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Functional consequences of HSV-1 infection of dendritic cells

A thesis submitted by

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Abstract

Herpes simplex virus-1 (HSV-1) infects a significant proportion of the population, causing widespread morbidity and occasional mortality. An understanding of the interaction of this virus with the immune system will aid in the development of vaccines to reduce the prevalence of this infection, and in evaluating its potential role as a vector in immunotherapy. Dendritic cells (DC) are the critical initiators of adaptive immune responses to HSV-1 and are likely to encounter the virus early after infection of the skin or mucosa. This thesis aims to describe the functional consequences of the interaction between HSV-1 and DC.

DCs are readily infected by HSV-1. The function of these cells is affected in a multitude of ways. The capacity of infected DC to stimulate T cell proliferation is reduced, following changes in morphology, IL-12 secretion and surface phenotype. However, the infection of DC by HSV-1 also results in activation of both infected and uninfected cells. The secretion and paracrine activity of type I IFN is responsible for the activation of bystander DC and plays an important amplifying role in the secretion of IL-12 by these cells. In contrast, HSV-1 envelope structures bind to the surface of DC and induce maturation directly. The binding of HSV-1 glycoprotein D is critical to the acquisition of this mature phenotype.

In the context of natural HSV-1 infection, these findings suggest the hypothesis that the coupling of viral entry to the activation of DC signalling pathways is counterbalanced by viral disruption of DC maturation. This delays the generation of the immune responses to HSV-1. Nonetheless, the parallel release of type I IFN, resulting in paracrine activation, enables other DC to mount an antiviral immune response

To my family, for always being there

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Publications

Data and concepts in this thesis have contributed to the following publications (attached at the end of the thesis):

- **Pollara G**, Jones M, Handley ME, Rajpopat M, Kwan A, Coffin RS, Foster G, Chain B, Katz DR.
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- **Pollara G**, Katz DR, Chain BM.
  The host response to herpes simplex virus infection.
  Curr Opin Infect Dis. 2004 Jun;17(3):199-203.

- **Pollara G**, Speidel K, Samady L, Rajpopat M, McGrath Y, Ledermann J, Coffin RS, Katz DR, Chain B.
  Herpes simplex virus infection of dendritic cells: balance among activation, inhibition, and immunity.
  J Infect Dis. 2003 Jan 15;187(2):165-78.

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Abbreviations

AAF	IFNalpha-associated factor
AdV	Adenovirus
APC	Antigen presenting cell
ARE	AU-rich elements
ATL	Adult T cell leukaemia
ConA	ConcanavalinA
DC	Dendritic cell
DRG	Dorsal root ganglia
dsRNA	double stranded RNA
E	Early
EBV	Epstein Barr Virus
EM	Electron microscopy
ER	Endoplasmic reticulum
FAK	Focal adhesion kinase
FCS	Foetal calf serum
FIX-HSV	Formaldehyde-inactivated HSV
FN	Fibronectin
FSC	Forward scatter

GAS	IFNgamma-activated-site
gB	Glycoprotein B
HBSS	Hank's balanced salt solution
HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HEV	High endothelial venules
HIgR	Herpesvirus Ig receptor
HIV	Human immunodeficiency virus
HS	Heparan sulphate
HSP	Heat shock protein
HSV-1	Herpes simplex virus 1
HTLV-1	Human T-cell lymphotropic virus type 1
HUVEC	Human umbilical vein endothelial cells
HVEM	Herpesvirus entry mediator
IE	Immediate early
IFN	Interferon
IFNAR	Type I IFN receptor
IRF	Interferon response factor
IRFE	IRF-1 elements
ISG	Interferon-stimulated gene

ISGF3	IFN-stimulated gene factor-3
ISRE	Interferon-stimulated regulatory elements
JAK	Janus kinase
L	Late
LAM	Lipoarabinomannan
LN	Lymph node
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
mAb	monoclonal antibody
ManLAM	Mannose-capped lipoarabinomannan
MAPK	Mitogen-Activated Protein Kinase
MCM	Monocyte conditioned medium
MCMV	Murine cytomegalovirus
MDC	Myeloid dendritic cell
MDDC	Monocyte derived dendritic cell
MF F	MV fusion glycoprotein
MLR	Mixed lymphocyte reaction
MMTV	Murine mammary tumour virus
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MV	Measles virus

MV HA	MV haemagluttinin glycoprotein
MV NP	MV nucleoprotein
MVA	Modified vaccinia virus Ankara
MyD88	Myeloid differentiation factor 88
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cells
PDC	Plasmacytoid dendritic cell
pfu	plaque forming units
PGN	Peptidoglycan
PI	Propidium iodide
PIV	Parainfluenza virus
Poly(I:C)	Polyriboinosinic polyribocytidylic acid
PPD	Purified protein derivative
PRD	Positive regulatory domain
PRR	Pattern recognition receptors
PS	Phosphatidylserine
RSV	Respiratory syncitial virus
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
siRNA	short interfering RNA

SSC	Side scatter
ssRNA	single stranded RNA
STAT	Signal transducer and activator of transcription
supCON-DC	supernatant from uninfected DC
supHSV-DC	supernatant from HSV-1 infected DC
TAP	Transporter associated with antigen processing
TIR	Toll-interleukin 1 receptor-resistance
TIRAP	TIR domain-containing adaptor protein
TLR	Toll like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TRAF	TNFR-associated factor
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
TRIF	TIR-domain-containing adaptor inducing interferon-beta
UV-HSV	UV-inactivated HSV
VSV	Vesicular stomatitis virus
VZV	Varicella zoster virus
WT-HSV	Wild type HSV

Chapter 1

Introduction

1.1 Natural history of HSV-1 infection in man

Primary infection with HSV-1 occurs predominantly in children. It is often asymptomatic. However, HSV-1 infection can result in a wide spectrum of clinical presentation, ranging from asymptomatic infection, to ulcerative and vesicular lesions of the skin, and to systemic infection of large organs. Furthermore, symptoms differ between primary and recurrent lesions.

When symptoms do occur, vesicular lesions are usually intraoral, affecting the gingival and buccal mucosa (gingivostomatitis and pharyngitis). The vesicles formed then ulcerate, exposing infectious particles to the environment and therefore other individuals. Over the following 1-2 weeks, any symptomatic peripheral lesion in the mucosa usually resolve. During primary infection, some viral particles enter the endings of peripheral sensory nerves (predominantly c-type nerve fibres) and the virus travels retrogradely to the dorsal root ganglion (DRG) of the innervating dermatome. For oral infection this is the trigeminal ganglion, and it is here that a latent infection occurs, a common ability of all herpesviruses. Oral HSV-1 infection can result in latent infection of up to 40-60% of sensory nerves innervating the infection site (Jones, 2003).

Periodically, the virus reactivates from latency. The factors responsible for this reactivation are still unclear, but include other febrile illnesses, direct sunlight (predominantly UV rays), psychological stress, and systemic immunocompromised states (both iatrogenic and infectious). After reactivation, viral particles travel down the same peripheral nerves and reinfect the innervated area of skin. The most common sites for reactivation include the lips and adjoining skin (herpes labialis). Clinically, secondary infection is characterised by a prodrome of pain, burning, tingling, or itching, followed by macular lesion formation within 24 hours, which later become vesicular and then pustular. The lesion is then most often cleared within 2 weeks.

In the majority of cases, local peripheral HSV infections are not life threatening, although the morbidity can be both physically and psychologically debilitating. This is particularly the case in the instance of complications of HSV-1 infection, such as

herpes keratitis (the second most common cause of non-traumatic corneal blindness), herpetic whitlow and eczema herpeticum, which may result from auto-inoculation from the oral lesion. HSV can also rarely result in life-threatening encephalitis, although the cause is not clear. It should also be noted that HSV-1 infection is not restricted to the oral region. An increasing number (up to 40%) of genital herpes infections, particularly in women under 25 years old, are now caused by HSV-1 (Malkin, 2004), and shedding genital herpes during child birth increases the risk of neonatal infection during birth. This has a high mortality rate and incidence of neurologic sequelae in survivors (Whitley, 2004).

Therefore, the clinical manifestation of HSV-1 disease demonstrate that despite being non-life threatening in the majority of immunocompetent individuals, this virus infection still carries considerable disease burden to the population. A more detailed understanding of its pathogenesis and immunobiology will aid towards successful vaccine design.

1.2 Biology of HSV-1

The virus name comes from the Greek *'herpein'* which means 'to creep', referring either to the chronic, latent or recurrent infections that occur or to the spreading nature of the visualised skin lesions. The epidemiology of HSV infections puzzled clinicians for many years and it was not until 1950 that Burnet and Buddingh first showed that HSV could become latent after a primary infection and reactivate after later provocation. Subsequent studies with other members of the herpesvirus family confirmed that latency is a shared property amongst them.

1.2.1 Virus structure

The HSV virion consists of a large double-stranded DNA genome (152 kbp long, with 85 open reading frames) wound around a proteinaceous core. The capsid surrounds the core and outside the capsid is the tegument, a protein-filled region that appears amorphous in electron micrographs. On the outside of the viral particle is the envelope,

which is derived from the outer leaflets of the Golgi apparatus of infected cells and contains numerous glycoproteins.

1.2.2 Mechanism of viral infection

Like other members of the herpesvirus family, HSV-1 is an enveloped virus and enters target cell cytoplasms following envelope fusion with the cell surface membrane. The initial interaction is attachment of virions to glycosaminoglycans (preferentially heparan sulphate) on the cell surface, followed by binding to and entry via specific entry receptors. The viral factors involved in this process have been well characterised and are described in more detail below.

1.2.2.1 Heparan sulphate

The role of heparan sulphate (HS) in HSV binding and entry has been studied extensively. Several lines of evidence have alluded to the role of HS as an initial receptor for HSV infection. Firstly, HS proteoglycans are frequently found on the surface of many cell types, including ones susceptible to HSV infection (WuDunn and Spear, 1989). Secondly, removal of HS from the cell surface, enzymatically or in HS deficient cells, renders the cells partially resistant to HSV infection by reducing surface binding of the virus (Shieh et al., 1992). Thirdly, heparin, a molecule chemically similar to HS (Kjellen and Lindahl, 1991), has been shown to prevent HSV infection by masking HS binding domains on the virus envelope (Herold et al., 1995).

The viral mediators of HS attachment are glycoprotein B (gB) and gC, as they can bind to immobilised heparin columns (Herold et al., 1991), and are the only HS binding proteins encoded by HSV-1 (Laquerre et al., 1998). Deletion mutants for gB and gC have demonstrated reduced virus binding to the cell surface (Herold et al., 1994), to a similar degree as infection of cell lines deficient for HS (Gruenheid et al., 1993). Furthermore, gB and gC deletion mutants can still penetrate cells, albeit at a reduced rate (Herold et al., 1991; Laquerre et al., 1998), suggesting that HS attachment may only be required to increase the efficiency of binding to a cellular surface receptor or to

induce changes in the bound particle envelope that facilitates the process of envelope binding to surface receptors and fusion with the cell surface membrane.

1.2.2.2 Entry receptors

There are currently three classes of entry receptors known, a member of the tumour necrosis factor receptor (TNFR) family, immunoglobulin superfamily members and specific enzyme modified moieties in heparan sulphate.

- ***Herpesvirus entry mediator (HVEM)*** - Located on human chromosome 1 (Kwon et al., 1997), this molecule is a member of the large TNFR superfamily. The expression of HVEM, also known as HveA, on non-permissive cells allows HSV-1 entry (Montgomery et al., 1996) after interaction with HSV-1 gD (Whitbeck et al., 1997). Like many other members of the TNFR family, the cytoplasmic tail of this receptor can bind the TNFR-associated factor (TRAF) family members TRAF1, 2, 3 and 5, but not 6 (Marsters et al., 1997). As a result, HVEM is capable of activating NF-kB and AP-1 signalling pathways (Marsters et al., 1997). Interestingly, abrogation of the cytoplasmic tail of HVEM, such that no TRAF binding sequences are present, does not interfere with viral entry, excluding the role of intracellular signalling via this receptor as being necessary for viral entry (Montgomery et al., 1996). However, this does not rule out that signalling induced by viral ligation of the receptor may be important in conditioning the cell for viral replication.

- ***Nectin-1*** – this molecule is a member of the immunoglobulin superfamily of nectins, that function as intercellular adhesion molecules by co-localising with cadherins in adherens junctions. HSV-1 gD can also bind this receptor (also known as poliovirus receptor-related protein 1 or HveC) (Geraghty et al., 1998; Krummenacher et al., 1998). This receptor is expressed on both epithelial and nervous tissue cells, and thus could be an important in vivo mechanism for viral infection of the local tissues and establishment of latency in the nervous system. Indeed, blocking viral-nectin-1 interaction can prevent HSV mucosal infection totally in a murine model of vaginal infection (Linehan et al., 2004). Related members of the nectin family can also bind gD; these include nectin-2

(Eberle et al., 1995; Connolly et al., 2001) and the herpesvirus Ig receptor (HIgR), which shares the same ectodomain as nectin-1 (Cocchi et al., 1998b; Cocchi et al., 1998a). However, HSV-1 has been shown not to require nectin-2 for cell entry (Warner et al., 1998; Krummenacher et al., 2004) and the role of HIgR in HSV-1 entry has not been defined.

The availability of nectins for viral binding may determine the relative susceptibility of epithelial cell layers to HSV-1 infection (Spear, 2002). Nectin-1 co-localises with E-cadherin at adherens junctions in epithelial cells and therefore, is relatively inaccessible to HSV-1 virions. Indeed, disruption of these junctions renders epithelial cells more susceptible to infection (Yoon and Spear, 2002; Marozin et al., 2004). As a result, it is likely that HSV-1 can only infect the host efficiently in the event of a previous break in the epithelial cell layer. The consequential advantage to the virus is that it can infect the more susceptible basal side of epithelial layers (Schelhaas et al., 2003; Marozin et al., 2004), as well as less differentiated epithelial cells in the wound site, on which most nectins are not sequestered in adherens junctions and thus, are available for viral entry (Spear, 2002)

- ***3-0-sulfotransferases*** - Specific glucosamine residues in heparan sulphate can be sulphated by 3-0-sulfotransferases to provide binding sites for gD, which are of similar affinity as the above entry receptors, and are alone sufficient to permit viral entry (Shukla et al., 1999). However, the necessity for enzyme modification of heparan sulphate prior to infection makes it difficult to determine the relative importance of this receptor in vivo.

Although the receptors described above are structurally unrelated, they can all bind gD directly (Whitbeck et al., 1997; Krummenacher et al., 1998; Nicola et al., 1998; Shukla et al., 1999). Therefore, coupled with the near ubiquitous distribution of heparan sulphate for viral attachment, the relative susceptibility of different cells to HSV-1 infection is likely to be dependent on gD receptor expression. An extension of this hypothesis is that polymorphisms in the sequence of some of the HSV entry receptors may determine infection susceptibility and disease outcome, but studies that have

attempted to address this question have not yet observed such a correlation (Struyf et al., 2002).

Following binding to the entry receptor(s), HSV-1 envelope fuses with the cell membrane and the tegument and capsid are released into the cytoplasm. In the majority of cells, this can occur at neutral pH on the surface of the cell (Wittels and Spear, 1991), whereas in a few cell types, acidification in endosomes is required to enable envelope-membrane fusion (Nicola et al., 2003). Envelope-membrane fusion requires gB, gH and gL, as well as gD binding to entry receptors. However, the mechanisms employed by these glycoproteins are still unclear. One possibility is that conformational changes in the entry receptors allow interaction with gB, gH or gL (Krummenacher et al., 2002). Alternatively there may be receptors for gB, gH and gL on the cell surface that are as yet unspecified.

1.2.3 Mechanisms of HSV-1 replication

After penetrating the surface membrane, tegument proteins disperse into the cytoplasm, whereas viral capsids travel along microtubules via dynein motors to the nucleus (Sodeik et al., 1997). This occurs by direct HSV-1 interaction with the motor ATPase dynein complex responsible for the transport of the virus to the nucleus (Ye et al., 2000; Mabit et al., 2002). The core enters via a nuclear pore (Dohner et al., 2002). The genome is subsequently circularised and can begin transcription using host RNA polymerase II. Like all other herpesvirus genomes, HSV-1 has a unique long (UL) and a unique short (US) region, bounded by inverted repeats. The repeats allow rearrangements of the unique regions and, as a result, herpesvirus genomes exist as a mixture of 4 isomers. Gene expression occurs in a cascade function, with immediate early genes (IE), important for activating early (E) genes, which are involved in viral DNA replication (e.g. thymidine kinase). Late (L) genes encode structural proteins and this occurs in tandem with E gene expression (Weir, 2001).

All IE promoters contain a common *cis*-acting sequence (TAATGARAT) that is required for VP16 mediated trans-activation. VP16 is a tegument protein that is delivered to the nucleus, where it interacts with two cellular proteins, Oct-1 and HCF,

to induce IE promoter activity efficiently (Wysocka and Herr, 2003). Although HCF is expressed in the nucleus of most cells, it is mostly found in the cytoplasm of neurones (Kristie et al., 1999), suggesting a mechanism for reduced gene expression in neurones and allowing the establishment of latency. However, other host factors may also play a role (Jones, 2003). One of these may be the role of inhibitory transcription factors. One such family is Oct-2, which is present as five different isoforms, two of which are neurone specific, Oct 2.4 and 2.5. In vitro transfection studies have shown that these factors can repress Oct-1-VP16 mediated expression of IE genes (Lillycrop et al., 1993). However, the expression of these inhibitory factors remains controversial. Some studies have detected their expression in rat adult DRG (Wood et al., 1992), but this has not been reproduced by all groups (Hagmann et al., 1995b; Turner et al., 1996), suggesting a role for other proteins that can regulate VP16 mediated transcription. It remains to be seen whether host mediated repression of IE gene expression plays a significant role in vivo in preventing the lytic replication cycle in neurones infected by HSV, and subsequently promoting latency.

One notable exception to the repression of HSV gene transcription in latently infected neurones is the LAT genes, whose RNA transcript accumulates in these cells. Although a functional protein product has not been detected (Stevens et al., 1987), one important function of these genes could be their ability to prevent apoptosis in latently infected neurones (Perng et al., 2000; Ahmed et al., 2002). Maintaining cell viability may be critical in maintaining the virus' ability to reactivate from latency (Inman et al., 2001).

1.3 Role of immune system in neuronal HSV infection

The lack of viral gene expression, other than the elusive LAT genes, led investigators to conclude that immunological recognition of viral proteins during latency did not occur. However, this concept is in disagreement with histological data of both animal models of HSV latency and human studies, showing an inflammatory cell infiltrate, including antigen specific CD8+ T cells, in latently infected ganglia (Gebhardt and Hill, 1990; Liu et al., 1996; Theil et al., 2003). This has been hypothesised to signify that low grade viral antigen expression occurs in latently infected neurones, and it has

been confirmed in several studies that have used RT-PCR to detect IE gene transcripts (Kramer and Coen, 1995; Chen et al., 1997; Chen et al., 2002). However, the assay does not give an estimate of the number of neurones expressing these genes and the RT-PCR may mask high-grade expression by a small number of cells. Indeed, recent studies using in situ hybridisation and immunofluorescence has described that one neurone in every 10-17 trigeminal ganglia expressed levels of IE and E transcripts and proteins at levels equivalent to that of lytically infected cells (Feldman et al., 2002; Sawtell, 2003). Furthermore, it was these neurones that were surrounded by an inflammatory cell infiltrate, suggesting that immune mediated control of latency reactivation may not be occurring globally (e.g. by cytokines), but rather in a focal antigen-specific mechanism on reactivating neurones (Feldman et al., 2002).

Currently, it is still unclear how the infiltrating inflammatory cells can regulate activation from latency. Significant levels of interferon γ (IFNγ) and tumour necrosis factor α (TNFα) are expressed in TG, suggesting that T cells present are activated following viral DNA replication (i.e. from the expression of the required proteins) (Cantin et al., 1995; Halford et al., 1996; Halford et al., 1997). The infiltrating T cells are CD8+ T cells and can block viral reactivation in an ex vivo model (Liu et al., 2000). They are antigen specific, although this study was only carried out in mice models where the HSV response is highly focused towards one epitope of gB (Khanna et al., 2003). The mechanism involves direct contact with infected neurons and IFNγ secretion (Khanna et al., 2003). The prevention of reactivation by T cell derived cytokines is supported by latency studies carried out in IFNγ and TNFα knockout mice, which demonstrated increased levels of HSV reactivation, though it is not possible to exclude that the immune defect in the periphery allowed a greater number of virions to enter latency per neurone, predisposing to increased frequency of reactivation (Cantin et al., 1999; Minami et al., 2002).

It is likely that the immunological control described in animal models can be extended to the human system. Histology of human DRG sections shows a CD8 T cell infiltrate and the presence of inflammatory cytokines (Theil et al., 2003). Thus, it seems that HSV latency is not only an intrinsic property of the virus, but is also actively ~~promoted~~ regulated by the host immune system. This might well explain the increased frequency of

reactivations in immunocompromised individuals (Schacker et al., 1998b). Furthermore, the buildup of IE gene transcript may explain the advantage of LAT gene expression, to prevent this stimulus promoting apoptosis of the neurone (Inman et al., 2001; Sanfilippo et al., 2004).

1.4 Role of the immune system in the control of peripheral HSV-1 disease

The immunological control of peripheral infection has been studied in greater detail than that of the central nervous system. The host response to infection occurs in two phases, the innate and adaptive immune responses. The prior is likely to be critical in limiting the spread of infection, although only the cellular adaptive immune responses is more strongly associated with resolution of HSV lesions (Siegal et al., 1981).

1.4.1 Innate immune system

1.4.1.1 Epithelial cells

In the context of a cutaneous/mucosal model of HSV infection, the first cell type to be infected is likely to be an epithelial cell or keratinocyte. Therefore, the signals secreted from this cell type will determine the nature and kinetics of the ensuing immune response. Indeed the cytokines found in herpetic lesion fluid within the first day of infection in humans, namely IL-1α, IL-1β, IL-6 and IL-10, are also secreted by keratinocytes following HSV-1 infection in vitro (Mikloska et al., 1998). Infected keratinocytes also secrete CCL3, CCL4 and CCL5, which are important in recruiting other inflammatory cells, including dendritic cell (DC) precursors and activated T cells (Taub et al., 1993; Yoneyama et al., 2004). In turn, infiltrating T cells can secrete IFNγ that upregulates HLA-DR expression on keratinocytes (Mikloska et al., 1996).

1.4.1.2 NK cells

NK cells may be protective in primary HSV infections (Pereira et al., 2001). Mouse models have also shown that they are an early source of IFNγ in the lesion, although

this is not critical for the clearance of vaginal infection with an attenuated HSV-2 strain (Milligan and Bernstein, 1997). Their role in humans has been extrapolated from a child with a deficiency in NK cells who succumbed to early and systemic herpesvirus infections (Biron et al., 1989), and an adult with disseminated HSV-2 disease in the absence of systemic NK cells (Dalloul et al., 2004).

Recently, NKT cells have also been suggested to play a protective role against HSV infection (Grubor-Bauk et al., 2003). However protection by this cell type, as determined by viral infection of the nervous system, was only apparent 7 days after infection. The delay in protection implies that this cell type, although sharing surface molecules with NK cells, should be considered part of the anti-HSV adaptive immune response (Grubor-Bauk et al., 2003).

1.4.1.3 Complement system

Complement can enhance the humoral immune response to HSV (Verschoor et al., 2001). A chimaeric mouse model demonstrated that local C3 production by myeloid BM-derived cells was sufficient to restore the humoral response to infectious HSV-1 in C3-deficient mice (Verschoor et al., 2003). The HSV strategy to avoid complement activation (section 1.5.1) may therefore result not only from an attempt to neutralise the complement activation cascade directly, but also to abolish the generation of an antibody response.

1.4.2 Adaptive immune system

1.4.2.1 Antibody response

The presence of neutralising antibodies to HSV has been observed for many years. The clearest example of their functionality comes in neonatal infections, whereby maternal antibodies protect from transmission to the newborn during delivery (Brown et al., 1997). The antibody targets are very broad, including envelope glycoproteins and viral capsid components (Ashley et al., 1985; Ashley et al., 1994), demonstrating that a wide variety of viral antigens are accessible to the immune system to sample.

The efficacy of the in vivo humoral response in adults is less clear. Murine studies have shown that antibodies can offer protection in the early stages of infection, when viral titres are low (Kuklin et al., 1998). Natural IgM antibodies may play a role in limiting low dose inocula spreading to the CNS (Deshpande et al., 2000), although the relevance to humans is unclear. Passive immunisation with HSV-specific IgG can also reduce lesion severity after vaginal infection (Parr and Parr, 1997), emphasising that antibodies are important in limiting the spread of infection in the early stages (the first 20 hours), whereas T cell responses peak later, independent of antibody activity (Parr and Parr, 2000). However, the role of B cells and antibodies remains controversial, as immunised B cell deficient mice can clear HSV infection equally well as wild type mice (Kuklin et al., 1998). Furthermore, large-scale clinical trials using recombinant glycoprotein subunit vaccines have showed that seroconversion did not correlate with subsequent disease severity (Corey et al., 1999). It should also be noted that the lack of correlation between antibody and protection from infection in man may be related to one of the immune evasion mechanisms of HSV-1 and –2, the gE-gI dimer that binds human, but not mouse, IgG, and neutralises its function (Johansson et al., 1985) (see section 1.5.2).

1.4.2.2 CD4+ T cell response

Approximately 0.2% of all CD4+ T cells in immune individuals are specific for HSV antigens, as detected by intracellular IFNγ staining (Asanuma et al., 2000). CD4+ T cells are the initial T cells infiltrating into herpetic lesions and can secrete a variety of cytokines (Mikloska et al., 1998). The most important of these is IFNγ and CD4+ T cells are the predominant source of this cytokine in peripheral blood mononuclear cells (PBMC) from patients with recurring herpetic lesions (Cunningham and Merigan, 1984; Cunningham et al., 1985a). This cytokine plays a role in partially reversing the HSV-induced downregulation of MHC class I and inducing MHC class II expression in keratinocytes, rendering the cells more detectable by both CD4+ and CD8+ T cells (Mikloska et al., 1996).

In mouse studies, CD4+ T cells confer greatest protection in vaginal challenge with HSV-2 (Kuklin et al., 1998). In humans, although increased IFNγ secretion by HSV

specific PBMC correlates weakly with reduced frequency of herpes labialis (Spruance et al., 1995), there has not been a study definitively showing a correlation between the strength and breadth of CD4+ responses and clinical severity (Posavad et al., 1997). This may relate to technical limitations of the assays used to detect CD4+ T cells. Alternatively CD4+ responses may be more important locally, either in the lymph nodes (LNs) for providing help to CD8+ T cells, or in the periphery for IFNγ secretion. Furthermore, HSV specific CD4+ T cells can be cytotoxic (Mikloska et al., 1996; Koelle et al., 1998b) and thus differences in circulating antigen specific CD4+ T cells, as detected by IFNγ secretion upon restimulation, may not necessarily correlate with their true role and distribution in vivo.

Recent focus has been directed on defining the antigenic epitopes of the host immune response, in order to identify desired vaccine targets. These studies observed that while keratinocytes can be induced to express HLA-DR after IFNγ stimulation (Mikloska et al., 1996), CD4+ T cell clones identified are also restricted by HLA-DP and –DQ (Koelle et al., 1998a). These are likely to be stimulated by professional APC in the draining LN, rather than keratinocytes in the lesion site. This underlies the importance of professional APC, such as DC, in the immune response to HSV.

1.4.2.3 CD8+ T cell response

Although CD8+ cells follow CD4+ T cells into lesions (Cunningham et al., 1985b), the role of CD8+ T cells in the control of HSV infections has been most clearly demonstrated in a cross-sectional study demonstrating an inverse correlation between CD8+ T cell number and the severity of genital herpes lesions (Posavad et al., 1997). Furthermore, serial histological studies of lesion biopsies demonstrated that the clearance of virus correlates best with the infiltration of CD8+ T cells (Koelle et al., 1998b). It should be noted that these studies were carried out on biopsies of HSV-2 infection of genital mucosa, and although likely to be similar, no direct studies of HSV-1 lesions have yet been performed. Nevertheless, a recent study demonstrated that CD8+ T cell responses could only prevent infection of peripheral nerve endings within 24 hours of epidermal inoculation (van Lint et al., 2004), emphasising that the

predominant role of CD8+ T cells in vivo is likely to be to resolve the lesion rather than prevent HSV spread to the nervous system.

Similarly to CD4+ T cells, recent studies have focused on understanding the breadth of the CD8+ T cell repertoire. Tetramer binding can give an estimate of individual TCR frequencies, and in vitro restimulation of T cells can generate clones that can identify antigen targets. However, the latter approach involves two rounds of antigen stimulation, which may bias the relative frequency of epitope-specific T cells. Recent studies using only one round of antigen stimulation have identified subpopulations of HSV-specific CD8 T cells with a broader range of reactivity than previously observed (Koelle et al., 2003). The relative immunodominance of the novel epitopes is still unspecified, but the data demonstrates clearly that despite the immune evasion mechanisms the virus utilises (see section 1.5), the host is still able to mount a potent and broad CD8+ T cell response.

1.5 HSV immune evasion

The suggested importance of the role of the immune system in the control of HSV infections has been based upon histological analysis of lesions and knockout mouse studies. However, the evolution of proteins that enable the virus to evade specific components of the immune system also implies that immunity plays an important role in the HSV life cycle. Below are the components of the immune response that the virus is known to interfere with.

1.5.1 Complement

HSV-1 gC is a receptor for C3b (Friedman et al., 1984) and prevents host activation of the alternative complement pathway (Fries et al., 1986). In part this occurs through accelerated decay of C3 convertase, but gC also has a domain that prevents the binding of C3b to C5 and properdin (Fries et al., 1986). It is the C3 binding domain that constitutes most of the virulence in vivo (Lubinski et al., 1999), as demonstrated by infections using gC mutant viruses (Lubinski et al., 1998; Lubinski et al., 1999). Importantly, this occurs largely independent of the presence of neutralising antibodies

(Friedman et al., 1996), demonstrating a specific evasion of the activation of the complement cascade by this virus and the effectiveness of this complement in control of HSV infection.

1.5.2 Antibody responses

A heterodimer formed between HSV-1 gE and gI functions as an IgG Fc receptor (Johnson et al., 1988). Virus specific antibodies can form antibody bridges between the target antigen via the F(ab')2 domain and the gE/gI receptor via the Fc domain of the antibody (Frank and Friedman, 1989). This can occur both on the surface of infected cells as well as on the viral envelope, preventing C1q binding and IgG binding to FcγR, inhibiting antibody-dependent cellular cytotoxicity (Dubin et al., 1991; Van Vliet et al., 1992). It is worth noting that as gE can prevent C1q binding, this is another mechanism of preventing complement activation, distinct from the actions of gC, which inhibits complement independent of antibody (section 1.5.1).

Murine models of infection with HSV-1 gE mutants possessing abrogated FcR activity suggest that this evasion mechanism is translated into a viral advantage in vivo (Nagashunmugam et al., 1998). Furthermore, similar IgG binding glycoproteins are encoded by HSV-2, varicella zoster virus (VZV) and human cytomegalovirus (HCMV), suggesting a conserved property within this virus family that confers a survival advantage (Keller et al., 1976; Para et al., 1982; Litwin et al., 1992).

1.5.3 Antigen processing

As noted earlier, it is the cellular immune response that correlates with resolution of the infection. Therefore, it is unsurprising that HSV-1 has also evolved mechanisms to prevent recognition by CD8+ T cells, by reducing the expression of (viral peptide presenting)-MHC class I molecules on the surface of infected cells.

The US12 gene of HSV-1 encodes for the ICP47 protein that can interfere with peptide loading of MHC class I molecules by binding to the cytosolic side of the transporter associated with antigen processing (TAP) molecules on the ER. The result is a lower

expression of MHC class I molecules (including ones presenting viral antigens) on the surface of infected cells. Indeed, deletion of ICP47 renders infected cells more susceptible to CTL mediated lysis (York et al., 1994; Tomazin et al., 1996). It is difficult to determine the role of ICP47 in vivo, as this protein does not bind and inhibit the function of murine TAP as efficiently as its human counterpart (Ahn et al., 1996; Tomazin et al., 1996; Tomazin et al., 1998). Nevertheless, ICP47 mutant viruses still display decreased neurovirulence in mice, a property lost in the absence of a CD8 T cell response, demonstrating the important role this viral protein can have in masking CD8 T cell targets in vivo (Goldsmith et al., 1998).

1.5.4 Cytotoxic T cell activity

HSV has evolved several mechanisms to prevent apoptosis (Koyama and Miwa, 1997) (see chapter 4), presumably with the intent of keeping the cell alive a sufficiently long time to complete an efficient replication cycle and produce ample viral progeny. One possible apoptosis inducing-signal is that originating from CTL, either by Fas ligation on the surface of infected cells or by granzyme activity. Indeed, HSV infected cells are resistant to CTL induced apoptosis (Jerome et al., 1998) owed in part to the expression of the US5 gene product, gJ (Jerome et al., 2001). However, the modulation of CTL activity is more complex, as HSV infected cells expressing the US3 protein kinase can also inactivate CTL that make direct contact with the infected cell (Sloan et al., 2003). Furthermore, in the event of an activated T cell being infected, presumably after coming in contact with an infected non-lymphocyte, it renders them more susceptible to die by Fas-mediated fratricide mediated by other HSV-specific CTL (Raftery et al., 1999).

1.6 Generation of adaptive immune responses: the role of dendritic cells

As a result of the data pointing to a role of cellular immunity in the control of HSV infection, recent efforts have concentrated on understanding the process that generates these responses. It has been appreciated for some time now that the generation of adaptive cellular immune responses is dependent on the stimulation of T cells by

professional antigen presenting cells (APC). Specifically, the initial activation of antigen specific naïve T cells in vivo is carried out predominantly by DC. This section will review briefly the major aspects of DC biology, and then consider DC in the context of viral immunity, focusing specifically on the role of immunity to HSV-1.

What makes a mature DC a more potent stimulator of T cells? This has been an important question, not only in understanding DC physiology, but also for providing clues to host responses and mechanisms that pathogens may disturb. Originally identified on the basis of their morphology (Steinman and Cohn, 1973), these cells are now accepted as the most potent APC, particularly in their capacity to activate naïve T cells. Several of their cell biological properties, such as the stability of MHC-peptide complexes, explain this role (Cella et al., 1997; Zehn et al., 2004). Other important factors include the cytokine cocktail secreted at the time of T cell contact, and the motile and plastic DC cytoskeleton. Recent intravital dynamic imaging in lymph nodes has shown that rapid dendrite extensions by DC permit scanning of around 5000 T cells per hour (Miller et al., 2004). The interaction time between DC and T cells is increased both by the presence of antigen bearing DC and the maturation state of DC, such that mature DC induced formation of stable immunological synapses that last for >8 minutes at a time (Bousso and Robey, 2003; Stoll et al., 2002; Miller et al., 2002; Benvenuti et al., 2004b). The increased length of interaction ensures that T cells receive appropriate stimulatory signals from the DC, both in the form of surface molecule interaction and via the paracrine activity of DC-secreted cytokines.

However, a snapshot of their interaction with T cells belittles their central role in the generation of antigen specific immune responses. DC exist in two functional states, immature and mature, although this simple model has recently been expanded on (see section 1.9). In the immature state, DC form a network in the body's periphery: skin, mucosa and most other organs where pathogen entry can occur. In this state, DC are relatively poor T cell stimulators. Their predominant role is sampling the antigenic environment surrounding them. This occurs through non-specific uptake in the fluid phase, by macropinocytosis, or through phagocytosis of molecules binding to specific receptors on the DC surface, such as c-type lectins or FcR. The antigenic contents of this uptake is degraded and processed for presentation on MHC molecules. The model

predicts that in the steady state, in the absence of a surrounding infection, the DC remains in the periphery and continues to sample its microenvironment.

However, upon encounter of certain (combinations of) stimuli, DC undergo a process of maturation, which involves rapid downregulation of antigen sampling and a parallel increase in presentation of the endocytosed antigens. This increase in peptide loaded MHC molecules is coupled to an increase in expression of co-stimulatory molecules, such as CD80 or CD86, critical in the activation of T cells. In parallel, DC migrate out of the peripheral tissues and into draining LNs. However, there is increasing evidence that the maturation response to a variety of pathogens occurs in a non-overlapping fashion by different DC subsets. Therefore, their origin and function will be discussed below before considering the ligands that stimulate DC.

1.7 Dendritic cell subsets

It was known for many years that two types of antigen presenting cells reside in lymphoid organs, one with a pronounced dendritic morphology, and another with a more round shape. By electron microscopy (EM), this second cell type has plasma cell like features, with a smooth outline and a large amount of ER in the cytoplasm (Grouard et al., 1997) and was thus termed the "plasmacytoid monocyte" or "plasmacytoid T cell". Concurrently, it was known that a small proportion of cells in peripheral blood could produce large amounts of type I IFN when stimulated with viruses and these were named "natural interferon producing cells" (NIPC) (Sandberg et al., 1989). In the last few years, it has been established that the plasmacytoid cells are in fact the NIPC (Siegal et al., 1999) and therefore the precursor of plasmacytoid DC (PDC) (Siegal et al., 1999). It has been hypothesised that the extensive ER is necessary for production of large quantities of type I IFN after viral stimulation, and that this exposure to virus promotes their differentiation into mature DC, with dendritic morphology and capacity to activate T cells (Grouard et al., 1997; Cella et al., 2000; Bauer et al., 2001; Yonezawa et al., 2003). It is important to note that the nomenclature used in this field is still inconsistent. For the purpose of this thesis these cells will be referred to as PDC at all stages of their differentiation.

In humans, the DC subsets circulating in blood have been described most extensively and have been classified as 'classical' myeloid DC (MDC) and PDC. The terminology comes from the definitive myeloid origin of classical DC (e.g. CD13+), whereas PDC originally were believed to derive from lymphoid progenitors, as they lack myeloid markers, and expressed T and B cell molecules, such as the preTCRα and Spi-B (Blom et al., 2002). However, the developmental origin appears to be not so rigid, as thymic MDC and PDC can arise from progenitors distinct from those committed to T cell development (Fohrer et al., 2004). In the mouse, easier access to lymphoid organs has allowed the definition of a large number of subsets (Shortman and Liu, 2002). However, the ontogeny of many of these cell types is also still unspecified. Mouse DC subsets had originally been defined on the basis of CD8α expression, and this was also believed to signify a distinction between those of myeloid and lymphoid origin. However, as both subsets could be reconstituted by either myeloid or lymphoid progenitor cells, this hypothesis is probably incorrect (Akashi et al., 2000; Traver et al., 2000). Furthermore, tissue-derived migrating myeloid CD11b+ DC may differentiate into CD8α+ DC in the LN (Moron et al., 2002). As the murine equivalent to human PDC can also be reconstituted from both myeloid- and lymphoid-committed progenitors (Shigematsu et al., 2004), a more plastic model of DC differentiation is now accepted in both mouse and man.

Irrespective of their ontogeny, the presence of DC subsets with divergent functions is likely to represent an evolutionary advantage to the host. Different organs are populated with varying proportions of DC subsets, and therefore their function must be analysed in the correct anatomical context in relation to the viral route of entry/transmission, in order to understand the relative roles of these two cell types. Infections can be subclassified broadly into those that enter via mucosae or skin and those that enter directly into the blood stream. In the resting state, MDC are ubiquitous in the skin, respiratory and gut mucosa, as well as peripheral blood and lymphoid organs. PDC, on the other hand, have only been found in peripheral blood and lymphoid organs (Ebner et al., 2004; Yoneyama et al., 2004). Few, if any, PDC are present in the skin or mucosae in the resting state (Jahnsen et al., 2000; Farkas et al., 2001; Wollenberg et al., 2002; Bangert et al., 2003). Furthermore, their migration pattern is different to that of MDC. Whereas the latter circulate from peripheral sites

via afferent lymphatics to draining lymphoid organs (Banchereau and Steinman, 1998), PDC enter lymph nodes predominantly from blood through high endothelial venules (HEV) (Cella et al., 1999a). Like MDC, they can also migrate into areas of inflammation in the periphery (Farkas et al., 2001; Wollenberg et al., 2002; Bangert et al., 2003; Zhao et al., 2003). Therefore, it is likely that viral interaction with both subsets is relevant, but only at appropriate times after infection.

In the initial stages of a skin/mucosal infection, MDC are likely to be the predominant DC subtype to be exposed to the invading virus. PDC could come into contact with the virus at later time points, in the periphery following inflammation-mediated migration (Jahnsen et al., 2000; Farkas et al., 2001; Wollenberg et al., 2002) and in secondary lymphoid organs, where cells, such as MDC, could be productively infected with a virus (Fugier-Vivier et al., 1997; Ho et al., 2001). However, for viruses that are transmissible via the blood route, PDC interaction may be more relevant at the earliest stages of infection. The in vitro observations that this cell type can secrete very large quantities of type I IFN may reflect such an in vivo scenario. The large quantities of this cytokine that are secreted may compensate for the dilutional effect of many litres of blood, achieving a sufficient functional concentration of type I IFN systemically. The importance of a PDC responses in human systemic viral infections has recently been proposed (Pichyangkul et al., 2003; Hishizawa et al., 2004), emphasising the critical role this DC subset plays in control of viral infections in the appropriate context.

When PDC are matured with virus, CD40L or a Toll like receptor (TLR) ligand, they differentiate into potent APC capable of stimulating memory responses (Fonteneau et al., 2003; Krug et al., 2003). However, the relative potency of mature PDC in stimulating naïve T cells remains controversial. Murine studies demonstrate that mature PDC are inferior to MDC in this capacity (Krug et al., 2003), whereas others observed that mature human PDC are only inferior in stimulating naïve T cells to exogenous, but not endogenous antigens (Salio et al., 2004). This implies an inefficiency in cross-presentation by this subset. Thus, in the early stages of infection, the main role of uninfected PDC may not be to cross-present antigen, but rather to amplify MDC activation, most probably through the secretion of type I IFN (Dalod et

al., 2003), which in turn may amplify cross-priming to exogenous antigens by MDC (Le Bon et al., 2003).

1.8 Dendritic ceil activating ligands

DC are likely to encounter multiple stimulatory ligands in their microenvironment, and this may be important in determining the degree of DC activation. Lipopolysaccharide (LPS)-containing Gram negative bacteria may activate DC fully, whereas other pathogens may not possess as much stimulating material per se, but may induce the secretion of cytokines that also promote the maturation of DC (e.g. TNFα (Sallusto and Lanzavecchia, 1994)). Thus, several relatively weak stimuli may add up to increase the maturation, or activation, state of the DC. In recent years, the number of ligands described that activate DC has greatly increased. Initial studies examined the ability of these ligands to upregulate the expression of costimulatory molecules on DC, thus inducing 'maturation'. However, recent work has demonstrated that this process is more complex and that the ligands the DC encounters can affect DC physiology differentially, particularly reflected in the cytokines secreted, and subsequently tailor the immune response appropriately for the pathogen encountered. The presence of DC subsets that detect pathogen structures in non-overlapping fashion has added further complexity to the field of DC recognition of the environment. However, in the context of ligands that activate DC, a unifying hypothesis has been proposed to explain how the innate immune system, including DC, is alerted to pathogen infection.

Charles Janeway first suggested that the immune system is activated via receptors, which he named pattern recognition receptors (PRRs), that recognise conserved pathogen associated molecular patterns (PAMPs) (Janeway, Jr., 1992). Much evidence has accrued to back up this theory, particularly following the identification of TLRs and c-type lectins that recognise specific components of a wide variety of pathogens. This hypothesis was extended to suggest that the adaptive immune system, for example via DC, can also be activated by mediators released during inflammation and invasion induced by pathogen infection (Ibrahim et al., 1995; Matzinger, 1998). In this way, the ligands that activate DC could also be endogenous and host derived, rather than unique to the pathogen.

There is now experimental data to support the function of both exogenous and endogenous activators, which may act to complement each other. However for the purpose of this introduction, PAMPs and endogenous ligands that activate DC will be discussed in separate sections.

1.8.1 Pattern recognition receptors

1.8.1.1 TLR family

This family of receptor was identified initially on the basis of its homology to the drosophila Toll protein (Medzhitov et al., 1997). There are currently 11 known TLR, and the number of ligands for each receptor is constantly growing. Notably, not all the structures recognised are pathogen specific, with a growing list of endogenous ligands being described, which will be discussed in section 1.8.2.2. These are summarised in table 1.1.

TLR	Pathogen associated ligand	Endogenous ligand
1	Mycobacterial lipoproteins, triacylated lipopeptides (Takeuchi et al., 2002)	-
2	PGN, LTA(Schwandner et al., 1999) MV + HCMV envelope protein (Bieback et al., 2002; Compton et al., 2003)	Heat shock proteins* (Vabulas et al., 2002)
3	dsRNA (Alexopoulou et al., 2001)	siRNA (Kariko et al., 2004a) mRNA (Kariko et al., 2004b)
4	LPS (Poltorak et al., 1998b) RSV envelope protein (Kurt-Jones et al., 2000)	Heat shock proteins* (Palliser et al., 2004) Hyaluronan* (Termeer et al., 2002)
5	Flagellin (Hayashi et al., 2001)	-
6	Cooperates with TLR2 recognition of PGN and other diacylated lipopeptides (Ozinsky et al., 2000; Takeuchi et al., 2001)	-
7	ssRNA (Diebold et al., 2004; Heil et al., 2004)	
8	ssRNA (Heil et al., 2004)	
9	CpG DNA (Hemmi et al., 2000)	Chromatin IgG complexes (Leadbetter et al., 2002)
10	-	-
11	Uropathogenic bacteria (Zhang et al., 2004)	

(*) The complete absence of microbial contaminants that could bind TLR2 and TLR4 in the preparation of these proteins could not be confirmed, and therefore, the role of these proteins as endogenous TLR ligands remains controversial.

Table 1.1 TLR ligands. Peptidoglycan (PGN), Lipoteichoic acid (LTA), Measles virus (MV), Respiratory syncytial virus (RSV), double stranded RNA (dsRNA), single stranded RNA (ssRNA), short interfering RNA (siRNA)

One effect of TLR ligation on DC may be the regulation of antigen uptake and processing. Two recent studies have demonstrated that TLR ligation, through p38 activation, promote uptake of bacteria in macrophages. This occurs through upregulation of expression of scavenger receptors, such as SR-1 and MARCO, and the mannose receptor, that ultimately increases uptake efficiency of bacteria (Doyle et al., 2004) and through maturation of the phagosome (Blander and Medzhitov, 2004). In DC, this TLR signalling effect may induce the delivery of degraded antigen to MHC class II loading compartments. It is interesting that MARCO is also involved in cytoskeletal rearrangement in DC (Granucci et al., 2003), suggesting that TLR4 ligation may also prime DC for emigration from the periphery.

Despite both activation of NF-kB after ligation of all TLR and the close association between the activation of this pathway with DC maturation (Verhasselt et al., 1999; Neumann et al., 2000; Yoshimura et al., 2001), it has become evident that the parallel activation of other signalling pathways by different TLR results in diverse functional outcomes. The initial TLR ligation can condition the DC for the signals it will pass on to engaging T cells, in the forms of cytokines and surface molecule expression. An example of the latter includes the Th1 promoting ICAM-1 (Salomon and Bluestone, 1998), whereas OX40 binding on T cells by OX40L on DC promotes Th2 responses (Ohshima et al., 1998). However, the most important DC derived signals include cytokines secreted, particularly IL-12 and type I IFNs.

IL-12 is made up of a 35kDa and a 40kDa chain (IL-12 p35 and p40 respectively) that heterodimerise to form the functional IL-12 p70 (Trinchieri, 2003). IL-12 exerts its actions on the cell through the IL-12 receptor (IL-12R), which activates the Janus Kinase (JAK)-STAT (signal transducer and activator of transcription) pathway, and through the activation of STAT4, can induce Th1 differentiation (Thierfelder et al., 1996; Kaplan et al., 1996; Cho et al., 1996). Recently, other members of the IL-12 family of cytokines have been described, IL-23 (Oppmann et al., 2000) and IL-27 (Pflanz et al., 2002). These cytokines utilise the IL-12 p40 chain as a common component. IL-23 binds to one of the IL-12R chains and induces similar downstream signals, including STAT4 activation and as a result also favours Th1 responses, albeit not as efficiently as IL-12 (Oppmann et al., 2000). IL-27 can also promote Th1

differentiation of T cells, although its receptor and signalling consequences are still less well defined (Pflanz et al., 2002).

Among the many actions of **type I IFNs** that will be discussed in greater detail in chapter 5, this family of cytokines can also skew T cell responses towards Th1 (Brinkmann et al., 1993; Kadowaki et al., 2000). Type I IFNs signal through the type I IFN receptor (IFNAR) and also activate the JAK-STAT pathway, specifically STAT4 required for Th1 differentiation (Cho et al., 1996; Rogge et al., 1997), although recent studies suggest that this effect is less potent than that of IL-12 (Athie-Morales et al., 2004).

DC can secrete cytokines that prevent Th1 activation, such as the chemokine CCL2 that can promote Th2 differentiation (Gu et al., 2000) and IL-10, which has regulatory functions irrespective of the Th bias (Zeller et al., 1999). However, most TLR ligands promote DC to induce a Th1 phenotype, as demonstrated by TLR3 (de Jong et al., 2002), TLR4 (Boonstra et al., 2003), TLR7 (Ito et al., 2002) and TLR9 (Hemmi et al., 2000). It is notable that the expression of these TLR is not ubiquitous on all DC subsets (Krug et al., 2001), and as a result, each cell responds to the stimulus differently. This is exemplified by TLR7, which induces IL-12 secretion upon ligation in MDC, whereas type I IFN is secreted in PDC. However, the end result is that that both TLR7 stimulated MDC and PDC promote Th1 responses (Ito et al., 2002; Lore et al., 2003).

In contrast, the effects of TLR2 ligation on DC are different, resulting in little IL-12 p70 secretion (Weigt et al., 2003) and skewing the immune response towards Th2 (Qi et al., 2003). The recent observation that HSV-1 can elicit TLR2 signalling raises interesting questions relating to the relevance of this interaction in the in vivo pathogenesis of HSV infection (Kurt-Jones et al., 2004), and is discussed further in chapter 6. Mechanistically, the TLR2 capacity to induce Th2 responses stems from the increased ERK activation after receptor ligation, relative to other Th1-inducing TLRs (Agrawal et al., 2003; Dillon et al., 2004). Human in vitro studies suggest that the consequential absence of IL-12 secretion is solely responsible for the Th2 shift (Agrawal et al., 2003), whereas murine studies suggest that the increased ERK

activation also results in increased IL-10 secretion (Dillon et al., 2004), which in turn can suppress IL-12 secretion (Xia and Kao, 2003).

Although many of the conclusions described above have been corroborated in both the mouse and human system, there are also some notable discrepancies. For example, TLR5 ligation in human cells induces IL-12 secretion and Th1 development (Agrawal et al., 2003), whereas in the mouse no IL-12 secretion and Th2 skewed responses follow (Didierlaurent et al., 2004). Secondly, some mouse TLRs may be non-functional (e.g. TLR8 (Jurk et al., 2002)). The issue of disparate TLR signalling between the two species will be revisited in chapter 5.

The difficulty in comparing the effects of different TLRs should also be noted, as the signal from the TLR can be not only qualitative, but also quantitative. For example, low dose LPS can promote Th2 development (Eisenbarth et al., 2002), whereas more commonly used larger amounts promote Th1 (Boonstra et al., 2003). Furthermore, Th decisions may also stem from the antigen dose, as low doses again promote Th2 development, whereas Th1 skew is supported at higher doses (Boonstra et al., 2003). The physiological relevance of this non-pathogen driven Th decision is currently still unclear and may relate to genetic background (Guler et al., 1997) or in vitro conditions that promote Th2 responses artificially even in the steady state (e.g. IL-4 in the culture medium). It is also important to note that different Th responses can be induced by both the two main DC subsets, MDC and PDC (Boonstra et al., 2003). Originally, it was believed that the two subsets induced Th1 and Th2 responses respectively (Rissoan et al., 1999). However, this observation may have been related to differences in antigen dose required to elicit Th1 responses by the different subsets (Boonstra et al., 2003). It is also important to remember that single ligand stimulation presents an artificial snapshot of the immune response. For example, ligation of TLR4 on DC can upregulate expression of TLR2, TLR4 and TLR9 (Visintin et al., 2001; An et al., 2002; Nilsen et al., 2004), and thus the regulation of expression of Th1 and Th2 biasing TLR may be either amplificatory or immunoregulatory depending on the appropriate context (i.e. in the presence of pathogens that possess multiple TLR stimulating ligands).

Studies analysing the association between polymorphisms in TLR sequences and disease susceptibility have demonstrated the protective role of TLRs in vivo. For example, a role for TLR5 is demonstrated by the increase in *Legionella pneumophilia* infection in individuals with a common polymorphism that introduces a stop codon into the TLR5 gene (Hawn et al., 2003). Polymorphisms in the TLR4 gene have also been associated with increased susceptibility to RSV infection and gram negative bacterial infections (Tal et al., 2004; Agnese et al., 2002). It is also possible that pathogens could exploit TLRs to generate non-resolving Th2 biased responses. Some murine studies suggest that infection with *Yersinia enterocolitica*, a pathogen that can activate TLR2, is cleared more efficiently in the absence of TLR2 (Sing et al., 2002), although no human data to date is available to backup this observation and is in contradiction with the protective effect of TLR2 in *Borrelia burgdorferi* and *Staphylococcus aureus* infection (Wooten et al., 2002; Takeuchi et al., 2000). The concept of pathogen exploitation of innate immune recognition will be discussed further in chapter 6.

1.8.1.2 c-type lectin family

This family of receptors is important for the recognition of a wide range of carbohydrate structures. These receptors play a role in facilitating phagocytosis and endocytosis, and hence also enhancing antigen processing and presentation. However, recent evidence suggests that the downstream signalling events they induce may also play a significant role in shaping the ensuing immune response. The receptors include the mannose receptor (MR), DEC-205, DC-SIGN, BDCA-2, Dectin-1 and the LC specific receptor, Langerin. Most of these are expressed on MDDC, although this does not correlate with the expression on circulating MDC, suggesting differential expression at various stages of DC development. Furthermore, some are specific to certain DC subsets, such as Langerin on Langerhans cells, whereas others are noted by their absence, such as the lack of expression of DC-SIGN on PDC. They all possess at least one carbohydrate recognition domain and recognise sugars in a variety of secondary and tertiary structures (Geijtenbeek et al., 2004).

Like for TLRs, c-type lectin ligands can be endogenous, such as ICAM-2 and ICAM-3 for DC-SIGN (Geijtenbeek et al., 2000b), but recent work has focused on the field of pathogen recognition. Initial studies utilised soluble sugars, such as mannan, to compete for binding to the MR, but this approach is not selective, notably also preventing interaction with DC-SIGN (Geijtenbeek et al., 2003). As a result, only studies using neutralising antibodies to these receptors can be interpreted as showing an interaction definitively.

Many bacteria and fungi can interact with c-type lectins (Geijtenbeek et al., 2004). For virus recognition, the MR may play a role in binding to HSV-1 (Milone and Fitzgerald-Bocarsly, 1998b; Rong et al., 2003) and influenza virus (Reading et al., 2000), although the interaction with either of these viruses has not been proved definitively. On the other hand, DC-SIGN binds to envelope glycoproteins of human immunodeficiency virus (HIV) (Geijtenbeek et al., 2000a), Ebola virus (Alvarez et al., 2002), HCMV (Halary et al., 2002), hepatitis C virus (HCV) (Lozach et al., 2003) and DV (Tassaneetrithep et al., 2003). Notably, a HSV-1 clinical isolate tested did not bind to DC-SIGN (Halary et al., 2002), although the sensitivity of this receptor to variability in glycosylation suggests that different strains of HSV-1 may yet bind DC-SIGN. This is because the interaction is dependent on different envelope glycosylation patterns that may also be affected by the cell type in which the virus is passaged (Lin et al., 2003). Functionally, it has been suggested that viral DC-SIGN binding will protect viruses from degradation and promotes efficient trans infection of other target cells (Geijtenbeek et al., 2000a; Halary et al., 2002).

The signalling events downstream of c-type lectin receptors have attracted interest recently. Dectin-1 and TLR2 can co-operate to increase zymosan induced signalling and cytokine secretion (Gantner et al., 2003). Cross-linking the mannose receptor, despite inducing upregulation of co-stimulatory molecules, inhibits IL-12 secretion and induces secretion of IL-10 (Nigou et al., 2001; Chieppa et al., 2003). Cross-linking BDCA-2 also inhibits IFNα production by PDC (Dzionek et al., 2001), whereas *Mycobacterium tuberculosis* capsid sugar, mannose-capped lipoarabinomannan (ManLAM), binding to DC-SIGN can inhibit IL-12 secretion by DC stimulated with LPS (Geijtenbeek et al., 2003). This demonstrates how pathogens may have evolved to

bind c-type lectin receptors with the purpose of subverting host immune responses. This issue will be explored further in chapter 6.

1.8.1.3 Other pattern recognition receptors

Recent studies have identified a pentraxin protein, PTX3, which plays an important role in the clearance of Aspergillus infection in mice and is secreted by MDC. Interestingly, PTX3 associates with various TLR and this may be a host mechanism to expand the repertoire of microbial structures that are recognised (Garlanda et al., 2002; Doni et al., 2003).

TLRs and c-type lectin receptors recognise PAMPs in the extracellular space, but many pathogens reach the cell cytoplasm so that hosts have had to evolve mechanisms to detect this process. For virus infections, a good example of this is the detection of dsRNA in the cytoplasm by protein kinase R (PKR) activation. The importance of this host recognition is exemplified by the presence of the influenza virus protein NS1 that can mask the viral dsRNA genome and prevent PKR activation (Diebold et al., 2003). For bacterial infections, the NOD family of proteins are intracellular receptors of bacterial lipids (Girardin et al., 2003a; Girardin et al., 2003b) and may play a role in shaping the cell response to cytoplasmic invasion by *Listeria monocytogenes* (McCaffrey et al., 2004). NOD proteins may have evolved to complement the detection of pathogens through intracellular recognition.

1.8.2 Endogenous ligands

1.8.2.1 Cytokines

The immature DC's immediate environment is the critical factor in determining the functional outcome of the cellular response. As well as to pathogens, DC can also respond to non-pathogen derived molecules. These include cytokines secreted by other cells, including non-immune cells (Lebre et al., 2003). Upregulation of MHC and co-stimulatory molecules is induced by TNFα (Sallusto and Lanzavecchia, 1994) and type I IFN (Padovan et al., 2002). However, it should be noted that, although most responses are dose-dependent, cytokine stimulation of DC is generally a weaker signal

than TLR stimulation, but that the activation signals are likely to be additive (or synergistic), and cytokines can amplify the responses to other maturation stimuli. For example, cytokines secreted by monocytes adherent to human IgG coated-plastic, known as monocyte conditioned medium (MCM), can amplify the response to trace amounts of LPS to induce full DC maturation (Nersting et al., 2003). Despite the uniform effect on phenotype, cytokines can also skew T cell responses towards Th1 or Th2. For example, IFNα can enhance IL-12 secretion in response to CD40L (Luft et al., 2002b), aiding the generation of Th1 responses (Brinkmann et al., 1993), whereas PGE_2, one of the active components of MCM, can skew towards Th2 responses (Kalinski et al., 1998). Alternatively, cytokines may have no effect on DC phenotype, yet prime for increased IL-12 secretion and Th1 development, as seen for IL-1β (Luft et al., 2002a).

DC can also respond to signals emanating from the T cell interaction. These include T cell surface ligands, such as CD40L (Sallusto and Lanzavecchia, 1994), LIGHT (Morel et al., 2003) or 4-1BBL (Futagawa et al., 2002; Wilcox et al., 2002)). Cytokines secreted by T cells can also affect DC. Interestingly, both IFNγ and IL-4 can synergise with CD40L to increase IL-12 secretion by the DC (Snijders et al., 1998; Ebner et al., 2001). In order to prevent permanent Th1 switching, it is likely that other cytokines are secreted to prevent excess IL-12 secretion.

1.8.2.2 Molecules derived from tissue damage

There is evidence that the immune system can respond to molecules released from areas of inflammation. These include lipid mediators, such as prostaglandins, which can skew responses to other TLR ligands towards a Th2 bias (Kalinski et al., 1998; Gosset et al., 2003). Similarly, extracellular adenosine (Panther et al., 2003) and its derivative ATP (Wilkin et al., 2002) can also have Th2 inducing effects. Intriguingly both these molecules and PGE_2 can also promote DC migration (Panther et al., 2003; Scandella et al., 2002; Schnurr et al., 2004). There is also some evidence that DC can respond to proteins released from cell injury, such as hyaluronan (Termeer et al., 2002), uric acid (Shi et al., 2003) and heat shock proteins (HSP) (Palliser et al., 2004) to skew immune responses towards Th1. It should be noted that stringent precaution to

ensure the absence of microbial contaminants in the preparation of these products are necessary, as it may be difficult to exclude that they are merely potentiating the effects on DC of microbial molecules.

1.9 Regulation of dendritic cell migration

An important aspect of the DC lifecycle is the migration from periphery to LN. This emigration from tissues is tightly regulated by chemotactic gradients, in particular by the chemokines CCL19 and CCL21, which are constitutively expressed in LNs (Gunn et al., 1998; Ngo et al., 1998). The regulatory step is at the DC level, specifically the expression of the receptor for these ligands, CCR7 (Dieu et al., 1998). CCR7 is a G-protein coupled receptor with 7-transmemebrane domains and induces Ca2+ signalling upon ligation by appropriate ligands (Sato et al., 2001). Mice deficient in CCR7 expression display impaired DC migration to lymph nodes, although it should be noted that lymph node architecture is also disrupted in these mice (Forster et al., 1999).

Recent work has identified that CCR7 mediated signalling has important functional effects on DC other than just inducing migration. CCR7 mediated intracellular signals activate the Rho GTPases, Rac and Cdc42 (Yanagawa and Onoe, 2003) and induce membrane ruffling, although curiously this only occurred in response to CCL19, not CCL21, and at concentrations greater than those needed to induce migration (Yanagawa and Onoe, 2002). The morphological changes in that study were interpreted as dendrite extension, but they bore no resemblance to dendrites observed on a fibronectin (FN) substratum (Swetman et al., 2002), highlighting how the morphological changes in response to these stimuli may be dependent on the substratum the cell is in contact with. Nevertheless, changes in morphology following activation of Rho GTPase are consistent with the regulation of the DC cytoskeleton by these molecules. The high concentration of CCL19 required to induce these changes may reflect the response of mature DC in the lymph nodes to assume a dendritic shape during antigen presentation to T cells (Benvenuti et al., 2004a). The recent findings that CCL19 and CCL21 transmit anti-apoptotic signals, via NF-kB and PI3K activation, are consistent with the notion that chemokine ligation of mature DC not

only promotes their migration to lymph nodes, but also aids their survival during antigen presentation (Sanchez-Sanchez et al., 2004).

Intriguingly, parallel studies have also revealed that CCL19 and CCL21 stimulation of mature DC induced a rapid increase in endocytosis (Yanagawa and Onoe, 2003). The physiological relevance of this study may relate to an increase in antigen uptake prior to emigration from the tissue. In that way, a DC leaving the periphery is induced to make one last efficient 'sweep' of its environment before presenting this content in the LN. Interestingly, CCL3, responsible for migration of DC precursors into the dermis (Yoneyama et al., 2004), also induces DC endocytosis, indicating again how chemokines can govern DC antigen uptake as well as migration (Yanagawa and Onoe, 2003).

