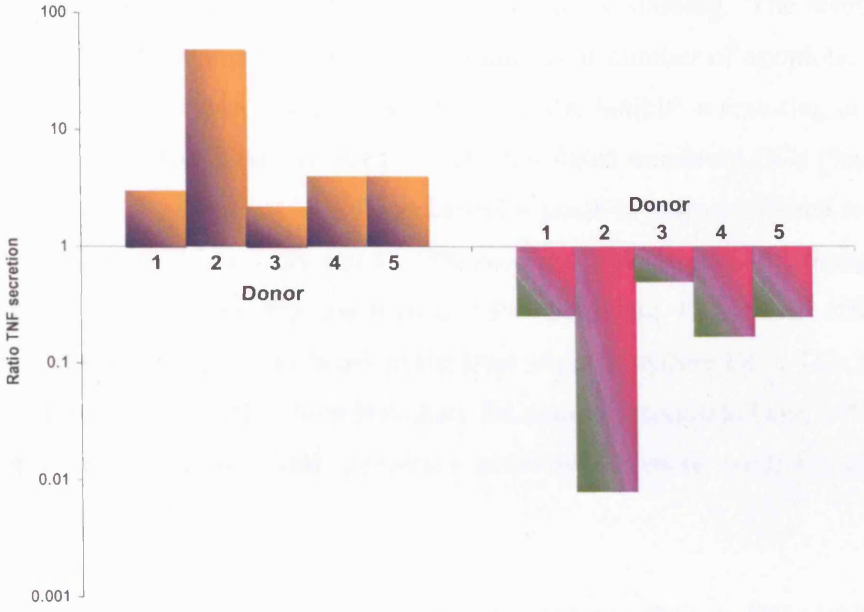


Figure 3.29 Donor variability effects on TNFα secretion

DCs were either untreated or pre-treated with SB. DCs were then mock-stimulated or further stimulated with LPS for 24 hours in culture. DC culture supernatants were harvested after 24 hours, and levels of TNFα were measured by a sandwich immunoassay for TNFα. Ratios of TNFα production (y-axis) were generated between mock-stimulated SB-treated or untreated DCs (orange bars), and LPS-stimulated untreated and SB-treated DCs (purple bars). This is shown on a $\log_{10}$ scale.
Orange bars show increase in TNFα production as a result of p38 inhibition in mock-stimulated DCs, and purple bars show reduction in TNFα production as a result of p38 inhibition in LPS-stimulated DCs.

3.2.10.4 Role of ERK in DC viability

The effects of SB and PD inhibitors on DC viability was also investigated. Forward and side scatter plots of DCs suggest that the size and geometry of the cell population was altered in the presence of PD treatment (data not shown). This was further investigated with Annexin V and propidium iodide staining to identify cells undergoing apoptosis. To identify effects of p38 and ERK inhibition in immature and mature DCs, viability was measured in DCs in various conditions (Figure 3.30A). DCs were pre-treated with SB or PD, and were "mock-stimulated ("mock-stimulated" panel in Figure 3.30B), or stimulated with LPS ("Early" panel in Figure 3.30B). Effects of p38 and ERK inhibition in maturing DCs were investigated by adding the inhibitors 10 hours after DCs were stimulated with LPS ("Late" panel in Figure 3.30B). DCs were harvested after 24 hours, and early apoptotic cells were identified by Annexin V staining. The results show that the addition of PD to the cells results in an increased number of apoptotic (Annexin V positive) cells in all three groups. The effect of ERK inhibition resulting in the increase in apoptotic DCs was most evident in mock-stimulated immature DCs ("control mock-stimulated" group contained 15.37% Annexin V positive cells compared to "PD mock-stimulated" group which contained 53.75% Annexin V positive cells), though this effect was also maintained in PD pre-treated LPS-stimulated DCs. The effect of ERK inhibition does not seem significant at the later stage in mature DCs. This suggests that ERK has a role in protecting both immature DCs (mock-stimulated and LPS-stimulated) from apoptosis, and the ERK signalling pathway therefore mediates an early pro-survival signal to DCs.

Addition of SB to immature and maturing DCs had no effect on DC viability (data not shown).

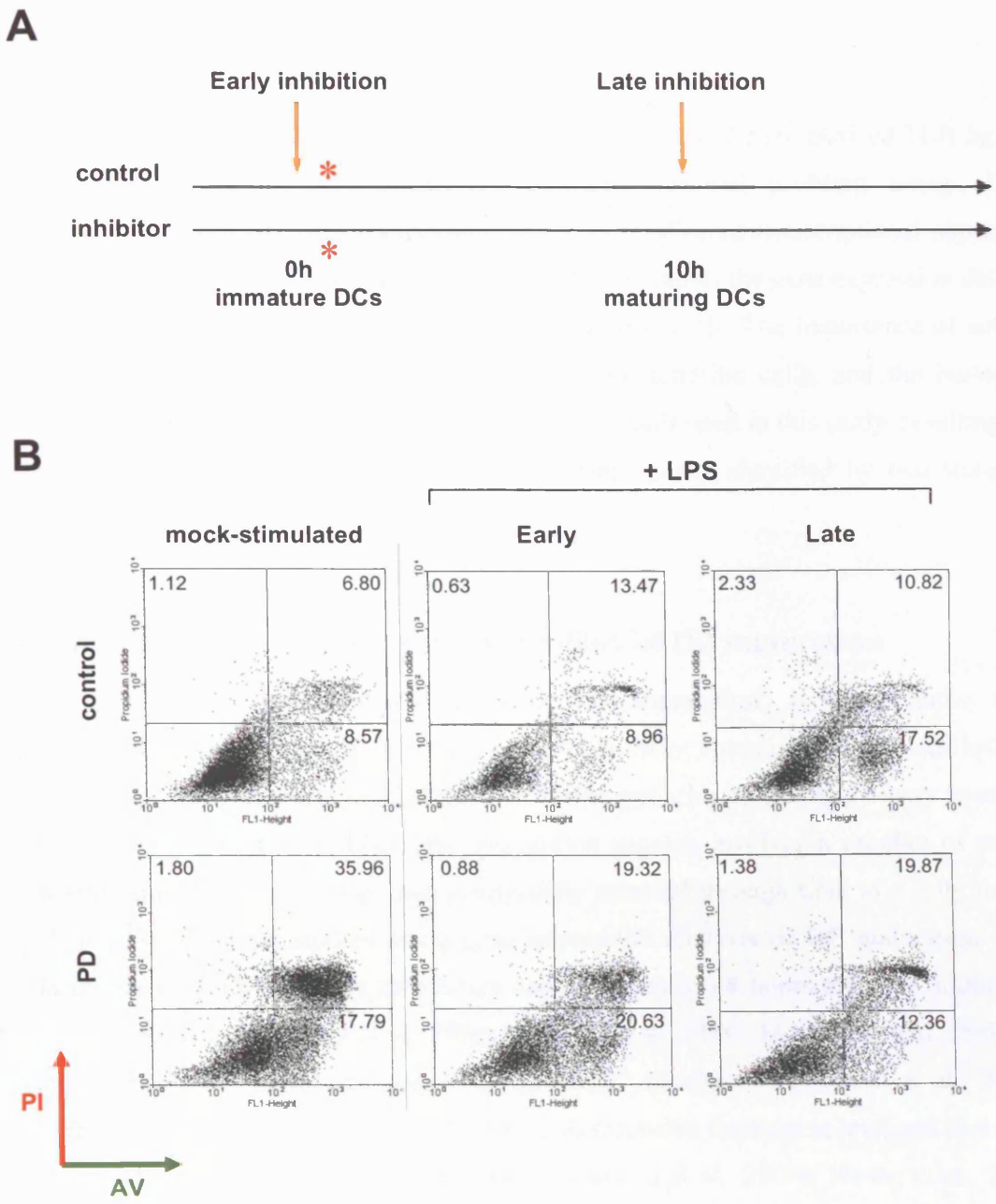


Figure 3.30 DCs treated with PD98059 have increased susceptibility to apoptosis

A Control and inhibitor-treated DCs were stimulated with LPS at the point marked *, or were left mock-stimulated. Inhibitors SB or PD were either added to immature DCs 1 hour prior to LPS stimulation ("early"), or inhibitors were added at 10 hours ("late") following LPS stimulation. Cells were harvested for flow cytometry at 24 hours.
B Representative AnnexinV (AV) and Propidium iodide (PI) staining of control DCs (top panel) or PD-treated DCs (bottom panel). The numbers in the quadrants indicate percentages of the total cell population. In the comparisons between untreated and PD-treated DCs, PD-treatment of mock-stimulated immature DCs and LPS-stimulated DCs resulted in greater numbers of Annexin V positive cells, indicating a larger proportion of cells undergoing apoptosis in the presence of PD inhibitor.

Discussion

The dendritic cell maturation response, induced by two well-characterised TLR ligands LPS and poly(I:C) was investigated by transcriptional profiling using cDNA microrrays. Sampling over an extended timecourse allowed transcriptional signatures DC maturation stages to be identified. This greatly expands the gene expression data for the maturation and differentiation of human dendritic cells. The importance of antigen dosage, donor and biological variability between dendritic cells, and the biological relevance of sampling at pertinent timepoints were addressed in this study, resulting in a robust dataset of core DC "maturation"-regulated genes identified by two statistical methods.

Time-dependent transcriptional profiles of DC maturation

The process of antigen-initiated dendritic cell "maturation" is a continuous time-dependent process where dendritic cells change from an immature immune sentinel to a mature antigen presenting cell. The two states do not arise by a simple binary transition from one to the other. Rather, this maturation process involves a number of stages, where immature DCs change and functionally remodel through time to a fully mature phenotype. Previous studies using gene expression analysis of DC maturation range from sampling DCs at time zero hours and 24 hours or 48 hours after the addition of maturation stimulus (Dietz et al., 2000; Messmer et al., 2003; Moschella et al., 2001), to studies that include various single "intermediate" timepoints (Granucci et al., 2001b; Matsunaga et al., 2002; Tureci et al., 2003), to extensive timecourse analyses that cover the timeframe relevant for DC maturation (Granucci et al., 2001a; Huang et al., 2001). Therefore, only the studies of Granucci and Huang produce definitive datasets for mouse and human dendritic cell maturation respectively.

The study by Huang *et al* identified a core response comprising only 166 genes regulated by dendritic cells in response to *E. coli*, Influenza, and *C. albicans*. These genes were therefore termed pathogen-independent. Most of the genes identified had known functions relating to DC biology, and the induction of these genes followed a temporal order. However, this set of core response genes is clearly an underestimate, as Influenza infection of DCs results in a productive infection with cytopathic effects, and Influenza also results in a significant transcriptional downregulation of a number of

genes (Chapter 4). These effects of Influenza infection in dendritic cells were not addressed in Huang's study, and other pathogen-specific effects may be similarly overlooked. Temporal ordering of genes regulated in the DC core response to pathogen in Huang's study used a self-organising map algorithm to cluster genes. In contrast, the work here used two statistical methods to validate genes involved in the transcriptional stages of DC maturation. Significantly regulated genes identified by the two statistical methods totalled 1055 genes out of 4321 genes analysed, a proportion of nearly 25% of genes expressed in DCs that comprised the core activation and differentiation response.

Transcriptional profiles defining stages of DC maturation were suggested for murine dendritic cells (Ricciardi-Castagnoli and Granucci, 2002), based on gene expression studies in a murine DC line stimulated with *E. coli* (Granucci et al., 2001a). Granucci's study defined temporal ordering of gene expression based on cluster analysis of genes divided into functional families. The validity of the clustering was tested by comparison to established markers of DC function where the kinetics of gene regulation were known. This functional classification of genes therefore relies on information already known about DCs, and additional information inferred from this potentially limits the amount of new biological information that can be found. The statistical approach used in this study therefore produces a wider-encompassing set of genes that are involved in the DC maturation programme.

Kinetic analysis of gene expression of dendritic cells responses to pathogens has also identified the differences between activation and differentiation, both processes occurring under the broader term of DC "maturation". Activatory responses are defined as transient (Huang et al., 2001; Ricciardi-Castagnoli and Granucci, 2002), where transcript levels undergo transient induction or repression, followed by return to basal levels. This type of response is particularly well-illustrated in the transcriptional induction of pro-inflammatory cytokines and chemokines that are identified in the transitional phase of DC "maturation". In contrast, differentiation responses are sustained, and result in DCs reaching a new steady state. These responses are seen in the group of genes statistically identified as significantly regulated over two stages of maturation, in particular the transitional and mature phase (Figure 3.13B). These genes comprise transcription factors and other immune response and interferon-inducible genes, highlighting the functional differentiation of DCs from an innate immune sentinel to an effective antigen presenting cell as part of the maturation process.

Components of the MAPK pathway identified in this work as being differentially temporally regulated may also fit this model of activation and differentiation. There is a transient requirement for p38 in the early phase of DC maturation involved in upregulating surface expression of CD86 and HLA-DR, and production of TNFα. This suggests that the activation of p38 is a result of "activation" stimulus, as this requirement for p38 is not sustained. In contrast, modulation of ERK activity, as reflected at the transcript level, is required throughout DC maturation to regulate DC phenotype and survival, which may suggest a role in mediating DC differentiation.

Whereas cascades of transcriptional changes have been suggested for human dendritic cells (Huang et al., 2001; Tureci et al., 2003), no studies have specifically use statistical methods to define significantly regulated genes during the maturation process of human dendritic cells in response to two well-characterised maturation stimuli. Work presented here on the human dendritic cell maturation process is a result of detailed transcriptional profiling throughout the 24-hour DC maturation timecourse and reveals statistically distinct transcriptional stages of DC maturation.

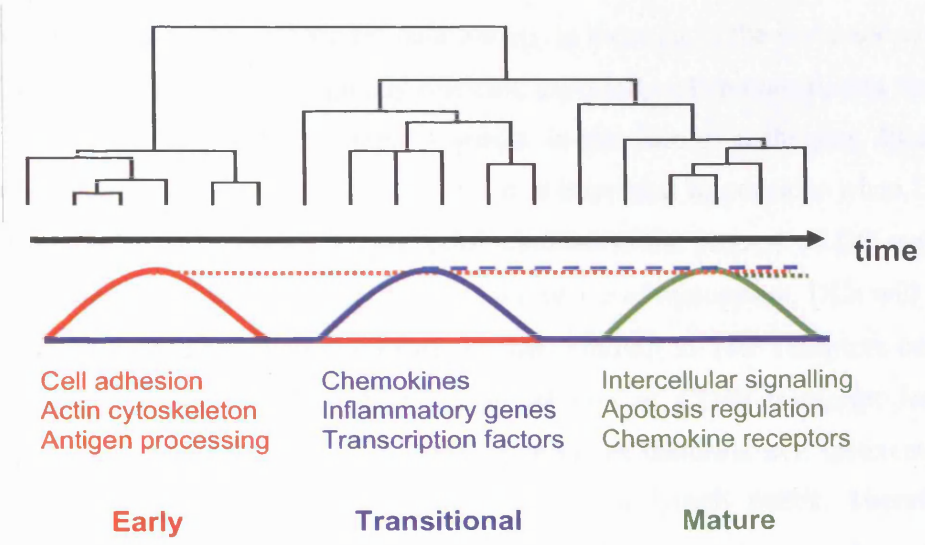


Figure 3.31 Transcriptional stages of DC "maturation"

The transcriptional profile of DC activation and differentiation in response to LPS and poly(I:C) can be divided into distinct stages that correspond to early, transitional and mature DCs. The transcriptional signatures are typified by genes that are strongly expressed at various stages of DC activation and differentiation, and delineate between temporary (dotted lines) and sustained (solid lines) induction of genes.

The genes that are regulated as the DCs traverse this maturation pathway reflect the biology and function of maturing DCs. The similarity between the responses to poly(I:C) and LPS suggest that the TLR3 and TLR4 signalling pathways functionally converge, to a large extent. TLR3 and TLR4 are unique among the TLR family in that in addition to a MyD88-dependent pathway shared by all TLRs, both utilise a MyD88-independent signalling pathway (Alexopoulou et al., 2001; Kaisho et al., 2001). This allows TLR3 and TLR4 ligands to stimulate an interferon response through the activation of the adaptor molecule TRIF resulting in the activation of IRF-3 (Doyle et al., 2002). The integration of the signalling pathways therefore determines common transcriptional and phenotypic responses, both of which are seen in this analysis. In this study, DCs therefore regulate a dominant time-dependent response rather than a stimulus-specific response.

LPS and poly(I:C) are simple antigen components that DCs functioning as immune sentinels should recognise as representative of gram negative bacteria and intracellular virus respectively. Using these antigens makes the study of downstream transcriptional responses more relevant to the biological and physiological setting where immature DCs encounter pathogens and regulate a maturation response. This is in contrast to studies that use conditioned medium, cytokine cocktails or members of the TNF family (e.g. TNFα or CD40L) to induce DC maturation. In these cases the maturation response of DCs is not necessarily biologically relevant, especially when considering the role of DCs which is to initiate the immune response in the face of pathogens. In addition, within the context of the immune response, it is important to consider when DCs will encounter TNFα and CD40L. As an initial stimulus to the process of DC maturation, DCs will primarily see antigen; in the transient phase of maturation, DCs will produce TNFα which should act in an autocrine manner, binding to TNF receptors on DCs to further propagate downstream effects. Upregulation of CD40 transcript leading to surface upregulation of CD40 in the mature phase of dendritic cell differentiation is necessary for optimising DC-T cell interactions in lymph nodes. Therefore, the relevance of using CD40L and TNFα to stimulate DC maturation can be questioned. The temporal context should always be an important consideration when deciding upon maturation-inducing stimuli in order to gain biologically relevant insight into DC function and immune responses.

Transcriptional regulation of signalling pathways

The dataset of core genes regulated by DCs have also revealed differential modulation of the MAPK pathway. This signalling pathway has been demonstrated to be important in modulating wide-ranging effects in the cell, including dendritic cells. The use of the gene annotation tool DAVID (Dennis et al., 2003) allowed visualisation of the large sets of genes identified as significantly regulated, that relate to biologically functional and relevant pathways.

Study of signalling pathways, MAPK included, have classically involved comparison of phosphorylated and unphosphorylated forms of protein kinases to assess the activity of a particular pathway. However, microarray analysis allows an overview of the transcriptional regulation of components of such signalling pathways, and offers a view of the gene regulatory control of signalling pathways. The caveat that transcriptional induction of such genes actually represents the normal activity of a pathway rather than an induction of a pathway in response to stimulus has to be considered. However, the overall "tone" of an integrated signalling pathway can be assessed by focussing on specific endpoints that are consistently up- or down-regulated. We found that serum response factor (SRF) and c-fos, transcription factors downstream of ERK, were signficantly upregulated at transitional and mature stages of DC differentiation; p53, downstream of p38 signalling, was downregulated at the transitional and mature phase of DC differentiation. This therefore highlighted ERK and p38 for further study, where both have been shown to be active in immature DCs in modulating appropriate responses in terms of phenotype, cytokine secretion, and maintenance of survival. p38 has been shown to be necessary for phenotypic maturation of DCs in response to LPS and TNFα (Arrighi et al., 2001), and this was also shown here using p38 inhibitor which impaired DC phenotype maturation in response to LPS. ERK has been shown to function as a negative regulator of DC maturation, where inhibition of ERK results in enhanced surface expression of antigen presenting molecules, costimulatory molecules, and greater production of IL-12 upon stimulation of DCs with LPS or TNFα (Puig-Kroger et al., 2001). It was further identified that ERK has a role in mediating DC survival in immature mock-stimulated and LPS-stimulated DCs, which has not been previously reported. In mature DCs ERK inhibition has been shown not to affect DC survival (Ardeshna et al., 2000), and this is in agreement with the data shown here. Therefore, the continued transcriptional upregulation of SRF and c-fos at the mature

phase of DC differentiation indicates a role for ERK signalling at this late stage, and may contribute to other aspects of DC function.

The modulation of DC responses by ERK and p38 has potential importance in affecting how DCs polarise appropriate adaptive immune responses. The use of MAPK inhibitors may have therapeutic potential. As p38 is a positive regulator of functional and phenotypic DC maturation, inhibition of p38 may be useful in autoimmune diseases which arise from an overactive immune response. In support of this, pyridinyl imidazoles that are closely related to SB203580 have shown efficacy in animal models of chronic inflammatory disease such as rheumatoid arthritis (Jackson and Bullington, 2002; Revesz et al., 2004). Anti-TNFα therapy is used for rheumatoid arthritis, further suggesting a potential role for inhibition of p38 (McLay et al., 2001; Westra et al., 2004). It will be interesting to look at the function of p38 and ERK in DC responses to antigens that induce a T_H2 response such as prostaglandin E and cholera toxin (Kapsenberg, 2003).

An important consideration for therapeutic intervention with MAPK inhibitors is variability between individuals. This variability was evident in the studies involving p38 and ERK inhibitor, where different donors' cells responded differently to a fixed dose of inhibitor and to LPS. This is a widely accepted problem in the study of dendritic cells, and the variability is tackled by increasing the number of donors in order to observe reproducible trends. In the case of responsiveness to SB203580 and PD98059, there is a trend that correlates with responsiveness to LPS. This suggests that levels of p38 and ERK varies between individuals, corresponding to individual "set-points" for DC activation and differentiation. This highlights the importance of obtaining patient-specific optimal therapeutic doses if such inhibitors are used to treat immunological diseases, as the basal levels of p38 and ERK are variable between individuals.

The array experiments in this chapter form a basis for extending the hypothesis of DC transcriptional plasticity to antigens. Although differential responses were limited, their presence at early timepoints suggested that DCs do have an ability to differentiate pathogen components. Having identified a statistically significant core set of maturation response genes, DC responses extending to whole pathogens can be investigated.

Chapter 4

Plasticity of Dendritic cell responses to Viruses

Introduction

In the previous chapter, dendritic cell responses to defined pathogen components were characterised. Transcriptional profiling revealed a defined, core "activation" and "differentiation" programme. This programme was initiated through both TLR4 (lipopolysaccharide) and TLR3 (dsRNA) and downstream signalling through these receptors. The core maturation programme is very similar between the two stimuli, presumably as they are simple antigens, essentially pathogen components. However, subtle differences can be seen in DC transcriptional responses to LPS and poly(I:C) (Figure 3.13A, Chapter 3). This is consistent with previous work with diverse antigen exposure to DCs (Huang et al., 2001). Here we investigate the LPS- and poly(I:C)-induced differential responses in DCs in more detail. The hypothesis that DCs are plastic in their response to antigens was then further investigated with more biologically relevant and complex antigens by using whole viruses, Influenza and Rhinovirus.

Both Influenza and Rhinovirus are RNA viruses. Influenza viruses are enveloped viruses with a segmented single-stranded RNA (ssRNA) genome that is "negative sense", with viral messenger RNAs (mRNAs) being transcribed from the viral RNA segments. Rhinoviruses are non-enveloped viruses with a ssRNA genome that is positive (mRNA) sense. Influenza and Rhinovirus are both respiratory viruses that infect airway epithelial cells. Importantly, Influenza has been shown to be able to productively infect dendritic cells, a fact not controlled for in an earlier study of DC transcriptional responses to diverse pathogens (Huang et al., 2001). To distinguish between effects of DC-virus interaction at the cell surface and effects of active viral replication, DC responses to exposure with inactivated virus was also investigated.

Results

4.1 LPS-specific transcriptional responses

In addition to the dendritic cell core activation and differentiation response to LPS and poly(I:C), DCs also regulate LPS- and poly(I:C)-specific genes (Figure 3.13A and 4.1A). These genes were determined to be significantly up- or down-regulated by SAM and MWU (Section 3.2.7). In response to LPS-stimulation, DCs transcriptionally regulate a larger number of genes at the early phase between 0 and 2 hours after LPS-stimulation compared to poly(I:C)-stimulation. These results were further explored by focusing on sustained differential responses, genes that are differentially regulated at early timepoints which are also similarly up- or down-regulated at later phases of DC maturation. The gene expression differences described below are sustained throughout 10 hours or 24 hours of the maturation programme. The number of genes that were consistently differentially regulated throughout DC maturation in response to LPS- or poly(I:C)-stimulation are shown in Figure 4.1B.

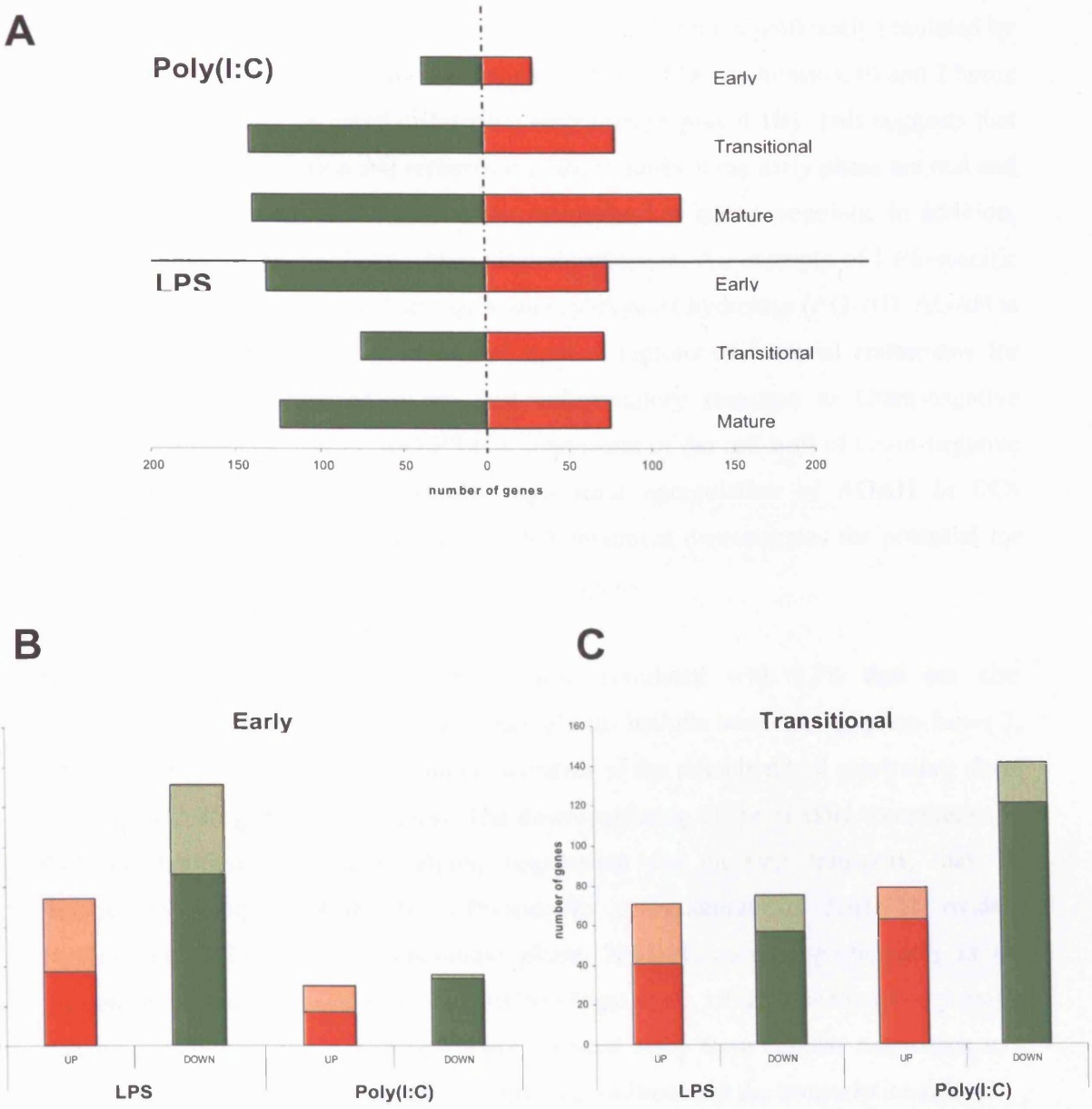


Figure 4.1 Genes differentially regulated in DCs in response to LPS and dsRNA

A Genes differentially regulated by DCs in response to LPS or poly(I:C) that are not part of the core response commonly induced in DCs in response to both stimuli, at the defined maturation stages of Early, Transitional and Mature. Genes in green are transcriptionally downregulated, genes in red are transcriptionally upregulated.
B Number of genes significantly differentially regulated at the early phase of DC maturation in response to LPS and poly(I:C). The solid bars indicate genes unique to the early phase, hatched bars indicate genes common to early and transitional, or early and mature phases of DC maturation.
C Number of genes significantly differentially regulated at the transitional phase of DC maturation in response to LPS and poly(I:C).The sold bars indicate genes unique to the transitional phase, hatched bars indicate genes common to transitional and mature phases of DC maturation.

This analysis shows that approximately 40% of genes that are significantly regulated by DCs in response to LPS-stimulation at the early phase of DC maturation, (0 and 2 hours post-stimulation), are sustained differential responses (Figure 4.1B). This suggests that the transcriptional induction and repression of these genes at the early phase are real and biologically significant, as the effects are maintained at later timepoints. In addition, functional analysis also indicates biological significance. An example of LPS-specific gene regulation is the sustained upregulation acyloxyacyl hydrolase (AOAH). AOAH is a lipase that targets fatty acyl chains of lipid A regions of bacterial endotoxins for hydrolysis, and may modulate the host inflammatory response to Gram-negative bacteria (Hagen et al., 1991). As LPS is a component of the cell wall of Gram-negative bacteria, the specific and statistically significant upregulation of AOAH in DCs responding to LPS-treatment and not dsRNA-treatment demonstrates the potential for functional bias in DC responses to particular antigens.

Downregulated genes in early phase DCs stimulated with LPS that are also downregulated at the transitional or mature phases include basic transcription factor 3, c-myc binding protein, RAP1A, and components of the mitochondrial respiratory chain (NADH dehydrogenase complexes). The downregulation of the NADH components in the mitochondrial respiratory chain, responsible for electron transport, may be functionally coupled with the LPS-specific upregulation of NADPH oxidase (cytochrome b-245) at the transitional phase. NADPH oxidase participates in the generation of reactive oxygen species (ROS) (Segal et al., 1992). It is therefore possible that the function of the mitochondria are diverted away from cellular respiration to a directed microbicidal response, and that this is coordinated at the transcriptional level.

4.2 dsRNA-specific responses

Transcriptional responses regulated by DCs specifically to dsRNA do not seem to be as consistently regulated throughout the different phases of maturation. Genes that are regulated by DCs in response to poly(I:C) at early or transitional phases do not retain this poly(I:C)-specificity at later stages. For example, genes that are "dsRNA-specific" at the transitional phase of DC maturation converge to being commonly regulated by LPS and poly(I:C) at the mature phase. This may demonstrate downstream integration of signalling pathways of TLR3 and TLR4. Alternatively, initial differences between regulated genes at the early stage in response to LPS and poly(I:C) may reflect different signalling strengths of the doses of stimuli used, where LPS may be a more "robust" initial stimulus for its receptor, and poly(I:C) a "weaker" initial stimulus. Early transcriptional responses are therefore more marked for LPS-stimulated DCs compared to poly(I:C)-stimulated DCs. However, the number of genes significantly regulated by the two stimuli at later transitional and mature stages are comparable between LPS and poly(I:C) (Figure 4.1A).

DC transcriptional responses specific to dsRNA are further explored later in this chapter in comparison with DC responses to viral antigens. As the motif of dsRNA is believed to be plentiful in RNA virus replication, transcriptional responses of DCs to dsRNA serve as a useful comparison between DC responses to viruses.

These results demonstrate that at the level of simple single-component antigens, dendritic cells are transcriptionally plastic in their responses. However, presumably because of the cross-talk and commonality in the signalling pathways of TLR3 and TLR4, DC responses to dsRNA and LPS are largely similar, and the differences observed are subtle and difficult to interpret. In order to view more substantial and biologically relevant transcriptional regulation, we decided to use more complex antigens in the form of whole virus to investigate dendritic cell plasticity.

Although only sustained differential responses (over more than one maturation stage) are investigated here, transient changes affected by LPS and dsRNA were seen in the DC activation and differentiaton response, in particular the rapid induction of pro-inflammatory cytokines that return to basal levels. Therefore, differential LPS- and poly(I:C)-induced transient changes also merit further investigation.

4.3 Establishing a method for detecting viral infection in DCs

4.3.1 Intracellular staining for cathepsin E

In order to compare between DC responses to different viruses, it was necessary to standardise methods for measuring the actual number of DCs exposed to a give virus titre. This is to ensure that DCs are exposed to a high multiplicity of infection (MOI), thereby synchronising the DC population response. In the case of Influenza, which is able to replicate in DCs, a high MOI will also help ensure that replication is synchronous. This is an important consideration, as the effects of virus spreading and downstream secondary effects will increase variation and hinder interpretation of DC responses to replicating virus. For inactivated viruses, comparisons between DC responses are only valid if DCs exposed to different virus treatments "see" comparable amounts of virus particles.

A robust and reliable method was developed to monitor for the presence of viral proteins inside infected dendritic cells, by intracellular staining for viral antigens and flow cytometry for quantification. Cathepsin E was used as the intracellular antigen in DCs to establish and standardise the technique. Cathepsin E staining was confirmed for permeabilised cells by flow cytometry, with positive staining greatly reduced for non-permeabilised cells (Figure 4.2). This is seen as the high median fluorescence intensity (MFI) for cathepsin E in permeablised DCs (intracellular), and low background MFI levels of cathepsin E in non-permeabilised DCs (extracellular).

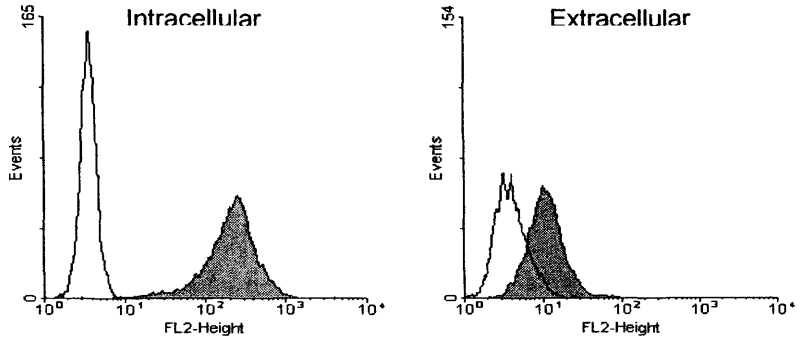


Figure 4.2 Cathepsin E staining in dendritic cells

DCs were stained for cathepsin E, in the presence (intracellular) or absence (extracellular) of prior permeabilisation. Shaded histogram show DCs stained with cathepsin E, black histogram show staining with isotype IgM.

4.3.2 Detection of Influenza nucleoprotein in Influenza-infected DCs

Influenza nucleoprotein (NP) is a viral protein that is necessary to encapsidate the virus genome for RNA transcription, replication and packaging, and is produced in infected cells during active viral replication. NP-encapsidated virus genome forms a ribonucleoprotein (RNP) complex, in which segments of genomic RNA are bound to stoichiometric quantities of NP monomers. Having established a method for detecting intracellular antigen in DCs, Influenza infection in DCs could be monitored by staining for Influenza NP. The proportion of cells positive for NP staining should correlate with the titre of Influenza virus, showing the proportion of DCs infected with Influenza.

Human Influenza virus A/Puerto Rico/8/34 (A/PR/8/34, H1N1) was a gift from Dr W Barclay (University of Reading, UK). This virus was grown in Madin-Darby canine kidney (MDCK) cells, and sucrose gradient density purified. Virus stocks, in aliquots of 100μl, were supplied at titres of 10^9 plaque-forming units (pfu)/ml. Plaque assays were carried out in confluent monolayers of MDCK cells.

Infection of DCs with different Influenza MOI (as determined by MDCK titre) resulted in a titration of the percentage of cells which stained positive for NP (Figure 4.3). At a MOI of 0.3, approximately 75% of DCs are positive for NP; at MOI of 1 and 3, over 95% of cells are positive for NP, as determined by flow cytometry. From this data, it appears that the infectious dose of Influenza as determined on MDCK cells underestimates the infectious dose for DCs. A true MOI of 1 would give approximately 68% infected cells as calculated by the formula below, based on the Poisson distribution.

$$P(n) = \frac{m^n \bullet e^{-m}}{n!}$$

where:
m = multiplicity of infection (MOI)
n = number of virus infecting a cell
P(n) = probability cell will be infected with n virus

This suggests that MOIs as determined on MDCK cells may actually correspond to higher MOIs in DCs, and that DCs may be more susceptible to Influenza infection than

MDCK cells. For clarity the stated MOI corresponding to the MDCK virus titre determination is used.

DCs infected with Influenza at a MOI of 1 showed two DC populations with different NP levels. At a MOI of 3, the levels at which NP was expressed in DCs were higher, and showed a more uniform population of NP-positive staining. This is likely to be a direct reflection of multiple infections of Influenza at this high MOI. Though it is not known how many virus particles a DC will encounter *in vivo*, an average of 5 infectious virus particles per DC results from a MOI of 3 as calculated by the Poisson distribution. Although this is likely to be an underestimate, this nevertheless seemed a reasonable exposure level, and this was therefore chosen as the MOI for further work with Influenza infection of DCs.

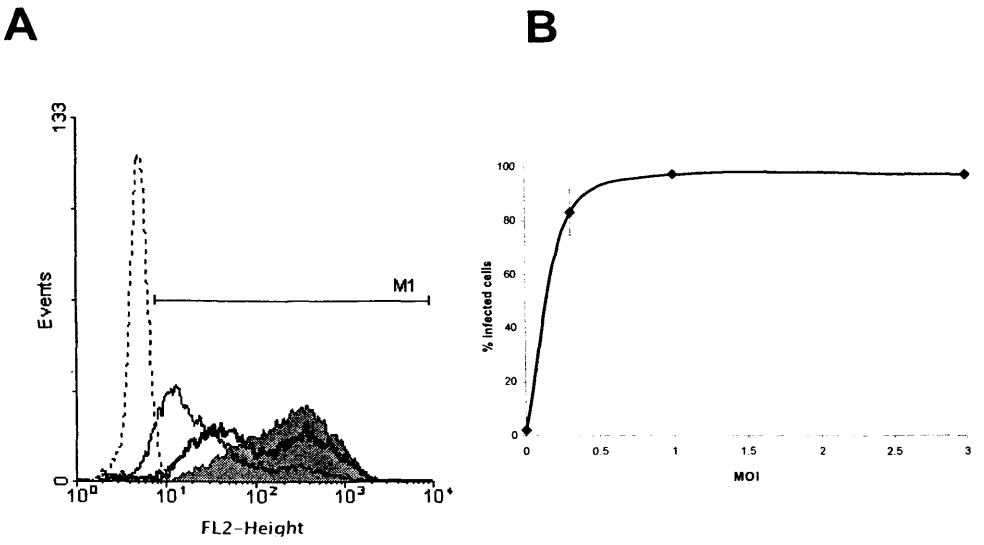


Figure 4.3 Influenza nucleoprotein staining in Influenza-infected DCs

A DCs were infected with Influenza virus at a range of MOIs. After 1 hour for virus adherence, DCs were washed, and cultured in complete medium with GM-CSF and IL-4 for a further 18 hours. Infected DCs were harvested, permeabilised, and stained for Influenza nucleoprotein (NP). NP expression was assessed by flow cytometry. M1 indicates the region of positive fluorescence intensity, which includes 2% of events counted for negative isotype control IgG stained DCs. Shaded histogram MOI=3, thick black histogram MOI=1, unfilled lack histogram MOI=0.3, dotted histogram isotype control.
B Relationship of median fluorescence intensity and MOI from flow cytometric analysis of MOI titration of Influenza infection of DC (n=3).