The regulation of DC migration may be important both in the induction of immunity and the maintenance of peripheral tolerance. Recent data has shown that immature DC can also acquire CCR7 expression and migrate to draining LN (Geissmann et al., 2002). Thus, the expression of CCR7 and the migration potential of DC are independent of the maturation state of the cell (Llodra et al., 2004). The implication of these findings is that both immature and mature DC can enter draining LNs. Immature DC still express low levels of antigen loaded MHC molecules and co-stimulatory molecules, and this has been proposed as one way to maintain peripheral tolerance. T cells that encounter antigen on immature DC do not receive sufficient signal to differentiate into effectors and are either deleted or become anergic. In this way T cells reactive to the host's peripheral microenvironment are excluded from the T cell repertoire and autoimmunity avoided (Hawiger et al., 2001; Bonifaz et al., 2002).

A further layer of complexity to the regulation of DC migration is observed by the fact that CCR7 expression is not predictive of the migration capacity of the DC, as the signalling machinery can be uncoupled from the receptor (Penna et al., 2001). Important factors that may link the two systems include inflammatory mediators, such as PGE2 (Scandella et al., 2002) and adenosine (Robbiani et al., 2000) and ectoenzymes, such as CD38, that enhance extracellular derived Ca2+ fluxes, that are critical for the migration of both DC precursors into skin and mature DC emanating

from areas of inflammation into LN (Partida-Sanchez et al., 2004). This regulation may enable DC to migrate more appropriately in response to inflammatory environmental cues.

Human PDC are predominantly found in the blood and their migration pattern is likely to differ from that of MDC. Although recruited to some areas of inflammation in the periphery, the chemokines required for this have not been characterised. Mouse footpad infection with HSV recruits MDC, but not PDC, into the dermis. Concurrently, both MDC and PDC are recruited to LN. PDC enter in a process dependent on E-selectin expression on HEV and the chemokine CXCL9, acting via CXCR3 expressed on PDC (Yoneyama et al., 2004). Thus, this in vivo study demonstrates how the migration pattern of the two subsets is diverse and may be suited to their function: recruitment of MDC into area of inflammation and PDC into LN, possibly to amplify immune responses through type I IFN secretion (Dalod et al., 2003).

1.10 Signalling pathways involved in dendritic cell physiology

The regulation of DC maturation and migration lies at the level of intracellular signalling. Different PAMPs, as well as viruses can activate these pathways and alter DC function. Furthermore, viruses may regulate the activation of these pathways for their own survival, making them critical to the understanding of the functional outcome to the DC-virus interaction. Owing to the cell-specific nature of signalling regulation of cellular responses, the literature review below will focus specifically on studies carried out on DC. Data relating to signalling regulation in other cell types is also discussed where that may prove insightful for extrapolating function in DC.

1.10.1 NF-kB

The family of NF-kB transcription factors is made up of 5 members: p65 (RelA), p50/p105, c-Rel, p52/p100, and RelB. The classical regulation of these members is quite similar. They are retained in the cytoplasm by the inhibitory proteins IkBα and IkBβ. Phosphorylation of these proteins results in ubiquitination and rapid proteosomal

degradation. Phosphorylation of the IkB proteins is in turn regulated by IKK family (Karin and Ben Neriah, 2000).

DC maturation is preceded by an increase in DNA binding activity of RelB, p50, p52, and c-Rel (Verhasselt et al., 1999; Neumann et al., 2000), suggesting that NF-kB activation positively regulates DC maturation. Furthermore, transfecting DC with IkBα expressing adenoviral vectors prevented both the upregulation of molecules associated with maturation and the secretion of inflammatory cytokines, including IL-12, IL-6 and TNFα (Yoshimura et al., 2001). The role of the p50 subunit in IL-12 secretion by DC in response to LPS has been formally demonstrated using siRNA (Laderach et al., 2003). It should be noted that the pathways become increasingly divergent and stimulus specific upstream of the effector molecules/transcription factors, as exemplified by the role of IKK2 in CD40L but not LPS induced maturation of DC (Andreakos et al., 2003). In this respect, it is interesting that PKCε is phosphorylated rapidly (within 15 min) after LPS stimulation of DC, and promotes TNFα and IL-12 secretion by facilitating degradation of IkB and therefore NF-kB access to the nucleus. However, this enzyme does not play a role in the upregulation of costimulatory molecules by LPS and subsequent inhibition of its activity only prevented Th1 skewing of co-cultured T cells, but not their proliferation, demonstrating how NF-kB can differentially activate effector pathways (Aksoy et al., 2002). Indeed, NF-kB subunits are differentially recruited upon DC maturation by various stimuli (Neumann et al., 2000; Hofer et al., 2001). The role of individual subunits has only been addressed in the murine system using knockout mice. Interestingly, large degree of redundancy has been observed. Mice lacking c-Rel showed no impairment in IL-12 secretion (Mason et al., 2002), whereas mice deficient in both p50 and c-Rel demonstrated reduced IL-12 secretion, despite DC from these mice expressing normal levels of MHC and costimulatory molecules (Ouaaz et al., 2002). It should also be noted that the role of the NF-kB subunits may vary between DC subsets, as c-Rel is important in IL-12 p35 expression in CD8α+ DC (Grumont et al., 2001), but not in BMDC (Mason et al., 2002).

NF-kB is also involved in maintaining DC viability, such as after Fas ligation, an event that is pro-apoptotic in most cell types. Fas induced NF-kB activation in DC prevents

these cells from dying when DC encounter FasL on T cells (Rescigno et al., 2000; Guo et al., 2003). Interestingly, this is a pathway that may be targeted by viruses, as MV infection renders DC susceptible to T cell mediated induced apoptosis by FasL (Servet-Delprat et al., 2000a).

1.10.2 p38 MAPK

DC stimulation with LPS or CD40L induces the phosphorylation of p38 Mitogen-Activated Protein Kinase (MAPK) (Aicher et al., 1999; Ardeshna et al., 2000). This occurs rapidly (within 30 minutes) and is transient (phosphorylation is absent after 2 hours) (Handley M, submitted for publication). Studies using inhibitors of p38 MAPK have shown that this activation is necessary for important physiological changes during DC maturation. The upregulation of co-stimulatory molecules, CD80 and CD86, is p38 dependent, as is the transcription and secretion of IL-12, and both these factors contribute to enhancing T cell stimulation (Aicher et al., 1999; Lu et al., 1999; Ardeshna et al., 2000; Vidalain et al., 2000b; Puig-Kroger et al., 2001). It is worth noting that there are inconsistencies in the literature on the relative contribution of the p38 pathway in the regulation of MHC class II expression (Ardeshna et al., 2000), which may stem from the varying length of exposure to the inhibitor and the concentrations of inhibitor used. The latter is particularly important as excessive amounts of inhibitor may also affect pathways other than the one intended.

Although p38 MAPK can phosphorylate and activate several transcription factors directly (e.g. ATF2, CREB), it is also important in positively regulating DNA binding activity of NF-kB during DC maturation (Puig-Kroger et al., 2001). A model for the role of p38 in DC maturation was proposed, whereby p38 is important for phosphorylating histones, inducing nucleosomal rearrangement, and thus exposing promoter sites of some genes to greater access by NF-kB subunits (Saccani et al., 2002). This model can apply to genes whose promoters lie within nucleosomes and may be regulated in this way, such as IL-12p40 (Weinmann et al., 1999). However, some genes, such as TNFα, are not regulated in this way, yet the secretion of TNFα and presence of its mRNA is dependent on p38 MAPK activity (Arrighi et al., 2001),

suggesting that different mechanisms may be responsible, such as stabilisation of the mRNA transcript (Dean et al., 1999).

1.10.3 ERK MAPK

The ERK signalling pathway is antagonistic to the activity of p38. It prevents the upregulation of MHC and costimulatory molecules (Arrighi et al., 2001; Puig-Kroger et al., 2001). With regards to IL-12, ERK induced IL-10 secretion inhibits IL-12 production (Xia and Kao, 2003). This is likely to be an endogenous negative feedback mechanism that limits IL-12 secretion to avoid immunopathology, and some pathogens, such as *Leishmania* amastigotes, may exploit this regulation by skewing towards ERK activation, instead of p38 MAPK, after CD40 stimulation (Mathur et al., 2004). Furthermore, different TLR ligands differentially activate p38 and ERK to varying degrees. Specifically, TLR2 ligation induces greater degree of ERK activation, which in turn activates c-Fos that is responsible for inhibiting IL-12 and increasing IL-10 secretion (Agrawal et al., 2003; Dillon et al., 2004). Therefore, the relative activation of the ERK pathway, through concomitant activation of c-Fos, is an important regulator of IL-12 secretion in DC.

Like NF-kB, the ERK pathway is also involved in maintaining DC viability after stimulation. Studies in murine DC suggested that ERK promotes DC survival (Rescigno et al., 1998), and experiments using an ERK inhibitor in human DC support a role for this MAPK in maintaining DC survival after maturation (Yu et al., 2004).

1.10.4 PI3K

PI3K is activated by most TLRs and members of the TNFR family that signal via TRAF6. By activating the pro-survival kinase, Akt, PI3K prevents cell death after DC maturation (Yu et al., 2004; Ardeshna et al., 2000) but also plays an important role in IL-12 secretion, although with disparate effect between mouse and man. PI3K potentates IL-12 secretion in humans (Re and Strominger, 2001; Yu et al., 2004), whereas it negatively regulates its secretion in the mouse (Fukao et al., 2002). Although possibly intrinsic to species differences, the differential role of PI3K may

also relate to the DC models used in the two systems. Nevertheless, if PI3K does play an important role in the regulation of IL-12 secretion, then it complicates the comparison of studies between mice and humans (see chapter 5).

1.11 Role of dendritic cells in the control of HSV infections

Sections 1.6-1.10 demonstrate the evolving understanding of DC physiology, and how this involves many layers of complexity in order to shape an appropriate effective immune response in the face of maintaining immune tolerance to self. Several pieces of evidence have indicated that DCs play an important role in vivo in the context of HSV infection. Mice deficient in the costimulatory molecules, CD80 and CD86 control HSV infections poorly (Thebeau and Morrison, 2002). Conversely, vaccination of mice with either DC loaded with HSV antigens (Schon et al., 2001), or DNA encoding CCL19 or CCL21 (Lee et al., 2003b), offer protection against HSV infection in these mice.

These studies serve to highlight the potency of DCs in generating T cell responses that are protective for HSV. They do not specifically highlight the role that DCs and their subsets play in the natural course of infection. This has been more directly approached by studies demonstrating that the severity of cutaneous HSV infection inversely correlates with the number of CD1a+ LC in the epidermis (Sprecher and Becker, 1986; Sprecher and Becker, 1989; Yasumoto et al., 1986). It is interesting that this DC subset may not directly play an important role in antigen presentation following cutaneous/mucosal infection (Zhao et al., 2003; Allan et al., 2003), but may be important in the role of antigen transfer to LN resident DC subsets, such as the CD8α+ DC that can activate CD8+ T cells (Iyoda et al., 2002; Schulz and Reis e Sousa, 2002).

Further in vivo studies have attempted to study the role of different DC subsets in the initiation of anti-HSV T cell responses by isolating different subpopulations from LN. However, in none of these models were any DC subsets depleted prior to HSV infection. Thus, while CD8α+ DC may stimulate anti-HSV CD8 T cell responses and CD4 T cell responses initiated by submucosal CD11b+ DC (Allan et al., 2003; Zhao et al., 2003), it is not clear if either of these subsets are essential, or whether redundancy

exists within the DC system. Such an interpretation would be further complicated by the unresolved questions regarding ontogeny of DC subsets (Shortman and Liu, 2002) and the possibility that DC subsets may differentiate into each other, as defined by the expression of surface molecules. For example, it is possible that 'migrating' myeloid CD11b+ DC may differentiate into 'resident' CD8α+ DC in the LN (Moron et al., 2002). Despite their accumulation in lymph nodes after peripheral infection (Smith et al., 2003b; Yoneyama et al., 2004), PDC were excluded from playing a role in T cell activation after peripheral HSV infection (Zhao et al., 2003), so their precise function in vivo remains unclear. Recently, a blunted PDC response was associated with more severe dengue virus (DV) disease (Pichyangkul et al., 2003), but such a correlation may not be present for a more peripheral infection, such as that caused by HSV-1.

There has not as yet been any evidence to show that DCs are infected by HSV-1 in vivo in humans. However, a combination of the evidence from mice studies, the critical function of DC in initiating any primary T cell response and the similar susceptibility of human DC in vitro to HSV-1 infection as epithelial cells (see chapter 3), suggest that the interaction between this cell and the virus is a relevant one in vivo.

1.12 Role of dendritic cells in the control of viral infections

The role of DC in inducing immunity suggests that, in general, it would be an evolutionary advantage for viruses to subvert this system. Preventing or delaying the presentation of viral antigens by DC may permit the virus sufficient time to establish in the host and replicate to transmissible titres, and perhaps even to establish latency. As a result, prior to, during and after the completion of the work presented in this thesis, the infection of DC by many other viruses has also been studied and the functional outcomes analysed.

The frequency of all DC subsets in peripheral blood is between 0.1% and 2%, depending on criteria used (Vuckovic et al., 2004), but it is possible to obtain large numbers of MDC in vitro from monocyte precursors (Sallusto and Lanzavecchia, 1994), a differentiation event that also occurs in vivo (Randolph et al., 1999).

Therefore, as summarised in table 1.2, the vast majority of studies have analysed the interaction of viruses with monocyte derived dendritic cells (MDDC).

Enhanced	*No change*	*Impaired*
Dengue virus (Ho et al., 2001) Hantavirus (Raftery et al., 2002) Influenza virus (Cella et al., 1999b)	HIV-1* (Sapp et al., 1999) HCV* (Longman et al., 2004)	Varicella Zoster Virus (Abendroth et al., 2001) Human Cytomegalovirus (Moutaftsi et al., 2002) HHV-6 (Kakimoto et al., 2002) Measles Virus (Grosjean et al., 1997) Parainfluenza Virus (Plotnicky-Gilquin et al., 2001) Respiratory Syncytial Virus (Bartz et al., 2003) Adenovirus (Tuettenberg et al., 2004) Vaccinia virus (Engelmayer et al., 1999) Ebola virus (Mahanty et al., 2003) Lassa fever (Mahanty et al., 2003) HIV-1* (Donaghy et al., 2003) HCV* (Kanto et al., 1999)

(*) – Refers to the function of MDDC from patients with chronic or long term infection, not MDDC infected in vitro with the relevant virus.

Table 1.2 Functional consequences of viral infection of MDDC. The capacity of virus infected DC to activate T cells is compared to uninfected DC.

It is evident that some viruses do not impair DC function following infection, whereas many others hinder the DC's ability to stimulate T cells. A wide variety of DC parameters are affected that may impinge on the ability to stimulate T cells. These include the morphology of the DC, the cytokine secretion profile, the viability and the expression of surface molecules, such as MHC and costimulatory ones. These will all be analysed in relation to HSV-1 in this thesis, and comparison between the effect of HSV-1 and other viruses on DC will be made in the appropriate sections.

The absence of an in vitro differentiation model, and the paucity of ex vivo derived PDC have limited the number of studies detailing their interaction with viruses. One parameter that has been studied is the number of PDC circulating in peripheral blood. However, this may not give a clear indication of the direct effects of the viral infection, as it may result from peripheral or central migration of these cells to areas of infection or antigen presentation respectively. Nevertheless, impaired PDC IFNα secretion or T cell stimulation has been observed in patients with adult T cell leukaemia (ATL) caused by human T-cell lymphotropic virus type 1 (HTLV-1) infection (Hishizawa et al., 2004), chronic HCV infection (Goutagny et al., 2004) and HIV-1 infection (Donaghy et al., 2003), suggesting that viruses may also alter the function of this subset of DC.

1.13 Hypotheses of thesis

In this introduction, the main features of the cell biology of DC and the central role they play in the interaction with viruses, and in the generation of antiviral responses have been discussed. On this basis, two main hypotheses will be investigated in this thesis:

1. HSV-1 infects DC and disrupts their function as APC.
2. The host has evolved mechanisms to bypass this DC-based viral evasion and is therefore able to generate a strong antiviral response capable of resolving the infection.

1.14 Aims and objectives of thesis

The aim of this thesis will be to test these two hypotheses using an in vitro culture system of human MDDC and a laboratory strain of HSV-1. The investigation will be divided into four main objectives, each discussed in a separate chapter:

1. Characterisation of the interaction between HSV-1 and DC, defining the normal range of parameters that may be affected by the virus.
2. Functions that define DC, namely changes in T cell stimulation, cytokine secretion and surface phenotype, will be assessed following HSV-1 infection.
3. Host mechanisms to enhance immune responses after HSV-1 infection will be investigated.
4. The functional consequence of interaction between HSV-1 and the DC surface will be investigated with a view to understanding the co-evolution between the virus and the host that permits both these species to co-exist.

In the concluding chapter of this thesis, these findings will be discussed in the broader context of the role of DC in the control of human viral infections, along with a model of a peripheral herpetic lesion, with DC playing a central role both in its establishment and resolution. Finally, the data acquired will be placed into a clinical setting, and its application to the fields of HSV vaccinology and HSV-based gene therapy will be considered.

Chapter 2

Materials & Methods

2.1 Materials

All reagents were of the highest grade commercially available. Unless otherwise stated, all chemicals and reagents were obtained from Sigma (UK) Ltd. (Poole, Dorset, UK). Disposable pipettes were from Philip Harris (Leicestershire, UK). Pipette tips were from Elkay (Hampshire, UK). All culture plates, flasks and centrifuge tubes were from Nunc (Rochester, NY, USA), unless otherwise stated.

2.2 Cell and Tissue Culture

2.2.1 Safety and sterile conditions

All work involving human tissues were performed in a M.D.H. InterMed class 2 safety cabinet, using sterile techniques. All short and long term cell cultures were carried out at a temperature of 37°C in an atmosphere compromising 95% air and 5% CO_2.

2.2.2 Culture medium

Unless otherwise stated, all cell cultures were conducted in Roswell Park Memorial Institute medium-1640 (RPMI-1640) (Gibco BRL, Paisley, UK) that was supplemented with 100U/ml penicillin, 100μg/ml streptomycin, 2mM L-glutamine (all from Clare Hall Laboratories, Cancer Research UK, London, UK) and 10% v/v heat inactivated foetal calf serum (FCS) (PAA Laboratories, Linz, Austria). The combined medium was denoted as complete medium (CM).

2.2.3 Culture condition

Unless otherwise stated, all incubations at 37°C were carried out in an incubator at 5% CO_2. Incubations at 4°C were carried out on ice.

2.2.4 Cell counting

All cell samples were counted using a Neubauer haemocytometer. 10μl of cell suspension was mixed thoroughly with an equal volume of 0.04% trypan blue stain

(Gibco). This technique allowed a rapid assessment of cell viability, since trypan blue is excluded from live cells.

2.3 Cell lines: characteristics and growth conditions

2.3.1 BHK cells

Baby Hamster Kidney 21 clone 13 cells (BHK) were a gift from Y. McGrath and grown in 1x Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) FCS, 100 IU/ml penicillin and 100 μg/ml streptomycin. The combined medium was denoted as BHK complete medium (BCM).

BHK cells were grown in 175 cm^2 flasks and were passaged when they were 80-90% confluent. Cells were washed with Hank's balanced salt solution (HBSS) (Gibco) at room temperature, detached from the surface of the flask with trypsin/versene (1:10) (Gibco) at room temperature, and disaggregated. The reaction was stopped by adding BCM and cells were aliquoted in 1:10 ratio in new flasks containing BCM.

2.3.2 HeLa cells

HeLa cells were a gift from A. Kwan and grown in CM in 80 cm^2 flasks and passaged in the same way as BHK cells.

2.3.3 Storage of cell lines

For long term storage, cells were resuspended in 10% (v/v) DMSO in FCS at an approximate concentration of $5x10^5$ cells/ml. The freezing vials were slowly cooled in a polystyrene box to -70°C and then transferred to liquid nitrogen for longer term storage. For recovery, cells from one vial were thawed rapidly and transferred into a 25 cm^2 flask containing the appropriate selection medium. Cells were passaged within 2 days depending on confluence.

All cell lines were regularly tested and shown to be negative for the presence of both mycoplasma antigens and DNA.

2.4 Primary cells

2.4.1 Isolation of peripheral blood mononuclear cells

PBMC were isolated from healthy volunteers by density gradient centrifugation. Peripheral blood (60 or 120ml) was obtained by venesection using a 21g butterfly needle into a heparanised syringe (original heparin concentration 5000IU/ml - C.P. Pharmaceuticals Ltd. Rexam, UK). This was diluted into an equal volume of HBSS. 30 ml of this diluted solution were layered carefully over 17.5 ml of Lymphoprep 1077 (Nycomed Pharma, Oslo, Norway) in a 50 ml tissue culture tube (Sterilin) and centrifuged for 30 minutes at 600g (brake off) at room temperature. The interface over the Lymphoprep contained the PBMC and was aspirated and washed three times with HBSS.

2.4.2 Derivation of monocyte-derived dendritic cells

The PBMC fraction from 120ml of blood was resuspended in 36 ml of CM and incubated (3ml per well) in 6 well tissue culture plates (Falcon, Becton-Dickinson, Mountain view, CA, USA) for 2 hours. Non-adherent cells were removed and the adherent cells were cultured in fresh CM supplemented with 100 ng/ml human recombinant GM-CSF and 50 ng/ml IL-4 (both gifts from Schering-Plough Research Institute, Kenilworth, New Jersey). Monocytes subsequently differentiated into MDDC.

MDDC were purified from any other non-adherent cells on day 4 of cell culture, according to the protocol described previously (Alderman et al., 2002). Loosely adherent cells were collected and layered carefully over 5ml of Lymphoprep. Cells were centrifuged at 600g for 30 minutes at room temperature (brake off) to remove dead cells and debris. The Lymphoprep interface was removed and washed three times in HBSS before being resuspended in 2ml ice cold CM and incubated on ice for 30 minutes in the presence of mouse anti-human monoclonal antibodies (mAb) that recognised the major contaminating cell populations: CD19 mAb removed B cells, CD3 T cells, and CD2 T cells and NK cells. Excess antibody was washed off twice in

ice cold HBSS and the cells were resuspended in ice cold CM. 10µl of immunomagnetic beads coated with sheep anti-mouse Ig (Dynal, Merseyside, UK) per 10^6 non-DC (as determined by cell size by microscopy). The cell-bead mixture was rotated for 45 minutes at 4°C. Contaminating cells and excess beads were removed by placing sample tubes adjacent to a magnet for 2 minutes. Supernatants were transferred to fresh tubes and placed adjacent to a magnet for a further 2 minutes. The supernatants, which contained the purified DC, were aspirated, and the cells cultured for a further 3 days in CM with fresh GM-CSF and IL-4 at a concentration of 5×10^5 DC/ml. They were used as a pure population of immature DC.

2.4.3 Preparation of T cells

Following 2 hour culture, non-adherent PBMC were removed and resuspended at approximately $5x10^6$ cells/ml in 10% (v/v) DMSO in FCS. Samples were transferred to 1ml cryotubes (Nunc) and frozen at -80°C in a polystyrene freezing box.

When required, frozen vials were thawed rapidly in a water bath at 37°C and then washed three times in HBSS. T cells were purified by negative immunomagnetic depletion as described for DC in section 2.4.2. The only difference was that the antibodies used to deplete contaminating cells were HLA-DR (monocytes, DC, B cells, activated T cells), CD14 (monocytes) and CD19 (B cells). This procedure removed about 10% of T cells that express HLA-DR, and therefore considered to be activated. At the end of purification the T cells were >95% viable as determined by trypan blue exclusion.

2.5 HSV-1 preparation

2.5.1 Virus growth

The HSV-1 construct used in this study was derived from HSV-1 strain 17+, and contained a cassette consisting of the CMV IE gene promoter driving expression of green fluorescent protein (GFP) (Clontech, Hampshire, UK). This cassette was inserted

into the UL43 gene as reported previously (Coffin et al., 1996). This virus was a gift from R.S. Coffin.

For large-scale viral culture, 10x175 cm^2 tissue culture flasks containing 90% confluent BHK cells were infected at a multiplicity of infection of MOI 0.01-0.03 and grown in fresh media for up to 48 hours until complete cytopathic effects were observed. Flasks were frozen immediately at -80°C. After thawing, the cellular debris was removed by centrifugation at 3500 rpm for 45 minutes at 4°C. The supernatant was removed immediately, filtered through a 5μm, followed by a 0.45μm filter, and spun at 12000 rpm for 2 hours at 4°C in a GS12 rotor. The supernatant was discarded and the viral pellet was resuspended gently in 1ml HBSS. The resuspended pellet was then sonicated 3 times for 10 seconds in a water bath sonicator and stored at -70°C. The virus stock was titred by the viral infectivity assay (see section 2.5.2). The usual titre achieved was 10^9 plaque forming units (pfu)/ml in 1 ml. All virus stocks used were tested and shown to be negative for the presence of both mycoplasma antigens and DNA.

2.5.2 Viral infectivity assay

Viral infectivity, and therefore titre, was determined from the ability of HSV-1 to form plaques on cell monolayer. Serial dilutions (10^{-3} to 10^{-8} pfu) of virus in DMEM without FCS were prepared and added to 80% confluent BHK cells in 6 well plates in triplicates. After 1 hour incubation at 37°C, 2.5ml of 1:2 (v/v) of 1.6% (w/v) carboxymethyl cellulose:DMEM supplemented with 10% FCS were added to each well. Cells were then incubated for 48 hours at 37°C and the number of plaques in each well were counted using GFP fluorescence in order to determine the virus titre in pfu/ml.

2.5.3 Viral inactivation

UV-inactivation was carried out by exposing the HSV-1 preparation (10^7 pfu/ml) to UV light at a distance of 6 cm, with a short wavelength UV-B light source delivering 20 mW/cm^2 (R-52 Grid Lamp, UVP, Upland, USA). A titration of time of exposure to

UV light and GFP expression by infected DC is shown in fig. 3.6C. For the experiments in chapter 6, 20 minutes of UV-inactivation were used.

Formaldehyde fixation was carried out by neutralising HSV-1 (10^9 pfu/ml) with equal volume of 2% formaldehyde (BDH, Poole, UK). The virus stock was diluted to 10^7 pfu/ml with RPMI and excess formaldehyde neutralised by adding 0.175% (w/v) sodium bisulphite solution (final concentration). Exposure of DC to the equivalent concentrations of fixative and neutralising agent (formaldehyde and sodium bisulphite) did not alter DC surface phenotype, excluding indirect effects on DC by the viral treatment protocol (fig. 6.8). Neutralisation of viral gB, gC or gD was carried out by co-incubating equal volumes of virus (@ $2x10^7$ pfu/ml) and LP2 or AP7 antibody +/- 12.5 μg/ml heparin (C.P. Pharmaceuticals Ltd.) at 37°C for 15 minutes, and then used to infect DC (see section 2.6). A titration of heparin addition is shown in fig. 6.2B. A titration of LP2 titration is shown in fig. 2.1. The antibody concentration of the neat supernatant of AP7 was approximately four times lower than that of LP2 (S. Bell, personal communication). Therefore, LP2 antibody was diluted 1 in 4 in BCM before addition to HSV-1. This concentration of antibody was still sufficient to neutralise the virus (fig. 2.1) and also controlled for the antibody concentration of AP7.

2.6 HSV-1 infection of DC

For all experiments other than infection of FN-adherent DC (see below), appropriate numbers of DC were centrifuged in a 15 ml tube at 1400 rpm for 5 minutes. Then the cell pellet was infected with appropriate pfu of HSV-1, depending on the required MOI. The virus-DC mixture was incubated at 37°C for 1 h. The cells were washed once and re-plated at $5x10^5$ cells/ml in CM with fresh GM-CSF and IL-4.

For all virus infections, uninfected groups received RPMI treated in the same manner as the virus. Previous experiments had determined that mock viral preparations had equivalent effects on DC as RPMI (fig. 2.2). The presence of significant stimulating non-viral contaminants in the preparation was excluded by neutralisation with anti-gD mAb (LP2 clone) in fig. 6.14.

For infections of FN-adherent DC, $5x10^4$ DC were allowed to adhere to FN for 2 hours in CM supplemented with GM-CSF and IL-4 (see section 2.10). RPMI or HSV-1 was then added at a MOI of 1 for 1 hour. Any unbound virus was then removed by washing twice with 1ml CM, before adding 500µl CM supplemented with GM-CSF + IL-4. The DCs were incubated at 37°C for 8 hours and the morphology analysed by confocal microscopy.

2.7 HSV-1 serology

This method was adapted from that devised by G. Simpson (PhD thesis). To test for the presence of neutralising antibodies to HSV-1, sera were collected from volunteers at the same time as venepuncture for DC and T cell isolation, heat inactivated to remove the effect of complement components and incubated at 37°C with 10^5 pfu/ml HSV-1 for 15 minutes. DMEM supplemented with 10% FCS was used as a negative control. The resulting HSV-sera mixture was titrated on BHK in a viral infectivity assay as described in section 2.5.2. A greater than 3 log decrease in plaque formation was interpreted as reflecting the presence of neutralising anti-HSV antibodies in the serum sample. Neutralising antibodies can be type common, and this assay does not therefore distinguish between previous infection with HSV-1 and HSV-2 (Gorander et al., 2003).

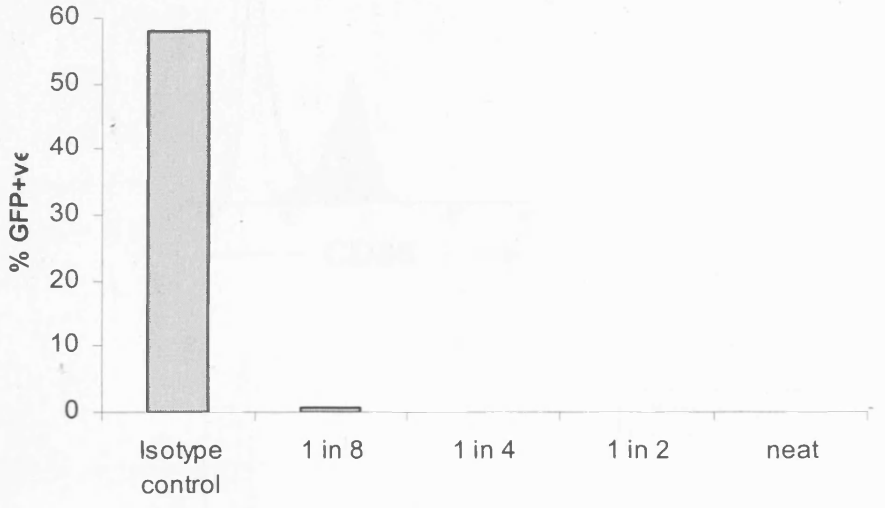

Figure 2.1 Neutralisation of HSV-1 by LP2 clone anti-gD mAb.
HSV-1 was neutralised with a range of concentrations of mAb and used to infect DC. After 16 hours of culture, expression of GFP was assessed. Representative of two independent experiments.

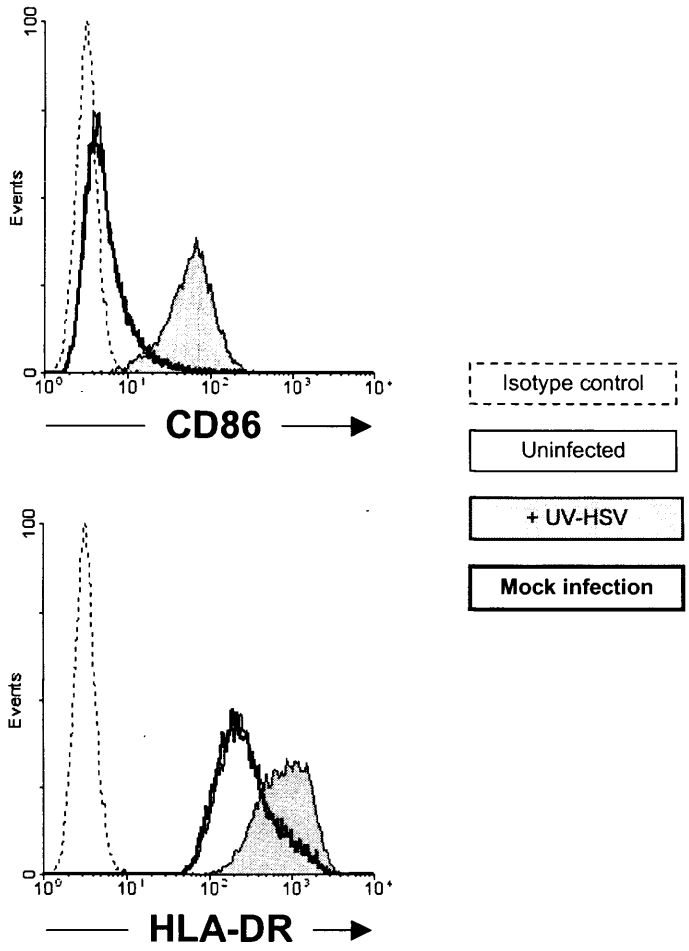

Figure 2.2 Effect of mock viral preparation on DC phenotype.
DC were infected with RPMI (uninfected group), a mock viral preparation, or UV-inactivated HSV (UV-HSV), and cultured for a further 16 hours. Representative of at least two independent experiments.

2.8 HSV-1 replication in DC and HeLa cells

$5x10^5$ DC and HeLa cells were infected at a MOI of 1 and aliquots of the supernatants and the infected cells were harvested at 0, 24, 48, 72 and 96 hours post-infection (at the same cell concentration) and stored at -80°C until use. Harvested samples were thawed, disrupting the cells, and the virus yield was assessed by plaque assay

2.9 DC stimulation

In some experiments control and infected DC were stimulated on day 7 by addition to the wells of LPS, polyriboinosinic polyribocytidylic acid (Poly(I:C)), IFNα2c (Roche, Welwyn Garden City, UK) or gD(285t) (gift from Dr. C. Krummenacher, University of Philadelphia, Philadelphia, USA) and cultured for 16 hours. All stimuli were added in a 1:100 dilution, so as not to disturb the total volume and cytokine concentrations in the CM.

2.10 Assessment of DC morphology

In some experiments DC were plated on FN-coated glass cover slips, which had been pre-coated overnight in 20 μg/ml FN (Sigma) in HBSS. After 2 hours of adhesion, DC were stimulated with 100 ng/ml LPS or infected with HSV-1 as described in section 2.6.

2.11 Microscopy

2.11.1 Confocal microscopy

All confocal microscopy was carried out by a confocal microscope (Bio-Rad Confocal microscope, Hercules, California, USA). GFP fluorescence was recorded by a 488nm excitation laser and detected in the fluorescence channel by a 522 ± 32 nm emission filter. The images were analysed using Confocal Assistant and Adobe Photoshop software.

2.11.2 Electron microscopy

Uninfected or HSV-1 infected DC were harvested 16 hours after infection, and pelleted by centrifugation at 4500 RPM in a bench top centrifuge in sterile eppendorf tubes. Cell pellets were fixed carefully in 3% buffered gluteraldehyde (0.1 M, pH 7.4) while avoiding disruption or dispersion of cells from the pellet. Pellets were embedded in resin and sections were cut on a Leica Ultracut E ultramicrotome. Ultra-thin sections were cut using a Diatome diamond knife and collected on naked 300 mesh copper grids. These were stained with a solution of 25% uranyl acetate in 100% methyl alcohol for 20 minutes, followed by Reynold's lead citrate staining for 20 minutes. The grids were examined in the Jeol 1200EX and Jeol 120CX electron microscopes at a magnification specified on the micrographs.

2.12 Secreted cytokine measurement

After 16 hours of treatment or infection, supernatants were harvested and used to assay for various cytokines. Secreted type I IFN was measured by a collaborating group using a bioassay as described (Foster et al., 2000). Secreted IL-12 was measured by ELISA: IL-12 p40 (R&D Systems Europe, Abingdon, UK) and IL-12 p70 (eBioscience, San Diego, USA). Secreted TNFα was measured by ELISA (eBioscience). Representative standard curves for ELISAs are shown in fig. 2.3.

2.13 Supernatant transfer studies

In some experiments, the effect of supernatant of HSV-1 infected DC on autologous uninfected DC was tested. Supernatants from uninfected and infected DC were taken at 16 hours incubation, centrifuged in a minifuge at 16000g for 30 minutes and passed through a 0.2-μm-pore-size filter (Sartorius AG, Goettingen, Germany), to remove any viral particles and cell debris, before adding to autologous DC. Direct assay of these supernatants found no remaining live virus, both by plaque assay (data not shown) and by GFP expression (fig. 5.1). Supernatant-treated DC were then incubated for 16h and examined for surface phenotype changes and IL-12 secretion.

2.14 Detection of antigens

2.14.1 Extracellular immunofluorescence staining

Within individual experiments, the number of cells stained in all samples was the same to ensure a constant cell:antibody ratio. The usual number of cells stained was $5x10^4$ – 10^5 cells. These cells were harvested and resuspended in 50µl of blocking buffer for 15 minutes at 4°C. The blocking buffer was HBSS containing 0.1% NaN_3 and 10% rabbit or foetal calf serum depending on whether mouse or rabbit primary antibodies were used respectively. 50µl of primary mAb (supernatants or determined optimum concentrations of purified antibody) were added to the cell suspension in a 96-well round-bottom plate (Nunc), and then incubated for a further 30 min at 4°C. For experiments utilising primary antibodies not directly conjugated to fluorochromes, cells were washed twice with blocking buffer, to remove any unbound antibody, and then incubated for a further 30 minutes at 4°C with the relevant secondary antibody. For mouse primary antibodies, either a fluorescein isothiocyanate (FITC) conjugated rabbit anti-mouse IgG (Dako, Glostreup, Denmark) diluted 1:20 in blocking buffer or a R-phycoerythrein (PE)-conjugated goat anti-mouse IgG (Dako) diluted 1:25 in blocking buffer were used. For rabbit primary antibodies, a FITC- or R-PE-conjugated goat anti-rabbit IgG (Southern Biotech Associates, Birmingham, USA) diluted 1:20 in blocking buffer were used. Finally, cells were washed twice in HBSS containing 0.1% NaN3, agitated and fixed by adding 50µl of HBSS containing 0.1% NaN_3, followed by 100µl of HBSS containing 3.7% (v/v) formaldehyde (BDH, Poole, UK). Samples were stored at 4°C in the dark and analysed within 24 hours.

Detection of opsonised HSV-1 particles was achieved by harvesting infected cells and blocking non-specific antibody binding with blocking buffer for 15 minutes at 4°C. R-PE-conjugated goat anti-mouse IgG (Southern Biotech) or R-PE goat anti-rabbit IgG (Southern Biotech) was added subsequently to the cell suspension for 30 minutes at 4°C. The cells were then washed and fixed as detailed above.

2.14.2 Intracellular Immunofluorescence staining

For the staining of intracellular targets, cells were harvested and fixed in 4% formaldehyde for 10 min at 4°C at a concentration of $\leq 10^6$ cells/ml. This was followed by a wash in HBSS supplemented with 2% FCS and 0.1% NaN_3 (FACS buffer). All subsequent washes and incubations were carried out in FACS buffer supplemented with 0.5% (w/v) saponin (PERM buffer). Non-specific antibody binding was prevented by incubating cell suspensions with 10% (v/v) goat serum for 15 min at 4°C. Subsequently, the primary antibody was added and incubated for a further 30 min at 4°C. Cells were washed twice in PERM buffer and then incubated for a further 30 min at 4°C with the secondary antibody, R-PE-conjugated goat anti-mouse IgG (Dako). Cells were washed twice more with PERM buffer and once with FACS buffer prior to immediate examination by flow cytometry.

2.14.3 Intracellular cytokine staining

After HSV-1 infection (MOI = 3), DC were cultured in CM supplemented with 2μM monensin (Sigma) for a maximum of 12 hours and then fixed, permeabilised and stained as detailed in section 2.14.2.

2.14.4 Flow cytometry

Stained cells were acquired on a FACScan (Becton-Dickinson) using CellQuest software version 3.3 (Becton-Dickinson). For each sample at least 5000 events were acquired. Analysis of the data was carried out using WinMDI software (Joseph Trotter, Scripps Research Institute). A measure of the amount of fluorescence emitted by the cells in the appropriate channels was determined by the median fluorescence intensity (MFI). Where appropriate, a marker was set such that <2% of negative control cells gave a fluorescence signal beyond this level. The percentage fluorescence (% +ve) refers to the percentage of cells with fluorescence above this marker.

2.15 Proliferation assays

All proliferation assays were conducted using 10^5 purified T cells incubated with increasing numbers of DC. Experiments were performed in 96-well flat bottom plates (Nunc). Quantification of cell proliferation was by [methyl-^{3}H] thymidine (ICN Pharmaceuticals Inc., CA, USA) incorporation. Cells were pulsed with 10μl of 100μCi/ml [methyl-^{3}H] thymidine for the final 16 hours of incubation (1μCi/well). All proliferation assays were performed in triplicate wells.

Cells were harvested, and DNA was transferred from wells on to glass fibre filters (Wallac, Turkum Finland) using a Tomtec cell harvester. "Melt-on" scintillant (Wallac) was added to the filters, and incorporation of radiolabel into cells was quantified using a 1450 Microbeta liquid scintillant counter (Wallac). Results are expressed as counts per minute (cpm).

2.15.1 Autologous recall assay

Titrations of DC from each condition were incubated with autologous T cells with or without 500 U/ml (final concentration) purified protein derivative (PPD) of M. Tuberculosis (Evans Medical Limited, Leatherhead, UK). The cell cultures were incubated for 6 days and then pulsed with 1 μCi ^{3}H thymidine (ICN Biomedical, High Wycombe, UK) for a further 16 hours.

2.15.2 Allogeneic mixed lymphocyte reaction

Titrations of DC from each condition were incubated with allogeneic T cells for 6 days and then pulsed with 1 μCi ^{3}H thymidine (ICN Biomedical, High Wycombe, UK) for a further 16 hours.

2.15.3 Autologous concanavalin-A dependent assay

Titrations of DC from each condition were incubated with autologous T cells with or without 1.25 μg/ml (final concentration) of concanavalinA (ConA). The cell cultures

were incubated for 2 days and then pulsed with 1 μCi ^{3}H thymidine (ICN Biomedical, High Wycombe, UK) for a further 16 hours.

2.16 Detection of cell viability

DC were infected and cultured for 16h and 40h at 37°C. 10^5 DC per treatment condition were cultured in triplicates in a 96 well plate with 20μl 5mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) at 37°C for 4 hours. Following this, 100μl of 10% sodium dodecyl sulphate (SDS) (Sigma) was added and the plate incubated at 37°C/5% CO_2 overnight. Subsequently, the plate was read in a microplate reader at a wavelength of 570 nm with wavelength correction at 630 nm. Results are expressed as mean optical density.

Historically, this assay has been accepted as measuring the ability of the cell to reduce the MTT dye by mitochondrial enzymes (Mosmann, 1983). Recently this notion has been challenged by studies demonstrating that MTT reduction occurs in endosomes (Liu et al., 1997) and that increased exocytosis reduces MTT reduction (Liu and Schubert, 1997). However, endocytosis would still be reduced in dead cells, and succinate dehydrogenase inhibitors still prevent some MTT reduction (Abe and Saito, 1998). Therefore, despite the controversy surrounding its precise scientific basis, this assay is still a suitable assessment of cell viability.

2.17 Detection of apoptosis

To identify whether or not the cells had undergone apoptosis, DC were washed once and resuspended in HBSS to give 10^6 cells/ml. Aliquots were fixed by dropwise addition of an equal volume of 70% ethanol (stored at -20 °C) to give a final concentration of 35% ethanol. The cells were then left on ice for 30 min, washed twice, and treated with DNAse-free RNAse A (final concentration, 500 μg/ml) at 37°C for 10 min. Propidium iodide (PI) (Sigma) was added (final concentration, 50 μg/ml) at 37°C for 30 min and the cells were analysed immediately by flow cytometry.

2.18 Neutralisation of DC surface receptors

DC were incubated with an optimum final concentration of antibody or antisera for 30 min on ice before being infected with virus (section 2.6) or appropriately stimulated (section 2.9). The antibody concentrations used were extrapolated from saturating amount of antibody required for extracellular staining.

2.19 Analysis of intracellular signalling

2.19.1 Western blotting

DC were incubated in CM that contained 0.5% FCS v/v for at least 24 hours prior to treatment. Total cell extracts were prepared by resuspending the cells in 50µl reducing sample buffer (2% SDS, 10% glycerol (BDH), 2% β-mercaptoethanol (Gibco), 60mM Tris-HCl (pH 6.8; Calbiochem CA, USA), and bromophenol blue) and stored at -20°C. Before loading, lysates were sonicated and boiled for 5 minutes, and then resolved by running 25µl on a SDS/12.5% PAGE gel and transferred to ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Bucks, UK). Immunoblotting was performed by standard procedures. HRP-conjugated rabbit anti-mouse IgG or swine anti-rabbit IgG detection antibodies (both Dako) were used in combination with ECL detection reagent according to manufacturer's guidelines (Amersham Pharmacia Biotech). Densitometry analysis of the blots was performed using GeneSnap software (Syngene). The ratios of the densities of the phospho-p38 and total-p38 were calculated to give an indication of the proportion of p38 phosphorylated in each condition.

Where necessary, membranes were stripped of bound antibodies by incubating at 65°C for 1 hour in stripping buffer (see section 2.22). The membranes were then washed 5 times in TTBS before immunoblotting was carried out as above.

2.19.2 SB203580 inhibitor studies

In some studies, the role of p38 MAPK in the maturation of DC was determined. This was carried out using the p38 MAPK inhibitor, SB203580 (Sigma) (Tong et al., 1997).

Prior to FIX-HSV, IFNα or LPS stimulation, 3μM of SB203580 was added to half the DC culture on day 6 and the cells cultured for a further 24 hours. On day 7, DC from the two groups were removed from the culture dish with no additional wash steps, spun down and resuspended in fresh CM containing GM-CSF and IL-4 in the presence or absence of 3μM SB203580. This approach permitted counting of the two groups, without changing the concentration of SB203580 inside DC significantly. There was no difference in DC yield or cell viability in the presence of SB203580 (data not shown). The DC were subsequently plated in a 24 well plate at $5x10^5$ DC/ml in the appropriate concentration of inhibitor and stimulated with FIX-HSV (equivalent MOI of 3), IFNα or LPS, and cultured for a further 16 hours prior to assessment of changes in surface phenotype. In this assay, the virus was added directly to DC plated out, and not washed off after 1 hour as for normal protocol. This was again to avoid affecting the extracellular concentration of SB203580.

2.20 Statistical analysis

Where appropriate, the means of paired groups were analysed by a 2-tailed Student's t test, using Microsoft Excel software.

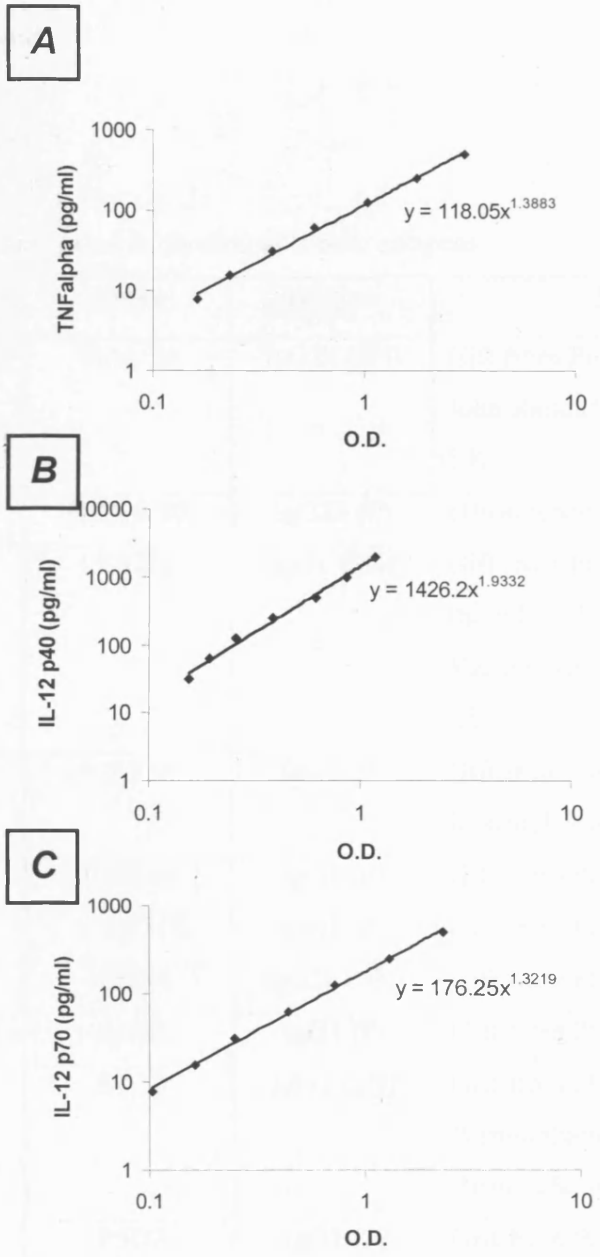

Figure 2.3 Standard curves for TNFα, IL-12 p40 and p70 ELISAs. Representative of at least three independent experiments.

2.21 List of antibodies

All antibodies are mouse monoclonal unless otherwise stated.

(AF) = Ascites fluid

(P) = Purified

(SN) = Supernatant

Table 2.1 Non-conjugated antibodies to human antigens

Specificity	Clone	Isotype	Source
CD1a	NA1/34	IgG2a (SN)	Gift from Prof. A. McMichael, John Radcliffe Hospital, Oxford, UK
CD2	RPA-2.10	IgG2b (P)	eBioscience
CD3	UCHT1	IgG1 (SN)	Gift from Prof P.C.L. Beverley, the Edward Jenner Institute for Vaccine Research, Newbury, UK
CD11a	ICRF38	IgG1 (P)	Gift from Prof. N. Hogg, Cancer Research UK, London, UK
CD11b	ICRF44	IgG1 (P)	Gift from Prof. N. Hogg
CD11c	ICRF319	IgG1 (P)	Gift from Prof. N. Hogg
CD14	HB246	IgG2b (SN)	Gift from Prof P.C.L. Beverley
CD18	B1/18	IgG1 (P)	Gift from Prof. N. Hogg
CD19	BU12	IgG1 (SN)	Gift from D. Hardie, Birmingham University, Birmingham, UK
CD29	P5D2	IgG1 (P)	Gift from Prof. N. Hogg
CD49d	7.2R	IgG1 (P)	Gift from Prof. N. Hogg
CD86	BU63	IgG1 (SN)	Gift from D. Hardie
HLA-ABC	W6/32	IgG2a (P)	Serotec UK, Oxford, UK

HLA-DQ	Ia3	IgG2a (SN)	Gift from Prof R. Winchester, New York University School of Medicine, NY, USA
HLA-DR	L243	IgG2a (SN)	Gift from Prof P.C.L. Beverley
Cathepsin E	CE1.1	IgM (SN)	Prof B. Chain
IFNAR2	MMHAR-2	IgG2a (P)	EMD Biosciences, San Diego, USA
IFNα (recognises largest number of IFNα subtypes)	MMHA-11	IgG1 (P)	IDS Ltd., Boldon, UK
TLR2	TL2.1	IgG2a (P)	eBioscience
HVEM	122	IgG1 (P)	Labvision, Fremont, USA
HVEM	-	Rabbit antiserum	Gift from P. Spear, Northwestern University, Pittsburgh, USA
Nectin-1	CK41	IgG1 (P)	Gift from Dr. C Krummenacher, University of Philadelphia, Philadelphia, USA

Table 2.2 Conjugated antibodies to human antigens

Specificity	**Clone**	**Isotype**	Source
CD3-APC	UCHT1	IgG1 (P)	BD Pharmingen, Oxford, UK
CD13-FITC	WM15	IgG1 (P)	DPC Biermann, Bad Nauheim, Germany
CD86-PE	HA5.2B7	IgG2b (P)	Beckman Coulter, High Wycombe, UK
HLA-ABC-PE	W6/32	IgG2a (P)	eBioscience
HLA-DR-PE	B8.12.2	IgG2b (P)	Beckman Coulter, High Wycombe, UK

Table 2.3 Antibodies to viral antigens

Specificity	Clone	Isotype	Source
VP16	LP1	IgG1 (AF)	Gift from Prof T Minson, Cambridge University, Cambridge, UK
gD (neutralising)	LP2	IgG2a (SN)	Gift from Prof T Minson
gD (non-neutralising)	AP7	IgG2a (SN)	Gift from Prof T Minson

Table 2.4 Control antibodies

Specificity	Clone	Isotype	Source
Aspergillus niger glucose oxidase	DAK-GO1	IgG1 (P)	DakoCytomation, Ely, UK
	679.1Mc7 (PE conjugated)	IgG1 (P)	Beckman Coulter, High Wycombe, UK.
Mouse MHC class I	TIB92	IgG2a (P)	ATCC, Manassas, USA
	HB38	IgM (SN)	Prof B.Chain
-	-	Pre-immune rabbit serum	Gift from P. Spear, Northwestern University, Pittsburgh, USA

Table 2.5 Antibodies to western blot targets

Specificity	Product no.	Isotype	Source
Phospho-p38 MAPK	9211	Rabbit polyclonal (P)	Cell Signalling Technology, Beverly, MA, USA
Total-p38 MAPK	9212	Rabbit polyclonal (P)	Cell Signalling Technology, Beverly, MA, USA
IkBα	Sc-371	Rabbit polyclonal (P)	Santa Cruz Biotechnology, Santa Cruz, CA, USA

2.22 List of buffers

Buffer	Components
TBS	8g NaCl, 0.2 g KCl, 3g Tris-Base in 1L of distilled water – adjusted to pH 7.5
TTBS	TBS supplemented with 0.1% Tween 20
Running buffer	14.4g Glycine, 3g Tris-base in 1L of distilled water.
Transfer buffer	800 ml running buffer, 200 ml methanol (Analar, BDH)
Blocking buffer for immunoblotting	2.5g milk powder (Tesco, Cheshunt, Hertfordshire, UK) in 50 ml TTBS
Antibody staining buffer	0.1 g milk powder (Tesco) in 10 ml TTBS
Stripping buffer	40 ml 10% SDS solution, 12.5 ml 1M Tris pH 6.7, 1.45ml 2-ME

Chapter 3

Characterisation of HSV-1 infection of DC

3.1 Introduction

As outlined in chapter 1, several lines of evidence allude to the important role of DC in the pathogenesis of HSV infections. As a result, the interaction between myeloid DC and HSV-1 was investigated in this thesis. This chapter will establish the models that will be used to carry out the functional studies later in the thesis.

It is possible to isolate myeloid DC directly from peripheral blood using commercially available bead systems that involve both positive and negative antibody selection criteria. However, the yield of DC is relatively poor, as all DC subtypes in blood make up between 0.1-2% of all PBMC, depending on the criteria used (Vuckovic et al., 2004). In order to study the DC-HSV interaction in sufficient detail, larger yields of DC are needed. Therefore, in this thesis a well-characterised DC model system was used, based on the differentiation of DC from monocyte precursors in the presence of GM-CSF and IL-4 for 7 days (Sallusto and Lanzavecchia, 1994). In the last few years, this protocol has been modified to ensure purity of the population, eliminating contamination with other non-DC PBMC (Alderman et al., 2002).

There is evidence that this in vitro DC model is relevant to the in vivo scenario. Tissue resident myeloid DC, which are the most relevant DC in models of a peripheral herpetic lesion, have been shown to be differentiated from monocytes in models of transendothelial migration (Randolph et al., 1998). Furthermore, the function of monocyte-derived DC (MDDC) is comparable to that of myeloid DC isolated directly from blood, in terms of the ability to stimulate T cell proliferation (Leon et al., 2004). Therefore, MDDC were used as the DC population in this study to assess the functional consequences of HSV-1 infection of DC.

The HSV-1 strain available for these studies was 17+, which has been well-characterised and used extensively by collaborating laboratories. Furthermore, the virus used has been engineered to contain an expression cassette, consisting of a CMV promoter driving expression of green fluorescent protein (GFP), inserted into the UL43 gene, as described previously (Coffin et al., 1996). UL43 is a late viral gene of unclear function that may play a role in the assembly of viral capsids in the nucleus (Ward et

al., 1996; Carter et al., 1996). HSV-1 lacking this gene show the same growth characteristics both in vivo and in vitro as the wild-type virus (MacLean et al., 1991). No role has been attributed to UL43 with regards to the in vivo pathogenesis of HSV-1, but the possibility remains that its deletion may affect the functional consequences of DC infection in unexpected ways. Nevertheless, the advantages of utilising GFP to track and gate on infected cells outweighed these known drawbacks, and this virus was used for all the studies.

The objectives of this chapter are:

- To define the purity of the DC population as well as its "normal" morphology and phenotype, in order to determine the parameters that HSV-1 infection will affect in chapters 4, 5 and 6.
- To assess the susceptibility of DC to HSV-1 infection and permissiveness for viral replication.
- To evaluate the suitability of using GFP transgene expression as an accurate quantification of DC infection efficiency.

3.2 Results

3.2.1 DC morphology

As detailed, in chapter 1, one of the defining features of DC is their morphology (Steinman and Cohn, 1973). After 7-day differentiation from monocytes, DC were non-adherent to plastic and assumed a "veiled" appearance, and possessed a characteristic ruffled appearance, with many fine membrane protrusions (fig. 3.1A), as previously described in vivo (Lens et al., 1983). This was confirmed by EM (fig. 3.1B).

Despite their non-adherence to plastic, immature DC can adhere to a FN substratum via β1 integrins (Swetman, C., submitted for publication) and assume an elongated dendritic shape (fig 3.2A). In contrast to the fine processes seen on DC in suspension, the number of processes seen on FN-adherent DC were low (on average 2 or 3 per cell) but they extended to several times the length of the cell body (fig 3.2), as described recently (Swetman et al., 2002). Two hours after seeding on FN, DC adherence to FN reached a state of equilibrium, with a significant proportion of cells adhering to and spreading on the substratum at any one time.

In order to be able to gauge whether HSV-1 infection would have a maturation-like effect on DC morphology, it was important to characterise the DC changes in response to a maturation stimulus. This was modelled by exposing DC to 100 ng/ml LPS. Although mature DC adhere poorly to fibronectin (Brand et al., 1998; Shutt et al., 2000; Puig-Kroger et al., 2000; Swetman et al., 2002), addition of LPS to DC cultures already adherent to FN had the opposite effect. 8 hours after the addition of LPS, the proportion of DC adherent to the FN increased dramatically (fig. 3.2B; quantification in fig. 4.5). There was also an increase in dendrite length of the adherent DC (fig 3.2B). This phenomenon of increased DC adherence and dendrite length was not unique to LPS, as Poly(I:C) stimulation of DC induced similar changes (data not shown). Therefore, from these experiments, one can conclude that the most dramatic consequence of exposing FN-adherent DC to maturation stimuli was the increase in the

proportion of DC adherent to a FN substratum and the length of the dendrites extended.

3.2.2 DC surface phenotype

3.2.2.1 Purity of DC population

To characterise the DC further, the surface expression of several molecules was assessed. Despite many years of study on DC biology, a single surface molecule that defines these cells conclusively, in a manner analogous, for example, to CD3 for T ceils or CD19 for B cells, has not yet been identified. This ambiguity has been further exacerbated by different in vitro DC model systems described and by the increasing number of DC subsets that have been proposed (Shortman and Liu, 2002).

Nevertheless, DC can still be characterised by the surface expression of a combination of surface molecules. Immature MDDC express CD1a at high levels (Pickl et al., 1996), a non-classical MHC molecule involved in non-protein antigen presentation (Porcelli et al., 1998). In conjunction, MDDC express CD14, the LPS-LPS binding protein receptor, at low levels on the cell surface (Sallusto and Lanzavecchia, 1994; Pickl et al., 1996). The combination of high CD1a and low CD14 expression in the MDDC cultures in these studies confirmed the purity of this cell population (fig. 3.3A) and excluded the presence of macrophages, that possess a similar FSC/SSC profile on the flow cytometer and can also be derived from monocytes in vitro under modified cytokine conditions (Chapuis et al., 1997).

To confirm the absence of contamination from other non-DC PBMC, DC cultures were stained for the expression of CD3, CD14 and CD19 (T cells, monocytes and B cells respectively). The expression of these molecules was analysed without a FSC/SSC gate, so as not to exclude any smaller contaminating cells. In comparison to the percentage of these cell types in untreated PBMC, the DC cultures were devoid of CD3+ T cells, CD19+ B cells, and $CD14^{hi}$ monocytes (fig. 3.3B). T and B cells were removed by immunodepletion, whereas the monocytes had either differentiated into

DC or died and therefore been removed on the ficoll gradient during the second round of DC purification on day 4.

In the light of the recent literature describing non-myeloid DC subsets (Shortman and Liu, 2002), it was important to exclude the contamination with these cells, namely PDC, from peripheral blood. The presence of these cells was unlikely, as their survival in culture is abolished by IL-4 (ubiquitous in MDDC cultures) (Rissoan et al., 1999) and their low expression of CD2 (Cella et al., 1999a), would have resulted in their depletion in the DC purification protocol used. Nevertheless, the myeloid derivation of the entire DC population was confirmed by the expression of CD13 and CD11c (fig. 3.3A). CD13 is an aminopeptidase which is expressed exclusively on cells of myeloid origin and is absent on PDC (Cella et al., 1999a). Furthermore, CD11c is the αX chain of β2 integrins, that has been used as a specific marker of DC in vivo. Although it is expressed at lower levels on murine PDC, it is absent on human PDC (Grouard et al., 1997; Asselin-Paturel et al., 2001). Flow cytometry revealed that >99% of MDDC expressed both CD13 and CD11c. Furthermore, CD11b, the αM chain of β2 integrin, absent on both mouse and human PDC (Grouard et al., 1997; Asselin-Paturel et al., 2001), was expressed on >99% of MDDC in these cultures (fig. 4.6). These data confirmed the homogenous nature of the MDDC population used for the subsequent studies in this thesis, which was devoid of contaminating non-DC and PDC.

3.2.2.2 DC maturation state

Although the in vitro derivation of DC from monocytes has been described to generate cells with an immature phenotype, this resting state can easily be disturbed by a cytokine imbalance (Alderman, C., PhD thesis), contamination with DC maturation stimuli (Xie et al., 2003; Rotta et al., 2003), or even excess mechanical agitation in the preparation protocol. As the immature to mature transition of DC is regarded as a terminal differentiation, the loss of an immature phenotype could result in insensitivity to further DC maturation. This was assessed by the addition of 100 ng/ml LPS to day 7 immature DC for a further 16 hours. The resultant changes in surface expression of markers of DC maturation were determined by flow cytometry (fig. 3.3C). In agreement with many other studies, MHC class I and II, as well as the co-stimulatory

molecule CD86 were upregulated in response to LPS (fig. 3.3C), confirming the immature state of DC used in this study.

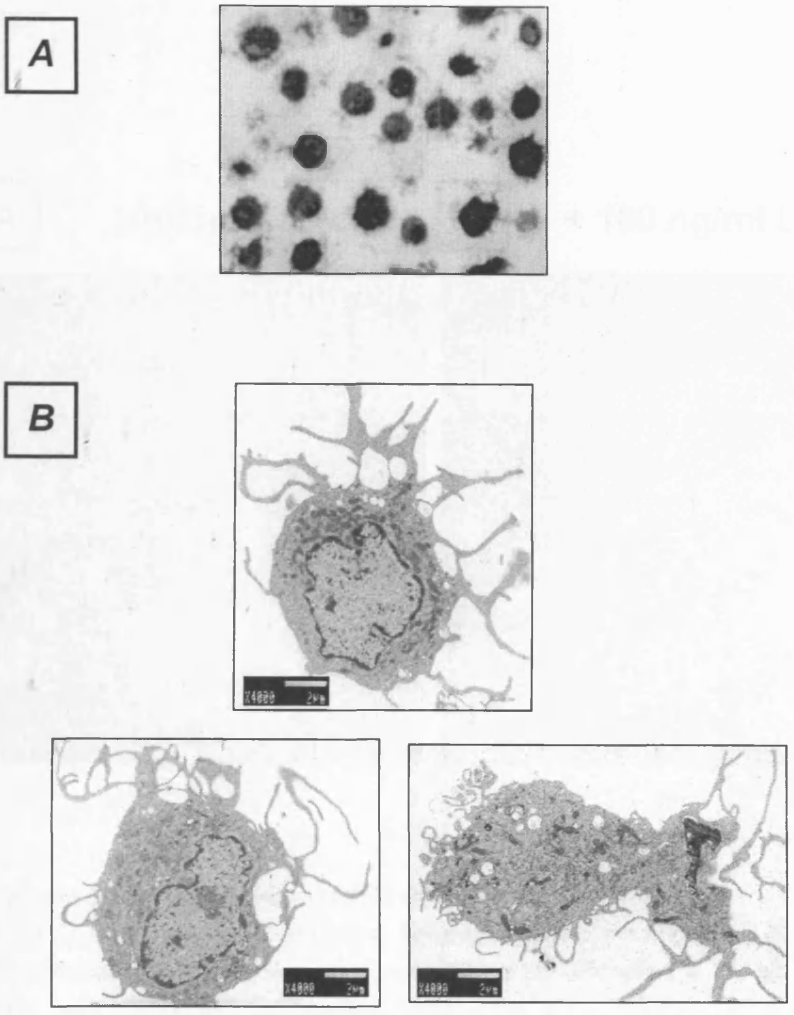

Figure 3.1 Morphology of non-adherent DC
Immature DC were fixed in 3% gluteraldehyde, prepared as described in materials & methods section 2.11, and visualised by confocal microscopy (A) or electron microscopy (B). Representative fields of two independent experiments.

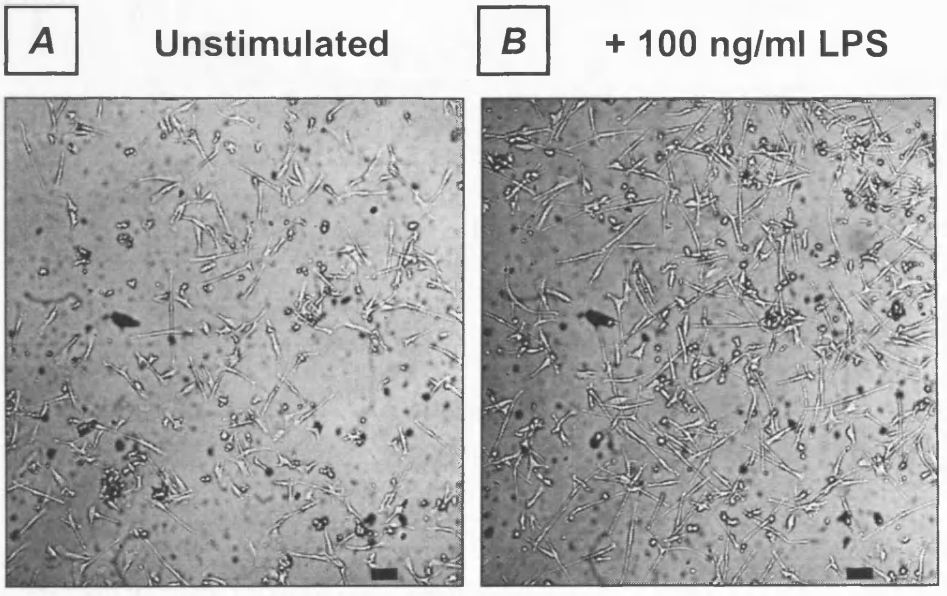

Figure 3.2 DC morphology on a FN substratum.
(A) DC were allowed to adhere to FN-coated glass coverslips for 2 hours and morphology assessed 8 hours later. (B) 100 ng/ml LPS added to DC adherent to FN and morphology assessed 8 hours later. Representative of at least 3 independent experiments. Scale bar represents 50μm.

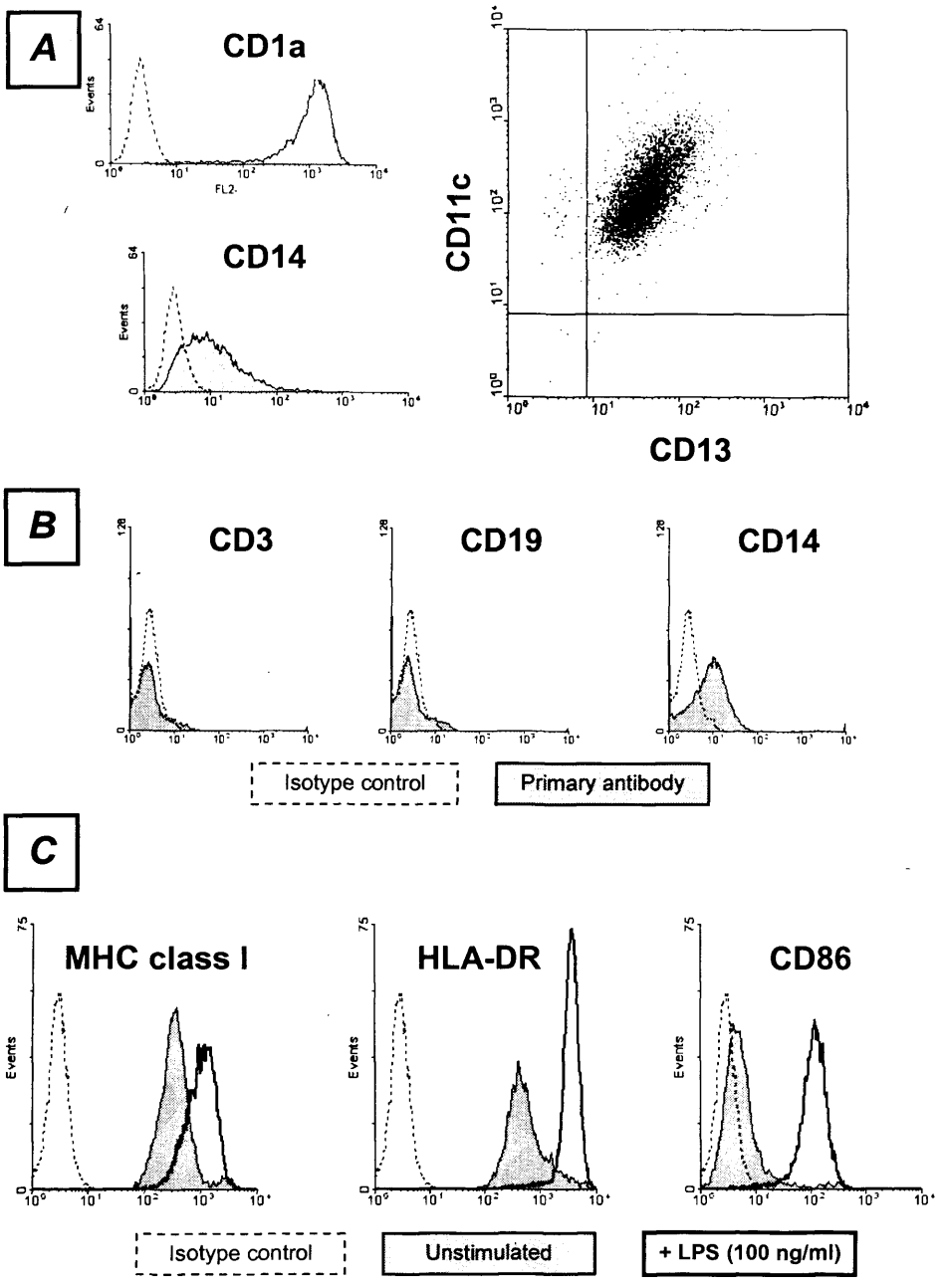

Figure 3.3 Phenotype and purity of the DC population.
(A) The expression of DC and myeloid markers by the MDDC was assessed by flow cytometry. (B) Day 7 DC cultures were stained for CD3, CD19 and CD14, to detect contaminating T cells, B cells and monocytes. Data were analysed ungated to avoid excluding smaller contaminating cells. (C) 100 ng/ml LPS was added to immature DC and changes in the expression of MHC class I , HLA-DR and CD86 were assessed 16 hours later. Representative of at least 3 independent experiments.

3.2.3 DC susceptibility to HSV-1 infection

3.2.3.1 HSV-1 entry receptor expression

The susceptibility to HSV-1 infection is determined by the expression of receptors required for viral entry (Spear et al., 2000). The two most critical for the entry of HSV-1 strain 17+ into cells are HVEM and nectin-1 (Krummenacher et al., 2004). Using mouse mAb, >75% of DC expressed these receptors (fig. 3.4). The expression in this homogenous population of cells is likely to be ubiquitous, and the <100% expression is most likely to reflect non-specific background staining by the isotype control mAb, rather than DC that did not express any nectin-1 or HVEM. This phenomenon was demonstrated clearly when anti-HVEM rabbit sera was used to stain DC and only 12% of DC appeared positive for the expression of this molecule, relative to the staining of pre-immune rabbit sera control (fig. 3.4). These staining data were in agreement with the expression observed in a previous study (Salio et al., 1999), predicting that DC would be susceptible to HSV-1 infection.

3.2.3.2 Quantifying HSV-1 infection of DC

After viral exposure, infected DC could be detected by the intracellular expression of GFP. This fluorescence could be detected not only by fluorescence microscopy (fig. 3.5A), but also by flow cytometry for quantification (fig. 3.5B). Infection of DC at a low MOI range (0.3 to 3) resulted in high infection efficiency, increasing in a dose dependent manner (31% GFP+ve DC at MOI of 0.3, compared with 78% GFP+ve DC at MOI of 3). However, it was important to ensure that the GFP expressed in DC was a direct consequence of viral gene expression and was, therefore, an accurate assessment of infection in DC. Despite the small volumes of concentrated virus used to infect DC, it was possible that the GFP detected was derived from the endocytosis of soluble GFP in the viral preparation, as opposed to the consequence of viral gene expression. This possibility was excluded in two ways.

Firstly, the kinetics of GFP expression was assessed over a 24-hour time course (fig. 3.6A). Endocytic uptake of GFP would have predicted a rapid increase of GFP to levels near those seen after overnight infection (Neil, S., unpublished observation), in

manner analogous to rapid macropinocytosis in vitro (Sallusto et al., 1995). However, in contrast, GFP was not detected until 4 hours post-infection and steadily increased until 16 hours post-infection, after which GFP expression plateaued (fig 3.6A+B). The kinetics of this slow increase in GFP expression were consistent with the immediate early CMV promoter driving the GFP transgene and subsequent protein accumulation. The clear distinction between the GFP-ve and GFP+ve population further excluded a pan-uptake of soluble GFP by all DC.

Secondly, UV inactivation of HSV-1 was used to block GFP transgene expression. UV light is able to inhibit DNA expression by generating dimers between adjacent pyrimidines, with relative sparing of protein structure (Tornaletti and Pfeifer, 1996). Consistent with the kinetic data above, GFP expression was almost totally abrogated after exposure to UV light for >5 min, excluding the possibility that the GFP inside DC derived from exogenous protein in the viral preparation (fig. 3.6C).

Although GFP expression was confirmed as a good index of viral infection in the cells expressing this transgene, it was also important to exclude the presence of infected DC not expressing GFP. The transcription of the CMV IE promoter driving the GFP gene is dependent on host cell factors, such as NF-kB activation and cAMP concentration (Sambucetti et al., 1989; Stamminger et al., 1990), although this was likely to be less of an issue in the homogenous, non-dividing DC population used in this study. Nevertheless, infected DC were counter-stained for two late viral proteins to confirm that GFP accumulation correlated with HSV-1 protein production.

VP16 is a viral tegument protein that functions as a transactivator of HSV-1 immediate early gene expression (Wysocka and Herr, 2003). As it is also an important structural component of the virus, it is incorporated into the viral progeny inside cells and can be detected intracellularly (fig. 3.7B). Glycoprotein D is an integral envelope component important for viral entry (Spear et al., 2000). As a result, infected cells express this protein during viral replication and it can be detected extracellularly (fig. 3.7C).

As the target molecules in these experiments ought to have been co-expressed only on GFP expressing DC and were not expressed in uninfected DC, positive staining

controls were needed for uninfected DC to establish the correct compensation settings on the flow cytometer. For intracellular staining, cathepsin E was used, as DC contain large quantities of this enzyme that can be detected by flow cytometry (Medd, P., PhD thesis) (fig. 3.7A). The data clearly demonstrate that all DC expressing GFP expressed both VP16 and gD (fig. 3.7B+C). An important observation was the absence of the two single positive populations, confirming that GFP was indeed an accurate marker of infection efficiency, with all cells infected expressing late HSV proteins.

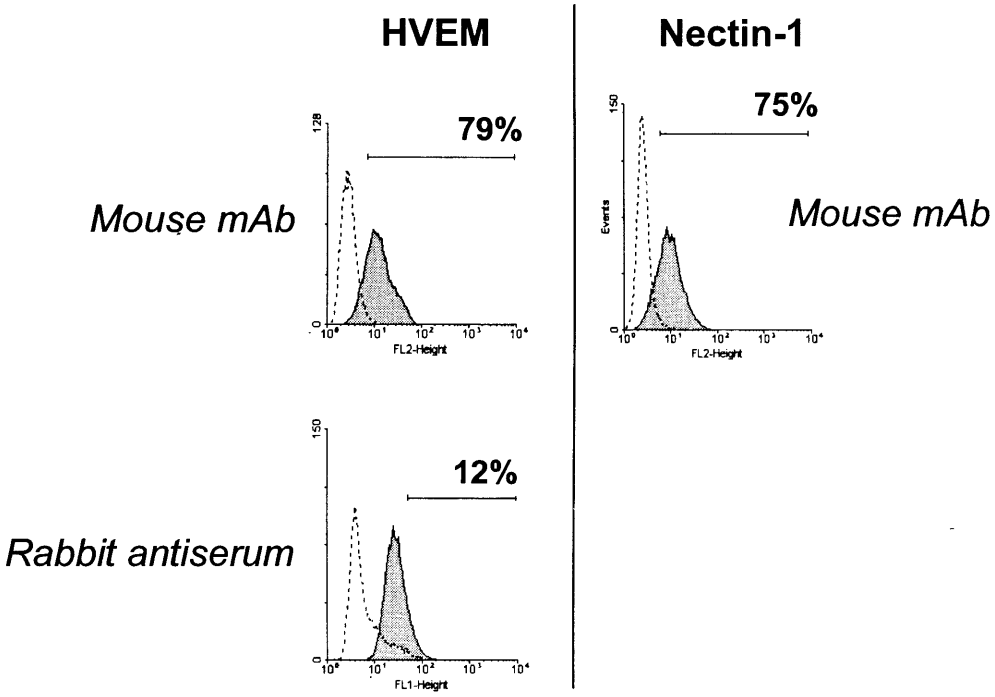

Figure 3.4 DC expression of HSV-1 entry receptors.
DC were stained for the presence of HVEM using a mouse monoclonal antibody *(lower left panel)* or rabbit anti-HVEM antiserum *(upper left panel),* and for nectin-1 *(right panel)*. Gates and percentages relate to <2% staining of isotype control or non-immune sera. Representative of three independent experiments.

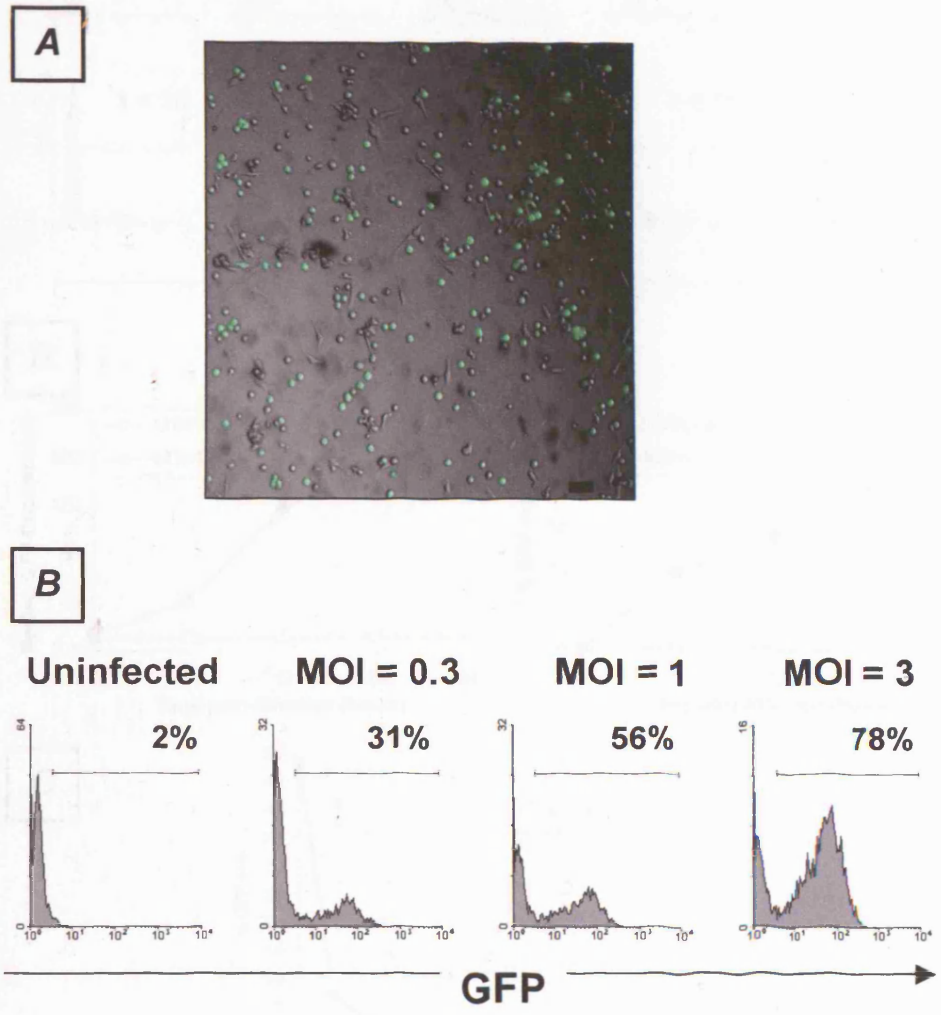

Figure 3.5 HSV-1 infection efficiency of DC.
DC were infected with HSV-1 at MOI of 1 in (A) or at the specified MOI in (B), and cultured for a further 16 hours. The expression of GFP was assessed by light microscopy (A) or flow cytometry (B). Scale bar represents 50μm. Representative of at least 3 independent experiments.

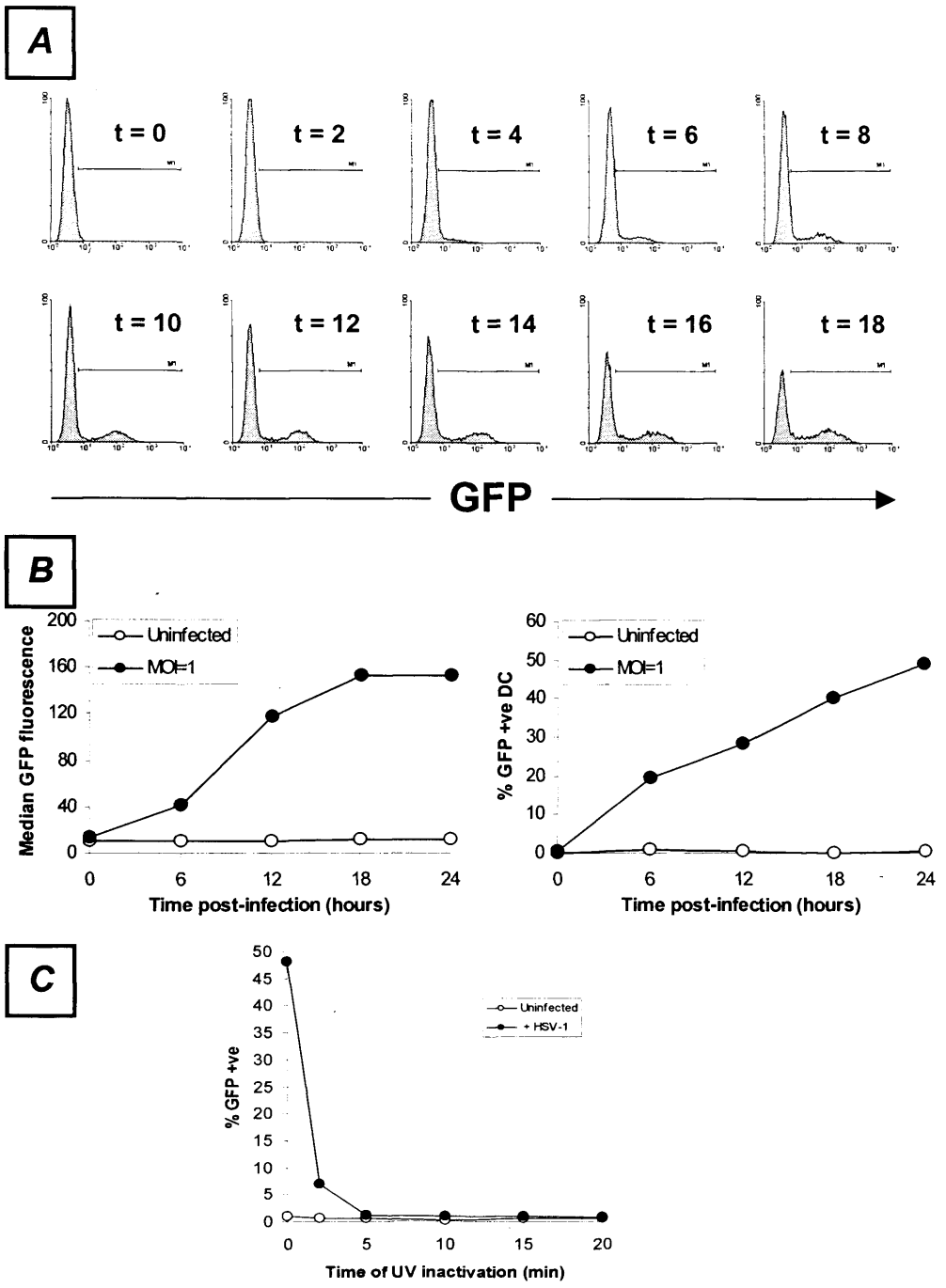

Figure 3.6 Quantification and mechanism of GFP expression.

(A) DC were infected at MOI of 1 for various times and expression of GFP assessed by flow cytometry. This was quantified in (B) both for MFI of GFP expression of GFP+ve cells, and percentage of DC expressing GFP at appropriate time points. (C) HSV-1 was exposed to UV light for various times, prior to infection of DC. DC were cultured for a further 16 hours and GFP expression assessed by flow cytometry. Representative of at least 3 independent experiments.

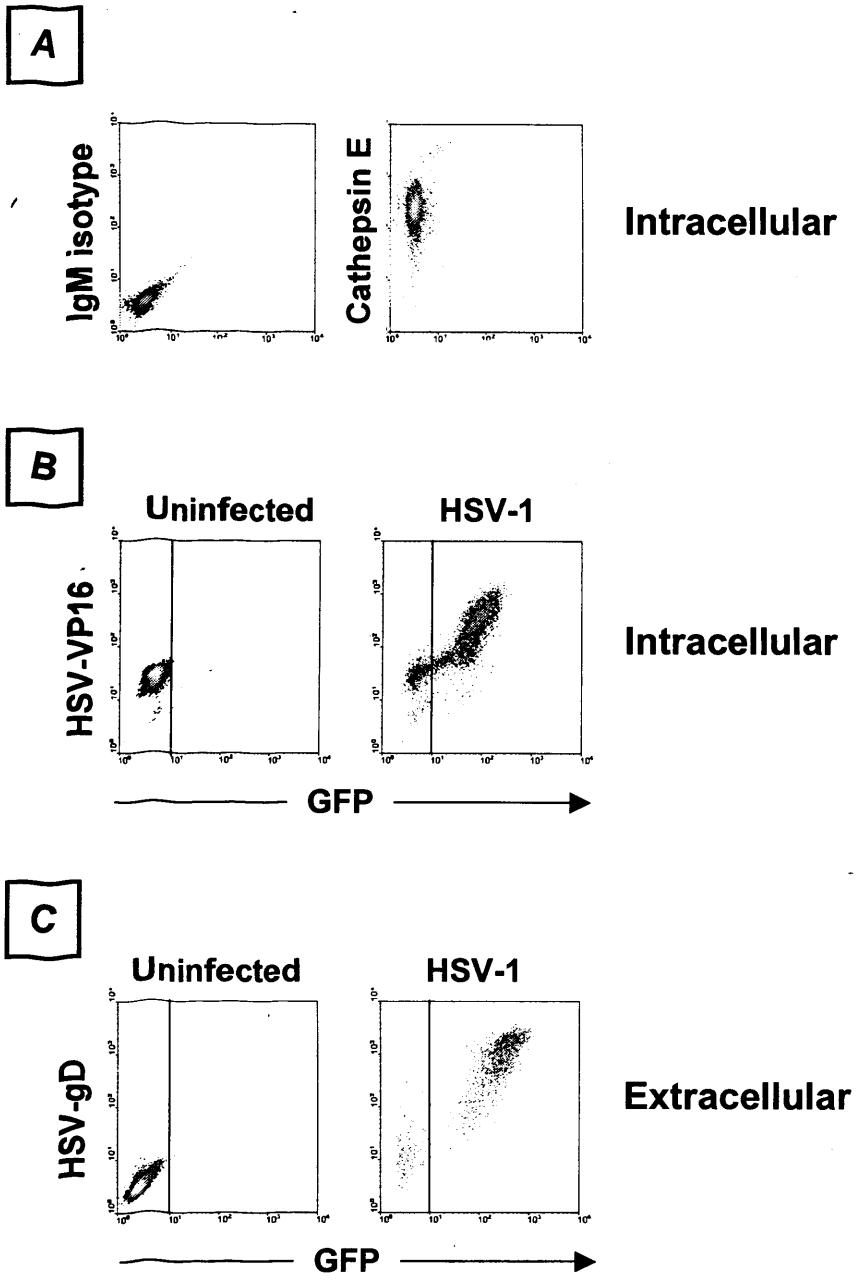

Figure 3.7 Assessment of GFP expression as marker of DC infection.
(A) DC were stained for the intracellular expression of cathepsin E *(right panel)* and the relevant isotype control *(left panel)*. (B+C) DC were infected with HSV-1 at MOI of 1 and stained for intracellular VP16 (B) and extracellular gD (C). Representative of at least 3 independent experiments.

3.2.4 HSV-1 replication in DC

The ability to express viral genes is not always followed by the production of infectious particles in all cell types. Therefore, it was important to relate the susceptibility to infection observed in section 3.2.3, to the replication competence of HSV-1 in DC. Furthermore, production of significant quantities of infectious particles may result in secondary infection of DC in long term cultures.

The production of infectious virus particles was measured by viral infectivity assay, as described in section 2.5.2. HSV-1 has evolved its fusogenic properties to allow intracellular replication and intracytosplasmic infection of neighbouring cells, excluding the need for the extracellular route of infection (Spear et al., 2000). As a result, both virus in the supernatant and intracellular infectious virus (by harvesting cell lysates) were assayed for 4 days following infection. HSV-1 is tropic for neuronal and epithelial cells, but it is in the latter that the greatest productive infection is seen, as evidenced by oral and mucosal herpetic lesions. Therefore, the ability of HSV-1 to replicate in an epithelial cell line (HeLa) was assessed in parallel to gauge the efficiency of replication in DC relative to a cell that permits efficient viral replication.

To ensure a comparable assessment of replication efficiency, both DC and HeLa were infected with the same viral load (MOI of 1), which resulted in similar infection efficiency, as detected by confocal microscopy and flow cytometry (fig. 3.8A vs. fig. 3.5). The data showed that virus production was different in the two cell types (fig. 3.8B). After the first day of infection, there was little difference between virus secreted from DC and HeLa cells. However, from 2 days post infection, there was significantly more virus in the supernatant of HeLa cells than DC, with nearly a 3 log difference after 4 days. This suggested strongly that HSV-1 could replicate more efficiently in HeLa than DC. The data for the production of intracellular virus reflected similar differences between HeLa cells and DC. Noticeable was that the disparity between the two cell types occurred one day earlier for intracellular virus as extracellular virus, as expected. Therefore, from fig. 3.8, it was concluded that expression of late viral genes seen in fig. 3.7 was indeed indicative of a productive viral replication in DC but that this was less efficient than that seen in epithelial cells.

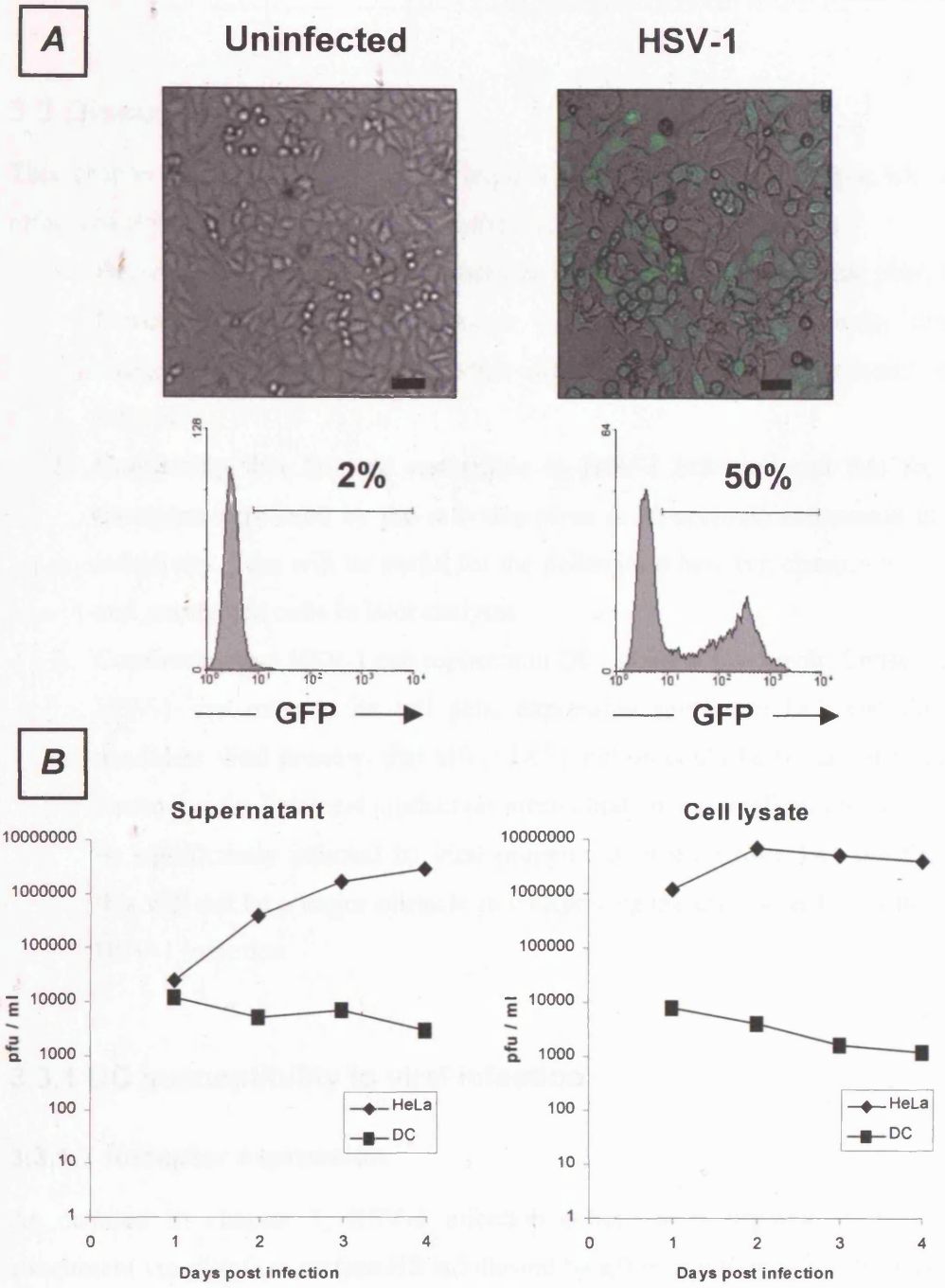

Figure 3.8 Quantification of HSV-1 replication in DC.
(A) HeLa cells were infected with HSV-1 at MOI of 1 and GFP expression was assessed by confocal microscopy (upper panel) and flow cytometry (lower panel). Scale bar represents 50μm. (B) DC and HeLa cells were infected with HSV-1 at MOI of 1 and supernatant and cell lysates were collected for 4 days following infection. Infectious virus was quantified by viral infectivity assay. Representative of 3 independent experiments.

3.3 Discussion

This chapter has set out to establish important setpoint conditions from which the effects of HSV on DC can be gauged in the later chapters. These include:

1. Detailing the "normal" DC parameters in terms of morphology and phenotype. Deviation from these descriptions will be interpreted as virally induced changes, which could occur either directly or indirectly as a result of the infection.
2. Confirming that DC are susceptible to HSV-1 infection and that the GFP transgene expressed by the infecting virus is an accurate assessment of viral infectivity. This will be useful for the delineation between changes in infected and uninfected cells in later analyses.
3. Confirming that HSV-1 can replicate in DC, albeit at low levels. Consequently, HSV-1 can execute its full gene expression profile in DC, and therefore candidate viral proteins that affect DC function could be predicted from this. Secondly, the low viral production means that only a small number of DC will be significantly infected by viral progeny emanating from DC and therefore this will not be a major obstacle in interpreting the effects on DC of the initial HSV-1 infection.

3.3.1 DC susceptibility to viral infection

3.3.1.1 Receptor expression

As detailed in chapter 1, HSV-1 infection occurs in a stepwise process. Viral attachment via gB/gC to surface HS is followed by gD interaction with entry receptors, which then induces the envelope-cell membrane fusion process (Spear et al., 2000). As HS is ubiquitous on all cell types, it is the expression of the entry receptors that determines the susceptibility of cells to HSV-1 infection. HSV-1 utilises HVEM and nectin-1 as entry receptors (Montgomery et al., 1996; Geraghty et al., 1998), but not nectin-2 (Warner et al., 1998). There are however strain differences in receptor usage. Recent studies have confirmed that the 17+ strain used in these studies does indeed use HVEM and nectin-1, and not nectin-2, to any significant degree (Krummenacher et al., 2004).

Consistent with the receptor expression data, it was confirmed that HSV-1 does indeed infect DC. This was not a novel finding, as DC susceptibility has been described in studies carried out both prior to and in parallel with the data above (Coffin et al., 1998; Salio et al., 1999; Mikloska et al., 2001).

Herpesviruses use different cellular entry receptors, but it is interesting to note that many of them can infect DC with similar efficiency (MOI of 1 can infect around 50% of cells (fig. 3.5) (Moutaftsi et al., 2002; Abendroth et al., 2001)). This may reflect one aspect of herpesvirus infection that is well conserved: attachment to surface heparan sulphate prior to interaction with entry receptors (Compton et al., 1993; Zhu et al., 1995; Laquerre et al., 1998). HS binding may increase the proximity of the virus to the entry receptors and form the basis for the similar infection efficiency seen for viruses that exploit this entry strategy. The importance of HS interaction for HSV-1 infection is considered further in chapter 6. Studies detailing the relative role of the entry receptors, both in the infection of DC and any downstream signalling consequences, will be described in chapter 6.

3.3.1.2 Quantification of infection efficiency

The advantage of utilising a GFP expressing virus was that it allowed rapid assessment of infectivity. Furthermore, it permits the gating of infected cells by flow cytometry without additional steps to stain for viral proteins. However, it was important in this setting, to confirm that GFP expression was an accurate correlate of infection efficiency. This was done by staining infected DC for VP16, the viral IE gene transactivator, and gD (fig. 3.7). The data confirmed that GFP expression closely correlated with the expression of both these proteins in DC and that the transgene was therefore an accurate gauge of infection efficiency.

In these studies, the linear correlation between GFP and VP16/gD expression was noticeable. This was consistent with the notion that the expression of these proteins was dependent on the number of HSV virions infecting the cells, and thus the number of viral gene copies inside DC. Furthermore, the correlation between early (GFP) and

late (VP16/gD) protein accumulation confirmed that infected DC are able to express the full repertoire of viral genes, excluding that the infection is abortive at the early gene stage, as is the case in vaccinia virus (VV) infection (Engelmayer et al., 1999).

The data in fig. 3.7 also illustrated the important absence of certain populations. A GFP+ VP16- or GFP+ gD- population DC would have represented DC infected with HSV-1 but in the absence of significant viral gene expression despite transgene transcription. This would have suggested that only the CMV IE promoter was being expressed and that HSV-1 IE gene transcription was not initiated. Such an abortive infection has been described for HSV-1 infection of some cell types (Kemp et al., 1990; Bruun et al., 1998). Secondly, the absence of GFP- VP16+ or GFP- gD+ DC confirmed that GFP was expressed in all cells that underwent HSV-1 replication, ensuring that GFP-ve DC in culture were not infected, and therefore under intracellular control by the virus.

3.3.2 DC permissiveness to viral replication

3.3.2.1 In vitro replication of HSV-1 replication in DC

Measuring virus in the supernatants and the cell lysates of the cells, allowed the assessment of both the quantity of infectious viral particles and consequently the replicative capacity of HSV-1 in DC. The data demonstrated that although DC permit viral replication, this happens to a low degree, compared both to the production in HeLa cells and relative to the input of virus (fig. 3.8). Generally, a productive viral infection can be defined as one that yields more virus than the input amount in one round of replication. At a MOI of 1, $5x10^5$ pfu of HSV-1 were added to DC, but viral production never rose above 10^4 pfu (the experiments were carried out in 1 ml volumes). This figure is also important as it can be deduced that insufficient virus was present in the supernatant after 1 day of infection of DC at an MOI of 1 to infect a significant number of uninfected DC remaining in the culture. At MOI of 1, with approximately 40% of DC remaining uninfected (fig 3.5), there would be 10^4 pfu of virus to infect $0.2x10^6$ DC (60% of initial $0.5x10^6$ DC are already infected). This results in a net MOI of 0.05, which would infect <2.5% of DC (data not shown). The

infection efficiency is likely to be even lower as the infection would occur in the larger volume of a DC culture, rather than the concentrated conditions used for 1 hour infection in all experiments. This calculation is important, because it confirms that the population of GFP+ve cells observed after one day of infection, is derived almost exclusively from the inoculum of the initial infection, and not as a result of a secondary infection from new virions made following replication in DC.

3.3.2.2 In vivo implications of HSV-1 replication in DC

The low permissiveness for viral replication in DC means that this cell type is unlikely to contribute significantly to the generation of a transmissible titre of the virus to infect a new host. However, the low viral production may still be relevant and significant in the DC's own microenvironment, as the LC and dermal DC network is surrounded by epithelial cells or fibroblasts. Perhaps more significant is the close anatomical relationship between DC and peripheral nerve endings in the skin, especially between LC and c-fibre nerve endings in the epidermis, that may provide a convenient entry point into the nervous system for LC-derived virus (Hosoi et al., 1993). The implications of this hypothesis are discussed further in the general discussion chapter 7.

3.3.2.3 Mechanisms and consequences of DC permissiveness to HSV-1 replication

The reasons behind the low replication permissiveness in DC relative to HeLa cells are unclear. As HSV-1 gene expression occurs in a cascade fashion of viral gene groups (Weir, 2001), a critical initiation step in replication is transcription of immediate early genes. The viral protein VP16 is important in this process, as it can form multi-protein transcriptional regulatory complex (the VP16 complex) with Oct-1 and HCF-1, two host cell factors, on the viral IE promoters. This process requires first the interaction with HCF-1, priming the dimer for interaction with Oct-1 transcription factor which targets 'TAATGARAT' sequences on the viral IE promoters (Wysocka and Herr, 2003). Therefore, viral gene expression and replication is dependent on the host cell environment.

One factor that may play a role is the presence of inhibitory transcription factors that can prevent Oct-1 binding to the target promoters. These include some Oct-2 isoforms that prevent IE gene expression, a strategy the virus may exploit to prevent initiation of a lytic cycle in neurones and consequently establish latency (Lillycrop et al., 1993). HeLa cells do not express Oct-2 transcription factors (Hagmann et al., 1995a), whereas immature DC only do so at low levels (Neumann et al., 2000). The presence of Oct-2 may quantitatively reduce VP16 complex binding to viral promoters in DC and result in lower gene expression and replication. However, while both monocytes and macrophages express high levels of Oct-2 (Neumann et al., 2003), HSV-1 replication is only observed in macrophages (Bruun et al., 1998), suggesting that other host factors may be more important in determining the expression of viral genes.

An important difference between DC and HeLa cells is that the latter are dividing cells. HCF-1 promotes proliferation (Wysocka and Herr, 2003) and may therefore be more abundant in HeLa cells. Indeed, in non-proliferating sensory neurons, HCF-1 is sequestered in the cytoplasm and only translocates VP16 to the nucleus upon induction of lytic infection (Kristie et al., 1999). However, recently an important role for the PKR pathway in determining the permissiveness of HSV-1 infected cells has also been described. Phosphorylation of PKR results in the phosphorylation of the translation initiation factor eIF-2α, which reduces viral protein translation (Clemens and Elia, 1997). The HSV-1 gene ICP34.5 can prevent this process (Chou et al., 1995), but was not a factor in these experiments as the same virus was used for infecting both DC and HeLa cells (i.e. no ICP34.5 mutants were used). However, an important endogenous negative regulator of the phosphorylation of PKR is the Ras pathway, whereby Ras activation prevents the phosphorylation of PKR. The net result is that in cells where the Ras pathway is most active, viral replication is enhanced (Farassati et al., 2001). Therefore, the difference in HSV-1 replication may lie at the level of Ras and PKR activation.

Being a transformed, replicating cell line, HeLa cells are likely to express elevated levels of active Ras (Dobrowolski et al., 1994) and this may have played a major role in permitting greater viral replication in these cells by preventing PKR activity.

However, it is also possible that PKR activation in DC is quantitatively greater and the subsequent inhibition of viral protein synthesis more potent in DC than in HeLa cells. Unfortunately, few studies have compared replication of other viruses in DC relative to other cells directly, and therefore it is difficult to determine the relative role of these two pathways. This could only be carried out by measuring Ras activity and PKR phosphorylation in parallel after infection of cells. However, the observation that maturation of DC renders the cells less susceptible to viral induced changes in phenotype (Salio et al., 1999) supports the hypothesis that DC have an innate protection to viral replication. Therefore, if HSV-1 infection were to elicit DC maturation, the cells may be able to switch to a more efficient antiviral state than HeLa cells and intrinsically hinder viral replication more effectively.

3.3.3 Role of replication in DC in viral pathogenesis

With regards to the general permissiveness of DC to viral infections, it should be noted that measuring the growth of virus in individual cells might mask the true events that occur in vivo. Although replication of MV and HIV in DC cultures alone is low or negligible, upon interaction with T cells (e.g. CD40L), this can be elevated to extremely high levels (Fugier-Vivier et al., 1997; Granelli-Piperno et al., 1999; MacDougall et al., 2002). This "inducible" form of viral replication demonstrates how some viruses may have evolved to exploit DC to serve as a reservoir of large virus production at appropriate time points in the virus lifecycle and pathogenesis, so as to produce a large progeny in close proximity to its target cells (e.g. CD4+ T cells for HIV).

The consequences of viral infection on DC's ability to initiate T cell activation will be discussed at greater length in the next chapter. Rapid viral infection, replication and lysis of DC is one potential strategy to disrupt the APC function of these cells. However, replication in DC does not correlate with the effects on DC induction of T cell proliferation. For example, HSV-1 and VV impair DC equally well despite the latter being an abortive infection (figs. 3.8 + 4.1) (Salio et al., 1999; Engelmayer et al., 1999). DC infected with strains of HCMV that differ in their ability to replicate, still result in equal inhibition of T cell stimulation (Beck et al., 2003). Furthermore, the

time necessary for viral replication varies. For example, HCMV requires up to four days to undergo a full replicative cycle, by which time the infected DC would have encountered responding T cells (Riegler et al., 2000). Therefore, viral replication per se is unlikely to be a predominant strategy to disable DC function, but rather reflects an in-built programme of the viral lifecycle in human cells. It is more likely that other aspects of DC physiology are specifically targeted by viral immunomodulatory genes, and these are discussed in the following chapter.

It is also worth mentioning that a critical factor in the study of viral egress from DC may relate more to the timing and context rather than the quantity of virus production. In recent years, the ability of DC to "capture" viruses by binding it to the c-type lectin, DC-SIGN, has generated much interest, with HIV-1 being the prototypic example (Geijtenbeek et al., 2000a). The pathogenesis model proposes that following viral interaction with the DC in the periphery, the cell "carries" the virus to the lymph node, where it can infect T cells in trans after interaction between the two cell types. Therefore, although this scenario proposes that no viral replication has taken place, the DC has facilitated the infection of a neighbouring target cell, albeit with a low virus inoculum, demonstrating an extra layer of complexity in the in vivo relevance of viral egress from infected DC.

3.4 Conclusions

This chapter has characterised the DC that will be used for the remainder of the studies described in this thesis. Determining the morphology and phenotype of DC has established the "normal" conditions which virus infection might offset. Data in this chapter have also characterised the HSV-1 strain that will be used for future study. Notably, it was concluded that GFP is an efficient method to quantify infection efficiency in vitro and will aid in discerning the effects of uninfected and infected DC. The degree of HSV-1 replication in DC was also assessed, and it excluded that DC infection results in the production of large quantities of infectious virus, which should not hinder the interpretation of assays used in the remainder of the thesis. The rest of the work presented will now build on these findings, by analysing in greater detail the impact of HSV-1 infection on the ability of DC to function as an APC, focusing on

viral mechanisms that affect the cell and the host's response to detect and counter the effects of the infection.

Chapter 4

HSV-1 mediated impairment of DC function

4.1 Introduction

Chapter 1 detailed how DC play an important role in the generation of antiviral T cells to HSV infection. It would be in the virus' interest to prevent DC from stimulating resolving T cell responses. The ubiquitous location of immature DC at the sites of potential viral entry, including the blood, provide a hypothetical motive for the virus to disrupt the ability of DC to function as APC. Subsequent hindrance to the onset of immunity may delay clearance of viral infection, allowing the virus to establish itself, replicate and reach transmissible titres in target organs. Some viruses could exploit this scenario to gain time to establish themselves in a latent state.

However, DC activation and the resultant enhancement of the immune response may promote and even exacerbate immunopathology, and this may generate outlets for viral transmission. Therefore, the viral-DC interaction can have important functional outcomes in the context of the natural history of the disease.

The most important function of DC that can be affected by HSV-1 is their ability to stimulate naïve T cell proliferation. Disruption of many aspects of DC physiology could impinge on the outcome of this interaction between DC and T cells. Changes on DC morphology would affect both the ability to migrate to secondary lymphoid organs (Swetman et al., 2002) and to present antigen to responding T cells (Kobayashi et al., 2001; Benvenuti et al., 2004a). Interference with the DC maturation process, including secretion of relevant cytokines, would prevent the generation of potent mature APC, possibly skewing towards a tolerogenic immune response (Hawiger et al., 2001). Finally, by inducing DC death, the virus would reduce the time of interaction between the DC and the responding T cell, preventing necessary activation for generating a potent multi-epitope anti-viral T cell response.

In this chapter, the aim is to determine how HSV-1 influences the DC ability to stimulate T cells and to define which DC functions are affected that could account for these changes. The focus will be on the effect on DC morphology, cytokine secretion, cell viability and surface phenotype.

4.2 Results

4.2.1 T cell stimulation by HSV-1 infected DC

The ultimate function of a DC is to stimulate T cell proliferation. Therefore, this was the first DC property measured, and evaluated to see how it was affected by HSV-1 infection.

4.2.1.1 HSV recall responses

Resolution of HSV infections requires a cellular immune response. This presence of HSV-specific T cells correlates with serostatus in most cases (Posavad et al., 2003). Therefore, it should be possible to measure memory responses in those individuals who had been previously infected with HSV-1. Indeed, HSV-1 infected DC stimulated autologous T cell proliferation, in the absence of any other antigen, in a proportion of individuals tested (fig. 4.1). In all, 10 individuals were tested, both for the presence of an HSV-specific T cell proliferative response, and for the presence of neutralising antibodies to HSV in serum (6 individuals shown in fig. 4.1). All the 5 responders were seropositive, while all 5 non-responders were seronegative. There were no discrepancies between responder status and seropositivity. This close relationship between serostatus to HSV and T cell proliferation supported the interpretation that the T cell response represented a memory response to the virus.

A noteworthy feature of the HSV recall responses was the reverse dose-dependent nature of the responses observed, with the greatest proliferation observed at the lowest MOI (i.e. in the conditions where the greatest number of uninfected DC were present). However, this response diminished with increasing numbers of DC infected (fig. 4.1).

4.2.1.2 PPD recall responses

All individuals tested had been vaccinated with BCG. The ability of infected DC to activate memory T cells was assessed by a recall antigen assay to PPD. As a proportion of individuals tested possessed HSV-1 recall responses (fig. 4.1), proliferation to PPD could only be analysed in those individuals that showed no such

responses (fig. 4.2A). This showed that the ability to elicit PPD responses was impaired in infected DC populations in a dose dependent manner, with the greatest inhibition observed at the highest MOI.

4.2.1.3 Allogeneic MLR proliferation

The capacity of HSV-1 infected DC to elicit primary T cell proliferation was tested in an allogeneic mixed lymphocyte reaction (MLR). Fig. 4.2B shows that HSV-1 infection resulted in an impaired ability to induce proliferation of naïve allogeneic T cells, with the greatest inhibition again seen at the highest infection rates. The data shown are a mean of six individuals, three with an HSV recall response and three without (see chapter 5). The ability to elicit an autologous HSV response did not correlate with the degree of inhibition observed in the allogeneic response.

4.2.1.4 ConA stimulated autologous proliferation

The impaired stimulation of naïve T cells in section 4.2.1.3 was assessed by 6 day assays. During this time, the viability of infected DC could have been affected (see section 4.2.4). Using a mitogen reduces the contribution of DC death in a shorter assay, excludes variability introduced due to differing degrees of allogeneic recognition, and also avoids HSV recall responses (chapter 5) and potential fratricide within the T cell population (Raftery et al., 1999).

The ability of HSV-1 infected DC to stimulate the proliferation of ConA-primed autologous naïve T cells in this assay was severely impaired. The degree of inhibition again correlated with the number of DC infected (fig 4.2C).

4.2.2 T cell infection by HSV-1

One possible explanation for the reduced T cell stimulating capacity was direct infection of T cells by virus emanating from infected DC in the coculture. T cell proliferation could be hindered by this infection. T cells could be unable to respond to DC stimuli, or susceptible to CTL lysis (Raftery et al., 1999). T cell infection by DC-

derived herpesvirus has been described (Abendroth et al., 2001), and it has been proposed that activated, not resting, T cells are permissive to HSV (Ito et al., 1997; Raftery et al., 1999). One of these previous studies used ConA stimulation to render the T cells susceptible to infection (Raftery et al., 1999). Therefore, T cells were pretreated with 1.25μg/ml ConA (the optimum concentration used in the proliferation assays in fig. 4.2C), prior to HSV-1 infection. Only (1.2%) of T cells expressed the GFP transgene, in comparison to 0.8% in the absence of ConA. This result questioned the efficiency of ConA to render T cells susceptible to HSV-1 infection, but also confirmed both that the CMV-driven GFP transgene could be expressed in T cells (Barry et al., 2000) and also that some T cells could be infected by HSV-1.

The number of infected T cells was also assessed in an allogeneic MLR. No significant numbers of CD3+ GFP+ cells could be detected 1, 3 or 5 days from the beginning of the co-culture (fig. 4.3). These data excluded the possibility that the inability for HSV-1 infected DC to stimulate T cell proliferation was secondary to trans infection of T cells.

Thus, based upon the results in sections 4.2.1 and 4.2.2, HSV-1 infection impairs DC ability to stimulate both naïve and memory T cell proliferation. The absence of T cell infection implies that the inhibitory effect was dependent predominantly on the viral effects on DC function. Hence, the remainder of this chapter will investigate the cellular processes affected in greater detail. The viral mechanisms responsible are analysed further in chapter 6.

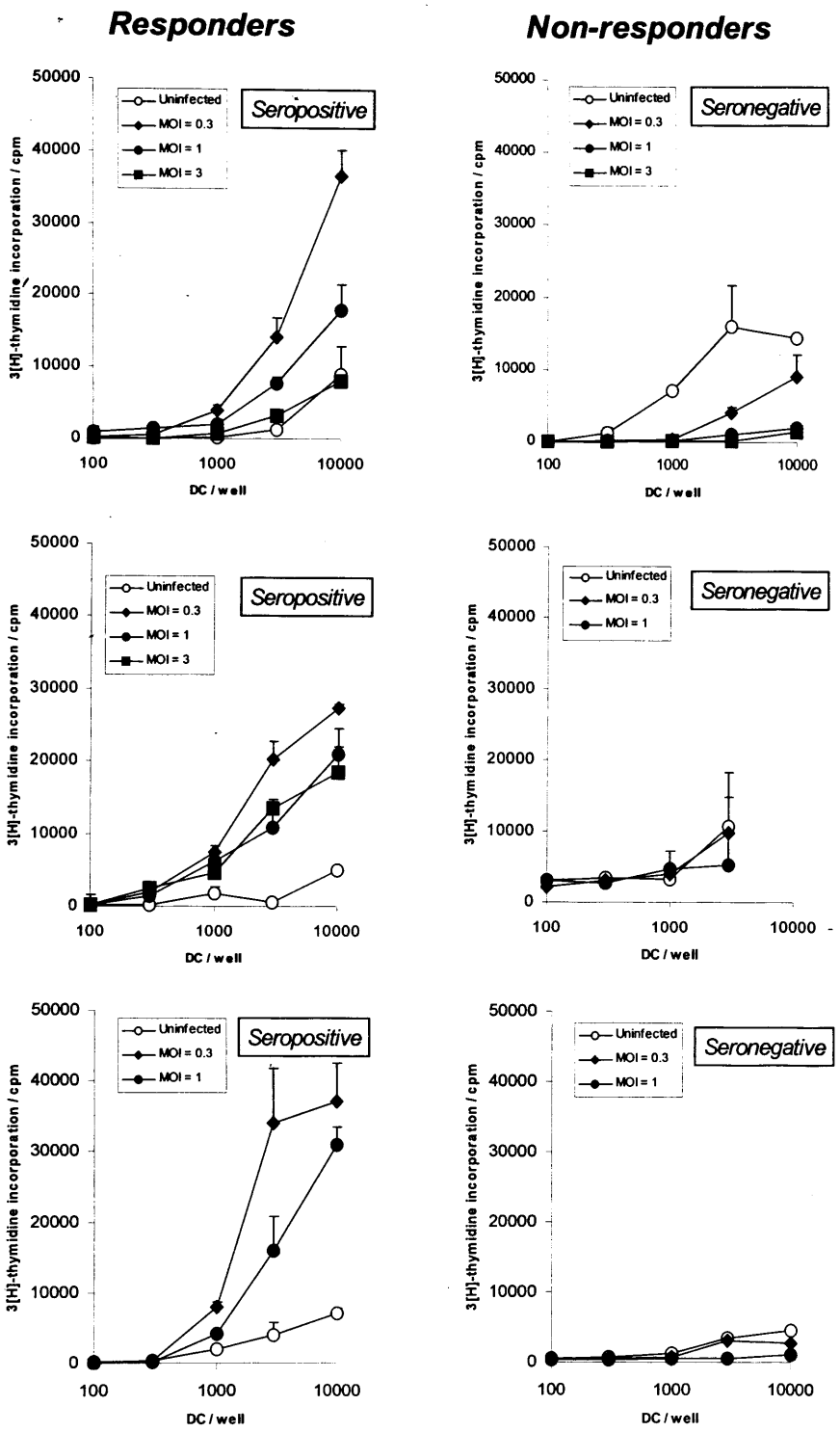

Figure 4.1 Autologous T cell responses in the presence of HSV-1 infected DC.
DC were infected with HSV-1 and cultured for 16 hours prior to addition of autologous T cells (10^5) and proliferation assessed by incorporation of ^{3}H-thymidine. Results expressed as the mean ± SEM for triplicate cultures from individual experiments. HSV serostatus of individuals indicated in box inserts.

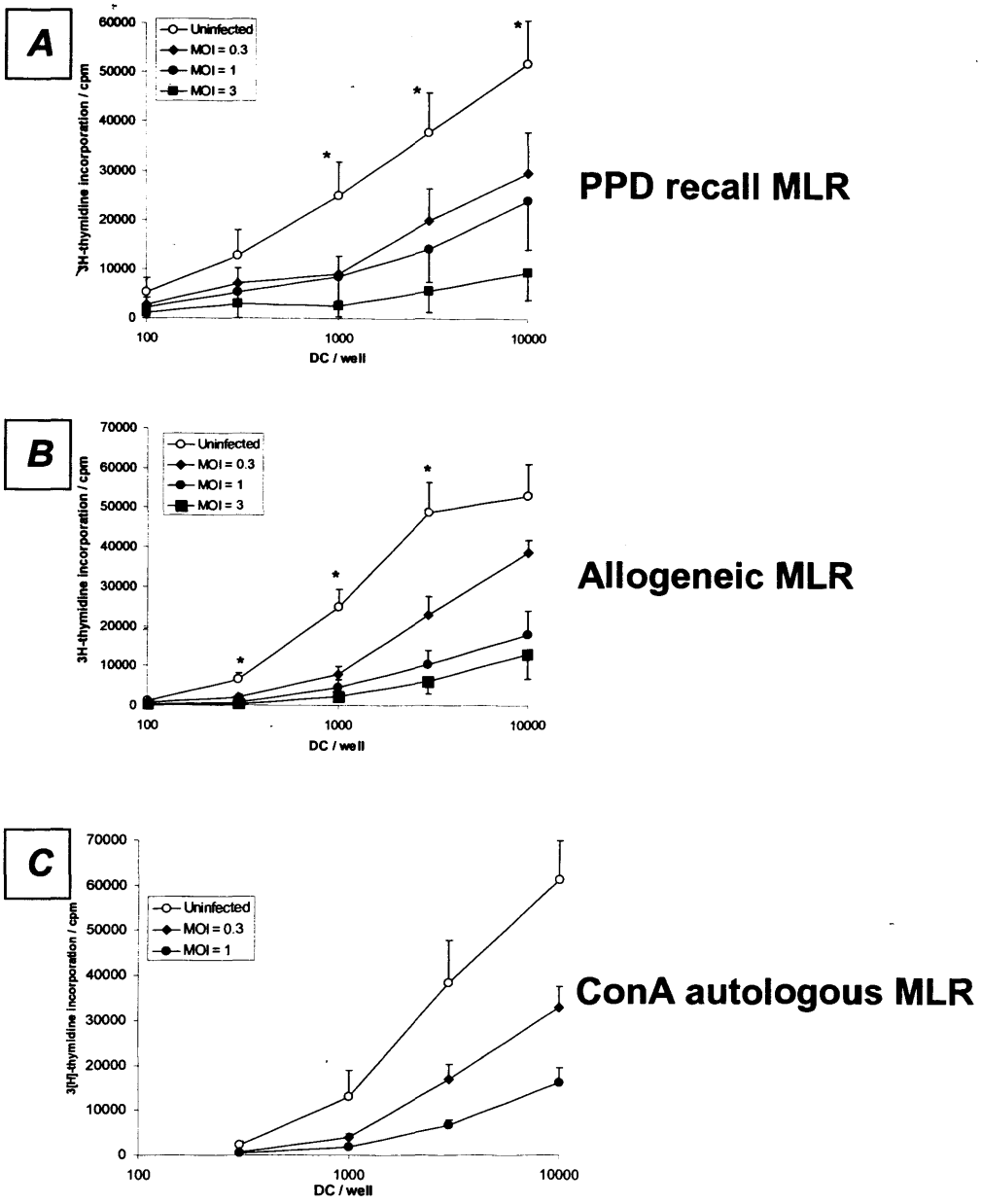

Figure 4.2 Memory and primary T cell proliferation elicited by HSV-1 infected DC. (A) DC were infected and cultured for 16 hours prior to addition of autologous T cells (10^5) in the presence of 500 U/ml PPD. Results expressed as mean ± SEM (n = 7). (B) DC were infected and cultured for 16 hours prior to addition of allogeneic T cells (10^5). Results expressed as mean ± SEM (n = 6). (C) DC were infected and cultured for 16 hours prior to addition of autologous T cells (10^5) in the presence of 1.25 μg/ml ConA. Results expressed as mean ± SEM (n = 4). Proliferation was assessed by incorporation of ^{3}H-thymidine. DC only proliferation 56 ± 10 cpm (mean ± SEM; n = 6). T cell only proliferation 57 ± 11 (mean ± SEM; n = 6). * = $p<0.05$ is the proliferation in presence of uninfected DC relative to all other conditions. Statistical analysis by Student's *t* test.

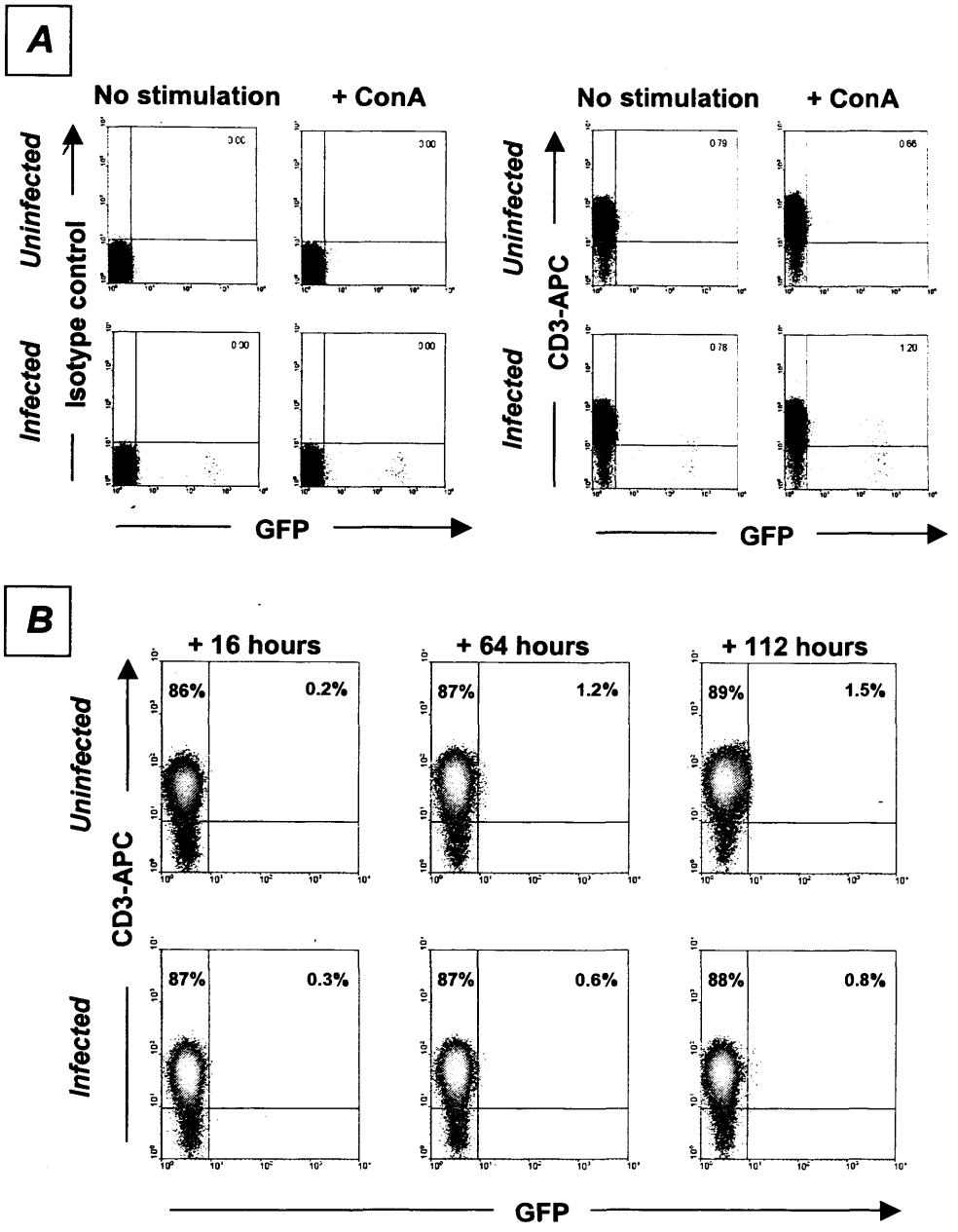

Figure 4.3 Infection of T cells by HSV-1.