4.3.3 UV-inactivation of Influenza

To compare the effects on DCs between those mediated by initial DC-virus interactions at the cell surface and those of active virus replication, Influenza virus replication was blocked. Influenza was inactivated by exposure to UV-light, which results in cross-linking of viral nucleic acids and virus inactivation. Direct exposure of the virus to short wave UV resulted in inactivation of the virus, as confirmed by the abrogation of NP expression in DCs exposed to inactivated Influenza. This was seen for a range of virus exposure times to UV, from 30 seconds to 10 minutes, on an initial MOI of 1. UV exposure for 2 minutes was chosen to be effective for Influenza inactivation, with inactivated Influenza exposed-DCs having the same NP background staining as uninfected DCs (Figure 4.4).

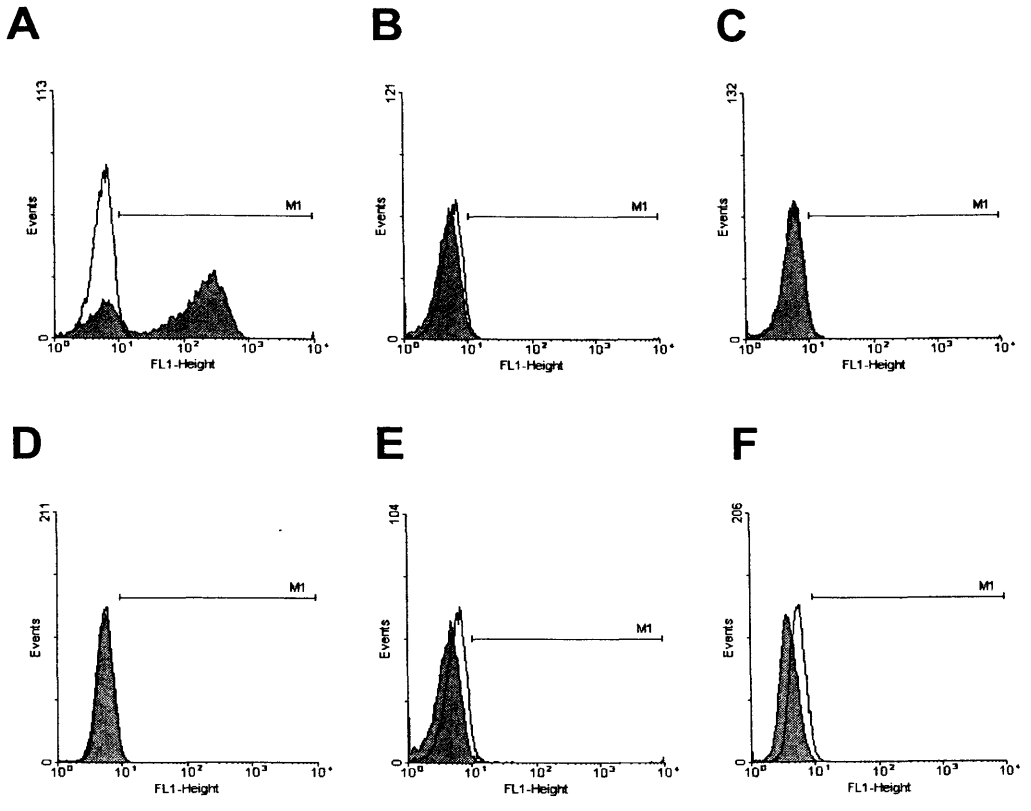


Figure 4.4 Intracellular staining for Influenza NP after different UV-exposure times

DCs were infected with Influenza, washed after 1 hour for viral adherence, and further cultured for 18 hours in complete medium with GM-CSF and IL-4. DCs were harvested, permeablised, and stained for Influenza NP. Shaded histogram shows NP staining in infected cells, unfilled black histogram shows NP staining in uninfected cells.
A DCs infected with Influenza MOI=1, **B** DCs exposed to UV-inactivated (30 seconds) Influenza, **C** DCs exposed to UV-inactivated (1 minute) Influenza, **D** DCs exposed to UV-inactivated (2 minutes) Influenza, **E** DCs exposed to UV-inactivated (5 minutes) Influenza, **F** DCs exposed to UV-inactivated (10 minutes) Influenza.

4.4 Rhinovirus interaction with DCs

4.4.1 Detection of Rhinovirus entry receptor on DCs

Human Rhinovirus serotype 16 (HRV16) was a gift from Dr T Tuthill and Prof D J Rowlands (University of Leeds, UK). This virus was grown in HeLa cells (Ohio strain, which have increased surface expression of ICAM1), and sucrose gradient density purified. Virus stocks in aliquots of 50µl were supplied at titres of 3×10^9 plaque-forming units (pfu)/ml. Plaque assays were carried out in confluent monolayers of Ohio HeLa cells.

Major group Rhinoviruses utilise the intercellular adhesion molecule ICAM1 (CD54) as a receptor for entry into cells (Greve et al., 1989; Staunton et al., 1989). As there were no reports of Rhinovirus establishing a productive infection in DCs, the presence of the HRV16 entry receptor on DCs was first confirmed. Flow cytometric analysis using an antibody to ICAM1 clearly shows the presence of this molecule on the surface of DCs (Figure 4.5). This suggests that HRV16 will be able to bind and possibly infect DCs.

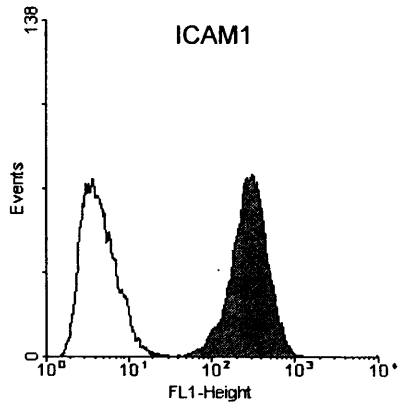


Figure 4.5 Extracellular staining of ICAM1 on immature DCs

Immature DCs were harvested and stained for extracellular ICAM1 (CD54). Shaded histogram show DCs stained for ICAM1, black unfilled histogram show DCs stained for isotype control IgG_1.

4.4.2 Detection of Rhinovirus particles in Rhinovirus-exposed DCs

The rhinovirus genome is a single positive-strand RNA genome. Once the viral RNA enters the cell cytoplasm, it is translated to provide viral proteins essential for genome replication and the production of new virus particles. Using the same method for intracellular staining as with Influenza-infected DCs, polyclonal sera to whole Rhinovirus was used to detect intracellular Rhinovirus particles in DCs exposed to live Rhinovirus. However, Rhinovirus particles were not detected in permeabilised DCs exposed to live Rhinovirus (Figure 4.6).

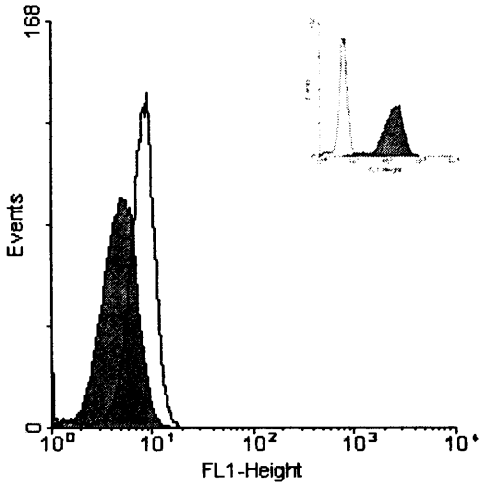


Figure 4.6 Intracellular staining for Rhinovirus particles inside Rhinovirus-exposed DCs

DCs were exposed to HRV16 at MOI of 10. Virus adherence was carried out for 1 hour, the cells were washed, and cultured in complete medium with GM-CSF and IL-4 for a further 24 hours. These DCs were harvested, permeabilised, and stained for whole Rhinovirus particles. Shaded histogram show cells stained with anti-HRV16 antiserum, black unfilled histogram show cells stained with control guinea pig antiserum. **Inset**, the same cells staining for cathepsin E, to demonstrate effective permeabilisation.

This suggests that DCs do not support active Rhinovirus replication, as whole Rhinovirus particles are not present in Rhinovirus-exposed DCs. Alternatively, the lack of a positive control means that it remains possible that the intracellular staining method was not functional for measuring HRV particles in DCs using this particular antisera. However, other groups have also reported the lack of Rhinovirus infection of monocytes and airway macrophages (Gern et al., 1996a) and dendritic cells (Kirchberger et al., 2003).

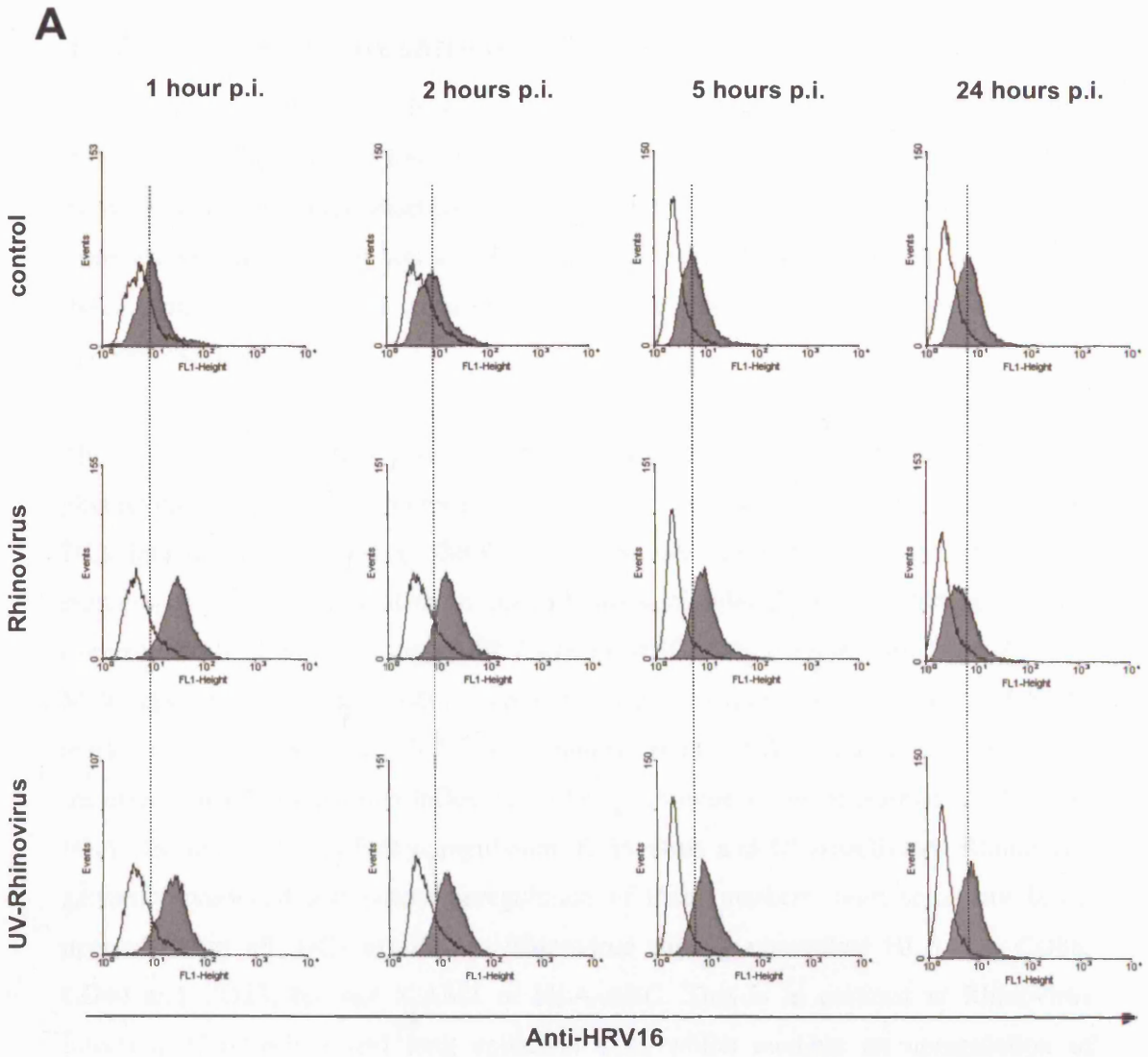
4.4.3 Detection of bound Rhinovirus particles on surface of DCs

To determine if Rhinovirus attached to DCs via its entry receptor ICAM1, extracellular staining for bound Rhinovirus particles to DCs was carried out using the same polyclonal antisera to whole Rhinovirus particles. After 1 hour of viral adherence, DCs were washed vigorously, stained with the antisera for Rhinovirus particles, and analysed by flow cytometry. The presence of bound Rhinovirus particles was monitored following washing and replating at an additional three timepoints (Figure 4.7). Rhinovirus particles clearly bound to the surface of DCs one hour after virus adherence, and this extracellular staining was shown to slowly decrease over time. This suggests that Rhinovirus particles are able to bind their entry receptor ICAM1 on the surface of DCs, and possibly mediate entry by triggering endocytosis. The same pattern of extracellular virus binding and decreased staining over time was also found for DCs exposed to UV-inactivated Rhinovirus (Figure 4.7), showing that the UV-inactivation procedure does not alter the virus' ability to bind ICAM1. The UV-inactivation procedure is the same as that for Influenza, namely 2 minutes direct exposure to UV-radiation. UV-inactivation of Rhinovirus was effective at reducing the infectious titre by more than 6 logs, from 10^9 pfu/ml to less than 10^3 pfu/ml, resulting in no plaque formation on Ohio HeLa cells (10^3 pfu/ml was the detection limit of the plaque assay). The plaque assays were carried out by Dr T Tuthill.

Figure 4.7 Timecourse of binding of Rhinovirus particles to surface of DCs (next page)

A DCs were exposed to Rhinovirus, inactivated Rhinovirus, or mock-stimulated (control). After 1 hour for viral adherence, cells were washed, and were either replated in complete medium with GM-CSF and IL-4 for indicated times (2 hours, 5 hours, and 24 hours after initial virus exposure), or stained for Rhinovirus particles with polyclonal antisera to whole virus. The same staining procedure was carried out at later timepoints. Shaded histogram show cells stained with anti-Rhinovirus antiserum, black unfilled histogram show cells stained with control guinea pig serum.

B Relationship between median fluorescence intensity (MFI) and time post-infection in cells exposed to live Rhinovirus (grey ■), UV-inactivated Rhinovirus (purple ▲), and control mock-stimulated cells (blue ♦), stained with anti-Rhinovirus antiserum.


A
1 hour p.i.
2 hours p.i.
5 hours p.i.
24 hours p.i.
control
Rhinovirus
UV-Rhinovirus
Events
FL1-Height
Anti-HRV16
B
uninfected
HRV16 MOI=10
UV-HRV16 MOI=10
MFI
time post infection (hours)
0
5
10
15
20
25
30
35

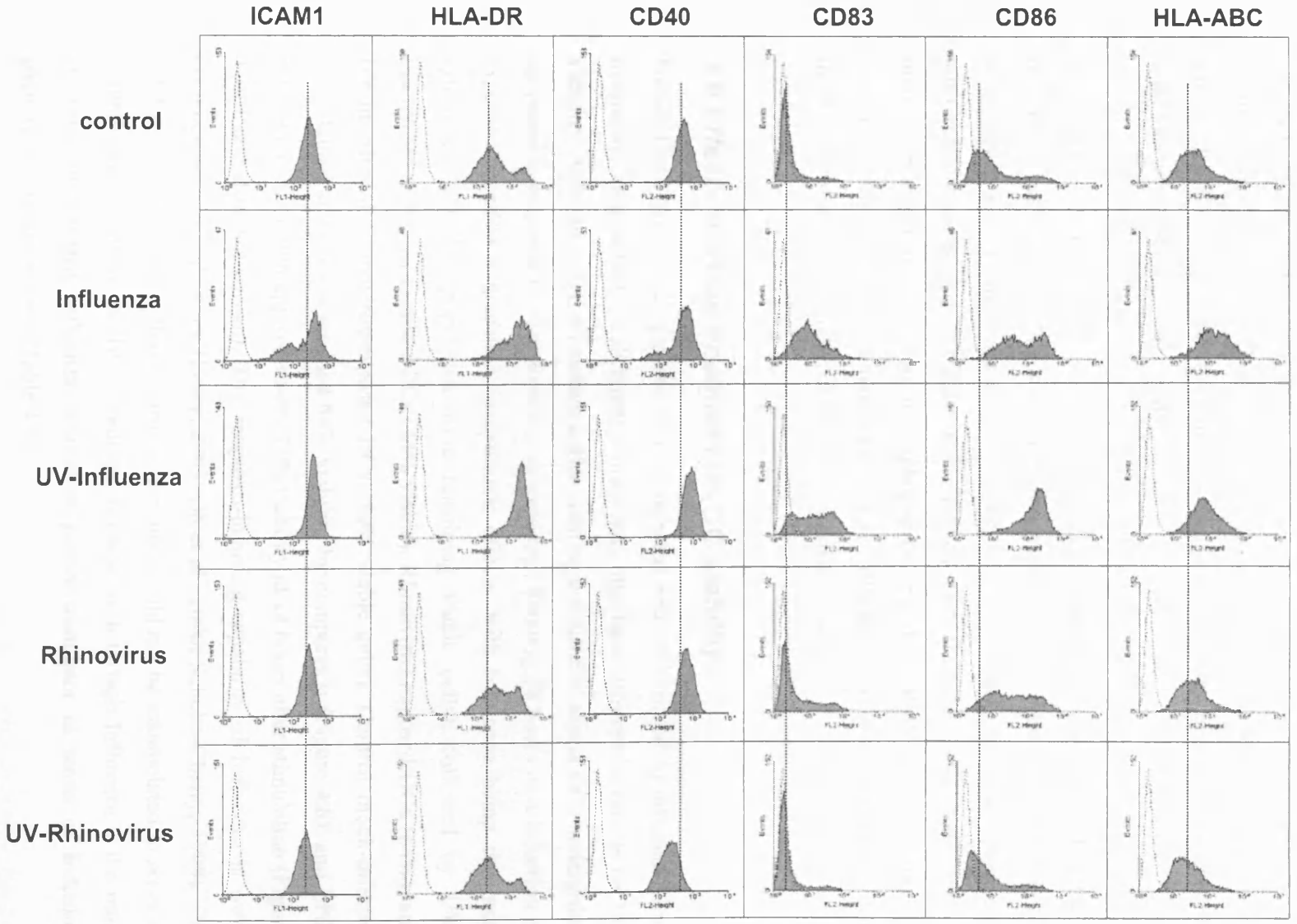
4.5 Effect of virus treatment on DC phenotype

The effects of the different virus exposures (live Influenza, inactivated Influenza, live Rhinovirus and inactivated Rhinovirus) on dendritic cell phenotype in terms of surface expression of maturation markers HLA-DR, HLA-ABC, CD86, CD40, and ICAM1, were investigated. Rhinovirus exposure of DCs at MOI of 10 was chosen, as this induced phenotypic changes consistent with DC maturation, without compromising DC viability (Section 4.6).

The exposure or infection with the different virus treatments resulted in differential phenotypic changes in DCs in terms of cell surface expression of a range of molecules. DCs infected with Influenza (MOI = 3) generally upregulated all the phenotypic markers of maturation relative to control mock-stimulated DCs, including ICAM1 (interacts with LFA-1 on T cells), HLA-DR (a MHC class II molecule), HLA-ABC (a MHC class I molecule), CD40 (receptor for CD40L expressed on T cells), CD83 (a marker of mature DCs), and CD86 (a co-stimulatory molecule). Compared to Influenza, the effects of UV-inactivated Influenza on DC phenotype are more marked for ICAM1, HLA-DR and CD86 surface upregulation. Rhinovirus and UV-inactivated Rhinovirus generally produced less potent upregulation of these markers, with some not being upregulated at all. DCs exposed to Rhinovirus slightly upregulate HLA-DR, CD86, CD40 and CD83, but not ICAM1 or HLA-ABC. This is in contrast to Rhinovirus infection of bronchial and lung epithelial cells which mediate an upregulation of ICAM1 surface expression (Papi and Johnston, 1999). DCs exposed to UV-inactivated Rhinovirus do not seem to upregulate any markers. To summarise, DCs infected with Influenza and DCs exposed to inactivated Influenza seem to undergo the most dramatic phenotypic changes, whereas DCs exposed to Rhinovirus undergo a less dramatic "maturation" response with slight upregulation of HLA-DR and CD86. DCs exposed to inactivated Rhinovirus do not seem to undergo any phenotypic changes at all, relative to control mock-stimulated DCs.

Figure 4.8 Immunophenotype of DCs treated with the different viruses (next page)

DCs were infected or exposed to the indicated viruses or mock-stimulated (control), and after 1 hour for virus adherence, the cells were washed and replated in complete medium with GM-CSF and IL-4. After 24 hours, DCs were harvested, and stained for the indicated markers. Grey shaded histograms show cells stained with the indicated markers, unshaded histograms show cells stained with the corresponding isotype controls.


ICAM1
HLA-DR
CD40
CD83
CD86
HLA-ABC
control
Influenza
UV-Influenza
Rhinovirus
UV-Rhinovirus

Influenza-infected DCs have been shown to be potent stimulators of T cells, inducing strong proliferative and cytolytic responses from CTLs (Bhardwaj et al., 1994). This has also been shown for DCs exposed to UV-inactivated Influenza, which can also induce strong Influenza-specific CTL responses (Bender et al., 1995). This indicates that *de novo* synthesis of viral proteins due to viral replication is not necessary to charge MHC class I molecules to elicit CTL responses.

By contrast, Rhinovirus has not been shown to be able to productively infect DCs, despite the expression of Rhinovirus entry receptor ICAM1 on these cells (Kirchberger et al., 2003). HRV16 has been shown to enter monocytes and airway macrophages *in vitro* and activate these cells without active replication of the virus (Gern et al., 1996a), inducing non-specific activation of lymphocytes (Gern et al., 1996c) and inhibition of antigen-specific T cell proliferation (Gern et al., 1996b; Stockl et al., 1999). To date there are no studies of Rhinovirus effects on dendritic cells.

4.6 Effects of virus treatment on DC viability

Forward and side scatter profiles of DCs infected with or exposed to different virus treatments suggest that the different viruses may also have differential effects on DC viability. Annexin V and propidium iodide staining was used to assess DCs undergoing apoptosis in response to virus infection or exposure. Staining 18 hours post-infection or exposure revealed a gradation in cytotoxic effects, with Influenza being the most cytotoxic (23% DC population were remaining viable cells), followed by UV-inactivated Influenza-exposed DCs (44% viable), Rhinovirus-exposed DCs (64%), and UV-inactivated Rhinovirus-exposed DCs (80% viable cells). Control mock-infected cells stained at 18 hours retained 84% viability by comparison (Figure 4.8), and LPS-stimulated DCs retain approximately 75% viability at 18 hours after stimulation (Figure 3.30B and data not shown). The apoptosis-inducing capability of Influenza is well-documented in the literature (Lowy, 2003; Oh et al., 2000; Schultz-Cherry, 1998). The cytotoxic effects of the different virus treatments should not be extrapolated to serve as an indicator for effects on DC phenotype, because even though Influenza is the most cytotoxic, inactivated Influenza is a more potent stimulator in terms of inducing phenotypic changes in DCs (Table 4.1).

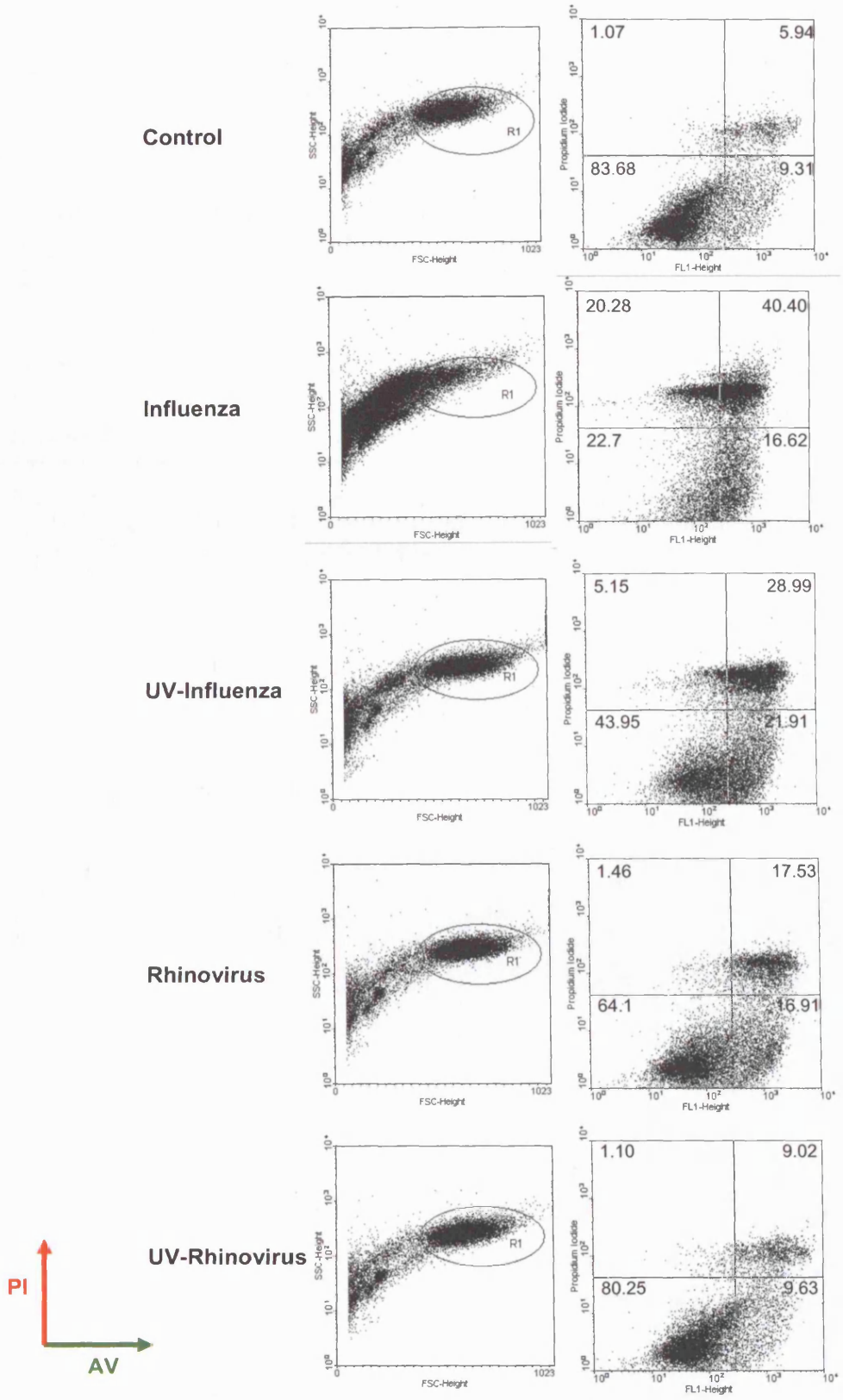


Figure 4.9 Viability of mock-infected and virus-treated DCs

DCs were infected with or exposed to different virus treatments, or mock-stimulated (control). Following 1 hour viral adherence, cells were washed and replated in complete medium with GM-CSF and IL-4. DCs were harvested 18 hours post-infection for Annexin V / Propidium iodide staining. The numbers in the quadrants represent percentage of the gated cell population (R1).

Table 4.1 Summary of effects of virus treatments on DC viability and phenotype†

	Control	*Influenza*	*Inactivated Influenza*	*Rhinovirus*	*Inactivated Rhinovirus*
Viability	84%	23%	44%	64%	80%
ICAM1	-	+	+	-	-
HLA-DR	-	++	+++	+	-
CD40	-	+	++	+	-
CD83	-	++	+++	+	-
CD86	-	++	+++	+	-
HLA-ABC	-	++	+	-	-

†Qualitative summary of the effects of virus treatment on DC phenotype, relative to control mock-stimulated DCs (pictorially shown in Figure 4.8). Summary of data from phenotype and viability experiments performed in triplicates.

4.7 Gene expression analysis of DC response to Influenza and Rhinovirus

Analysis of dendritic cell transcriptional responses to core maturation-stimuli LPS and dsRNA has revealed a core activation and differentiation programme that comprise clearly defined transcriptional signatures corresponding to three phases of DC maturation. Though DC transcriptional responses to LPS and dsRNA are largely similar, there are some differences that are stimulus-specific, suggesting functional plasticity of DC responses to antigens. This plasticity has been suggested for DC responses to three distinct pathogens: bacteria (*E. coli*), virus (Influenza) and yeast (*C. albicans*) (Huang et al., 2001). However, Huang's study did not differentiate between responses regulated by DCs to virus exposure and virus replication. This is an important distinction, as virus replication events invariably cause transcriptional changes in host cells, and not controlling for virus replication-induced responses will lead to incorrect identification of genes that are regulated by DCs in response to virus stimulation. Influenza virus infection and exposure results in differential effects in DCs in terms of surface expression of classical markers (Table 4.1). This differential effect was further investigated on the transcriptional level, using gene expression arrays to monitor DC responses to virus treatments over time. As inactivated Rhinovirus exposure did not affect a phenotypic response in DCs, array experiments were not performed for DCs exposed to inactivated Rhinovirus at this time.

4.7.1 Sample preparation

In total 3 timcourses were performed, one for each virus condition (Influenza, inactivated Influenza, Rhinovirus, Table 4.2). Total RNA was extracted as before, DNase treated, quantified, mRNA purified and reverse transcribed to cDNA labelled with Cy5. The same Cy3-labelled common reference used for LPS- and poly(I:C)-stimulated DC arrays (Section 3.2.2) was used for virus-stimulated DC arrays. The Cy5- and Cy3-labelled cDNA was mixed in equal amounts, and hybridised to HGMP (version 1 or version 2) cDNA arrays.

Table 4.2 DC timecourse experiments of virus infection and virus exposure

Buffy coat	*Stimulus*	*Timepoints*	*HGMP array*	*Reference batch*
4	Influenza	0, 2, 5, 10, 18, 24h	Human cDNA array version 1	3
5	Inactivated Influenza	2, 5, 10, 18, 24h	Human cDNA array version 1	3
6	Rhinovirus	0, 2, 5, 10, 18, 24h	Human cDNA arrav version 2	3
7	Inactivated Influenza	0h	Human cDNA arrav version 2	3

4.7.2 Data extraction

Scanned array images from GenePix Pro were extracted and processed as in Materials and Methods (Section 2.4.5). Low signal to noise ratio (SNR) data was filtered as described in Section 3.2.3. Genes in each independent array was filtered using the averaged signal-to-noise ratios of defined negative control genes as the SNR cut-off. A total of 18 arrays comprised the timecourse of dendritic cell responses to Influenza, inactivated Influenza, and Rhinovirus.

4.7.3 Cluster analysis of DC responses to Influenza and Rhinovirus

Hierarchical clustering was used as an exploratory data analysis method to assess the similarity in gene expression of dendritic cells infected or exposed to Influenza and Rhinovirus. As UV-inactivated Rhinovirus did not induce any marked phenotypic changes in DCs, array experiments were not performed at this time, although it would be interesting to examine transcription responses of DCs to inactivated Rhinovirus. The 18 virus-DC array experiments were clustered together with the 9 arrays of DC responses to poly(I:C). This was used to provide a means for comparison between DC responses to different virus treatments and the core maturation program (Chapter 3). In addition, this allowed comparison of virus-induced processes that may involve DC responses to dsRNA produced during the replication of Influenza or present as secondary structures within the virus genomes. The $\log_2$ expression ratios of the combined 27 arrays were compiled, filtered and normalised using Cluster (Eisen et al., 1998) (Section 3.2.4). In total, 1713 genes passed the SNR filtering criteria in all 27 array experiments. Following construction of a one-dimensional self-organising map for the genes, hierarchical clustering produced the sample cluster dendogram in Figure 4.10.

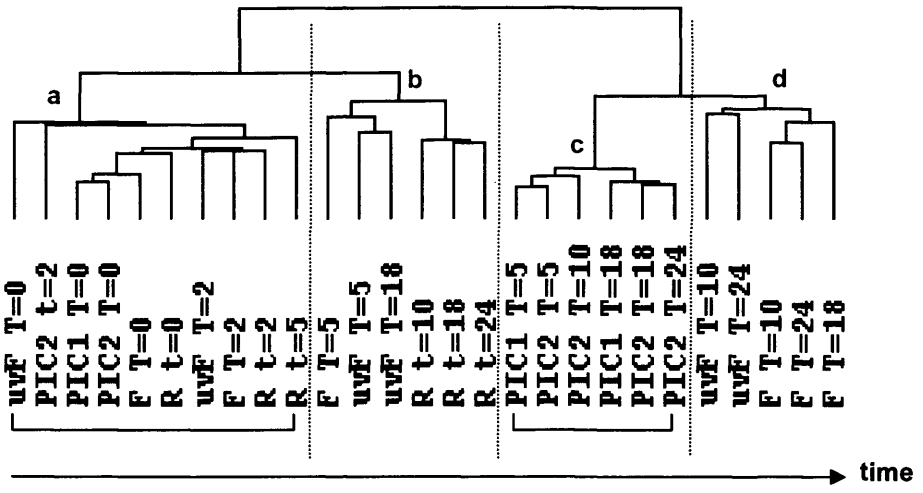


Figure 4.10 Cluster dendogram of virus and poly(I:C) arrays

Hierarchical clustering of 9 poly(I:C)-stimulated DC arrays (PIC1 and PIC2), 6 Influenza-infected DC arrays (F), 6 UV-inactivated Influenza-exposed DC arrays (uvF), and 6 Rhinovirus-exposed DC arrays (R), over a 24-hour timecourse generated this sample dendogram in Cluster. Letters refer to the major sample groups discussed further in the text below.

The cluster dendogram shows two main branches at the highest level (Figure 4.10, branches **a** and **b** separated from branches **c** and **d**). These branches discriminated between DCs exposed to all viruses at early timepoints and the complete Rhinovirus timecourse, and DCs responding to poly(I:C) and DCs responding to UV-inactivated Influenza and Influenza at later timepoints. We have shown that DCs respond to LPS and poly(I:C) in a time-dependent manner, suggesting that gene expression correlates well with DC activation and differentiation over time. DC responses to viruses are also broadly time-dependent, allowing an ordering of the arrays over time. However, clustering also reveals differential DC responses that are stimulus-specific.

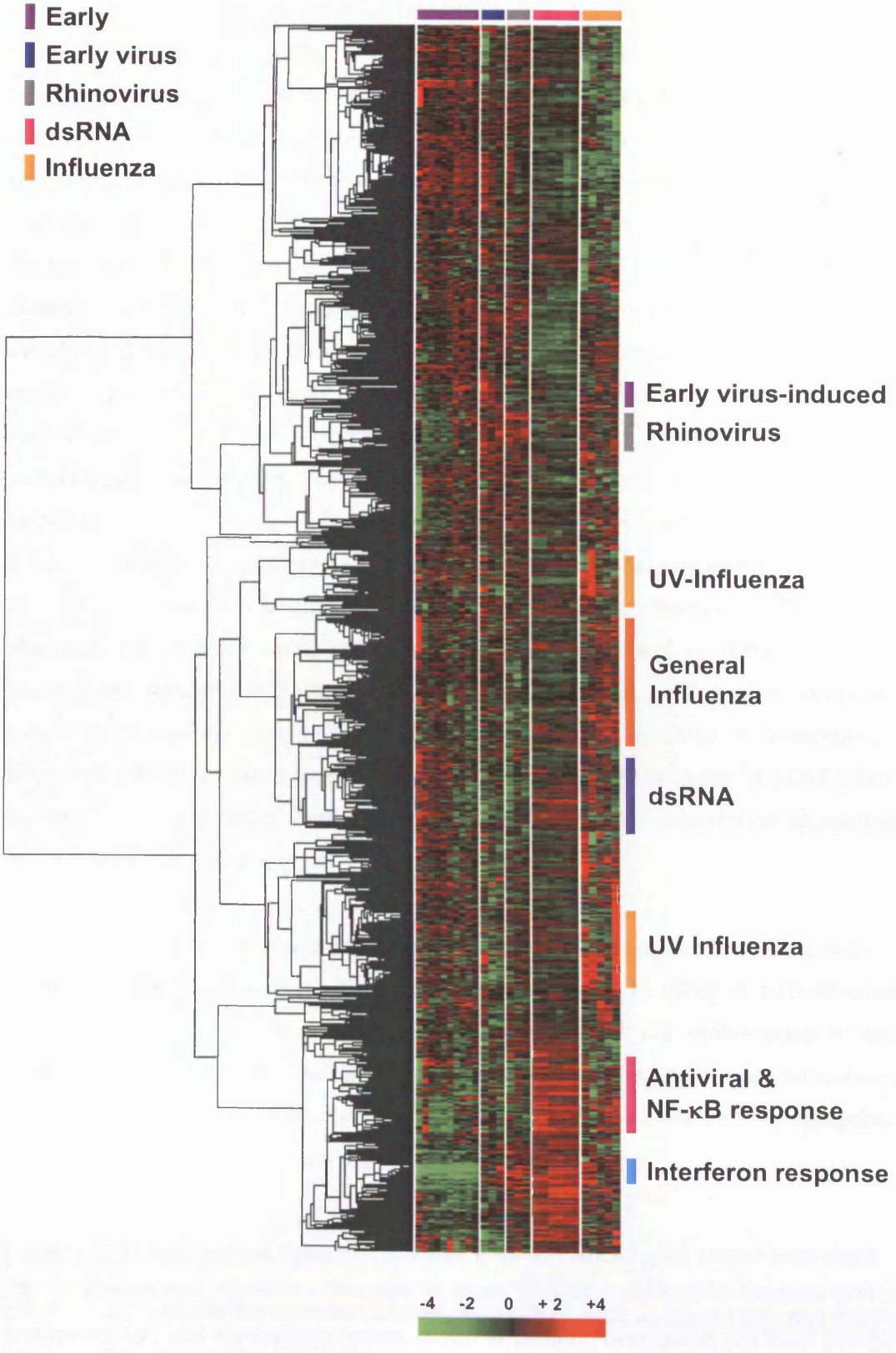
From the left (**a** in Figure 4.10), the first broad cluster groups together all DC arrays at early timepoints, 0 and 2 hours after stimulation with Influenza (F), UV-inactivated Influenza (uvF), Rhinovirus (R), and poly(I:C) (PIC). This group also contains the array of DCs exposed to Rhinovirus at 5 hours. This suggests that DC transcriptional activation by Rhinovirus is delayed relative to the other stimuli. The second cluster (**b**) groups together arrays of DCs infected with Influenza and exposed to UV-inactivated Influenza at 5 hours, and UV-inactivated Influenza at 18 hours, and also DCs exposed to Rhinovirus at 10, 18, and 24 hours. Again the gene expression profile of DC response to Rhinovirus is more similar to gene expression of DCs at early timepoints post-virus

infection or exposure, rather than at later timepoints. This further supports the fact that Rhinovirus exposure leads to delayed DC maturation. This correlates with the flow cytometry data, where Rhinovirus-induced DC surface upregulation HLA-DR and CD86 are not as great as those induced by Influenza and UV-inactivated Influenza. The presence of the array of DCs exposed to UV-inactivated Influenza at 18 hours in this group is difficult to explain, and is the only array to cluster aberrantly in the whole study of 45 arrays.