(A) T cells were infected with HSV-1 (MOI = 1) and cultured for 16 hours. (B) DC were infected with HSV-1 (MOI = 1) and cultured for 16 hours prior to addition of allogeneic T cells (10^5). DC-T mixtures were co-cultured for the times indicated and GFP expression by CD3+ T cells assessed. Representative of three independent experiments.

4.2.3 Morphological consequences of HSV-1 infection of DC

An intact and flexible DC cytoskeleton is important in maintaining DC morphology during antigen sampling in the periphery, DC migration to secondary lymphoid organs and for the presentation of antigen to T cells. Disruption of this cytoskeleton, and therefore cell shape, could affect the ability to stimulate T cells (Kobayashi et al., 2001; Benvenuti et al., 2004a). Indeed, after HSV-1 infection, the most striking changes seen following infection were in the morphology of the DC.

4.2.3.1 Morphological changes of non-adherent DC

Following HSV-1 infection, the veiled DC appearance was lost and the cells assumed a more rounded morphology. Infected cells displayed a heterogeneous morphology. Many of the DC lost the membrane processes observed in chapter 3 (fig. 4.4A). Another feature was the large number of non-adherent homotypic clusters. GFP fluorescence revealed that these comprised both uninfected and infected DC (fig. 4.4B). Homotypic clustering is observed commonly in vitro in response to some maturation stimuli (e.g. LPS), but these are often of different character, being adherent and larger in size than those observed after HSV-1 infection. HSV-induced clusters were 10-20 cells or less (fig. 4.4B). Occasionally, multi-nucleate giant cells were visible, particularly within or near DC homotypic clusters. The multicellular origin of these structures was confirmed by EM, as determined by the presence of two or more nuclei in cells significantly larger than normal DC (fig. 4.4C)

4.2.3.2 Morphological changes of fibronectin-adherent DC

In the context of a cutaneous/mucosal infection in vivo, HSV-1 is unlikely to come into contact with non-adherent DC, but rather DC adhered and spread in their surrounding tissue microenvironment. Therefore, to model this scenario, DC were adhered to a FN substratum for 2 hours, prior to infection with HSV-1. After 1 hour of infection, any unbound virus was washed off and the morphology of the DC was analysed 8 hours later. Preliminary experiments showed that this was the earliest time that the fluorescent channel of the confocal microscope could detect sufficient GFP

fluorescence to distinguish between uninfected and infected DC. Even at this stage of the infection, significant morphological changes could be seen. GFP+ve DC rapidly lost their dendritic morphology, but remained adherent to FN (figs. 4.5A+B). At higher DC confluence, evidence of clustering could be seen, raising the possibility that infected DC could influence uninfected DC functionally through direct cell-cell contact (fig. 4.5A).

As shown in the previous chapter, one of the ways in which DC respond to maturation stimuli (such as LPS) is to increase adherence to FN (fig. 3.2). Therefore, the effect of HSV-1 infection on the morphological response to LPS was assessed. After infection, FN-adherent DC were cultured in the presence or absence of 100 ng/ml LPS. As seen in fig. 4.5B, uninfected DC responded by increasing the number of adherent DC with dendritic morphology. However, LPS did not increase the number of infected, GFP+ve, DC with a dendritic shape. In sharp contrast, GFP-ve DC responded similarly to uninfected DC cultures, consistent with them being uninfected, and therefore free from the inhibitory effects of the virus (fig. 4.5B). These data argue against a scenario in which viral infection resulted in the secretion of soluble factors that prevented uninfected DC in the culture responding to maturation stimuli.

4.2.3.3 Changes in integrin expression after HSV-1 infection

DC interact with and adhere to FN via β1 integrins (Le Varlet et al., 1992; D'amico et al., 1998), and thus the inability of HSV-1 infected DC to spread on FN could have been attributed to change in expression of these integrins. Expression of both β1 and β2 integrins was assessed by flow cytometry after HSV-1 infection. The β1 integrins (CD29 and CD49d) were downregulated after infection (GFP+ve gate), but β2 integrin (CD11a, CD11b, CD11c and CD18) expression was not altered (fig. 4.6). Hence, the loss in dendritic morphology on FN after HSV-1 infection could be attributed at least in part to the selective downregulation of β1 integrins on the surface of the infected DC.

Section 4.2.3 has shown that HSV-1 infection results in the loss of dendritic morphology, and prevents DC responding to exogenous maturation stimuli by altering

their shape. Selective downregulation of β1 integrin expression may have contributed to this. Functionally, this may impair DC migration out of the periphery and may also play a role in the loss of T cell stimulatory capacity of HSV-1 infected DC.

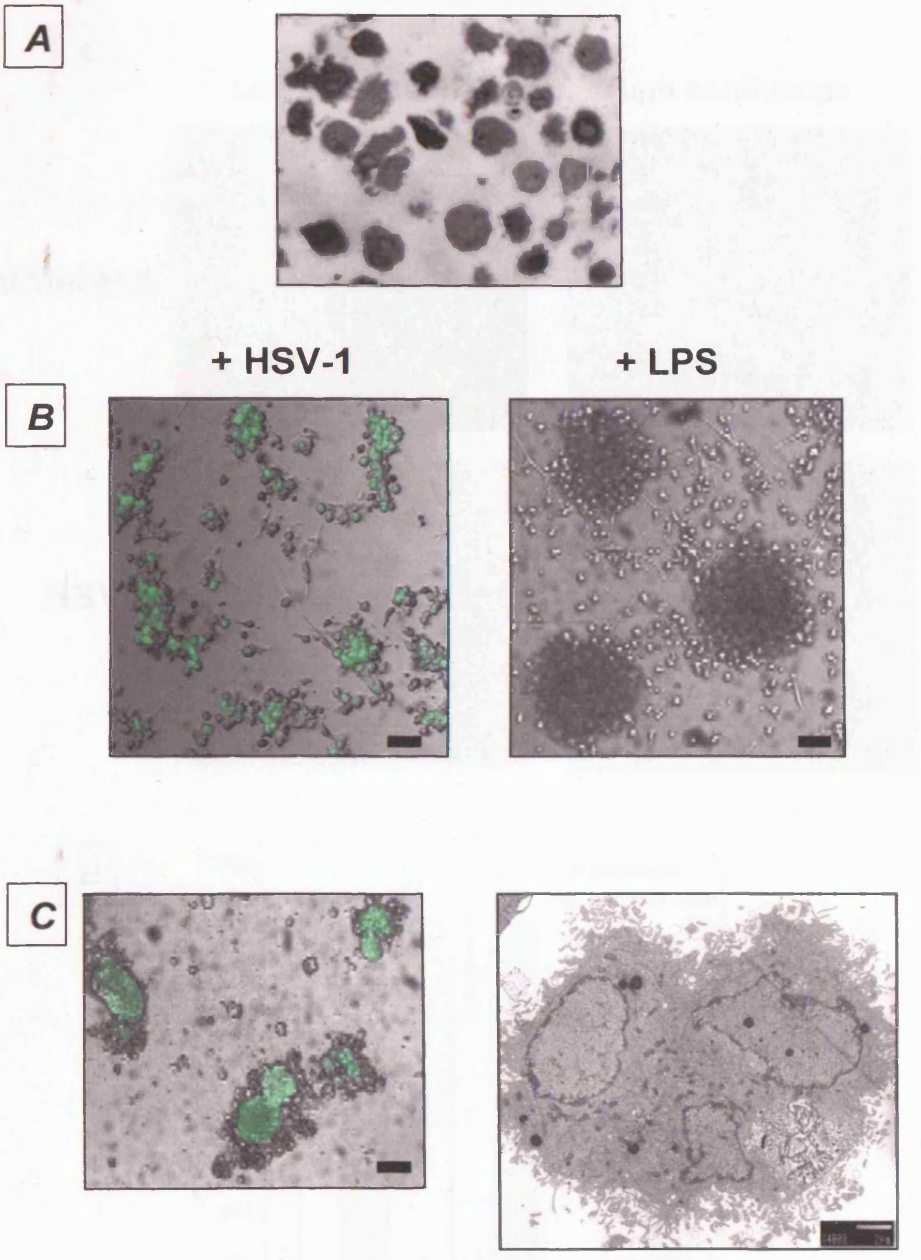

Figure 4.4 Morphology of HSV-1 infected DC in suspension.
DC were infected with HSV-1 (MOI = 1) and (A) fixed with 2% gluteraldehyde and viewed on slides, or (B) viewed in tissue culture suspension by confocal microscopy, revealing small DC-DC clusters compared to those after LPS stimulation. (C) Analysis also revealed occasional syncytia formation (*Left panel* – confocal microscopy; *right panel* – EM). Scale bar represents 50μm. Representative fields of at least three independent experiments.

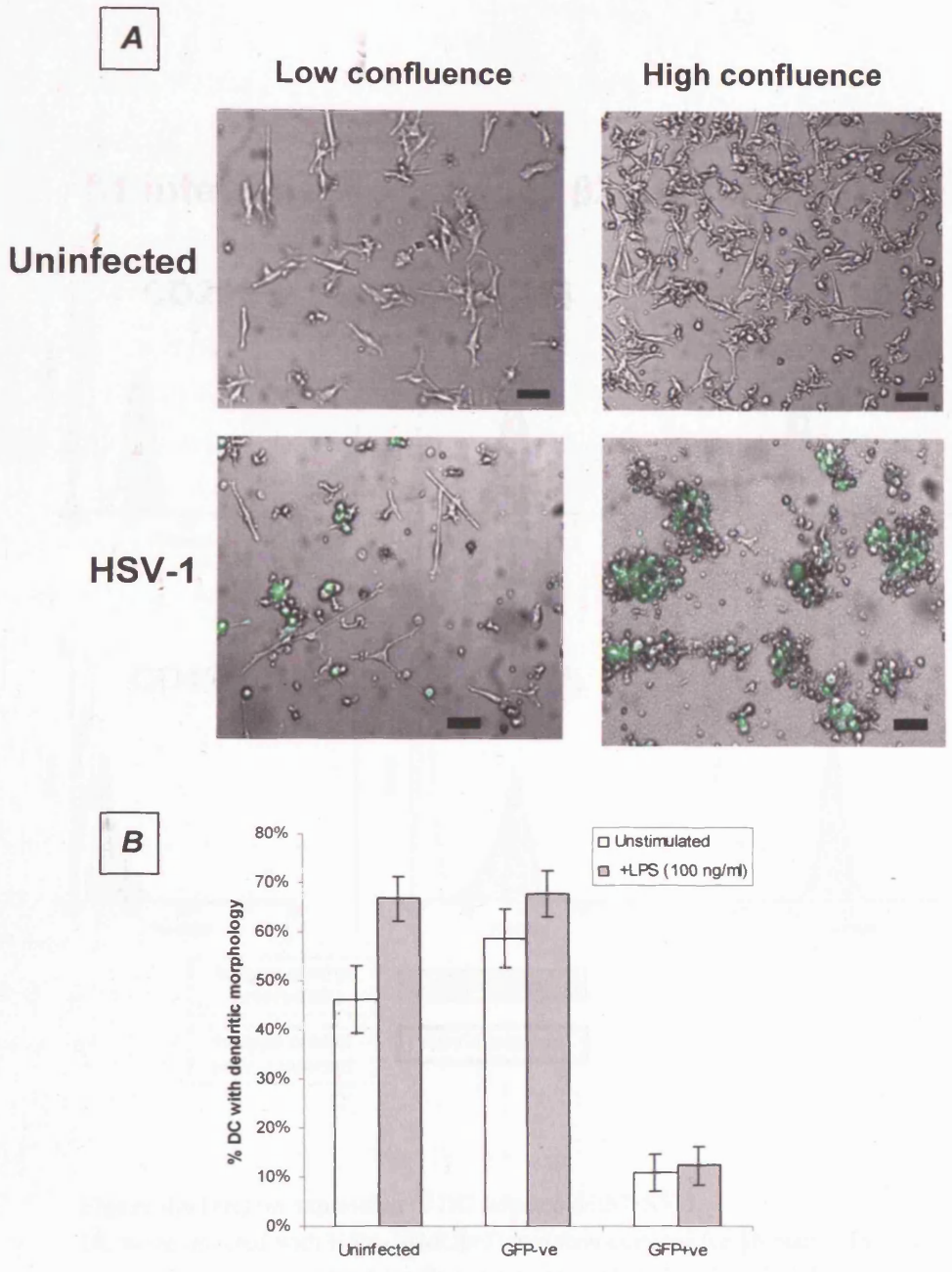

Figure 4.5 Morphology of FN-adherent DC infected with HSV-1.
DC were allowed to adhere to a FN substratum for 2 hours and then infected with HSV-1 (MOI = 0.3). Cell morphology was assessed 8 hours after infection by light microscopy (A) and quantified from a total of 4 random fields from 2 independent experiments (B). Results expressed as mean ± SEM. Scale bar represents 50μm.

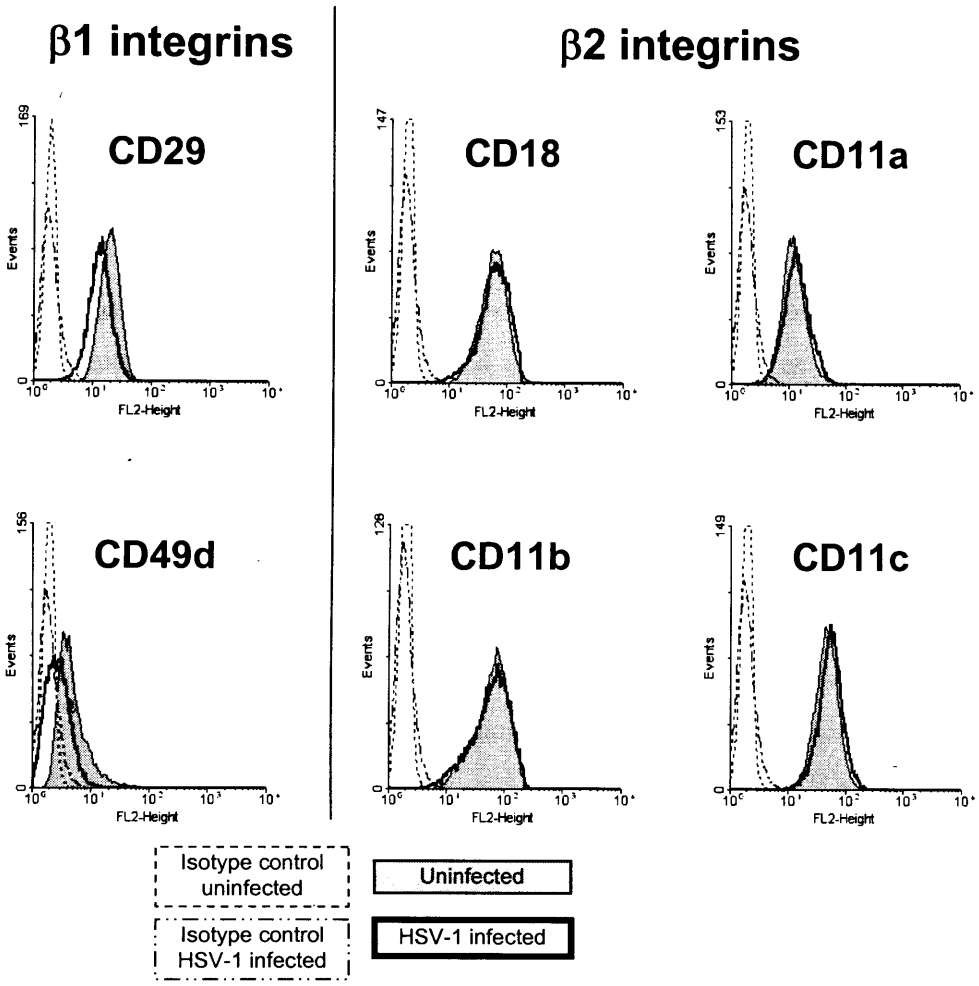

Figure 4.6 Integrin expression of DC infected with HSV-1.
DC were infected with HSV-1 (MOI=1) and then cultured for 16 hours. Integrin expression was determined by flow cytometry. HSV-1 infected cells gated on GFP+ve. Representative of at least two independent experiments.

4.2.4 DC viability following HSV-1 infection

Both the rapid changes in morphology described in section 4.2.3 and the dramatic functional impairment in section 4.2.1, might be a result of rapid cell death after infection. A crude assessment by flow cytometry, analysing the percentage of cells detected within the customary live gate of DC, showed that, 40 hours after infection, there was a significant fall in the number of live DC (fig. 4.7). However, this assay is not a direct measure of cell viability, but rather an assessment of change in shape and granularity of cells after fixation with formaldehyde. Infection may render the cells more susceptible to fixation artefacts, and therefore shape changes detectable on the flow cytometer may not necessarily be indicative of cell death. Therefore, several assays were used to determine DC viability directly.

Previous studies using trypan blue exclusion revealed no difference in DC viability 16 hours following infection, with only a slight reduction after 40 hours (Mikloska et al., 2001; Samady et al., 2003). This was confirmed qualitatively during cell counting prior to setting up the functional studies described in section 4.2.1 (data not shown). However, the assessment of viability was also undertaken by measuring MTT reduction as a measure of overall cell metabolic activity (see section 2.16). DC demonstrated a non-significant trend in reduction in viability 16 hours after HSV-1 infection. The degree of cell death was significantly increased after a further day of culture, such that MTT reduction by infected cultures was >50% lower than that of uninfected DC (fig. 4.8).

The MTT reduction assay did not give any information regarding the type of cell death occurring. However, reduction in cell size, in conjunction with loss of cell viability, suggested apoptosis. EM of HSV-1 infected DC found several cells that fulfilled apoptotic criteria, namely cell rounding (fig. 4.9) and nuclear chromatin condensation, both with intact (fig. 4.9A) and lost (fig. 4.9B) nuclear envelope. There was also evidence of apoptotic DC that, in the absence of phagocytic removal, underwent secondary necrosis, as evidenced by cells with apoptotic morphology that had lost cell membrane integrity (fig. 4.9C). Occasionally, it was possible to observe HSV-1

capsids in the nuclei of these cells, confirming apoptosis in DC with clear evidence of HSV-1 infection (fig. 4.9B+C).

For quantitative assessment of apoptosis in these cultures, flow cytometry was used. A defining criterion of apoptotic cell death is the degradation of nuclear DNA. DNA fragments elute out of permeabilised cells and the remaining DNA stain less brightly with the DNA intercalating dye PI (Rad et al., 2003). Permeabilising DC with ethanol and staining them with PI will therefore allow assessment of the DNA content of the cell. DC are non-replicating cells, so all live cells were in the G0 phase of the cell cycle and the apoptotic hypodiploid population are the "sub-G0" population. A sub-G0 population was observed at 12 hours of infection, accounting for 6% of cells over the background rate of DC apoptosis in the cultures (fig. 4.10A). There was a progressive increase in the number of cells falling in the sub-G0 gate over time, but there was no distinct peak, as seen in other systems (Rad et al., 2003). This reflected the asynchronous induction of apoptosis in DC. Unfortunately, ethanol fixation impaired GFP detection, and as a result the data could not be gated on infected DC. However, the number of sub-G0 DC increased with MOI, strongly suggesting that it was infected DC that died by apoptosis (fig. 4.10B). It was noteworthy that even at 24 hours, only approximately 25% of DC infected at MOI of 1 (i.e. about half the number infected) were apoptotic, demonstrating that not all infected DC underwent apoptosis.

Section 4.2.4 shows that HSV-1 infection of DC does indeed result in cell death, which occurs partly by apoptosis. However, this is delayed and occurs asynchronously, suggesting that reduced T cell stimulatory capacity of DC cannot be attributed solely to virus induced cell death. Therefore, two facets of the DC-T cell interaction that have an important bearing on T cell activation, namely cytokine secretion and the maturation state of the DC, were studied in greater detail.

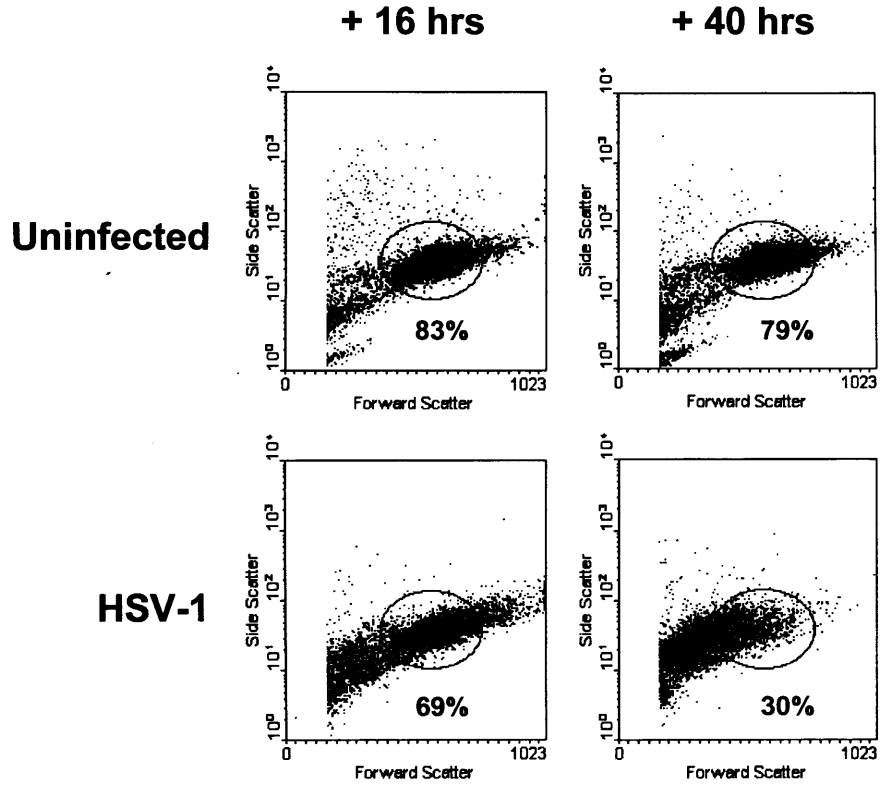

Figure 4.7 Change in DC size following HSV-1 infection.
DC were infected at MOI of 1 and cultured for 16, 40 and 64 hours. A typical FSC/SSC gate that includes the majority of DC is shown. Representative experiment shown (n = 3).

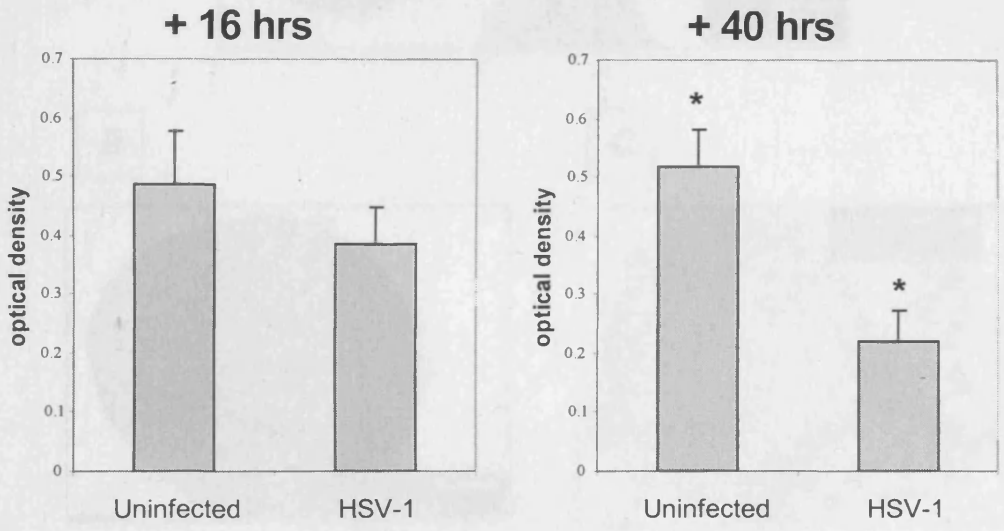

Figure 4.8 Changes in DC viability following HSV-1 infection.
DC were infected at MOI of 1 and cultured for 16 or 40 hours. MTT reduction was assessed as described in Materials and Methods. * = $p<0.02$ is the mean optical density of uninfected DC relative to infected cultures. Results expressed as mean optical density ± SEM (n = 3). Statistical analysis by Student's *t* test.

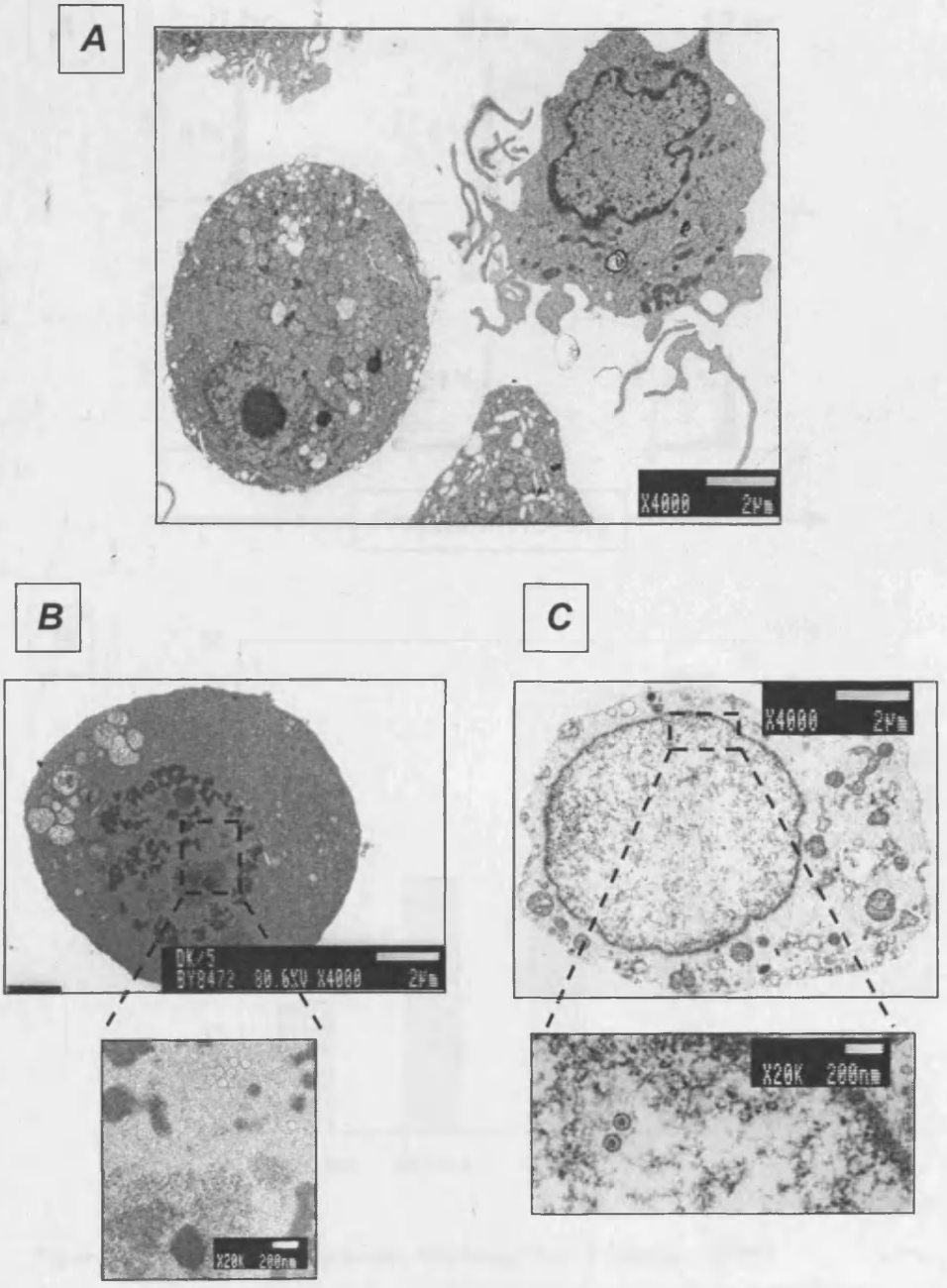

Figure 4.9 Ultrastructural changes in DC morphology after HSV-1 infection.
DC were infected with HSV-1 (MOI = 1) for 16 hours, fixed in 2% gluteraldehyde and prepared for analysis by EM (see materials & methods). Unaffected DC were observed (A), as well as apoptotic DC (A+B) and secondary necrotic DC (C). Some cells displayed evidence of HSV-1 infection (inserts in B+C). Representative cells shown.

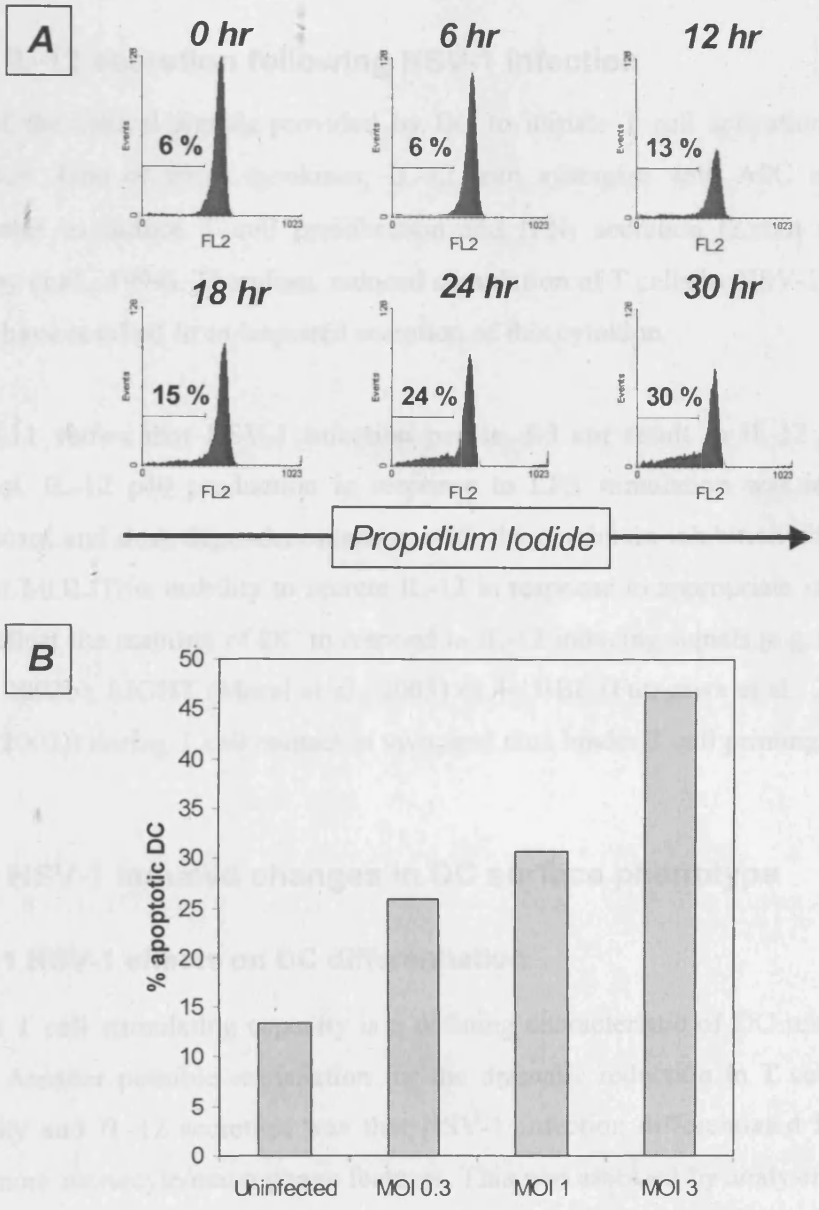

Figure 4.10 Induction of apoptosis following HSV-1 infection of DC. DC were infected at MOI of 1. The proportion of cells undergoing apoptosis was defined as the number of cells in the sub-G0 phase by flow cytometry (gate shown). (A) Representative experiment shown (n = 3). (B) Mean percentage of DC in sub-G0 after 40 hours of infection. Results expressed as mean percentage (n = 3).

4.2.5 IL-12 secretion following HSV-1 infection

One of the critical signals provided by DC to initiate T cell activation is cytokine secretion. One of these cytokines, IL-12, can synergise with APC costimulatory molecules to induce T cell proliferation and IFNγ secretion (Kubin et al., 1994; Murphy et al., 1994). Therefore, reduced stimulation of T cells by HSV-1 infected DC might have resulted from impaired secretion of this cytokine.

Fig. 4.11 shows that HSV-1 infection per se did not result in IL-12 secretion. In contrast, IL-12 p40 production in response to LPS stimulation was impaired in a significant and dose-dependent manner, with the maximum inhibition observed at the highest MOI. This inability to secrete IL-12 in response to appropriate stimuli may in turn reflect the inability of DC to respond to IL-12 inducing signals (e.g. CD40L (Luft et al., 2002b), LIGHT (Morel et al., 2003) or 4-1BBL (Futagawa et al., 2002; Wilcox et al., 2002)) during T cell contact in vivo, and thus hinder T cell priming.

4.2.6 HSV-1 induced changes in DC surface phenotype

4.2.6.1 HSV-1 effects on DC differentiation

Potent T cell stimulating capacity is a defining characteristic of DC relative to other APC. Another possible explanation for the dramatic reduction in T cell stimulatory capacity and IL-12 secretion was that HSV-1 infection differentiated DC into cells with more monocyte/macrophage features. This was assessed by analysing the surface expression of CD1a and CD14 following infection. The expression of both these molecules did not change following infection (fig. 4.12). Thus, DC did not de-differentiate following infection.

4.2.6.2 HSV-1 effects on surface phenotype

The other major signal a T cell receives from a DC during contact are co-stimulatory signals which act in conjunction with TCR ligation by the MHC-peptide complex. Therefore, the MHC and co-stimulatory molecule expression of infected DC was assessed (fig. 4.12).

Immature DC were infected and the surface phenotype of these DC analysed after 16 hours of culture. Gating on GFP-ve and GFP+ve DC following infection distinguished between uninfected and infected DC populations respectively. DC were infected at a MOI of 0.3 to ensure the presence of a substantial number of uninfected DC. Fig. 4.12 shows that HSV-1 infection results in significant phenotypic maturation, as demonstrated most obviously by the increased expression of CD86, in both GFP-ve and GFP+ve DC. HLA-DQ and HLA-DR (fig. 6.11) expression on the surface of DC was also consistently higher in infected cultures. MHC class I levels were elevated only on GFP-ve DC. The data suggest that both infected and uninfected DC in these culture were induced to mature by HSV-1 infection.

When infected DC were cultured in the presence of 100 ng/ml of LPS (fig. 4.12), strikingly only GFP-ve DC were able to further upregulate CD86 above the level induced by HSV alone, whereas no increase occurred in GFP+ve DC. This difference was even more apparent for MHC class I expression. Interestingly, HLA-DQ (and HLA-DR) expression was not affected in this way. Virus infection did not affect the response to LPS of both GFP-ve and GFP+ve DC, permitting an increased expression of these molecules.

Therefore, section 4.2.6 demonstrated that HSV-1 infection did not differentiate DC into cells of a known lower T cell stimulatory capacity, such as macrophages. Concurrently, viral infection induced maturation of infected DC, which could have rendered these cells more potent APC. However, the refractory nature of the phenotype change to LPS suggests that infected DC cannot respond fully to maturation stimuli in their microenvironment. This could impair their ability to turn into potent mature APC, and thus explain the poor T cell stimulatory capacity of these cells (figs. 4.1 + 4.2).

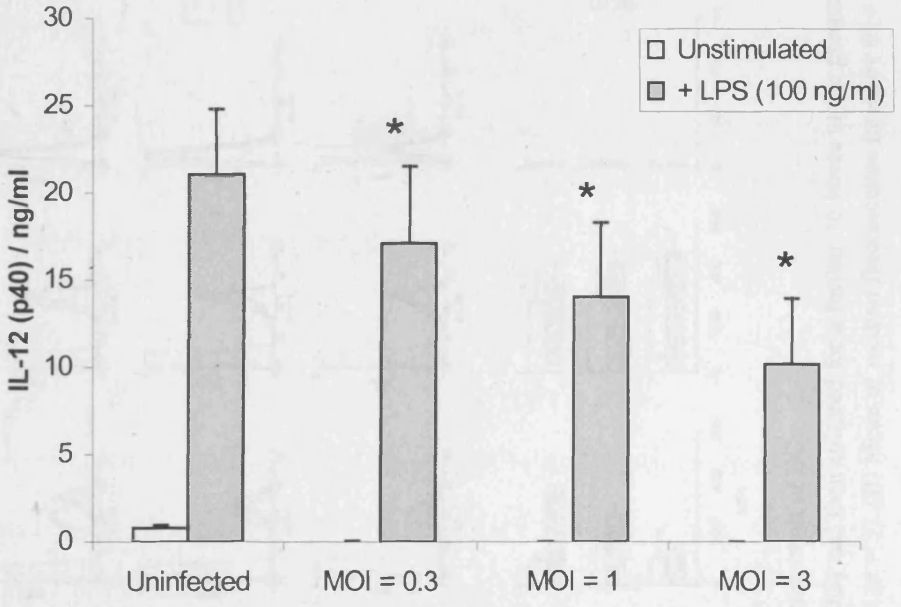

Figure 4.11 IL-12 secretion by DC infected with HSV-1.
DC were infected HSV-1 and cultured for 40 hours in the presence or absence of LPS (100ng/ml). IL-12 (p40) levels in the supernatant were measured by ELISA. * $p<0.03$ is uninfected DC relative to all other groups. Results expressed as mean ± SEM (n = 4). Statistical analysis by Student's *t* test.

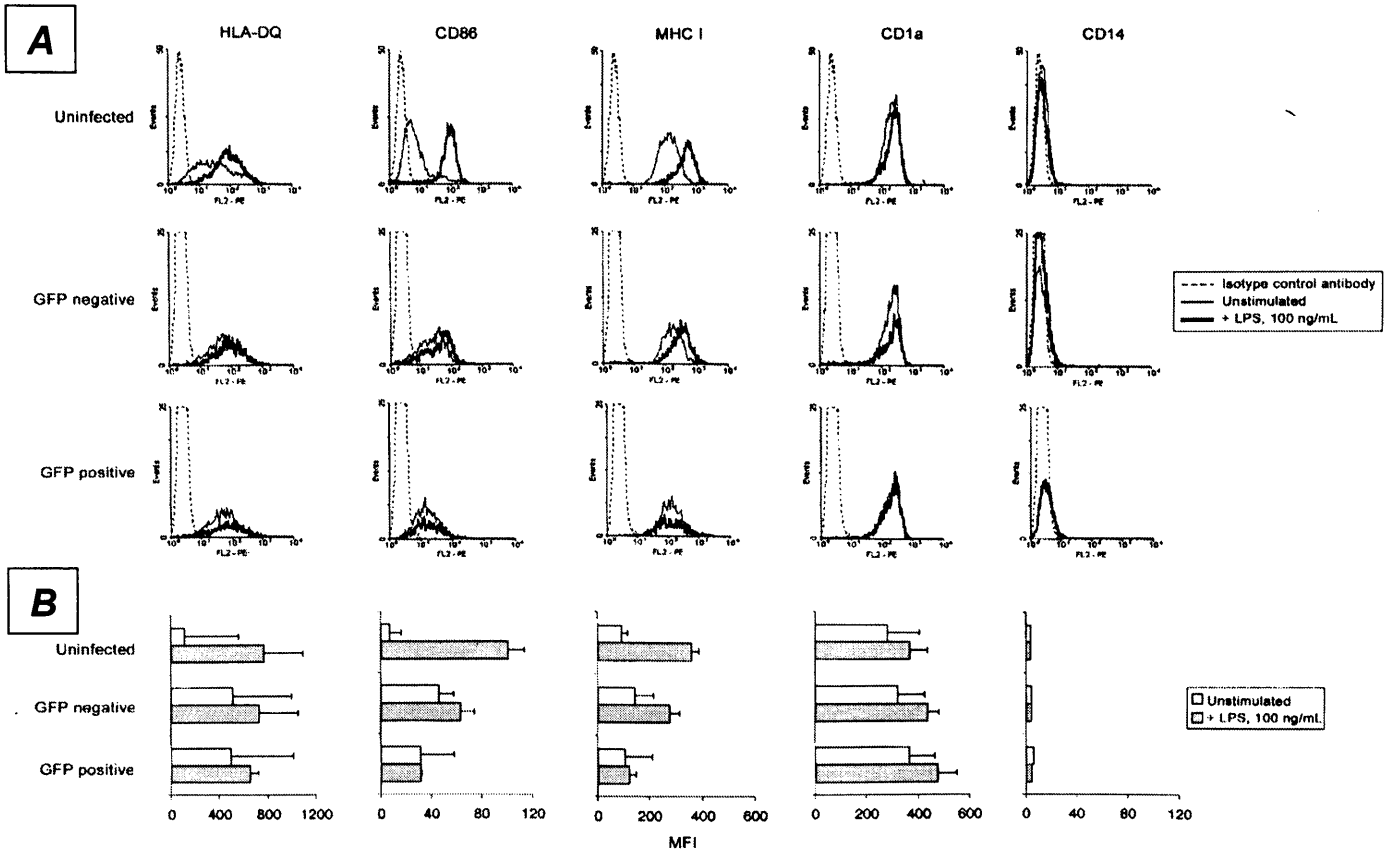

Figure 4.12 Phenotypic changes following HSV-1 infection of DC.
Immature DC were infected with HSV-1 (MOI = 0.3) and then cultured for a further 16 hours in the presence or absence of LPS (100 ng/ml). (A) Representative experiment (n = 3). (B) Mean of median fluorescence intensity (n = 3). Error bars represent SEM.

4.3 Discussion

This chapter has detailed the effects of HSV-1 infection on DC-mediated T cell stimulation, morphology, viability, IL-12 secretion and surface phenotype. The data paint a negative picture with regards to the effects of HSV-1 on DC physiology. However, based on changes in surface phenotype, both infected and uninfected DC also show evidence of activation in response to signals from the infection, and this will form the basis of further study (see chapters 5 and 6). The present discussion will aim to integrate the effects of the virus seen here with the effects of HSV-1 on other cell types, and to evaluate how these changes can explain the functional consequences. Furthermore, the data are placed in the context of the ability of the host to generate anti-HSV responses in vivo, and how they may relate to the control of viral infections in general.

4.3.1 HSV-1 effects on DC-mediated T cell proliferation

The data in figs. 4.1 and 4.2 clearly demonstrate that HSV-1 infection of DC inhibits their ability to stimulate T cell proliferation. Reduced activation of naïve T cells was determined by allogeneic MLR and ConA driven autologous MLR assays. These assays are non-physiological in nature in relation to an in vivo HSV response, and therefore, the ability to stimulate a memory T cell recall response was assessed by measuring proliferation in response to PPD exposure. Although this assay requires conventional antigen capture, processing and presentation, it did not directly assess the ability to elicit HSV-specific responses. Indeed, in a proportion of individuals, HSV-1 infected DC did elicit such memory responses, but these were difficult to assess quantitatively without a positive control.

4.3.1.1 HSV recall responses

The proliferative responses obtained from the coculture of infected DC and autologous T cells were strongly indicative of a memory response to HSV-1. The proportion of individuals responding was consistent with serological data suggesting that approximately 50% of the UK population has been previously exposed to HSV-1.

However, there was the possibility that the virus infection stimulated a non-specific response, perhaps to exogenous antigens such as proteins in the FCS in the medium. Therefore, the serostatus of the volunteers tested was analysed. Owing to administrative difficulties in obtaining a diagnostic laboratory reading, an antiviral assay was used. Although it was very sensitive and the data clear-cut, it is not possible to say whether the antibodies in the serum were specific for HSV-1 or were HSV-2 antibodies that cross-reacted for HSV-1 (Gorander et al., 2003). There is evidence that some T cell responses can be type specific (Eriksson et al., 2004), although the assays used in this thesis could not determine this. Nonetheless, the perfect correlation between the proliferative response and serostatus suggested that these were indeed memory responses to HSV, and that proliferation resulted from response to HSV-1 antigens in the DC-T cell coculture.

The mechanisms involved in initiating these recall responses were not clear, as fig. 4.2 indicated that DC directly infected with HSV-1 had very poor APC function. It was possible that CD4+ T cells controlled viral replication within the DC, as seen for HCMV, thereby permitting direct antigen presentation by infected DC (Le Roy et al., 2002). However, this was an unlikely scenario because the observed proliferation would have correlated directly with MOI of DC, such that the more DC infected, the greater would have been the responses. Furthermore, as the proliferation assays were set up 16 hours after DC infection, the viral effects on DC would have been exerted already. Therefore, it was unlikely that the APC driving T cell proliferation were the infected DC themselves.

In the absence of direct presentation, uninfected APC cross-presenting HSV antigens from dead/dying DC were most likely to be responsible for stimulating the T cells. It was possible that this was being mediated by contaminating APC in the T cell preparation. This was unlikely, not only from the purity of the T cell population (chapter 2), but also because the HSV recall assays were carried out in parallel with the PPD assays in fig. 4.2. In those experiments, T cells alone cultured in the presence of PPD did not proliferate, suggesting that this population of cells did not contain appreciable number of APC (data not shown). The only other population that could cross-present HSV antigens were the uninfected DC. This scenario would be consistent

with the inverse correlation seen between the number of DC infected and the degree of response. The proliferation was greatest at the lowest MOI, that is, in the presence of the greatest number of uninfected DC left in co-culture.

4.3.1.2 Cross-presentation in anti-viral responses

The role of cross-presentation in the stimulation of anti-viral T cell responses has been studied in detail recently. DC can take up exogenous viral proteins or apoptotic cells that have been infected with virus, present viral antigens on MHC class I molecules and stimulate CD8+ T cell responses (Bachmann et al., 1996; Albert et al., 1998b). This has also been shown recently for the herpesviruses HCMV and Epstein Barr Virus (EBV) (Arrode et al., 2000; Tabi et al., 2001; Bickham et al., 2003). Mechanistically, the accumulation of MHC class I processing machinery to phagosomes is believed to provide a suitable environment for the generation of MHC class I peptides for loading and presentation on the surface (Guermonprez et al., 2003; Ackerman et al., 2003). Although recent evidence suggests that other APC also possess this machinery to cross-present exogenous antigens (Ramirez and Sigal, 2002), the potency of DC as APC renders this cell type critical for generating T cell responses in this way (Jung et al., 2002).

From the evidence above, it is hypothesised that uninfected DC in HSV-1 infected cultures could have acquired HSV antigens from infected DC and stimulated the T cell responses seen in fig. 4.1. Although DC can acquire antigen from live cells (Harshyne et al., 2001), a significant source of antigen was also likely to have been apoptotic infected cells, as these cells could be targeted to DC for uptake (Albert et al., 1998a). Recent evidence also suggests that DC apoptosis in vivo enhances the immunogenicity of its antigens, and uptake of virally infected DC in the LN in this way may be important in enhancing cross-priming (Racanelli et al., 2004). This may also serve as a useful mechanism to transfer antigenic material in DC emigrated from the periphery to lymphoid organs (Huang et al., 2000; Belz et al., 2004).

The in vivo relevance of cross-presentation pathway in the generation of anti-HSV immunity has been recently demonstrated in mouse models, whereby stimulation of

CD8+ T cell responses is carried out by uninfected CD8α+ LN-resident DC (Smith et al., 2003b; Allan et al., 2003). The absence of viral DNA in these cells confirmed that infected DC were not responsible for the presentation. However, it is not yet clear whether the CD8α+ DC in LN are tissue resident DC or whether migrating DC can differentiate into these cells (Moron et al., 2002). The implication from the latter scenario is that the presenting DC are uninfected bystander DC at the infection site. In support of this notion, anti-HSV CD4+ T cells are stimulated by uninfected CD11b+ DC derived from the submucosa of infected sites (Zhao et al., 2003). Furthermore, excising the infection site reduces the length of time of HSV-1 antigen presentation and subsequent CTL activation (Stock et al., 2004).

4.3.1.3 HSV-1 mediated inhibition of T cell stimulation

The ability to stimulate T cell proliferation is a property reserved for a small number of cell types, professional APC. Studies carried out prior to and in parallel to the data presented here have shown that immature DC infected with HSV-1 have reduced capacity to stimulate allogeneic T cell proliferation (Salio et al., 1999). This inhibition is mirrored in HSV-1 infected macrophages (Hoves et al., 2001) and B cells (Barcy and Corey, 2001). Interestingly, mature DC infected with HSV-1 also demonstrated impaired ability to elicit T cell stimulation, despite HSV-1 having little effect on the surface phenotype of these cells (Kruse et al., 2000). Furthermore, the reduced ability of infected immature DC to stimulate T cells seems paradoxical in view of the more "mature" phenotype of infected DC (fig. 4.12) (Salio et al., 1999). This indicates that changes in surface phenotype are not the only determinants of infected DC ability to activate T cells. This issue is discussed in section 4.3.5.

In the general context of viral infections, disruption of DC function, and subsequently delaying the generation of a cellular immune response, would be a selective advantage to the virus' survival in the host. Indeed, many viral infections of DC result in an inhibition of the cell's ability to elicit T cell proliferation (see table 1.2). This concept will be discussed in more detail in chapter 7.

One level at which the virus could impair the DC's function is during the interaction with T cells. It is possible that the virus blocks an essential "back-signal" by T cells into the DC. There are many signals the DC receives from T cells, such as CD40L or 4-1BBL. Although the data in this chapter have shown that infected DC are refractory to further phenotype changes, IL-12 secretion and morphological changes in response to LPS, analysis of studies with other infections demonstrate that this conclusion cannot be extended to all ligands. Whereas, responses to LPS and CD40L are equally impaired following HCMV infection of DC (Moutaftsi et al., 2002), MV and parainfluenza virus (PIV) infected DC show impaired responses to CD40L but additive maturation with LPS or P(I:C) (Fugier-Vivier et al., 1997; Schnorr et al., 1997; Bartz et al., 2003). Therefore, the impaired response to LPS stimulation here suggests, but does not confirm, that DC may also be refractory to T cell-derived stimuli.

HSV-1 -infected

Theoretically viruses could also affect the DC ability to simulate T cells by exploiting the plastic differentiation pathway of myeloid cells to differentiate DC into cells with less potent APC potential, such as macrophages or back to their monocyte precursors (Chapuis et al., 1997; Palucka et al., 1998; Akashi et al., 2000). There is evidence that some viruses can affect the differentiation of monocytes into DC (Li et al., 2002; Gredmark and Soderberg-Naucler, 2003). However, the data presented here demonstrate that, at least within a day after HSV-1 infection, DC differentiation is maintained. Time points beyond this would be obscured either by cell death or the DC migration to lymph nodes and interaction with T cells, a process that can itself maintain DC viability (Bjorck et al., 1997).

4.3.1.4 HSV-1 effects on T cell function

As well as affecting the response of DC to the environment, the virus could have prevented T cell proliferation by having a direct inhibitory effect on the T cells themselves, either by inducing secretion of inhibitory cytokines, or through expression on the DC surface of immunosuppressive proteins that can subsequently interact with T cells. Subsequent to the completion of this work, HSV-1 gD has also been reported to have immunosuppressive actions on T cell proliferation, possibly by competing for the endogenous ligand of HVEM on T cells, LIGHT (La et al., 2002). This function

was only tested in the context of immobilised anti-CD3 mediated polyclonal T cell stimulation and it is not clear whether gD could mediate similar effects in a more conventional APC-T cell interaction. MV HA and MV F glycoprotein expression on DC is important for the inhibition of T cell proliferation (Klagge et al., 2000; Dubois et al., 2001). It has been hypothesised that the generalised immunosuppression observed after MV infection in vivo results from the non-specific inhibition of T cell responses by these glycoproteins. In contrast, the immunostimulatory properties of gD on other cell types (e.g. other DC - see chapter 6) may compensate for its immunomodulatory function. Alternatively, the more peripheral and localised nature of HSV infections in vivo restricts significantly its systemic access and functional consequence.

It is also possible that infected DC express endogenous inhibitory proteins. Following HCMV infection of DC, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) expression is upregulated, turning DC cytotoxic and killing neighbouring T cells (Raftery et al., 2001). Recent studies suggest that TRAIL expression is also upregulated on DC after HSV-1 infection, although this was only demonstrated by western blot (Muller et al., 2004). Therefore, an untested hypothesis is that TRAIL expression may also render HSV-1 infected DC cytotoxic and that the inhibition in T cell proliferation observed may have resulted from killing of T cells.

One other possibility was that virus replicating in DC (fig. 3.8) could have infected T cells directly. HSV-1 infection of activated T cells renders these cells susceptible to apoptosis (Raftery et al., 1999), possibly explaining the functional defect observed. In certain viral infections, especially HIV-1, DC-T cell transfer has formed the basis of models to explain the establishment of the infection in vivo. Virus picked up by DC in the periphery (e.g. in the genital mucosa) is "carried" by DC migration to the target CD4+ T cells (Geijtenbeek et al., 2000a). VZV infection and replication in DC permits trans infection of T cells (Abendroth et al., 2001), but this was not observed here with HSV-1. This may be because DC were washed prior to addition of T cells 16 hours after infection, and viral production after this time is minimal (fig. 3.8). Alternatively, T cells in co-culture with infected DC were insufficiently activated to render them susceptible to HSV-1 infection, as activating T cells by mAb cross linking CD3 does increase these cells' permissiveness (La et al., 2002). However, it is also possible that

HSV trans infection of T cells is not a property of this virus, as HSV is a more localised disease and does not require systemic spread of infection.

4.3.2 Morphological changes after infection

The physiological advantages of a dendritic morphology remain an area of debate. Hypothetically, it may be advantageous to both the main functions of DC. The large membrane surface area created by the processes allows a larger sampling volume for sensing danger in the surrounding environment. Equally, it also provides a larger stage for the interaction with T cells in secondary lymphoid organs. Surprisingly, there is little experimental data to support these theories, although recently, Rho GTPase modulation of the actin cytoskeleton in DC has been shown to affect DC migration, and contact and subsequent activation of T cells (Kobayashi et al., 2001; Swetman et al., 2002; Benvenuti et al., 2004a). From the virus' perspective, reducing both antigen sampling and presentation would be beneficial in order to impair the generation of a virus specific cellular response, and hence favour virus survival.

HSV-1 infection resulted in striking changes in the morphology of DC, most noticeably causing the loss of the characteristic membrane processes on non-adherent cells and dendrite formation on FN-adherent cells. These changes occurred in parallel with development of homotypic clusters between infected and uninfected DC and with occasional syncytia formation. Several explanations could account for these changes. In the light of reduced DC viability after infection, an important factor to take into account is cell death. However, it is also important to study the viral-host interplay in the regulation of changes in cell shape. Finally, changes in adhesion molecule expression can also affect cellular morphology on a substratum. These issues will be discussed in turn below.

4.3.2.1 Morphological changes vs. cell death effects

Apoptosis of any cell also incorporates a cell rounding stage and a loss of locomotive capacity on the underlying substratum. Given that HSV-1 infection of DC can ultimately result in apoptosis (figs. 4.9 and 4.10), this is an important issue. However,

morphological changes were apparent within 8 hours of infection, when cell viability was not yet affected. No significant changes in MTT reduction were seen until 16 hours after infection and hypodiploid apoptotic cells were only seen from 12 hours after infection. Furthermore, cell rounding induced by viruses can also occur in a caspase independent manner (Carthy et al., 1998). Therefore, it was likely that the morphological changes observed were secondary to specific viral induced changes rather than cell death alone.

It is worth noting that HSV-1 is a cytopathic virus, and one of the known steps in cytopathic effects is cell rounding. This raises interesting questions regarding the cell specificity of the effects observed here (i.e. whether HSV targets the cytoskeleton of DC specifically). Although it is unlikely that HSV has evolved to target DC morphology, the question could be addressed from another perspective: are DC particularly sensitive to the cytoskeleton modulating effects of HSV-1? Few cells have as elongated and delicate structures as DC, which are also more dynamic than they first appear. Changes in temperature, as well as short-term exposure (3 hours) to the protein synthesis inhibitor, cycloheximide, can result in rapid loss of these processes (data not shown). It is therefore likely that small changes in the cytoskeleton or upstream signalling in cells such as DC, would be observed at earlier times after HSV-1 infection. In support of this theory, HeLa cell morphology infection is remarkably unaffected 16 hours after infection (fig. 3.8). Furthermore, Vero cells infected with HSV-1 show an accumulation of cytoplasmic G-actin within 4 hours of infection, but do not show loss of integrity of actin microfilaments and cell morphology until 16 hours after infection. As there was no increase in the total actin concentration in these cells, the conversion of filamentous (F)-actin to globular (G)-actin in the early stages of infection was insufficient to result in gross morphological and cytoskeletal changes in those cells (Bedows et al., 1983). Therefore, it is possible that DC are more susceptible to equivalent changes in F-actin distribution/integrity, explaining the early changes in morphology seen.

4.3.2.2 Viral induced changes in DC cytoskeleton

The family of small Rho GTPases are important in regulating the turnover of the actin cytoskeleton, and therefore maintaining and inducing changes in cell morphology (Hall, 1998). Recently, their role in the regulation of the DC cytoskeleton has been investigated. DC endocytosis and macropinocytosis are dependent on the activity of Cdc42 and Rac (Garrett et al., 2000; West et al., 2000). These signalling molecules may regulate the formation of membrane ruffles and lamellipodia necessary to carry out these DC functions (Burns et al., 2001; Swetman et al., 2002). Viral interference with these signalling pathways could affect the formation of such structures, and therefore alter the morphology of the cell overall. In addition, cytoskeletal rearrangement is important for chemokine-mediated migration (Yanagawa and Onoe, 2002; Dong et al., 2003), and for antigen processing and presentation (Al Alwan et al., 2001; Al Alwan et al., 2003; Dong et al., 2003; Benvenuti et al., 2004a). Impairment of actin-mediated polarisation of surface molecules into the immunological synapse may also have contributed to the impaired T cell stimulation in these studies (Al Alwan et al., 2001; Al Alwan et al., 2003). Such altered cytoskeletal rearrangement was proposed to explain the equivalent functional inhibition by HSV-infected B lymphoblastoid cells (Barcy and Corey, 2001).

The viral effector molecules that mediate changes in the cytoskeleton are currently unclear. One candidate molecule is the protein product of the US3 gene. US3 from HSV-2 can prevent Rac- and Cdc42-mediated phosphorylation of one of the downstream regulators, PAK (Murata et al., 2000), which is implicated in regulating the actin cytoskeleton and cell morphological changes (Sells et al., 1997; Daniels et al., 1998). Both HSV-1 and HSV-2 US3 are highly homologous to PAK (Murata et al., 2000), and may prevent PAK phosphorylation by competing for interaction with guanine-nucleotide exchange factors. Inhibition of Cdc42- and Rac-regulated PAK activity would be consistent with the morphological changes seen after HSV-1 infection, as microinjection of dominant negative Rac or Cdc42 redistributes F-actin and induces a rapid retraction of dendrites in DC adhered to a FN substratum (Swetman et al., 2002). Recent microarray analysis has revealed that US3 is expressed as early as 2 hours post-infection (Stingley et al., 2000). The redistribution of F-actin 3 hours after infection (Heeg et al., 1986), and the accumulation of G-actin within 4

hours of infection (Bedows et al., 1983) are consistent with an important role for US3 in early changes in the cytoskeleton of HSV-1 infected cells.

In vivo studies carried out with US3 deleted viruses result in severely attenuated footpad and vaginal infection (Nishiyama et al., 1992; Inagaki-Ohara et al., 2001). The reduced virulence did not stem from an impaired ability of the virus to replicate in cells, but rather was secondary to a more potent local inflammatory cell infiltrate, including DC and T cells, with increased secretion of IL-12 and IFNγ. It is possible that US3 deleted viruses were not able to prevent apoptosis in infected epithelial cells (Leopardi et al., 1997) (see section 4.3.3.2), but that would not explain the normal replication kinetics in the infected tissues (Inagaki-Ohara et al., 2001). On the other hand, it is tempting to speculate that failure of the virus to disrupt the DC cytoskeleton allowed infected DC to present viral antigens rapidly. Coupled with the cross-presentation pathway, a quantitative increase in antigen presentation capacity may have elicited a more potent immune response that could clear the peripheral infection more effectively.

4.3.2.3 Integrin mediated change in morphology

Integrins are heterodimeric molecules composed of an α and β subunit. DC express both β1 and β2 integrins (fig. 4.6) (McCarthy et al., 1997; Woodhead et al., 1998; Ammon et al., 2000; Puig-Kroger et al., 2000). These molecules are important for DC migration to and from the blood and lymphatics, via peripheral tissues. Transendothelial migration requires adhesion to surface of endothelial cells by both β1 and β2 integrins (D'amico et al., 1998), whereas in the tissues, DC adhesion to and migration on the ECM occurs via β1, but not β2, integrins (Le Varlet et al., 1992; D'amico et al., 1998).

The only two known β1 associating α chains expressed on MDDC are α4 (CD49d) and α5 (CD49e) (D'amico et al., 1998). These integrin heterodimerise with β1 integrins to form VLA-4 and VLA-5 respectively. Both of these molecules are important in the adhesion of DC to FN, a major component of the ECM (Puig-Kroger et al., 2000). The β2 integrin heterodimers that are important in DC migration across

endothelial barriers are αLβ2 (CD11a/CD18), αMβ2 (CD11b/CD18) and αXβ2 (CD11c/CD18) (D'amico et al., 1998; Fiorini et al., 2002).

Following HSV-1 infection, the expression of the β1 integrin (CD29) was downregulated on infected DC, whereas the β2 integrin (CD18) levels were unaffected. The downregulation of CD29 has also been observed after HSV-1 infection of fibroblasts (Ito et al., 1995), and may therefore constitute a general HSV-1 modulatory mechanism. Interestingly, not all members of the herpesvirus family affect β1 integrin expression in the same way. Whereas VZV also downregulates CD29 expression, HCMV results in upregulation of CD29 on fibroblasts (Ito et al., 1995).

Similarly to CD29, CD49d expression was also downregulated after HSV infection of DC. Unfortunately, no suitable CD49e antibodies were available for this study. As integrins exist in a dimeric form, changes in expression could be mediated by regulation of either subunit, although the antibodies used here only detected single integrin chains, giving no direct assessment of the quantity of functional heterodimer present on the surface. In that respect, it is noteworthy that CD29 expression is unaffected by DC maturation (Puig-Kroger et al., 2000), whereas CD49d is upregulated 48 hr after DC stimulation, in a p38 and NF-kB dependent manner (Aiba et al., 1993; Puig-Kroger et al., 2000). Reduced expression of both molecules suggests regulation by the virus, but the factors involved are unclear. CD49d is predominantly controlled at the transcriptional level in the steady state (Postigo et al., 1997; Puig-Kroger et al., 2000), and its levels could be controlled by changes in transcription rate during infection, as well as post-transcriptionally, by the viral proteins vhs and ICP27 (Kwong and Frenkel, 1987; Hardwicke and Sandri-Goldin, 1994). The observed differential regulation of β1 and β2 integrins is currently difficult to explain. Further study may elucidate differential regulatory mechanisms involved in the expression of these molecules.