The next cluster (**c**) groups together arrays of DCs responding to poly(I:C), from 5 to 24 hours post exposure. This shows that DC responses to poly(I:C) are more similar to each other, across time, than to gene expression of DCs responding to the virus treatments. Together, this indicates that at later timepoints (10 to 24 hours after virus exposure or infection), differential responses of DCs to various stimuli are easily recognisable. This is further supported by the next cluster (**d**), that groups together later timepoints of DCs responding to UV-inactivated Influenza (10 and 24 hours) and DCs infected with Influenza (10, 18, and 24 hours). Even though these 5 arrays are grouped in one cluster, the responses to Influenza are distinct from the responses to UV-inactivated Influenza. Such grouping of Influenza and UV-inactivated Influenza DC arrays may also reflect transcriptional effects of apoptosis and cell death in DCs infected with Influenza and exposed to inactivated Influenza (Section 4.6, Table 4.1). In summary, we have shown that DCs can regulate differential transcriptional responses to virus stimuli, which contrasts and extends the general, stimulus-independent responses of DCs to LPS and poly(I:C) for maturation, activation and differentiation.

Figure 4.11 Hierarchical clustering of virus- and dsRNA-stimulated DC arrays (next page)

Genes in the 27 virus- and dsRNA-stimulated DC arrays were filtered for genes that passed the SNR filter in all 27 arrays. The resulting 1713 genes and 27 arrays were clustered together with hierarchical clustering. The dendrogram on the left represents the relationship between genes in terms of their gene expression patterns. Gene expression is shown as a pseudo-coloured representation of $\log_2$ expression ratio with red being above and green below the row/column median level of expression (set to 0) as shown by the scale. The coloured bars to the right mark the position of gene expression signatures which are particularly upregulated in DCs exposed to the indicated stimuli. These gene expression signatures are discussed in the following sections.


Early
Early virus
Rhinovirus
dsRNA
Influenza
Early virus-induced
Rhinovirus
UV-Influenza
General
Influenza
dsRNA
UV Influenza
Antiviral &
NF-κB response
Interferon response
-4
-2
0
+2
+4

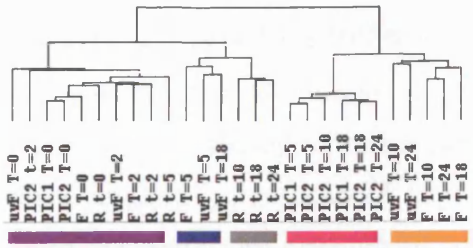
4.7.3.1 Early virus-induced responses

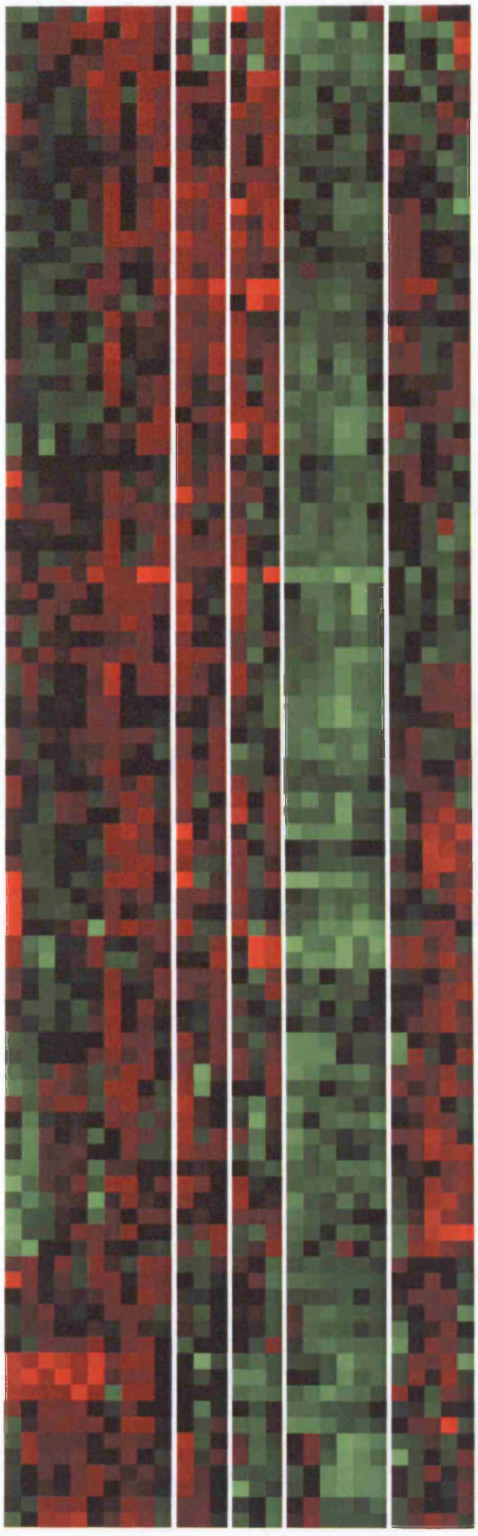
Genes involved in the early virus-induced response are upregulated between 2 and 5 hours after DC exposure to inactivated-Influenza, Rhinovirus, and infection with Influenza (Figure 4.11, 4.12). These genes are largely involved with intracellular transport: in the transport of vesicles (RAB5C, vesicle amine transport protein 1 homolog, hippocalcin-like 1, copine III), transport of proteins (endoplasmic reticulum glycoprotein, solute carrier family 25 and 16, KDEL endoplasmic reticulum protein retention receptor 1, exportin 1), transport of lipids (sterol carrier protein 2), and transport of ions that participate in intracellular signalling (voltage-dependent anion channel 1-3, ATPase, ATP synthase). There is also evidence of mobilisation of the actin cytoskeleton that facilitate these processes, reflected in the upregulation of genes that encode proteins that regulate the cytoskeleton and cellular motility (cofilin 1, calmodulin 3, integrin beta 4 binding protein, myosin, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein). Genes involved in signal transduction include phosphodiesterase, cAMP-dependent protein kinase A, RAB5C, protein phosphatase 2, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, and protein tyrosine phosphatase 11. This cluster also includes genes involved in nucleic acid metabolism and synthesis, including guanine monophosphate synthetase, dUTP pyrophosphatase, uridine monophosphate kinase, RNA binding proteins, DNA helicase, and genes involved in transcription, including activating transcription factor 7, TAF12 RNA polymerase II (TATA-box binding protein-associated factor), LIM domain binding 1, transcription elongation factor B, and SET translocation.

This cluster of genes, upregulated at early timepoints after exposure to viruses, but not by dsRNA, demonstrates a specific virus-induced response, resulting in a coordinated upregulation of genes involved in intracellular transport and mobilisation of the cytoskeleton. There is also upregulated genes involved in nucleic acid metabolism, suggesting early events downstream of virus entry stimulates the DCs to upregulate these genes to facilitate downstream virus replication.

Figure 4.12 Transcriptional signature of the early virus-induced gene cluster (next page)

This gene expression signature is enriched for genes involved in intracellular transport (genes shown in red), cytoskeleton regulation and cellular motility (genes shown in blue), and nucleic acid metabolism and transcription (genes shown in green). These genes are most strongly induced in DCs at early timepoints after exposure to virus.





CLU	Clusterin
MRPL3	Mitochondrial ribosomal protein L3
GMPS	Guanine monophosphate synthetase
FANCA	Fanconi anemia, completemtation group G
LAPTM5	Lysosomal-associated mutispanning membrane protein-5
CALM3	Calmodulin 3
CALM3	Calmodulin 3
ITGB4BP	Integrin beta 4 binding protein
MYL4	Myosin, light polypeptide 4, alkali; atrial, embryonic
KDELR1	KDEL endoplasmic reticulum (ER) protein retention receptor 1
LAPTM5	Lysosomal-associated multispanning membrane protein-5
CDC37	Cell division cycle 37 homolog
CDC37	Cell division cycle 37 homolog
HCS	Cytochrome c
TCEB1	Transcription elongation factor B
VDAC1	Voltage dependent anion channel 1
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
YWHAH	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
PPP2R1A	Protein phosphatase 2A regulatory subunit
ATF7	Activating transcription factor 7
CFL1	Cofilin 1
CFL1	Cofilin 1
G22P1	Thyroid autoantigen 70kD (Ku antigen)
VDAC2	Voltage dependent anion channel 2
HPCAL1	Hippocalcin-like 1
ALDOC	Aldolase C, fructose-bisphosphate
ALDOC	Aldolase C, fructose-bisphosphate
H04816	Putative membrane protein
SEP15	15kDa selenoprotein
SEP15	15kDa selenoprotein
NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2
VDAC3	Voltage dependent anion channel 3
SLC25A3	Solute carrier family 25 (mitochondrial)
SLC25A3	Solute carrier family 25 (mitochondrial)
RPL11	Ribosomal protein L11
RPL11	Ribosomal protein L11
RBM10	RNA binding motif protein 10
ACHE	Acetylcholinesterase (YT blood group)
ATP5F1	ATP synthase, H+ transporting, mitochondrial
VATI	Vesicle amine transport protein 1 homolog
VATI	Vesicle amine transport protein 1 homolog
PRKACA	Protein kinase A, catalytic subunit
PRKACA	Protein kinase A, catalytic subunit
eIF3S7	Eukaryotic translation initiation factor 3, subunit 7
eIF3S7	Eukaryotic translation initiation factor 3, subunit 7
RAB5C	Member of RAS oncogene family
GP36B	Endoplasmic reticulum glycoprotein
KIAA0530	
KIAA0530	
FKBP4	FK506 binding protein 4, 59kDa
PCTK1	PCTAIRE protein kinase 1
KIAA0606	
FKBP4	FK506 binding protein 4, 59kDa
eIF4H	Eukaryotic translation initiation factor 4
eIF4H	Eukaryotic translation initiation factor 4
CD37	CD37 antigen
ST14	Suppressor of tumorigenicity 14
ST14	Suppressor of tumorigenicity 14
G22P1	Thyroid autoantigen 70kD (Ku antigen)
VDAC2	Voltage dependent anion channel 2
SUPT5H	Suppressor of Ty5 homolog (S cerevisiae)
SUPT5H	Suppressor of Ty5 homolog (S cerevisiae)
SET	SET translocation (myeloid leukemia-associated)
SET	SET translocation (myeloid leukemia-associated)
UMPK	Uridine monophosphate kinase
SACS	Spastic ataxia of Charlevoix-Saguenay (sacsin)
SLC16A1	Solute carrier family 16 (monocarboxylic acid transporters)
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
COMMD3	COMM domain containing 3
COMMD3	COMM domain containing 3
NDUFA2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 2
SACS	Spastic ataxia of Charlevoix-Saguenay (sacsin)
SHMT1	Serine hydroxymethyltransferase 1 (soluble)
MDC1	Mediator of DNA damage checkpoint 1
UMPK	Uridine monophosphate kinase
XPO1	Exportin 1 (CRM1, yeast, homolog)
ATP6V0A1	Vacuolar ATPase
ATP6V0A1	Vacuolar ATPase
SEC6	Similar to S. cerevisiae Sec6p
ARHGEF1	Rho guanine nucleotide exchange factor (GEF) 1
ARHGEF1	Rho guanine nucleotide exchange factor (GEF) 1
EBNA1BP2	Nucleolar protein p40
PDE2A	Phosphodiesterase 2A
PDE2A	Phosphodiesterase 2A
EphB1	EPH receptor B1
TAF12	TATA box binding protein (TBP)-associated factor
LDB1	LIM domain binding 1
DAP	Death-associated protein
DAP	Death-associated protein
ATP6AP2	ATPase, H+ transporting, lysosomal
ATP6AP2	ATPase, H+ transporting, lysosomal
SCP2	Sterol carrier protein 2
CPNE3	Copine III

4.7.3.2 Antiviral response

The induction of this cluster of genes is a common response in DCs to dsRNA, Rhinovirus, inactivated Influenza and live Influenza (Figure 4.13). We define this here as the core antiviral response. A large number of genes upregulated in response to virus treatments were also upregulated in DCs as part of the core maturation response (Section 3.2.8). These genes include members of the STAT family of transcription factors (STAT1, STAT3, STAT5A), IRF-2, immunoproteasome subunits, IFITM1 and IFITM2, MyD88, OAS1, CD40, BIRC2, BAK1, CFLAR, SEMA4D, and metallothioneins (MT2A, MT1L, MT3). A highly correlated sub-cluster (correlation coefficient 0.94) comprise a subset of 5 genes in this antiviral response (indicated in Figure 4.13). The 5 genes, IFITM1 and IFITM2, insulin-like growth factor 1 receptor (IGF1R), ubiquitin conjugating enzyme E2L 6 (UBE2L6), and apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3B (APOBEC3B) are strongly co-expressed. While IFITM1 and IFITM2 are integral membrane proteins induced by alpha and gamma interferons, and IGF1R is induced in response to mitogenic stimuli, the inclusion of APOBEC3B and UBE2L6 in this cluster suggests their role in antiviral immunity.

APOBEC3B is a member of the apolipoprotein B (ApoB) mRNA editing protein family. The prototype member APOBEC1 (for "apoB editing catalytic subunit 1") functions as a cytidine deaminase by catalysing the conversion of cytidines to uracils. DNA deamination in the host is targeted to endogenous immunoglobulin loci to generate antibody diversity in the adaptive immune system. However, several recent papers have shown that one mechanism of innate immunity to retroviruses is achieved by direct deamination of deoxycytidine to deoxyuracil in retroviral first (-)strand cDNA, resulting in guanine to adenine hypermutation of the (+)sense viral DNA, and leading to inactivation of viral functions (Harris et al., 2003; Mangeat et al., 2003; Zhang et al., 2003a). The enzyme APOBEC3G is packaged in virions and, during reverse transcription, deaminates viral cDNA. APOBEC3G has been shown to have a role in determining HIV-1 infectivity in human and primate cell lines (Sheehy et al., 2002; Shindo et al., 2003). Most lentiviruses encounter the antiviral effects of APOBEC3G by virally encoded *vif*, which targets APOBEC3G for ubiquitin-mediated proteasomal degradation, thereby preventing its incorporation into virus particles (Yu et al., 2003). There is also evidence that APOBEC3G can edit DNA and affect HBV infectivity

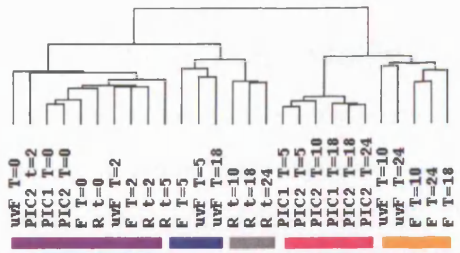
(Turelli et al., 2004). Another member of the family, APOBEC3F, has also been shown to deaminate deoxycytidine to deoxyuracil during HIV (-)strand cDNA synthesis, thereby causing guanine to adenine mutations in the viral genome (Zheng et al., 2004). However, APOBEC3B has not been characterised to have immune functions. It is attractive to speculate that the induction of APOBEC3B in DCs in response to Influenza, Rhinovirus and dsRNA reflects an antiviral role for APOBEC3B that represents an interferon-inducible component of innate antiviral defense, and as such, the function of members of the APOBEC family are a general antiviral mechanism in addition to defense against retroviruses.

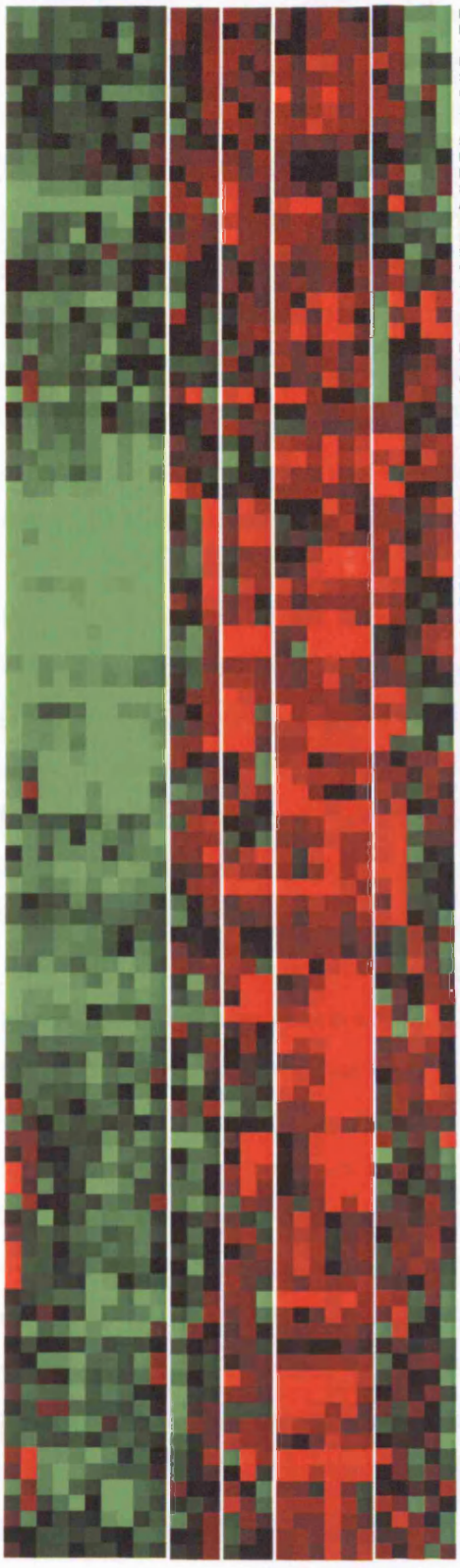
The presence of UBE2L6 in this cluster also suggests an interferon-induced antiviral role. Ubiquitination is a process that is integral to protein degradation, and involves three classes of enzymes: E1 ubiquitin-*activating* enzymes, E2 ubiquitin-*conjugating* enzymes, and E3 ubiquitin protein *ligases*. Protein degradation involves covalent attachment of the protein to ubiquitin, which is a three-step process. Ubiquitin is first activated on the C-terminal glycine by E1. Activated ubiquitin is then transferred by E2 to a member of the E3 family, to which the protein is bound. E3 enzymes then catalyse the covalent attachment of ubiquitin to the target protein. After the biosynthesis of a target protein-anchored polyubiquitin chain, the complex is bound to a ubiqutin receptor on the 26S proteasome, allowing degradation of the target protein (Hershko and Ciechanover, 1998).

The biological activites of the ubiquitin system include cell cycle regulation, activation or degradation of transcription factors, and antigen presentation (Hershko and Ciechanover, 1998). For example, STAT1 is inactivated by the ubiquitin-proteasome pathway (Kim and Maniatis, 1996), whereas NF-κB is activated by the ubiquitination and degradation of its inhibitor IκB. Ubiquitination features heavily in the regulation of immune functions (Reinstein, 2004), and proteome analysis has revealed ubiquitin-conjugating enzymes UBE2E1, 2E2, and 2D1 to be upregulated by IFNα (Nyman et al., 2000). The presence of UBE2L6 in the antiviral cluster indicates that this E2 enzyme may also be involved in antiviral functions and be regulated by IFNα.

Figure 4.13 Transcriptional signature of the core antiviral response (next page)

This group of genes characterises the proposed core antiviral response that DCs induce in response to the 3 different virus treatments and dsRNA. The genes shown in red are genes that were similarly upregulated in DCs in response to LPS and dsRNA as part of the core maturation, activation and differentiation response (Section 3.2.8). Genes in blue correspond to the highly correlated sub-cluster. The genes are very strongly correlated in their expression in DCs in response to the different virus treatments (correlation coefficient 0.94).





PC4 Activated RNA polymerase II transcription cofactor 4
PC4 Activated RNA polymerase II transcription cofactor 4
TXNDC4 Thioredoxin domain containing 4 (endoplasmic reticulum)
PSMB10 Proteasome (prosome, macropain) subunit, beta type, 10
SAP18 sin3-associated polypeptide, 18kD
C1orf38 Chromosome 1 open reading frame 38 (basement membrane-induced gene
PSMB10 Proteasome (prosome, macropain) subunit, beta type, 10
TRPC4AP Transient receptor potential cation channel subfamily C member 4 associated protein
SRP54 Signal recognition particle 54kD
EIF2S2 Eukaryotic translation initiation factor 2, subunit 2 (beta, 38kD)
EIF2S2 Eukaryotic translation initiation factor 2, subunit 2 (beta, 38kD)
SSB Sjogren syndrome antigen B
ADM Adrenomedullin
PSMA3 Proteasome (prosome, macropain) subunit, alpha type, 3
PSMA3 Proteasome (prosome, macropain) subunit, alpha type, 3
MTHFD2 Methyl tetrahydrofolate dehydrogenase (NAD+ dependent)
PRKCD Protein kinase C, delta
PRKCD Protein kinase C, delta
SEMA4D Semaphorin 4D
BIRC2 Baculoviral IAP repeat-containing 2
BIRC2 Baculoviral IAP repeat-containing 2
HIRA HIR (histone cell cycle regulation defective homolog A)
TRAF2 TNF receptor-associated factor 2
GRSF1 G-rich RNA sequence binding factor 1
TNFRSF5 TNF receptor superfamily, member 5 (CD40)
SLC20A1 Solute carrier family 20 (phosphate transporter), member 1
SLC20A1 Solute carrier family 20 (phosphate transporter), member 1
FLN29 FLN29 gene product
TOR1B Torsin family 1, member B
FLN29 FLN29 gene product
MYD88 Myeloid differentiation primary response gene (88)
IFITM1 Interferon induced transmembrane protein 1 (9-27kD)
IFITM2 Interferon induced transmembrane protein 2 (1-8kD)
IFITM2 Interferon induced transmembrane protein 2 (1-8kD)
IGF1R Insulin-like growth factor 1 receptor
IGF1R Insulin-like growth factor 1 receptor
UBE2L6 Ubiquitin-conjugating enzyme E2L 6
UBE2L6 Ubiquitin-conjugating enzyme E2L 6
APOBEC3B ApoB mRNA editing enzyme, catalytic polypeptide-like 3B
MT2A Metallothionein 2A
MT2A Metallothionein 2A
PSME2 Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)
PSME2 Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)
STAT1 Signal transducer and activator of transcription 1, 91kD
NMI N-myc (and STAT) interactor
TCTEL1 t-complex-associated-testis-expressed 1-like 1
TCTEL1 t-complex-associated-testis-expressed 1-like 1
TAP1 ATP-binding cassette, subfamily B (MDR/TAP), member 2
TAP1 ATP-binding cassette, subfamily B (MDR/TAP), member 2
TNFRSF5 TNF receptor superfamily, member 5 (CD40)
GCH1 GTP cyclohydrolase 1 (dopa-responsive dystonia)
IRF2 Interferon regulatory factor 2
IRF2 Interferon regulatory factor 2
OAS1 2',5'-oligoadenylate synthetase 1 (40-46kD)
LGALS9 Lectin, galactoside-binding, soluble, 9 (galectin 9)
STAT1 Signal transducer and activator of transcription 1, 91kD
NAPA N-ethylmaleimide-sensitive factor attachment protein, alpha
NAPA N-ethylmaleimide-sensitive factor attachment protein, alpha
DNAJA1 DnaJ (Hsp40) homolog, subfamily A, member 1
DNAJA1 DnaJ (Hsp40) homolog, subfamily A, member 1
KIAA0040 KIAA0040 gene product
MT3 Metallothionein 3 (growth inhibitory factor (neurotrophic))
MT3 Metallothionein 3 (growth inhibitory factor (neurotrophic))
MT1L Metallothionein 1L
IL15RA Interleukin 15 receptor, alpha
KIAA0040 KIAA0040 gene product
HBLD2 HESB like domain containing 2
CHST2 Carbohydrate (chondroitin 6/keratan) sulfotransferase 2
CHST2 Carbohydrate (chondroitin 6/keratan) sulfotransferase 2
HBLD2 HESB like domain containing 2
STAT5A Signal transducer and activator of transcription 5A
KIAA0664 Putative eukaryotic translation initiation factor 3 subunit
KIAA0664 Putative eukaryotic translation initiation factor 3 subunit
TGM2 Transglutaminase 2
TGM2 Transglutaminase 2
R47951 Hypothetical protein DKFZp761G1923
BAK1 Bcl2-antagonist / killer 1
BAK1 Bcl2-antagonist / killer 1
BAK1 Bcl2-antagonist / killer 1
BAK1 Bcl2-antagonist / killer 1
THBS2 *Thrombospondin 2*
TNFSF13B TNF ligand superfamily, member 13B (BAFF, BLyS, THANK)
UBE1L Ubiquitin-activating enzyme E1-like
VRK2 Vaccinia related kinase 2
WS-3 Novel RGD-containing protein
BTG3 BTG family, member 3
NBS1 Nijmegen breakage syndrome 1 (nibrin)
NBS1 Nijmegen breakage syndrome 1 (nibrin)
ZCWCC3 Zinc finger, CW-type with coil-coil domain 3
MTHFD2 Methyl tetrahydrofolate dehydrogenase (NAD+ dependent)
CUL1 Cullin 1
CFLAR CASP8 and FADD-like apoptosis regulator (FLIP)
CFLAR CASP8 and FADD-like apoptosis regulator (FLIP)
CCL8 Chemokine (C-C motif) ligand 8 (monocyte chemoattractant protein 2)
CCL8 Chemokine (C-C motif) ligand 8 (monocyte chemoattractant protein 2)
HIRA HIR (histone cell cycle regulation defective homolog A)
STAT3 Signal transducer and activator of transcription 3 (acute-phase response factor)
STAT3 Signal transducer and activator of transcription 3 (acute-phase response factor)

4.7.3.3 Rhinovirus-specific responses

As shown in the sample cluster dendogram (Figure 4.10), gene expression of DCs in response to Rhinovirus exposure suggests a delayed activation relative to the response to other virus and dsRNA stimuli. This is demonstrated by the positioning of the Rhinovirus-specific cluster, sharing a higher branch point with DC samples at 5 hours after Influenza infection and 5 hours after inactivated Influenza exposure (branch **b** in Figure 4.10), and adjacent to DC samples at early timepoints (branch **a** in Figure 4.10). This indicates that the gene expression profile of DCs exposed to Rhinovirus at 10 to 24 hours is more similar to the gene expression profile of DCs exposed to inactivated Influenza and infected with Influenza at 5 hours after virus exposure. Exposure to Rhinovirus may therefore affect an "intermediate" activation of DCs. This may be a specific strategy of Rhinovirus in escaping the immune response by inducing minimal phenotypic changes in DCs (Section 4.5) other than the core antiviral response (Section 4.7.3.2). This intermediate transcriptional response may also result from an inability of Rhinovirus to replicate in DCs.

Matrix metalloproteinase 2 (MMP2) is strongly upregulated in DCs exposed to Rhinovirus, especially at later timepoints, 10 hours to 24h post-exposure. This upregulation is not as marked in DCs responding to Influenza, inactivated Influenza, or dsRNA (Figure 4.14). MMP2 is a zinc-dependent enzyme involved in the degradation of extracellular matrix, and is important in tumour invasion and cell migration. MMP2 has also been shown to be important in the migration of Langerhans cells and dermal DCs from human skin (Ratzinger et al., 2002). The induction of MMP2 by DCs responding to Rhinovirus, which contains a zinc-dependent proteinase ($2A^{pro}$), may indicate an interesting interaction between the virus and host cell in utilising zinc-dependent enzymes to facilitate virus replication and mobilise an immune response.

Protein modification enzymes and inhibiting factors are heavily represented in this cluster, with cathepsins, cystatins, ubiquitin conjugating enzymes and enzymes involved in protein biosynthesis upregulated by DCs responding to Rhinovirus throughout the timecourse (Figure 4.14). Actin-binding proteins (thymosin beta 10 and beta 4, and lymphocyte cytosolic protein 1), proteins involved in regulating the actin cytoskeleton, as well as actin and vimentin are also encoded by genes represented in this cluster. Again there are genes involved in signal transduction (RAP2A, GDP dissociation

inhibitor 1, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, glucose regulated protein GRP58, FYN tyrosine kinase, Bruton agammaglobulinemia tyrosine kinase), cell cycle regulation (microtubule-associated protein 4, soluble galactoside-binding lectin 3), transcription factors (GATA-binding protein 6, transcription elongation factor TCEB1) and translation factors (eIF). It is clear that although Rhinovirus does not replicate in dendritic cells, there is significant transcriptional responses in DCs to the virus stimulus, probably reflecting downstream events triggered by Rhinovirus and dendritic cell interactions via cellular receptors and endocytosis.

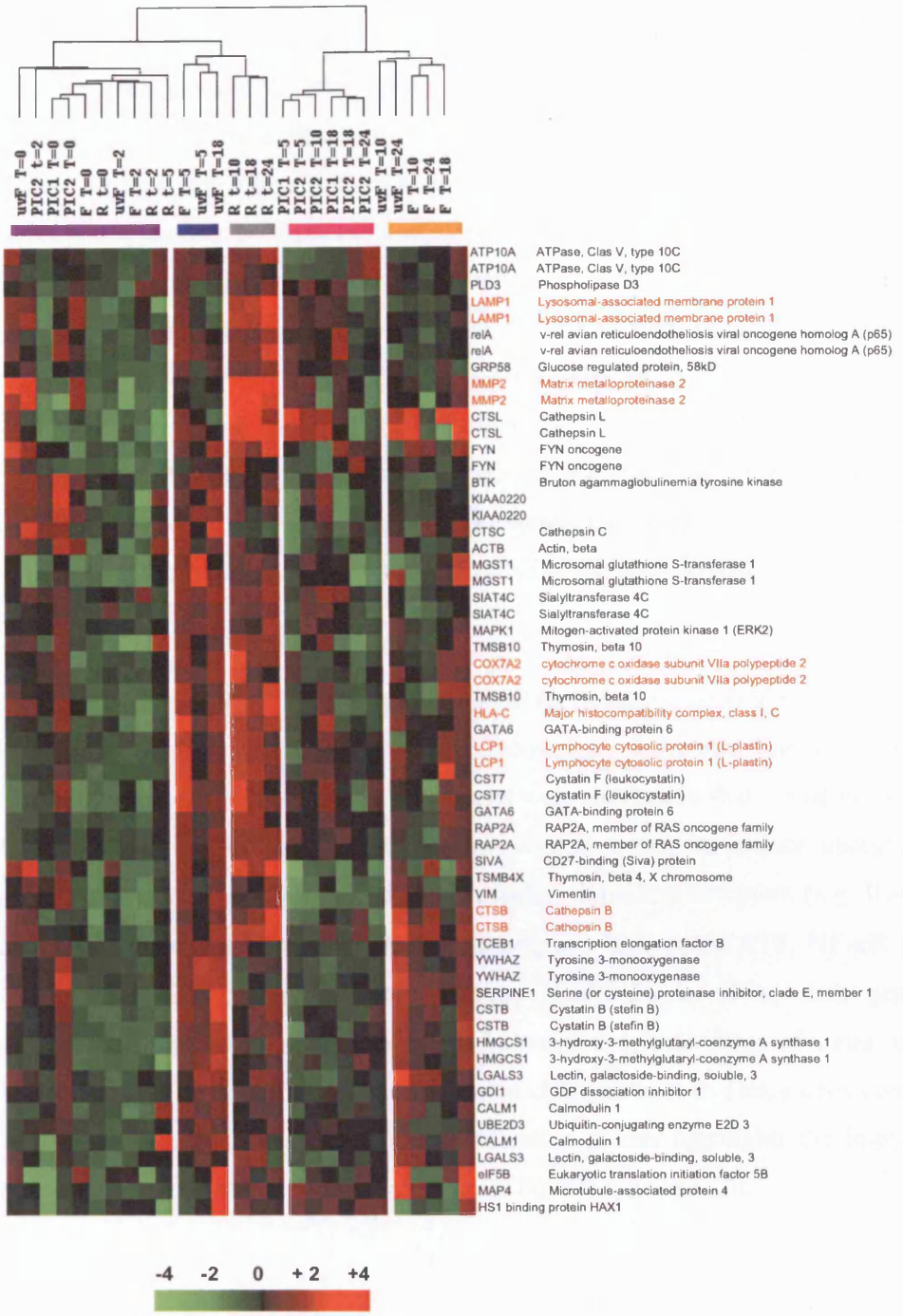


Figure 4.14 Transcriptional signature of DCs responding to Rhinovirus

This group of genes are more strongly upregulated in DCs responding to Rhinovirus compared to other virus treatments (particularly genes shown in red). However, this profile also includes genes that are upregulated in DCs in response to early and later timepoints after Influenza and inactivated-Influenza exposure. This upregulation in expression is generally not seen in DCs responding to dsRNA, similar to the early virus-induced responses (Section 4.7.3.1).

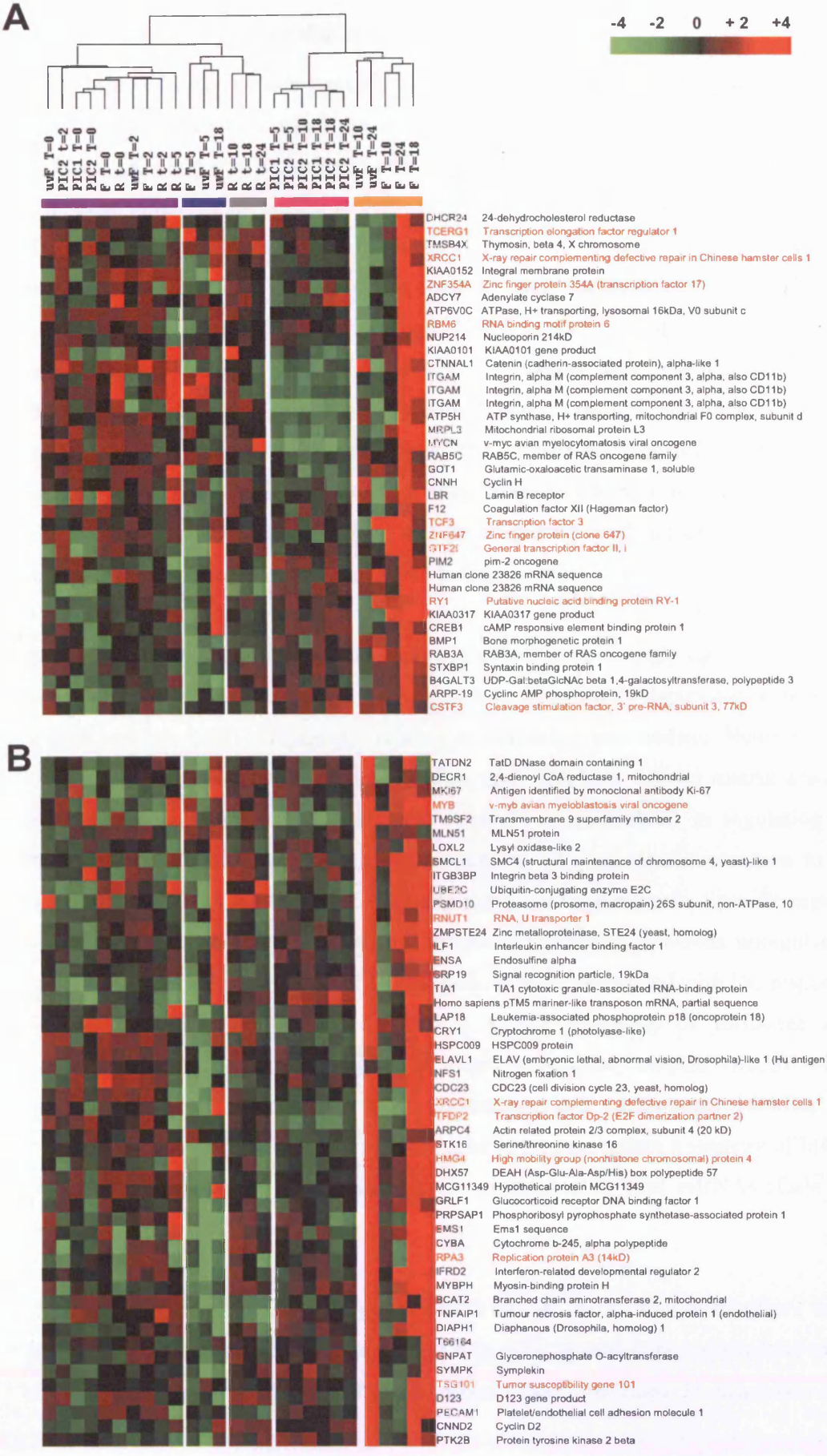
4.7.3.4 Inactivated Influenza-specific responses

The small cluster of strongly upregulated genes in DCs exposed to UV-inactivated Influenza are mainly involved in the regulation of DNA transcription, especially linking the control of basal transcription to the cell cycle (Figure 4.15A). Some of these genes are also strongly upregulated at 18 hours after infection with Influenza (Figure 4.15B). These genes encode proteins associated with nuclear transcription. This shows that a non-replicating viral stimulus may be able to strongly induce dendritic cells to upregulate a transcriptional program, in the absence of viral replication. Therefore, the initial stimulus mediated by surface interactions between Influenza virus and dendritic cell and subsequent virus entry may be sufficient to trigger such downstream responses. This has been shown for HIV-1, where gp120 (a glycoprotein present on the envelope of HIV-1) treatment of PBMCs and macrophages stimulates the induction of nuclear transcription factors and proteins associated with the actin cytoskeleton (Cicala et al., 2002).

Investigations of HeLa cells has also revealed replication-independent and replication-dependent cellular transcriptional events induced by Influenza (Geiss et al., 2001), though transcriptional changes on HeLa cells were distinct to that found in DCs in this study. In HeLa cells, Influenza replication was necessary to induce upregulation of genes which encode cytokines and growth factor signalling receptors (e.g. IL-6, IL-15, insulin-like growth factor 2 receptor), transcription factors (STAT5, NF-κB p65) and proteins involved in the ubiquitin pathway (PSME2). In DCs, such genes were upregulated as part of the proposed antiviral response, regardless of virus replication (Figure 4.13). The disparity in transcriptional changes seen in HeLa cells compared to DCs in response to live and inactivated Influenza further highlights the importance of recognising certain transcriptional changes to be cell-type specific.

Figure 4.15 Transcriptional signature of DCs responding to UV-Influenza (next page)

This group of genes are more strongly upregulated in DCs responding to UV-inactivated Influenza compared to other virus treatments. **A** shows genes that are more strongly upregulated in DCs in response inactivated Influenza compared to Influenza, and **B** shows genes that are more similarly upregulated by DCs in response to both inactivated and live Influenza, at late timepoints. Genes involved in nuclear transcription are shown in red.


A
-4 -2 0 +2 +4
uvF T=0
PIC2 t=2
PIC1 T=0
PIC2 T=0
F T=0
R t=0
uvF T=2
F T=2
R t=2
R t=5
F T=5
uvF T=5
uvF T=18
R t=10
R t=18
R t=24
PIC1 T=5
PIC2 T=5
PIC2 T=10
PIC1 T=18
PIC2 T=18
PIC2 T=24
uvF T=10
uvF T=24
F T=10
F T=24
F T=18
DHCR24 24-dehydrocholesterol reductase
TCERG1 Transcription elongation factor regulator 1
TMSB4X Thymosin, beta 4, X chromosome
XRCC1 X-ray repair complementing defective repair in Chinese hamster cells 1
KIAA0152 Integral membrane protein
ZNF354A Zinc finger protein 354A (transcription factor 17)
ADCY7 Adenylate cyclase 7
ATP6V0C ATPase, H+ transporting, lysosomal 16kDa, V0 subunit c
RBM6 RNA binding motif protein 6
NUP214 Nucleoporin 214kD
KIAA0101 KIAA0101 gene product
CTNNAL1 Catenin (cadherin-associated protein), alpha-like 1
ITGAM Integrin, alpha M (complement component 3, alpha, also CD11b)
ITGAM Integrin, alpha M (complement component 3, alpha, also CD11b)
ITGAM Integrin, alpha M (complement component 3, alpha, also CD11b)
ATP5H ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d
MRPL3 Mitochondrial ribosomal protein L3
MYCN v-myc avian myelocytomatosis viral oncogene
RAB5C RAB5C, member of RAS oncogene family
GOT1 Glutamic-oxaloacetic transaminase 1, soluble
CNNH Cyclin H
LBR Lamin B receptor
F12 Coagulation factor XII (Hageman factor)
TCF3 Transcription factor 3
ZNF647 Zinc finger protein (clone 647)
GTF2I General transcription factor II, i
PIM2 pim-2 oncogene
Human clone 23826 mRNA sequence
Human clone 23826 mRNA sequence
RY1 Putative nucleic acid binding protein RY-1
KIAA0317 KIAA0317 gene product
CREB1 cAMP responsive element binding protein 1
BMP1 Bone morphogenetic protein 1
RAB3A RAB3A, member of RAS oncogene family
STXBP1 Syntaxin binding protein 1
B4GALT3 UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 3
ARPP-19 Cyclinc AMP phosphoprotein, 19kD
CSTF3 Cleavage stimulation factor, 3' pre-RNA, subunit 3, 77kD
B
TATDN2 TatD DNase domain containing 1
DECR1 2,4-dienoyl CoA reductase 1, mitochondrial
MKI67 Antigen identified by monoclonal antibody Ki-67
MYB v-myb avian myeloblastosis viral oncogene
TM9SF2 Transmembrane 9 superfamily member 2
MLN51 MLN51 protein
LOXL2 Lysyl oxidase-like 2
SMCL1 SMC4 (structural maintenance of chromosome 4, yeast)-like 1
ITGB3BP Integrin beta 3 binding protein
UBE2C Ubiquitin-conjugating enzyme E2C
PSMD10 Proteasome (prosome, macropain) 26S subunit, non-ATPase, 10
RNUT1 RNA, U transporter 1
ZMPSTE24 Zinc metalloproteinase, STE24 (yeast, homolog)
ILF1 Interleukin enhancer binding factor 1
ENSA Endosulfine alpha
SRP19 Signal recognition particle, 19kDa
TIA1 TIA1 cytotoxic granule-associated RNA-binding protein
Homo sapiens pTM5 mariner-like transposon mRNA, partial sequence
LAP18 Leukemia-associated phosphoprotein p18 (oncoprotein 18)
CRY1 Cryptochrome 1 (photolyase-like)
HSPC009 HSPC009 protein
ELAVL1 ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)
NFS1 Nitrogen fixation 1
CDC23 CDC23 (cell division cycle 23, yeast, homolog)
XRCC1 X-ray repair complementing defective repair in Chinese hamster cells 1
TFDP2 Transcription factor Dp-2 (E2F dimerization partner 2)
ARPC4 Actin related protein 2/3 complex, subunit 4 (20 kD)
STK16 Serine/threonine kinase 16
HMG4 High mobility group (nonhistone chromosomal) protein 4
DHX57 DEAH (Asp-Glu-Ala-Asp/His) box polypeptide 57
MCG11349 Hypothetical protein MCG11349
GRLF1 Glucocorticoid receptor DNA binding factor 1
PRPSAP1 Phosphoribosyl pyrophosphate synthetase-associated protein 1
EMS1 Ems1 sequence
CYBA Cytochrome b-245, alpha polypeptide
RPA3 Replication protein A3 (14kD)
IFRD2 Interferon-related developmental regulator 2
MYBPH Myosin-binding protein H
BCAT2 Branched chain aminotransferase 2, mitochondrial
TNFAIP1 Tumour necrosis factor, alpha-induced protein 1 (endothelial)
DIAPH1 Diaphanous (Drosophila, homolog) 1
T66164
GNPAT Glyceronephosphate O-acyltransferase
SYMPK Symplekin
TSG101 Tumor susceptibility gene 101
D123 D123 gene product
PECAM1 Platelet/endothelial cell adhesion molecule 1
CNND2 Cyclin D2
PTK2B Protein tyrosine kinase 2 beta

4.7.3.5 Influenza-specific responses

This group of genes encode proteins that are involved in cell growth, maintenance, and nucleic acid metabolism, which are strongly upregulated in late timepoints in Influenza-infected DCs and also at 24 hours after inactivated Influenza exposure (Figure 4.16). The gene functions are similar to those observed in the cluster of upregulated genes by DCs responding to inactivated Influenza (Figure 4.15). Genes involved in protein metabolism, including protein processing and degradation (PSMD10) and ubiquitination (UBE2C), biosynthesis and phosphorylation, are also upregulated, which are common between DC responses to Influenza and inactivated Influenza. Modification of the host response that involves protein synthesis and metabolism appear to be a general feature of Influenza virus. The conflict between Influenza modulating conditions to favour the translation of viral mRNAs (de la Luna et al., 1995) and dendritic cells' increased processing activity to present viral antigens to T cells may explain the upregulation of these genes in Influenza-exposed DCs.

In addition, this cluster also includes genes that are more strongly upregulated by DCs responding to Influenza infection. These genes are presumably dependent on replication events, such as THO complex 2, involved in stabilising intermediate filament proteins as part of the transcription/export complex, and SWI/SNF related matrix associated actin dependent regulator of chromatin (SMARCA2), involved in regulating DNA transcription. These genes bind to chromatin, to alter chromatin structure to allow transcriptional activation of genes normally repressed by chromatin. Specific regulation of cellular transcription is affected by Influenza replication, whereas upregulation of genes involved in regulating basal transcription is more associated with DC responses to inactivated Influenza (Section 4.7.3.4). A further influence of Influenza in DC transcription include the upregulation of origin recognition complex ORC3L which is necessary for the initiation of DNA replication, and nucleoporin 160kDa, which regulates mRNA export from the nucleus. The latter may reflect a strategy of Influenza to block translation of cellular mRNAs in preference to viral mRNAs (Gale et al., 2000).

The combination of modulating genes which encode for protein metabolism, together with selective transcriptional regulation of host cells, appear to be a distinctive effect of Influenza infection in DCs. Influenza protein NS1 functions to sequester dsRNA

thereby preventing PKR activation. The activation of PKR would result in a block to all protein synthesis. In addition to degrading cellular mRNAs in the nucleus (Katze and Krug, 1984), Influenza has been shown to block nuclear export of cellular RNAs (Chen and Krug, 2000), whilst favouring viral mRNA translation. This serves to provide a virus-specific defence mechanism against non-specific inhibition of translation mediated by PKR.

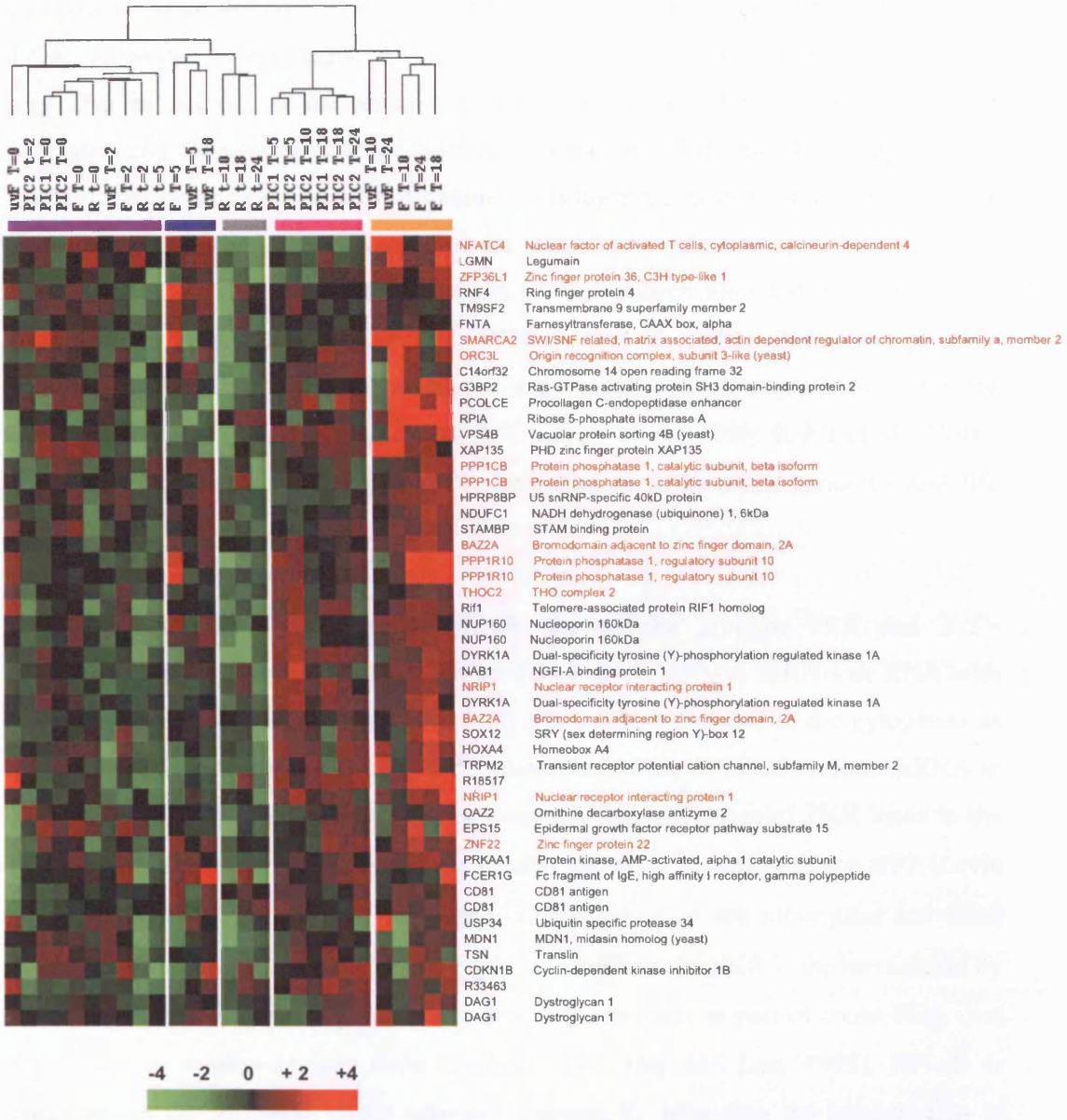


Figure 4.16 Transcriptional signature of DCs responding to Influenza

This group of genes are strongly upregulated in Influenza-infected DCs at late timepoints, though a few are also upregulated by DCs at late timepoints in response to inactivated Influenza. Genes affecting nuclear transcription and chromatin remodelling are shown in red.

4.7.3.6 dsRNA-specific responses

The dsRNA motif is one that is commonly found during virus replication (Jacobs and Langland, 1996). Minute quantities of dsRNA, as little as one molecule per cell, can have profound effects on cellular physiology (Marcus and Sekellick, 1977). For ssRNA viruses, the dsRNA motif can be found as the replicative intermediate in infected cells. The presence of both positive-sense and negative-sense RNAs that are synthesized in infected cells has been detected for a number of viruses, including encephalomyocarditis virus (EMCV) (Gribaudo et al., 1991) and Rubella (Lee et al., 1994). However, virus-specific RNA molecules that contain double-stranded regions may also be present in the absence of viral replication. The ssRNA genomes of Influenza and Rhinovirus contain secondary structures that may be recognised as dsRNA. The negative strand ssRNA genome of Influenza contains panhandle structures in the terminal noncoding regions that are partially double-stranded. This structure serves to regulate initiation and termination of viral transcription and polyadenylation (Desselberger et al., 1980; Fodor et al., 1994; Luo et al., 1991). Rhinovirus positive strand ssRNA genome contain cloverleaf structures that direct host proteins to regulate translation or replication (Racaniello, 2001; Mellits et al., 1998; Rohll et al., 1994). Therefore dsRNA motifs are potentially abundant in RNA virus genomes and life cycles.

In addition to TLR3 that detects dsRNA, the cellular proteins PKR and 2',5'-oligoadenylate synthetase 1 (OAS1) can also bind specifically to dsRNA or RNA with secondary structures, and be activated. PKR and OAS1 are found in the cytoplasm as well as the nucleus (Jeffrey et al., 1995; Rosenblum et al., 1988), therefore dsRNA in Influenza and Rhinovirus should both be detected. The activation of PKR leads to the phosphorylation of the α subunit of the protein synthesis initiation factor eIF2 (Levin and London, 1978) and IκB, which leads to IκB degradation and subsequent activation of NF-κB (Kumar et al., 1994). Phosphorylation of eIF-2α by PKR is the mechanism by which interferon mediates the cessation of protein synthesis as part of controlling viral replication in virally-infected cells (Barber, 2001; Der and Lau, 1995). NF-κB is important in the initiation of the antiviral response by activating the transcription of IFNβ (Visvanathan and Goodbourn, 1989), in addition to a range of other cytokines, chemokines, adhesion molecules and MHC molecules (Baeuerle and Henkel, 1994). The activation of OAS1 by dsRNA leads to ATP polymerisation to 2',5'-linked

oligoadenylates. These oligoadenylates can then activate a ribonuclease RNaseL that cleaves ssRNA (Floyd-Smith et al., 1981). Both negative and positive sense picornaviral RNA have been found bound to OAS1 in EMCV-infected cells (Gribaudo et al., 1991), and reduction of EMCV RNA shown to be mediated by RNaseL (Li et al., 1998), consistent with the activation of this pathway in picornavirus-infected cells.

Genes upregulated by dendritic cells in response to dsRNA are distinct to DC responses to virus treatments (Figure 4.17). This dsRNA-specific response is initiated as soon as 5 hours after stimulation, and the poly(I:C)-stimulated DC samples cluster closely together from 5 to 24 hours post stimulation. These upregulated genes are largely a part of the core DC maturation response (Chapter 3). These include genes encoding proteins of the TNF family (TRADD, TNFSF13), proteins that act downstream of TNF (LPS-induced TNF factor), cytokine-mediated signal transduction pathways (STAM binding protein), TGFβ receptor signalling pathway (farnesyltransferase), integrin-mediated signalling pathway (a disintegrin and metalloproteinase domain 11), wnt receptor signalling pathway (transcription factor 7-like 2), G-protein coupled receptor protein signaling pathway (endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 4), BNIP family (Bcl2/adenovirus E1B 19kDa interacting protein 3-like), intracellular signalling cascades (stathmin-like 2, autocrine motility factor receptor) and apoptosis regulation (presenilin 1, cold autoinflammatory syndrome 1, cold inducible RNA binding protein, death-associated protein kinase 1). Insulin-like growth factor binding protein 4 (IGFBP4) is also strongly upregulated, which functions to prolong the action of insulin-like growth factors.

Interestingly, this subset of genes is largely specific to dsRNA, and not shared between DC responses to Rhinovirus or Influenza. This difference could be a result of the differentiation of the DC core maturation response (dsRNA and LPS) and specifically virus-tailored responses. Another possibility for this difference is that viruses are able to downmodulate this activatory response in DCs, as these core genes are not upregulated in DCs responding to Rhinovirus exposure or Influenza infection. However, some of these dsRNA-induced upregulated genes are also found in DC responses to inactivated Influenza, like IGFBP4, TRADD, cyclin G2, RBMS2 (RNA binding motif), and complement factor C2 (Figure 4.17). This suggests that the dsRNA motif as part of secondary structures in inactivated Influenza genome is present and is detected by dendritic cells. In contrast, replication-competent Influenza does not cause DCs to

upregulated these genes, suggesting that Influenza is able to suppress a range of the host immune responses to dsRNA. Interestingly, although there is no evidence that Rhinovirus can replicate in DCs in this system, DC responses to non-replicating Rhinovirus is distinct to DC responses to inactivated Influenza. This further suggests that DC responses are virus-specific, in addition responses dependent on the replicative ability of the virus.

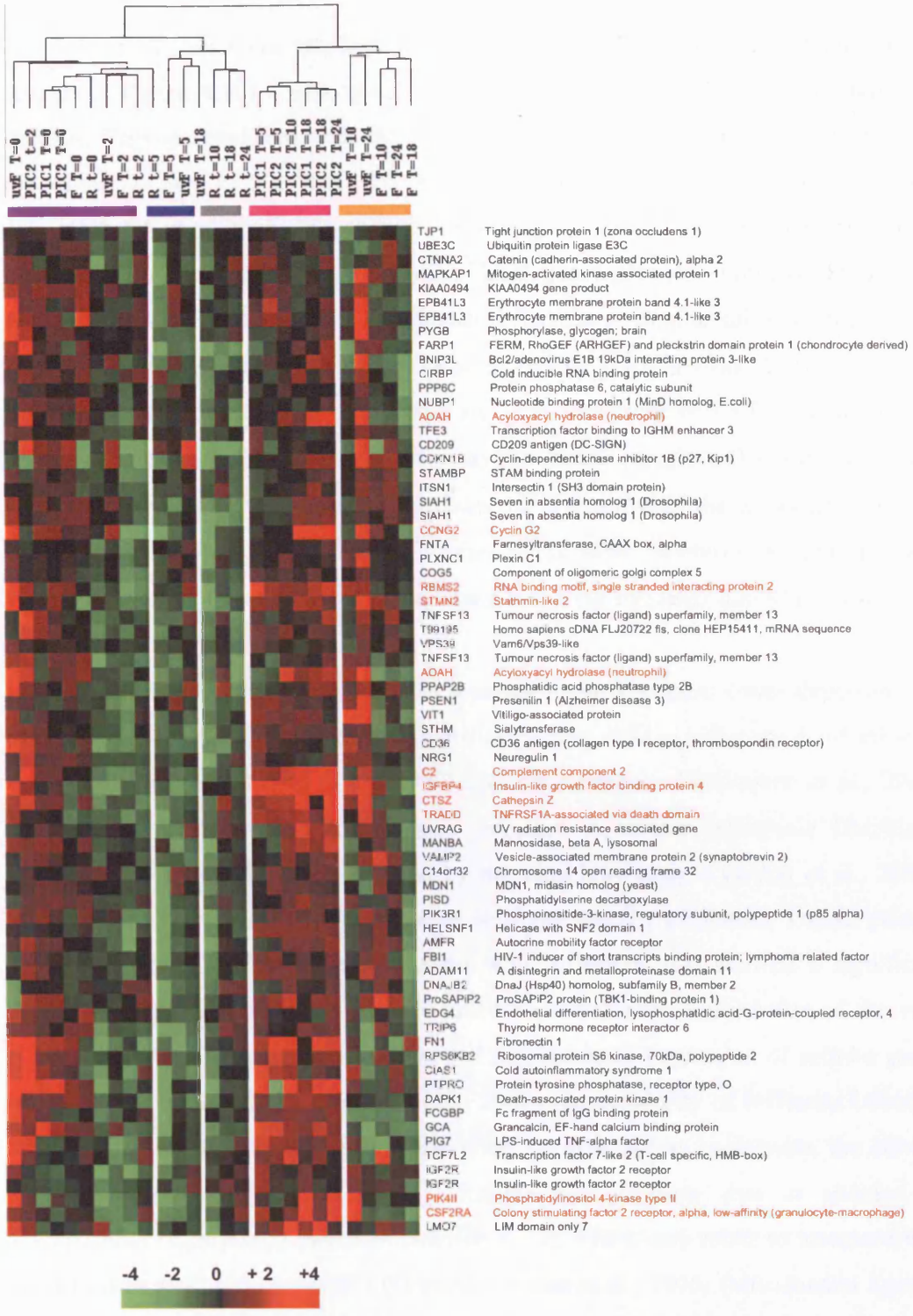


Figure 4.17 Transcriptional signature of DCs responding to dsRNA

This group of genes are strongly upregulated by DCs responding to dsRNA, compared to Rhinovirus and Influenza. These genes reflect a subset of the core maturation program in DCs responding to LPS and dsRNA, and are not upregulated in DCs responding to viruses. There are genes that are similarly upregulated by DCs in response to dsRNA and inactivated Influenza (genes shown in red).

4.7.3.7 NF-κB response

This group of genes are involved in the induction and maintenance of the NF-κB response (Figure 4.18). These genes are strongly upregulated in DCs responding to dsRNA. This is consistent with the results from Chapter 3 where these genes were classified as part of the core DC activation and differentiation response, as the activation of numerous intracellular signalling pathways downstream of TLR3 culminates in the activation of NF-κB. These genes are also upregulated in DCs responding to live Influenza and inactivated Influenza. A smaller subset of the NF-κB response genes are also upregulated in DCs responding to Rhinovirus. Activation of this transcriptional program may be a result of TLR signalling or PKR activation as a response to dsRNA, as well as other virus-stimulated receptors that induce NF-κB activation. The ability of viruses to activate NF-κB suggests the importance of this transcription factor in the life cycle of viruses (Pahl, 1999; Santoro et al., 2003). Many viruses are known to induce NF-κB responses, including Influenza and Rhinovirus.

Influenza's ability to activate NF-κB as reported in the literature varies depending on the cell type in which the experiments were carried out. Where Influenza A infection of lung epithelial cells (Ronni et al., 1997) and macrophages (Matikainen et al., 2000) showed activation of NF-κB, Influenza infection in mouse embryonic fibroblasts (MEFs) showed that this was inhibited by the viral NS1 protein (Wang et al., 2000). This further emphasises the importance of cell-type specific responses. Transcriptional profiling of lung epithelial cells infected with Influenza also showed a significant induction of genes involved in the IFN and NF-κB pathway; and deletion of the viral NS1 gene further increased the number and magnitude of expression of cellular genes implicated in these pathways (Geiss et al., 2002). Susceptibility of Influenza infection has been shown to vary in cells which relate to their ability to activate the NF-κB pathway (Nimmerjahn et al., 2004). Results here indicate that in addition to susceptibility to Influenza infection (Section 4.3.2), which may relate to increased NF-κB activities and proteins in DCs (Granelli-Piperno et al., 1995) (Introduction Section 1.2.3.1), Influenza infection of DCs results in the transcriptional activation of NF-κB (Figure 4.18). As inactivated Influenza exposure to DCs also transcriptionally activates this cluster of genes, active Influenza replication is not required for NF-κB activation. Binding of the viral particle to its receptor may be sufficient to trigger membrane-

proximal signalling cascades that activate NF-κB, and entry of inactivated Influenza, and detection of Influenza dsRNA by PKR and TLR3 also activates NF-κB (Alexopoulou et al., 2001; Kumar et al., 1994).

Rhinovirus is able to activate NF-κB in a range of respiratory epithelial cells, resulting in the induction of various cytokine (IL-6) and chemokine (CXCL8) transcripts (Funkhouser et al., 2004; Kim et al., 2000; Zhu et al., 1996) and upregulation of surface expression of ICAM1 (Papi and Johnston, 1999). Rhinovirus exposure in DCs however, results in the induction of a small subset of the NF-κB response genes (Figure 4.18).

In addition to activating core immune response genes which include cytokines and chemokines, regulators of motility, migration and cell adhesion, and antigen presenting functions (Chapter 3), NF-κB also activates genes involved in apoptosis regulation. These genes include proapoptotic genes caspase 4, tumour protein p53 binding protein 2, serine/threonine kinase 17b, and anti-apoptotic genes growth arrest and DNA-damage-inducible beta (GADD45β), TNF alpha-induced protein 3 (TNFAIP3), and baculoviral IAP repeat-containing 3 (BIRC3). The activation of such apoptosis regulatory genes downstream of NF-κB clearly make the regulation and timing of NF-κB activation important in determining whether the virus-infected cells are protected from or undergo apoptosis (Bose et al., 2003). The regulation of apoptosis may also be important for DC responses to Influenza as the induction of apoptosis by Influenza is an important mechanism of Influenza-induced cell death (Hinshaw et al., 1994). In addition to activating NF-κB and downstream pro-apoptotic genes, PKR also mediates apoptosis via eIF-2α (Gil et al., 1999; Srivastava et al., 1998) by inhibiting translation of cellular proteins. As Influenza antagonises the activity of PKR by sequestering dsRNA, the virus may also indirectly regulate apoptosis mediated by PKR activity. The mechanisms by which Influenza induces apoptosis in cells are ill defined, and cell-specific considerations make data from experiments performed in various cell types difficult to extrapolate. In DCs, cell viability experiments clearly showed that Influenza infection induced a significant amount of cell death (Section 4.6) (Oh et al., 2000) compared to DC exposure to inactivated Influenza and Rhinovirus. Together with the marked induction of apoptosis-regulating genes, this suggests that transcriptional regulation of NF-κB and therefore its downstream genes by Influenza-infected DCs may be a mechanism by which Influenza mediates its apoptotic effects.

So far, the upregulation of NF-κB responsive genes (Figure 4.18) mediated by DCs in response to Influenza infection in particular has been interpreted as a response to viral infection, as these genes have important immune functions (cytokines, chemokines, apoptosis regulation, and antigen presentation). However, a target for PKR is eIF-2α, and phosphorylation of the α subunit results in the inhibition of cellular translation initiation. The upregulation of such NF-κB response genes may be a reflection of transcript accumulation of such induced genes as translation is inhibited. Therefore, gene expression data should be interpreted within the confines of transcriptional activity, and that this does not necessarily translate to protein activity.

Figure 4.18 Transcriptional signature of the NF-κB response (next page)

This cluster shows a number of genes that are thought to be regulated by NF-κB. These genes are upregulated by DCs most strongly in response to dsRNA, as demonstrated in Chapter 3 (Section 3.2.8) as part of the core DC maturation program. However, Influenza-infected DCs also seem to upregulate the transcripts of NF-κB genes (shown in red), suggesting that Influenza also activates NF-κB as part of its replication cycle in DCs.


-4 -2 0 +2 +4
uvF T=0
PIC2 t=2
PIC1 T=0
PIC2 T=0
F T=0
R t=0
uvF T=2
F T=2
R t=2
R t=5
F T=5
uvF T=5
uvF T=18
R t=10
R t=18
R t=24
PIC1 T=5
PIC2 T=5
PIC2 T=10
PIC1 T=18
PIC2 T=18
PIC2 T=24
uvF T=10
uvF T=24
F T=10
F T=24
F T=18
FOLR2 Folate receptor 2 (foetal)
FOLR2 Folate receptor 2 (foetal)
FGL2 Fibrinogen-like 2
GCLM Glutamate-cysteine ligase, modifier subunit
CCL15 Chemokine (C-C motif) ligand 15
CD209L CD209 antigen-like (dendritic cell-specific ICAM3-grabbing nonintegrin 2)
ACE Angiotensin converting enzyme
ACE Angiotensin converting enzyme
CD209L CD209 antigen-like (dendritic cell-specific ICAM3-grabbing nonintegrin 2)
FCGR3B Low affinity immunoglobulin gamma Fc receptor III
FCGR3B Low affinity immunoglobulin gamma Fc receptor III
R66325 Cytokine receptor beta chain precursor
CLN5 Ceroid lipofuscinosis, neuronal 5
ACP2 Acid phosphatase 2, lysosomal
ACP2 Acid phosphatase 2, lysosomal
RBMS1 RNA binding motif, single stranded interacting protein 1
R10398 Interleukin-7 alpha chain precursor
R10398 Interleukin-7 alpha chain precursor
RBMS1 RNA binding motif, single stranded interacting protein 1
CD86 CD86 (CD28 antigen ligand 2, B7-2 antigen)
ARHG Ras homolog gene family, member G (RhoG)
RAB35 RAB35, member of RAS oncogene family
FCER1G High affinity IgE receptor I, Fc fragment
N4BP1 Nedd 4 binding protein 1
MAP3K7IP2 TAK-1 binding protein 2
MAP3K7IP2 TAK-1 binding protein 2
BTN2A1 Butyrophilin
NUMB NUMB homolog (Drosophila)
STX4A Syntaxin 4A (placental)
CCL7 Chemokine (C-C motif) ligand 7
SYCP2 Synaptonemal complex protein 2
CD86 CD86 (CD28 antigen ligand 2, B7-2 antigen)
CCL7 Chemokine (C-C motif) ligand 7
MAOA Monoamine oxidase A
H42888 Interleukin 3 receptor alpha chain precursor
G2 G2 protein
PCAF p300/CBP-associated factor
KIAA0143 Hypothetical protein KIAA0143
NUP153 Nucleoporin 153kD
DUSP5 Dual specificity phosphatase 5
BTN2A1 Butyrophilin
SPP1 Secreted phosphoprotein 1 (osteopontin, early T-lymphocyte activation 1)
C2 Complement component C2
ADCY2 Adenylate cyclase 2
ADCY2 Adenylate cyclase 2
CSF2RA Colony stimulating factor 2 receptor, alpha, low affinity (granulocyte-macrophage)
H42888 Interleukin 3 receptor alpha chain precursor
DUSP5 Dual specificity phosphatase 5
SIAT9 Sialyltransferase 9
SIAT9 Sialyltransferase 9
SLC7A7 Solute carrier family 7, member 7
LYN v-yes-1 Yamaguchi sarcoma viral related oncogene homolog
CIC Capicua homolog (Drosophila)
NFKBIA Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
NFKBIA Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha
CXCL2 Chemokine (C-X-C motif) ligand 2
CXCL2 Chemokine (C-X-C motif) ligand 2
KBTBD2 Kelch repeat and BTB (POZ) domain containing 2
PLAUR Plasminogen activator, urokinase receptor
CASP4 Caspase 4, apoptosis-related cysteine protease
NXF1 Nuclear RNA export factor 1
JAK2 Janus kinase 2 (a protein tyrosine kinase)
BLZF1 Basic leucine zipper nuclear factor 1 (JEM-1)
NFKB1 Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
CD83 CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)
NINJ1 Ninjurin 1
NFKB1 Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)
BIRC3 Baculoviral IAP repeat containing 3
RUNX3 Runt-related transcription factor 3
INHBA Inhibin, beta A (activin A, activin AB alpha polypeptide)
SRF Serum response factor (c-fos response element-binding transcription factor)
SIAH2 Seven in absentia homolog 2 (Drosophila)
SIAH2 Seven in absentia homolog 2 (Drosophila)
TANK TRAF family member-associated NFKB activator
TANK TRAF family member-associated NFKB activator
CD83 CD83 antigen (activated B lymphocytes, immunoglobulin superfamily)
PHF1 PHD finger protein 1
TNFAIP3 Tumour necrosis factor, alpha-induced protein 3
ICAM1 Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
CCL4 Chemokine (C-C motif) ligand 4
CCL4 Chemokine (C-C motif) ligand 4
IL1RN Interleukin-1 receptor antagonist
TNFAIP2 Tumour necrosis factor, alpha-induced protein 2
TNFAIP2 Tumour necrosis factor, alpha-induced protein 2
ERH Enhancer of rudimentary homolog (Drosophila)
ERH Enhancer of rudimentary homolog (Drosophila)
WTAP Wilms' tumour 1-associating protein
WTAP Wilms' tumour 1-associating protein
FGFR3 Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)
FGFR3 Fibroblast growth factor receptor 3 (achondroplasia, thanatophoric dwarfism)
DPF2 D4, zinc and double PHD fingers family 2
EZH2 Enhancer of zeste homolog 2 (Drosophila)
EZH2 Enhancer of zeste homolog 2 (Drosophila)
CCL2 Chemokine (C-C motif) ligand 2
GPR109B G protein-coupled receptor 109B
GPR109B G protein-coupled receptor 109B
IL1A Interleukin-1, alpha
TNF Tumour necrosis factor (TNF superfamily, member 2)
TNF Tumour necrosis factor (TNF superfamily, member 2)
TRAF1 TNF receptor-associated factor 1
ICAM1 Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
ICAM1 Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
ICAM1 Intercellular adhesion molecule 1 (CD54), human rhinovirus receptor
PLEK Pleckstrin
PLEK Pleckstrin
DNAJB6 DnaJ (Hsp40) homolog, subfamily B, member 6
MCL1 Myeloid cell leukemia sequence 1 (Bcl2-related)
KBTBD2 Kelch repeat and BTB (POZ) domain containing 2
HPRP3P U4/U6-associated RNA splicing factor
NIT1 Nitrilase 1
HDAC6 Histone deacetylase 6
TP53BP2 Tumour protein 53 binding protein, 2
TP53BP2 Tumour protein 53 binding protein, 2
CENTD2 Centaurin, delta 2
STK17B Serine/threonine kinase 17b (apoptosis-inducing)
GADD45B Growth arrest and DNA-damage-inducible beta

Discussion

The theme of DC plasticity to antigens was further explored in this chapter. LPS- and dsRNA-specific responses from the core activation and differentiation program alluded to antigen-specific responses that were subtle and difficult to interpret. The use of more complex antigens in the form of whole viruses allowed DC plasticity to be seen more clearly. Both live and inactivated forms of Influenza and Rhinovirus induced differential phenotypic responses in DCs at the level of surface expression of a range of relevant markers. Exposure to viruses also differentially affected DC viability. This was supported by the observed transcriptional plasticity in DC responses to different virus treatments.

Currently there are no studies of the global transcriptional effects of different viruses on DCs. DC responses to different viruses are usually investigated within a particular facet of the DC response (Lopez et al., 2003), whereas global changes affected by virus infections are usually investigated in stromal cell lines (Geiss et al., 2001; Geiss et al., 2002). In particular, effects of Influenza and Rhinovirus infections have been investigated in lung and bronchial epithelial cells, as both are respiratory viruses that cause pathogenesis in the lung.

A number of microarray studies have addressed the effects of Influenza A infection in cells, in particular the effects of viral NS1 and active replication in mediating host antiviral responses. Effects of NS1 in cell lines include targeting of PKR to counteract replication inhibition (Bergmann et al., 2000) and translation shutoff (Salvatore et al., 2002), inhibiting the activation of NF-κB and IFNα/β (Wang et al., 2000), inhibiting the activation of IRF-3 (Talon et al., 2000), and inhibition of JNK and AP1 transcription factors (Ludwig et al., 2002). Anti-Influenza responses mediated by different cell-types are largely cell-type specific, as seen by differential NF-κB activation induced by Influenza in different cell types (Section 4.7.3.7). The effect of Influenza infection in respiratory epithelial cells are important in the *in vivo* infective context and in considering the pathogenesis of Influenza, as stromal cell detection and responses to viruses contribute to antiviral immunity (Sato and Iwasaki, 2004). However, DCs are also targeted and are susceptible to Influenza infection. Downstream consequences of Influenza-infected DCs shape the adaptive immune response, making DC responses to Influenza important to understand.

Immune responses to Rhinovirus have been investigated particularly in the context of asthma exacerbation in atopic patients (Papadopoulos et al., 2004) and the contribution to chemokine induction in the airways to affect immunopathology (Gern et al., 2003). Rhinovirus does not seem to induce a clearly polarised T cell response (Papadopoulos et al., 2002). Interaction with monocytes results in a broad non-specific activation of lymphocytes (Gern et al., 1996c) and inhibition of antigen-specific T cell proliferation (Gern et al., 1996b; Stockl et al., 1999). The lack of DC stimulation by Rhinovirus relative to Influenza observed here may also be linked to the lack of T cell responses to Rhinovirus. The inability to establish productive infection in monocytes and macrophages (Gern et al., 1996a) and DCs (Kirchberger et al., 2003) is in agreement with the finding here that Rhinovirus particles were not detected in Rhinovirus-exposed DCs.

Transcriptional profiling analysis has revealed that DCs are plastic in their responses to live and inactivated Influenza and Rhinovirus, and DCs are able to regulate specific responses to each virus treatment. In addition to virus-specific regulation, subsets of genes appear to be commonly regulated. The early virus-induced response (Section 4.7.3.1) is seen at early timepoints after virus exposure. These responses are not shared by DCs responding to dsRNA. The genes in this cluster encode proteins that function in intracellular transport, which may be mediated specifically by viruses in the process of viral entry and uncoating.

In contrast, the late antiviral response (Section 4.7.3.2) is induced in DCs responding to all three virus treatments as well as dsRNA. This suggests that the antiviral response shares similarity to the core activation and differentiation response of DCs, possibly mediated by IRF-3, the transcription factor that participates in mediating the antiviral program (Doyle et al., 2002). However, even within the common antiviral cluster, genes are differentially regulated by DCs responding to different viruses, in particular the lower transcript levels of the antiviral genes expressed by Influenza-infected DCs (Figure 4.13), suggesting the ability of live Influenza to downmodulate this host defence mechanism.

The lack of evidence for Rhinovirus replication in DCs suggests that downstream transcriptional events specific to Rhinovirus-exposed DCs are a result of Rhinovirus interaction with ICAM1 or other receptors on DCs, and possibly viral entry by endocytosis. Upregulated genes include MMP2, cathepsins associated with antigen processing, and HLA-C upregulated for antigen presentation (Figure 4.14). Genes associated with actin remodelling (LCP1, ACTB) are also upregulated. Such genes induced at late timepoints in Rhinovirus-exposed DCs suggests that Rhinovirus induces a delayed activation response in DCs.