As β1 integrins on MDDC are FN receptors, viral downregulation could have played a role in the loss of dendritic morphology in infected DC. It is unclear what degree of β1 integrin downregulation is required to have a significant functional effect on cell morphology. The rapid turnover of the cytoskeleton at the point of adhesion with the

ECM means that even a small reduction in turnover of adhesion molecules may result in the loss of cellular attachment and hence retraction of the dendrite. Furthermore, it should be noted that the data acquired in this chapter relate to whole cell expression of these molecules by flow cytometry. CD29 expression is critical to dendrite formation on FN by focal accumulation at the end of dendritic processes and attachment to the ECM (Swetman et al, submitted for publication). Therefore, the downregulation of expression may be more specific than has hitherto been suggested, and may thus have more dramatic effects on FN-adherent DC morphology.

The only other virus where DC morphology has been documented following infection is MV. The MV-infected cells were also rounded, but there was no data on the effect on the morphology of FN-adherent DC (Grosjean et al., 1997; Fugier-Vivier et al., 1997). Furthermore, as it is currently unknown whether any MV protein can interact with or affect the function of RhoGTPases, it is difficult to extrapolate relevant parallels with this virus to the effects seen here with HSV-1.

Another observed phenomenon that may be related to expression of adhesion molecules was the increased clustering of infected and uninfected DC. The absence of upregulation of β1 or β2 integrins might imply that the increased adhesion between DC was being mediated through the increased expression of alternative intercellular adhesion molecules, such as nectins (Takai and Nakanishi, 2003), but it is also possible that clusters were formed by the increased migration of uninfected DC towards infected ones, without quantitative or qualitative changes in adhesion molecules. This may explain the clusters seen in highly confluent FN-adherent DC, but cannot account for the many non-adherent clusters that were also seen. Furthermore, the functional relevance of such "heterotypic" clustering is unclear, because in this scenario it would be difficult to specify whether it is a virus induced effect or a host response to viral infection. The close proximity of infected and uninfected cells allows the intrinsic fusogenic ability of HSV-1 to form syncytia (Spear et al., 2000).

4.3.2.4 Effects of HSV-1 infection on DC migration

One other aspect of viral modulation of DC morphology is how it impinges on the ability of the cell to migrate. Emigration from the skin and entry into appropriate areas of lymphoid organs require locomotion in response to chemotactic gradients. One previous study raised this issue by determining the Ca2+ flux response to CCL19, the CCR7 ligand important in mediating mature DC migration to lymph nodes. The flux was abrogated in HSV-1 infected cells (Salio et al., 1999). However, the assay was carried out 24 hours after infection, by which time, under normal stimulatory circumstances, the majority of DC would have already migrated to a draining lymph node (de Vries et al., 2003). It would be interesting to study the migration response of DC very early after infection, as upregulation of chemokine receptors, especially CCR7, may occur in parallel to that of CD86 and MHC class II (fig. 4.12). This may be a critical step as CCR7 expression is inhibited following HCMV infection, corresponding to a decreased migration potential of these infected DC (Moutaftsi et al., 2004). However, chemokine receptor expression does not always necessarily correlate with migration potential due to uncoupling from downstream signalling pathways (Sozzani et al., 1997; D'amico et al., 2000; Penna et al., 2001). Furthermore, some viruses have evolved to secrete antagonistic chemokine analogues, which would disrupt chemokine-mediated migration even in the presence of functional chemokine receptors (Seet et al., 2003). Preliminary studies revealed that HSV-1 infected DC could not extend dendrites and migrate on FN like uninfected DC (data not shown) (Swetman et al., 2002)). Therefore, future studies should use chemokine gradients to test the migration potential of virally infected DC (Moutaftsi et al., 2004).

4.3.3 DC viability after HSV-1 infection

Viral induction of DC death represents a very direct strategy to annihilate the capacity to stimulate anti-viral immunity. However, both the two main forms of cell death, necrosis and apoptosis, create a dilemma for viruses.

4.3.3.1 HSV-1 and necrotic cell death

Necrotic cell death is uncontrolled, resulting in dispersal of a large amount of viral antigens in a proinflammatory environment. This can activate neighbouring DC, which alerts the immune system to the presence of infection, and may enhance DC cross-presentation of exogenous viral material to T cells (Sauter et al., 2000). It is interesting that HCMV replicates at low levels in DC, which has the effect of minimising necrotic cell death (Raftery et al., 2001). Similar findings are presented here for HSV-1, and low replication in lymphoid tissues may provide a selective advantage.

4.3.3.2 HSV-1 prevention and induction of apoptosis

Viral induction of apoptotic cell death, although self-contained and immunologically more silent (Sauter et al., 2000), does conflict with the evolutionary requirements of viruses to replicate. Therefore, many have developed sophisticated anti-apoptotic pathways to keep infected cells alive long enough to complete a full replicative cycle (Dobbelstein and Shenk, 1996; Lukac and Alwine, 1999; Aubert and Blaho, 2001). HSV-1 can prevent apoptosis in a number of cells it infects using several mechanisms designed to protect against a variety of death stimuli. These include glycoprotein J (gJ) (Jerome et al., 1999; Zhou et al., 2000), gD (Zhou et al., 2000; Medici et al., 2003), ICP4 (Leopardi and Roizman, 1996), ICP27 (Aubert and Blaho, 1999) and the US3 protein kinase (Leopardi et al., 1997; Hagglund et al., 2002). Loss of gJ or US3 renders cells susceptible to CTL induced apoptosis (Jerome et al., 2001), loss of ICP4 leads to mitochondria dysfunction and DNA fragmentation (Galvan et al., 1999), whereas loss of ICP27 results in the activation of caspase 3 and DNA damage (Aubert et al., 1999).

Anti-apoptotic mechanisms are also needed to protect infected cells from endogenous apoptosis inducing signals. For HSV, the precise identity of these components remains controversial. Recent studies have suggested that the apoptotic stimulus is dependent on IE gene transcription (Koyama and Adachi, 1997; Aubert et al., 1999; Sanfilippo et al., 2004). However, this does not explain apoptosis induced by gD deleted viruses, which cannot infect cells normally or express any viral genes (Zhou et al., 2000). In this scenario, only the endocytosed virion components could be the apoptotic ligands. It is also not yet clear whether these apoptotic stimuli render the cells more susceptible

to environmental apoptosis-inducing stimuli or whether they activate endogenous apoptotic pathways directly. Nevertheless, pro-apoptotic viral components are likely to have played a part in the evolution of some of the anti-apoptotic mechanisms described above.

4.3.3.3 HSV-1 induced apoptosis in DC

Despite the extensive list of anti-apoptotic proteins, it is intriguing that HSV-1 can prevent apoptosis following infection in some (Chou and Roizman, 1992; Leopardi et al., 1997; Jerome et al., 1999), but not other cell types (Galvan and Roizman, 1998). In cells of haematopoietic origin, apoptosis has been observed after HSV-1 infection of both PBMC (Tropea et al., 1995) and activated T cells (Ito et al., 1997), but not macrophages (Hoves et al., 2001). Other studies of HSV-1 infection of immature DC either did not address the issue of viability (Salio et al., 1999) or did not find a gross difference, as assessed by trypan blue exclusion assay (Mikloska et al., 2001; Samady et al., 2003). However this assay is not very sensitive, as it relies on the structural integrity of the host cell membrane. Therefore, DC viability was assessed by a MTT reduction assay, which demonstrated a substantial reduction in viability of HSV-1 infected DC 40 hours post infection. Although the changes in FSC/SSC affected the majority of the cells (fig. 4.7), this only translated into a 50% decrease in MTT reduction. One possible explanation could be that changes in cell size on the flow cytometer overestimate cell death due to increased susceptibility of HSV-1 infected cells to shrinkage after formaldehyde fixation.

The ultrastructural changes seen by EM (cell rounding, nuclear chromatin condensation) and the reduction in cell size detected by flow cytometry, indicate that apoptotic cell death was primarily responsible for the loss of cell viability. In contrast, necrosis would have caused an increase in cell size, followed by cell rupture and loss of structural integrity. This was not seen in the majority of DC infected with HSV-1. An assay commonly used for the quantitative assessment of apoptosis is the detection of phosphatidylserine (PS) leaflets on the extracellular portion of the apoptotic cell by annexin V binding. However, in HSV-1 infected cells, PS expression does not necessarily correlate with cell death, whereas DNA degradation does (Jerome et al.,

1998). Therefore, the loss of DNA integrity was detected by the presence of decreased PI staining by flow cytometry, a sub-G0 population. The detection of this population post-infection confirmed that HSV-1 infection did result in apoptosis of DC. However, entry into apoptosis was extremely slow and asynchronous, with only very slight changes seen one day after infection, and only 30% cells affected after 40 hours, even though transgene expression was detected within four hours of infection. EM studies confirmed that there was viral infection of some apoptotic cells. Unfortunately, the ethanol fixation used in this PI staining protocol did not prevent GFP from washing out of the cells, and therefore a direct 2-colour analysis of the viability of infected cells could not be carried out. However, the correlation between the number of infected DC and the degree of apoptosis strongly suggested that viral infection was the stimulus for apoptosis.

The number of DC in the sub-G0 region was consistently lower than the percentage of cells infected, suggesting that not all infected DC died by apoptosis. Some cell death could be attributed to rare necrotic cell death or to syncytia formation. Alternatively, some infected cells did not die. This may have occurred in a subpopulation of more mature DC in the DC cultures. This was observed occasionally by flow cytometry as a population (up to 10%) of cells expressing high levels of CD86 or MHC molecules, equivalent to those induced by LPS stimulation. This conclusion is consistent with the relative resistance of mature DC to HSV-1 induced cell death (Kruse et al., 2000).

4.3.3.4 Mechanisms responsible for HSV-1 induced apoptosis

The signalling pathways affected by virus infection that can induce apoptosis have not been elucidated. Recent studies have suggested that the level of JNK activation correlates with DC apoptosis following stimulation with H_2O_2 (Handley et al, submitted for publication). Activation of this pathway may also be important in DC apoptosis after HSV-1 infection, as the activation of JNK correlates with the degree of apoptosis in HSV-1 infected neurones in vivo (Perkins et al., 2003). Furthermore, HSV encodes a protein kinase, US3, which attenuates the activation of JNK and prevents apoptosis in infected cells (Murata et al., 2000), underlying the advantage to the virus to prevent cell apoptosis via this pathway.

Other signalling pathways that may be important in inducing apoptosis include the NFAT, ERK and p38 pathways. Firstly, HSV-1 protein mediated inhibition of NFAT nuclear translocation (Scott et al., 2001) could promote apoptosis (Pyrzynska et al., 2001; Mosieniak et al., 1998), although in some cell types it may have the opposite effect (Srivastava et al., 1999). Secondly, HSV-1 expression of the protein kinase ICP10 activates ERK and subsequently prevents apoptosis in infected cells (Perkins et al., 2002). Finally, although no correlation has been found between the levels of p38 MAPK activation and apoptosis induction by HSV-1, p38 MAPK activation has been associated with the induction of apoptosis in some scenarios (Torcia et al., 2001; Tikhomirov and Carpenter, 2004). Indeed, DC pre-treated with the p38 MAPK inhibitor, SB203580, demonstrated an increase in FSC on the flow cytometer compared to infected cells not treated with the inhibitor (data not shown). Although not a formal assessment of cell viability, this preliminary observation is suggestive that the activation of p38 MAPK by HSV-1 is a pro-apoptotic event in DC, in contrast to no effect on H_2O_2 induced apoptosis (Handley et al, submitted for publication). Therefore, the cellular pathways involved in inducing cell death may be stimulus specific.

A possible host effector mechanism for the induction of apoptosis by HSV-1 in DC has recently been described. Early viral gene products rendered DC susceptible to autocrine/paracrine TRAIL and TNFα mediated apoptosis by downregulating the pro-caspase 8 inhibitory protein, c-FLIP, and up-regulating p53 (Muller et al., 2004; Muller et al., 2004). The downregulation of c-FLIP is likely to be an important effector pathway in HSV-1 mediated apoptosis in general. Macrophage viability is not affected by HSV-1 infection and the level of expression of c-FLIP in these cells is also unaffected (Hoves et al., 2001). Conversely HSV-1 infection of human umbilical vein endothelial cells (HUVEC) induces apoptosis at a similar rate as seen in immature DC and a similar reduction in c-FLIP expression was seen (Muller et al., 2004). These observations demonstrate the cell-specific nature of HSV-1 regulation of apoptosis. Furthermore, it suggests that HSV-1 is unlikely to have evolved specific mechanisms to induce apoptosis in immature DC and that DC are not more susceptible to HSV-1 induced apoptosis than other cell types. Of note, c-FLIP expression is elevated in

mature DC (Franchi et al., 2003; Lundqvist et al., 2002; Rescigno et al., 2000). Therefore, it is possible that HSV-1 cannot reduce c-FLIP expression in mature DC to levels that render the cells susceptible to apoptosis, explaining the preservation of viability by mature DC infected with HSV-1 (Kruse et al., 2000).

The discussion above does not explain the delayed kinetics of death or why the viral anti-apoptotic mechanisms do not rescue DC from HSV-1 induced apoptosis. One hypothesis revolves around the low levels of replication observed in DC. As viral replication in DC is lower than in a more permissive cell line, it suggests that viral gene expression would also be lower in DC. Consequently, it may take longer for sufficient quantities of the apoptotic IE gene transcripts to accumulate and trigger apoptosis in DC than in a more permissive cell (Sanfilippo et al., 2004). Concurrently, however, the lower gene expression in DC may also result in insufficient synthesis of viral anti-apoptotic proteins and the cell eventually undergoes apoptosis. Such a scenario demonstrates the delicate balance between pro- and anti-apoptotic signals that is also seen in other viral infections(Cella et al., 1999b). VV has apoptosis inducing components (Ramsey-Ewing and Moss, 1998) and induces apoptosis in DC (Engelmayer et al., 1999). Since the virus only abortively replicates in DC (Engelmayer et al., 1999), insufficient expression of anti-apoptotic proteins may allow apoptosis to ensue. Influenza virus components can also trigger apoptosis (Schultz-Cherry et al., 2001; Chen et al., 2001) and apoptotic cell death does occur after replication and infection in DC (Cella et al., 1999b).

Furthermore, VV, influenza virus and HSV-1 also demonstrate how the relationship between DC viability and T cell stimulation is not a straightforward one (Engelmayer et al., 1999; Cella et al., 1999b). Similar rates of apoptosis are seen for both infections, yet influenza virus infected DC stimulate T cell proliferation more efficiently than uninfected DC, whereas vaccinia virus DC are impaired, similar to the effects of HSV-1 infection. Influenza virus, but not VV, induces DC maturation, which may partly explain the difference in T cell stimulatory capacity, but HSV-1 also induces maturation without enhancing T cell stimulation. Although in vivo many other factors, such as migration to LN, would also need to be taken into consideration, the discriminating factor may relate to the timing of DC apoptosis in relation to T cell

contact. Indeed, MV may have evolved to exploit that interaction specifically, as DC infected by this virus upregulate Fas expression, rendering them susceptible to T cell FasL-mediated apoptosis (Servet-Delprat et al., 2000a), despite the antiapoptotic CD40-CD40L interaction with activated T cells (Bjorck et al., 1997). This "induced" cell death is a very efficient way to target the death of DC at its most critical moment, during the DC-T cell interaction.

The discussion above highlights that cell death cannot be the sole component responsible for the inhibition of T cell stimulation by HSV-1 infected DC. Shorter functional assays of DC-T cell activation (ConA autologous assays – fig. 4.2C), which reduced the contribution of cell death during the assay, also showed impaired proliferative responses. Furthermore, despite little change in DC viability, a pronounced inhibition in T cell proliferation is seen following HSV-1 infection of mature DC (Salio et al., 1999; Kruse et al., 2000). The role of cell death could ultimately be addressed experimentally by artificially preventing apoptosis, perhaps using small pharmacological inhibitors of caspases, which may keep the DC alive throughout the length of the functional assays.

4.3.4 Impact on DC IL-12 secretion by HSV-1

Clearance of most viral infections is dependent on a Th1 skewed CD4+ and CD8+ T cell response. One of the key determinants is the cytokine context at the time of initial interaction between DC and T cells. IL-12 appears to be the initial signal for many Th1 responses (Trinchieri, 2003). In the context of HSV infection in vivo, mouse studies have implicated IL-12 in the control of the infection, acting predominantly upstream of the initiation of IFNγ-mediated protective T cell responses (Malmgaard and Paludan, 2003; Vollstedt et al., 2004). As a result, the protective effects of IL-12 have recently been harnessed therapeutically to design vaccination strategies for HSV in mice models (Sin et al., 1999; Lee et al., 2003a).

The cellular source for IL-12 secretion in vivo is critical to understanding how this cytokine regulates the ensuing immune response. Studies of peripheral lesions in mice have suggested that this cytokine is secreted by inflammatory cells and not by

parenchymal cells of the infected tissue (e.g. epithelial cells) (Kumaraguru and Rouse, 2002; Mikloska et al., 1998). Cells that can secrete IL-12 in vivo include neutrophils and macrophages, and they may play a role in these models (Trinchieri, 2003). However, DC can also secrete large amounts of IL-12 following appropriate stimulation via TLR ligands, as well as members of the TNF family, including CD40L, LIGHT and 4-1BBL on T cells (Schulz et al., 2000; Morel et al., 2003; Futagawa et al., 2002; Wilcox et al., 2002). HSV-1 DNA contains many hypomethylated CpG motifs (Honess et al., 1989) that are sufficient stimuli for IL-12 secretion in DC (Hemmi et al., 2000). However, HSV-1 infection alone was insufficient to initiate IL-12 secretion by DC in this system, consistent with previous findings in human myeloid DC (Ghanekar et al., 1996; Salio et al., 1999).

In vivo, the relative paucity of IL-12 inducing stimuli may be important to ensure that DC secrete this cytokine only under appropriate stimulation. However, this does not explain why viruses (including HSV) that possess known IL-12 inducing ligands (CpG DNA and dsRNA) do not always induce IL-12 secretion after infection (Moutaftsi et al., 2002; Plotnicky-Gilquin et al., 2001; Servet-Delprat et al., 2000b; Libraty et al., 2001). It is possible that a viral infection in vitro provides DC with insufficient quantities of stimulatory ligand, whereas in vivo DC respond to greater concentrations of extracellular ligand in the infection site. Alternatively, the expression of appropriate receptors may be an important variable. Human myeloid DC do not express TLR9 and this may explain the unresponsiveness to HSV DNA containing hypomethylated CpG motifs (Krug et al., 2001). In contrast, human PDC (Krug et al., 2001) and mouse macrophages (Hemmi et al., 2000) do express high levels of TLR9 and secrete IL-12 after HSV-1 infection (Krug et al., 2004; Malmgaard et al., 2000). However, the literature is not consistent with respect to the TLR9 dependence of IL-12 secretion. In the mouse, myeloid DC cultured from bone marrow, unlike their human counterparts, do express TLR9 (Boonstra et al., 2003). However, HSV-1 and HSV-2 infection of these cells does not induce IL-12 secretion (Jones et al., 2003), although there may be upregulation of IL-12 p40 mRNA synthesis (Kanangat et al., 1996). This suggests that hypomethylated CpG DNA may not be sufficient as an IL-12 inducing stimulus from HSV-1. Efficient replication in some cells may generate sufficient dsRNA intermediates to stimulate TLR3 induced IL-12 secretion (Alexopoulou et al., 2001),

whereas the low replication of HSV-1 in MDDC (fig. 3.8) may prevent sufficient quantities of stimulatory dsRNA to accumulate and activate TLR3 expressed in these cells (Krug et al., 2001). HSV-1 may also possess other IL-12 inducing ligands, independent of replication products that are detected by receptors not ubiquitously expressed. Consistent with this, UV-inactivation of HSV prior to infection of mouse macrophages abrogated IL-12 secretion (Malmgaard et al., 2000), whereas it remained intact following PDC infection (Krug et al., 2004). Therefore, the ability to secrete IL-12 may relate to both viral replication within cells, as well as receptor availability for appropriate stimulatory ligands.

Lack of IL-12 induction by viruses may also result from viral inhibitory mechanisms. IL-12 secretion in response to LPS by HSV-1 infected DC was impaired, in agreement with other studies (Salio et al., 1999; Jones et al., 2003). As most IL-12 is secreted 8-12 hours following DC stimulation (Langenkamp et al., 2000), the inhibition is unlikely to result simply from cell death. Similarly to HSV, DC infected with HCMV demonstrate reduced IL-12 secretion when simultaneously activated with an IL-12 inducing stimulus (e.g. LPS or CD40L) (Moutaftsi et al., 2002). The ability to secrete other cytokines (type I IFN) following infection, while IL-12 secretion is suppressed (Chapter 5) (Moutaftsi et al., 2002; Ho et al., 2001), supports a theory of targeted regulation of this cytokine, as does the specific inhibition of IL-12 secretion by post-transcriptional regulation by another herpesvirus, HHV-6 (Smith et al., 2003a).

The complexity of regulation of IL-12 secretion presents several levels where viral intervention may be important, including transcriptional, translational and post-translational (Trinchieri, 2003). The viral mechanisms affecting IL-12 secretion may differ dramatically between virus families. MV mediated inhibition has been attributed to the actions of MV nucleoprotein (MV NP) and the glycoproteins MV HA and MV F. Therefore, MV gene expression is not essential to reproduce the inhibition (Klagge et al., 2000; Marie et al., 2001; Dubois et al., 2001). In contrast, UV-inactivation of some viruses (e.g. HCMV and HSV-1) removes the inhibition of IL-12 secretion ((Moutaftsi et al., 2002), data not shown), reflecting regulation of this cytokine by a viral gene product. In addition, inhibition of IL-12 secretion may be specific to the stimulus. For example, MV infection of DC specifically inhibits CD40L induced IL-12

secretion, while enhancing it in response to LPS stimulation (Schnorr et al., 1997; Fugier-Vivier et al., 1997).

One transcription factor that has recently been implicated in the regulation of IL-12 p40 is nuclear factor of activated T cells (NFAT), which can bind the IL-12 p40 promoter and enhance its transcription (Zhu et al., 2003). In the resting state, NFAT resides in the cytoplasm and in response to an increase in intracellular [Ca2+], it is dephosphorylated by calcineurin, unmasking its nuclear localisation signal and allowing nuclear import. The ability of HSV to specifically prevent the nuclear translocation of NFAT may play a role in the inhibition of IL-12 secretion (Scott et al., 2001).

The reduced IL-12 secretion may have contributed to the impaired ability of HSV-1 infected DC to induce T cell proliferation. However, HSV-1 may also impair the secretion of other cytokines, such as IL-2, that are equally critical in the initiation of T cell proliferation, as seen in murine CMV (MCMV) infection of DC (Granucci et al., 2001; Andrews et al., 2001). It should also be noted that the data in fig. 4.11 only demonstrated inhibition in the secretion of IL-12 p40 by the entire DC population. The absolute dependence of the IL-12 p70 heterodimer, as well as the related cytokines, IL-23 and IL-27, for the p40 chain implies that the secretion of these functional cytokines would also be inhibited (Trinchieri, 2003). However, preliminary studies analysing the secretion of the functional heterodimer reveal that its secretion is only inhibited at much higher MOI than seen for IL-12 p40 (data not shown). This likely reflects a drawback of ELISA measurement of cytokines, as data in chapter 5 reveals that IL-12 p70 secretion by uninfected DC in those cultures could be enhanced greatly by paracrine type I IFN secreted and this may mask the inhibition in virus infected cells. Future studies should analyse the presence of IL-12 p70 intracellularly by flow cytometry, gating on both infected and uninfected cells, in order to definitively conclude the degree of inhibition seen for IL-12 secretion.

4.3.5 Changes in DC surface phenotype

The switch of DC functional state from immature to mature, represents a profound change in physiology. Therefore, not only is it tightly regulated, but it also presents itself as an attractive target for infecting viruses. Inhibiting the maturation of DC would render the antigen presenting cells incapable of initiating a potent antiviral immune response. Indeed antigen presentation to T cells by DC in an immature or semi-mature state, in the absence of sufficient costimulation or cytokine secretion, has been proposed as a mechanism for establishing tolerance or anergy in the T cell pool (Hawiger et al., 2001; Albert et al., 2001), a strategy that would clearly be advantageous for viral survival.

Two striking observations were made in this chapter regarding the change in expression of surface molecules in DC. Firstly, after HSV-1 infection, both infected and uninfected DC displayed more mature phenotypes relative to uninfected DC. This maturation response was indicative that virus infection resulted in activatory signals for both these cell populations. This will be discussed in more detail in chapters 5 and 6.

Secondly, despite the activated phenotype of infected cells, the expression of molecules such as MHC class I and CD86 was not as high as that seen following LPS stimulation alone, and infected DC were refractory to further maturation by LPS. In vivo, DC that acquire a maturation signal in the periphery (of any origin) would migrate to draining lymph nodes and are likely to receive further maturation stimuli from T cells and cytokines they encounter there(Wilcox et al., 2002). In the system used here, the second maturation signal was modelled with LPS, as this is a very well characterised stimulus. However, it is important to bear in mind that each stimulus signals in a receptor-specific manner, and thus changes seen in response to LPS may be specific to TLR4-induced signalling and difficult to extrapolate to other stimuli. Furthermore, the viral mechanisms responsible for the refractory response to LPS are still ill defined. There are several well-characterised immunomodulatory molecules in HSV-1 that could be responsible for the changes observed, but their relative expression and specific function in DC are currently unclear.

4.3.5.1 HSV-1 changes in MHC class I expression

The US12 gene of HSV-1 encodes for the ICP47 protein which interferes with peptide loading of MHC class I molecules by binding to the cytosolic side of the transporter associated with antigen processing (TAP) molecules on the ER. The result is lower expression of MHC class I molecules, including those presenting viral antigens on the surface of infected cells (York et al., 1994). However, there is no discernable change in the expression of MHC class I on the surface of DC after HSV-1 infection. The efficacy of ICP47 is cell-type dependent (York et al., 1994) and it is possible that ICP47 does not exert significant functional effects in DC. As potent APC, these cells may express larger amounts of TAP than in other cell types. Alternatively, maturation after infection may render the cells more resistant to the effects of ICP47. It is also possible that other viral proteins play a role in regulating the expression of MHC class I. Studies have shown that vhs prevents the recognition of infected targets by CD8+ T cells, suggesting an effect on MHC class I expression (Tigges et al., 1996). However, as vhs is a ribonuclease, its actions may be non-specific. The precise role of ICP47, vhs or any other candidate molecules, can only be determined by the use of viruses lacking functional genes and/or proteins.

4.3.5.2 HSV-1 changes in CD86 expression

The expression of CD86 in DC after infection follows a similar pattern to that of MHC class I, particularly the inhibition to further upregulation by LPS. Although the two proteins are transcribed from separate genes, the transcriptional control governing this process may be very similar, as both p38 MAPK and NF-kB are important (Yoshimura et al., 2001; Ardeshna et al., 2000). As well as affecting DC morphology, changes in Rac activation by HSV-1 US3 may also play a role in regulating expression of both MHC class I and CD86 (Murata et al., 2000; Jaksits et al., 2004). Therefore, changes in cell signalling induced by the virus may affect the expression of both these molecules, and explain the similar refractory response to LPS. Alternatively, virus infection induced the secretion of cytokines that inhibit the maturation of DC, such as TGFβ (Geissmann et al., 1999), as seen after HCMV infection of fibroblasts (Arrode et al., 2002).

It is also possible that the virus expresses proteins that modulate the expression of CD86 (and possibly also other members of the B7 family) in immature DC specifically, in a manner analogous to the relationship between ICP47 and MHC class I. This is an unexplored hypothesis, but it is strengthened by recent studies of HSV-1 infection of mature DC. These cells are more resistant to HSV-1 induced changes than immature DC. The expression of MHC and co-stimulatory is unaffected after infection, whereas CD83 is selectively downregulated (Salio et al., 1999; Kruse et al., 2000), suggesting that HSV-1 may have evolved mechanisms to downregulate the expression of this molecule. It is interesting that HCMV and VV also specifically downregulate CD83 expression on mature DC (Hertel et al., 2003; Jenne et al., 2000). The mechanisms that regulate CD83 expression are unclear and it is not possible to exclude that CD83 may be targeted 'inadvertently' by viral mechanisms that modulate the surface expression of other non-APC specific molecules. Indeed, some viral proteins can modulate the expression of structurally unrelated host molecules (Tomazin et al., 1999).

4.3.5.3 HSV-1 changes in MHC class II expression

MHC class II molecule expression was affected differently to that of CD86 and MHC class I. It was elevated by infection, and in most experiments, this expression was increased further by LPS stimulation. There was no evidence of specific downregulation or refractoriness in the expression of MHC class II in HSV-1 infected DC. This observation may be best explained by the cellular regulation of MHC class II expression in DC, which differs from MHC class I and CD86. MHC class II molecules are 'stored' in endosomes in immature DC and, upon maturation, associate with the relevant peptides, and are shuttled to the surface (Inaba et al., 2000). As this process occurs simultaneously with transcriptional downregulation of MHC class II (Landmann et al., 2001), the regulation of these molecules could escape both transcriptional and post-transcriptional regulation by HSV-1 proteins (e.g. by vhs or ICP27). However, recent data has suggested that HSV-1 can disrupt MHC class II loading, by exporting HLA-DR molecules from the ER, thereby preventing their surface expression (Neumann et al., 2003). As HLA-DR expression increases to maximal levels in response to LPS within 5 hours of stimulation (data not shown), gB

may not affect the expression of this molecule on the surface of DC rapidly enough. This does not exclude the possibility that gB may affect the antigenic repertoire expressed by the DC after this time point. This protein may have played a role specifically in the reduced stimulation of PPD-specific memory CD4+ T cells (fig. 4.2A) (Pathan et al., 2001; Li et al., 1998). Similarly, protein antigen presentation is impaired in DC infected with HHV-6, whereas presentation of pre-processed peptides was intact, confirming that viruses may inhibit DC antigen presentation at the processing level (Kakimoto et al., 2002), possibly also mediated by disrupting the cell's cytoskeleton (Dong et al., 2003).

However, it is also a distinct possibility that the virus cannot synthesise sufficient quantities of gB to affect DC expression of MHC class II-peptide complexes, which are more stably expressed on the surface than on other cells (Cella et al., 1997). Therefore, although targeting of MHC class II pathway suggests a viral adaptation to target the function of APC specifically, it is also possible that the main evolutionary drive for this molecule is the inducible, but low, expression of MHC class II on infected epithelial cells (Albanesi et al., 1998), protecting these cells from CD4+ T cell recognition.

4.3.5.4 Responses of the uninfected GFP-ve population

Although GFP-ve DC upregulated both MHC class I and CD86 after LPS stimulation, it was noticeable that the expression of these molecules did not reach those of uninfected DC treated with LPS alone. Several reasons could explain this. Infected DC could express inhibitory molecules that prevent a complete response to maturation stimuli by neighbouring DC. Other viruses do indeed possess candidate inhibitory molecules (e.g. MV NP (Marie et al., 2001)). The only known HSV candidate molecule is gD, as this glycoprotein can also suppress T cell proliferation (La et al., 2002) and is expressed on the surface of DC (chapter 3). However, data in chapter 6 demonstrate that gD has stimulatory, not inhibitory activity on DC function. Other candidate molecules that can be excluded are other viral envelope glycoproteins, as their interaction with the DC surface do not prevent upregulation of CD86 to LPS stimulation (chapter 6). Nevertheless, despite these provisos, the behaviour of the

GFP-ve DC population was clearly different from that of infected cells and appeared, at least in part, free from some of the viral regulatory mechanisms seen in GFP+ve cells.

4.3.5.5 Relationship between phenotype changes and T cell stimulation

The changes in phenotype need to be analysed in relation to the functional inhibition seen in fig. 4.2. Alterations in the expression of MHC and co-stimulatory molecules would be predicted to have an impact on the ability to stimulate T cells. Preventing MHC molecule expression would hamper the activation of T cells both quantitatively and kinetically. Furthermore, the absence of co-stimulatory molecules may also induce tolerance or regulatory T cells (Jonuleit et al., 2000; Hawiger et al., 2001).

Studies of other viral infections of DC demonstrate that the relationship between surface phenotype, T cell responses and infection resolution is not so straightforward. Infections that prevent DC responses to maturation stimuli, such as LPS or CD40L, do indeed result in impaired stimulatory function (Salio et al., 1999; Moutaftsi et al., 2002; Engelmayer et al., 1999). It is interesting that it is only the large DNA viruses of the herpesvirus and poxvirus families that possess this function, suggesting that this is a highly evolved and complex viral strategy. However, it is equally noteworthy that DC maturation following infection per se does not generate more potent APC ubiquitously (Schnorr et al., 1997; Plotnicky-Gilquin et al., 2001). DC maturation following infection may contribute both to the disease pathogenesis (Raftery et al., 2002; Ho et al., 2001; Libraty et al., 2001), and also risk generating potent DC that present viral antigens. The spectre of fatal immunopathology to the host and/or rapid clearance of the virus may have selected for the evolution of viral mechanisms to curb the function of DC (Raftery et al., 2001; Moutaftsi et al., 2002; Marie et al., 2001).

4.4 Conclusions

HSV-1 infection of DC renders these cells poor stimulators of naïve and memory T cell proliferation. This chapter has attempted to elucidate some of the DC functions that are disrupted by the virus that may be responsible for the loss in antigen

presentation capacity seen. HSV-1 infection results in a rapid loss in dendritic morphology, and this loss in cytoskeletal plasticity may hamper their migration as well as their interaction with T cells. DC also undergo delayed apoptosis after infection, although its contribution to the functional impairment is difficult to discern. Noticeable, however, was the inability of infected DC to respond to further maturation stimuli after infection. This was demonstrated in the reduced ability to secrete IL-12, and the absence of this cytokine may be responsible for the inhibition in T cell proliferation. The inability to upregulate MHC class I and costimulatory molecules in response to further maturation stimuli may result in functional impairment of DC in vivo. This may include decreased or delayed migration to secondary lymphoid organs, as well as defective DC-T cell interaction.

The data presented in this chapter are consistent with the hypothesis that HSV-1 infection of DC impairs the ability of these cells to directly present and activate HSV-specific CD4 and CD8 T cells. This would subsequently delay the generation of the antiviral cellular and humoral response, perhaps long enough to allow the virus to establish itself in the host. The full implication of how this fits into a model of a peripheral herpetic lesion will be discussed in more detail in the final chapter of this thesis. However, this conclusion leaves unanswered two important questions: how are effective anti-HSV immune responses, present in all seropositive individuals, generated in vivo if DC are functionally impaired? Secondly, how can the knowledge gained from the viral-DC interaction be exploited therapeutically? The changes in surface phenotype after HSV infection may suggest answers for both these questions.

The maturation of uninfected DC in the culture suggests one potential mechanism to overcome the functional block on DC function after infection. The factors responsible for this bystander maturation will be the subject of investigation in the next chapter. On the other hand, the direct maturation in response to viral infection, albeit sub-maximal, implies a host response to the virus. The ability of critical cells of the innate immune system, such as DC, to recognise HSV-1 infection warrants further investigation. Identifying the immunostimulatory components of the virus may play an important role in vaccine design and will be the subject of the subsequent chapter.

Chapter 5

Type I IFN: autocrine and paracrine priming factor

5.1 Introduction

The previous chapter detailed the functional consequences of HSV-1 infection of DC. Through inhibition of a variety of aspects of DC physiology, the virus prevented the ability of DC to stimulate T cell proliferation. However, HSV specific T cell memory responses could be initiated from individuals who had previously been exposed to the virus. The possible resolution of this paradox is that, in vivo, the onus for antigen presentation lies with bystander uninfected DC in the periphery of the infection. It is critical that these DC present HSV antigens to T cells with sufficient potency to stimulate effector cells, rather than induce tolerance or anergy in the T cell pool towards HSV antigens. One key determinant of this outcome is the maturation state of DC, such that presentation of antigen by immature DC can result in tolerance (Jonuleit et al., 2000; Hawiger et al., 2001). Important determinants of the infection outcome are the soluble factors secreted by infected DC, and how bystander DC respond to these. The rest of this chapter will attempt to characterise and detail these further.

The prime candidate mechanism is that of an immunostimulatory factor secreted after infection of DC, which can affect neighbouring uninfected DC. One major piece of evidence in support of this hypothesis was that uninfected GFP-ve DC in infected cultures displayed a more mature phenotype than the original immature population that was infected. It was possible that these activation signals originated from direct cell contact with infected DC. However, the activated phenotype was observed irrespective of the confluence in the tissue culture plate, suggesting that a soluble factor(s) secreted after infection was responsible for "bystander activation" of uninfected DC. This is an attractive model as, if extrapolated in vivo, it would allow DC neighbouring the infection site to respond to danger signals without migrating into direct contact with infected cells and facing the risk of infection. Therefore, the objectives of this chapter will be to characterise the soluble factor(s) secreted after infection that is responsible for bystander DC activation in vitro, and determining how it may play a role in vivo in overcoming the block in DC function by HSV-1 infection. Other activities of this soluble factor on DC responses to TLR ligands will also be investigated and discussed.

5.2 Results

5.2.1 Bystander DC activation by HSV-1 infection

5.2.1.1 Phenotype changes

The changes in phenotype observed in the previous chapter (fig. 4.12) demonstrated that uninfected GFP-ve DC demonstrated elevated levels of CD86 and MHC class II relative to DC from cultures not exposed to virus. The possibility that this population represented infected DC not expressing GFP was excluded in chapter 3 (fig. 3.7). The addition of LPS to these cultures elevated the expression of CD86 and MHC class I in GFP-ve DC but not GFP+ve DC, further delineating the difference between the two populations (fig. 4.12).

To investigate the possibility that a soluble factor secreted after infection was responsible for the maturation of GFP-ve DC, supernatants were harvested from infected DC cultures and used to culture autologous uninfected DC (fig. 5.1). Although the production of HSV-1 in infected DC was low, it was important to remove any virus from these supernatants. This was carried out by ultracentrifuging the supernatant, and then filtering it through a 0.2μm filter. As a control, the supernatant from uninfected DC was treated in the same manner and was defined as supCON-DC. Supernatant from infected DC was defined as supHSV-DC. To confirm that supHSV-DC was devoid of infectious virus, it was titred on BHK cells in a viral infectivity assay. No infectious virus was detected (data not shown). Furthermore, culture of autologous DC with supHSV-DC yielded no GFP+ve DC (fig. 5.1A).

The most noticeable observation was that DC cultured in supHSV-DC upregulated both CD86 and HLA-DR (fig. 5.1B). Consistent with the upregulation of these molecules seen in GFP-ve DC, this was not as elevated as the effects seen with LPS (fig. 5.1B). Furthermore, this upregulation was also not refractory to further LPS stimulation (fig. 5.1B), excluding the presence of viral inhibitory molecules that rendered DC refractory to further stimulation as seen for infected DC (fig. 4.12). As a control, culturing DC with supCON-DC did not upregulate CD86 or HLA-DR

expression, excluding the possibility that the maturation signal was ubiquitous in all supernatants from one-day old DC cultures.

Culturing DC in supHSV-DC had no effect on cell viability (data not shown). Therefore, the reduced viability observed in HSV-1 infected DC cultures (fig. 4.8) was likely to result from the direct effects of viral infection, rather than the release of soluble cytotoxic mediators.

5.2.1.2 Effects on IL-12 secretion

The secretion of IL-12 from DC cultured in supHSV-DC was measured next. Although supHSV-DC was not per se a stimulus for IL-12 secretion, it synergised with LPS and induced elevated levels of IL-12 p40 and p70 secretion relative to that seen with LPS stimulation of DC cultured in supCON-DC (fig. 5.2). Therefore, the soluble factors that mediated the changes in phenotype above also primed DC for elevated IL-12 secretion.

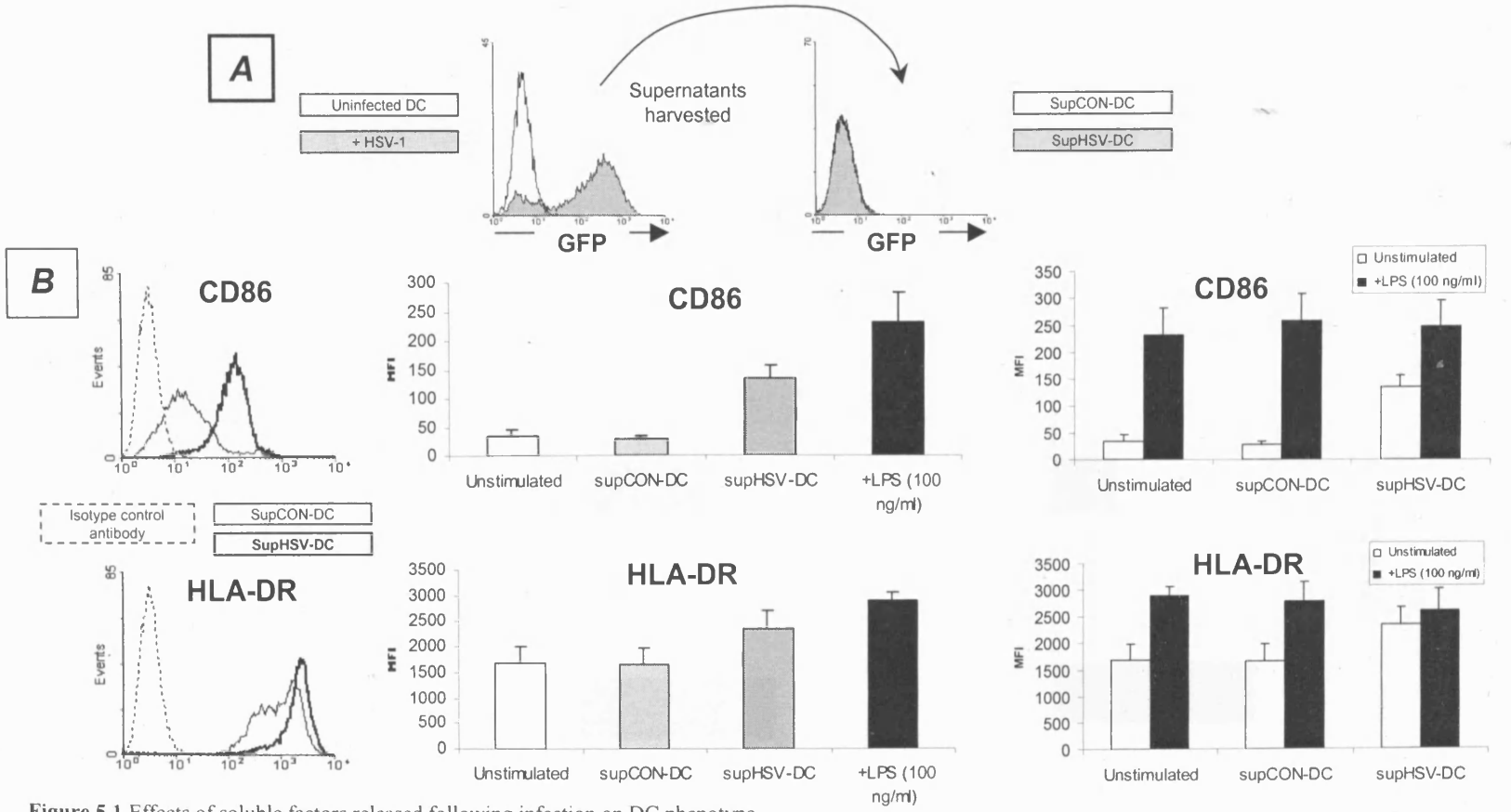

Figure 5.1 Effects of soluble factors released following infection on DC phenotype.

DC were infected with HSV-1 (MOI = 1) and supernatants were collected after 16 hours. The supernatants from both uninfected and HSV-1 infected DC were then treated as described in materials and methods (supCON-DC and supHSV-DC respectively), and added to fresh autologous DC. Flow cytometry detected the expression of GFP (A), and CD86 and HLA-DR (B). *(Left panel)* Representative experiment. *(Middle panel)* Mean of three independent experiments. *(Right panel)* LPS (100 ng/ml) was added to DC cultured in supCON-DC or supHSV-DC and the expression of CD86 and HLA-DR assessed. Mean of three independent experiments.

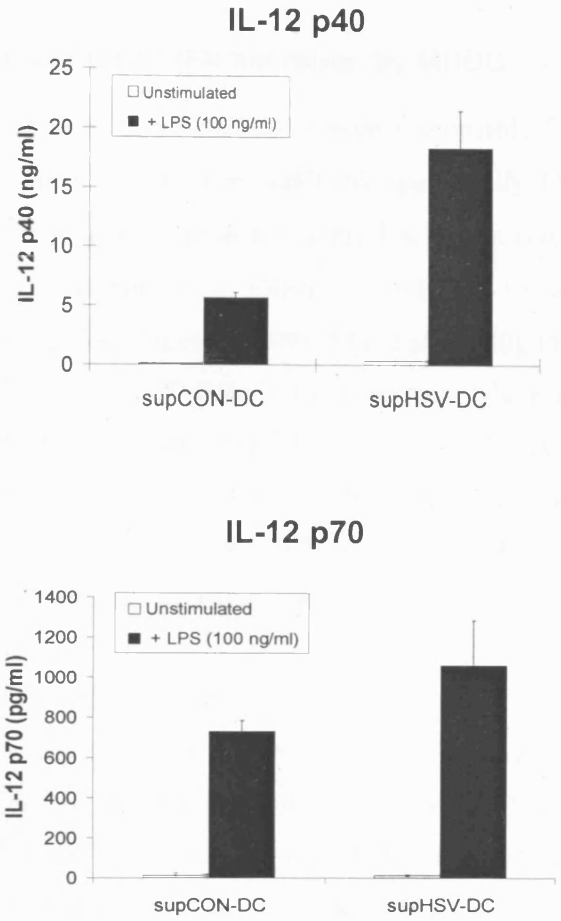

Figure 5.2 Effects of soluble factors released following HSV-1 infection on DC IL-12 secretion.

DC were infected with HSV-1 (MOI = 1) and supernatants were collected after 16 hours. The supernatants from both groups were then treated as described in materials and methods (supCON-DC and supHSV-DC) and added in the presence or absence of 100ng/ml LPS to fresh autologous DC. Secretion of (A) IL-12 p40 or (B) IL-12 p70 was measured by ELISA. Results expressed as mean ± SEM (n = 3). * denotes $p<0.01$ relative to supCON-DC.

5.2.2 Characterisation of the soluble factor

5.2.2.1 Confirmation of type I IFN secretion by MDDC

Although a secreted viral protein could have been responsible for the above effects, cytokines secreted by infected DC were analysed, specifically TNFα and type I IFN (fig. 5.3A). TNFα was secreted, but at relatively low levels compared to those seen after LPS stimulation. The ability of TNFα to inhibit IL-12 secretion made it an unlikely candidate (Hodge-Dufour et al., 1998; Ma et al., 2000). On the other hand, the amount of type I IFN secreted (270 IU/ml) was consistent with previous studies of the effects of recombinant IFNα on upregulation co-stimulatory molecules on DC (Padovan et al., 2002) and on priming CD40-induced IL-12 p70 secretion (Luft et al., 2002b). Therefore, the presence of type I IFN in supHSV-DC was a likely candidate for the effects observed, and this hypothesis was tested further.

The source of type I IFN from DC cultures has been the subject of recent investigations, particularly since the identification of "natural interferon producing cells", or PDC, that can secrete large quantities of type I IFN when stimulated with many viruses, including HSV-1 (Siegal et al., 1999). Therefore, despite the purity of the myeloid DC population used in this study (fig. 3.3), it was important to demonstrate that the type I IFN released following HSV-1 infection did not derive from small numbers of contaminating PDC. This was confirmed by intracellular staining for IFNα (fig. 5.3B+C). The increase in intracellular IFN was mediated by a shift in fluorescence of the entire DC population, not by a subset of IFN-secreting DC (fig. 5.3B+C). As the type I IFN secretion system responds to a well described autocrine and paracrine positive feedback loop (Taniguchi and Takaoka, 2002), DC in these experiments were infected at a high MOI of 3. The resulting infection efficiency was near 90%, reducing to a minimum the number of uninfected DC, and ensuring that the stimulus for IFN secretion came directly from the infection process. Therefore, type I IFN detected in the supernatant of HSV-1 infected MDDC was derived directly from these cells.

5.2.2.2 Confirmation of antiviral activity of type I IFN

It has been known for many years that type I IFN can prevent HSV-1 replication by preventing the transcription of IE genes (Mittnacht et al., 1988; Oberman and Panet, 1988). In the context of a herpetic lesion, the release and paracrine activity of this family of cytokines would not only have immunostimulatory activity on bystander DC, but may also protect them from viral infection. Therefore, DC were cultured in the presence of 1000 IU/ml of IFNα for 24 hours prior to HSV-1 infection and then for a further 16 hours, also in the presence of IFNα. Consistent with antiviral activity, IFNα pretreatment prevented GFP expression (fig. 5.4A). Although this gene is controlled by the CMV IE promoter, IFNα has similar inhibitory effects on the transcription of this heterologous promoter as on HSV-1 IE genes (Nicholl and Preston, 1996). Therefore, although not directly assessed, the antiviral effect of type I IFN on GFP expression could confidently be extrapolated to HSV-1 genes. IFNα pretreated DC infected with HSV-1 also displayed a more activated phenotype than either untreated DC or IFN pretreated DC alone (fig. 5.4B), suggesting that infection induced activation of DC additional to that induced by IFNα alone (fig. 5.5A). Direct viral activation of DC, independent of type I IFN, is discussed in greater detail in chapter 6.

5.2.2.3 Effect of type I IFN on MDDC phenotype

To demonstrate that type I IFN in supHSV-DC could account for DC maturation, DC were cultured in recombinant IFNα, and the changes in surface phenotype assessed. IFNα induced the upregulation of CD86 and HLA-DR in a dose-dependent manner (Fig. 5.5A). Similarly to supHSV-DC, the upregulation of these molecules was less than the levels induced by LPS stimulation. When the two stimuli were added together, the upregulation of CD86 and HLA-DR was no higher than LPS alone (fig. 5.5B), analogous to the effects of supHSV-DC (fig. 5.1B).

One way to confirm that type I IFNs were indeed the active factors was to neutralise their activity in the supernatants. Type I IFNs are a family of cytokines, comprising many subtypes, but which all bind one receptor. Although neutralising antibodies are available that can bind many subtypes, a more straightforward approach was to use a

blocking antibody against the receptor, which is comprised of two chains: IFNAR1 and IFNAR2. A neutralising monoclonal antibody that bound IFNAR2 was used. Initial experiments demonstrated that, consistent with previous findings (Padovan et al., 2002; Gauzzi et al., 2002), DC express this receptor, albeit at low levels (fig. 5.6A). To test its neutralizing ability, DC preincubated with the antibody, were cultured in recombinant 1000IU/ml IFNα, and upregulation of CD86 was assessed. This cytokine concentration was chosen to confirm that the antibody would neutralise amounts of type I IFN in excess of the concentration found in infected cultures (fig. 5.3A). The ability of IFNα to upregulate CD86 was totally abrogated by this antibody, confirming its ability to neutralise the biological activity of type I IFN in this system (figs. 5.6B (right panel), 5.9B+C and 5.10). Finally, addition of the antibody almost completely abrogated the ability of supHSV-DC to upregulate CD86, confirming that this cytokine was predominantly responsible for the activity in supHSV-DC (fig. 5.6B).

5.2.2.4 Confirmation of type I IFN effect on MDDC secretion of IL-12

Unfortunately, limitations in DC numbers precluded experiments to neutralise type I IFN activity and assessing its role in the IL-12 priming effect. However, in order to confirm that type I IFNs were able to enhance IL-12 secretion, DC were cultured in the presence of recombinant IFNα, as above, and simultaneously stimulated with LPS. Consistent with the effects of supHSV-DC, IFNα itself was not a stimulus for DC secretion of IL-12 p40 but could prime DC for increased LPS-mediated IL-12 p40 secretion (fig. 5.7A). This synergistic action was also observed for IL-12 p70 secretion (fig. 5.7B). Of note, the synergistic effect of IFNα on IL-12 p70 secretion was greater than that on IL-12 p40 secretion, inducing a 81% increase in IL-12 p70 over that of LPS alone, in comparison to a 61% increase for IL-12 p40. This was reflected in the increase in the IL-12 p70:p40 ratio, increasing the proportion of functional heterodimer secreted (fig. 5.7C).

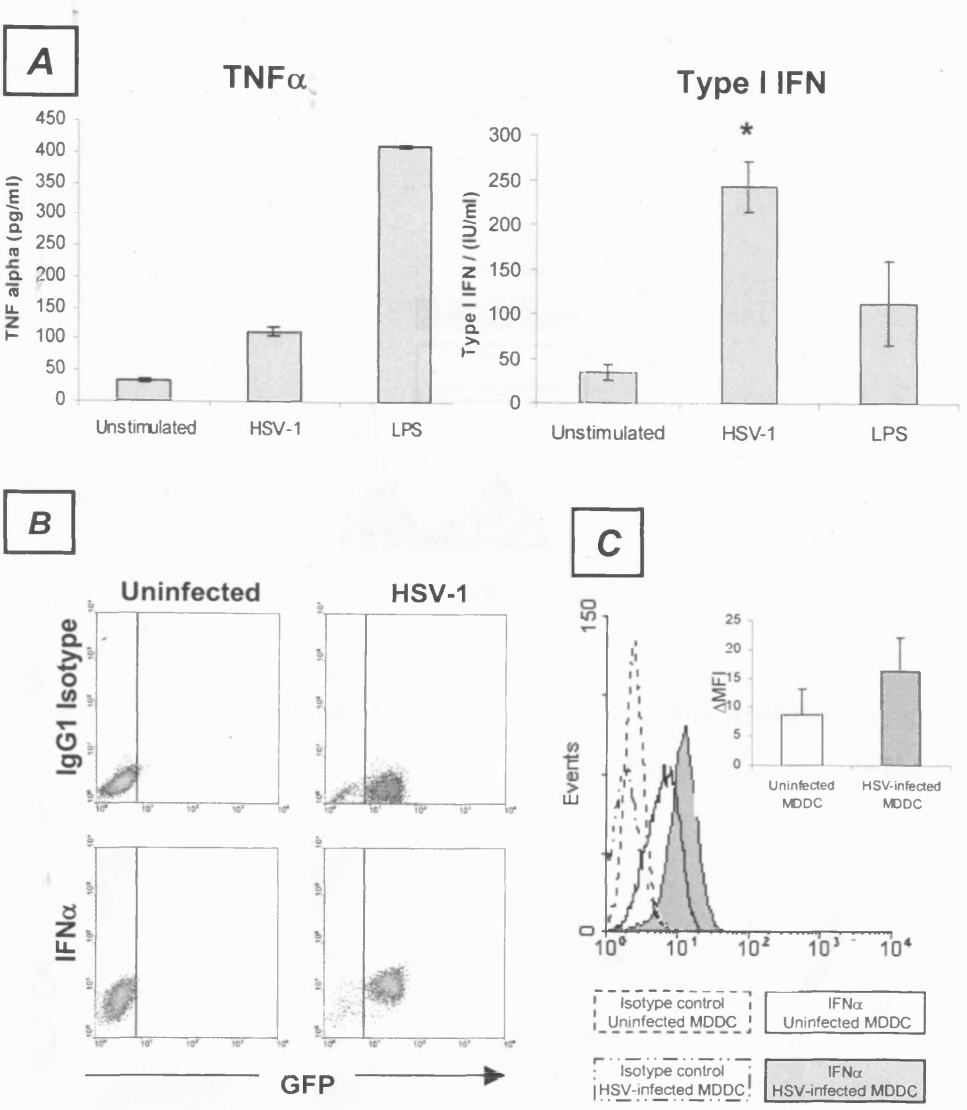

Figure 5.3 Type I IFN secreted by MDDC infected with HSV-1.
(A) Supernatants from uninfected and HSV-1 infected DC, and DC stimulated with 100 ng/ml LPS were harvested 16 hours after infection and the secretion of TNFα and type I IFN secretion determined by ELISA and an antiviral bioassay respectively. The data are the mean of two and four independent experiments respectively. Error bars represent SEM. * denotes $p<0.01$ relative to uninfected DC. (B) MDDC were infected with HSV-1 at MOI of 3 and incubated for 12 hours in the presence of 2 μM monensin. Production of IFNα was assessed by intracellular staining. Representative of three independent experiments. (C) As in (B) but displayed as frequency histogram. Representative of three independent experiments. *Insert:* bar chart represents mean ΔMFI (MFI IFNα - MFI isotype control) staining of three independent experiments. Error bars represent SEM.

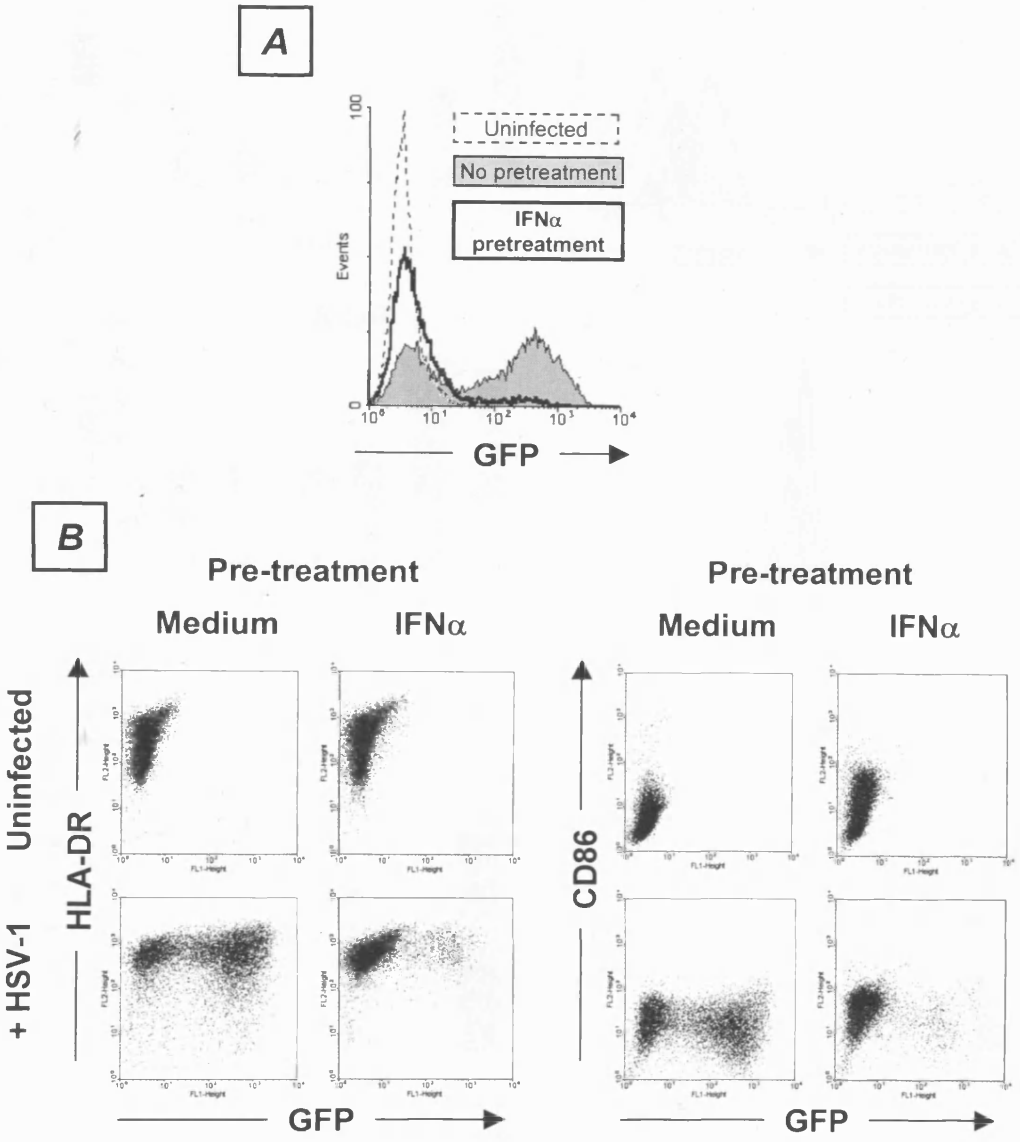

Figure 5.4 Antiviral activity of IFNα on HSV-1 infection.
DC were precultured in the presence or absence of 1000 IU/ml IFNα for 24 hours prior to infection with HSV-1. After 16 hours of culture in same conditions, the expression of (A) GFP, and (B) HLA DR and CD86 was assessed. Representative of two independent experiments.

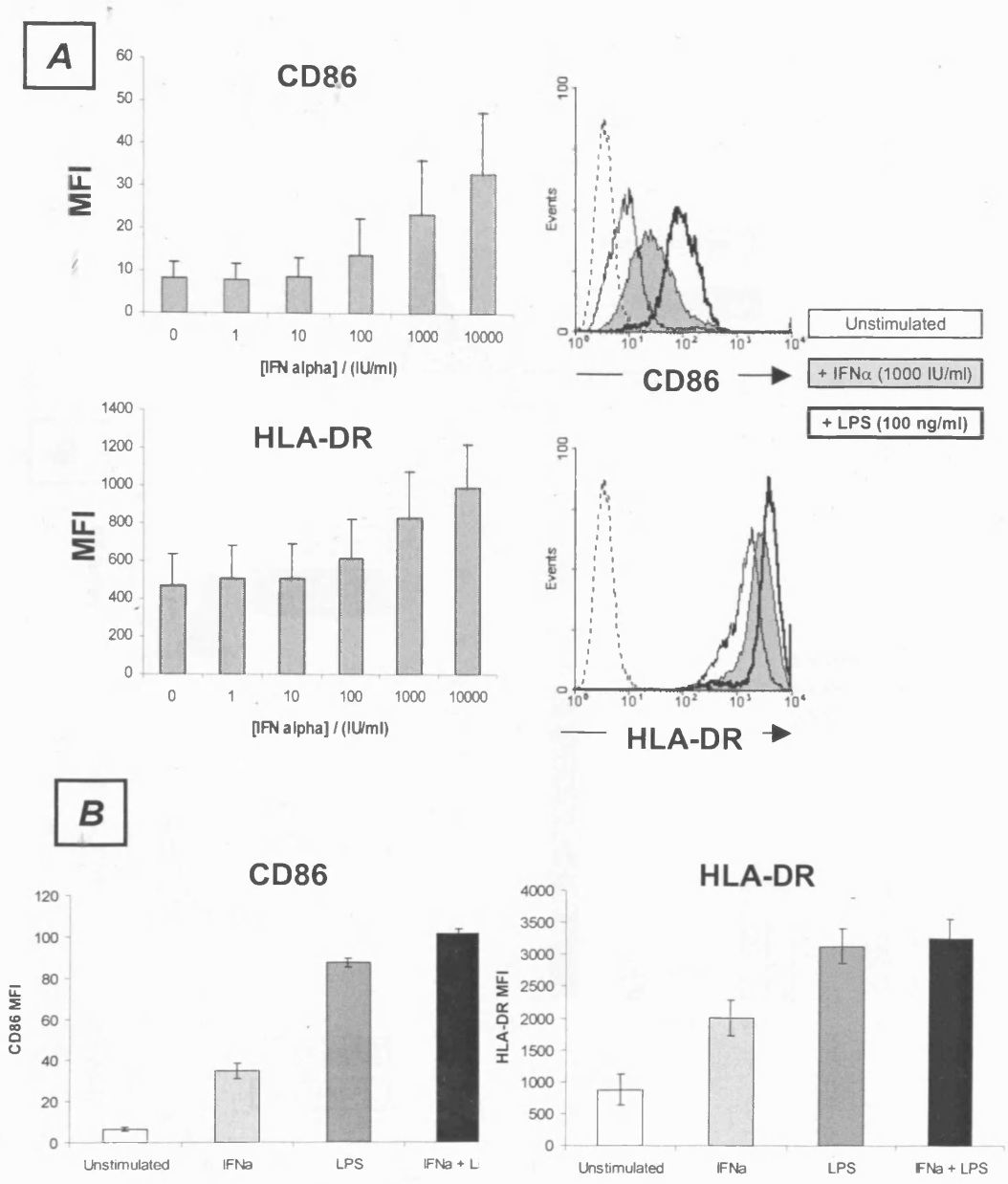

Figure 5.5 Effect of IFNα on DC phenotype.
(A) *(Left panel)* MDDC were cultured for 16 hours in a range of concentrations of IFNα and changes in surface phenotype assessed. Data are mean MFI of three independent experiments. Error bars represent SEM. *(Right panel)* MDDC were cultured for 16 hours in 1000 IU/ml IFNα or 100 ng/ml LPS and changes in surface phenotype were assessed. Representative of three independent experiments. (B) As for (A) but in some groups DC were also co-cultured with both 1000 IU/ml IFNα and 100 ng/ml LPS. Mean of three independent experiments. Error bars represent SEM.

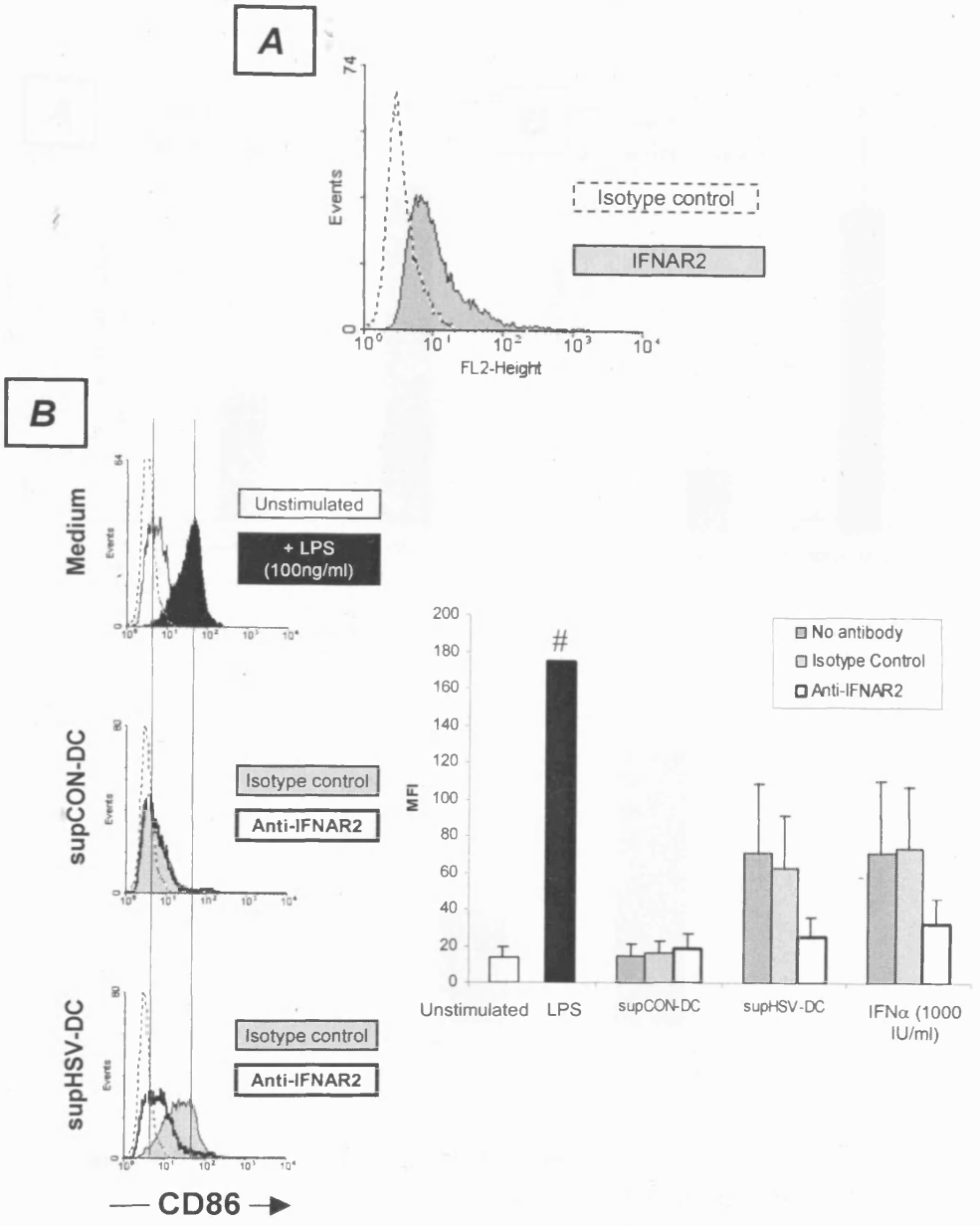

Figure 5.6 Role of type I IFN in supHSV-DC in inducing DC upregulation of CD86.

(A) Expression of IFNAR2 on DC. Representative of two independent experiments. (B) DC were cultured in supCON-DC, supHSV-DC or 1000 IU/ml IFNα for 16 hours in the presence of anti-IFNAR2 mAb or an isotype control and changes in CD86 expression were assessed. *(Left panel)* Representative experiment. *(Right panel)* Mean MFI of three independent experiments. Error bars represent SEM. # = S.E.M. = +/- 100 MFI.

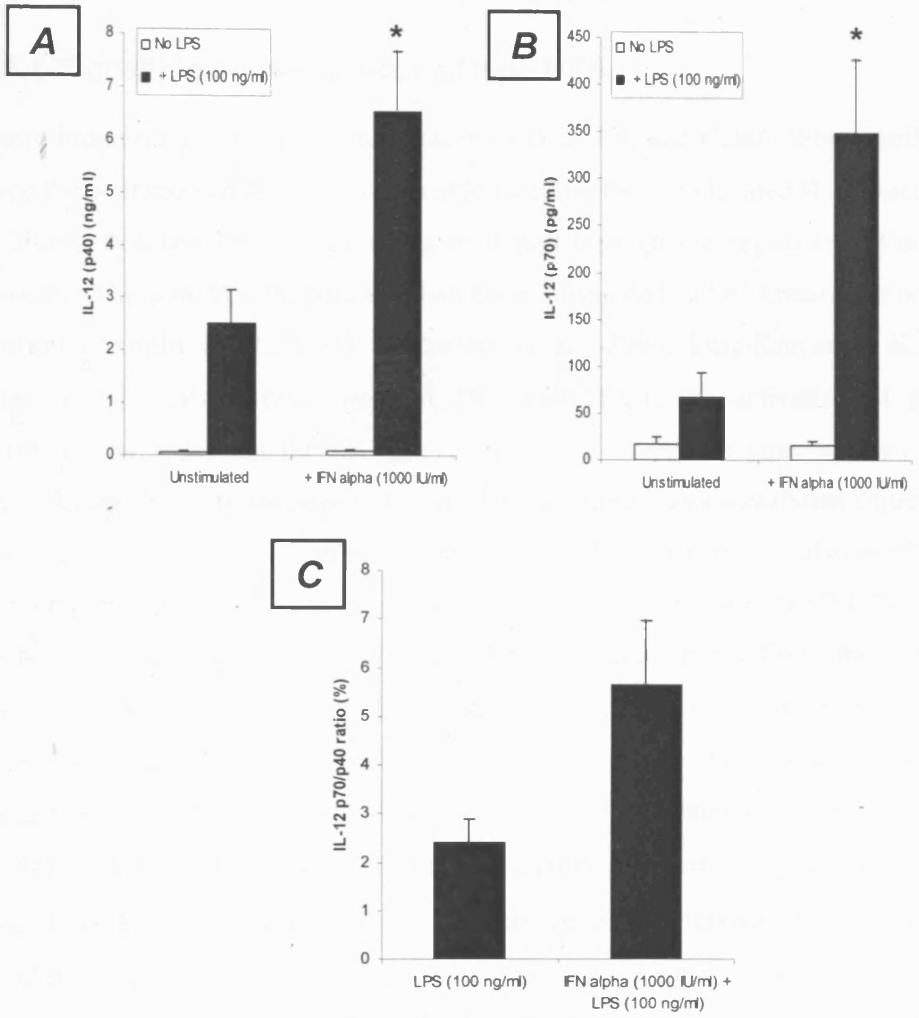

Figure 5.7 Effect of IFNα on DC IL-12 secretion.
DC were cultured in the presence or absence of 1000 IU/ml IFNα +/- 100 ng/ml LPS for 16 hours. Supernatants were assayed for IL-12 p40 (A) or IL-12 p70 (B) by ELISA. Results are mean of three independent experiments. Error bars represent SEM. * denotes $p<0.01$ relative to LPS only stimulated DC. (C) IL-12 p70/IL-12 p40 ratio calculated from (A) and (B) displayed as a bar chart.

5.2.3 Mechanism of type I IFN activity on DC

5.2.3.1 Signalling consequences of type I IFN

Despite inducing a moderate upregulation of HLA-DR and CD86, IFNα itself did not induce the secretion of IL-12, but was able to prime DC for elevated IL-12 secretion. It was likely that this effect was being mediated through the regulation of signalling pathways. The p38 MAPK pathway has been implicated in DC maturation and IL-12 secretion (Arrighi et al., 2001; Ardeshna et al., 2000; Puig-Kroger et al., 2001). Therefore, after short-term pulse of DC with IFNα, the activation of p38 was determined by assessing the phosphorylation state of this enzyme, which correlates with its kinase activity (Raingeaud et al., 1995). There was a significant degree of p38 phosphorylation, albeit less than that observed following LPS stimulation (fig 5.8A). Another pathway involved in maturation and IL-12 secretion is the NF-kB pathway (Yoshimura et al., 2001; Laderach et al., 2003). Its activation follows the degradation of IkBα, a cytosolic inhibitor of the NF-kB subunits. Loss of the IkBα protein permits NF-kB transcription factor subunits to translocate to the nucleus and induce transcription of NF-kB controlled genes (Karin and Ben Neriah, 2000; Yoshimura et al., 2001). IFNα induced some IkBα degradation, but this was again less than that induced by LPS stimulation alone. The degree of p38 phosphorylation and IkBα degradation induced by IFNα and LPS individually correlated with the resultant degree of upregulation of CD86 and MHC class II (fig. 5.5A), and may therefore have been a key determinant. Furthermore, culture with both IFNα and LPS resulted in little difference in the activation of p38, but a greater IkBα degradation relative to LPS stimulation alone (fig 5.8A). This additive increase in NF-kB activation could contribute to the elevated IL-12 secretion seen when DC were pulsed concurrently with the two stimuli (fig. 5.7).

5.2.3.2 Role of p38 MAPK activation in IFNα mediated increase in CD86 expression

The relationship between p38 activation and upregulation of CD86 by either IFNα or LPS, prompted the investigation of the role of this kinase in this change. DC were pre-

treated with the specific p38 kinase inhibitor, SB203580 (Tong et al., 1997) overnight prior to stimulation with IFNα or LPS and then for a further 16 hours. Upregulation of CD86 by IFNα occurred independently of p38 activity. In contrast, LPS induced upregulation was partially dependent on the activity of p38 MAPK (fig. 5.8B). Noticeably expression of CD86 under these conditions (i.e. in the absence of p38 activity) was similar to that induced by IFNα alone. Therefore, this section demonstrates that CD86 expression is regulated at two levels; lower expression is independent of p38 MAPK activity, whereas maximal expression is dependent on p38 MAPK.

5.2.4 Role of type I IFN in mediating TLR-dependent DC activation

5.2.4.1 Phenotype changes

Previous data has indicated that TLR ligation in myeloid DC can induce secretion of type I IFN (Cella et al., 1999b; Coccia et al., 2004). In agreement with this, 100 ng/ml of LPS induced DC to secrete approximately 100 IU/ml of type I IFN (fig. 5.3A). This concentration of IFNα could exert a functional effect on DC phenotype (fig. 5.5). Therefore, it was possible that a proportion of DC phenotype changes in response to LPS were mediated by the autocrine activity of secreted type I IFN (fig. 5.9A). This was tested by stimulating DC with 100 ng/ml LPS in the presence of neutralising anti-IFNAR2 antibody, as used in fig. 5.6. To confirm that the amount of antibody used in these assays was saturating, DC were cultured in parallel with 1000 IU/ml of IFNα (i.e. an excess relative to the amount secreted by DC stimulated with 100 ng/ml LPS) in the presence of the IFNAR2 antibody. This abrogated the upregulation of CD86 induced by IFNα, confirming that saturating quantities of antibody were used in these assays. However, upregulation of CD86 by LPS was unaffected by removing the effect of type I IFN. Thus, TLR4 induced maturation of DC phenotype was independent of the effects of type I IFN (fig. 5.9B).

To assess whether other TLRs induced maturation of DC which was dependent on type I IFN, the actions of the TLR3 ligand, double stranded RNA (dsRNA), were assessed. In vitro dsRNA can be mimicked by commercial preparation of Poly(I:C), which

ligates TLR3 and induces maturation of DC through the activation of NF-kB (Alexopoulou et al., 2001). Poly(I:C), like LPS, was used at the concentration that induced maximal upregulation of maturation markers (25μg/ml, data not shown). When 25μg/ml of Poly(I:C) were used to stimulate DC in the presence of neutralising anti-IFNAR2 antibodies, upregulation of CD86 was partially abrogated (fig. 5.9C). Although the DC appeared to be a uniform population (at least by CD1a+ expression), only a subset of DC was inhibited. Therefore, TLR3 mediated upregulation of CD86, in contrast to TLR4, was partially dependent on the autocrine / paracrine activity of type I IFN.

5.2.4.2 Cytokine secretion

Although TLR4-mediated upregulation of CD86 was independent of type I IFN activity, TLR4 ligation also induces secretion of proinflammatory cytokines. Two of these are IL-12 and TNFα (Banchereau and Steinman, 1998). The supernatants from the experiments in fig. 5.9B were analysed for the presence of TNFα and IL-12 p70 by ELISA. Surprisingly, while type I IFNs were not required for the increase in CD86 induced by LPS, they had a significant role in the secretion of both TNFα and IL-12 p70. Neutralising the activity of type I IFN, reduced the secretion of both these cytokines (fig. 5.10). It is difficult to determine precisely the relative contribution of IFN due to the variability in cytokine secretion between individuals. However, it was clear that the secretion of both these cytokines was not totally dependent on type I IFN, but that this factor played a significant role in ensuring maximal secretion.

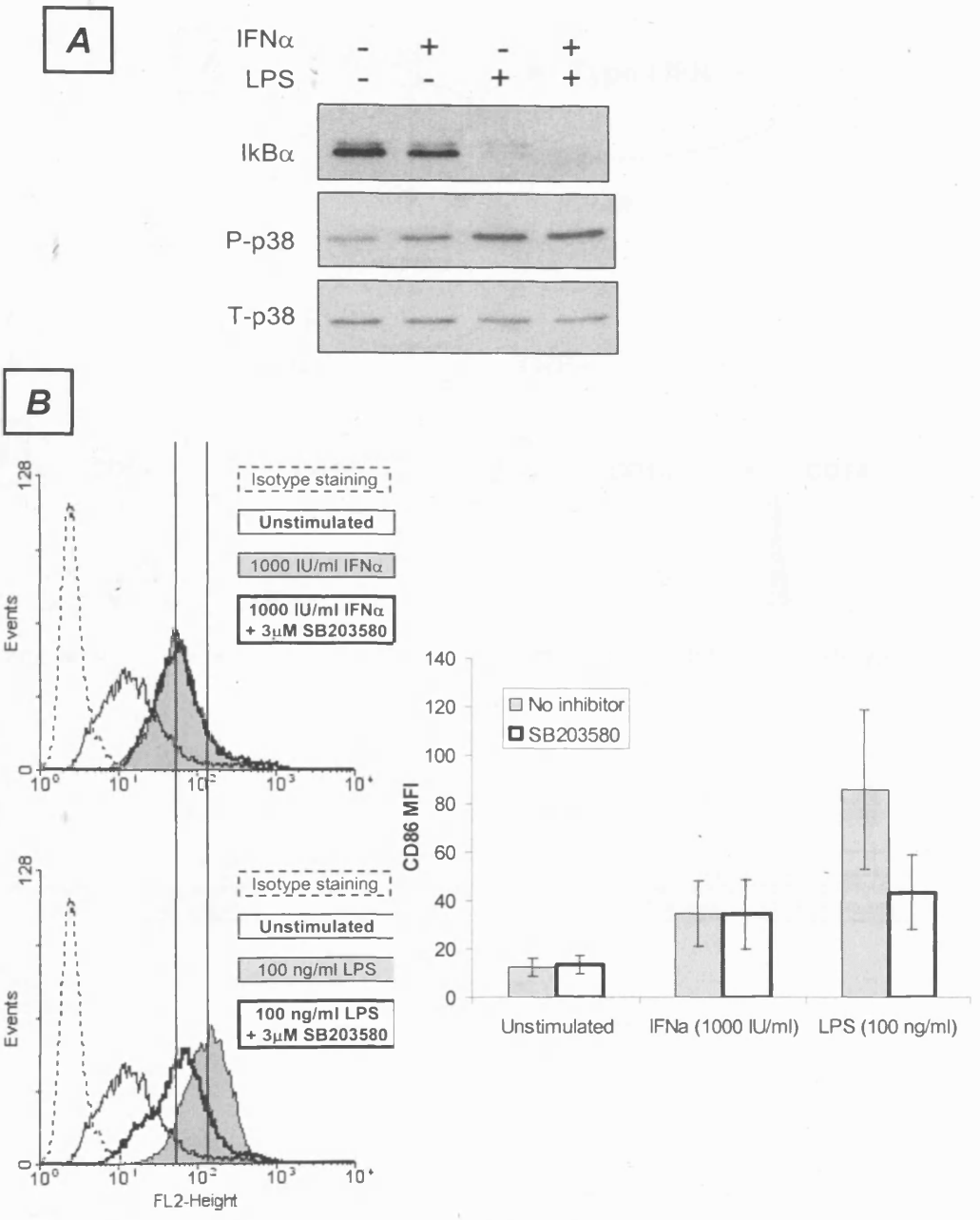

Figure 5.8 Intracellular signals activated by IFNα in DC

(A) DC were cultured in the presence or absence of recombinant 1000 IU/ml IFNα +/- 100 ng/ml LPS for 60 minutes. Cell lysates were harvested and assayed for IkBα, phospho- and total-p38 MAPK by western blotting. Representative of two independent experiments. (B) DC were cultured in the presence or absence of the p38 MAPK inhibitor, SB203580, for 24 hours prior to stimulation with 1000 IU/ml IFNα or 100 ng/ml LPS and cultured for a further 16 hours +/- 3μM SB203580. *(Left panel)* Representative experiment. *(Right panel)* Mean of three independent experiments.

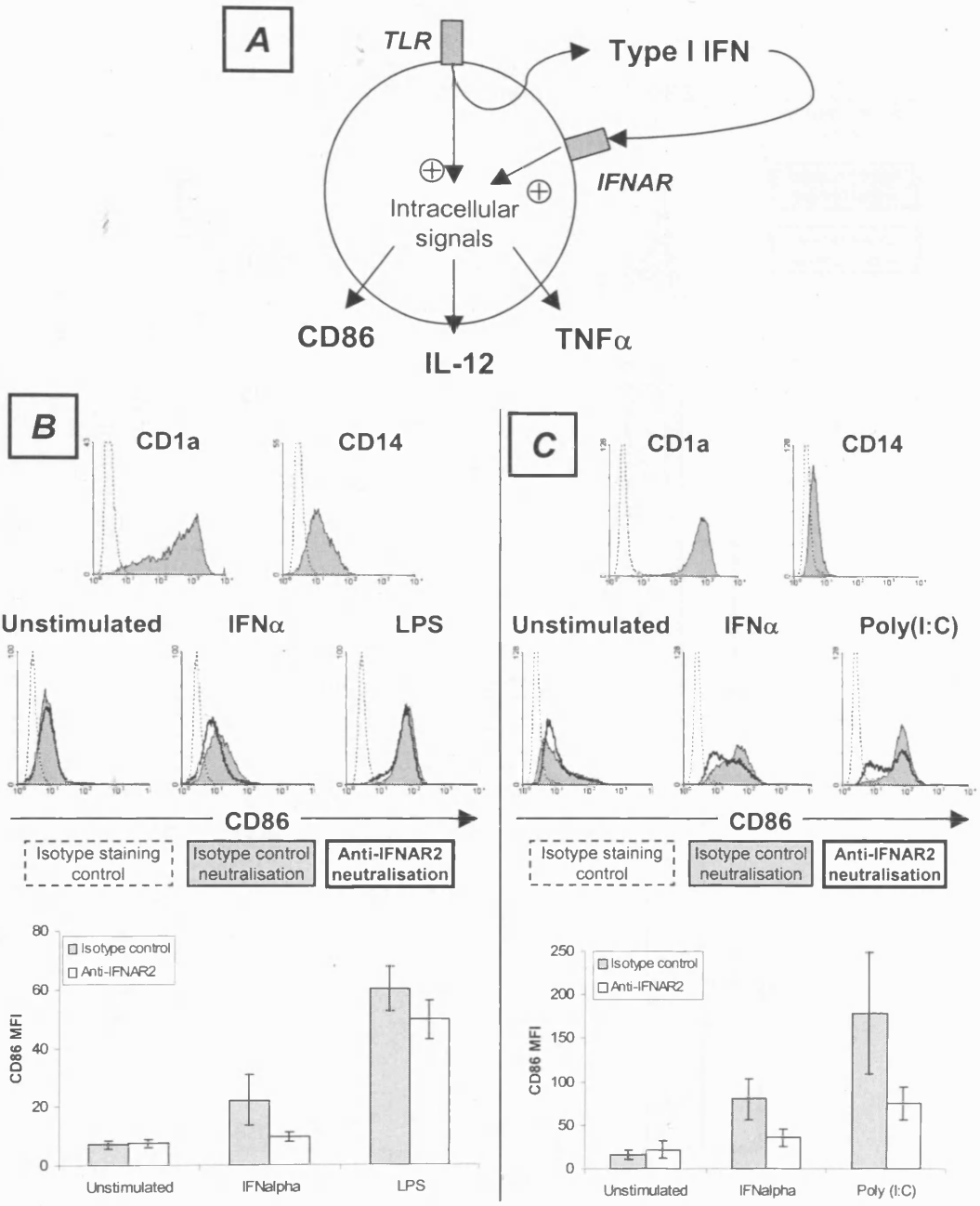

Figure 5.9 Role of autocrine/paracrine type I IFN on DC phenotype changes by LPS and Poly(I:C)

(A) Proposed model for autocrine/paracrine activation of DC by type I IFN after TLR ligation. (B+C) DC were pretreated with anti-IFNAR2 mAb or an isotype control, and then stimulated with 1000 IU/ml IFNα, 100 ng/ml LPS (B) or 25μg/ml Poly(I:C) (C). *(Upper panel)* Representative experiment. *(Lower panel)* Mean of three independent experiments. Error bars represent SEM.

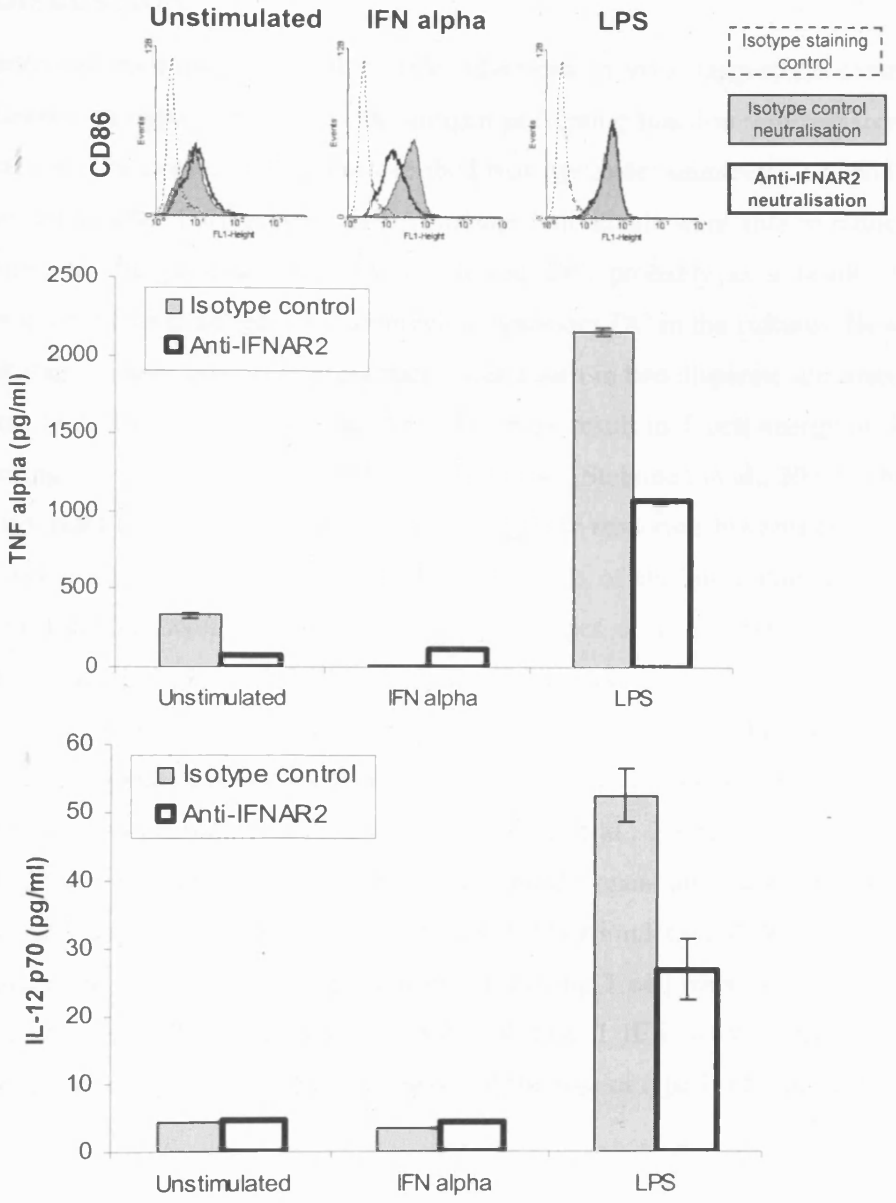

Figure 5.10 Role of autocrine/paracrine type I IFN on LPS induced secretion of TNFα and IL-12.
Supernatants from the experiments in figure 5.9 *(upper panel)* were harvested and assayed for secretion of TNFα *(middle panel)* or IL-12 p70 *(lower panel)*. Data are representative of at least three independent experiments.

5.3 Discussion

The antiviral immunity seen after HSV infections in vivo suggests the existence of mechanisms to bypass the loss in DC antigen presenting function resulting from HSV-1 infection. The previous chapter described that bystander uninfected DC had a more mature phenotype. Furthermore, HSV immune individuals were able to induce recall responses in the presence of HSV-1 infected DC, probably as a result of cross-presentation of viral antigens by uninfected bystander DC in the cultures. However, it is important to note that cross-presentation can result in two disparate scenarios: cross-priming or cross-tolerance and that the latter may result in T cell anergy or deletion, preventing the generation of an anti-viral response (Steinman et al., 2000). Therefore, it is important to understand the processes that skew responses towards cross-priming. One important determinant is the maturation state of the presenting DC, whereby cross-priming is favoured by mature DC (Hawiger et al., 2001). As well as the increased upregulation of MHC and co-stimulatory molecules, the secretion of cytokines, such as IL-12, is also important to ensure a potent T cell response (Albert et al., 2001). Recent data suggests that type I IFN plays an important role in skewing towards a cross-priming response in vivo (Dalod et al., 2003; Le Bon et al., 2003), possibly by acting on DC in secondary lymphoid organs and skewing towards Th1 responses (Foster et al., 2000). However, type I IFN stimulation of DC derived from an infectious site may be equally relevant in stimulating T cell responses. The remainder of this chapter will focus on two aspects of type I IFN activity: the response of bystander DC at the site of HSV infection and the role of type I IFNs in TLR-mediated activation of DC.

5.3.1 DC secretion of type I IFN

A prime candidate for the stimulatory factor secreted by HSV-1 infected DC was type I IFN, a family of cytokines made up of many subtypes (Taniguchi and Takaoka, 2002). The antiviral properties of type I IFN (fig. 5.4) have been appreciated for many years (Isaacs and Lindenmann, 1957), and are believed to play a major role in the control of HSV infections in vivo (Leib et al., 1999; Murphy et al., 2003). Most cell types can act as a source of type I IFN. However, a subset of DC, PDC, secrete high

levels of type I IFN when appropriately stimulated with certain viruses. It has been suggested that this discriminates PDC from myeloid DC (MDC) (Siegal et al., 1999; Foster et al., 2000; Cella et al., 1999b; Giacomini et al., 2001), although this notion has recently been challenged, since appropriate stimulation of myeloid DC can also induce secretion of large quantities of type I IFN, equivalent to that produced by PDC (Diebold et al., 2003).

Therefore, it was important to exclude the presence of PDC from the myeloid DC preparations used in the current study. The culture conditions used made the presence of PDC unlikely. IL-4, which is present in the DC differentiation cultures, has been shown to have an inhibitory effect on PDC survival (Rissoan et al., 1999). Furthermore, PDC express CD2 (Cella et al., 1999a), a marker used to deplete contaminating T and NK cells in the experimental protocol. The combination of myeloid markers expression in >99% of these DC and intracellular staining for IFNα confirmed that the source of type I IFN secreted into the supernatant of HSV infected cultures was undeniably MDDC (fig. 5.3). Thus, MDDC can secrete sufficient functionally active type I IFN following natural infection by the DNA virus HSV-1.

Other studies have attempted to quantify the secretion of type I IFN from MDDC after HSV-1 infection. Some have failed to detect any (Siegal et al., 1999; Mohty et al., 2003), whereas others have detected small amounts (Izaguirre et al., 2003). Independent of variation in experimental protocol, DC generation and HSV strain used, one common difference between this study and some others that have found no IFN, is the detection method for type I IFN. The bioassay used is more sensitive than ELISAs used by others. Furthermore, it is likely to detect a wider range of IFN subtypes than ELISAs, which are limited to the specificity of the antibodies used. This is an important distinction with regards to the mechanisms that regulate the secretion of type I IFN. Viral induction of IFNβ transcription and secretion is followed by autocrine signalling through the IFNAR, and subsequent transcription of IFNα genes (see section 5.3.2). Consistent with the type I IFN detected in this thesis, IFNβ transcription is increased in MDDC after HSV-1 infection (Izaguirre et al., 2003). However, the same study detected smaller increases in IFNα transcription and in a smaller number of IFNα subtypes than seen for PDC (Izaguirre et al., 2003). This

would explain the small shift in IFNα intracellular staining in fig. 5.3. Thus, the widespread use of IFNα ELISA for the detection of type I IFN is likely to generate negative data for MDDC secretion of type I IFN after HSV-1 infection.

The IFN secreted by MDDC is less than the amount secreted by PDC in response to HSV-1 (Siegal et al., 1999). However, as the amount secreted by MDDC is nonetheless sufficient to elicit functional changes in DC (figs. 5.1 and 5.2), this raises interesting questions about the in vivo advantage to the host of such potent IFN producing cells. This is discussed in greater detail in chapter 7.

The mechanisms responsible for type I IFN secretion by MDDC are also of interest. One viral component that may induce IFN secretion by MDDC is gD (fig. 6.17). This is discussed further in chapter 6. However, it is unclear what determines the differential quantity of IFN secreted by PDC compared to MDC. The recently described capacity of MDC to secrete large amount of IFN only when exposed to dsRNA in the cytoplasm (Diebold et al., 2003), suggests that PDC can detect components of HSV infection which MDC cannot, rather than an intrinsic inability of MDC to secrete type I IFN. This is supported by PDC secretion of IFNα in response to HSV independent of an IFNβ autocrine feedback through the IFNAR (Barchet et al., 2002). A likely ligand that fits this model is HSV-1 DNA itself, which contains a high frequency of hypomethylated CpG motifs (Honess et al., 1989). The receptor for this ligand is TLR9, which in humans is expressed in PDC but not MDC (Krug et al., 2001), and exposure of PDC to HSV DNA promotes type I IFN production (Lund et al., 2003). The ability of PDC to secrete IFNα in response to stimulation with UV-inactivated HSV is consistent with recognition of the viral genome (Siegal et al., 1999; Barchet et al., 2002). The secretion of IFN by MDDC is also independent of viral replication, but this is likely to involve recognition of different viral components, such as gD (chapter 6).

In support of the plasticity of viral recognition by MDC in the appropriate context, recent data has demonstrated that monocytes differentiated into DC in the presence of GM-CSF and IFNα (instead of IL-4), acquire the capacity to secrete IFNα in response to HSV-1 infection, although this is still inferior to the amount secreted by PDC

(Mohty et al., 2003). The response to HSV correlates with the expression of TLR7 in these cells. The ligand for this receptor is ssRNA, possibly with some specificity for viral sequence (Diebold et al., 2004; Heil et al., 2004). Thus, DC can acquire the capacity to detect products of viral replication in the correct environmental context (i.e. high concentration of IFNα), although HSV may also possess a novel TLR7 binding ligand. A model whereby monocytes are recruited to an infection site rich in IFNα that may prime these cells for recognition of HSV infection is an attractive one and further explored in chapter 7.

5.3.2 Role of type I IFN in DC physiology

The actions of type I IFN are mediated by interferon-stimulated gene (ISG) products. The transcription of these genes is regulated by interferon-stimulated regulatory elements (ISREs) within ISG promoters. Cell stimulation (by TLR or viral infection), results in the phosphorylation of interferon response factor-3 (IRF-3), and induces translocation of this cytoplasmic protein to the nucleus, where it binds to the promoters of some ISGs and in the IFN-β promoter. The subsequent secretion of IFNβ results in autocrine stimulation of the IFNAR. In turn, this induces the formation of the IFN-stimulated gene factor-3 (ISGF3) or IFNα-associated factor (AAF). ISGF3 activates transcription of more ISGs and IRF-7, the latter promoting the transcription of IFNα genes, which can amplify the cell's ISG response further. AAF binds IFNγ-activated-site (GAS) elements which promote transcription of IRF-1, which in turn can bind to IRF-1 elements (IRFE) on target gene promoters (Taniguchi and Takaoka, 2002).

The most recognised actions of the IFN system are its antiviral properties (Isaacs and Lindenmann, 1957), but recent studies using mice lacking genes for IFNAR and various IRFs have suggested a role for type I IFNs in regulating several aspects of DC physiology. DC isolated from spleens of IFNAR KO mice possess a more immature phenotype, do not undergo further spontaneous maturation in vitro and are impaired in stimulating T cells (Montoya et al., 2002). These data imply that type I IFNs, acting in an autocrine fashion, are important in enhancing the APC capacity of DC. Furthermore, it is likely that type I IFNs also play a role in the differentiation of precursors into DC, as mice lacking IRF-2 or IRF-8 show selective deficiencies in

several, non-overlapping DC subsets. Furthermore, the absence of these transcription factors also impairs maturation of the remaining DC subsets, further supporting a role for type I IFN in DC responses to various stimuli (Tsujimura et al., 2003; Schiavoni et al., 2002; Aliberti et al., 2003; Ichikawa et al., 2004).

5.3.2.1 Type I IFN effects on immature DC phenotype

Previous studies have demonstrated that IFNα induces sub-maximal DC maturation (Lehner et al., 2001; Padovan et al., 2002; Luft et al., 2002b), and the data in this chapter extends this by demonstrating that the response is dose-dependent. It is important to note that the amount of type I IFN secreted following HSV-1 infection (about 250 IU/ml) closely reproduced the sub-maximal upregulation of CD86 and HLA-DR observed in DC cultured in supHSV-DC. In vivo, the localised concentration of IFN-α achieved may be much higher, and drive full DC maturation, although this is likely to occur in conjunction with other stimuli in the environment.

As well as affecting DC maturation, the local release of type I IFN may impinge on differentiation of DC precursors. The ability of IFNα from SLE patient sera to induce spontaneous monocyte differentiation into DC suggests this is a relevant process in vivo (Blanco et al., 2001). Subsequent in vitro studies have investigated this phenomenon, in particular comparing with DC generated in GM-CSF and IL-4. Replacement of IL-4 with IFNα induces a proportion of monocytes to develop characteristics of more mature DC, as determined by surface phenotype and T cell stimulating capacity (Paquette et al., 1998; Wang et al., 1999a; Santini et al., 2000; Parlato et al., 2001). However, other studies have observed that similar treatment does not induce mature DC development (Dauer et al., 2003; Mohty et al., 2003), and the absence of IL-4 in the culture may also render the DC refractory to further stimulation (Dauer et al., 2003). The different times at which DC phenotype and function were analysed in these studies make direct comparison difficult.

The number of DC recovered in GM-CSF + IFNα cultures is lower than in the presence of GM-CSF and IL-4 (Paquette et al., 1998; Wang et al., 1999a; Dauer et al., 2003; Della et al., 2004). Furthermore, addition of IFNβ to monocyte cultures

supplemented with GM-CSF, IL-4 and TNFα, conditions which normally generate mature DC, reduced the number of DC recovered (McRae et al., 2000a). It is possible that "over-accumulation" of activation signals induces DC death. In similar fashion, addition of IFNα and LPS to monocytes cultured in GM-CSF and IL-4 also induces rapid apoptosis (Lehner et al., 2001), but no such synergy in death occurs at equivalent LPS and IFNα concentrations in the immature DC cultures (data not shown and in (Lehner et al., 2001)). This may be due to different relative susceptibility to activation-induced cell death of differentiating and immature DC respectively. Alternatively, the length of time of stimulation may be important, as monocyte death is often analysed at least 3 days after culture in the various conditions, whereas DC phenotype changes are analysed within 24 hours of stimulation.

It is also important to remember that monocytes can differentiate into both macrophages and DC. The cytokine environment is important in influencing this outcome (Chapuis et al., 1997; Palucka et al., 1998). For example, both IL-10 and IFNγ can push towards macrophages (Allavena et al., 1998; Delneste et al., 2003), whereas IL-4 is important in enhancing differentiation into DC (Sallusto and Lanzavecchia, 1994). The absence of DC-specific markers makes it difficult to define cells as "non-DC", but the increased expression of CD14 in DC generated in the presence of IFNα may suggest cells tending more towards macrophage properties than those classically generated in the presence of GM-CSF and IL-4 (Mohty et al., 2003). Furthermore, the similarity in signalling between type I and type II IFN receptors, may be consistent with the ability of IFNγ to skew towards macrophages (Allavena et al., 1998; Delneste et al., 2003).

5.3.2.2 Type I IFN effects on immature DC IL-12 secretion

It is critical that the DC system possesses mechanisms to overcome the block in IL-12 secretion that results from HSV-1 infection (fig. 4.11). One mechanism proposed from this chapter is the secretion of type I IFN from HSV-1 infected DC that can prime bystander uninfected DC for IL-12 secretion, in the context of another inflammatory stimulus. However, further studies detailed that type I IFNs are also involved in

inducing maximal secretion of IL-12 by LPS alone (fig. 5.10), further underlying the critical priming role of this family of cytokines.

Although the synergistic effects observed with IFNα in this study are similar to those reported by others (Luft et al., 2002b; Heystek et al., 2003), the regulation of IL-12 secretion by type I IFN remains an area of controversy, as the effects reflect both cell type and species differences. For example, IFNα inhibits LPS induced IL-12 p40 secretion from monocytes (Hermann et al., 1998) and negative regulation of IL-12 secretion by type I IFN has been reported in mice (Cousens et al., 1997). The latter may be explained through type I IFN mediated PI3K activation (Yang et al., 2001), as PI3K can potentiate IL-12 secretion in humans (Re and Strominger, 2001) in contrast to its negative effects in the mouse (Fukao et al., 2002). Other contentious issues relate to the subtypes of IFN and the precise timing of IFN exposure. In contrast to IFNα (fig. 5.7) (Luft et al., 2002b), IFN-β does not have a priming effect on DC IL-12 secretion and may even inhibit it (McRae et al., 2000b; van Seventer et al., 2002). It seems paradoxical that two cytokines that signal through the same receptor may give rise to disparate functional consequences. However, recent data suggest that different type I IFNs bind to different sites on the IFNAR2 chain (Chill et al., 2003). The kinetics of binding to the receptor may be critical in determining the outcome of the cytokine interaction with the cell and the downstream signalling.

It has also been shown that DC generated in the presence of IFNα or IFNβ during DC differentiation demonstrate impaired ability to secrete IL-12 (Paquette et al., 1998; Bartholome et al., 1999; Mohty et al., 2003). As the window for IL-12 secretion is narrow (between 8-12 hrs after stimulation), these cells may exhibit signs of "exhaustion" (Langenkamp et al., 2000). It is also possible that the absence of IL-4 in these cultures may deprive DC of the endogenous adjuvant activity of this cytokine for IL-12 secretion (Ebner et al., 2001). Indeed, in the mouse, DC do not secrete IL-12 p70 in response to a single stimulus (Schulz et al., 2000). The requirement to derive human DC from precursors in the presence of IL-4 may reduce the threshold for the secretion of IL-12. The species differences may also be intrinsic to the regulation of the transcription of the cytokine itself. For example, PI3K, may alter the threshold for secretion in humans compared to mice (Re and Strominger, 2001; Fukao et al., 2002).

In addition, the protein products of the IL-12 family may differ between the two species. Although both secrete excess IL-12 p40 compared to functional IL-12 p70 heterodimer, p40 from mouse cells can homodimerise and antagonise IL-12 p70 activity, whereas non-transfected human cells do not produce the homodimer, despite the presence of equivalently large excess quantities of IL-12 p40 secreted (Carra et al., 2000).

5.3.3 Role of type I IFN in TLR induced changes to DC

Given that previous studies have demonstrated that human DC stimulation by TLR ligands induces the secretion of type I IFN (Cella et al., 1999b), and that the secretion of type I IFN can affect the functional outcome of DC-pathogen interaction (Trottein et al., 2004), an immediate extension of the work was to assess the relative contribution of autocrine type I IFN activity on the changes in surface phenotype and cytokine secretion that occur during DC maturation by TLR ligands.

5.3.3.1 Type I IFN and phenotype changes to TLR3 and TLR4

All TLRs signal downstream via the Toll-interleukin 1 receptor-resistance (TIR) domain of the cytoplasmic tail of the receptor. This occurs through an adaptor protein, of which myeloid differentiation factor 88 (MyD88) is a prototype. However, LPS stimulation of DC deficient in MyD88 does not prevent phenotype changes (Kaisho et al., 2001). Another adaptor molecule that can associate with the TIR domain of TLR4 is TIR domain-containing adaptor protein (TIRAP), but this protein is also not essential in mediating LPS induced phenotypic maturation of DC (Yamamoto et al., 2002). However, absence of a third TLR adaptor molecule, TIR-domain-containing adaptor inducing interferon-beta (TRIF), does prevent this (Hoebe et al., 2003b). Noticeably, TRIF is important for LPS induced phosphorylation of IRF-3 (Fitzgerald et al., 2003), which in turn activates transcription of the IFNβ gene (Sakaguchi et al., 2003). Therefore, this raises the possibility that autocrine type I IFN could mediate some of the maturation induced by LPS.

This question has been studied in the murine system using mice lacking the type I IFN receptor. The data show that autocrine type I IFN is required to allow maximal upregulation of CD40 and CD86 in DC in response to LPS (Hoshino et al., 2002; Honda et al., 2003). It is interesting that macrophages from IFNAR KO mice are completely unable to upregulate CD86 and MHC class II in response to LPS (Hoebe et al., 2003b), in contrast to more moderate effects on DC (Hoshino et al., 2002; Honda et al., 2003). This cell type specific effects may be related to a variable contribution of the TRIF pathway to TLR4 induced gene expression in different leucocyte populations. A note of caution is required when comparing the surface phenotype changes between DC and macrophages because the primary output of macrophages in response to LPS may not be the upregulation of molecules involved in antigen presentation, but rather effector mechanisms involved in pathogen clearance, such as nitric oxide production (Gao et al., 1998).

There is little data available regarding the relative contribution of type I IFN in LPS mediated maturation of human DC. The data in this chapter suggests that type I IFN is not required in LPS induced upregulation of CD86 (fig. 5.9) and HLA-DR (data not shown), in agreement with another study (Gauzzi et al., 2002). The difference from mice may be related to the differential regulation of TLR4 between the two species. LPS stimulation of mouse macrophages downregulates TLR4 expression, whereas LPS stimulation of human macrophages results in upregulation (Matsuguchi et al., 2000; Poltorak et al., 1998a; Muzio et al., 2000). The reduced signalling through TLR4 may result in obligate amplification by a second stimulus in mouse cells to result in maximal upregulation of surface molecules. However, differences in DC models, and the culture conditions used might also contribute to the discrepancy, possibly priming human DC for stimulation at lower levels of LPS than mouse DC.

In contrast to LPS, dsRNA stimulation of DC is partly dependent on the autocrine activity of type I IFN. This is consistent with previous data in both DC and macrophages in mice (Honda et al., 2003; Hoebe et al., 2003b), although the contribution of IFN in mice is greater. The dependency on IFN is consistent with the recruitment of TRIF to TLR3 (Oshiumi et al., 2003), and the subsequent activation of IRF-3 and transcription of the IFNβ gene (Sakaguchi et al., 2003). However, the

requirement for IFN in the TLR3 response may also relate to the role of anther adaptor molecule, TRAM. This molecule can interact with TLR4 but not TLR3, possibly increasing the relative strength of the direct stimulus via TLR4 relative to TLR3 (Fitzgerald et al., 2003; Yamamoto et al., 2003).

Mechanistically, the role of type I IFN in Poly(I:C) induced DC maturation was peculiar, in that removing the autocrine activity of IFN did not reduce CD86 upregulation uniformly. Rather, two discrete populations of cells were observed, one that expressed CD86 as high as untreated cells, and another expressing lower CD86 (fig. 5.9). These data are not directly comparable to that from mice where the original DC population is often not as uniform as that of human MDDC (Honda et al., 2003). Nevertheless, the continuous dose-response of DC phenotype to IFN (fig. 5.5) excluded the possibility that a critical concentration of type I IFN was required to trigger sufficient signalling to upregulate CD86 or HLA-DR. The positive regulation of TLR3 expression by type I IFN (Miettinen et al., 2001; Heinz et al., 2003; Tanabe et al., 2003), suggests that abolishing the activity of IFN may prevent sufficiently high expression of TLR3 to permit a maximal response to Poly(I:C). This regulation of receptor expression may be particularly relevant to TLR3 because, in the resting state, very low levels of this receptor are expressed on the DC surface (Matsumoto et al., 2003). This may be an important control mechanism to ensure that immune responses in the presence of dsRNA are generated only in the context of an ensuing antiviral response, where the local concentration of type I IFN and extracellular dsRNA may be high. Furthermore, in the presence of a high concentration of dsRNA but low type I IFN, as may occur towards the end of a viral infection when multiple CTL lysis events release large quantities of dsRNA, the absence of significant viral replication would reduce the IFN released. This model proposes that the DC is able to curb the cellular response in this scenario by not upregulating TLR3, reducing its sensitivity to the dsRNA.

5.3.3.2 Role of autocrine type I IFN in LPS induced cytokine secretion

The secretion of several proinflammatory cytokines occurs during DC maturation by TLR ligands. Murine macrophage and DC secretion or mRNA induction of TNFα and

IL-12 to LPS are not dependent on autocrine type I IFN activity (Hoebe et al., 2003b; Honda et al., 2003; Trottein et al., 2004). Similarly, the absence of IRF-3 does not affect the mRNA levels of TNFα in response to LPS (Sakaguchi et al., 2003). Interestingly, the secretion of both of these cytokines was dependent on TRIF, demonstrating that in DC maturation, TRIF is not required solely to induce the secretion of IFNβ (Hoebe et al., 2003a). In contrast, the secretion of both TNFα and IL-12 by DC in this thesis was reduced when type I IFN activity was neutralised (fig. 5.10), despite the independence from autocrine type I IFN activity on changes in surface phenotype in response to LPS stimulation (figs. 5.9 and 5.10). The early downregulation of IFNAR after LPS stimulation suggests that the secretion of type I IFN occurred within 5 hours of LPS stimulation (Gauzzi et al., 2002). The one other study that has investigated this effect highlighted not only the complexity of signalling pathways downstream of TLR4, but also the cell specific regulation of these pathways. Transfection of human macrophages with adaptor molecule-expressing adenoviruses demonstrated that TIRAP is not required for the secretion of TNFα by macrophages in response to LPS. However, TIRAP was essential for LPS induced transcription of the IFNβ gene, independently of the phosphorylation of IRF-3 (Andreakos et al., 2004). Not only does this imply that IFNβ transcription does not result directly form IRF-3 activation, it also indicates that the secretion of TNFα by macrophages occurs independent of autocrine type I IFN activity, in contrast to the requirement in DC (fig. 5.10).

5.3.4 Signalling consequences of type I IFN on DC

5.3.4.1 Regulation of IL-12 and TNFα secretion

The priming effect of type I IFN for maximal IL-12 secretion may be explained by the signalling consequences of IFNAR ligation on DC. As IFNα alone is unable to induce IL-12 secretion, it is likely that the activation of p38 and NF-kB induced by this cytokine is insufficient to induce transcription of either subunit. Conversely, LPS activates these pathways sufficiently. Nevertheless, the synergistic secretion of this cytokine after co-stimulation with both IFNα and LPS suggests that IFNAR-derived signals play a role. IFNAR ligation induces the activation of NF-kB (fig. 5.8) (Yang et

al., 2000), as well as the generation of IRF-1, which can bind IRFE on gene promoters (see section 5.3.2). This is significant because both IL-12 subunit promoters contain NF-kB binding sites (Murphy et al., 1995; Grumont et al., 2001) and IRFEs, binding to which promotes gene transcription (Kollet et al., 2001; Maruyama et al., 2003; Liu et al., 2003). The activation of p38 MAPK by IFNα (fig. 5.8) is unlikely to play a role during co-stimulation, as LPS alone activates p38 with the same kinetics as IFNα (data not shown) and to maximal levels, as judged by the lack of further activation when co-stimulated with IFNα (fig. 5.8).

However, autocrine type I IFN signalling after LPS stimulation alone may affect secretion of IL-12 differently. IFNα may suppress activation of the ERK pathway (Romerio et al., 2000), which may in turn promote IL-12 transcription(Puig-Kroger et al., 2001). Alternatively, the activation of p38 MAPK following LPS stimulation will have subsided, and thus IFNα may reactivate this pathway at the time of feedback (fig. 5.8). As type I IFN induced p38 MAPK activation can result in histone phosphorylation (Li et al., 2004), this process may mark the IL-12 promoters for increased IFNAR-derived NF-kB and IRF-1 recruitment (Saccani et al., 2002), because in both IL-12 subunit promoters, transcription factor access to at least one NF-kB binding site and/or one IRFE is regulated by chromatin remodelling via histone phosphorylation or acetylation (Goriely et al., 2003; Liu et al., 2003; Weinmann et al., 1999; Maruyama et al., 2003). However, it is also possible that LPS induced p38 activation causes a permanent change in histone structure that permits greater direct access to NF-kB and IRF-1 induced by autocrine type I IFN. Indeed, cells can demonstrate such temporal memory, as IFNγ can prime macrophages for enhanced IL-12 secretion 16 hours prior to LPS exposure (Liu et al., 2003).

The signalling regulation of TNFα secretion by autocrine type I IFN may occur through similar mechanisms to those for IL-12, as TNFα transcription is positively regulated by p38 MAPK and NF-kB (Hoffmeyer et al., 1999; Zhu et al., 2000). One notable difference is that NF-kB recruitment to the TNFα promoter is not regulated by histone phosphorylation as for IL-12, although the study only analysed the role of one histone (Saccani et al., 2002).

A model whereby type I IFN provides a secondary stimulus that promotes NF-kB recruitment to target promoter sites, either by inducing changes in nucleosome conformation or by initiating greater NF-kB and IRF-1 activation, is consistent with delayed targeting of NF-kB to some promoters. Whereas some genes are targeted within 30 minutes, for others this only occurs over 2 hours after LPS stimulation (Saccani et al., 2001). Murine BMDC treated with Poly(I:C) also demonstrate two waves of nuclear NF-kB recruitment. The second of these occurred 4-6 hours after initial stimulation, and was absent in mice lacking IFNAR, implicating type I IFN in this effect (Honda et al., 2003).

To validate this model, it will be important to demonstrate that LPS stimulation does indeed induce a second wave of NF-kB, IRF-1 and/or p38 activation. Furthermore, IFNAR neutralisation experiments should ablate this secondary wave of signalling, and thus reduce IL-12 and TNFα secretion. Equally, addition of p38 or NF-kB inhibitors after the first wave of activation of these pathways by LPS (i.e. after 2 hours) should also reduce the secretion of IL-12 and TNFα.

Intriguingly, type I IFN may also promote IL-12 and TNFα secretion through post-transcriptional regulation. The activation of p38 MAPK stabilises TNFα mRNA (Dean et al., 1999; Wang et al., 1999b; Brook et al., 2000; Neininger et al., 2002), and IL-12 secretion may also be regulated at the level of mRNA stability (Du and Sriram, 1998). Analysing the expression of cytokine mRNA after LPS stimulation in the presence of neutralising anti-IFNAR2 antibodies may determine whether this is a significant regulation mechanism in this system.

5.3.4.2 Differential regulation of CD86 and IL-12

The regulation of the expression of CD86 contrasted that of IL-12. IFNα was able to upregulate CD86 independent of the activity of p38 MAPK, suggesting that weaker signalling activation is required to induce transcription of this protein compared to IL-12. Furthermore, LPS induced upregulation was only partly dependent on p38 activity. The p38 independent signalling is likely to be mediated by NF-kB (Li et al., 1999;

Yoshimura et al., 2001). It is currently not known whether CD86 expression is predominantly regulated at the transcriptional level and whether chromatin remodelling is important. If so, it would suggest that the CD86 promoter is constitutively more accessible to NF-kB access than either of the IL-12 subunit promoters.

The lower requirement for signalling activation for CD86 would also explain why LPS stimulation alone is sufficient for maximal upregulation, independent of signals from autocrine type I IFN. The differential signalling requirement between CD86 and IL-12 ~~proposes~~ suggests two thresholds of DC activation. Physiologically, this control may be important to ensure that cytokine secretion occurs only at the appropriate time and place, and under the correct stimulation (e.g. during contact with T cells).

5.4 Conclusions

This chapter aimed to investigate some of the mechanisms that DC use to bypass the HSV-1 induced inhibition of antigen presenting function described in chapter 4. Secretion and paracrine activity of type I IFN early after infection fits these criteria. Bystander maturation of neighbouring DC primes these cells for increased IL-12 secretion and induces sub-maximal activation. In this way, secretion of type I IFN in peripheral infection sites can serve two purposes. In a lesion where there is incapacity of DC in the core of the site to stimulate T cells, such as for HSV-1, type I IFN can skew the neighbouring DC that take up the infectious antigen to cross-prime instead of cross-tolerise. This is critical for the generation of an effective immune response that will resolve the infection.

Type I IFN also serves as an important extracellular second messenger to gauge the threat to the host. Although the DC system has evolved to recognise a large number of conserved pathogen molecules, it is important to tailor the amplitude of the immune response to the severity of the infection, in order to avoid undesirable immunopathology. Type I IFN secretion, also in response to non-viral stimuli, can serve such a function by amplifying the DC response where appropriate, but equally important, by not activating indiscriminately all neighbouring DC maximally. In the

event of the spread of infection, bystander cells are primed for stimulation by pathogen structures and can then undergo maximal maturation and secrete larger quantities of pro-inflammatory cytokines. This could occur both in the periphery and in the secondary lymphoid organs. The intracellular signals that regulate these properties may illustrate how DC have evolved to integrate and appropriately respond to endogenous and exogenous danger signals in their microenvironment.

Chapter 6

Mechanisms of HSV-1 activation and inhibition of DC

6.1 Introduction

Chapter 4 demonstrated that DC infected with HSV-1 expressed higher levels of CD86 and HLA DR molecules compared to uninfected immature DC. Irrespective of the subsequent inhibition in function and response to maturation stimuli, this finding indicates that the viral infection is a maturation stimulus for DC. The viral infection could result in the secretion of factors that can activate infected cells in an autocrine manner. The obvious candidate is type I IFN detailed in chapter 5, as this was the only significant stimulatory ligand in the supernatant of infected DC cultures. MV induced maturation of DC is dependent on autocrine type I IFN activity (Dubois et al., 2001). Similarly, the autocrine action of TNFα after adenovirus (AdV) infection of DC is responsible for the maturation of the cells (Trevejo et al., 2001; Philpott et al., 2004). Alternatively, virus products can stimulate DC directly and induce maturation, independent of the autocrine activity of secreted cytokines (Lopez et al., 2003). The viral ligands can either take the form of virion components that activate cells upon infection (Fantuzzi et al., 1996), or alternatively they can be the products of the viral replication cycle (e.g. dsRNA intermediates of viral replication (Jacobs and Langland, 1996)). The two mechanisms may be linked, as exemplified by AdV penton base that is required to induce TNFα secretion and subsequent maturation of DC (Philpott et al., 2004). However, for the most part, viral factors responsible for inducing DC maturation remain ill defined.

Although intracellular delivery of virion components may trigger a DC response, an important interface for virus-cell interaction is the cell surface and receptors that can bind to viral ligands. The expression of entry receptors and their downstream signalling pathways may be important in such a scenario. Alternatively, viruses may possess conserved structures that are recognised by PRRs, as detailed in chapter 1, which may induce intracellular signals. The receptors could be expressed on the cell surface to recognise viral envelope structures (Bieback et al., 2002; Compton et al., 2003) or alternatively they may be expressed in endosomal structures to detect the viral genome during uncoating. Indeed, ssRNA, dsRNA and hypomethylated CpG DNA are all recognised by, TLR7 and TLR8, TLR3 and TLR9 respectively (Diebold et al., 2004; Heil et al., 2004; Alexopoulou et al., 2001; Hemmi et al., 2000), receptors that

are expressed in endosomes (Heil et al., 2003; Matsumoto et al., 2003; Ahmad-Nejad et al., 2002)

In the context of HSV-1, viral binding to cell surface can elicit signalling that will induce activation of pathways with a functional output (Paludan et al., 2001; Cheshenko et al., 2003). Whether these events are sufficient to induce the maturation of DC has not yet been investigated. Alternatively, HSV-1 induction of IFN responses may result in DC maturation directly (Lopez et al., 2003) or through the autocrine activity of secreted type I IFN (chapter 5 + (Dubois et al., 2001)). Furthermore, HSV-1 replication in DC (fig. 3.8) may also generate sufficient dsRNA molecules (Jacquemont and Roizman, 1975) that can ligate TLR3 or activate PKR, and induce DC maturation or type I IFN secretion (Alexopoulou et al., 2001; Diebold et al., 2003).

This chapter will aim to determine the HSV-1 ligands that elicit activation of DC and induce signalling changes, through the use of progressively inactivated or neutralised viruses. This approach will allow not only the investigation of the activatory components of the virus, but also help identify some of the components responsible for the inhibitory effects on DC function that were detailed in chapter 4. The emphasis will be on viral envelope components, specifically gD, and how they interact with receptors on the surface of DC. The mechanistic understanding of this host-pathogen interaction will be discussed in the context of other viral infections and how it may impinge on disease pathogenesis.

6.2 Results

6.2.1 Type I IFN independent HSV-1 maturation of DC

It was possible that the mature phenotype observed by infected DC (fig 4.12) was dependent on the autocrine activity of type I IFN secreted (fig 5.3). Therefore, DC were infected in the presence of anti-IFNAR2, and the upregulation of CD86 was assessed. This molecule was elevated by HSV-1 infection most consistently. IFNα stimulation of DC was used as a positive control to confirm the neutralisation of IFN by this antibody. In the presence of the same concentration of anti-IFNAR2 that neutralised IFNα and the IFN activity in supHSV-DC in fig. 5.6, HSV-1 induced upregulation of CD86 was not affected (fig. 6.1). These data demonstrate that HSV-1 stimulated activation of DC directly, independent of the autocrine activity of secreted type I IFN after infection.

6.2.2 Role of viral gene expression in DC activation and inhibition

To determine the role of viral gene products in the activation of DC, HSV-1 virions were inactivated by ultraviolet (UV) light (UV-HSV). UV light disrupts viral gene expression by generating dimers between adjacent pyrimidines (Tornaletti and Pfeifer, 1996). However, the protein structure of the virions is left intact, rendering UV-HSV theoretically infective but unable to express any viral genes. Both of these properties were assessed before functional experiments were attempted.

6.2.2.1 Efficiency of UV inactivation of HSV-1

Several factors, including the viral size, structure and genome type may determine the length of time required to inactivate different viruses by UV-light (Kwan A, personal communication). The efficiency of HSV-1 inactivation was assessed in order to find the optimum time of UV light exposure. UV-inactivation (i.e. loss of viral genome integrity) was assessed by analysing the expression of GFP after infection. The time course of UV-light exposure is shown in chapter 3 (fig. 3.6C). In agreement with the time of exposure used in other studies (Paludan and Mogensen, 2001), total viral

inactivation was achieved after 20 min of UV-light exposure. This was the time used for the rest of the studies.

6.2.2.2 Binding of UV-inactivated HSV-1

It was possible that the UV light exposure could damage the ability of UV-HSV to infect DC. Therefore, the binding capacity of UV-HSV to the DC surface was compared to non-treated HSV (WT-HSV). The DC surface was stained for gD 1 hour post infection to detect bound particles and envelope gD that had fused with the DC surface membrane. Fig. 6.2A shows that UV-HSV and WT-HSV bound DC with equivalent efficiency. It remained possible that any soluble gD in the viral preparation might also have bound to DC, and generated a false positive. However, as HSV-1 entry is mediated by gB/gC attachment to heparan sulphate (HS), followed by binding of gD to entry receptors (Spear et al., 2000), it was possible to prevent the attachment step by preincubaitng HSV virions with heparin prior to infection (Herold et al., 1994). As expected, this process reduced the infection efficiency dramatically (fig. 6.2B). Therefore, to exclude the presence of significant soluble gD being the source of the false positive result in fig. 6.2A, heparin-coated HSV-1 was added to DC, and the cells then stained for gD on the surface (fig. 6.2C). This experiment showed that in the presence of heparin, gD staining was abrogated. Thus, no significant soluble gD was present in the viral prep, and the gD staining in fig 6.2A represented the degree of virion-derived gD on DC.