Influenza infection of DCs results in a markedly distinct transcriptional program compared to treatment of DCs with inactivated Influenza. This illustrates the importance of active viral modulation of host responses, and that Influenza virus is very active at downmodulating host immune responses. Whereas previous literature have attributed the immunomodulatory NS1 viral protein as responsible for counteracting host anti-Influenza defences, results here suggest the additional involvement of proteins involved in the process of Influenza replication.

Transcriptional plasticity mediated by DCs in response to different virus treatments demonstrates that DCs are able to distinguish and regulate responses between different viruses, at the level of specific virus and dendritic cell interactions (comparing inactivated Influenza- and Rhinovirus-induced responses) and replication-dependent events (comparing inactivated and live Influenza-induced responses).

Chapter 5

Pathogen-specific dendritic cell effector functions

Introduction

In the previous chapter, hierarchical clustering analysis of virus-treated dendritic cell microarrays resulted in a sample dendogram that was broadly ordered in time, within which virus-specific responses could be identified. This suggested that DCs are transcriptionally plastic to different antigens. Statistical methods for identifying differentially regulated genes do not easily apply to time-dependent data, unless there are multiple repeat arrays as in Chapter 3. We therefore needed to find a new filtering method that captured the time-dependent nature of the experiments, without the need for over-sampling at multiple timepoints. There is much evidence in the literature that emphasise the need for further development of statistical tools and analysis methods for time series microarray data (Bar-Joseph, 2004; Slonim, 2002). Currently, the prototype time-series analysis of microarray data involves using Fourier transformations to test for periodicity, phase shift and cyclical patterns in the gene expression monitored during the yeast cell cycle (Spellman et al., 1998). However, this is not applicable to dendritic cells, as the cells respond to stimuli and antigens by maturation and differentiation, and not simply cell cycling resulting in a return to the resting state. Mature and differentiated DCs have a very different phenotype compared to immature DCs, so the very nature of the biological event being a change and transition in state requires a new method of analysis for differential gene expression over time.

A novel analysis method based on vector algebra was devised that involved the modelling of gene expression profiles as gene expression vectors which incorporated temporal relationships between samples. Basic concepts of vector algebra and its applicability to microarray analysis were confirmed, and this new method was compared to hierarchical clustering by investigating the genes commonly and differentially regulated as identified by the two methods. Overall, the new method allowed the temporal relationship of the dendritic cell samples to be incorporated into the gene expression vectors. The comparison of such expression vectors in dendritic cells responding to different viruses allowed the capturing of the complex temporal aspects of the evolution of DC responses to antigen. Candidate genes that contributed to

the common and differential responses were selected for further investigation, on the transcript and protein level.

Results

5.1 Criteria for differential gene expression

In order to identify genes that fit certain criteria, the criteria first need to be defined. One criterion is to identify genes that were differentially regulated in DCs in response to different stimuli, for example gene **X** in Figure 5.1 that is differentially regulated in DCs responding to Influenza, inactivated-Influenza, and Rhinovirus. The second criterion is to identify genes that were induced or suppressed in time, in order to filter out non-changing genes, for example gene **X** regulated by Influenza-infected DCs in Figure 5.1. Hierarchical clustering and Treeview offer a global view of these results as a pseudo-coloured representation of $\log_2$ expression ratios, allowing a quick visualisation of the overall results. However, the ability to accurately partition the data based on defined conditions is difficult. In Chapter 3, the extensive over-sampling of different timepoints and the defined temporal ordering throughout the core maturation process made it possible to use statistical methods to identify differentially regulated genes. However, because the temporal ordering of different virus responses were more complex (compare Figure 3.8 to Figure 4.10), statistical methods are not easily applicable. Therefore, using two criteria defined above, a method adapted from vector algebraic-based global gene expression analysis was devised to allow genes that fit the criteria to be identified from two calculations.

5.2 Vector analysis

The concepts of vector algebra, including vector angle (the angle between two vectors) and the magnitude of vectors, have natural and powerful interpretations in the analysis of microarray data (Kuruvilla et al., 2002). Vector angles in particular offer a rigorous method for identifying "similarity" and "dissimilarity" in gene expression patterns. The layout of microarray data as a matrix of $\log_2$ expression ratios, where rows represent gene elements (n) and columns represent experiments or transcription profiles (p), make the complementary concepts of gene vectors (variation of gene expression ratios over p experiments) and experiment vectors (experimental variation over n genes) intuitive,

and the generation of these vectors a natural operation. The nature of timecourse array data naturally lends itself to this type of analysis, by the modelling of gene expression ratios as gene vectors, defined by coordinates which are the expression ratios in each experimental timepoint. The temporal nature of the data then becomes inherent in the definition of the gene vectors. For example, the expression of gene **X** in DCs exposed to different virus treatments (Figure 5.1A) can be visualised as a two-dimensional line graph of the variation of $\log_2$ gene expression ratios across the experiments (Figure 5.1B). Alternatively, the $\log_2$ expression ratios of genes can be visualised as vectors in experimental space, where each dimension of the vector is the gene expression ratio at a given experimental timepoint (Figure 5.1C, first three dimensions shown). Importantly, the fundamental basis of three-dimensional Euclidean space can be extended to *n*-dimensional space, and therefore the number of dimensions corresponding to the number of experiments can be extended. This makes it possible to determine vector angles between the *n*-dimensional vectors of each gene in the different dendritic cell treatments.

Figure 5.1 Transformation of gene expression to gene vectors (next page)

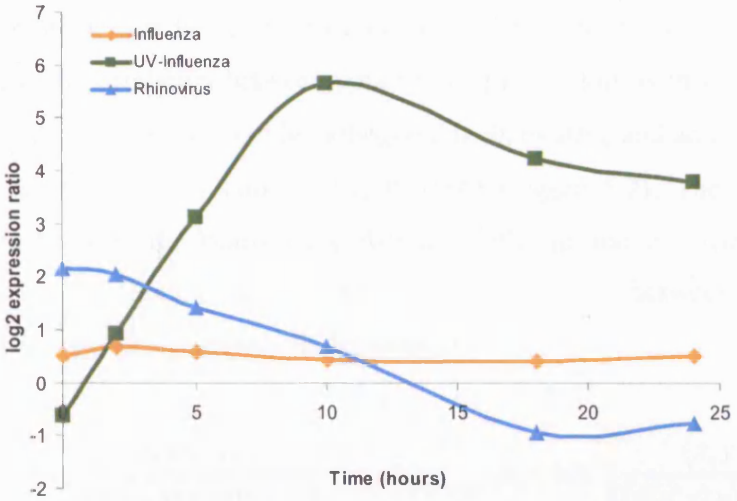
A Example of a data matrix table of a gene X as expressed in DCs treated with Influenza, UV-Influenza, and Rhinovirus, and the corresponding $\log_2$ gene expression ratios in 6 experiments across a 24 hour timecourse. Gene vectors then correspond to the variation in $\log_2$ gene expression ratios over time, where each element in the vector is the $\log_2$ gene expression ratio in one experiment.
B A line graph of $\log_2$ gene expression ratios for gene X in the 3 conditions across 6 experiments corresponding to the variation in expression over time.
C The corresponding 3 gene vectors (first three dimensions shown) transformed from $\log_2$ expression ratios plotted in 3-dimensional (experimental) space.

A

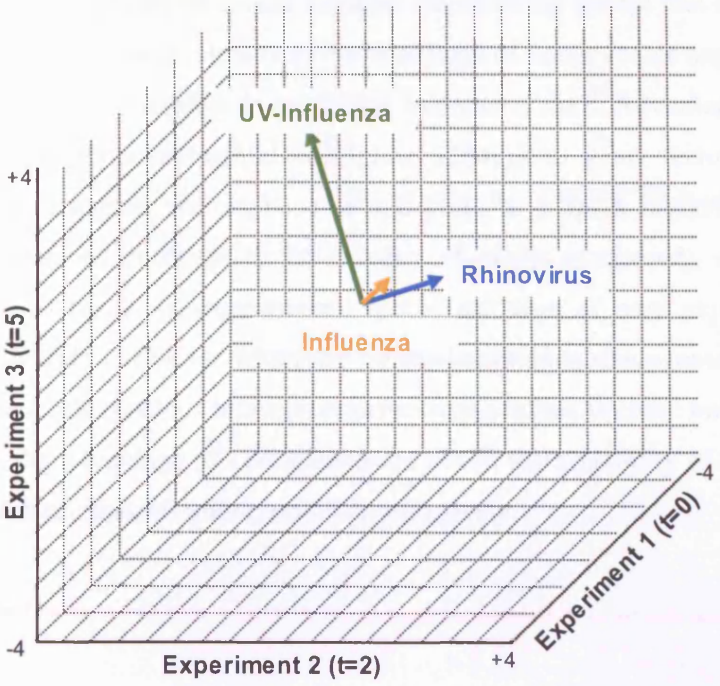
Gene X	Expt 1 (t=0)	Expt 2 (t=2)	Expt 3 (t=5)	Expt 4 (t=10)	Expt 5 (t=18)	Expt 6 (t=24)
Influenza	0.52	0.69	0.57	0.44	0.42	0.51
UV-Influenza	-0.62	0.92	3.12	5.64	4.23	3.78
Rhinovirus	2.15	2.03	1.42	0.67	-0.94	-0.77

Gene vectors

B



C



5.2.1 Vector angle and its counterpart

Hierarchical clustering of microarray data was originally devised to cluster correlation matrices (Eisen et al., 1998), and has become a popular method for microarray data analysis (Quackenbush, 2001; Slonim, 2002). The measure of similarity used in hierarchical clustering is the Pearson correlation coefficient (r), which is generated for all the pair-wise comparisons between genes or experiments and used in the subsequent production of experimental and gene dendograms (Section 1.4.4.3, Chapters 3 and 4). The complementary measure of correlation in the analysis of vectors is the concept of the vector angle (θ). If two vectors are pointing in the same direction in space they are highly correlated, and the corresponding angle between the vectors will be small. Therefore, high correlation between genes will give vectors with a small angle ($r \approx 1$, $\theta \approx 0°$). Uncorrelated vectors will be orthogonal ($r \approx 0$, $\theta \approx 90°$), and anti-correlated vectors will point in opposite directions ($r \approx 1$, $\theta \approx 180°$) (Figure 5.2). The similarity in the relationship between the Pearson correlation coefficient and the vector angle can be seen from their definitions, where cov(,) is the covariance between gene expression ratios, and $\langle , \rangle$ represents an inner or dot product:

$$r = \frac{\mathrm{cov}(x,y)}{\sqrt{\mathrm{cov}(x,x) \bullet \mathrm{cov}(y,y)}} \qquad \theta = \cos^{-1}\left(\frac{\langle x,y \rangle}{\sqrt{\langle x,x \rangle \bullet \langle y,y \rangle}} \right)$$

It is clear that r and inverse cosine function are identical, except that covariance and dot product are interchanged. However, the advantage of using vector angles is the ultimate application of vector algebra in modelling microarray data. Advantages of this method include modelling experimental vectors in addition to gene vectors utilised in this chapter. Experimental vectors become one point in a much higher-dimensional gene space (dimensions correlate to the number of genes monitored), compared to gene vectors which reside in experimental space. Addition of new experimental profiles means the addition of points where the dimension of gene space remains fixed (by gene number), and relationships between experimental profiles become much more apparent. Therefore, the advantage of vector analyses lies in the scalability of concepts to higher dimensions that captures microarray data effectively.

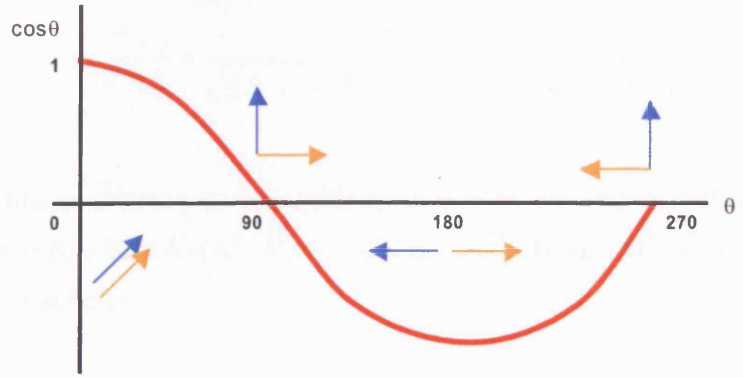


Figure 5.2 Function of cosineθ and its relationship to vector angles

The blue and orange arrows represent simplified pairs of 2-dimensional gene vectors. The relationship between gene vectors and the corresponding vector angle between the vectors relates to the cosine function, where small vector angles correspond to $\cos\theta \approx 1$, and large vector angles showing no correlation (90°, 270°) and anti-correlation (180°) correspond to $\cos\theta \approx 0$ and $\cos\theta \approx -1$ respectively. This directly relates to the Pearson correlation coefficient *r*.

5.2.2 Transforming gene expression data into vectors

The data matrix generated from Cluster gives filtered normalised $\log_2$ expression ratios. The 27 arrays used for this analysis were the 18 virus-stimulated and 9 poly(I:C)-stimulated DC samples over 24 hours. Filtered for genes that passed the signal-to-noise ratio cut-off of the negative genes for all 27 arrays, this produced 1713 gene elements for vector analysis. The data matrix was partitioned into the four conditions to be compared, and 6 pairwise comparisons for vector analysis were undertaken (Table 5.1).

To illustrate the transformation of gene expression ratios into gene vectors, let **P** and **Q** be two gene vectors, with three coordinates each. These Cartesian vectors are defined by $\mathbf{P} = (P^1, P^2, P^3)$; $\mathbf{Q} = (Q^1, Q^2, Q^3)$. The magnitude of a Cartesian vector is represented by the Cartesian scalar $|P|$ and $|Q|$, where:

$$|P| = [(P^1)^2+(P^2)^2+(P^3)^2]^{1/2}, \quad |Q| = [(Q^1)^2+(Q^2)^2+(Q^3)^2]^{1/2}$$

The inner product $\langle P,Q\rangle$ is defined by:

$$\langle P,Q\rangle = |P||Q|\cos\theta$$

Proof for the inner product is derived from the law of cosines. Rearrangement of the above formula will give the angle between vectors **P** and **Q**:

$$\cos\theta = \frac{\langle P,Q\rangle}{|P||Q|} = \frac{P^1Q^1 + P^2Q^2 + P^3Q^3}{\sqrt{[(P^1)^2 + (P^2)^2 + (P^3)^2][(Q^1)^2 + (Q^2)^2 + (Q^3)^2]}}$$

Extending this to microarray data, with 6 timepoints, the vector angle θ between gene vectors **A** and **B**, where **A**=(A^0, A^2, A^5, A^{10}, A^{18}, A^{24}); **B**=(B^0, B^2, B^5, B^{10}, B^{18}, B^{24}), can be calculated as below:

$$\cos\theta = \frac{A^0B^0 + A^2B^2 + A^5B^5 + A^{10}B^{10} + A^{18}B^{18} + A^{24}B^{24}}{\sqrt{[(A^0)^2 + (A^2)^2 + (A^5)^2 + (A^{10})^2 + (A^{18})^2 + (A^{24})^2][(B^0)^2 + (B^2)^2 + (B^5)^2 + (B^{10})^2 + (B^{18})^2 + (B^{24})^2]}}$$

If gene vector **A** represents the $\log_2$ expression ratio of a particular gene in dendritic cells responding to Influenza over time, and gene vector **B** represents the $\log_2$ expression ratio of the same gene in dendritic cells responding to inactivated Influenza over time, then the cosθ values generated would represent similarity in expression of that gene in DCs responding to Influenza and inactivated Influenza.

In practice, the vector angle of 45° was used as a cut-off for correlation; two vectors where the vector angle was smaller than 45° were deemed correlated and therefore similarly regulated by DCs responding to the different stimuli (criteria to filter at $\cos\theta > 2^{-½}$), and two vectors where the vector angle was larger than 45° were deemed differentially regulated.

5.2.3 Standard deviation of gene expression

A simple way of identifying genes that change their level of expression over time (across experiments) is to plot $\log_2$ expression ratios of n genes over time, as defined by the experiments. Genes that vary in time will have a larger standard deviation of $\log_2$ gene expression ratio over time, whereas non-changing genes will have a small standard deviation.

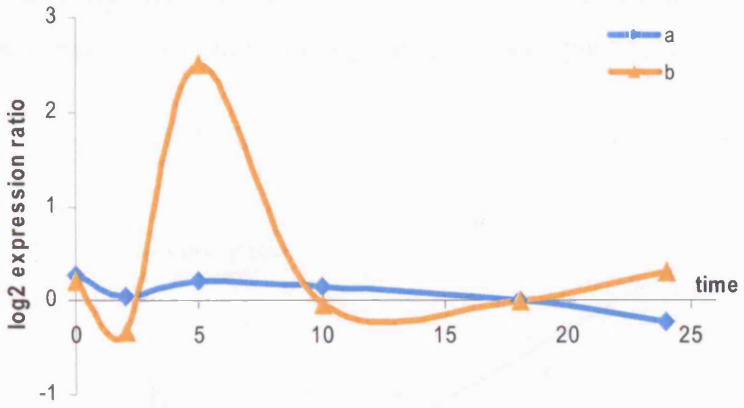


Figure 5.3 Expression ratios of two genes over time

An example of the change in expression ratios of two genes over time. The expression of gene A (blue) is non-changing, whereas gene B (orange) changes significantly. Accordingly, gene A will have a small standard deviation, and gene B a large standard deviation.

The definition of standard deviation is correlated to a second metric derived from treating gene expression as vectors, which is the ratio of magnitudes. Genes that are highly correlated, i.e. small vector angle between the vectors, may have different magnitudes. For example, two vectors defined by three coordinates (2,4,7) and (6,12,20) will have a small vector angle ($\cos\theta \approx 0.99$), but they are clearly of different magnitudes. If only correlation coefficients were used, as is the case for hierarchical clustering, this property of magnitude would be obscured. This information can be captured by the ratio of magnitudes (α) between vectors, namely the square root of the quotient of the scalar components of two vectors:

$$\alpha = \sqrt{\frac{|Q|}{|P|}} = \sqrt{\frac{[(Q^0)^2 + (Q^2)^2 + (Q^5)^2 + (Q^{10})^2 + (Q^{18})^2 + (Q^{24})^2]^{1/2}}{[(P^0)^2 + (P^2)^2 + (P^5)^2 + (P^{10})^2 + (P^{18})^2 + (P^{24})^2]^{1/2}}}$$

This ratio of magnitude (α) is an additional metric in cases where vector angles are small (close to 0°), in order to differentiate between the magnitude of the vectors. To confirm the relationship between α and standard deviation, 761 vector pairs with small vector angles (761 genes that were commonly regulated between different virus conditions, i.e. the sum of commonly regulated genes in the comparisons from Table 5.1), were compared to standard deviation values generated for the same gene vectors. There is a clear correlation between the standard deviations computed and the ratio of magnitudes α between the commonly regulated genes (Figure 5.4).

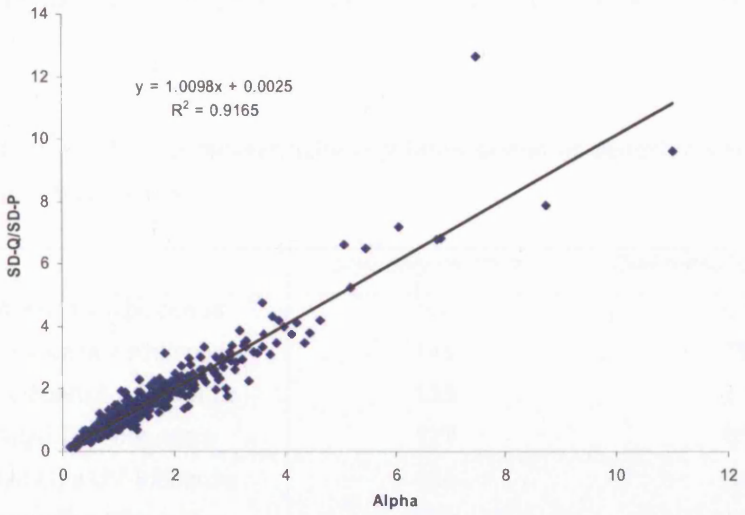


Figure 5.4 Relationship between standard deviations and ratio of magnitudes alpha

Standard deviations (SD) of expression ratios over time of similarly regulated genes were calculated for genes that are commonly regulated between virus treatments. The quotients of SDs between vector pairs were plotted on the *y*-axis, against the ratio of magnitudes α of the corresponding vector pairs on the *x*-axis. The correlation coefficient (*r*) between the quotients of SDs and α between the vector pairs was 0.957 (to 3 significant figures).

This shows that by defining standard deviations for gene vectors, the magnitude component of the gene vectors is also captured. However, the advantage of standard deviations is that non-changing genes can be filtered out, as each gene vector has a standard deviation value, compared to the ratio of magnitude which is defined for vector pairs. Therefore to identify differentially regulated genes between virus treatments over time, pairwise gene vector angles and standard deviations were calculated and filtered according to $\cos\theta < 2^{-½}$ and standard deviation of 1.5. Commonly regulated genes were filtered at a vector angle criteria of $\cos\theta > 2^{-½}$.

5.2.4 Analysis of virus-stimulated and dsRNA-stimulated DCs

An Excel workbook was designed to incorporate the filtered and normalised gene expression data from Cluster and transform these into gene vectors, and calculate the filtering parameters of vector angles and standard deviation. Considering the 4 different stimuli within these 27 arrays, 6 different binary comparisons were made to identify genes that were commonly and differentially regulated by dendritic cells over time in response to different stimuli (Table 5.1). This identified an average of 128 commonly regulated genes (n = 6, range = 45) in DCs responding to the different viruses and dsRNA treatment, and an average of 670 genes (n = 6, range = 501) that are differentially regulated in DCs responding to different virus and dsRNA treatments (Table 5.1).

Table 5.1 Commonly and differentially regulated genes in dendritic cells responding to different virus treatments

	Commonly regulated †	*Differentially regulated* ‡
Influenza v Rhinovirus	100	686
UV-Influenza v Rhinovirus	145	794
UV-Influenza v Influenza	135	716
Poly(I:C) v Influenza	127	652
Poly(I:C) v UV-Influenza	124	841
Poly(I:C) v Rhinovirus	130	339

†Commonly and ‡differentially regulated genes for the various binary comparisons are listed in Appendices 1-6 and 7-12 respectively (attached disk).

Vector analysis of gene expression ratios in DCs responding to the three virus treatments revealed genes that are commonly regulated in response to all three virus treatments (blue area in Venn diagram in Figure 5.5). Genes are also commonly regulated between pairs of virus treatments analysed, which indicate similarity between DC responses to different viruses. The two virus treatments with the most genes commonly regulated and most genes differentially regulated are inactivated Influenza and Rhinovirus, followed by inactivated Influenza and Influenza infection. Comparison between Influenza and Rhinovirus resulted in the least number of commonly regulated and differentially regulated genes. This may reflect on the absolute number of regulated genes by DCs in response to different virus treatments, where treatment with inactivated Influenza induces change in the largest number of genes in DCs, and infection with

Influenza induces change in the least number of genes. There are also genes that seem virus-specific, which are identified as differentially regulated for a particular virus treatment within the comparisons (e.g. 359 Influenza-specific genes that are common between 686 differentially regulated genes in DCs responding to Influenza and Rhinovirus, and 716 differentially regulated genes in DCs responding to Influenza and inactivated Influenza) (Figure 5.5).

Inactivated Influenza as a superior antigen compared to live Influenza was seen in phenotypic changes (Section 4.5, Table 4.2) and transcriptional responses in DCs (Section 4.7). Transcriptional regulation in lung epithelial cells responding to live Influenza and NS1-deleted Influenza (Geiss et al., 2002) also showed that live Influenza perturbed the expression of fewer number of cellular genes (84 genes significantly regulated, corresponding to ≈0.5-1% of all the cellular genes monitored on the array) compared to a NS1-deleted Influenza strain which resulted in the regulation of 115 cellular genes (corresponding to ≈0.7-1.4% of all the cellular genes monitored on the array). Results here show much larger numbers of regulated genes, for example DCs regulate a total of 719 genes in response to inactivated Influenza, which represents approximately 42% of genes that are analysed. As discussed in Chapter 4, comparison of DC responses to HeLa cell responses to live and inactivated Influenza (Geiss et al., 2001) show that genes regulated in DCs responding to inactivated Influenza are not regulated in HeLa cells, where in the latter, regulation of the same genes are dependent on Influenza replication. Both these comparisons indicate that DCs are more sensitive to antigenic stimuli, transcriptionally regulating larger numbers of genes to similar stimuli, and have a lower threshold of "activation" in that certain virus replication events are not required for the regulation of host genes.

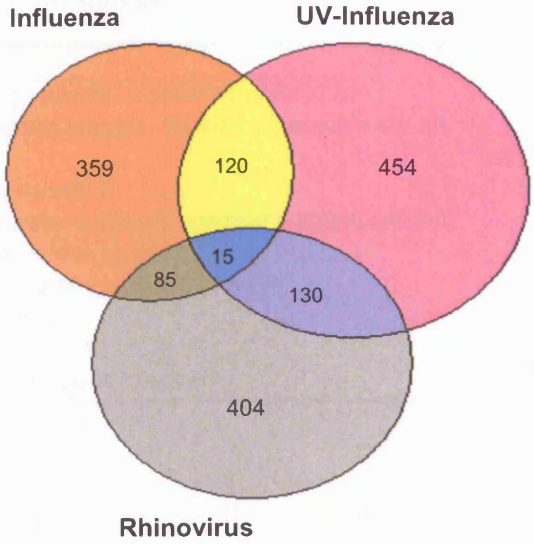


Figure 5.5 Distribution of regulated genes in dendritic cells responding to viruses

Log_2 expression ratios were compared using vector and standard deviation analyses, and commonly and differentially regulated genes were identified, giving rise to intersections of common response genes and virus-specific dendritic cell responses. Commonly regulated genes correspond to figures in Table 5.1, virus-specific genes derived from comparing differentially regulated genes between virus pairs.

5.3 Commonly regulated core antiviral response

A group of 15 genes was identified to be very closely correlated in terms of small vector angles in DC responses to all three virus stimuli (Figure 5.5). Of these 15 genes, 10 genes are commonly upregulated (Table 5.2, Figure 5.6), and 5 are commonly downregulated. All 10 upregulated genes are within the subset of the proposed "core antiviral response" identified by hierarchical clustering (Section 4.7.3.2, Figure 5.7). Of this group of 26 genes identified as commonly regulated by hierarchical clustering, over half of the genes (16 genes out of 26) are not defined as commonly regulated by the criteria of vector angles. This is because some of the genes (for example STAT1 and OAS1) are clearly downregulated by Influenza-infected DCs at late timepoints, and other genes (for example TOR1B and FLN29) are not upregulated by DCs in response to Rhinovirus (Figure 5.7). This illustrates the sensitivity of vector angles to identify more precisely co-expressed genes. The 10 genes identified as commonly upregulated as defined by the criteria of vector angles ($\cos\theta > 2^{1/2}$) are shown in Figure 5.6.

Table 5.2 Genes commonly upregulated by dendritic cells in response to viruses

Gene Name	*Gene Symbol*	*Genbank*
Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)	TAP1	H23432
Apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3B	APOBEC3B	T71211
Metallothionein 3	MT3	AI418147
Ubiquitin-conjugating enzyme E2L 6	UBE2L6	H93314
Tumour necrosis factor receptor subfamily, member 5 (CD40 antigen)	TNFRSF5	R49883
Interferon induced transmembrane protein 1 (9-27)	IFITM1	H00943
Interferon induced transmembrane protein 2 (1-8D)	IFITM2	W37877
Proteasome activator subunit 2	PSME2	AA029750
Insulin-like growth factor 1 receptor	IGF1R	H00849
DnaJ (Hsp40) homolog, subfamily A, member 1	DNAJA1	H06531

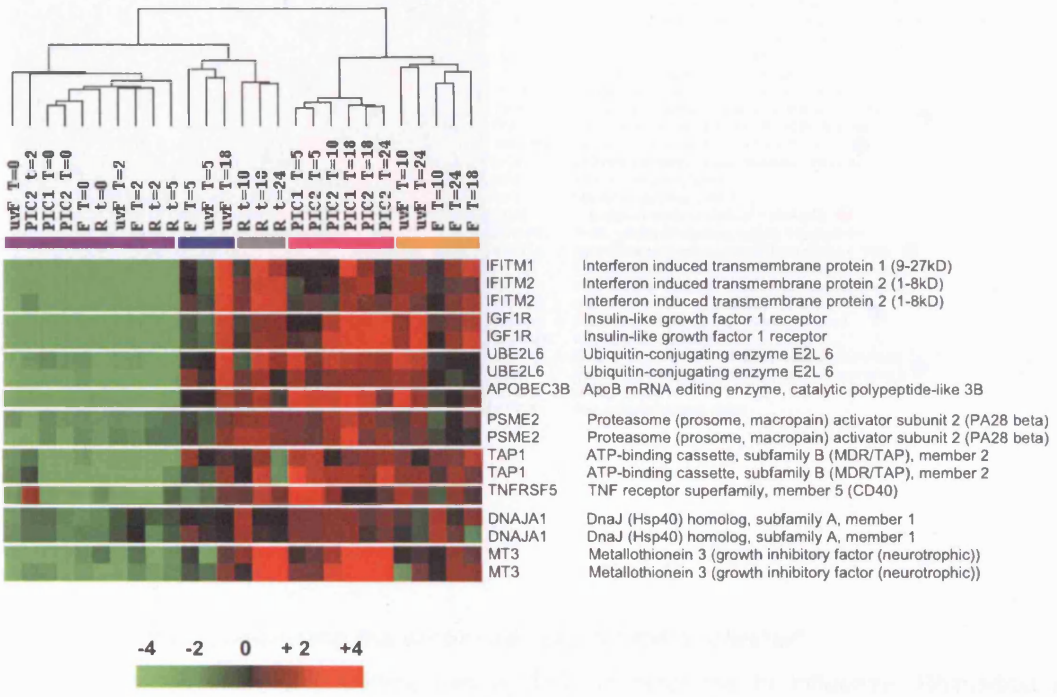


Figure 5.6 Commonly upregulated genes by DCs in response to viruses

Treeview visualisation of the 10 genes identified by vector analysis as being commonly upregulated in DCs responding to different virus treatments. This group of genes are within the proposed antiviral cluster identified in Section 4.7.3.2 (and shown in Figure 5.7).

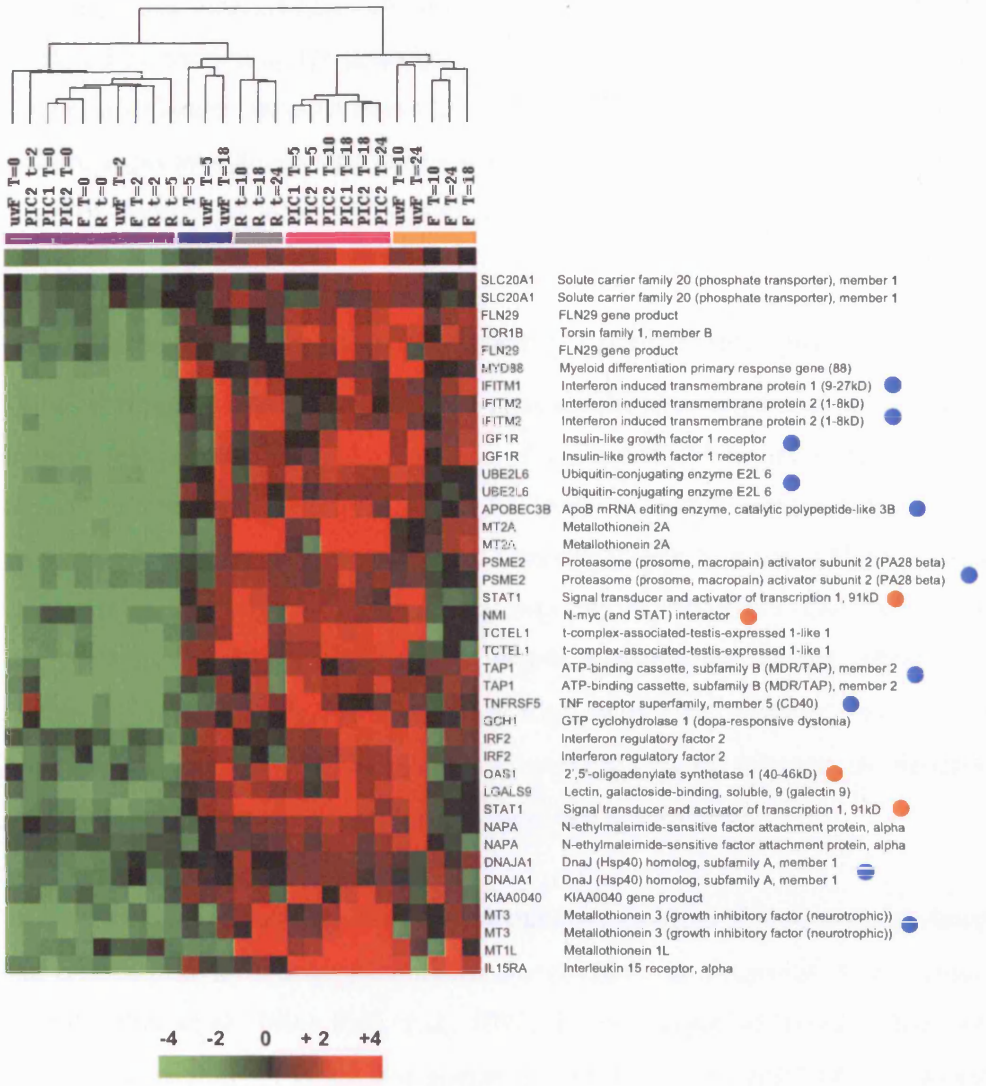


Figure 5.7 Genes comprising the proposed core "antiviral cluster"

Cluster of genes commonly upregulated by DCs in response to Influenza, Rhinovirus and dsRNA treatment. Blue dots indicate the 10 genes commonly upregulated by DCs responding to all three virus treatments (Figure 5.6) as identified by vector analysis. Orange dots indicate known interferon-response genes (see Section 5.3.2).

The 10 commonly upregulated genes showing a common expression pattern may be induced by a cytokine that is secreted by DCs in response to the virus treatments, resulting in the activation of common transcription factors, and the coordinated transcriptional upregulation of these genes (Figure 5.7). These possibilities were explored by examining the promoter regions of these genes to identify the presence of common promoter regions where transcription factors may bind, and measuring DC culture supernatants for secreted type I interferon, a known cytokine for modulating some of these genes as part of the antiviral response.

5.3.1 Genome analysis of commonly upregulated genes

Genes that are co-expressed may be co-regulated by the same transcription factor(s). To explore this possibility, DNA sequences of regions 1000 base pairs (bp) 5' upstream of the 10 commonly upregulated genes in Table 5.2 were extracted from the human genome and input to the Transcription Element Search Software (TESS) to search for transcription factor binding sites (TESS: http://www.cbil.upenn.edu/tess). In particular, the presence of DNA sequences to which transcription factors IRF-1 (interferon regulatory factor-1), ISGF-3 (interferon-stimulated gene factor-3), and NF-κB bind were noted (Figure 5.8), as these are probably most relevant in dendritic cells orchestrating a common response to various virus treatments.

IRF-1 functions to activate the IFNβ promoter and other interferon-stimulated genes, but is not a primary transcriptional activator of either, as it has only weak transcriptional activity (Pine et al., 1990; Reis et al., 1992). Known targets of IRF-1 include TAP1 and PSME2, as well as IFNβ. ISGF-3 comprises IRF-9, STAT1 and STAT2 (Qureshi et al., 1995), and functions to mediate downstream effects of IFN signalling and activates the interferon-stimulated genes (ISGs) (Kessler et al., 1990; Schindler et al., 1992). STAT1 itself has a binding site for ISGF-3 in its promoter sequence to enable positive feedback for interferon signalling. Although STAT1 was identified as part of the proposed antiviral cluster (Section 4.7.3.2), STAT1 is not further upregulated by Influenza-infected DCs at later timepoints (10 to 24 hours after virus infection), and was not identified by vector and standard deviation analyses as commonly upregulated by DCs (Figure 5.6). NF-κB activates numerous immune response genes, as discussed in the context of the DC core activation and differentiation program, and is also involved in

mediating the antiviral effects of interferon as well as participate in IFNβ gene regulation (Hiscott et al., 2003; Lenardo et al., 1989).

Of the 10 genes commonly upregulated by dendritic cells, 8 genes had DNA sequences in their promoter regions to which transcription factors IRF-1, ISGF-3, and NF-κB can bind (Figure 5.8). The two genes which did not have consensus sequences for these transcription factors in their promoter regions are IGF1R and IFITM2. No one transcription factor binding site is present in all of the 8 genes. Five out of 8 genes share consensus sequences for NF-κB, and 4 share consensus sequences for interferon-related transcription factors IRF-1 and ISGF-3. TAP1 and UBE2L6 share consensus sequences for both NF-κB and IRF-1.

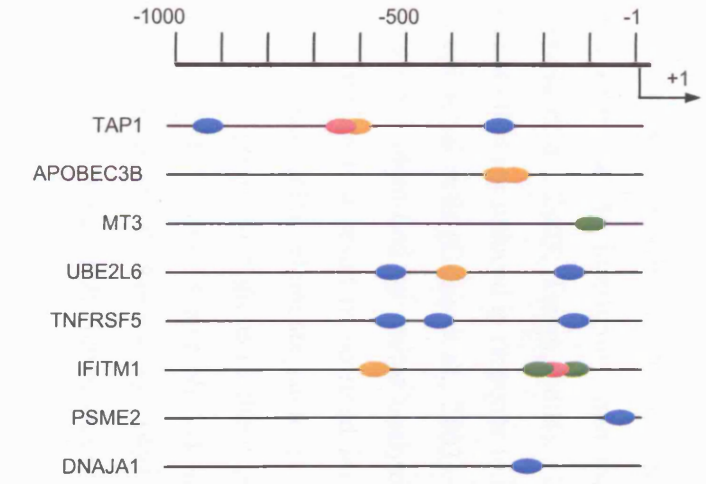
To explore the possibility of the role of IRF-3 and IRF-7 in the activation of the 10 commonly upregulated genes, consensus sequences that matched IRF-3 and IRF-7 binding sites were also searched in the promoter regions (Yan et al., 2004). These two IRF transcription factors is believed to be activated independently of interferon signalling, and IRF-3 activation directly downstream of virus entry has been demonstrated (Bose and Banerjee, 2003; Hiscott et al., 1999; Servant et al., 2002), resulting in the production of IFNβ. The production of IFNβ results in autocrine signalling via interferon receptors, and downstream signalling results in the activation of IRF-7, and the transcription of IFNα and further activation of IFNβ (Nakaya et al., 2001; Sato et al., 1998; Yang et al., 2004). However, only the promoter regions of TAP1 and IFITM1 had consensus sequences for IRF-3 and IRF-7 respectively (Figure 5.8).