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6.2.2.3 Infectivity of UV-inactivated HSV

The experiments in section 6.2.2.2 only demonstrated that UV-HSV could bind to DC as efficiently as WT-HSV. It was also important to assess that UV treatment did not damage their ability to enter DC. VP16 is a tegument protein involved in maintaining the structure of virions and is also a viral gene transactivator (Wysocka and Herr, 2003). Therefore, intracellular staining of DC 2 hours post-infection for the presence of VP16 allowed an estimate of the ability of HSV virions to bind and enter DC (Aubert et al., 1999). WT-HSV infection revealed a small but consistent increase in VP16 expression in DC. The VP16 expression profile of UV-HSV infected DC 2 hours

post infection was near identical to that of WT-HSV, confirming that UV-HSV was able to infect DC with equal efficiency as WT-HSV (fig. 6.3A). VP16 staining could not be correlated with GFP expression at 2hr post-infection, as the expression of GFP was low or undetectable (fig. 3.6). However, UV-HSV infected DC did not express any GFP 16 hours after infection, confirming the efficiency of inactivation by the UV-treatment protocol (fig. 6.3B).

The data in sections 6.2.2.2 and 6.2.2.3 confirm that UV treatment of HSV-1 prevented the expression of viral genes without impairing the ability of the virus to bind and enter DC. In this way, the effects on DC of viral binding and entry, but not gene expression, could be assessed.

6.2.3 Functional effects of UV-HSV infection of DC

6.2.3.1 T cell proliferation induced by UV-HSV infected DC

Fig. 6.4A demonstrates that UV-HSV infected DC can stimulate allogeneic T cell proliferation equally well as uninfected DC, in contrast to the strong inhibition seen with WT-HSV infected DC. Therefore, HSV-1 gene products are responsible for the majority of the inhibition in DC ability to stimulate T cell proliferation.

6.2.3.2 Cell viability of UV-HSV infected DC

Another striking effect of WT-HSV infection of DC was the loss of cell viability 40 hours after infection (fig 4.8). Using the MTT reduction assay again, fig. 6.4B demonstrates that UV-HSV infected DC maintained cell viability up to 40 hours after infection, in contrast to the effects of WT-HSV. Therefore, this suggests that viral gene products were responsible for the loss of DC viability after infection.

6.2.3.3 Morphology of UV-HSV infected DC

One of the earliest changes seen with WT-HSV infection of FN-adherent DC was the loss of dendritic morphology (fig. 4.5). This was not evident after UV-HSV infection, suggesting that viral gene products were responsible for the loss of dendritic shape

(fig. 6.4C). In contrast to treatment with LPS, UV-HSV infection did not increase the number of adherent dendritic shaped DC, and did not increase the length of the dendrites.

Another noticeable effect of WT-HSV infection was resistance to further DC stimulation by LPS. Again, this was most marked in effects on morphology. DC rounded up by WT-HSV were unable to regain a dendritic shape after LPS stimulation (fig 4.5). By contrast, UV-HSV infected DC, when further stimulated with LPS, increased DC adherence to FN and dendritic shape, in a manner indistinguishable from the effects of adding LPS alone to uninfected DC (fig. 6.4C). Therefore, viral gene products were responsible not only for the loss of dendritic shape, but also for the refractory morphological response to LPS.

6.2.3.4 Surface phenotype of UV-HSV infected DC

UV-HSV infection of DC resulted in similar changes in surface phenotype to those seen with WT-HSV, upregulating CD86 (fig. 6.5) and HLA-DR (data not shown) expression. However, in contrast to WT-HSV, LPS stimulation of UV-HSV infected DC resulted in further upregulation of CD86 and MHC class I (fig. 6.5).

In summary, section 6.2.3 showed that HSV-1 gene expression is responsible for the deleterious effects of HSV-1 infection on DC, rendering DC morphology and phenotype refractory to further stimulation by LPS, and also inducing cell death and preventing efficient T cell stimulation. However, the changes in phenotype also demonstrate that viral receptor binding and/or entry into DC is a maturation stimulus and this event will be dissected further below.

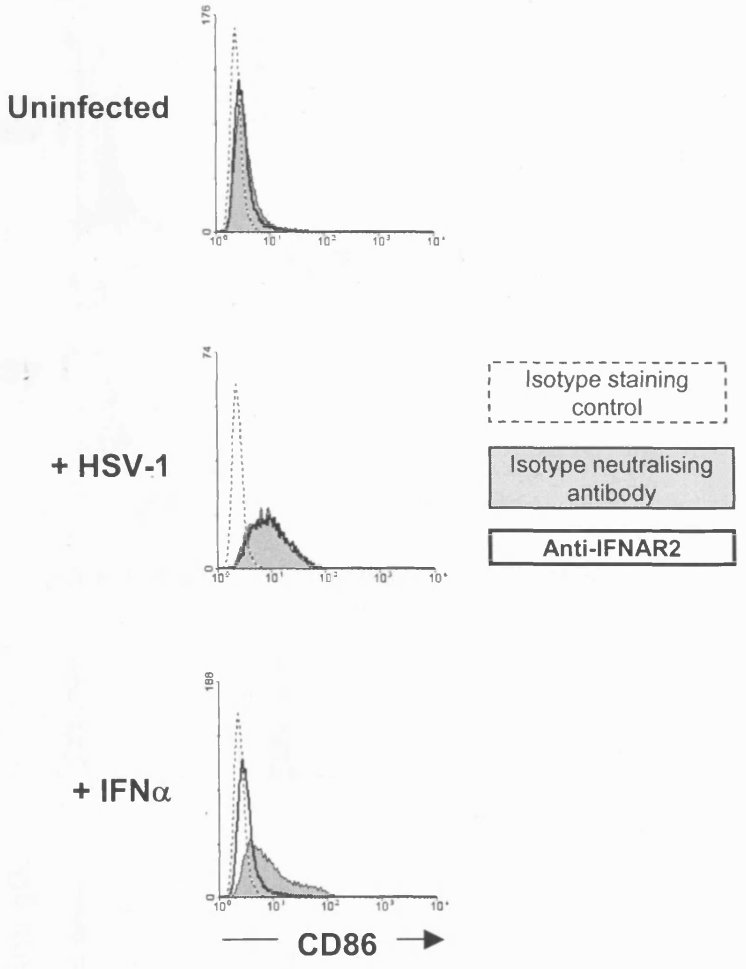

Figure 6.1 Role of autocrine type I IFN in upregulation of CD86 by HSV-1.

DC were infected with HSV-1 at MOI of 1 or treated with 1000 IU/ml IFNα in the presence of anti-IFNAR2 mAb or an isotype control antibody, and expression of CD86 assessed. WT-HSV data gated on GFP+ve DC. Representative of three independent experiments.

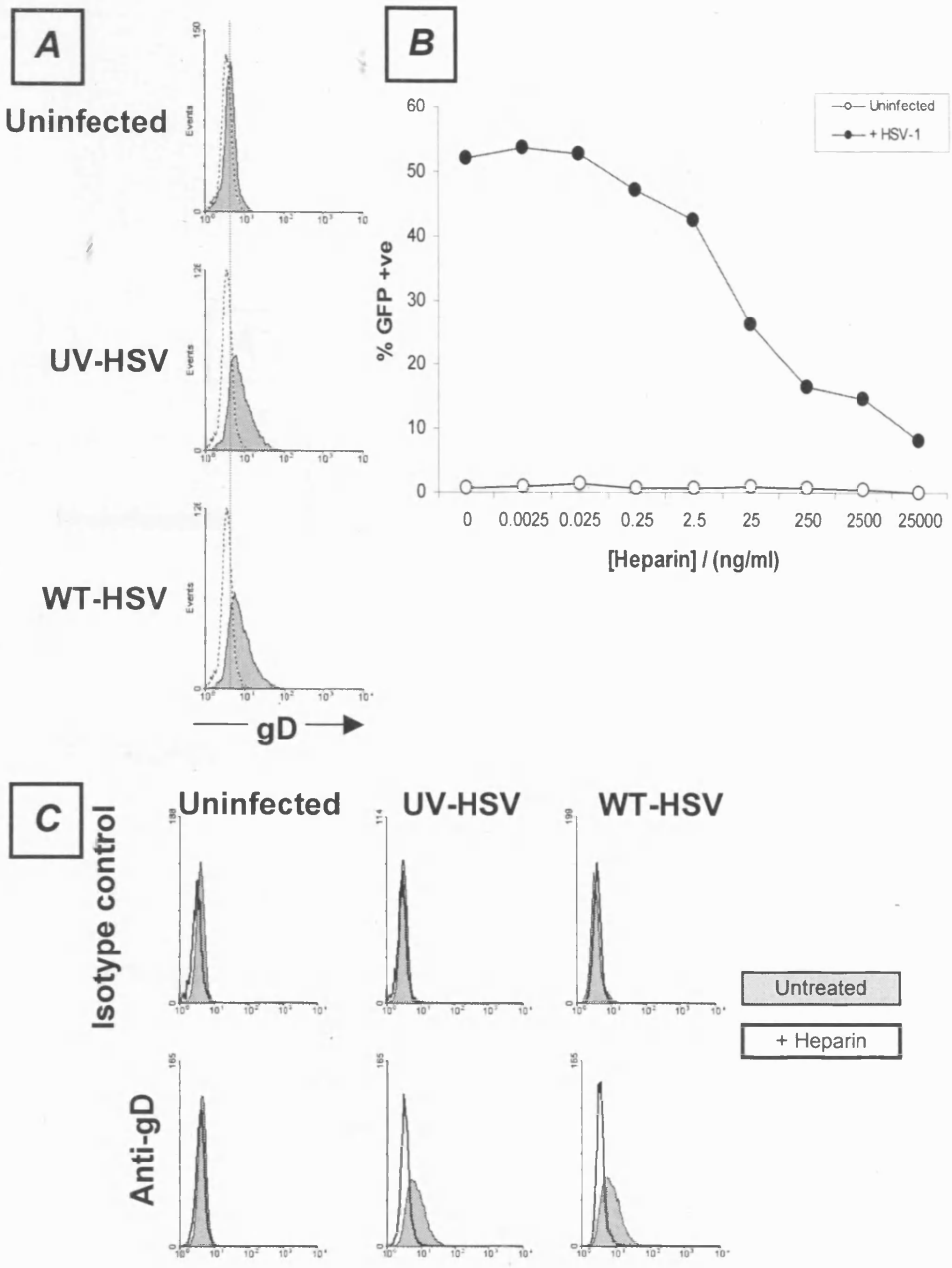

Figure 6.2 Assessment of gD on UV-HSV and WT-HSV binding to DC surface.
DC were infected with UV-HSV or WT-HSV for 1 hour and analysed for expression of gD. (B) WT-HSV was incubated with increasing concentration of heparin and used to infect DC at MOI of 1. 16 hours after infection, the percentage of DC expressing GFP was assessed. (C) UV-HSV was incubated in the presence or absence of 12.5 μg/ml of heparin before infecting DC at MOI of 1 for 1 hour, and the surface expression of gD analysed. All figures are representative of three independent experiments.

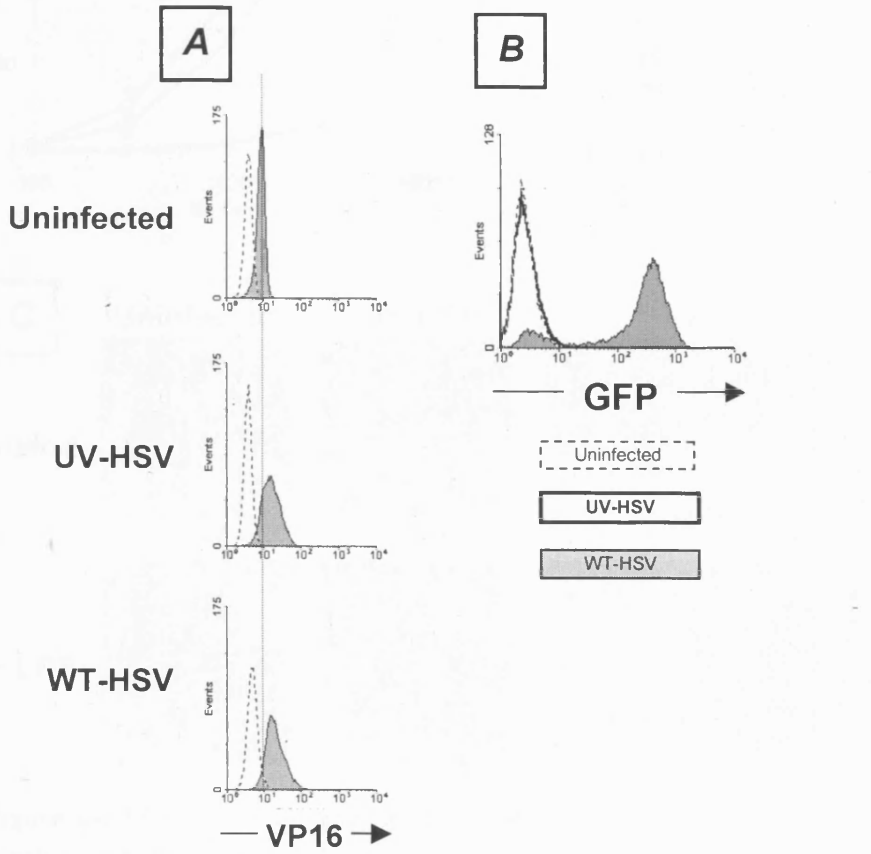

Figure 6.3 Assessment of infectivity and inactivation of UV-HSV.
(A) DC were infected with UV-HSV or WT-HSV (MOI =1) for 2 hours and stained for intracellular HSV-1 VP16. (B) DC infected with UV-HSV or WT-HSV (MOI =1) were analysed for GFP expression 16 hours after infection. All figures are representative of at least three independent experiments.

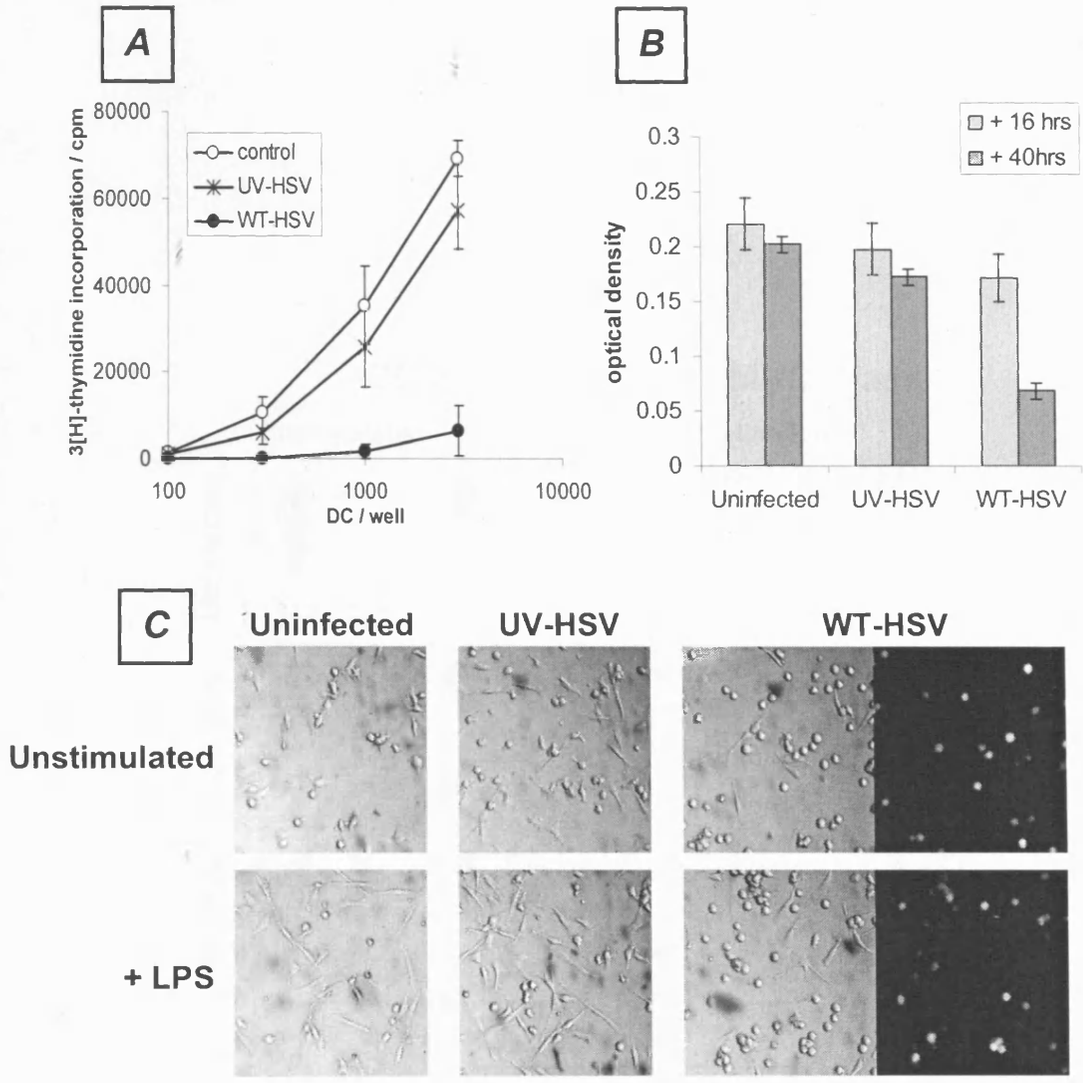

Figure 6.4 Effects of viral gene expression on HSV-1 mediated changes in DC function, viability, and morphology.

(A) DC infected with UV-HSV or WT-HSV (MOI=1) were used to stimulate allogeneic T cell proliferation. Data are mean ^{3}H-thymidine incorporation of three independent experiments. Error bars represent SEM. * denotes $p<0.01$ between WT-HSV and either UV-HSV or uninfected DC. (B) Viability of DC infected with UV-HSV or WT-HSV was analysed by MTT reduction assay 16 and 40 hours after infection. Data shown as mean optical density of four independent experiments. Error bars represent SEM. * denotes $p<0.01$. (C) DC adherent to fibronectin-coated glass coverslips were infected with UV-HSV or WT-HSV in the presence or absence of 100 ng/ml LPS. Morphology of DC was assessed 8 hours after infection. Phase contrast and GFP fluorescence displayed in separate panels for WT-HSV. Representative fields of at lease three independent experiments.

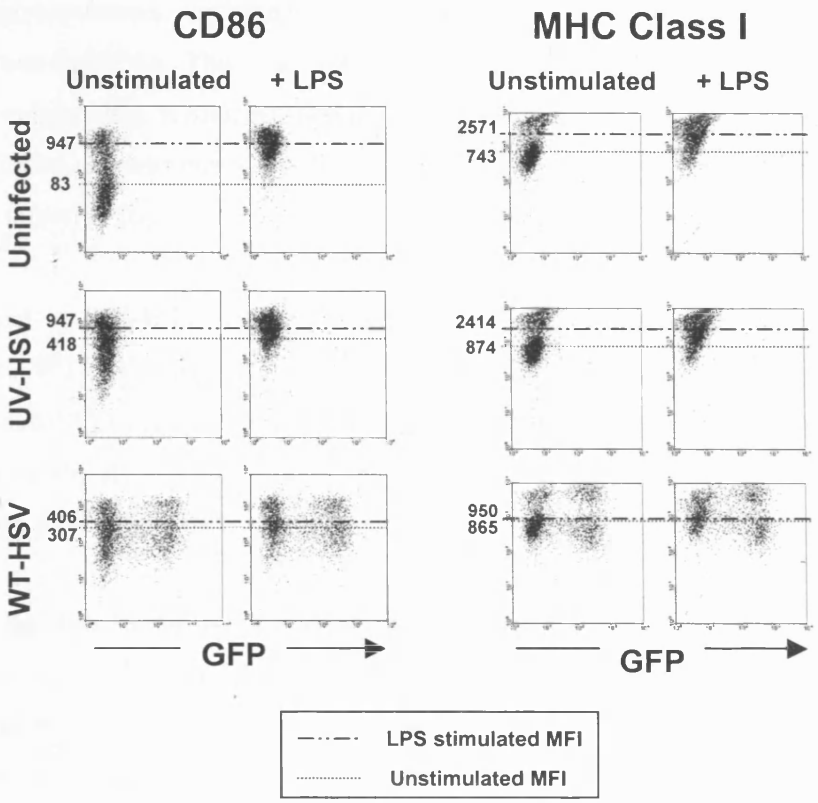

Figure 6.5 Effects of viral gene expression on HSV-1 mediated changes in DC phenotype.

DC infected with UV-HSV or WT-HSV for 16 hours in the presence or absence of 100 ng/ml LPS were analysed for expression of surface molecules. Numbers on dot plot represent MFI. Representative of three independent experiments.

6.2.4 Role of HSV binding to DC surface

The data in section 6.2.3 isolated the DC activation event as occurring prior to gene expression. This could have resulted either from virus binding to the DC surface, or from post entry events. To dissect these two possibilities, WT-HSV particles were modified further by formaldehyde fixation (FIX-HSV). This treatment cross-links the envelope glycoproteins, rendering them unable to fuse with the cell membrane and therefore non-infective. This was confirmed by the lack of GFP expression after overnight culture (fig. 6.6A). However, the virus' ability to bind to the DC surface remained intact, as determined by the presence of HSV-1 gD on the surface of DC 1 hour after infection (fig. 6.6B).

DC exposed to FIX-HSV upregulated the surface molecules, CD86 and HLA-DR similarly to WT-HSV (fig. 6.7A and 6.9+10+11). Furthermore, like UV-HSV, FIX-HSV infected DC were not refractory to further LPS stimulation, again in contrast to the effects of WT-HSV, as CD86 expression could be further elevated by simultaneous pulsing with LPS (fig. 6.7A).

Although the data in fig. 6.7A suggest that viral interaction with the DC surface is sufficient to induce DC maturation, the autocrine activity of type I IFN cannot be ruled out because the inhibitory effects of viral genes on type I IFN signalling are absent during FIX-HSV exposure (Yokota et al., 2001; Mossman et al., 2001; Yokota et al., 2004; Chee and Roizman, 2004). Therefore, DC 'infected' with FIX-HSV were treated with anti-IFNAR2 in the same way as in fig. 6.1, in order to determine the role of autocrine type I IFN in FIX-HSV induced DC maturation. Interestingly, the upregulation of CD86 by FIX-HSV was abrogated to a small degree by neutralising the activity of type I IFN (fig. 6.7B). These data suggest that type I IFN is secreted by DC stimulated with FIX-HSV, but that this cytokine is not wholly responsible for the mature phenotype of infected DC and that the cell receives a significant signal by virus binding to its surface.

In order to exclude that the changes in DC phenotype in fig. 6.7 occurred as a result of contaminants in the viral treatment protocol, DC were exposed to equivalent

concentrations of fixative and neutralising agent (formaldehyde and sodium bisulphite) in the absence of virus. This did not result in changes in CD86 expression (fig. 6.8 – upper row), in contrast to upregulation observed in the presence of WT-HSV (RPMI +/- sodium bisulphite groups) or FIX-HSV (formaldehyde + sodium bisulphite group) (fig. 6.8 – lower row).

6.2.5 Signalling consequences of HSV-1 binding to DC surface

6.2.5.1 Activation of signalling pathways by HSV-1

If HSV-1 binding to the cell surface alone was responsible for the increased expression of CD86 and HLA-DR, it was likely that this event was inducing the activation of signalling pathways inside the DC. As detailed in chapter 5, two effector pathways involved in the upregulation of CD86 are NF-kB and p38 MAPK. Although, HSV-1 infection can initiate NF-kB and p38 MAPK activation in other cell types (Patel et al., 1998; Zachos et al., 1999), this has not been demonstrated to result from early viral interaction with receptors on the cell surface.

Western blotting showed that WT-HSV infection of DC induced significant IkBα degradation within 30 minutes of infection (fig. 6.9A and data not shown). WT-HSV also induced significant phosphorylation of p38 after infection (fig. 6.9A) and this was quantified relative to total p38 by densitometry (fig. 6.9B). Interestingly, LPS mediated greater IkBα degradation and p38 activation than WT-HSV, correlating with the degree of maturation seen with LPS relative to the (sub-maximum) maturation observed following HSV-1 infection (fig. 4.12).

6.2.5.2 Role of p38 MAPK in HSV-1 induced DC activation

It was important to determine the significance of the activation of these proinflammatory pathways on DC activation by HSV-1. This can be carried out efficiently in vitro by preventing activation of the pathways described above and subsequently infecting DC with HSV-1. To study specifically the role of activation of these pathways emanating from the cell surface interaction with HSV-1, DC were

stimulated only with FIX-HSV in these assays. Other studies have shown that p38 and NF-kB can be activated at later times after infection with HSV-1 (Patel et al., 1998; Zachos et al., 1999), which would be difficult to distinguish from the effects of DC receptor ligation after infection.

Unfortunately, no inhibitor of NF-kB activity was available for these studies. On the other hand, the inhibitor SB203580, which prevents the kinase activity of phosphorylated p38, was used (Tong et al., 1997). DC treated with 100 ng/ml LPS were used as a positive control in these assays to ensure that the inhibitor prevented p38 MAPK activity sufficiently to observe effects on DC phenotype. Fig. 6.9C shows that 3μM SB203580 was able to partly prevent the upregulation of CD86 and HLA-DR following LPS stimulation (same data as in fig. 5.8). In parallel cultures, the upregulation of expression of these molecules on DC after FIX-HSV exposure was only prevented to a small degree. Therefore, these experiments demonstrate that p38 MAPK activation by viral interaction with DC surface receptors plays a minor role in the acquisition of a more activated phenotype seen with these DC. The implication and technical limitations of this experiment are considered in more detail in the discussion in section 6.3.3.3.

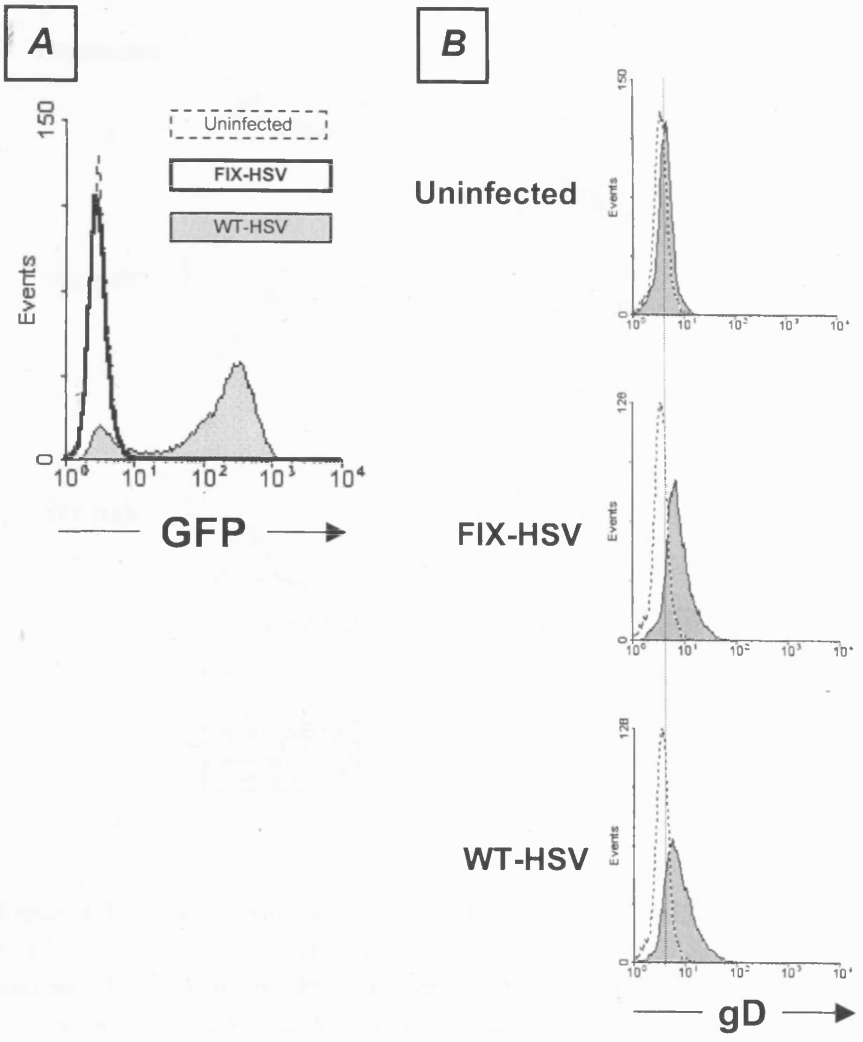

Figure 6.6 Inactivation and cell surface binding capacity of FIX-HSV.
(A) DC infected with FIX-HSV or WT-HSV were analysed for GFP expression 16 hours after infection. (B) DC infected with FIX-HSV or WT-HSV for 1 hour were analysed for expression of gD. WT-HSV data gated on GFP+ve DC. All figures are representative of three independent experiments.

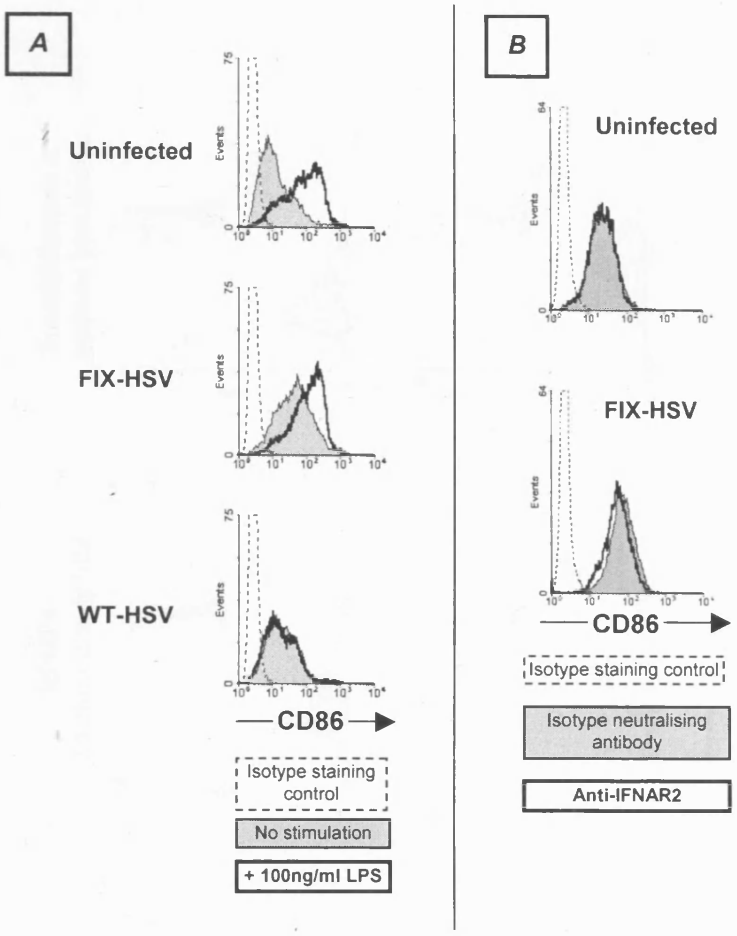

Figure 6.7 Surface phenotype changes on DC exposed to FIX-HSV.
(A) DC infected with FIX-HSV or WT-HSV for 16 hours in the presence or absence of (A) 100 ng/ml LPS or (B) anti-IFNAR-2 antibody were analysed for expression of CD86. WT-HSV data gated on GFP+ve DC.

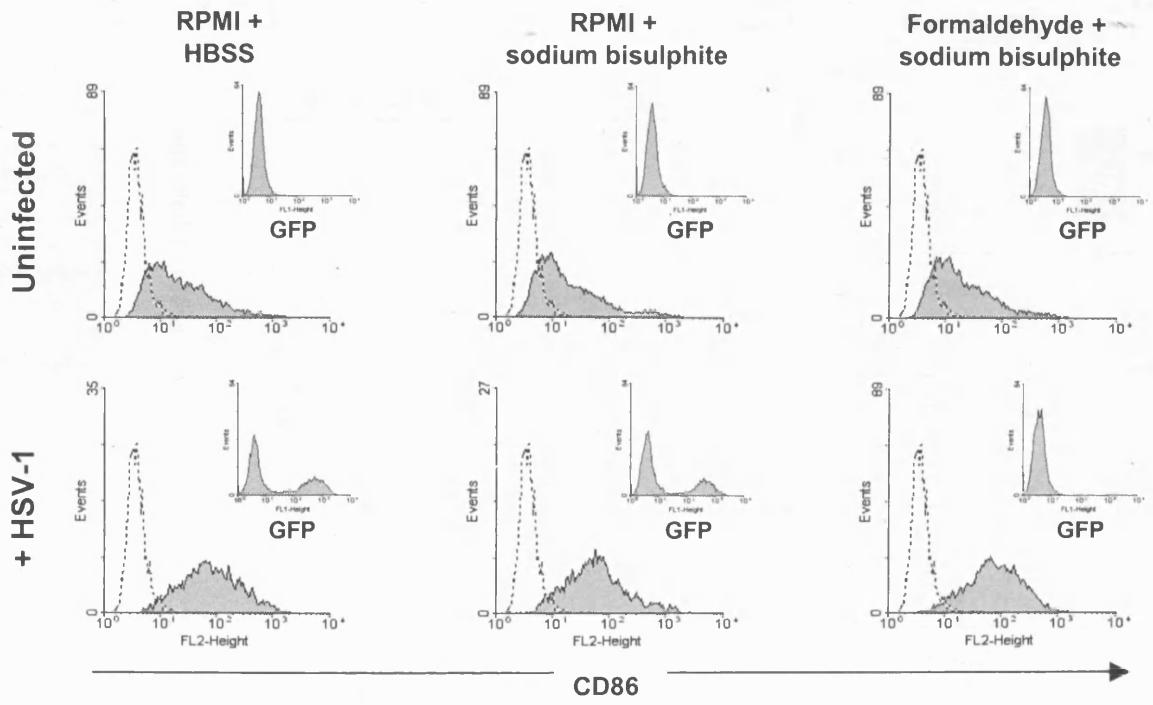

Figure 6.8 Effects of virus inactivation protocol on DC CD86 expression.
RPMI or HSV-1 were treated with various combination of RPMI or 3.8% formaldehyde, and HBSS or 17.5% sodium bisulphite prior to 'infection' of DC, and cultured for further 16 hours. Expression of CD86 (main panel) and GFP (insert) were assessed. All figures are representative of at least three independent experiments.

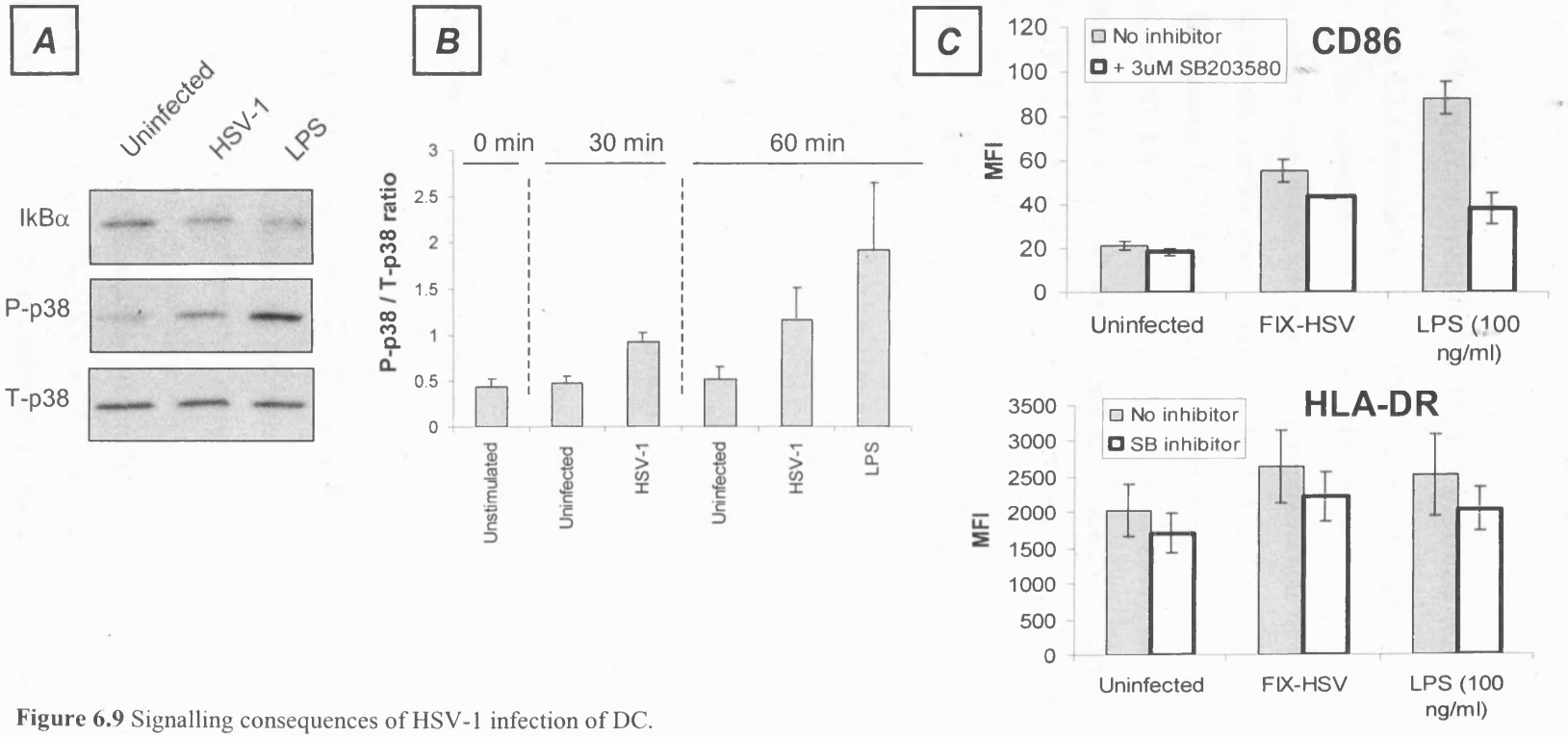

Figure 6.9 Signalling consequences of HSV-1 infection of DC.

(A) MDDC were infected with WT-HSV (MOI = 3) or stimulated with 100 ng/ml LPS for 60 minutes. Cell lysates were harvested and assayed by Western blot for IkBα, phospho-p38 and total p38. Representative of three independent experiments. (B) Data in (A) presented as ratio between band intensity of phospho- and total-p38 MAPK. Results are mean of three independent experiments. Error bars represent SEM. (C) DC were cultured in the presence or absence of 3μM SB203580 for 24 hours prior to stimulation with FIX-HSV or 100 ng/ml LPS. DC were cultured for a further 16 hours in the presence of SB203580 prior to assessment of CD86 and HLA-DR exposure. Results are mean of three independent experiments. Error bars represent SEM.

6.2.6 Receptors involved in HSV-1 induced activation of DC

Section 6.2.5 demonstrated that HSV-1 is able to ligate surface receptors on DC and induce the activation of p38 and NF-kB. An attempt to identify the receptors responsible was made using antibody neutralisation of candidate molecules. These included the known entry receptors, HVEM and nectin-1 (Spear et al., 2000) and TLR2, because HSV-1 may signal via this receptor (Kurt-Jones et al., 2004). HVEM activates NF-kB on some cells (Marsters et al., 1997), and TLR2 ligation can induce maturation of DC via NF-kB and p38 activation (Michelsen et al., 2001; Hertz et al., 2001; Re and Strominger, 2001). Fig. 3.4 (and data displayed again in figs. 6.11+12) documented that the two entry receptors are expressed on DC. The optimum amount of antibody used to neutralise the receptors was determined by utilising a saturating amount of antibody for surface staining. This approach was applied also for TLR2 and its expression is displayed in fig. 6.10A.

A caveat about such neutralisation experiments is that antibodies may not bind to ligand-binding sites, and thus may not prevent signal transduction. With regards to TLR2, this ambiguity was minimised by using a positive control TLR2 ligand, PGN, the activity of which can be partially neutralised in DC by the mAb used in these studies (Uehori et al., 2003). For the entry receptors, neutralisation of virus infection served as a useful indicator of prevention of viral ligand binding, the step that could be responsible for cell surface-derived signalling.

6.2.6.1 Role of TLR2 in DC activation by HSV-1

TLR2 is not known to be an entry receptor for HSV-1. This was confirmed in fig. 6.10B, which showed that this receptor is not essential for infection of DC, as determined by antibody blocking and subsequent GFP expression. When DCs were coated with anti-TLR2 antibody prior to HSV-1 infection, the upregulation of CD86 or HLA-DR by either FIX-HSV or WT-HSV was not prevented (fig 6.10C). This antibody also did not prevent maturation induced by the positive control PGN, which raises doubts about the ability of the mAb used for these experiments to prevent TLR2

ligand binding and signal transduction (fig. 6.10C). By extension, it is also difficult to conclude convincingly that HSV-1 does not activate DC via TLR2 ligation.

6.2.6.2 Role of nectin-1 in DC activation by HSV-1

There are many antibodies raised to nectin-1, but the clone used for these studies, CK41, can prevent infection of HSV-1 in some cell types (Krummenacher et al., 2000). However, saturating amounts of this antibody did not prevent HSV-1 infection of DC (fig. 6.11B). Furthermore, the antibody to nectin-1 did not prevent upregulation of CD86 or HLA-DR induced by either FIX-HSV or WT-HSV (fig. 6.11C). The saturating amounts of this antibody used in these studies should have ensured that HSV-1 could not interact with this receptor. However, the lack of neutralisation of infection questions the firm conclusion that all interactions with this receptor by the virus ~~was~~ were abrogated. Thus, while it appears unlikely that nectin-1 alone plays a significant role in DC maturation, this cannot be totally excluded by these data.

6.2.6.3 Role of HVEM in DC activation by HSV-1

There are few, if any, mAb to HVEM that have been demonstrated previously to neutralise infection. The "neutralising" one that was tested in this system, was not able to prevent infection (fig. 6.12A). Therefore, a neutralising rabbit polyclonal serum to HVEM was used to prevent HSV-1 infection by this receptor (Montgomery et al., 1996). The advantage of this approach was that the polyclonal nature made it more likely that all potential receptor ligation sites were covered, though the downside would be that any effect would make the binding site on the receptor difficult to map out directly or accurately.

Incubation of DC with anti-HVEM sera prior to HSV-1 exposure did reduce infectivity to a small degree (fig. 6.12B), consistent with previous reports (Salio et al., 1999). However, analogous to nectin-1, HVEM neutralisation did not prevent maturation by either FIX-HSV or WT-HSV (fig. 6.12C). As for nectin-1, the absence of a suitable positive control to check receptor neutralisation, and the inability to prevent infection significantly makes it difficult to exclude this receptor firmly from a role in DC

maturation by HSV-1. However, the partial neutralisation of infection suggested that saturating quantities of antibody were used, which could prevent virus-HVEM interaction and that the infected cells were likely to reflect redundancy in receptor usage (Krummenacher et al., 2004). Thus, the data in fig. 6.12 suggests that ligating HVEM is not essential for maturation of DC by HSV-1.

6.2.7 Receptor internalisation during infection process

The absent or incomplete neutralisation with TLR2, nectin-1 and HVEM antibodies seen in section 6.2.6 could have been due to the neutralised receptors undergoing rapid internalisation in the steady state, consequently exposing a large number of non-neutralised receptors for the virus to bind. The presence of "neutralising" antibody-bound receptors on infected cells would suggest that those cells had been infected independently of that receptor and its downstream signalling. Therefore, DC were stained 16 hours after infection with anti-mouse IgG (for TLR2 and nectin-1) or anti-rabbit IgG (for HVEM), to assess the expression of the receptors on infected DC that had originally been coated with antibody. While the expression of TLR2 and nectin-1 was not detectable at this time (fig. 6.13A), rabbit IgG bound HVEM was still present, both in uninfected and infected DC (fig. 6.13B). This excluded preferential downregulation of this entry receptor after infection, as previously described for some other viruses (Crise et al., 1990; Welstead et al., 2004), but also confirmed that DC had been infected despite the expression of saturating quantities of antibody. As this was a polyclonal antibody with neutralising capacity, this suggested that virus binding to HVEM was not essential in DC maturation by HSV-1.

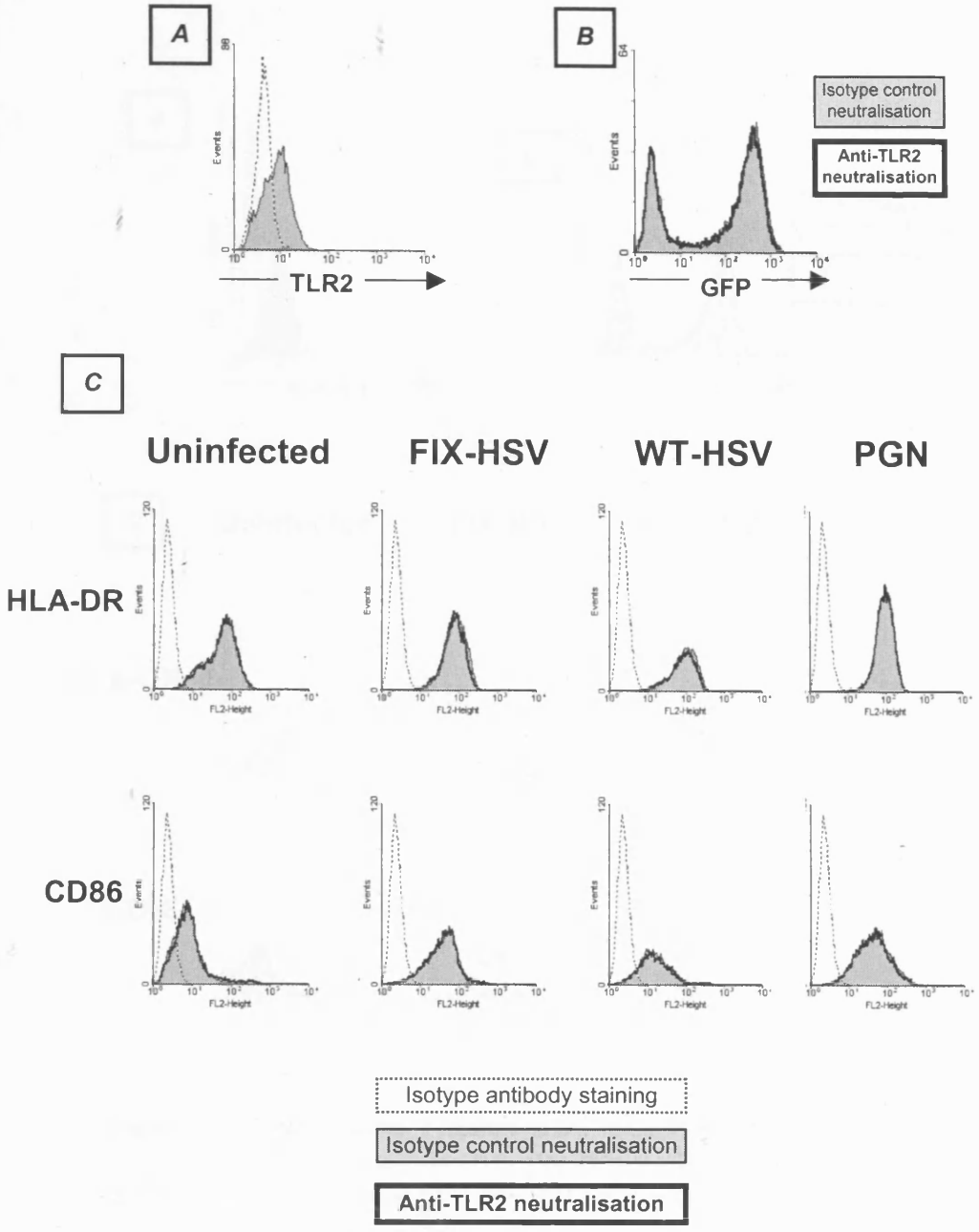

Figure 6.10 Effects of TLR2 ligation on DC stimulation by HSV-1.
(A) Expression of TLR2 on DC. (B) DC pre-treated with anti-TLR2 antibody were infected with WT-HSV and GFP expression assessed after 16 hours. (C) DC were pre-treated in the presence or absence of anti-TLR2 antibody and then stimulated or infected with FIX-HSV, WT-HSV or PGN. DC were cultured for further 16 hours, and the expression of HLA-DR and CD86 assessed. All figures are representative of at least three independent experiments.

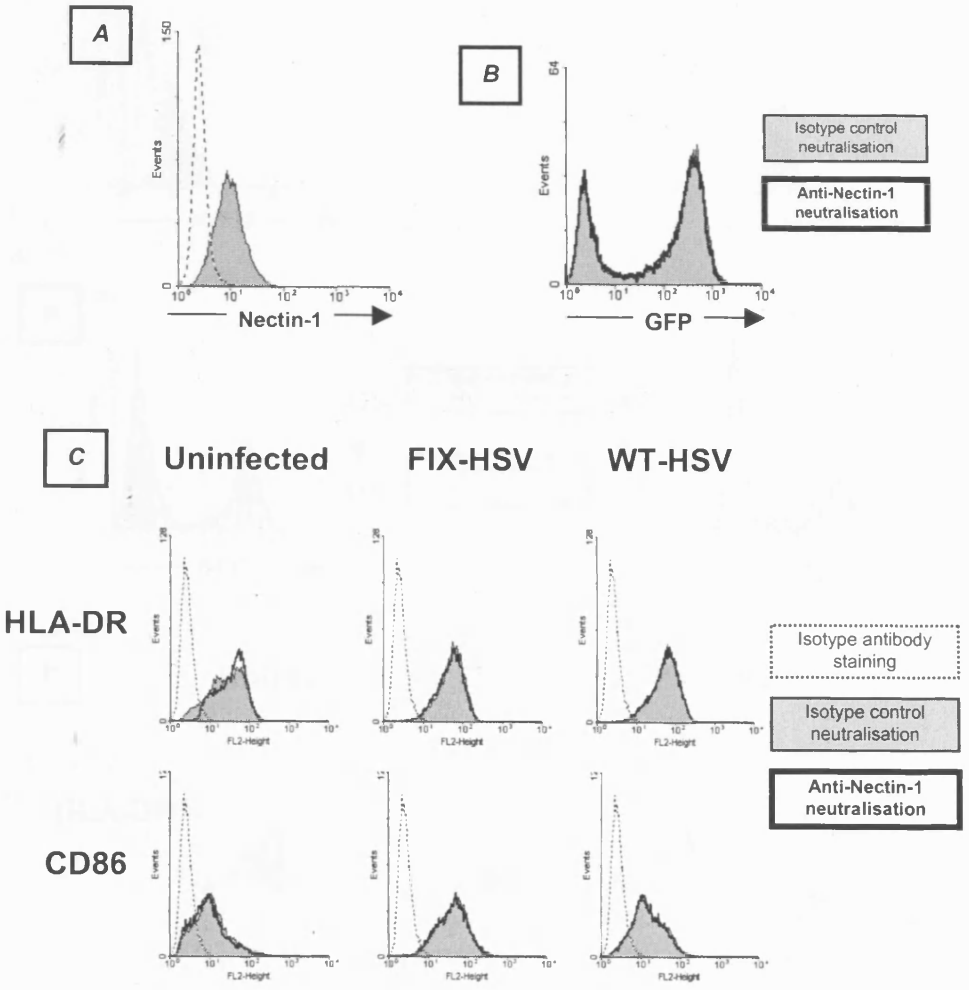

Figure 6.11 Effects of nectin-1 ligation on DC stimulation by HSV-1.
(A) Expression of nectin-1 on DC. (B) DC pre-treated with anti-nectin-1 antibody were infected with WT-HSV and GFP expression assessed after 16 hours. (C) DC were pre-treated in the presence or absence of anti-nectin-1 antibody and then stimulated or infected with FIX-HSV or WT-HSV. DC were cultured for further 16 hours, and the expression of HLA-DR and CD86 assessed. All figures are representative of at least three independent experiments.

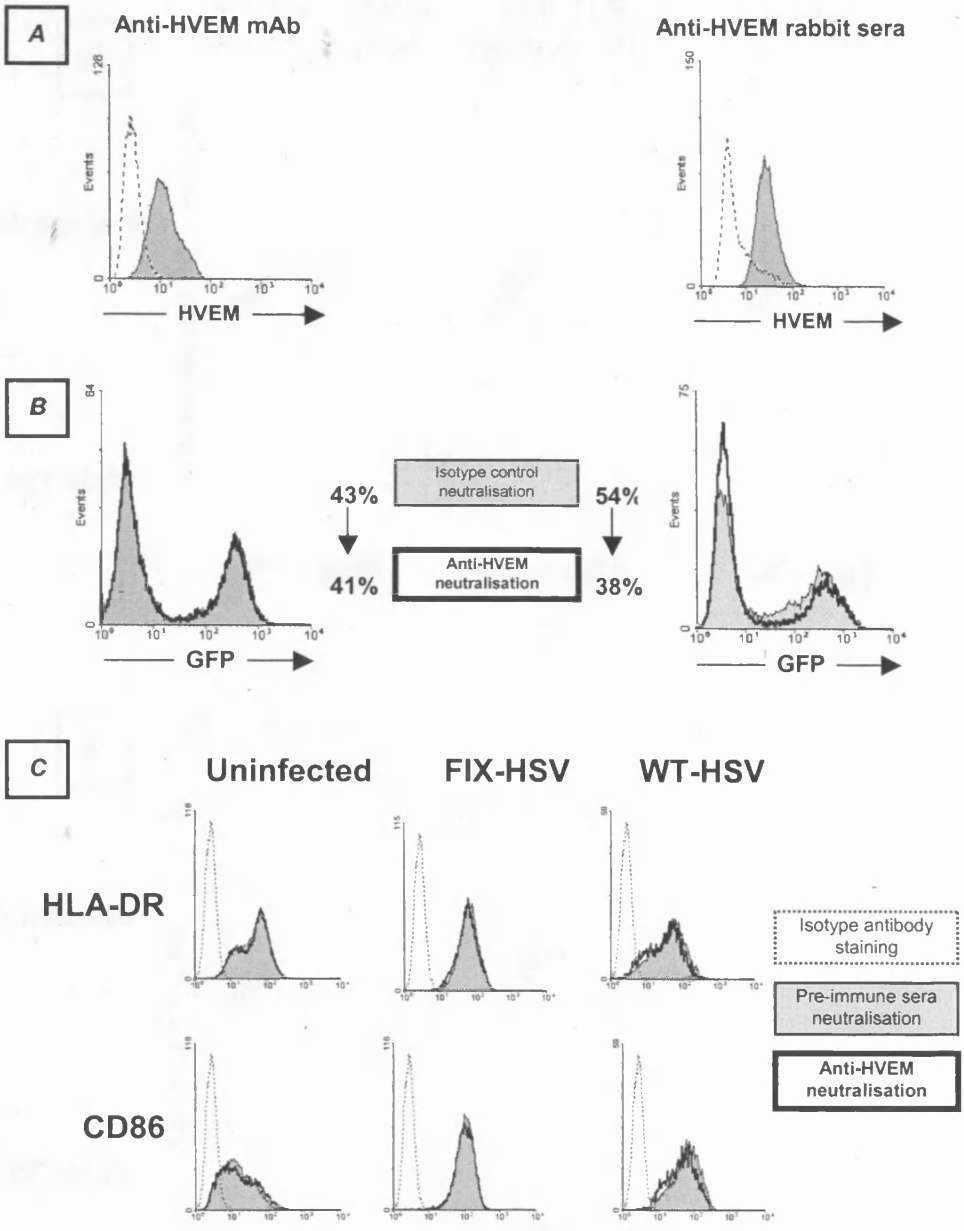

Figure 6.12 Effects of HVEM ligation on DC stimulation by HSV-1.
(A+B) Left panel anti-HVEM mAb, right panel anti-HVEM antisera (A) Expression of HVEM on DC. (B) DC pre-treated with anti-HVEM antibody or antisera were infected with WT-HSV and GFP expression assessed after 16 hours. (C) DC were pre-treated in the presence or absence of anti-HVEM antisera, and then stimulated or infected with FIX-HSV or WT-HSV. DC were cultured for further 16 hours, and the expression of HLA-DR and CD86 assessed. All figures are representative of at least three independent experiments.

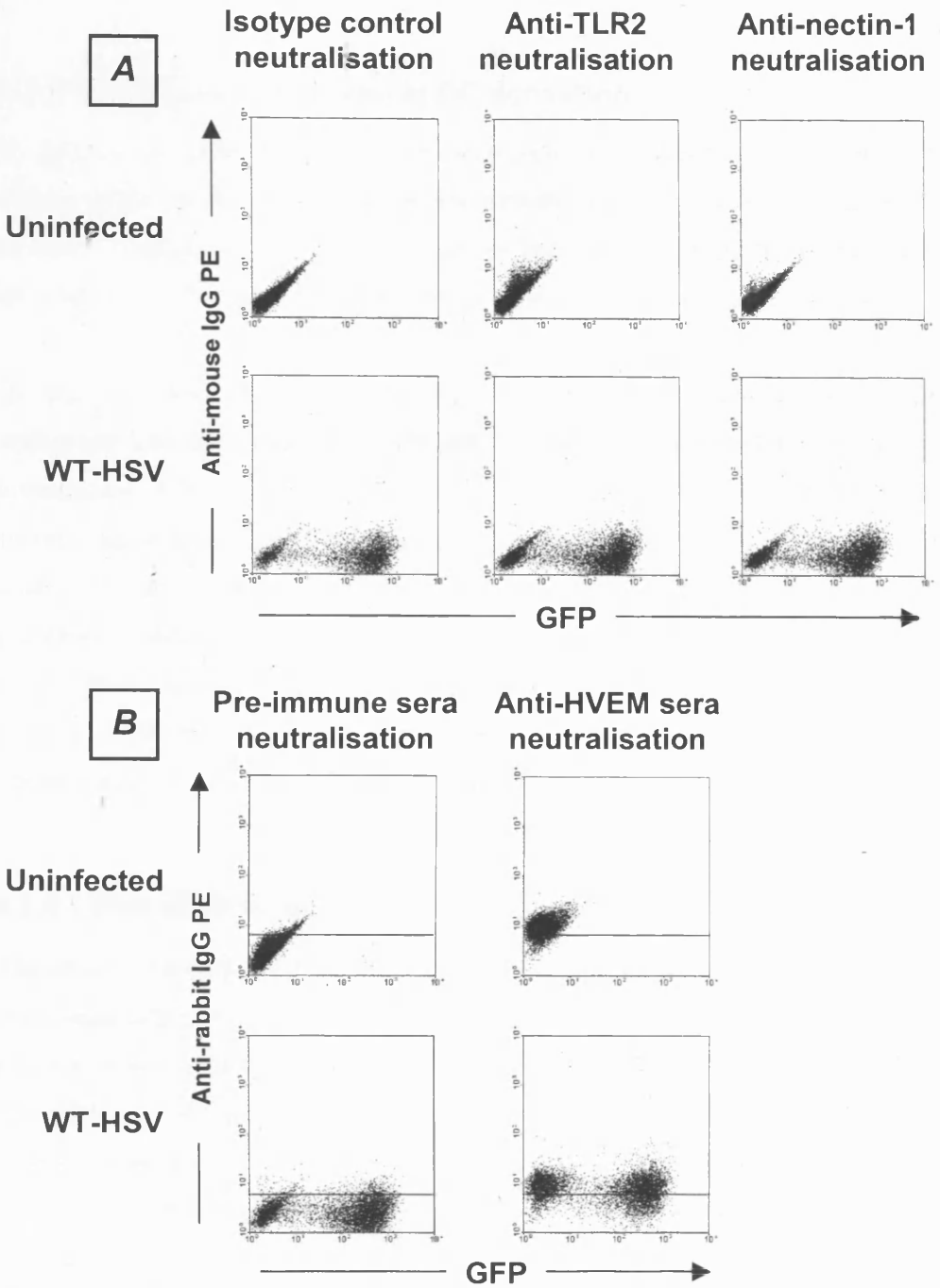

Figure 6.13 Internalisation of TLR2, Nectin-1 and HVEM on DC.
DC were pre-treated with anti-TLR2 mAb, anti-Nectin-1 mAb or anti-HVEM antisera, and cultured for 16 hours and then stained for the presence of antibody on the surface using PE-conjugated goat anti-mouse Ab (for TLR2 and Nectin-1) or PE-conjugated goat anti-rabbit Ab (for HVEM). All panels are representative of at least two independent experiments.

6.2.8 Viral ligands involved in DC activation

In light of the difficulty in defining the receptor(s) mediating DC activation, focus shifted towards defining the viral ligands, with the hope that candidate receptors would be easier to define in this way; and, that, perhaps addition of purified viral ligands to DC could provide a more robust system to identify the activatory receptor(s).

Having established that HSV-1 binding to the DC surface induces a degree of DC maturation and the activation of p38 and NF-kB pathways, envelope structures were investigated. HSV-1 entry into cells is mediated by gB and gC attachment to cell surface HS, followed by gD mediated entry via one of the entry receptors (Spear et al., 2000). The most likely ligand was gD, due to its critical role in binding surface receptors or entry into cells and its capacity to activate NF-kB in cells directly (Spear et al., 2000; Medici et al., 2003). Without ready access to viruses not expressing functional gD on their envelope, the role of gD in DC activation was determined by neutralisation of HSV-1 with a mAb to gD (clone LP2).

6.2.8.1 Role of gD in upregulation of CD86

The ability of LP2 to neutralise the infection of DC was assessed by measuring GFP expression after DC "infection" with neutralised virus. Fig. 6.14A shows that GFP expression was totally abrogated if HSV-1 was neutralised by gD. Treating UV-HSV, FIX-HSV and WT-HSV in this way abrogated the stimulus for the upregulation of CD86 (fig. 6.14B). The effect on FIX-HSV confirmed that extracellular binding of gD to a cell surface receptor was a sufficient activating signal to induce partial DC maturation seen following HSV-1 infection. To exclude the possibility that the presence of antibodies on the viral envelope could sterically hinder the interaction of viral ligands with the cell surface, HSV particles were also pre-coated with a non-neutralising anti-gD monoclonal antibody (AP7 clone) prior to addition to DC. As determined by GFP expression, this antibody did not prevent infection to a significant degree, in contrast to LP2 (fig. 6.15A). Furthermore, staining for the presence of mouse IgG on the surface of DC 16 hours after infection demonstrated that DC infected with AP7-coated HSV (i.e. the GFP+ve population), expressed mouse IgG on

the cell surface. This is likely to have originated from non-neutralised viral envelope that fused with the DC membrane.

DC exposed to FIX-HSV coated in AP7 demonstrated an equally mature phenotype as virus treated with an isotype control antibody, in contrast to the neutralising effect of LP2 (fig. 6.15B). Thus figs. 6.14 and 6.15 demonstrate that the ability of neutralising anti-gD antibodies to prevent DC maturation is likely to occur by preventing specific sites on gD from interacting with activatory surface receptors, rather than through non-specific steric effects or through interacting with inhibitory FcγR on DC.

The neutralisation of gD did not exclude the role of other viral structures in the activation of DC totally. Two of these are gB and gC, which attach HSV-1 virions to HS on the DC surface (Spear et al., 2000). It was possible that gD neutralisation also prevented HS binding and that this interaction was an important activation signal in DC, as suggested for HCMV infection (Song et al., 2001). To assess whether gD-neutralised virions could attach to DC, the binding of these opsonised virions was assessed by flow cytometry, by staining DC 1 hour post infection with a PE-conjugated anti-mouse IgG antibody. As seen in fig. 6.16, significant signal could be detected when neutralised virions were added to DC. To determine the mechanism of their binding, opsonised virions were also incubated with 12.5 μg/ml of heparin, prior to addition to DC and flow cytometry analysis. Fig. 6.16 clearly shows that the cell surface binding was sensitive to the heparin step, demonstrating that gD neutralisation of HSV-1 permitted virion attachment to HS on the cell surface and that attachment per se is not an activatory signal in DC.

6.2.8.2 Role of gD in type I IFN secretion by DC

Similar to the changes in surface phenotype, gD-neutralisation of WT-HSV with LP2 also abolished DC secretion of type I IFN (Fig 6.17). Equivalent IFN secretion to that seen with WT-HSV was also observed in 5 out of 7 individuals' DC infected with UV-HSV, and in 2 out of 3 individuals' DC exposed to FIX-HSV. Therefore, HSV-DC surface interaction is important for inducing IFN secretion, and viral envelope gD plays an important role in inducing this effect.