This analysis shows that the promoter regions for 10 genes commonly upregulated in dendritic cells do not share common transcription factors. There are several possible reasons for this. Firstly, it is possible that with the current state of knowledge, these 10 genes do share a common transcription factor that is not yet represented in the TRANSFAC database, or a transcription factor that is still unknown. Secondly, gene regulation is multiplex, and individual genes are regulated by multiple transcription factors, which together enhance or suppress transcription. Therefore, genes may be co-expressed as a result of a combination of effects of multiple transcription factors that need not be shared across all genes. Furthermore, transcription factors that bind to

enhancer regions may be more than 1000 bp upstream of the genes they regulate, which were not taken into account in this analysis. Cross-species promoter analysis may also improve promoter prediction by incorporating information about promoter sites that are conserved across other mammalian species, increasing the likelihood of such promoter sites being functional *in vivo* (Frazer et al., 2003; Sandelin et al., 2004). However, the presence of interferon-regulated transcription factors and NF-κB in the 10 upregulated genes strongly suggest that these genes are co-regulated. NF-κB can induce the transcription of IFNβ (Lenardo et al., 1989), and signalling downstream of the interferon receptor also results in the activation of NF-κB (Hoebe et al., 2003b). Therefore, the possibility of secreted interferon acting in an autocrine manner to mediate the induction of the 10 commonly upregulated genes in DCs responding to different virus treatments was investigated.

Figure 5.8 Promoter analysis of the commonly upregulated genes in DCs responding to different virus treatments (next page)

Nucleotide sequences 1000 base pairs 5' upstream of the 10 commonly upregulated genes derived from Ensembl (www.ensembl.org) were searched for consensus binding sites for transcription factors NF-κB, IRF-1, ISGF-3 in Transcription Element Search Software (TESS) Transfac database (http://www.cbil.upenn.edu/tess/). Binding sites for IRF-3 and IRF-7 were analysed individually with sequences from (Yan et al., 2004). The 8 genes whose upstream regions contained binding sites for the above transcription factors are shown. Degenerate nucleotides indicated are as follows: W=A/T, N=A/C/G/T, S=C/G, Y=C/T.



Consensus sequences

NF-κB	IRF1	IRF3/7	ISGF1/3
GGGAMTNYCC	CTTTCTCTTT	GAAASSGAAANY GAAWNYGAAANY	CTTTCAGTTT GCTTCAGTTT

TAP1	(-290)	GGGGaAAGTCCC (R)
	(-943)	GgAAAGTCCCC (R)
	(-605)	AAAGaGAAAG (R)
	(-607)	GAAASSGAAANY
APOBEC3B	(-263)	CTTTCTCTTT
	(-269)	CTTTCTCTTT
MT3	(-95)	AAACtGAAAG (R)
UBE2L6	(-500)	TgGGGGCTTCCCC
	(-393)	AAAGAGAAAG (R)
CD40	(-106)	GGGAATtTTC
	(-392)	GGGAAtTTCC
	(-500)	GGGAATTTCC
IFITM1	(-519)	GGGAATtTTC (R)
	(-171)	GGAAANtGAAACT
	(-135)	AAACTGAAAg (R)
	(-137)	GAAWNYGAAANY
PSME2	(-13)	CTTTCTcTTT
DNAJA1	(-201)	GgAGATTCCAC

5.3.2 Type I Interferon production by DCs

Type I interferons (IFNα/β) are produced in large quantities upon viral infection in many different cell types, and directly exert their antiviral functions in two ways: to induce antiviral activity in uninfected cells, and to induce apoptosis in infected cells. In this way, the inhibition of viral replication and the selective induction of apoptosis in virally infected cells constitute the first line of defence of the immune system against viruses. During viral infection, transcriptional induction of the IFNα/β genes is achieved through the activation of two transcription factors of the interferon-regulatory factor (IRF) family, IRF-3 and IRF-7 (Lin et al., 1998b; Sato et al., 1998). The virus-induced production of IFNα/β transmits signals to induce numerous target genes, termed the IFN-inducible genes (or IFN-stimulated genes ISGs). This elicits an antiviral response in both an autocrine and paracrine manner (Stark et al., 1998). The mechanisms of transcriptional activation of the IFN-inducible genes have been studied extensively (Stark et al., 1998).

Dendritic cells have been heavily investigated in terms of their ability to produce interferons, their responses to interferons, and the variety of stimuli for interferon production (Diebold et al., 2003; Tough, 2004). Global gene expression analysis has also identified genes that are induced in response to interferons in a variety of cell types, for example in epithelial cells (Geiss et al., 2003). The subset of 10 genes within the core antiviral response identified by vector analysis indicate the possibility that their transcriptional induction is a result of secreted interferon because of the presence of interferon-related transcription elements such as the interferon-stimulated response element (ISRE) in the promoter regions of these genes (Figure 5.8). Furthermore, the transcription factors IRF-1, ISGF-3 and NF-κB have been shown to be induced by interferons (Kessler et al., 1990; Sims et al., 1993; Yang et al., 2000). Therefore, we assayed virus-treated DC culture supernatants over 24 hours for the presence of type I interferons.

Culture supernatants from DCs infected with Influenza, exposed to inactivated Influenza, Rhinovirus, and inactivated Rhinovirus, were collected at 5 timepoints after virus exposure. Immunoassays for IFNβ and IFNα (13 subtypes) clearly showed differential IFN production in DCs that is dependent on the virus stimulus (Figure 5.9).

The highest levels of both IFNα and IFNβ was produced by DCs responding to inactivated Influenza, and the kinetics of production are such that the peak of IFNβ production is at 10 hours, in contrast to the levels of IFNα which steadily increased over 24 hours.

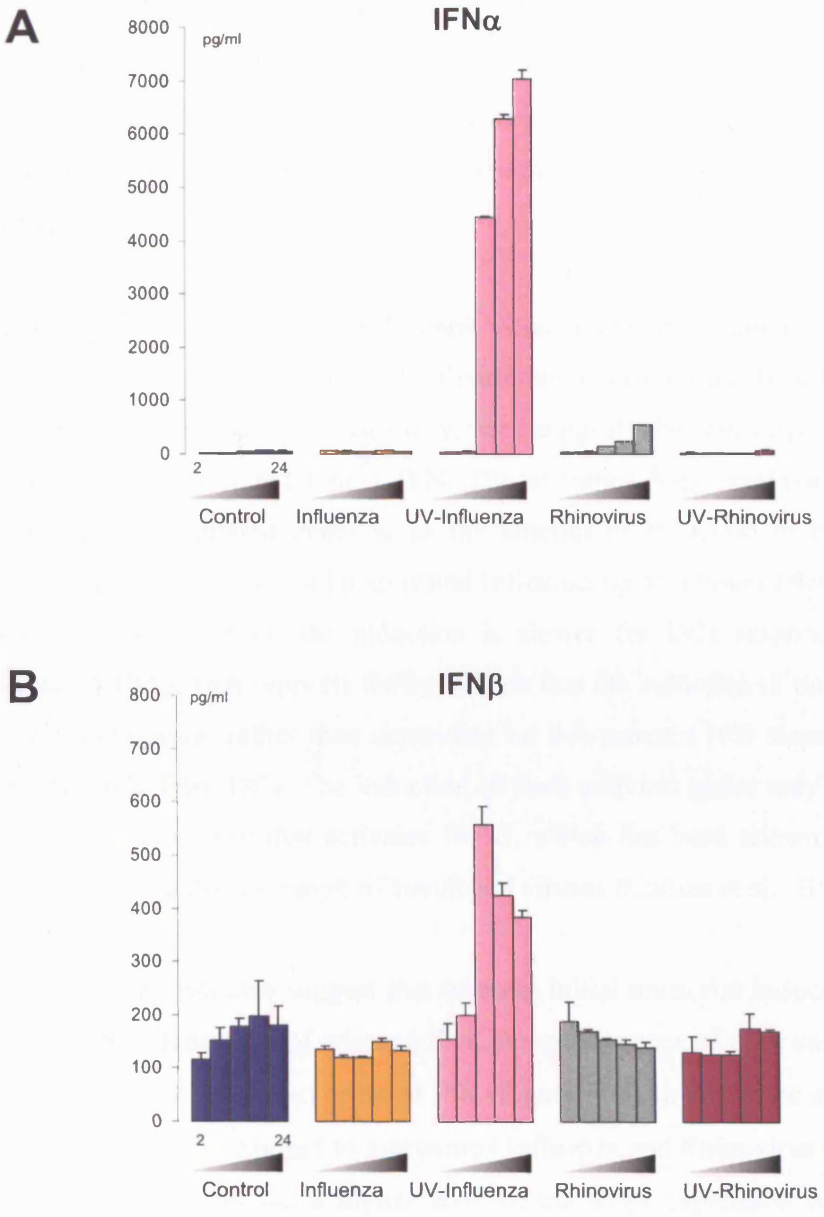


Figure 5.9 Production of IFNα and IFNβ in DCs responding to different virus treatments

Type I IFN was measured by two sandwich immunoassays, to detect 13 subspecies of IFNα (**A**) and IFNβ (**B**). DC culture supernatants were harvested at 5 timepoints after virus or mock-stimulation (2, 5, 10, 18, 24 hours). Bars indicate the levels of IFN (pg/ml) detected. Error bars were derived from the standard deviations of triplicates performed in the assay.

The results of the type I Interferon immunoassay suggests that DCs exposed to the different virus treatments do not all produce interferons to the same extent. DCs treated with inactivated Influenza produce the most IFNα; the levels of IFNα produced are almost 7 times higher than the levels produced by DCs exposed to Rhinovirus by 24 hours. DCs exposed to the other virus treatments, Influenza and inactivated Rhinovirus, do not produce significant amounts of IFNα relative to control mock-infected DCs. Interferon-β is also produced to the highest levels in DCs exposed to inactivated Influenza, and the levels of IFNβ reach maximum at 10 hours after virus exposure. In contrast, DCs exposed to other virus treatments do not produce significant levels of IFNβ.

Given that DCs exposed to the different virus treatments commonly upregulated the 10 putative "antiviral" genes, the lack of correlation between levels of IFN production and the DC transcriptional responses to viruses suggests the transcriptional response to be independent of secreted type I IFN. The averaged $\log_2$ expression ratios of the 10 commonly upregulated genes show the kinetics of induction of these genes in DCs responding to Influenza and inactivated Influenza up to 5 hours after virus exposure are very similar; however, the induction is slower for DCs responding to Rhinovirus (Figure 5.10A). This supports the hypothesis that the induction of these genes is specific to virus exposure, rather than dependent on downstream IFN signalling as a result of secreted IFN from DCs. The induction of such antiviral genes may be a result of early events in virus entry that activates IRF-3, which has been shown in lung fibroblasts responding to a diverse range of enveloped viruses (Collins et al., 2004).

However, the data also suggest that whereas initial transcript induction (between 0 and 5 hours) is independent of released IFN, the maintenance of gene expression (between 5 and 24 hours) is related to released IFN (Figure 5.10A), and more specifically to IFNα. For example, DCs exposed to inactivated Influenza and Rhinovirus both produce IFNα, and these DCs maintain a higher level of transcript expression of the 10 commonly upregulated genes, possibly as a result of IFN autocrine feedback and downstream signalling. DCs infected with Influenza do not secrete IFNα, and there is no further upregulation of these transcripts after 5 hours.

The hypothesis of IFN signalling functioning at later timepoints may be illustrated in the expression kinetics of known interferon-response genes that are clustered within the proposed antiviral cluster (orange dots in Figure 5.7). These genes include STAT1, IRF2, 2'5'-oligoadenylate synthetase 1 (OAS1), and N-myc and STAT interactor (NMI). These have all been shown to be induced by type I interferons (Benech et al., 1987; Lebrun et al., 1998; Shuai et al., 1994; Zhou et al., 2000). While the induction of the transcripts (between 0 and 5 hours) of interferon response genes is similar to the 10 commonly upregulated genes, being rapid for DCs exposed to Influenza and inactivated Influenza, but delayed for Rhinovirus, the levels of IFN-response genes after 5 hours show the same interferon-dependency seen for the 10 commonly upregulated genes. The effects of IFN signalling can be seen in the higher levels of expression of IFN-response genes in DCs treated with UV-Influenza at 5 hours to 10 hours. These transcripts steadily increase over 24 hours, reflecting increasing amounts of IFNα detected in culture supernatants samples of DCs exposed to inactivated-Influenza. In the case of replication-competent Influenza, the initial burst of transcript induction of IFN-response genes is not maintained. Finally, for DCs exposed to Rhinovirus, after a delay in transcript induction, the level of expression is maintained at later timepoints.

Taken together, the data suggests that induction of this proposed antiviral response, including upregulation of the 10 commonly upregulated genes in DCs responding to all three virus treatments, is downstream of early events of virus exposure and virus entry, and is not induced as a result of IFN feedback signalling. However, secreted type I interferons may play a role in the maintenance of this antiviral response, where IFN feedback signalling functions to sustain and amplify the transcript levels of these genes.

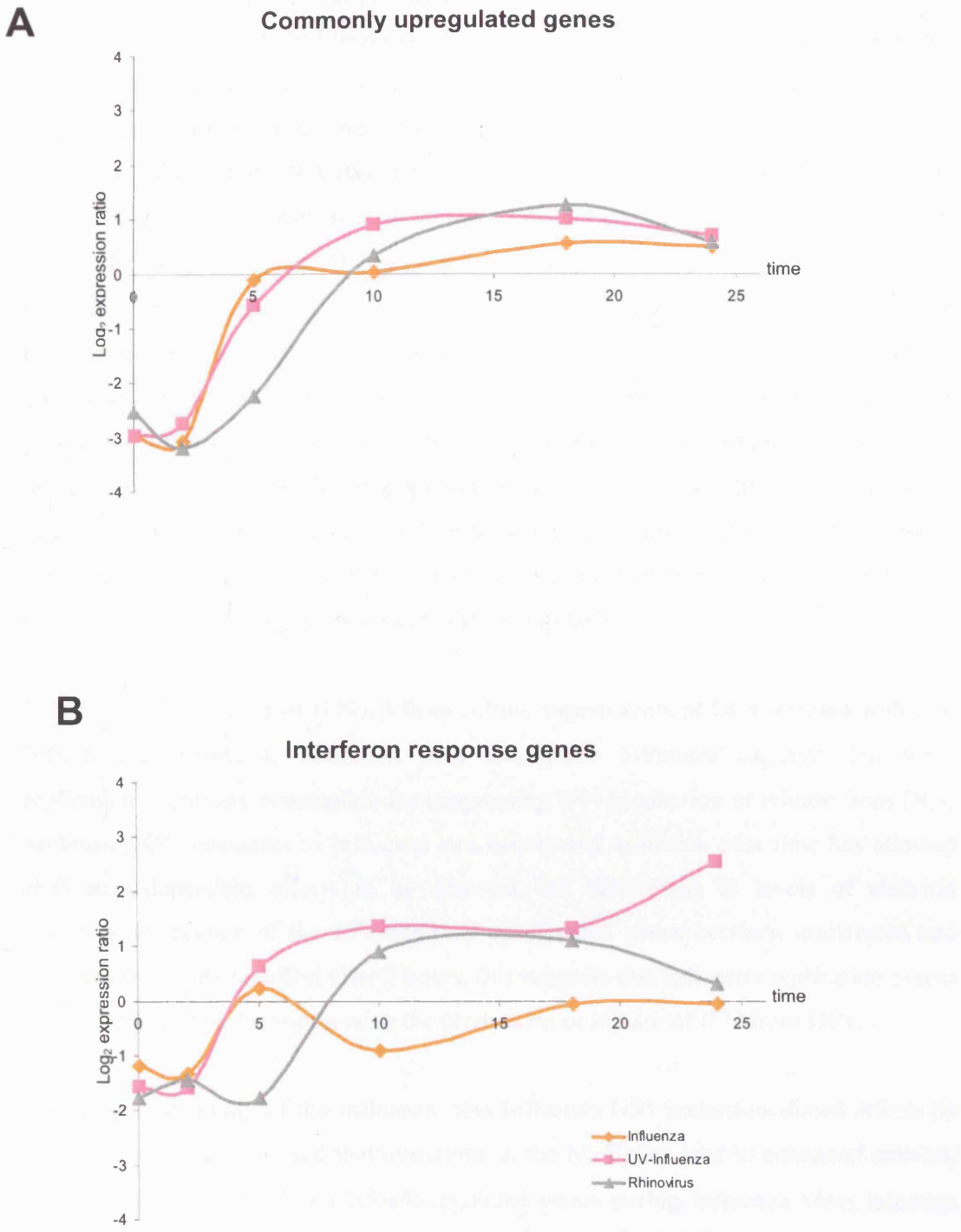


Figure 5.10 Transcript expression of IFN-independent and IFN-dependent genes

Normalised log_2 expression ratios of (**A**) the 10 commonly upregulated genes, and (**B**) four characterised interferon-response genes (indicated in Figure 5.7) were averaged and plotted over time to show their expression in DCs responding to Influenza infection (orange ♦), inactivated Influenza (pink ■) and Rhinovirus (grey ▲) exposure.

The kinetics of induction of the proposed antiviral genes suggest that even though this is classified as a core antiviral response, there are still subtle variations dependent on the initial virus stimulus. The similarity in the antiviral transcript induction in DCs responding to Influenza and inactivated Influenza, compared to the delayed antiviral transcript induction in DCs responding to Rhinovirus, suggests that this is a virus-dependent effect. The delay is evident and consistent for all the genes in this antiviral cluster. Rhinovirus-exposed DCs also secrete lower levels of IFNα. This may suggest that downstream events triggered in DCs by Rhinovirus binding and subsequent endocytosis is slower in DCs, and/or induces a less vigorous antiviral response compared to Influenza. It is still unclear whether Rhinovirus is capable of establishing a productive infection in our DC system; though Rhinovirus particles could not be detected in DCs, there is still the possibility that Rhinovirus particles are produced but not detected by the antiserum used. Alternatively, a slower induction of antiviral transcripts may suggest a slow replication cycle, and that later timepoints should be investigated for detecting Rhinovirus replication in DCs.

In contrast, the absence of IFNα/β from culture supernatants of DCs infected with live Influenza compared to treatment with inactivated Influenza suggests that virus replication events are responsible for suppressing IFN production or release from DCs. Measuring DC responses to Influenza and inactivated Influenza over time has allowed replication-dependent effects to be detected. As differences in levels of antiviral transcript expression of the 10 commonly upregulated genes between inactivated and live Influenza only manifest after 5 hours, this suggests that Influenza replication events may be responsible for suppressing the production or release of IFN from DCs.

The microarray study of the Influenza- and Influenza NS1 mutant-mediated effects on lung epithelial cells showed that mutations in the NS1 gene lead to enhanced antiviral gene expression of IFN and NF-κB-regulated genes during Influenza virus infection (Geiss et al., 2002). This is in agreement with our results which showed marked type I IFN production and greater expression levels of IFN-response genes in DCs exposed to inactivated Influenza compared to Influenza-infected DCs. This may be explained by the ability of NS1 to inhibit the activation of IRF-3 (Talon et al., 2000), and PKR (Bergmann et al., 2000) by sequestering dsRNA, and also inhibit downstream NF-κB activation and type I IFN induction (Wang et al., 2000).

5.4 Differentially regulated genes

Even in the core antiviral response, differential transcriptional regulation of genes by DCs responding to the three virus treatments was already evident. This strongly suggests that DCs are able to differentiate between virus stimuli. Analysis of genes that are classified as differentially expressed revealed TNFα to be differentially regulated between DC responses to Influenza and Rhinovirus, and between responses to Influenza and inactivated Influenza (Appendices 7-9, see attached disk). TNFα is thought to have antiviral functions by synergising with interferons to mediate an antiviral state (Seo and Webster, 2002). It is also one of the key mediators in the immunopathology associated with Influenza infection (Cheung et al., 2002). Viruses that encode factors which target TNF-dependent activities, for example poxviruses that encode soluble versions of TNF receptors (Introduction Section 1.2.3.2), and a viral TNFR gene encoded by myxoma virus (Schreiber et al., 1996), also supports an antiviral role for TNFα. Models of adenovirus infection in mice have also indicated the importance of TNFα in mediating early DC maturation and the activation of the T_H1 pathway (Trevejo et al., 2001).

TNFα is found in a cluster with NF-κB responsive genes identified in Section 4.7.3.7 (Figure 5.11). There are many known inducers of NF-κB activity, including TNFα itself, IL-1 and TLRs (Introduction Section 1.1.4). The clustering of TNFα and NF-κB, additional components downstream of the TNFα and NF-κB signalling pathway (e.g. TANK, TRAF1), and NF-κB-activated genes (e.g. BIRC3, CD83, ICAM-1), suggest that this cluster may represent the TNFα signalling pathway leading to activation of NF-κB and downstream responses. However, Influenza, Rhinovirus, and dsRNA may also induce NF-κB activity independently of TNFα (Alexopoulou et al., 2001; Ronni et al., 1997; Zhu et al., 1996). Vector analysis also showed NF-κB to be differentially regulated in DCs exposed to different viruses (Appendices 7-9, see attached disk). Therefore, TNFα transcript expression and production in DCs exposed to different virus treatments was investigated by real-time RT-PCR and TNFα immunoassay respectively.

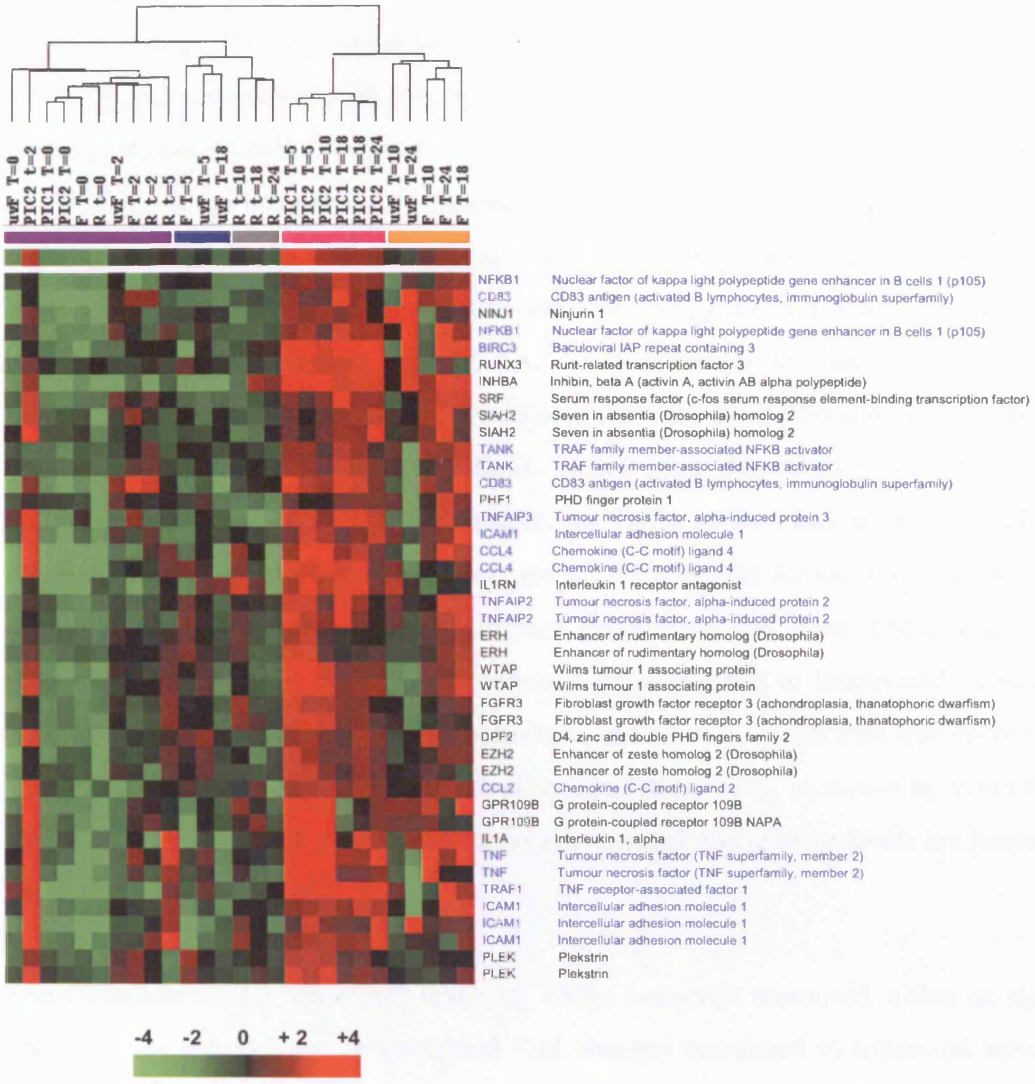


Figure 5.11 Virus-specific gene regulation in the NF-κB gene cluster

Cluster of genes in the NF-κB cluster, strongly upregulated by DCs exposed to dsRNA and Influenza, variably upregulated by DCs exposed to inactivated Influenenza, and showing minimal change in expression in DCs exposed to Rhinovirus. Genes in blue are associated with NF-κB signalling and NF-κB-induced genes

5.4.1 Production of TNFα by DCs

To measure the transcript levels of TNFα, real-time RT-PCR was performed using the Taqman system. The correlation between TNFα transcript levels as seen by real-time RT-PCR and microarrays is shown in Figure 5.12, comparing raw background-corrected signal intensities of sample (Cy5) TNFα from the microarrays to fold changes in TNFα transcript compared to timepoint zero by RT-PCR. It should be noted that the signal intensities for the TNFα duplicate spots for the array of Influenza-infected DCs at 10 hours post-infection (t=10) are not consistent, indicative of a noisy spot in one of the duplicate spots (circled in Figure 5.12C, 5.13). However, this inconsistency is not seen in the other virus arrays, as the signal intensities for the TNFα duplicate spots are all in good agreement (Figure 5.12C). When this discrepancy is taken into consideration, the raw Cy5 signal intensities for TNFα in the virus arrays shows high correlation between microarray and real-time RT-PCR results for the TNFα transcripts (Figure 5.13). DCs infected with Influenza accumulate the most TNFα transcript between 0 and 2 hours, which then decreases. DCs exposed to inactivated Influenza accumulate the most TNFα transcript between 2 and 5 hours, which then also decreases. There is lower TNFα expression in DCs exposed to Rhinovirus, as shown by lower Cy5 signal intensities and RT PCR transcript levels, although the relative levels are less well correlated.

The difference in the "absolute" levels of TNFα transcript measured, either as signal intensities by microarrays or calculated fold changes compared to timepoint zero by real-time RT-PCR may reflect the larger dynamic range of real-time RT-PCR in measuring transcript levels. However, comparison of the kinetics of TNFα transcript accumulation in DCs exposed to different virus treatments over time show much similarity between microarray results and real-time RT-PCR (Figure 5.13). The levels of TNFα protein were also determined by a sandwich immunoassay (Methods Section 2.7.1) which correlated with real-time RT-PCR results (Figure 5.14).

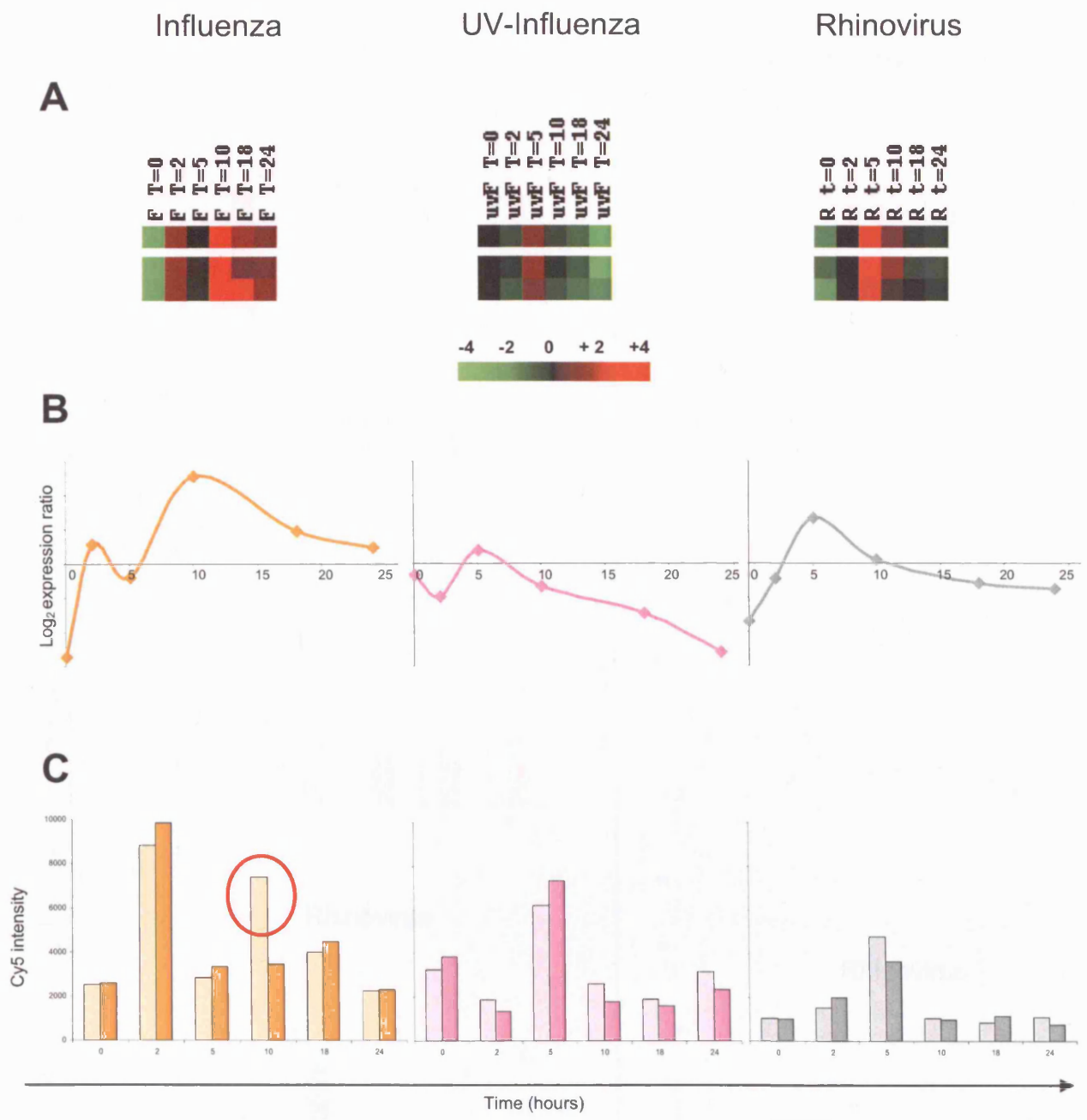


Figure 5.12 Microarray data for TNFα

A TreeView visualisation of normalised log_2 expression ratios for duplicate copies of TNFα across 18 virus arrays, showing pseudocoloured representation of up- (red) and down- (green) regulation of TNFα in DCs responding to different viruses over 24 hours.
B Corresponding line graphs of the averaged normalised log2 expression ratios for TNFα across virus arrays, showing expression of TNFα in Influenza-infected DCs (orange), inactivated Influenza-exposed DCs (pink), and Rhinovirus-exposed DCs (grey).
C Corresponding raw signal intensity values for Cy5 (sample) values of TNFα of the virus arrays. Raw signal intensity values for Cy5 (the fluorophore with which the sample RNA is labelled) TNFα were plotted for each of the duplicate spots, in virus-treated DC arrays (Influenza – orange, inactivated Influenza – pink, Rhinovirus – grey). Comparison between signal intensity of the duplicate spots indicates the duplicate spot correlation or background noise associated with the particular spot. The red circle indicates the lack of correlation in the duplicate spots for TNFα in the array hybridised with Influenza-infected DCs at 10 hours.

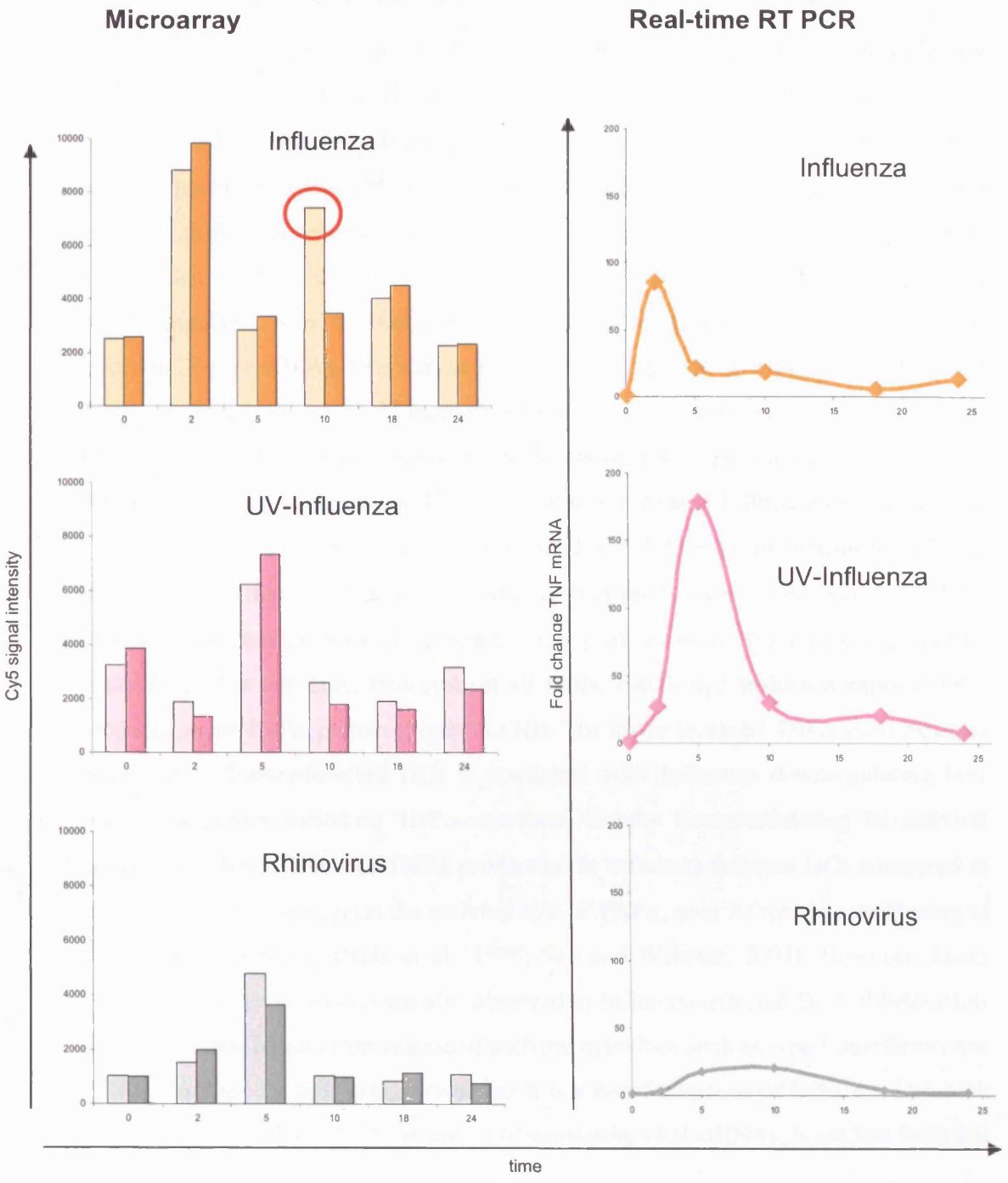


Figure 5.13 Comparison between microarray and real-time RT PCR data for TNFα

The sample raw Cy5 intensity values for the 18 virus-treated DC arrays from Figure 5.12C are shown on the left, compared to real-time Taqman results for TNFα on the right. This shows the transcript expression of TNFα in virus-treated DCs over time in response to Influenza (orange), inactivated Influenza (pink), and Rhinovirus (grey) as measured by microarrays and by real-time RT-PCR. Transcript levels for TNFα from real-time RT PCR results are derived from calculated fold-changes in TNFα transcript compared to levels of TNFα at 0 hours in corresponding virus-treated DCs.

5.4.1.1 Production of TNFα by DCs exposed to Influenza

At the transcript and protein level, both live Influenza and inactivated Influenza stimulate DCs to upregulate TNFα transcript which results in the release and detection of TNFα in DC culture supernatant (Figure 5.14). The kinetics of transcript induction and the absolute levels of TNFα transcript between Influenza and inactivated Influenza treatment differ. Inactivated Influenza triggers a slower induction of TNFα transcript in DCs, which reaches peak levels at 5 hours post-exposure. In contrast, live Influenza rapidly induces TNFα transcript in DCs reaching a maximum level 2 hours post-infection (Figure 5.14A). DCs exposed to inactivated Influenza induce higher levels of TNFα transcript, almost 2-fold more than DCs infected with Influenza. This difference in levels of TNFα transcript translates to levels of TNFα protein production that is detected in culture supernatants. DCs exposed to inactivated Influenza produce almost 2.5 times more TNFα compared to DCs infected with Influenza. In contrast to different transcript induction, the kinetics of TNFα protein production is very similar in DCs exposed to live and inactivated Influenza, where peak levels of TNFα are detected at 10 hours post-virus exposure. However, at all times, inactivated Influenza exposed DCs produced more TNFα protein (Figure 5.14B). The lower levels of TNFα transcript and protein in Influenza-infected DCs is consistent with Influenza downregulating host cellular responses including TNFα secretion, thereby downmodulating its antiviral activity. The lower levels of TNFα production in Influenza-infected DCs compared to inactivated Influenza support the antiviral role of TNFα, over its role in contributing to Influenza pathogenesis (Nain et al., 1990; Seo and Webster, 2002). However, lower levels of IFNα/β production are also observed in Influenza-infected DCs. Whether this is a specific inhibition of the release of antiviral cytokines such as type I interferons and TNFα mediated by Influenza, or whether it is a broad response of Influenza to inhibit translation of cellular mRNAs in favour of translating viral mRNAs, is unclear from this data.

5.4.1.2 Production of TNFα by DCs exposed to Rhinovirus

DCs exposed to Rhinovirus and inactivated Rhinovirus also induce some TNFα transcript, but to a much lower level (Figure 5.14A). Similar to the production of IFNα, DCs exposed to Rhinovirus induce TNFα transcript and protein with slower kinetics compared to Influenza-infected and inactivated Influenza-exposed DCs. This transcript induction of TNFα in Rhinovirus-exposed DCs translates to small levels of TNFα production in culture supernatants, which reaches peak levels at 10 hours post-virus exposure (Figure 5.14B). The delayed kinetics is evident, as Rhinovirus-exposed DCs produce the most TNFα between 5 to 10 hours, compared to Influenza-exposed DCs which produce the most TNFα between 2 to 5 hours (Figure 5.14B). The initial induction of TNFα transcript in DCs exposed to inactivated Rhinovirus does not translate to TNFα protein production, with TNFα levels similar to control unstimulated DC culture supernatants.

Overall, it is clear that in DCs exposed to purified virus preparations, the levels of TNFα transcript and protein are differentially regulated. Considering both the reduction of TNFα production and the lack of IFNα/β production in Influenza-infected DCs compared to inactivated Influenza, this suggests a hierarchy in the relative importance of suppressing functions of particular cytokines in order for Influenza to establish infection. There is a complete suppression of IFNα/β production, while levels of TNFα are reduced in Influenza-infected DCs compared to DCs exposed to inactivated Influenza. The Influenza NS1 protein can sequester dsRNA and inhibit the activation of IRF-3 (Talon et al., 2000), PKR (Bergmann et al., 2000) and NF-κB (Wang et al., 2000), thereby preventing IFNα/β production. However, other aspects of the virus entry and infection process may trigger activation of DCs and the subsequent production of TNFα independently of dsRNA. The absolute levels of TNFα production, higher in DCs exposed to inactivated Influenza compared to live Influenza, may suggest that Influenza broadly downmodulates host production of TNFα.

DCs exposed to Rhinovirus induced TNFα transcript with slower kinetics. This is similar to the induction of the core antiviral genes and IFNα production. However, the production of IFNα and TNFα in DCs exposed to Rhinovirus show that Rhinovirus does have a direct effect on DCs.

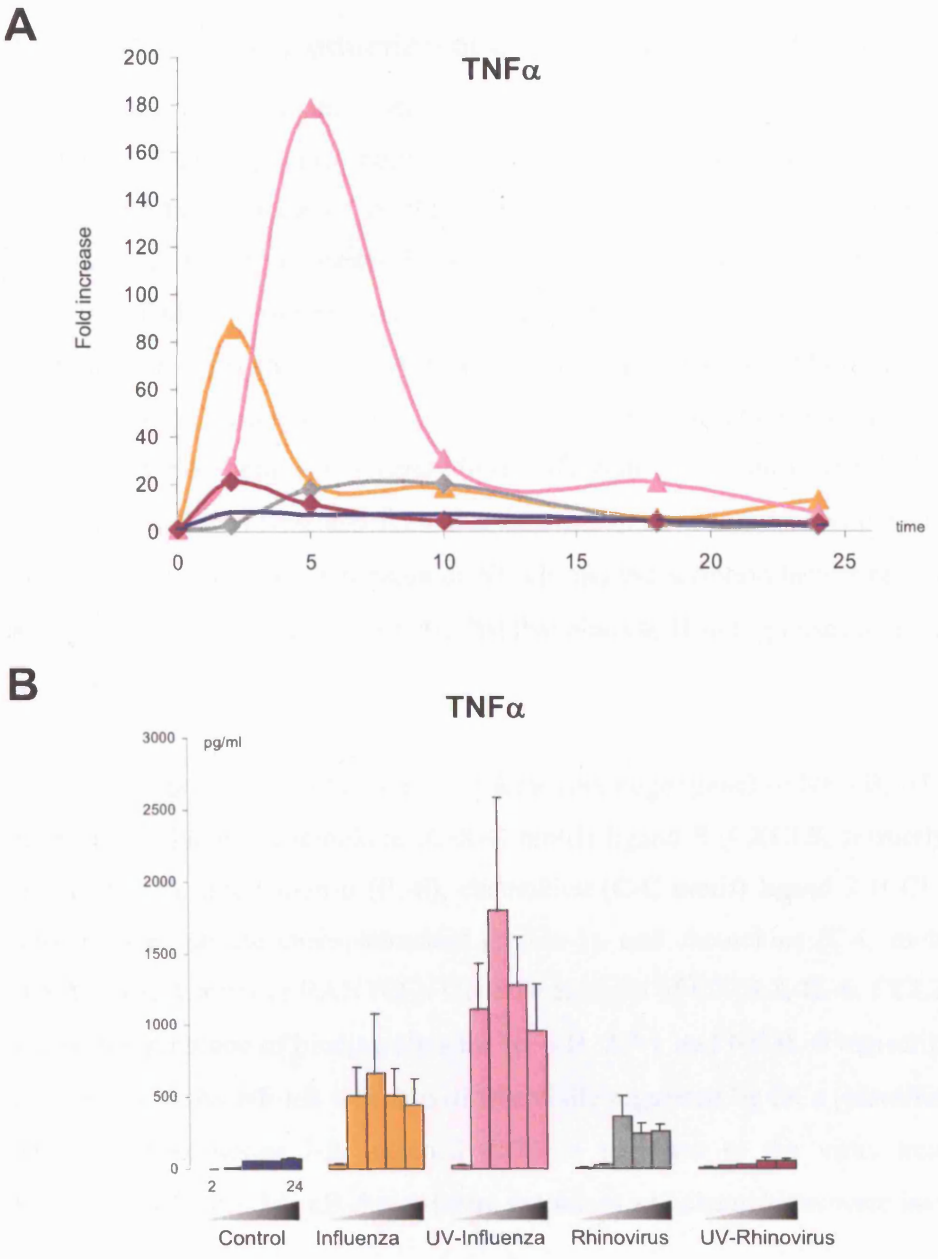


Figure 5.14 Transcript and protein measurement of TNFα

A TNFα transcript levels as measured by real-time RT PCR
Buffy coat DCs were stimulated with the different virus treatments, or mock-stimulated with medium. At the indicated timepoints (0, 2, 5, 10, 18, 24 hours) following infection or exposure, DCs were harvested, RNA extracted, and reverse transcribed to cDNA. This was used as template cDNA (50ng) for TaqMan RT-PCR, with commercially available Assays-On-Demand TNFα primers and FAM-labelled probe (Materials and Methods Section 2.6.2). The y-axis shows fold induction of TNFα mRNA compared to time zero for each virus condition. The colours correspond to the stimuli as indicated in **B**: control - blue, Influenza - orange, inactivated Influenza - pink, Rhinovirus - grey, inactivated Rhinovirus - purple.

B TNFα protein production as measured by ELISA
Culture supernatants from DCs stimulated with the different virus treatments, or mock-stimulated, were harvested at the indicated timepoints (2, 5, 10, 18, 24 hours) following infection or exposure. TNFα sandwich immunoassay was used to detect TNFα (pg/ml) in the culture supernatants.

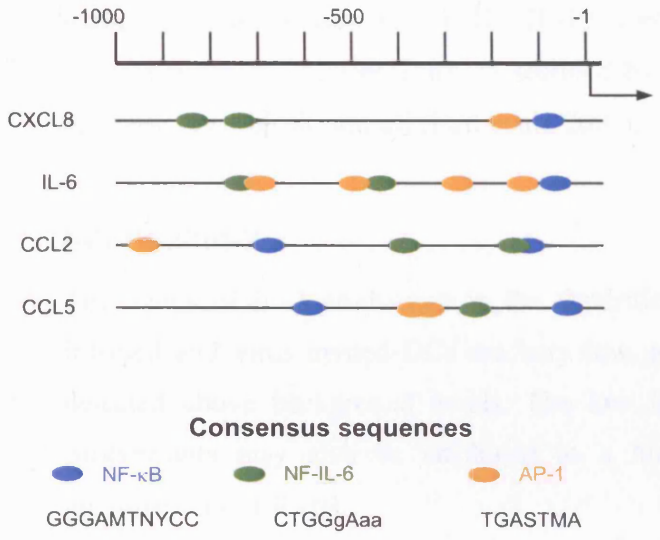
5.4.2 Differential production of chemokines and cytokines

Chemokines and cytokines play an important role in host defense against virus infections. Transcriptional studies of the core maturation response of dendritic cells highlighted the importance of the regulation of such proteins (Section 3.2.8.3) for coordinating the downstream immune response. The transcriptional regulation of cytokines and chemokines involve a large number of different transcription factors, including NF-κB (Pahl, 1999). Activation of DCs also leads to MAPK pathway activity, resulting in the formation of AP-1 (activating protein 1) transcription factor, consisting of Jun and Fos homo- and heterodimers (Curran and Franza, 1988). Inflammatory cytokines such as TNFα and IL-6 also function in an autocrine and paracrine fashion, resulting in downstream activation of NF-κB and transcription factors responsive to IL-6 (e.g. NF-IL-6 (also known as C/EBPβ) that binds to IL-6 response elements) (Akira et al., 1992).

A large number of chemokines and cytokines are target genes of NF-κB, AP-1, and NF-IL-6. These include chemokine (C-X-C motif) ligand 8 (CXCL8, formerly known as interleukin-8), interleukin-6 (IL-6), chemokine (C-C motif) ligand 2 (CCL2, formerly known as monocyte chemoattractant protein-1), and chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES). Genome analysis of CXCL8, IL-6, CCL2 and CCL5 reveal the presence of binding sites for NF-κB, AP-1 and NF-IL-6 transcription factors (Figure 5.15). As NF-κB was also differentially regulated by DCs (identified by vector analysis, Appendices 7-9, attached disk) in response to the virus treatments, the production of these NF-κB-downstream cytokines and chemokines were investigated

The FAST®Quant MicroSpot ELISA (Schleicher & Schuell BioScience, Germany) is a high throughput system for multiplex cytokine quantification. The FAST®Quant Human II system allows the detection of 10 different cytokines: CXCL8 (IL-8), GM-CSF, IL-10, IL-1β, IL-12p70, IL-2, CCL2 (or MCP-1), IL-4, CCL5 (or RANTES), and IL-6. The 10 cytokines are spotted in triplicate, along with 6 "dummy" controls, making 36 spots per reaction pad. Each assay consists of 4 slides containing 16 pads each, making up a total of 64 pads. This allows the assay of 56 individual samples along with 8 control samples (used to produce standard curves) allowing the simultaneous assay of 10 different cytokines in triplicate (Materials and Methods Section 2.7.2).

Of the 10 cytokines measured in the DC culture supernatants by this array system, the levels of GM-CSF and IL-4 detected were all above the maximum detection limit. This is due to the presence of GM-CSF and IL-4 in the culture medium of DCs. The results for IL-1β, IL-2, IL-10 and IL-12p70 were equivocal and difficult to interpret. In contrast, Influenza and Rhinovirus treatment of DCs both result in significant production of CXCL8, IL-6, CCL2 and CCL5.



CXCL8	(-80)	gGGAATTTCC
	(-853)	CTGGAA
	(-752)	CTGGAA
	(-170)	TTTCGTCA
IL-6	(-73)	GGGAcTTTCC
	(-762)	CTGGGA
	(-432)	CTGGAA
	(-745)	GGGTCA
	(-540)	AGATGACT
	(-284)	CTGAGTCA
	(-133)	CCAATCA
CCL2	(-670)	GGGAATTTtC
	(-135)	GGAAATCCcC
	(-394)	CTGGGA
	(-137)	CTGGAA
	(-953)	TAACTCA
CCL5	(-590)	AgGAAATTCC
	(-53)	GGAAAtTCCC
	(-254)	CTGGAA
	(-361)	TGACTCA
	(-353)	TGAGTAA

Figure 5.15 Promoter analysis of CXCL8, IL-6, CCL2, and CCL5

Nucleotide sequences 1000 bp 5'-upstream of the start codons (promoter regions) for the genes CXCL8, IL-6, CCL2 and CCL5 were derived from Ensembl (www.ensembl.org) were searched for consensus binding sites for transcription factors NF-κB, NF-IL6 and AP-1 in Transcription Element Search Software (TESS) Transfac database.
The degenerate nucleotides indicated: M=A/C, N=A/C/G/T, S=C/G, Y=C/T.

Interleukin-1β

Virus-exposed DCs do not seem to produce significant amounts of IL-1β compared to mock-infected DCs (Figure 5.16A). Studies in human monocytes show that stimulation with LPS results in the production of up to 3000 pg/ml of IL-1β between 4 and 8 hours post-stimulation (Kwak et al., 2000). Monocyte-derived DCs have also been shown to be able to secrete large amounts of IL-1β (Ruppert and Peters, 1991), both at basal levels and after stimulation with LPS (Luft et al., 2002). The levels of IL-1β detected with the FAST®Quant assay system in all DC samples, including post LPS- and poly(I:C)-stimulation (data not shown), are much lower, only up to 80 pg/ml (Influenza-infected DC at 24h, Figure 5.16A). This result may reflect a limitation of the protein cytokine assay in measuring IL-1β. It would therefore be useful to verify this with a traditional immunoassy for IL-1β. IL-1β contributes to Langerhan cell migration (Cumberbatch et al., 1997) and is secreted by DCs as an inflammatory cytokine in response to antigenic stimuli (Luft et al., 2002).

Interleukin-2

The levels of IL-2 production in the dendritic cell supernatants in both the mock-infected and virus treated DCs are very low, and IL-2 levels cannot be consistently detected above background levels. The low levels of IL-2 measured in these DC supernatants may also be attributed to a limitation of the assay, similar to the measurement of IL-1β.

Recent microarray studies have identified strictly defined kinetics of IL-2 production in murine DCs in response to bacterial encounter (Granucci et al., 2001a), where early IL-2 transcript expression between 2 to 8 hours after bacterial encounter helps sustain the growth of T, B and NK cells, and expression during the late phase contributes to regulation of the cell-mediated response by promoting activation-induced cell death (AICD) of effector T cells (Granucci et al., 2003b; Van Parijs et al., 1999). On the protein level, peak levels of IL-2 between 4 and 10 hours after bacterial encounter reached 200 pg/ml (Granucci et al., 2001a). The data here may also suggest a biphasic mode of IL-2 production in virus-exposed human DCs, but needs to be interpreted with caution.

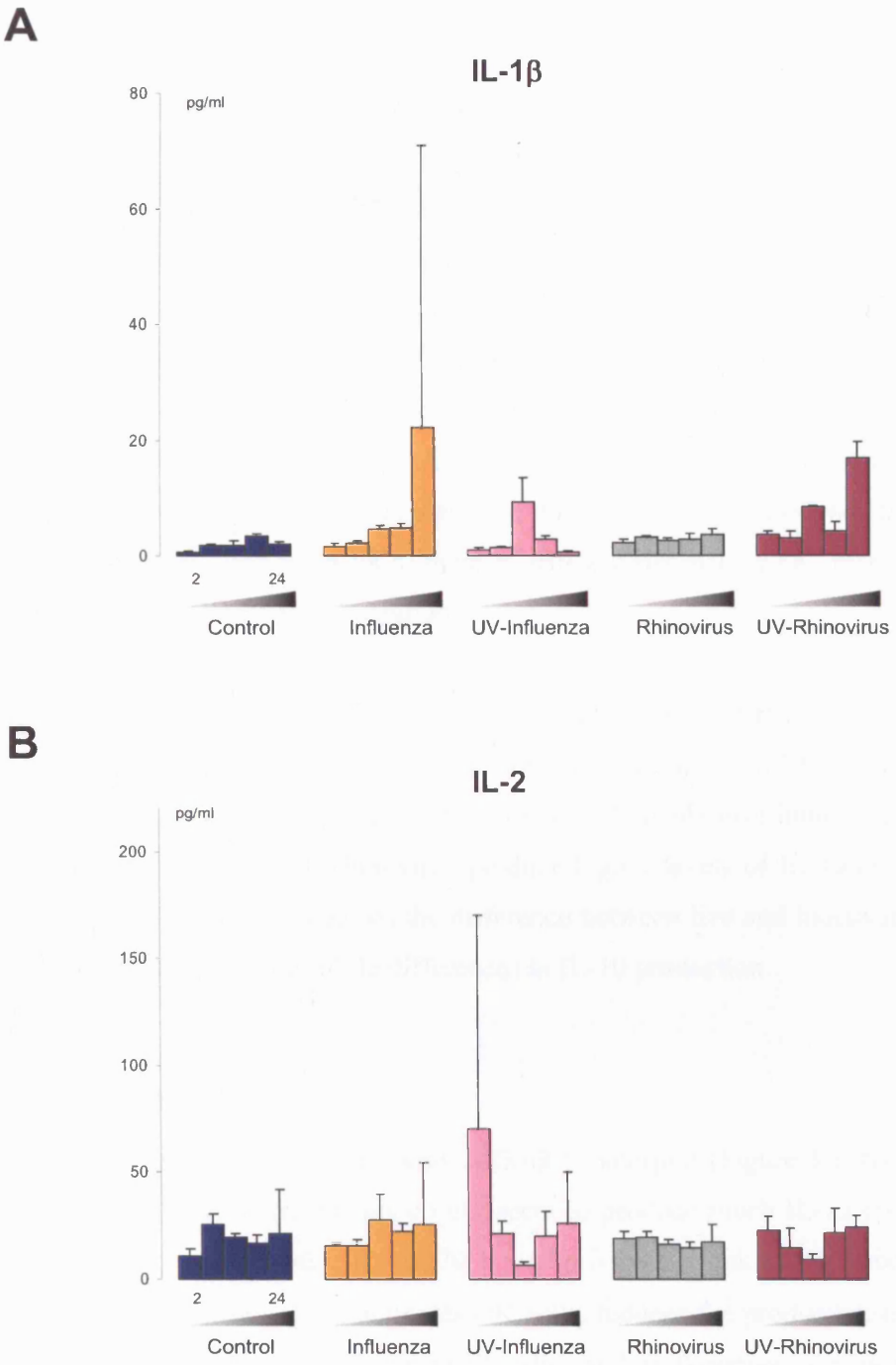


Figure 5.16 Production of IL-1β and IL-2 by DCs stimulated with viruses

DCs were exposed to different viruses or mock-stimulated, and the culture supernatants were collected at 5 different timepoints (2, 5, 10, 18, 24 hours) after virus stimulation. Culture supernatants were assayed for (**A**) IL-1β and (**B**) IL-2 production by the FAST®Quant ELISA array system. The error bars indicate standard deviation from triplicate results.

Interleukin-10

The results for IL-10 production are difficult to interpret for DCs exposed to live and inactivated Influenza, largely because of the large fluctuations at 24 hours and 10 hours post-virus exposure respectively (Figure 5.17A). DCs exposed to Rhinovirus seem to actively downregulate IL-10 production, as there are significantly lower levels of IL-10 in Rhinovirus-exposed DCs compared to mock-infected DCs at 24 hours (p=0.017, Figure 5.17A). In contrast, DCs exposed to inactivated Rhinovirus produce higher levels of IL-10 at 24 hours post-virus exposure (p=0.021, Figure 5.17A). The difference in IL-10 production in DCs exposed to live and inactivated Rhinovirus supports differential phenotypic changes in DCs responding to live and inactivated Rhinovirus from Chapter 4. This suggests that even though there is no evidence that Rhinovirus is capable of replication in DCs, there is still a difference in DC responses to live and inactivated Rhinovirus preparations.

IL-10 functions to sustain humoral immunity by inducing the production of the B cell chemoattractant CXCL13 in both myeloid and plasmacytoid DCs (Perrier et al., 2004), amplifying B cell recruitment in the initiation of an adaptive immune response. As DCs exposed to inactivated Rhinovirus produce higher levels of IL-10 compared to mock-stimulated DCs, this suggests the difference between live and inactivated Rhinovirus is an ability to modulate subtle differences in IL-10 production.

Interleukin-12

The results for IL-12p70 are also difficult to interpret (Figure 5.17B). DCs exposed to the different virus treatments do not seem to produce much IL-12 compared to mock-stimulated control DCs. IL-12p70 is an important cytokine for modulating adaptive immune responses. IL-12 activates NK cells, induces the production of IFNγ, promotes the differentiation of T_H1 CD4+ T cells, and is therefore a critical factor in viral immunity. IL-12 is secreted by dendritic cells and macrophages in response to pathogens. In the *in vivo* context, however, IL-12 production is enhanced by T cells due to the production of T cell-derived cytokines (IFNγ and IL-4) as well as CD40L-CD40 interactions (Trinchieri, 2003). The lack of secondary signals in this system may be a reason for the low levels of IL-12p70 detected.

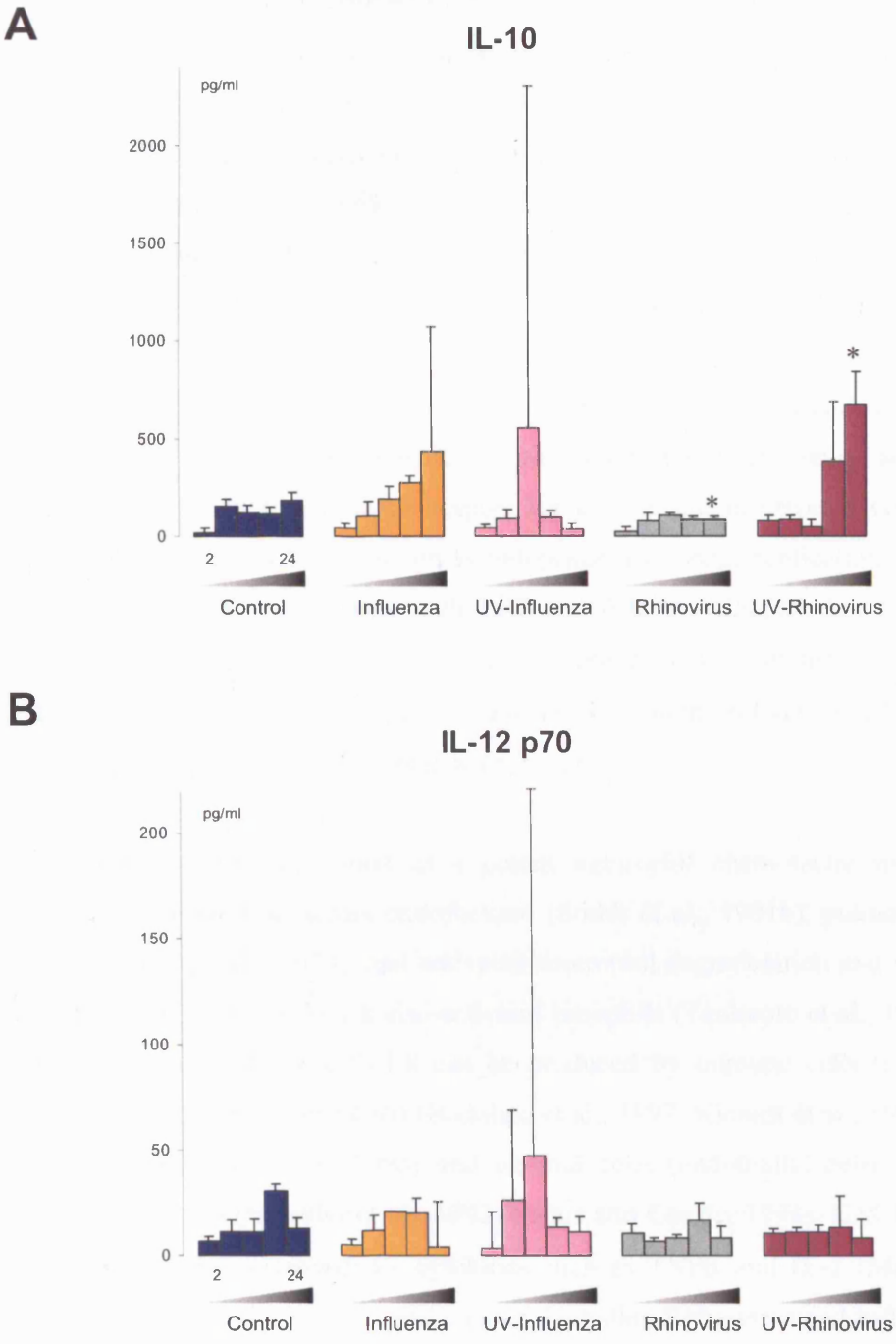


Figure 5.17 Production of IL-10 and IL-12 by DCs stimulated with viruses

DCs were exposed to different viruses or mock-stimulated, and the culture supernatants were collected at 5 different timepoints (2, 5, 10, 18, 24 hours) after virus stimulation. Culture supernatants were assayed for (**A**) IL-10 and (**B**) IL-12p70 production by the FAST®Quant ELISA array system. The error bars indicate standard deviation from triplicate results. Black asterixes indicate significance at $p<0.021$ compared to matching timepoints in mock-stimulated DC supernatants. *P*-values were calculated by the standard Student's paired *t*-test.

Chemokine (C-X-C motif) ligand 8

Virus-exposed DCs produce significantly higher levels of CXCL8 compared to mock-infected DCs (Figure 5.18A). The detection limit of the assay at 6000 pg/ml makes it difficult to distinguish between a plateau in levels of CXCL8 or whether production of CXCL8 still increases. Evidence in the literature suggests that CXCL8 production still increases after 24 hours in pulmonary epithelial cells infected with Rhinovirus (Johnston et al., 1998; Papadopoulos et al., 2001) and Influenza (Arndt et al., 2002).

The robust production of CXCL8 in DCs in response to Influenza and Rhinovirus seems likely to be a core response induced by virus contact and virus entry, and the similarity in CXCL8 production by DCs responding to live and inactivated virus preparations suggests that CXCL8 production is independent of virus replication. CXCL8 is also produced by DCs stimulated with LPS and dsRNA (Chapter 3, Figure 3.18). This suggests that production of CXCL8 is a broad acute response as a result of DC stimulation by pathogen components and viruses, via the activation of NF-κB and AP-1 or NF-IL-6 transcription factors (Figure 5.15).

CXCL8 was first described as a potent neutrophil chemotactic factor, promoting neutrophil migration across endothelium (Smith et al., 1991b), pulmonary epithelium (Smart and Casale, 1993), and activates neutrophil degranulation and respiratory burst (Walz et al., 1991). CXCL8 also activates basophils (Tanimoto et al., 1992) and T cells (Lippert et al., 2000). CXCL8 can be produced by immune cells (monocytes, DCs, macrophages, and neutrophils) (Badolato et al., 1997; Kienast et al., 1996; Konig et al., 1996; Verhasselt et al., 1998) and stromal cells (endothelial cells, fibroblasts, and epithelial cells) (Mauviel et al., 1992; Smart and Casale, 1993). CXCL8 production is induced by pro-inflammatory cytokines such as TNFα and IL-1 (Matsushima et al., 1989), as well as by bacteria and viruses, including Rhinovirus and Influenza (Aihara et al., 1997; Johnston et al., 1998; Johnston et al., 1997; Matsukura et al., 1996; Murayama et al., 1997). The accumulation of CXCL8 at the site of antigen encounter functions to recruit immune cells to this site and focuses the immune response.

Interleukin-6

Virus-exposed DCs produce significantly higher levels of interleukin-6 compared to mock-infected DCs (Figure 5.18B). Comparison between DCs exposed to live and inactivated Influenza suggest that live Influenza downmodulates the production of IL-6 in DCs. The levels of IL-6 produced from DCs exposed to inactivated Influenza are significantly higher compared to Influenza-infected DCs. This pattern is also seen in DCs exposed to Rhinovirus, where there are higher levels of IL-6 production in DCs exposed to inactivated Rhinovirus compared to live Rhinovirus. The peak levels of IL-6 produced at 10 hours in DCs exposed to live and inactivated Rhinovirus are higher than Influenza-infected DCs, yet lower than in DCs exposed to inactivated Influenza. This suggests that live Rhinovirus in DCs does not vigorously downmodulate the host response to IL-6 production compared to live Influenza, and that both live and inactivated Rhinovirus may not be as immunogenic as inactivated Influenza in stimulating IL-6 production in DCs.

Interleukin-6 is a pro-inflammatory cytokine that plays a central role in host defence due to its wide range of immune and haematopoietic activities, as well as its potent ability to induce the acute phase response (Poli and Cortese, 1989). Its broad-ranging effects reflect the key function of IL-6 as a systemic alarm signal that recruits diverse host defence mechanisms in order to limit tissue injury. Here, IL-6 production by DCs in response to viruses is varied, depending on virus type.

IL-6 has been shown to be produced in lung fibroblasts infected with Rhinovirus (Zhu et al., 1996), though this study found that UV-inactivated Rhinovirus did not induce IL-6 production in lung fibroblasts. Details of UV-inactivation procedures are relevant, as prolonged exposure to UV-light destroys virus particles and their ability to bind to their cognate receptors. Extracellular binding assays (Section 4.2.2) show that Rhinovirus exposed to UV-light for 20 minutes are unable to bind to DCs, whereas Rhinovirus exposed to UV-light for 2 minutes are able to bind to DCs, even though the titre is still effectively reduced by 6 logs (data not shown). Therefore, the lack of response reported in the literature for inactivated virus that infers cytokine secretion as dependent on virus replication should be interpreted in light of the inactivation procedure.

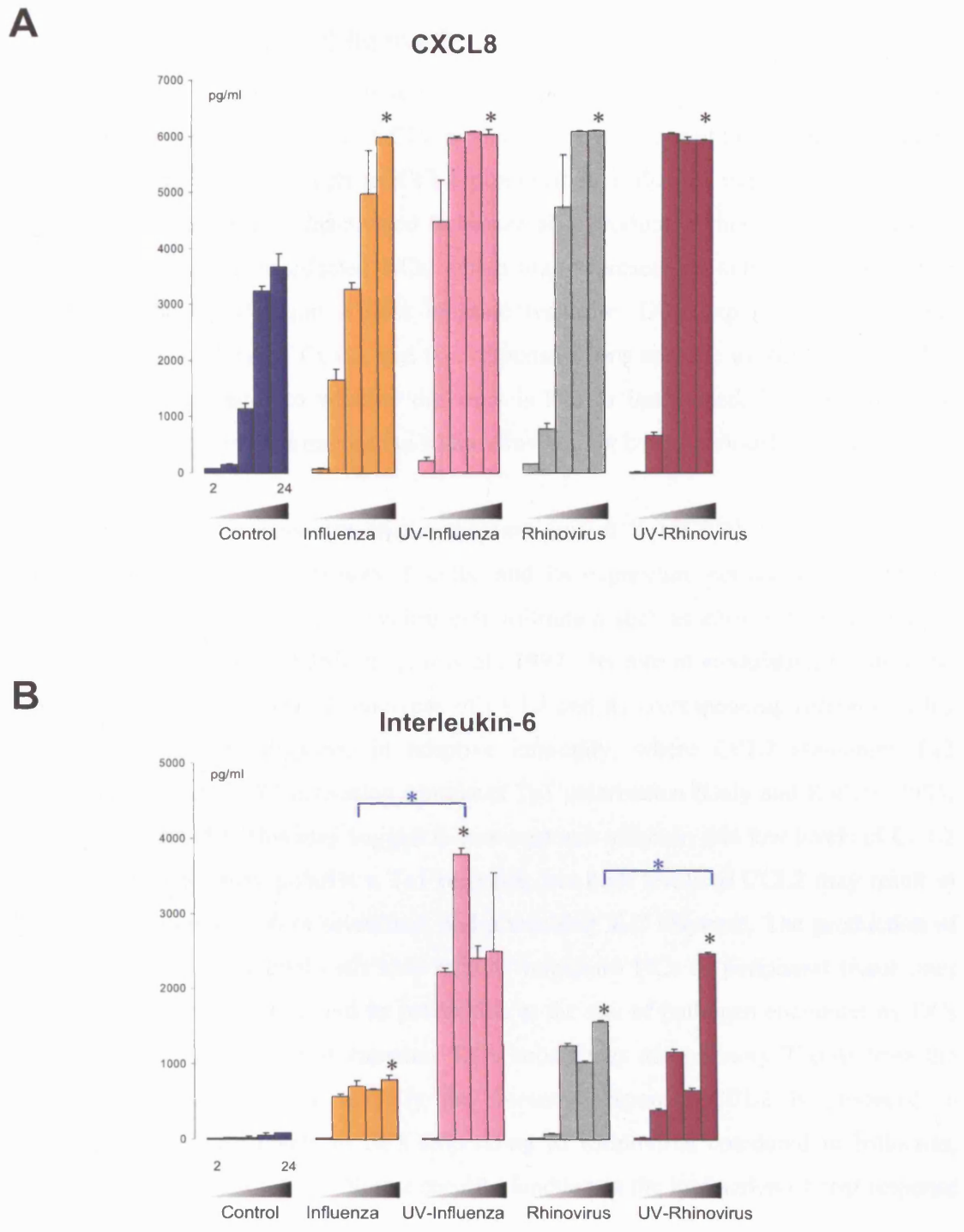


Figure 5.18 Production of CXCL8 and IL-6 by DCs stimulated with viruses

DCs were exposed to viruses or mock-stimulated, and the culture supernatants were collected at 5 different timepoints after (2, 5, 10, 18, 24 hours) virus stimulation. Culture supernatants were assayed for (**A**) CXCL8 and (**B**) IL-6 production by the FAST®Quant ELISA array system. The error bars indicate standard deviation from triplicate results. Black asterixes indicate significance at $p<0.001$ compared to matching timepoints in mock-stimulated DC supernatants. Blue asterixes indicate significance at $p<0.0005$ between virus-treated DCs at matching timepoints. *P*-values were calculated by the standard Student's paired *t*-test.

Chemokine (C-C motif) ligand 2

Production of CCL2 in DCs seem much more dependent on specific virus exposure than CXCL8 and IL-6. The levels of CCL2 produced in DCs exposed to live and inactivated Rhinovirus are twice as high as CCL2 produced in Influenza exposed DCs (Figure 5.19A). DCs exposed to inactivated Influenza also produce higher amounts of CCL2 compared to Influenza-infected DCs, which may represent an active process of live Influenza downmodulating a host immune response. DCs exposed to Rhinovirus produce higher levels of CCL2, and this response seems specific to Rhinovirus, and is not necessarily related to whether the virus is live or inactivated. This link between CCL2 production in DCs exposed to Rhinovirus has not been previously reported.

Formerly known as monocyte chemoattractant protein 1 (MCP-1), CCL2 specifically attracts monocytes and memory T cells, and its expression occurs in a variety of diseases characterised by mononuclear cell infiltration such as atherosclerosis, multiple sclerosis and rheumatoid arthritis (Gu et al., 1997). Its role in modulating the immune response is as yet unclear. Phenotypes of CCL2 and its corresponding receptor CCR2 knockout mice are disparate in adaptive immunity, where CCL2 stimulates T_H2 polarisation, and CCR2 activation stimulates T_H1 polarisation (Daly and Rollins, 2003; Sato et al., 2000b). This may suggest a dose-response effect in that low levels of CCL2 activating CCR2 may polarise a T_H1 response, but high levels of CCL2 may result in receptor desensitisation or saturation, and a resulting T_H2 response. The production of CCL2 by basal epithelial cells also recruits immature DCs to peripheral tissue sites (Vanbervliet et al., 2002), and its production at the site of pathogen encounter by DCs may serve to further recruit immature DCs, monocytes and memory T cells from the blood into these sites to amplify the immune response. CCL2 is produced to significantly higher levels in DCs responding to Rhinovirus compared to Influenza, which may suggest CCL2 to have a specific function in the interaction of host response and Rhinovirus.

Chemokine (C-C motif) ligand 5

Virus-exposed DCs secrete significant amounts of CCL5. DCs exposed to inactivated Influenza produce more CCL5 more rapidly compared to Influenza-infected DCs (Figure 5.19B). The rate of CCL5 production in Rhinovirus-exposed DCs is slower compared to Influenza-exposed DCs. This is another indicator of the slower replication

or infectious program mediated by Rhinovirus compared to Influenza in DCs, and suggests that CCL5 production in live and inactivated Rhinovirus-exposed DCs will continue after 24 hours. Rhinovirus infection of respiratory epithelial cells show that CCL5 continues to be produced between 24 and 48 hours after infection (Papadopoulos et al., 2001).

Influenza- and Rhinovirus-exposure in DCs clearly result in the differential production of CCL5. This indicates that in addition to its role in the core maturation response (Section 3.2.8.3), CCL5 may have an important antiviral role. Influenza actively downmodulates this host response by decreased CCL5 production compared to inactivated Influenza, which is not seen in the comparison between live and inactivated Rhinovirus. The trigger for CCL5 production may also be different, where the binding and entry of Influenza stimulates vigorous CCL5 production (DCs responding to inactivated Influenza), whereas this occurs later in DCs responding to Rhinovirus.

CCL5 recruits memory T_H cells, eosinophils and monocytes (Schall et al., 1990), and has been demonstrated to be involved in immune responses associated with viral diseases (Gross et al., 2003; Melchjorsen and Paludan, 2003). There is evidence that type I IFN and TNFα may also induce CCL5 production (Cremer et al., 2002; Lane et al., 1999), and the involvement of IRFs that bind to promoter regions of CCL5 (Casola et al., 2001; Genin et al., 2000) further support the importance of CCL5 in antiviral immunity. Both Influenza and Rhinovirus infection have been shown to cause CCL5 production in airway epithelial cells (Kujime et al., 2000; Matsukura et al., 1998; Schroth et al., 1999), and bronchial epithelial cells incubated with UV-inactivated Rhinovirus secreted significant amounts of CCL5 (Schroth et al., 1999). Though CCL5 contributes to antiviral immunity, in atopic patients with allergic airway disease, CCL5 may exacerbate allergic symptoms (Elliott et al., 2004; John et al., 2003; Tekkanat et al., 2002).

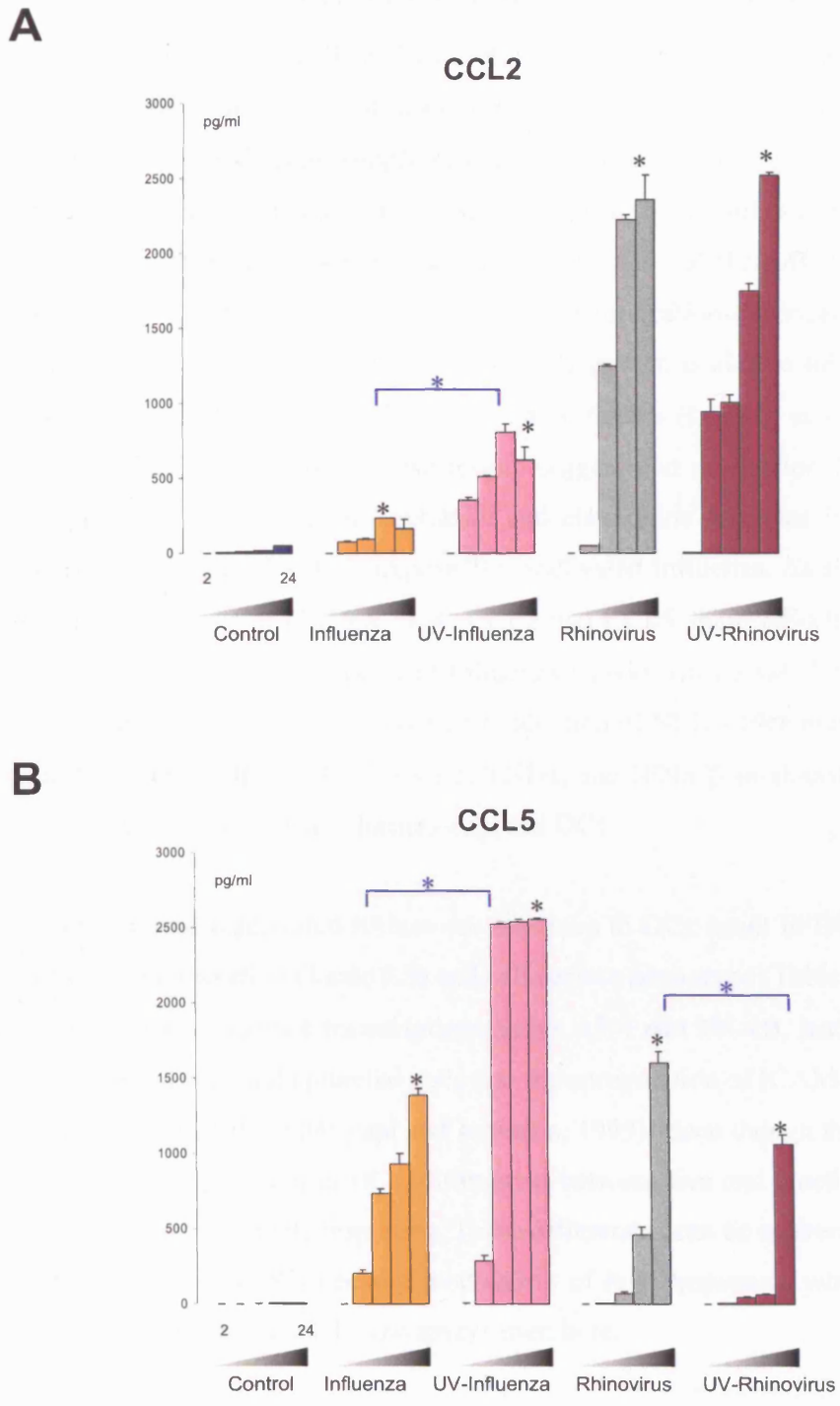


Figure 5.19 Production of CCL2 and CCL5 by DCs stimulated with viruses

DCs were exposed to viruses or mock-stimulated, and the culture supernatants were collected at 5 different timepoints (2, 5, 10, 18, 24 hours) after virus stimulation. The culture supernatants were assayed for (**A**) CCL2 and (**B**) CCL5 production by the FAST®Quant ELISA array system. The error bars indicate standard deviation from triplicate results. The black asterixes indicate significance at $p<0.001$ compared to matching timepoints in mock-stimulated DC supernatants. The blue asterixes indicate significance at $p<0.001$ between virus-treated DCs at matching timepoints. *P*-values were calculated by the standard Student's paired *t*-test.

Chemokines as effectors of dendritic cell responses to viruses

The levels of CXCL8, IL-6, CCL2 and CCL5 production, together with IFNα/β and TNFα, suggest that upon infection of DCs, Influenza actively downmodulates the host response by consistently suppressing the secretion of these cytokines and chemokines (Table 5.3). In comparison, DCs exposed to inactivated Influenza are able to mount a full robust immune response in secreting high levels of IFNα/β, TNFα, CXCL8, IL-6, CCL2 and CCL5, without "interference" from replication-dependent events (Table 5.3). Studies have shown that Influenza virus NS1 protein is able to inhibit the activation of AP-1, NF-κB, IRF-3 and IRF-7 transcription factors (Ludwig et al., 2002; Talon et al., 2000; Wang et al., 2000). These results suggest that replication-dependent events are responsible for a dampened cytokine and chemokine response in DCs infected with Influenza compared to DCs exposed to inactivated Influenza. As shown in Figure 5.15, promoter regions of CXCL8, IL-6, CCL2 and CCL5 share NF-κB, AP-1 and NF-IL-6 binding sites. Though inactivated Influenza should still contain NS1 protein, Influenza replication results in much increased production of NS1, which may explain differences in the levels of IL-6, CCL2, CCL5, TNFα, and IFNα/β produced between Influenza-infected and inactivated Influenza-exposed DCs.

Both live and inactivated Rhinovirus exposure in DCs result in DC activation in terms of cytokine secretion (Table 5.3) and cell surface phenotype (Table 4.2). Rhinovirus has been shown to activate transcription factors AP-1 and NF-κB, leading to production of CXCL8 in bronchial epithelial cells and the upregulation of ICAM-1 surface expression (Funkhouser et al., 2004; Papi and Johnston, 1999). Even though there is no evidence of Rhinovirus replication in DCs, differences between live and inactivated Rhinovirus are observed as varied DC responses. These differences can be a direct result of replication of Rhinovirus in DCs causing modulation of host responses, where the replication is abortive or undetectable by the assays used here.

In studies involving Rhinovirus infection in bronchial epithelial cells, sampling at 24 and 48 hours after infection showed that levels of CXCL8 and CCL5 continued to increase after 24 hours following virus exposure (Papadopoulos et al., 2001; Schroth et al., 1999). However, the levels of CXCL8 and CCL5 after incubation with RV16 for 48 hours reported in these studies were lower than the levels that DCs produced 24 hours following virus exposure. Therefore, the differences in DC responses in terms of

cytokine and chemokine production over 24 hours are probably indicative of early DC responses to live and inactivated Rhinovirus exposure, and the roles of CCL5 and CCL2 in mediating host immune responses to Rhinovirus warrant further investigation.

Chemokines CXCL8, CCL2 and CCL5 have been shown to be important in mediating immunopathology in relation to pulmonary diseases (Maus et al., 2003; Mukaida, 2003; Rose et al., 2003). This is largely due to their chemotactic properties in recruiting neutrophils (CXCL8), monocytes and T helper cells (CCL2 and CCL5) and eosinphils (CCL5), leading to pathological situations characterised by chronic inflammation. However, these studies focus on responses of stromal cells to infection and pathology. Dendritic cells also secrete large numbers of chemokines, and the role of DCs in the initiation and shaping of the adaptive immune response make chemokines crucial effectors. The varied responses of DCs in the production of IFNα/β, TNFα, CXCL8, IL-6, CCL2 and CCL5 demonstrate that DCs are also plastic in their responses at the level of protein production that is tailored to the viral antigen (Table 5.3).

Discussion

Transcriptional plasticity of dendritic cell responses to different virus stimuli were further investigated. Initial hierarchical clustering analysis of DC responses to Influenza and Rhinovirus, inactivated Influenza and dsRNA suggest that DCs are able to modulate differential gene responses depending on virus stimuli. Vector analysis robustly identified subsets of differentially and commonly regulated genes. These genes were further explored by promoter analysis and investigated at the transcript and protein level.

Novel gene expression analysis method

Time-series analysis applied to microarray data is an area that has received relatively little specific attention. Whereas statistical analysis methods for identifying differential expression in straightforward binary comparisons are plentiful, including SAM and Mann-Whitney U methods described in Chapter 3, these methods do not take into consideration the temporal relationships between experimental samples. From the microarray analysis of the dendritic cell "maturation" response, it is clear that temporal relationships are an inherent part of DC function, which make the temporal element crucial to analysing DC responses.

Hierarchical clustering of dendritic cell responses to different virus treatments revealed differential responses modulated by DCs that suggest virus-specificity. Though clustering analysis has provided potentially meaningful clusters that can be further explored, the limitations of this method for identifying commonly and differentially regulated genes means only a broad overview through pattern finding is achieved. To further identify genes that may be important in differentially regulating DC responses to viruses, a method that incorporates the temporal relationship between experimental samples was developed.

Commonly and differentially regulated responses were identified using a novel analysis method that involved modelling gene expression changes over time as multi-dimensional gene vectors. The number of dimensions correlates to the number of timepoints per virus treatment (i.e. 6 timepoints over 24 hours transforms into a 6-dimensional gene vector). As each timepoint is a dimension of the gene vector, this

allows the temporal relationship between experimental samples that relate to gene expression changes to be considered as an inherent property of the gene vector (i.e. gene expression), so that the temporal aspect of the data is retained. Modelling gene expression ratios as vectors is advantageous because it offers flexibility in the dimension of vectors that is analysed (no limitations on timepoints), and allows user-defined filtering criteria (vector angles and vector magnitudes). However, a drawback of this analysis method is that aberrantly variable data points (from inconsistent microarray signal measurements) would result in a vector which when compared to other vectors may give a falsely large vector angles. Vector magnitude is analogous to the standard deviation measurement used here, though this measurement is also sensitive to poor quality microarray spots. This indicates the importance of confirming original microarray data (Figure 5.12).

The scalability of this vector analysis method in its ability to capture high dimensional data also offers the option of modelling experimental vectors in addition to gene vectors. An experimental vector captures the same information contained in a scatterplot of gene vectors, and is subsequently of higher dimension. As experimental profiles are added to a microarray dataset, where the number of genes remains fixed, plotting experimental vectors allows more effective visualisation of the relationship between experiments.

Vector modelling allowed the use of vector angles as a metric for gene expression correlation over time, and the magnitude of gene expression changes over time to be assessed by standard deviations. This method is robust in that commonly correlated genes with small vector angles are identified that correlate to genes that are clustered together with high correlation coefficients by hierarchical clustering in Chapter 4. An additional advantage of this vector method of analysis is that it allows flexibility in the subsets of genes that are identified as commonly and differentially regulated, based on the criteria of vector angles. If a cut-off for common regulation is less stringent, i.e. a larger vector angle is considered, then the subset of genes identified as co-expressed will be larger, though this may incorporate more "noise" in the common regulation profile. Conversely, the subset of differentially regulated genes will be smaller, and more robust. This flexibility of vector angles allows tailoring a cut-off value that is suitable for the analysis. In addition, standard deviation filtering allows the selection of genes that change expression significantly with time. The measure of standard deviation

is an extension from the magnitude metric that is also considered in modelling gene expression as vectors, but allows the measure of magnitudes to relate to individual gene vectors and not the relationship between two vectors.

Differential production of cytokines and chemokines

Vector analysis identified a set of 10 genes that are commonly upregulated on the transcript level in DCs in response to all three different virus treatments – Influenza infection, inactivated Influenza exposure, and Rhinovirus exposure. These genes were also clustered together in the proposed antiviral cluster identified in Section 4.7.3.2. Co-expression of genes suggest co-regulation by common transcription factors, and promoter analysis showed the presence of NF-κB, IRF-1, IRF-3, IRF-7 and ISGF-3 binding sites that are probably most relevant to modulating the antiviral response (Katze et al., 2002; Li and Verma, 2002; Pfeffer et al., 2004; Taniguchi et al., 2001). Type I interferons are known to activate these transcription factors, and the hypothesis of IFNs mediating the activation of common transcription factors which upregulated the 10 commonly upregulated genes was tested. This was shown to be incorrect, as the levels of IFNα/β detected from dendritic cells exposed to viruses were variable and did not correlate to the common transcriptional upregulation of the 10 genes. IFNα/β levels may, however, be involved in the maintenance of the antiviral response. This strongly suggests that this core response is most likely independent of *de novo* protein synthesis, and its induction a response to core viral motifs that are recognised by dendritic cells and commonly triggered by viruses.

A large number of differentially responsive genes were identified, including TNFα and NF-κB. TNFα has been shown to be critical in affecting DC maturation in response to virus infection and downstream activation of adaptive immunity (Trevejo et al., 2001). Other cytokines and chemokines activated by NF-κB were also investigated. Influenza seems to commonly modulate a downregulation in cytokine production from DCs, in contrast to the vigorous response stimulated by inactivated Influenza. This difference between live and inactivated virus exposure is less clear for Rhinovirus. However, responses of DCs to Rhinovirus are clearly different to those induced by Influenza, including the absolute levels of cytokines produced and rate of cytokine production. This temporal delay of induction of DC responses to Rhinovirus should be addressed by

monitoring cytokine production at later timepoints, in order to allow for delayed effects. The possibility of Rhinovirus replication in DCs should not be excluded, as there is clearly a robust response to Rhinovirus exposure, and further investigations are needed to determine Rhinovirus replicative capacity in DCs, or if Rhinovirus establishes an abortive infection in DCs.

Both live and inactivated Rhinovirus-exposed DCs produce high levels of CCL2, significantly higher than the levels produced by Influenza-infected and inactivated Influenza-exposed DCs. The production of CCL2 from DCs has not been previously reported, and suggests a mechanism for monocyte and DC recruitment from the blood to the site of Rhinovirus encounter to further amplify the immune response. Additional downstream effects of CCL2 should also be further investigated, as it may have important implications for shaping downstream adaptive immunity. Rhinovirus-exposed DCs also produce high levels of CXCL8, IL-6 and CCL5. In contrast to other respiratory viruses (e.g. Influenza and adenovirus), it is believed that the manifestations of Rhinovirus-induced pathogenesis are the result of virus-induced mediators of inflammation (Zhu et al., 1996) rather than direct cytotoxic effects (Johnston et al., 1993).

Rhinovirus infection account for 80% of acute asthma exacerbations in school-aged children and half of all asthma exacerbations in adults (Yamaya and Sasaki, 2003). Numerous reports in the literature have shown that cytokines are produced in abundance in Rhinovirus-infected bronchial and pulmonary epithelial cells, and the resulting recruitment and activation of effector cells such as neutrophils, eosinophils and basophils to the airway mucosa is thought to contribute to the exacerbation of asthma (Papadopoulos et al., 2004). Interestingly, CCL2 is elevated in the airways of asthmatics (Sousa et al., 1994), and is thought to be involved in the chain of inflammatory events that promotes airway remodelling in asthma. Rhinovirus infection of asthmatic patients probably exacerbates the already present hyperreactivity and inflammatory response in asthmatic airways, and acts as a trigger in susceptible patients. Whether the asthma exacerbation is primarily caused by the viral infection resulting in the heightened inflammatory environment in the airways, or is secondary to the delayed clearance of the viral infection as a result of the T_H2-bias of the immune response of atopic patients is unknown.

Further investigations into functional DC plasticity in recognising different viruses, and how viruses interact with DCs, will allow insight into differential adaptive immune responses that are initiated by DCs in response to viral pathogens. Cytokines and chemokines are a link between cells of the innate and adaptive immune system, and have wide-ranging effects. Understanding how they are differentially regulated by DCs may provide an avenue for their modulation in therapeutics in order to affect clearance of viral infections.

Chapter 6

Summary and directions for future research

In this thesis I have used DNA arrays to explore transcriptional plasticity in dendritic cell responses to simple and complex antigens. The antigens used ranged from single pathogen components to two different RNA viruses which differ in their virion architecture and their capacity to establish infection in DCs.

The *in vitro* response of dendritic cells to antigens can be extrapolated to reflect the *in vivo* initiation of the wider immune response. The complexity of this response, the nature of which is dependent on the specific antigenic stimulus and the context in which the antigenic stimulus is met, is in part revealed by transcriptional profiling. Furthermore, sampling DC maturation at discrete timepoints can elucidate the temporal aspects of the evolution of the immune response to antigen. Consequently, to achieve a more complete model, consideration of the temporal and environmental context is necessary for the understanding of dendritic cell function.

6.1 Transcriptional states reflect biological function

Dendritic cell responses to component antigens LPS and dsRNA showed distinct cascades of transcriptional regulation, which corresponded to temporally distinct stages of dendritic cell maturation comprising of activation and differentiation, as well as dendritic cell specific functions (Figure 3.31). The various stages of DC maturation also reflect their migration from the periphery where the antigens are first encountered, initial DC effector responses which further recruit innate cells to the site of antigen encounter, and entry into draining lymph nodes where adaptive immune responses are initiated. Transcriptional states of maturing dendritic cells therefore reflect the generation of the innate and adaptive immune response both in time and place (Figure 6.1). The contribution of the temporal and spatial context to the dendritic cell maturation response is therefore inherent in the consideration of the downstream effects of dendritic cells to initiate the adaptive immune response. However, questions remain as to how much of this process of activation and differentiation is pre-programmed in DC biology and function, and how much is influenced by the type of antigenic stimuli

encountered, and the context within which such an immune response is initiated? In other words, are differences seen when different maturation stimuli are used?

6.2 Relevance of maturation stimuli

Culture systems that generate mature DCs from immature precursors generally induce maturation by the addition of TNFα, CD40 ligand (CD40L, or TNFSF5), a combination of inflammatory cytokines, or conditioned medium (Kato et al., 2001; Reddy et al., 1997). CD40 ligand is expressed on T cells, and its interaction with CD40 on DCs functions to enhance the DCs' capacity to stimulate T cells via the upregulation of co-stimulatory molecules and cytokine secretion (Cella et al., 1996; Schulz et al., 2000). However, CD40 ligation in immature DCs in the absence of priming from microbial stimuli does not determine the type of DC responses (Edwards et al., 2002), and the role of CD40L is probably limited to amplifying innate DC responses that are determined by the microbial signal. In addition, DCs do not generally undergo CD40 ligation until they are in draining lymph nodes and interacting with T cells, which questions the appropriateness of CD40L as a maturation stimulus for DCs.

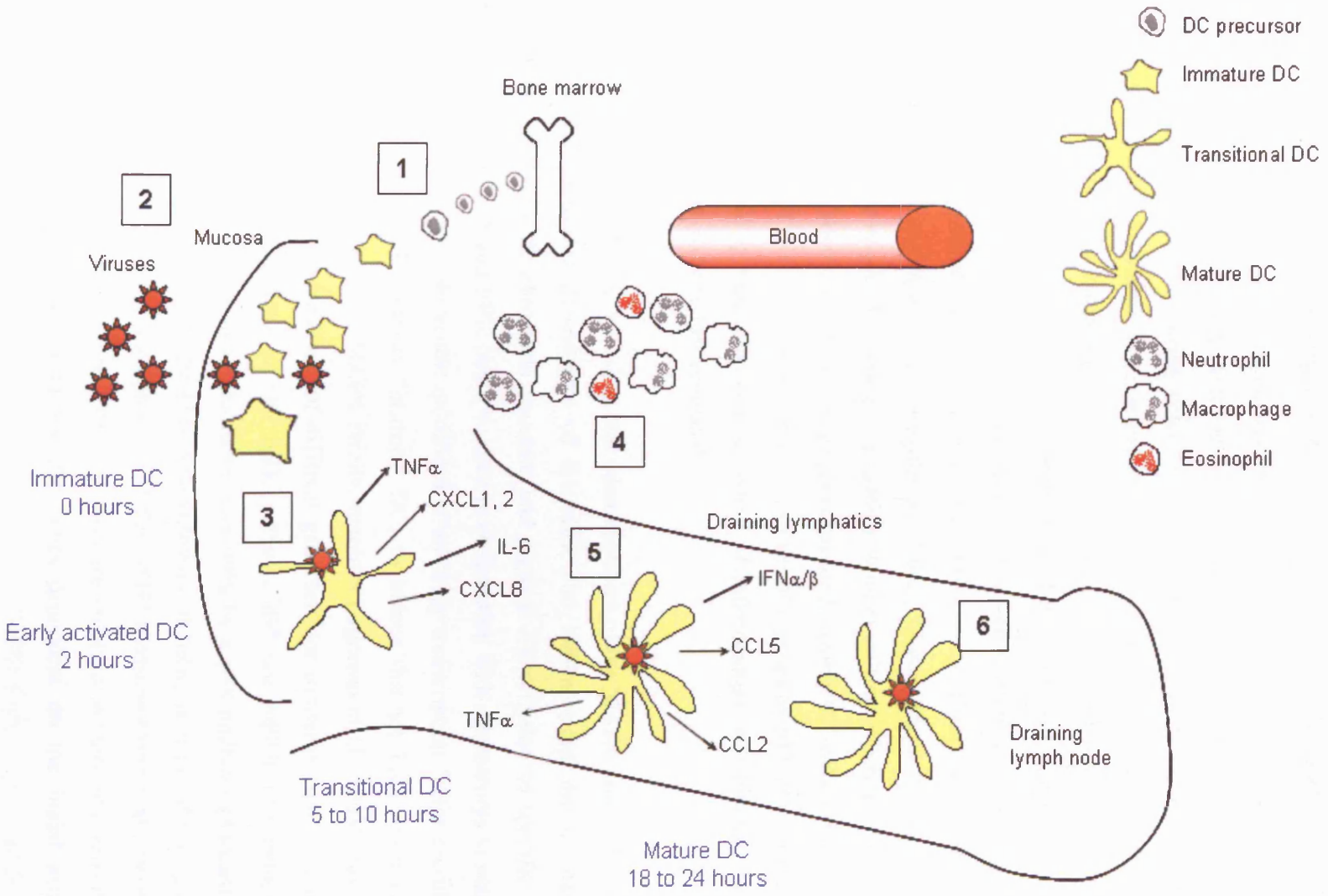
TNFα stimulation of immature DCs does not result in terminal DC maturation, but induces only a transient activated state (Nelson et al., 1999). This demonstrates that inflammatory cytokines may not necessarily cause DCs to differentiate into mature antigen presenting cells, and the appropriateness of maturation stimuli also needs to be considered. The concept of conditioned medium is interesting, as it considers the context in which DCs reside and are stimulated, implying the factors in such conditioned medium derived from various immune cells should more closely mimic physiological states. However, the difficulty in knowing the precise composition of conditioned media and issues with consistency currently limit the value of such an approach.

The relevance of maturation stimuli is of central importance, as DCs stimulated to mature with cocktails of inflammatory cytokines in the absence of pathogenic stimuli may actually mimic a detrimental maladaptive immune response similar to that seen in autoimmunity. In multiple sclerosis, IFNβ therapy has been shown to be beneficial in ameliorating the detrimental T_H1-biased responses that results in the immune system

targeting oligodendrocytes in the central nervous system. This is in contrast to IFNβ's well-recognised role in inducing T_H1-biased antiviral immunity. This paradoxical effect is linked to the timing of IFNβ exposure, where the presence of IFNβ during TNFα-induced DC maturation strongly augments T_H1-differentiation of naïve T_H cells, whereas the presence of IFNβ during mature DC-mediated primary stimulation of naïve T_H cells has the opposite effect of inhibiting T_H1 cell polarisation and promoting the generation of a IL-10-secreting T cell subset (Nagai et al., 2003). This study emphasises the importance of the timing in which immune cells are exposed to cytokines within the framework of the generation of the immune response, where cytokines may have conflicting polarising effects that may not be obvious if temporal aspects are not considered.

Figure 6.1 Schematic representation of the dendritic cell response to antigen

1) Bone marrow progenitors giving rise to DC precursors migrate from the bone marrow to peripheral sites such as the skin or mucosa. Here, precursors differentiate into immature DCs, their properties and location optimised for antigen capture. **2)** Antigens such as viruses may enter the body at breaches in barrier sites such as mucosae. DCs at such sites can capture the viruses, and may be susceptible to virus infection. **3)** Soon after activation by viruses, either viral infection or surface interaction with viruses, early activated DCs secrete a variety of effector cytokines such as TNFα, CXCL1 and CXCL2, and CXCL8. These early activated DCs also modulate expression of cytoskeletal genes to begin their migration out of the periphery. **4)** The secreted cytokines serve to further recruit additional innate cells from the blood to the site of antigen encounter. **5)** Transitional DCs further secrete additional cytokines and chemokines, which may signal in autocrine and paracrine ways to amplify signalling pathways and activate transcription factors that contribute to DC differentiation and maturation, and further influence the surroundings e.g. establishing an antiviral state through the action of IFNα/β. **6)** Mature DCs finally reach the draining lymph nodes, and are fully differentiated and optimised to interact with T cells to initiate the adaptive immune response.


DC precursor
Immature DC
Transitional DC
Mature DC
Neutrophil
Macrophage
Eosinophil
Bone marrow
Blood
Mucosa
Viruses
1
2
3
4
5
6
TNFα
CXCL1, 2
IL-6
CXCL8
IFNα/β
CCL5
CCL2
TNFα
Draining lymphatics
Immature DC
0 hours
Early activated DC
2 hours
Transitional DC
5 to 10 hours
Mature DC
18 to 24 hours
Draining
lymph node

6.3 Transcriptional regulation of signalling pathways

Components of the mitogen-activated protein kinase (MAPK) cascade, an important signal transduction pathway affecting dendritic cell activation and differentiation, were differentially regulated during dendritic cell maturation. p38 is clearly involved in aspects of DC activation and maturation, including activation of transcription factors and upregulation of surface expression of co-stimulatory molecules (Ardeshna et al., 2000; Arrighi et al., 2001). It has also been shown that p38 modifies chromatin structure by phosphorylation of histone H3, and regulating NF-κB recruitment to selected targets including inflammatory and immune response genes (Saccani et al., 2002). Furthermore, transcriptional downregulation of the p38 pathway at the later stages of DC maturation implies that there is a specific window of time where enhancement of NF-κB recruitment to inflammatory genes is required, namely the early activation stage. Modification of the chromatin environment of specific genes by p38 adds an additional regulatory level to transcriptional activation of inflammatory and immune response genes that needs to be tightly regulated.

It may be interesting to speculate that other members of the MAPK family that are co-ordinately activated downstream of microbial and inflammatory stimuli may also function to modify chromatin structure and regulate transcription of specific genes. Inhibitors of p38 and ERK could be used in conjunction with microarrays to map such genes that undergo chromatin modification allowing transcription factor recruitment. That different TLR agonists "instruct" DCs to induce distinct T_{helper} responses via differential modulation of MAPK family members (Agrawal et al., 2003) may be the result of selective recruitment of different gene sets for transcriptional induction by ERK and p38. Blockade of the ERK pathway did not inhibit LPS-induced H3 phosphorylation, which was reduced by over 90% by a p38 inhibitor (Saccani et al., 2002). In contrast, RSK-2, the kinase downstream of ERK, is required for epidermal growth factor (EGF)-activated phosphorylation of H3 (Sassone-Corsi et al., 1999). This suggests that both the p38 and ERK pathways are involved in chromatin remodelling, modifying the transcription of specific genes dependent on the initial activating stimulus.

Overall, the transcriptional regulation of signalling pathways further supports the importance of the temporal regulation of DC maturation, and their innate and adaptive functions including secretion of inflammatory and polarising cytokines.

6.4 Dendritic cell plasticity to antigens

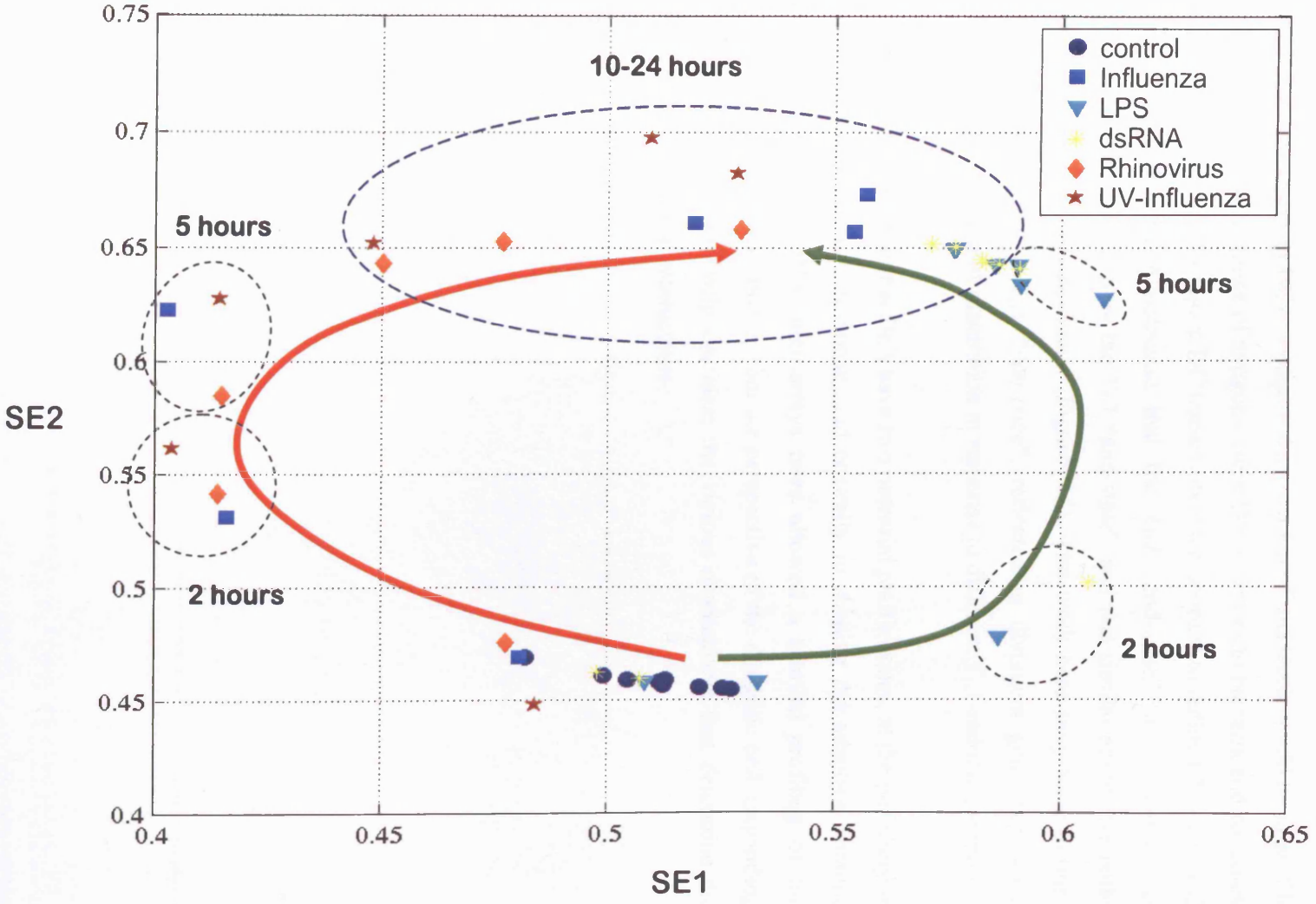
An understanding of the core dendritic cell activation and differentiation response formed the basis for further investigation of DC transcriptional responses to more complex antigens, whole viruses. Array results showed that although there was still a temporal ordering and presence of the core maturation response, these were within pathogen-specific transcriptional responses. This specificity depended on the type of virus and the capacity of the virus to replicate. Transcriptional plasticity demonstrated by dendritic cell responses to viruses was also evident on the protein level. Further characterisation of virally-challenged DC gene expression programmes, including the regulation of signalling pathways, transcription factors and innate and adaptive effector functions of DCs will facilitate understanding of how antiviral and virus-specific responses are modulated.

The presence of a core and pathogen-specific transcription programmes raises the question of how and when such plasticity is manifested in DC phenotype and function. The differences between the core DC transcriptional responses to pathogen components (LPS and dsRNA) and the virus-specific DC responses can be illustrated by the data visualisation technique of spring embedding. Spring embedding allows the visualisation of the relationships between all genes on each array to be examined by the strength of associations ("springs") based on gene expression profile correlation, a form of non-linear principal component analysis. This in effect collapses high-dimensional data (45 arrays, approximately 2000 genes) into a 2-dimensional output, the dimensions encompassing the largest variance in gene expression (Spring embedding (SE)1 and SE2 in Figure 6.2). Figure 6.2 shows the relationship between DC responses to the two pathogen components and the three virus treatments. The correlation between the arrays is represented by the distance between the arrays. There are three major conclusions that can be drawn from this. Firstly, the control mock-stimulated DCs and time zero arrays of stimulated DCs all cluster together, i.e. the gene expression of DCs at the beginning of the timecourse, and DCs that are mock-stimulated over 24 hours, are essentially the same. This is supported by hierarchical clustering in Chapter 3 and 4 which clustered

together mock-stimulated DCs and DCs at time zero. Secondly, by 24 hours, gene expression of mature DCs are also very similar, suggesting the common phenotype of a potent antigen presenting cell. Thirdly, core maturation and virus-stimulation results in markedly different gene expression changes, where LPS- and dsRNA-stimulated DCs respond by progressing in one direction (green arrow), and virus-stimulated DCs respond by progressing in a different direction (red arrow). This suggests that DCs activate and respond in very divergent paths to core pathogen components and viruses, and this difference is evident by 2 hours post-stimulation. Such paths then seem to converge, resulting in a mature antigen presenting cell by 24 hours after stimulation. This suggests that mature DCs induce certain characteristics that define an effective antigen presenting cell, but the way such characteristics are acquired are plastic, dependent on the stimulus.

Figure 6.2 Dendritic cell core and virus-specific maturation

Spring Embedding showing relationship between DC responses to LPS, dsRNA, Influenza, UV-inactivated Influenza, Rhinovirus, and control mock-stimulation. The two dimensions SE1 and SE2 refer to the dimensions that capture the two largest variations in gene expression profiles of the 45 arrays. (Figure courtesy of Tim Ebbels, Department of Computer Science, University College London)


0.75
0.7
0.65
0.6
0.55
0.5
0.45
0.4
0.4
0.45
0.5
0.55
0.6
0.65
SE2
SE1
10-24 hours
5 hours
5 hours
2 hours
2 hours
control
Influenza
LPS
dsRNA
Rhinovirus
UV-Influenza

However, Figure 6.2 does not clearly demonstrate DCs' ability to induce differential T cell responses, namely T_H1 and T_H2. This may be a result of the similarity in the range of antigens used, in that both LPS, dsRNA, and the two RNA viruses generally stimulate T_H1 responses in DCs. A natural continuation of this work would be to profile DC responses to a wider range of antigens, from DNA viruses to bacteria and parasites. It would be interesting to compare DC transcriptional responses to defined T_H1 and T_H2 polarising antigens. We hypothesise that the T_H2 "end-state" at 24 hours after stimulation will be distinct from the T_H1 "end-state", and that similar alternative paths to the T_H2 state will be determined (Figure 6.3). The work here may have defined subtler plasticity within a T_H1 "subspace", reflected in divergent gene expression profiles of the progression of maturation in response to different T_H1-inducing stimuli.

In summary, it can be seen that DCs have two potential plastic states, at the periphery in shaping the innate immune response, and centrally in shaping the adaptive immune response (Figure 6.3). DNA microarrays have allowed a detailed profiling of the evolution of the immune response from the perspective of the dendritic cell. Expanding the repertoire stimuli will help elucidate the various mechanisms that determine the outcome of host-pathogen interactions.

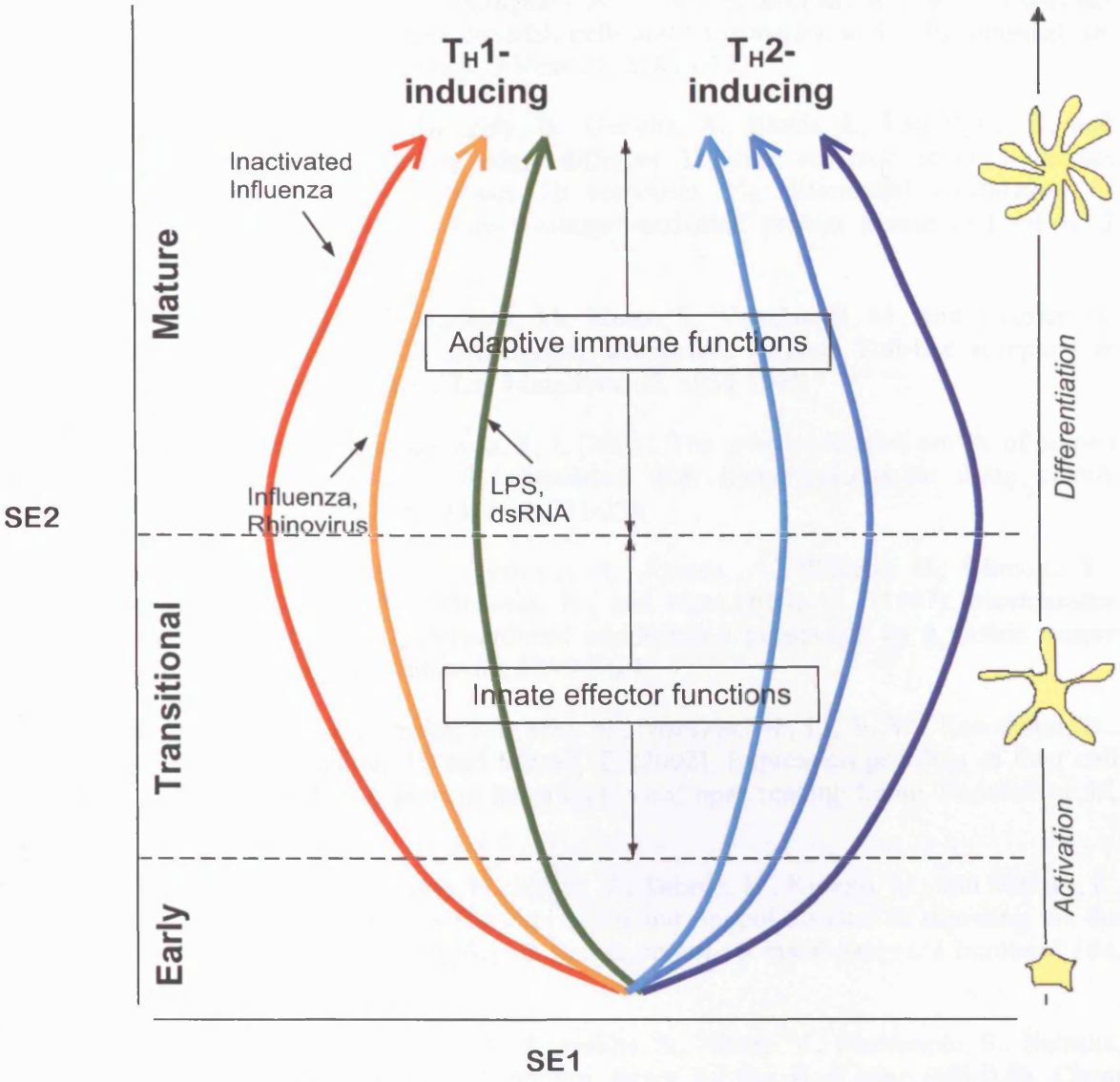


Figure 6.3 Proposed plasticity model for DC activation and differentiation

This is an extrapolated version of Figure 6.2 that includes hypothesised DC gene expression changes in response to T_H2-inducing stimuli, for example parasitic antigens. As DCs undergo the process of activation and differentiation in response to various stimuli, there is plasticity at the various stages of maturation. The activation of DCs depends on the initial stimuli and environmental context, which affects downstream effector cytokines that are secreted. These transitional DCs then differentiate into mature antigen presenting cells that can induce either T_H1 or T_H2 responses depending on the stimuli. The subtleties in transcriptional plasticity at the various stages are demonstrated by gene expression profiling of DC responses throughout maturation, giving rise to functional plasticity affecting differential host-pathogen immune responses.

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