6.2.8.3 Role of gD in bystander DC activation

Chapter 5 confirmed that type I IFN was the only secreted factor that could mediate bystander activation of DC. However, the immunostimulatory property of gD described above, combined with the high level of expression of gD on the surface of infected DC (fig. 3.7), made it possible that direct contact between infected and uninfected DC could also have played a role in the maturation of GFP-ve DC (fig. 4.12). Therefore, DC were infected with HSV-1 and then cultured in the presence of anti-gD antibodies (LP2 clone), in order to neutralise the activity of the gD expressed on infected DC. It was important to determine that sufficient antibody was added to neutralise all gD activity, and this was carried out in two ways. Firstly, DCs were stained 16 hours after infection with an anti-mouse IgG antibody, in order to confirm that all cells expressing gD had indeed bound the antibody (fig. 6.18A). The expression of gD in this way was similar to that seen in fig. 3.7, suggesting that all gD-expressing cells were saturated with antibody. Secondly, virus production was determined by viral infectivity assay of the supernatant of these cells. This showed that HSV-1 in the supernatant was neutralised totally, confirming that sufficient unbound antibody in the medium was present to prevent secondary stimulation of GFP-ve DC by gD on HSV-1. In this experimental system, the expression of CD86 and HLA-DR on GFP-ve DC was not reduced when infected DC were cultured in the presence of anti-gD mAb, excluding that bystander uninfected DC were significantly activated by direct contact with gD expressing infected DC (fig. 6.18B).

6.2.9 Direct activation of DC by gD

Finally, to determine whether gD was solely responsible for activating DC, cells were exposed to a recombinant form of gD and the changes in surface phenotype assessed. The gD used in these studies was derived from a baculovirus system, which produces proteins analogous to mammalian-produced forms of gD, as determined by reactivity to various mAb that recognise discontinuous epitopes of gD (Chiang et al., 1994; Rux et al., 1998). The gD used in these studies was a truncated form, gD(285t), missing 21 amino acids from the C-terminal (Rux et al., 1998). Although this deletion does not

alter the structure drastically relative to full length gD, this gD variant has a higher affinity for HVEM and nectin-1 (Rux et al., 1998),(Krummenacher et al., 1998) and may have been more likely to elicit an effect on DC. The data demonstrated a dose dependent increase in the expression of CD86 (fig. 6.19). Although it is difficult to make a direct comparison between the concentration of dimeric gD added in solution and envelope associated glycoprotein, it was noticeable that even 50μg/ml of gD did not upregulate CD86 to the maximal levels observed with LPS stimulation, analogous to the effects of WT-HSV, or more relevantly, FIX-HSV. If the purity of the protein preparation can be confirmed, then these experiments are confirmation that gD alone can activate DC, although not excluding that other envelope glycoproteins may improve the efficiency of this activation or are themselves stimulatory ligands. These issues are discussed in more detail below.

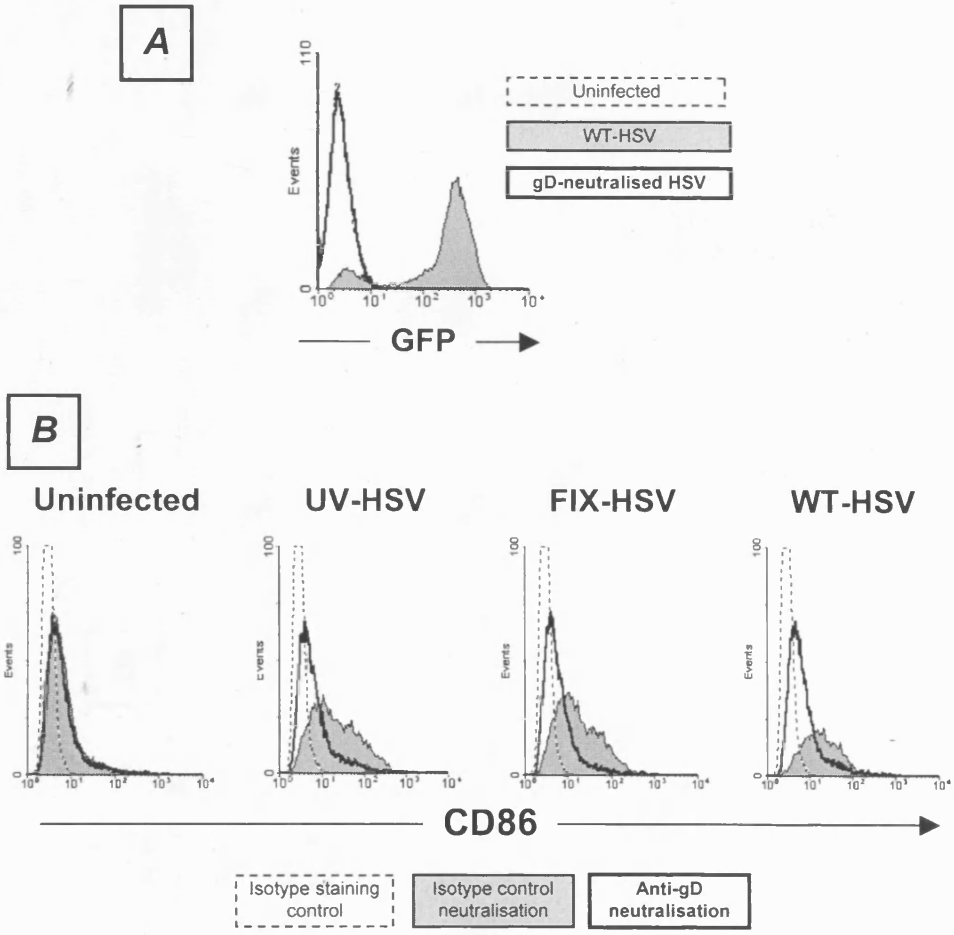

Figure 6.14 Role of gD interaction with DC on CD86 expression.
WT-HSV was neutralised with anti-gD mAb (LP2 clone) and used to infect DC (MOI = 3). DC were cultured for 16 hours and GFP expression assessed. Representative of three independent experiments. (B) UV-HSV, FIX-HSV or WT-HSV were neutralised with anti-gD mAb (LP2 clone) and then used to infect DC for 16 hours. Expression of CD86 was analysed by flow cytometry. WT-HSV data gated on GFP+ve DC. Representative of three independent experiments.

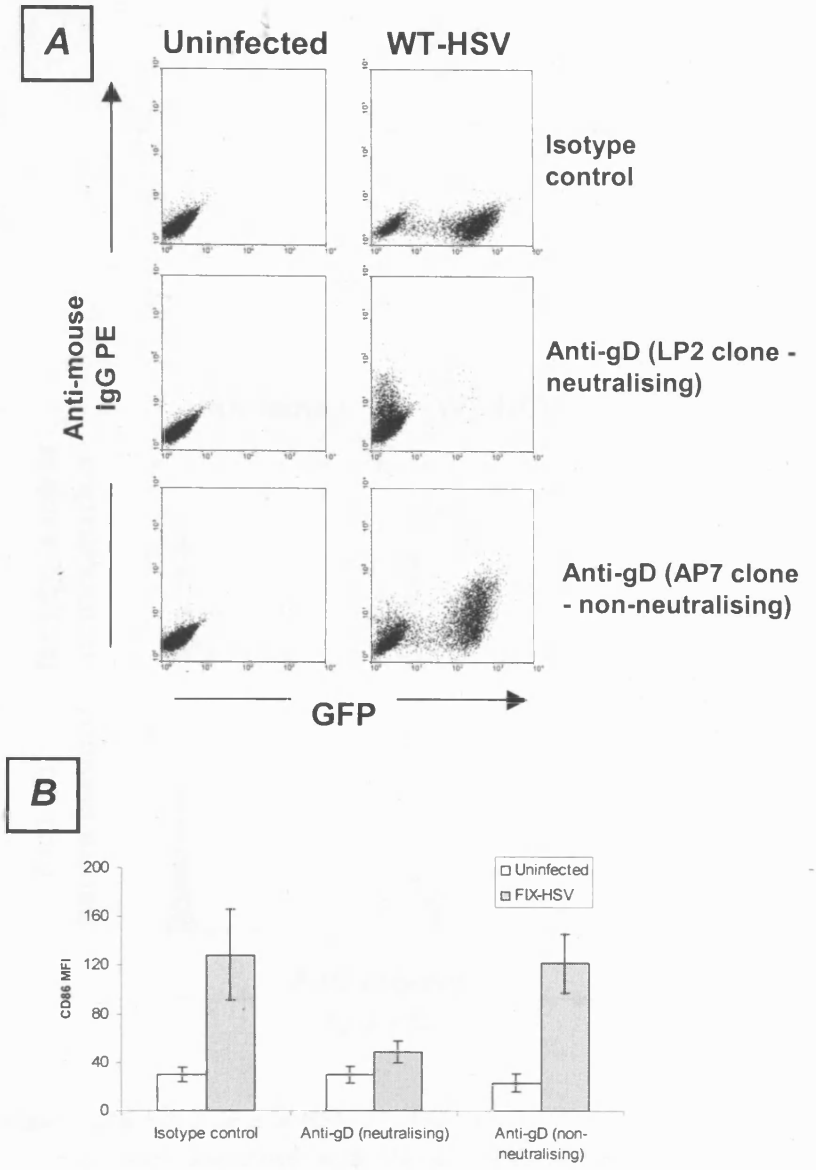

Figure 6.15 Specificity of gD interaction with DC

(A) DC infected with WT-HSV exposed to neutralising (LP2 clone) or non-neutralising (AP7 clone) anti-gD mAb for 16 hours. Expression of mouse IgG and GFP was assessed by flow cytometry. Representative of three independent experiments. (B) DC ~~infected~~ treated with FIX-HSV exposed to LP2 or AP7 clones anti-gD mAb were infected for 16 hours and expression of CD86 was assessed by flow cytometry and presented as mean CD86 MFI of three independent experiments. Error bars represent SEM.

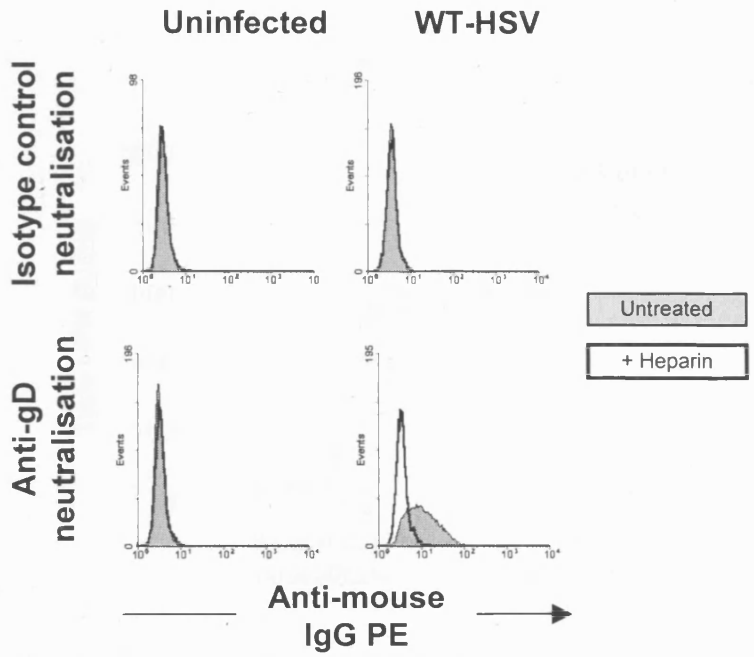

Figure 6.16 Attachment of HSV-1 to DC surface.
WT-HSV was neutralised with anti-gD mAb (lower panels) and then incubated in the presence or absence of 12.5 μg/ml heparin prior to infection of DC for 1 hour. DC were stained for the presence of opsonised viral particles on the surface with PE-conjugated goat anti-mouse Ab. Representative of three independent experiments.

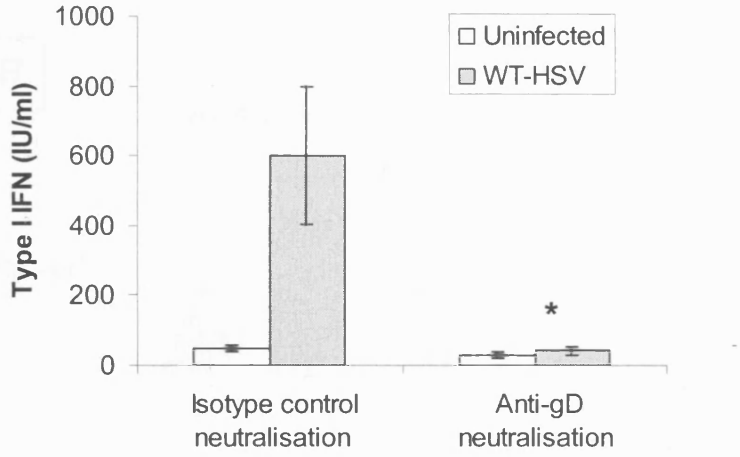

Figure 6.17 Role of gD in HSV-1 induced type I IFN secretion by DC. WT-HSV was neutralised with anti-gD mAb (LP2 clone) and then used to infect DC. Supernatants were harvested after 16 hours and type I IFN secretion determined by an antiviral bioassay. Results are mean of four independent experiments. Error bars represent SEM. * denotes $p<0.01$ relative to WT-HSV neutralised with an isotype control antibody.

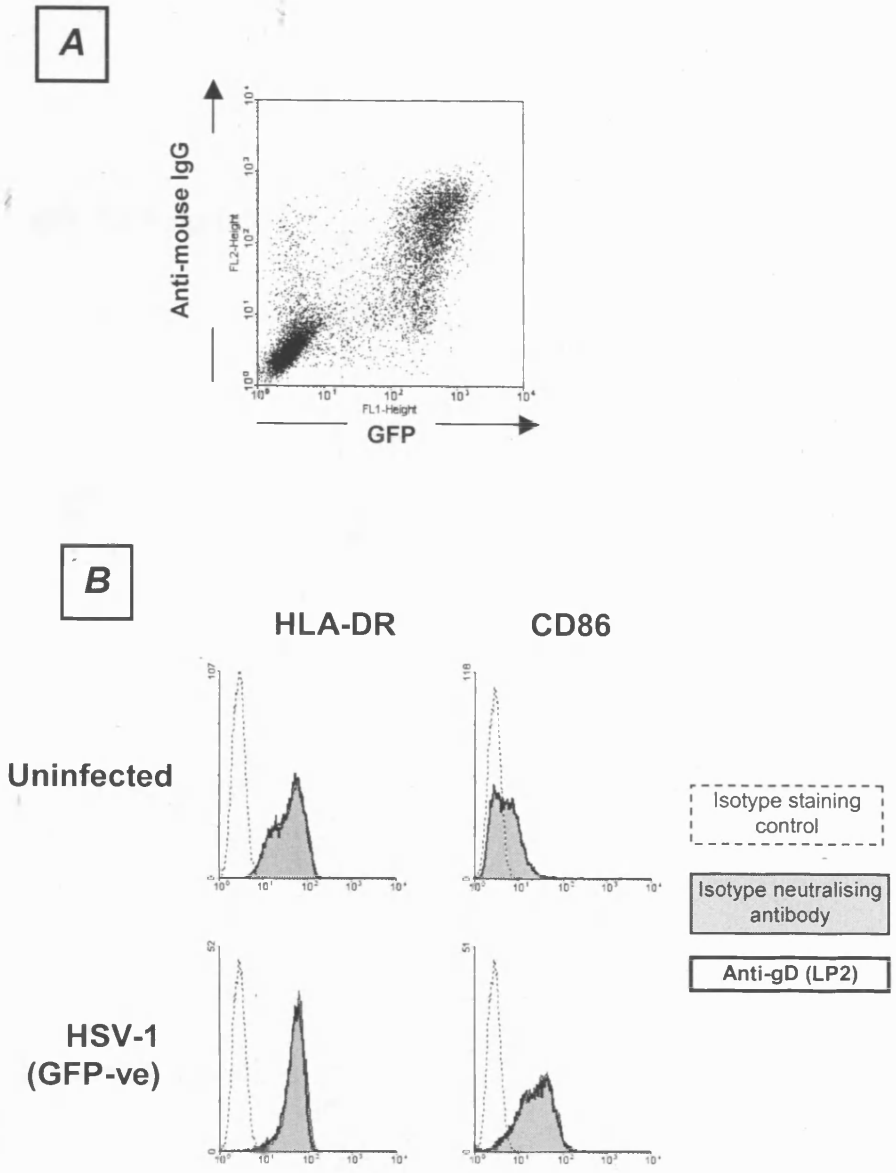

Figure 6.18 Role of cell surface gD in bystander DC activation.
DC were infected with WT-HSV (MOI =1) for 1 hour and cultured in the presence of anti-gD mAb (LP2 clone) for a further 16 hours. DC were stained with goat anti-mouse IgG (A), and HLA-DR and CD86 (B). WT-HSV data gated on GFP-ve DC. Representative of two independent experiments.

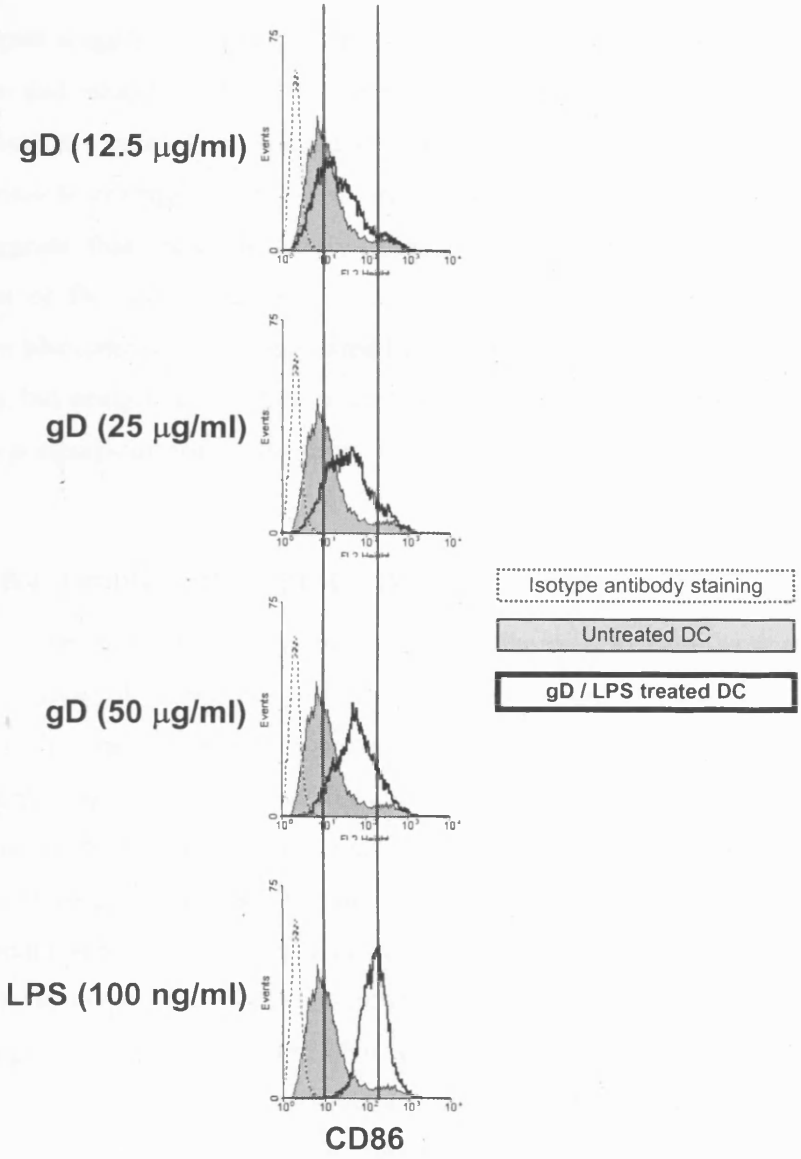

Figure 6.19 Changes in CD86 expression by recombinant gD.
DC were incubated with increasing concentrations of gD or 100 ng/ml LPS for 16 hours and the expression of CD86 was assessed. Representative of three independent experiments.

6.3 Discussion

This chapter sought to dissect the viral and host mechanisms responsible for both the activation and inhibition of DC following HSV-1 infection. Progressively inactivated and neutralised viruses demonstrated that the viral effects on DC morphology, viability and response to external stimuli were carried out predominantly by viral gene products. This suggests that these inhibitory mechanisms may have evolved to curb the activation of DC following early steps in the infection process. Dissection of the activation phenomena was complicated by inconclusive attempts to block DC surface receptors, but neutralisation of the activity of viral gD suggested that this glycoprotein may play a significant role in the direct activation of DC by HSV-1.

6.3.1 HSV-1 factors that inhibit DC functions

Infection of DC with MV impairs the function of the cells to a similar degree as HSV-1 does in chapter 4. However, a large amount of the inhibition has been attributed to the effects of immunosuppressive proteins interacting with DC and/or T cells (Klagge et al., 2000; Marie et al., 2001; Dubois et al., 2001). In contrast, de novo synthesis of viral gene products was predominantly responsible for the inhibitory effects seen following WT-HSV infection of immature DC. DC infected with UV-HSV remained viable, could stimulate T cell proliferation efficiently, had normal morphology and were able to respond to further maturation stimuli through changes in shape and through upregulation of CD86 and MHC class I to maximal levels, in sharp contrast to the effects seen with WT-HSV (chapter 4) (Salio et al., 1999) (figs. 6.4 and 6.5). These findings agreed with previous studies showing that UV-HSV infection of mature DC results in no loss of function (Kruse et al., 2000), but it is important to note that the phenotype of mature DC is more resistant to virus induced changes than immature DC (Salio et al., 1999), and therefore subtler effects of viral proteins might not have been observed. It is likely that DC maturation, in inducing a cellular state that enhances the APC function of the cell, also generates an antiviral state. The effector mechanisms are as yet unspecified, but are likely to inhibit viral gene expression at the transcriptional level (Bakri et al., 2001). The central role of the type I IFN signalling system both in DC maturation (chapter 5) and in antiviral activity is likely to be important in this

effect, either through the autocrine activity of type I IFN (chapter 5), or through activation of ISGs in the absence of IFN secretion, as demonstrated in the maturation of DC by some RNA viral infections (Lopez et al., 2003).

The specific viral components responsible for the changes observed have not been defined. A key role has been suggested for the tegument protein vhs, which inhibits protein synthesis by accelerating mRNA degradation (Kwong and Frenkel, 1987; Samady et al., 2003). However, the precise targets for vhs and other candidate viral immunomodulatory molecules remain to be elucidated. Recent studies have also suggested that vhs has specificity for mRNA with AU-rich elements (ARE) (Esclatine et al., 2004). In addition, a database of ARE containing mRNA has been set up, allowing candidate proteins for vhs-mediated degradation to be identified (http://rc.kfshrc.edu.sa/ared). These include IL-12 p35, IL-12 p40 and TNFα, possibly explaining the low secretion of these proteins by infected DC (figs. 4.11 and 5.3). The absence of IL-12 secretion may be particularly relevant because HSV-1 possess a ligand that could induce IL-12, gD. This is structurally related to LIGHT, a HVEM ligand that can induce IL-12 secretion from DC (Morel et al., 2003). Although HVEM is unlikely to play a major role in DC maturation by HSV-1 (fig. 6.12), it is also possible that ligation of this receptor may induce the secretion of IL-12 from DC primed for maturation by other receptor interactions, and that vhs prevents this response (fig. 4.11). Targeted IL-12 mRNA degradation by UV-inactivated HHV-6 raises the possibility that similar RNA degrading mechanisms are conserved among other herpesviruses (Smith et al., 2003a). Alternatively, if HSV-1 binds to DC via TLR2 (Kurt-Jones et al., 2004), then the absence of IL-12 secretion is consistent with the effects of ligating this receptor on DC (Weigt et al., 2003).

CD86 is an important negative in this discussion, as this molecule is upregulated by de novo protein synthesis (Li et al., 1999), but its mRNA does not contain ARE (http://rc.kfshrc.edu.sa/ared/). Therefore, its increased expression after HSV-1 infection may result from escape from vhs mediated degradation. However, the disparity between IL-12 secretion and CD86 expression is reminiscent of the effects of IFNα that were observed in chapter 5, and may similarly reflect an insufficient dose of

virus to ligate sufficient receptors to overcome p38 and NF-kB signalling thresholds controlling IL-12 secretion (see section 5.3.4.2).

6.3.2 Mechanism of HSV induced apoptosis

The absence of cell death after UV-HSV infection is consistent with the notion that viral IE gene expression is required to induce DC apoptosis after WT-HSV infection (Koyama and Adachi, 1997; Aubert et al., 1999; Sanfilippo et al., 2004). In the context of this chapter, it is also interesting to note that several studies have proposed that gD can prevent apoptosis. This may occur secondary to the accumulation of gD inside infected cells (Aubert et al., 2001), but gD binding to the cell surface, possibly also through autocrine activity of secreted gD (Murata et al., 2002), may be the critical anti-apoptotic step. In that respect, addition of exogenous HSV-1 gD can prevent apoptosis in cells infected with gD- virus (Zhou et al., 2000; Zhou and Roizman, 2001) and gD can also prevent Fas-induced apoptosis in U937 (Medici et al., 2003). The anti-apoptotic action is believed to occur through interaction with the mannose receptor inside endosomes (Zhou and Roizman, 2002), resulting in NF-kB activation and upregulation of c-FLIP expression (Medici et al., 2003). Concurrently, the activation of these pathways may be responsible for the maturation of DC seen (Rescigno et al., 2000; Yoshimura et al., 2001; Lundqvist et al., 2002; Franchi et al., 2003).

6.3.3 HSV-1 induced DC maturation

6.3.3.1 HSV-1 activation of DC independent of type I IFN

Some viral infections of DC induce secretion of cytokines that can feedback in an autocrine/paracrine manner and induce cytokine-dependent maturation of DC (Dubois et al., 2001; Trevejo et al., 2001). Given that chapter 5 detailed how type I IFN secretion from HSV-1 infected DC could induce maturation of bystander DC, it was important to determine the relative role of this cytokine in the acquisition of a mature phenotype by infected DC. Figs. 6.1 and 6.7 demonstrate that this cytokine was largely not required for this effect. As the data in chapter 5 suggested that type I IFNs were the only major cytokines secreted that were responsible for the activation of DC (fig. 5.6), these data were suggestive that the stimulus for DC maturation following HSV-1

infection derived from the virus itself. The downregulation of IFNAR after LPS induced maturation of DC may explain the insensitivity to type I IFN (Gauzzi et al., 2002). However, LPS and Poly(I:C) induced type I IFN secretion rapidly enough to exert a significant autocrine effect (figs. 5.9 and 5.10). Nevertheless, it was possible that HSV-1 infection downregulated the expression of IFNAR more rapidly and efficiently than after TLR3/4 stimulation, possibly through the activity of a viral gene product (Chee and Roizman, 2004).

Partly through the activation of NF-kB, viruses can induce antiviral states in cells independent of the activity of secreted type I IFN (Lopez et al., 2003; Bose et al., 2003), emphasising the importance of direct pathways of viral activation of the cell. However, the data cannot exclude the role of cytokines other than type I IFN secreted after infection that do not activate bystander DC, but that could do so on infected DC, possibly in concert with a priming signal from the infection itself (Trevejo et al., 2001; Bose et al., 2003). Excluding other factors is further complicated by the partial dependence on type I IFN of FIX-HSV induced maturation relative to the absolute independence of WT-HSV. This could have been attributed to the ability of HSV-1 to disrupt type I IFN signalling in infected cells, and demonstrates how the relative role of different factors may be driven by the virus as much as by the cell itself (Mossman et al., 2001; Yokota et al., 2001; Yokota et al., 2004; Chee and Roizman, 2004; Duerst and Morrison, 2004).

It should also be remembered that the regulation of cytokine secretion and surface molecule expression in DC is closely related and rarely independent of each other (chapter 5). Therefore, although the studies in this thesis have aimed to define the relative importance of one stimulus (the virus) over another (type I IFN) in the maturation of DC, it is likely that in vivo the multi-stimulus environment in which the DC encounters the virus will affect the cellular response. The activity of other stimulatory factors in the microenvironment, as well virus interaction and any cytokine secretion, may summate and drive towards full DC maturation. Therefore, defining that virus interaction with DC plays a role, if not an exclusive one, in inducing some degree of maturation of these cells may still have a significant effect in vivo.

6.3.3.2 Viral interaction with the cell surface

Upregulation of CD86 and HLA-DR in response to UV-HSV and FIX-HSV suggested that virus binding to the cell surface was responsible for the activation of DC. This conclusion is consistent with observations in other cell types. One common sequelae of receptor ligation is intracellular calcium fluxes and subsequent phosphorylation of host proteins. Indeed, within 2 min of infection with HSV-1 infection, calcium fluxes are seen, and within 5 minutes significant levels of tyrosine phosphorylation of cytoplasmic and membrane proteins can be observed (Qie et al., 1999; Cheshenko et al., 2003). The downstream effects of these signals are still unclear. Protein phosphorylation may be important for the rearrangement of cytoskeleton for viral infection, by phosphorylating focal adhesion kinase (FAK), similar to the events after *Shigella flexneri* penetration of cells (Dehio et al., 1995). Alternatively, Ca2+ fluxes and protein phosphorylation may also be important in promoting envelope fusion (Dimitrov et al., 1993; Briand et al., 1997), as observed for the other herpesviruses EBV and HCMV (Dugas et al., 1988; Keay and Baldwin, 1996), and may also promote phosphorylation of proteins important in the activation of certain signal transduction pathways, such as NF-kB and p38 (Kelher et al., 2003). Indeed, HCMV enters cells in a similar manner to HSV-1 (Wang et al., 2003), and binding of this virus to the cell surface initiates Ca2+ signalling and receptor phosphorylation (Keay et al., 1995; Keay and Baldwin, 1996) that are important in downstream activation of the Sp1 and NF-kB pathways (Yurochko et al., 1997).

6.3.3.3 HSV-1 activation of NF-kB and p38 pathways

DC maturation by FIX-HSV suggested that virus binding to the cell surface initiated the activation of signalling pathways that induced some aspects of DC maturation. Both the p38 and NF-kB pathways mediate DC maturation (Ardeshna et al., 2000; Yoshimura et al., 2001), and both were activated by HSV-1 interaction with the DC surface. It is interesting that there was correlation between activation of p38 and NF-kB, and the phenotype of HSV-1 infected DC compared to LPS, supporting the hypothesis that these pathways are important in the maturation of DC following HSV-1 infection. However, p38 inhibition had minimal effect on the upregulation of CD86 or HLA-DR by FIX-HSV. Similar to the effects of IFNα in fig. 5.8, this probably

reflected a p38 independent upregulation of CD86 and HLA-DR. The reduced activation of both p38 and NF-kB compared to LPS would also explain why some molecules, such as MHC class I, are not upregulated to a great degree by FIX-HSV (data not shown). Nevertheless, the partial, although insufficient, activation of p38 MAPK raises the activation state of the cell nearer a threshold that would be required to elevate expression of several surface molecules (e.g. CD86) to maximal levels seen with LPS. Therefore, as suggested above, the partial activation of these signalling pathways may not be sufficient per se to induce drastic effects on DC phenotype, but in the absence of viral intervention, could summate with other stimuli the DC receive, and induce full maturation.

Transient activation of NF-kB has been reported after HSV-1 binding to the cell surface, but de novo gene expression is required to sustain this to higher levels (Patel et al., 1998; Amici et al., 2001). This suggests that viral gene expression and/or its products provide further stimuli for NF-kB activation. Alternatively, HSV proteins target inhibitory proteins of the NF-kB pathway (Amici et al., 2001), as also seen for HCMV (DeMeritt et al., 2004). The mechanisms employed to activate the NF-kB pathway in the earlier wave may involve both triggering of host pathways by viral entry and active viral modulation of these signals. The most likely scenario is that receptor triggering activates the IKK-IkB cascade that triggers NF-kB translocation to the nucleus (see section 6.3.5). However, vhs may also target IkBα mRNA, which contains ARE (http://rc.kfshrc.edu.sa/ared/). In contrast, the elevated levels of NF-kB activation at later stages of infection (Patel et al., 1998) may result from host recognition of products of viral replication. Accumulation of replication intermediate dsRNA may trigger TLR3 or activate PKR to induce NF-kB (Zamanian-Daryoush et al., 2000), as can an ER or ribotoxic stress response (Pahl and Baeuerle, 1997; Bunyard et al., 2003). However, HSV-1 expression of IE genes can also induce NF-kB activation within 2 hours of infection in macrophages through ROI-induced release of Ca2+ from mitochondria (Mogensen et al., 2003). It is unclear whether this mechanism of response to intracellular stress is specific to macrophages, cells that have a greater propensity to generate ROI, or whether ROI are more ubiquitous intracellular messengers in response to viral infection.

Irrespective of the mechanism, the early activation of the NF-kB pathway is likely to be important for the DC response to viruses. For example, HCMV induces early IkBα degradation and NF-kB activation within 30 minutes of infection of fibroblasts and monocytes (Yurochko et al., 1995; Yurochko and Huang, 1999; DeMeritt et al., 2004). In monocytes, this early phase of NF-kB is accompanied by activation of p38 MAPK. It can induce transcription and translation of immunological proteins, such IL-1β, and can be mediated solely by glycoprotein interaction with surface receptors (Yurochko and Huang, 1999). As HCMV infection of fibroblasts does not result in the early wave of p38 MAPK seen in monocytes (Johnson et al., 2000), this demonstrates the importance of studying these early activation events in the appropriate cells. HSV-1 binding to DC may elicit similar signals to HCMV, but DC may possess lower requirements to activate particular signalling pathways in response to selected stimuli. The degree of NF-kB or p38 MAPK activation induced may also be critical. While prolonged NF-kB activation may be important for enhancing transcription of NF-kB responsive HSV genes (Rong et al., 1992) to enhance replication (Patel et al., 1998; Amici et al., 2001), lower levels of NF-kB activation induced by gD binding to the cell surface may be sufficient for the induction of a cellular antiviral response (Bose et al., 2003). In DC, this response is translated into maturation.

6.3.4 DC receptors involved in HSV-1 induced activation

Three candidate receptors responsible for initiating cell surface signalling were identified on the basis of their known properties either in response to or in the infection by HSV-1. TLR2 has been recently proposed as playing a role in cellular responses to HSV-1 (Kurt-Jones et al., 2004), whereas HVEM and nectin-1 are HSV-1 entry receptors (Montgomery et al., 1996; Geraghty et al., 1998). Of these three, TLR2 and HVEM have been shown to activate NF-kB in other cell types, consistent with being members of TLR and TNFR superfamilies respectively (Re and Strominger, 2001; Kurt-Jones et al., 2004; Marsters et al., 1997). TLR2 can also activate p38 (Agrawal et al., 2003), but recent studies suggest that HVEM may not be able to do so, as DC require TRAF6 for p38 activation (Mackey et al., 2003) and this factor does not associate with HVEM (Marsters et al., 1997). The central role of NF-kB and p38 activation in mediating DC activation suggested an important role for these receptors

(Clark et al., 1999; Neumann et al., 2000; Hofer et al., 2001; Yoshimura et al., 2001; Ardeshna et al., 2000). On the other hand, nectin-1 is a member of adhesion molecules and its downstream signalling is currently undefined.

Receptor neutralisation studies using monoclonal and polyclonal antibodies were inconclusive. Despite using saturating quantities of antibody, the maturation of DC by HSV-1 could not be attributed to one receptor. The efficacy of the TLR2 neutralising antibody could not be guaranteed, as the upregulation of CD86 and HLA-DR by PGN was not affected. However, as this antibody has only previously been shown to partially neutralise DC secretion of TNFα in response to PGN (Uehori et al., 2003), the expression of CD86 may not be as sensitive as TNFα secretion to incomplete reductions in signalling through TLR2 (see chapter 5). The same hypothesis may also explain the unaltered phenotype of DC exposed to HSV-1 in the presence of anti-TLR2 mAb. Future strategies will involve using another neutralising antibody to TLR2, clone 2392, which has been shown in side-by-side studies to have more potent neutralising capacity than a similar antibody clone to the one used in the experiments in this thesis (Iwaki et al., 2002).

For the entry receptors, the data is even more difficult to interpret in the absence of an absolute positive control and the redundancy in receptor usage by HSV-1 (Krummenacher et al., 2004). This is often bypassed by transfecting resistant cell lines with a single receptor. However, this is not a suitable control for DC. Therefore, the theoretical approach behind the experimental design used was that, despite the redundancy, DC coated with receptor antibody, but still infected, could be interpreted as having been infected independent of the neutralised receptor. This conclusion could be made with HVEM, as infected cells still expressed antibody-bound receptors 16 hours post-infection, but nectin-1 was internalised more rapidly. This may simply have reflected that anti-nectin-1 was a mAb, and therefore fewer antibodies were present initially on the DC surface. Nevertheless, the saturating amount of antibody added to the cells should have prevented interaction with nectin-1 by the virus, especially as this antibody prevents the critical interaction between gD and nectin-1 necessary for entry (Krummenacher et al., 2000). This suggested that nectin-1 was not involved in activating DC. The neutralisation of HVEM suggested a similar conclusion for this

receptor. Considered in conjunction with the polyclonal nature of the reagent used, this supported most strongly that HVEM was not solely responsible for the activation of DC.

The receptor neutralisation studies leave open two possibilities that might explain the inconclusive results. One of these is that HSV-1 induces DC maturation by binding to, and signalling via, receptors other than TLR2, HVEM or nectin-1. Indeed, gD interaction with chemokine receptors (Ankel et al., 1998) or the MR (Brunetti et al., 1994; Brunetti et al., 1995) can exert functional effects on cells (Ankel et al., 1998; Milone and Fitzgerald-Bocarsly, 1998a; Rong et al., 2003). The MR has also recently been shown to mature DC, though programming the cells to induce Th2 responses (Chieppa et al., 2003). Only neutralisation of these receptors could determine their role. An alternative scenario is that all (or two) of the receptors neutralised in these experiments contribute relatively similar weak activation signals to DC, and that HSV-1 ligates these receptors in a non-saturable manner. The titration of recombinant gD on DC supports this theory, as even at the highest concentration of 50 μg/ml, the expression of CD86 did not plateau, suggesting that a non-saturating dose of glycoprotein had been added (fig. 6.19). This model predicts that neutralisation of one receptor would result in compensatory ligation of heterologous 'empty' receptors. In this way, the signals emanating from these receptors could compensate for the loss of signal from the neutralised receptors, resulting in no change in surface phenotype. This hypothesis could be tested by using combinations of neutralising antibodies, thereby preventing ligation of more than one receptor at any one time.

6.3.5 Viral ligands responsible for DC activation

The ability of FIX-HSV to induce maturation of DC suggested that HSV-1 envelope structures were able to ligate receptors on the surface of DC and transmit activation signals. The virus envelope is made up of at least 11 glycoproteins (Handler et al., 1996), but the most likely candidates were viral glycoproteins that interact with receptors during viral entry. These include gB and gC that interact with HS, and gD that binds to entry receptors (Spear et al., 2000). Fig. 6.16 excluded the role of gB and gC binding to HS as providing a significant stimulus for activation of the cell. It has

previously been suggested that HCMV, a virus that utilises similar mechanism to enter cells as HSV-1, can attach HS and transmit signals that induce upregulation of HLA-ABC in fibroblasts (Song et al., 2001). However, HS is a surface glycomatrix with no precise anchoring or defined intracellular signalling machinery, and the study did not exclude that inactivated virus was binding to an activatory surface receptor (Song et al., 2001).

It is important to note, that although the data in this chapter rules out the role of gB and gC binding to HS on DC as providing maturation signals, they do not exclude the role for these glycoproteins completely. Although, no other receptors have been described that bind gC, gB plays a role in inducing envelope-membrane fusion, along with gH-gL, and this event may play an important role in the activation of DC (see section 6.3.5.2).

6.3.5.1 Role of gD in DC activation

The biological activity of viral glycoproteins binding to and inducing a response in infected cells has been previously described. EBV gp350 can induce TNFα alpha secretion in a NF-kB dependent mechanism (D'Addario et al., 2000). HIV-1 gp120 can also induce the secretion of a wide range of cytokines, depending on the cells stimulated and the microenvironment of the cells (Ameglio et al., 1994; Fantuzzi et al., 1996), and in DC, it may induce maturation (Fantuzzi et al., 2004).

Neutralisation of gD with LP2, rendered the virus particles non-infectious, but did not prevent virus attachment to the cell surface (figs. 6.14 and 6.16). From these findings, it was extrapolated that the neutralisation prevented the interaction of gD with receptors on DC. The loss of mature phenotype induced by both FIX-HSV and WT-HSV by LP2 neutralisation was suggestive of the role of gD in providing the initial maturation stimulus to DC. Previous studies analysing the ability of gD to bind to surface receptors back up a role for this glycoprotein in activating DC. Recombinant forms of gD alone are able to activate NF-kB in U937 monoblastoid cells (Medici et al., 2003), and induce secretion of TNFα from RAW 264.7 macrophage-like cell lines (Paludan and Mogensen, 2001) and type I IFN from PBMC (Ankel et al., 1998).

However, there are several caveats, both from the data presented here and from the literature, that currently precludes ~~from concluding~~ the conclusion that gD is solely responsible for providing activation signals to DC.

It was possible that LP2-neutralised virus prevented DC maturation by interacting with inhibitory FcR on the DC surface. This is unlikely because when FcγRII, the only FcγR expressed on human MDDC, are cross-linked, maturation of DC ensues, suggesting that this receptor possesses a cytoplasmic tail that can transmit positive rather than negative signals in DC (Banki et al., 2003). Furthermore, when HSV particles were coated with the non-neutralising anti-gD mAb, AP7, maturation was not prevented. On the assumption that AP7 coated virions had an equal possibility of interacting with FcγRII as those neutralised with LP2, then the interaction with FcγRII was unlikely to play a significant role in this system.

An alternative explanation for the effect of LP2 neutralisation on DC phenotype was that the antibody sterically hindered the binding of other viral components that were responsible for the activation of DC. AP7 binding did not prevent maturation, suggesting that the LP2 binding domain of gD was responsible for activation of DC. However, as AP7 is a non-neutralising antibody, it is likely to bind a different epitope of gD to LP2, one that is unlikely to prevent receptor interaction. Therefore, AP7 may not be the best control antibody to exclude steric hindrance. An antibody to a component closer to the receptor binding complex, such as gH, may be a more appropriate control.

The neutralisation and resultant loss in DC maturation by LP2 may assist in determining the receptor used for virus activation. Co-sedimentation of antibody-bound HSV-1 with truncated soluble forms of receptor demonstrated that LP2 binding to gD prevented viral interaction with nectin-1 but not HVEM (Nicola et al., 1998; Krummenacher et al., 1998). These data would appear to exclude the role of HVEM in DC maturation by HSV-1. However LP2 can prevent HSV infection in CHO cells (which are naturally resistant to HSV) transfected and expressing only HVEM (Nicola et al., 1998), suggesting that even though LP2 allows gD-HVEM binding, it may still affect receptor-induced conformational change of gD (Carfi et al., 2001) or fusion of

the viral envelope with the cell membrane. As both steps may be important in virus induced signalling (see section 6.3.3), then the role of HVEM in virus induced signalling in DC cannot be excluded definitively from the LP2 neutralising experiments.

In order to determine the direct role of gD in DC activation, recombinant gD was added directly to DC. This glycoprotein induced the upregulation of CD86 in a dose-dependent manner, suggesting that alone it could induce activation signals in DC directly (fig. 6.19). However, it is important to confirm that the effects observed were gD-specific. The glycoprotein was prepared from a baculovirus transfection system, and therefore should not have contained any bacterial contaminants. Furthermore, proteins purified from insect cells added to DC do not induce maturation (Chan et al., 2003). Nevertheless, it will be important to exclude any contaminants in the protein preparation. One approach may be to precipitate the protein out of the culture medium (prior to addition to DC) using anti-gD antibodies. Alternative approaches include attempting to neutralise the receptor that the protein may be ligating, which has the added advantage of suggesting which receptor HSV-1 binds to activate DC.

6.3.5.2 Role of non-gD viral components in DC activation

Evidence that viral components other than gD alone bind to receptors to elicit signalling from the cell surface has emanated from the use of deletion mutants. Ca2+ fluxes elicited by viral infection do not occur with gL- viruses, implying that gD receptor interaction alone is insufficient to induce tyrosine phosphorylation, and that post-binding events are required for activation (Cheshenko et al., 2003). These viruses are also less potent at eliciting TNFα production from macrophages (Paludan and Mogensen, 2001).

These findings appear to contradict the data presented in this thesis, specifically the activation of DC by FIX-HSV and recombinant gD alone. One possible explanation is that the gD binding to cells in the glycoprotein deletion mutants is aberrant. However, this is unlikely because the removal of individual glycoproteins from the viral envelope does not grossly affect the quantity and conformation of the remaining ones

(Rodger et al., 2001). Another possibility is that the activation of DC by recombinant gD was an artefact independent of any contaminants in the protein preparation. The gD used in fig. 6.19 was gD(285t), a truncated form of the full length glycoprotein that is missing the 21 amino acids from the C-terminal end (Rux et al., 1998). This form of gD binds to HVEM and nectin-1 with a 100-time greater affinity than the full length protein (Rux et al., 1998; Krummenacher et al., 1998). In this thesis, the truncated protein was used in order to determine whether an effect could be observed that would suggest that gD could activate DC directly. Use of the full-length protein may determine whether the difference in protein affinity impinges on the downstream signalling, as this has not been investigated previously. No effect on DC phenotype by the full length gD would also suggest that the gD(285)t preparation was free of contaminants, as both preparations are purified using the same gD column (Rux et al., 1998). In keeping with this hypothesis, fixation of the viral envelope may have cross-linked the closely juxtapositioned envelope glycoproteins into a conformation that can bind DC receptors with greater affinity than the wild type glycoproteins (Handler et al., 1996; Rodger et al., 2001). Thus, fixation may have generated a ligand that was artificially over-efficient at stimulating DC, perhaps in a similar manner to gD(285)t. This may also explain the common observation that FIX-HSV could elicit more potent maturation of DC than UV-HSV or WT-HSV (data not shown).

However, the limitations of the FIX-HSV model do not detract from the conclusion that virus-surface binding activated DC in a gD-dependent manner. It is likely, though, that gD can activate cells in concert with other viral ligands, possibly through their role in inducing a conformational change in gD and/or the bound receptors to induce signalling downstream (Carfi et al., 2001; Krummenacher et al., 2002). Consistent with this, HSV-1 virions that are unable to fuse with the cell membrane cannot initiate Ca2+ fluxes or tyrosine phosphorylation (Cheshenko et al., 2003; Qie et al., 1999). However, only alpha-herpesviruses possess gD-like molecules that are bone fide ligands for receptors, whereas beta- and gamma-herpesvirus entry glycoproteins also possess fusogenic capacity. Indeed, the fusogenic capacity of HCMV entry glycoprotein, gB, is required to activate an interferon response following surface binding (Netterwald et al., 2004).

One approach to determine the relative roles of these HSV-1 accessory glycoproteins would be to use neutralising antibodies to prevent their binding to the cell surface. Antibodies to gB may affect viral attachment to the HS on DC (Laquerre et al., 1998), but antibodies to gH or gL would prevent infection by preventing envelope-membrane fusion. Theoretically, this approach should permit gD ligation to surface receptors, excluding the role of non-gD proteins. It has also not been excluded that gH-L binds an unknown surface receptor, and the addition of recombinant gH-L may exclude this possibility and/or further address the issue of protein purity. The use of deletion viruses (e.g. gD- vs. gH- or gL- HSV-1) to "infect" DC would also aid in dissecting this scenario.

6.3.6 HSV-1 induced cytokine secretion

6.3.6.1 Secretion of type I IFN

The secretion of type I IFN induced by UV-HSV and FIX-HSV infection in the majority of individuals tested is consistent with replication-independent trigger for IFN secretion. Recent data shows that PDC can also respond to HSV-1 infection in this manner (Hochrein et al., 2004), although PDC may uniquely recognise ligands that induce greater type I IFN secretion (see section 5.3.1). The data presented in this chapter also indicate that HSV-1 gD plays an important role in type I IFN secretion by MDDC (fig. 6.17). The potential receptors responsible include CXCR4 and CCR3, neither of which are implicated in the HSV-1 infection process (Ankel et al., 1998). Alternatively, MR can bind the heavily glycosylated gD (Brunetti et al., 1994; Brunetti et al., 1995), and has been implicated in HSV-1 induced secretion of type I IFN (Milone and Fitzgerald-Bocarsly, 1998a; Rong et al., 2003). Irrespective of the receptor, the early role of PKC and Ca2+ fluxes in the induction of type I IFN secretion, supports the triggering of surface receptors by the virus to elicit this response (Li et al., 1996).

For the same technical limitations described in section 6.3.5, the loss of IFN secretion by LP2 anti-gD mAb does not prove that this glycoprotein is solely responsible for inducing type I IFN secretion. As for the effects on phenotype, it is likely that gD may

induce type I IFN secretion with the assistance of the gB, gH-L fusion complex. Indeed, the induction of IRF-3 by HSV-1 only occurs in the presence of both binding and fusing with the cell surface (Preston et al., 2001). Similarly, HCMV can triggering of IRF-3 activation and ISG transcription occurs through both binding of gB to the cell surface and subsequent post-attachment events (i.e. envelope fusion) (Preston et al., 2001; Boyle et al., 1999; Netterwald et al., 2004). Therefore, it appears that herpesviruses can trigger an IFN response early after infection, prior to viral gene expression that may modulate it. However, it is unclear whether this pathway relates to a host recognition of viral entry, analogous to recognition of products of viral replication (e.g. dsRNA), and whether it is a significant selective pressure for the evolution of HSV-1 mechanisms to inhibit signalling of the type I IFN system signalling (Nicholl et al., 2000; Mossman et al., 2001; Lin et al., 2004; Collins et al., 2004). This concept is discussed further in section 6.3.7.

6.3.6.2 Secretion of other cytokines

In a mouse macrophage cell line, HSV-1 replication induces NF-kB activation and IL-12 secretion (Malmgaard et al., 2000). In the same cells, HSV-2 also triggers TNFα secretion (Paludan et al., 2001), via binding of gD to the cell surface, viral DNA delivery to the nucleus and viral replication (Paludan and Mogensen, 2001). Similar multi-layered mechanisms have also been implicated in macrophage production of CCL5 in response to HSV infection. Viral replication is required to trigger production of this chemokine, through the activation of NF-kB, PKR and IRF-3 (Melchjorsen et al., 2002; Melchjorsen and Paludan, 2003). NF-kB activation in DC may have been sufficient to induce low degree of TNFα secretion, but insufficient for IL-12, further highlighting the cell-dependent outcomes of signalling activation.

6.3.7 Functional outcome of HSV-1 triggering of host signalling pathways

It is important to relate the mechanistic discussion in earlier sections to the outcome of infection. The phenotype changes induced by HSV-1 on DC are mild, and may not result per se in a significant increase in antigen presenting function of the DC, but may

condition the cell's Th skewing capacity. Equally, the viral induced signalling changes may also favour the survival and replication of the virus. The survival advantages of the host-pathogen interaction are discussed below.

6.3.7.1 Pathogen recognition: advantage to host survival

Recent studies have focused on the ability of the innate immune system, of which DC are a central component, to recognise PAMPs and swiftly initiate anti-pathogen responses (chapter 1). Considerable interest has been generated by the role of the evolutionarily conserved TLR family in the regulation of viral infections. Known viral ligands are detailed in table 1.1. The direct activation of DC by HSV-1 would appear to be consistent with the concept of host-pathogen recognition and indeed, some studies have shown that the binding of viral structures to TLR favours host survival. TLR9 and TLR3 are important in the control of murine cytomegalovirus infection (Tabeta et al., 2004). The hypomethylated CpG rich genome of HCMV, along with the dsRNA intermediate, are likely to be the PAMPs respectively. Recent studies have also suggested that TLR7 can recognise ssRNA (Heil et al., 2003; Diebold et al., 2004) and that this receptor is important for the clearance of the ssRNA virus, vesicular stomatitis virus (VSV) (Lund et al., 2004). Similar protection studies have been extended to the human pathogen, RSV. The resolution of bronchiolitis caused by this virus is TLR dependent, as the RSV F protein is recognised by TLR4 (Kurt-Jones et al., 2000; Haynes et al., 2001; Haeberle et al., 2002). Although it has also been suggested that the IL-12 receptor axis is more critical than TLR4 in some mice models (Ehl et al., 2004), polymorphisms in the human population have confirmed that TLR4 does play an important role in RSV infection in vivo (Tal et al., 2004). The evolutionary advantage to host immunity of TLR recognition in some infections is further highlighted by the expression of proteins by pox viruses that inhibit TLR signalling (Harte et al., 2003).

6.3.7.2 Receptor triggering: advantage to viral survival

The presence or expression of viral ligands that bind TLR may not necessarily correspond to a protective advantage to the host. TLR3 is not required for clearance of the dsRNA virus LCMV, and TLR9 expression does not impact on the replication or

clearance of HSV-1 after epidermal infection (Krug et al., 2004), despite the DNA genome of this virus being rich in CpG motifs that can ligate TLR9 (Honess et al., 1989; Lund et al., 2003). Furthermore, in some mouse models, the expression of TLRs, promotes the establishment of wild type viruses over more attenuated forms (Jude et al., 2003).

There is also now evidence to demonstrate that viruses have evolved to exploit TLR binding and signalling. Murine mammary tumour virus (MMTV) can bind TLR4 and upregulate expression of its own receptor (Rassa et al., 2002). Interestingly viral-TLR4 binding induces the maturation of DC, demonstrating how increased viral infectivity may be of sufficient advantage to trade off against activation of these cells. Similarly, MV HA binding to TLR2 also upregulates the MV receptor, CD150 (Murabayashi et al., 2002; Bieback et al., 2002). Interestingly, the attenuated vaccine strain of MV uses CD46 as an entry receptor, and does not bind TLR2 (Bieback et al., 2002).

Virus proteins may also bind non-TLR receptors to favour their own survival. MV NP can bind FcγR on DC, and suppress their IL-12 secretion (Marie et al., 2001). The ability of many viruses to bind to c-type lectins (see section 1.8.1.2) may also be exploited as evidenced by the Th2 conditioning initiated by MR cross-linking (Chieppa et al., 2003), and inhibition of DC maturation by *Mycobacterium tuberculosis* binding to DC-SIGN (Geijtenbeek et al., 2003).

Closer analysis of the biology of HSV-1 replication reveals how binding to stimulatory receptors may be advantageous. Calcium fluxes induced by HSV-1 binding may increase the efficiency of viral infection by aiding transport of VP16 to the nucleus, possibly through actin remodelling (Cheshenko et al., 2003). Activation of the NF-kB, and p38 MAPK pathways can prevent apoptosis (Goodkin et al., 2003) and enhance the efficiency of viral replication (Patel et al., 1998; Zachos et al., 1999), as many viral gene promoters are positively regulated by NF-kB (Rong et al., 1992). Consequently, it is conceivable that viral activation of NF-kB and p38 are desired events, analogous to the regulation of the ERK and JNK pathways, where the virus expresses proteins that specifically induce or prevent the activation of these pathways respectively, in order to prevent cellular apoptosis (Perkins et al., 2002; Murata et al., 2000).

Although a genetically stable virus, HSV-1 can evolve under selective pressure, as seen by the acquisition of acyclovir resistance (Danve-Szatanek et al., 2004). Therefore, this discussion suggests that HSV-1 has conserved stimulatory structures to activate the host cell signalling machinery to favour its own replication. As the activation of both p38 MAPK and NF-kB in DC primes these cells' maturation and subsequent initiation of antiviral responses, it is hypothesised that this activation is the driving force for the evolution of viral mechanisms to dampen the function of DC that are activated early by the initial infection process (chapter 4 + section 6.3.1). The interesting implication from this model is that the ability of DC to 'recognise' and mature in response to HSV-1 infection may result primarily from the dependence of the virus to activate certain signalling pathways for its own replicative advantage. Identification of the receptors involved would also aid in determining how these virus-induced signals affect the T cell stimulatory capacity. In that respect, the ability of HSV-1 to bind MR or TLR2 (Milone and Fitzgerald-Bocarsly, 1998a; Kurt-Jones et al., 2004) may skew towards Th2 responses (Chieppa et al., 2003; Agrawal et al., 2003).

6.4 Conclusions

This chapter has sought to detail the mechanisms involved in both the activation and inhibition of DC after infection with HSV-1. Although a precise study using mutant viruses is required to explore the latter issue fully, the data demonstrated that viral genes expressed, rather than virion components, were responsible for the suppression in DC function observed in chapter 4. In contrast, interaction of the viral envelope with the surface of DC induced the activation of pro-inflammatory signalling pathways and the upregulation of MHC class II and co-stimulatory molecules. A major player in this activation was gD, although its precise role and the receptors with which it interacts remains elusive. The virally induced activation of DC needs to be detailed further both qualitatively and quantitatively, as it may alter the accepted dogma of pathogen recognition by host receptors. Furthermore, the consequence of HSV-1 entry triggering changes in DC physiology has important implication both in a model of HSV-1

infection and for therapeutic uses of HSV-1 as a gene delivery vector. These issues will be discussed in the final chapter of this thesis

Chapter 7

General discussion

7.1 Introduction

The work in this thesis has addressed the interaction between HSV-1 and DC. Disruption of DC function would appear to be a candidate addition to the list of strategies this virus possesses to evade or delay the immune response. Furthermore, the pressures to escape the generation of immunity by DC that are activated by early events in the infection process, may have led to selection of a virus which meets the stringent requirement to interfere with as many DC functions as possible. However, this in vitro data must be reconciled with in vivo observations that potent and well-characterised cellular responses occur following HSV-1 infection. The interplay between virus and the host in a model of HSV-1 infection will be discussed in this chapter. The application of this model to a more clinical setting will also be assessed. Finally, the central role of DC in the pathogenesis of other viral infections will be discussed.

7.2 Model for establishment and resolution of peripheral HSV-1 lesion

7.2.1 Role of DC impairment

HSV-1 infects the epidermis, where the predominant cell type it encounters is keratinocytes. These cells allow efficient replication (Mikloska et al., 1996). DC are represented in the epidermis by LC, which may be infected at the earliest stages of infection. LC share many characteristics with the myeloid non-Langerhans DC described in this thesis: potent ability to stimulate naïve T cells, dendritic morphology and a similar expression profile of surface molecules (e.g. MHC class II, CD1a) (Caux et al., 1996; Ito et al., 1999; Canque et al., 2000). Therefore, LC may well be affected negatively by HSV-1 infection in a similar manner to these DCs. Infected LC are unlikely to be able to migrate out of the skin into draining LNs, and will not stimulate T cells effectively if they get there. The absence of detectable virus in draining lymph nodes of infected peripheral sites supports this model (Mueller et al., 2002; Zhao et al., 2003).

Impaired LC function inhibits the stimulatory capacity of the first APC encountered, which delays the generation of the adaptive immune response. The subsequent delay in infiltrating CD4+ and CD8+ T cells at the infection site allows the virus sufficient time to replicate and infect a second round of keratinocytes (and LC). This would not only increase the size of the lesion, but also increase the local virus titre, favouring successful transmission of the infection to the next individual. The absence of CD8+ T cells early in infection would also allow efficient infection of nerve endings (van Lint et al., 2004). Furthermore, LC infection may specifically enhance the efficiency of nervous system infection and subsequent establishment of latency. It is noticeable that there is a close anatomical association between LC and c-type nerve endings in the epidermis, such that 60% of these nerves are associated with LC (Hosoi et al., 1993). Although chapter 3 demonstrated that viral production by infected DC is low, the local concentration of produced virus may be sufficiently high to infect the nerve endings, allowing retrograde travel to the DRG and the establishment of latency (Jones, 2003). The relationship between LC and nerve endings also suggests that LC would be one of the first cells infected after reactivation from latency, favouring the establishment of secondary skin lesions.

The involvement of other skin DC subsets in this model is less well defined, as there is little data analysing the spread of lesions in vivo. Specifically, it is unclear whether a lytic lesion breaks through the epidermal-dermal barrier. Recent data analysing HSV-2 infection of the genital mucosa suggests that this represents an anatomical barrier to the infection (Zhao et al., 2003), but it is unclear whether this also occurs in the skin. As a result, it is unclear whether dermal DC would also be infected and affected by the virus. An important factor may be determined by the depth of the skin abrasion that allowed the initial viral breach of the epidermal barrier, and the dose of viral inoculum of the infection, which determines the number of cells infected initially and the subsequent rate of spread of the lytic infection.

7.2.2 Role of bystander DC

Anti-HSV cellular responses in vivo demonstrate that the host can bypass the initial block in antigen presentation and harness the potency of DC. The bystander activity of

type I IFN in this process was suggested in chapter 5 and is discussed in section 7.2.3. This model proposes that the epidermis surrounding the lesion, as well as the underlying dermal/submucosal tissue, will contain uninfected DC, and that while the function of the infected DC in the epidermis at the centre of the lesion may be impaired, uninfected "bystander" dermal/submucosal DC at the margin may take up dead or dying virally infected cells, and then cross-present the viral antigens in the draining LN (see section 4.3.1.2). The correlation in fig. 4.1 between uninfected DC in infected cultures and HSV recall proliferative response supports this hypothesis.

Data from murine models supports the role of uninfected bystander DC in initiating the antiviral response. Neither epidermal nor vaginal infection resulted in the presence of detectable HSV DNA in the draining LN (Mueller et al., 2002; Zhao et al., 2003), excluding both direct presentation by infected DC and direct viral drainage into the LN via lymphatics, as seen after footpad infection (Mueller et al., 2002). However, there are differences between the DC subsets involved in antigen presentation. Following epidermal HSV-1 infection, virus specific CD8+ T cells were shown to be activated exclusively by LN resident CD8α+ DC (Allan et al., 2003; Smith et al., 2003b). In contrast, after vaginal infection with HSV-2, virus specific CD4+ T cells were activated by CD11b+ submucosal DC (Zhao et al., 2003). It remains unclear whether this disparity relates to differences between CD4+ and CD8+ T cells (Pooley et al., 2001) or between HSV-1 and HSV-2 infection. Furthermore, the direct ontogeny of DC subsets is still unresolved (Shortman and Liu, 2002), and migrating myeloid CD11b+ DC may differentiate into CD8α+ DC in the LN (Moron et al., 2002). Nevertheless, the preferential role of mouse CD8α+ DC in cross-presentation to CD8+ T cells is consistent with the model of indirect presentation (Iyoda et al., 2002; Schulz and Reis e Sousa, 2002).

Early murine studies had demonstrated an inverse correlation between LC numbers in the skin of mice and the severity of the lesion after epidermal HSV-1 infection (Sprecher and Becker, 1989). However, both of the models above demonstrated that LC were not responsible for CD4+ or CD8+ T cell stimulation (Zhao et al., 2003; Allan et al., 2003). Therefore, in the context of an epidermal infection, the primary role of LC may be antigen transfer to draining LN rather than direct T cell stimulation.

Such an 'antigenic vector' role of DC from the periphery to LN has been proposed previously (Huang et al., 2000; Belz et al., 2004). The decline in LC numbers migrating to the LN after infection (Zhao et al., 2003) may reflect the speed of the spread of infection in the epidermis and/or the synchronous early emigration of uninfected LC from that compartment.

The antigen presenting role of PDC in this model also remains a mystery. PDC express TLR9, which can ligate HSV DNA that is rich in hypomethylated CpG motifs (Lund et al., 2003). However, abrogating this pathway does not hamper the control of viral replication or resolution of peripheral HSV-1 lesions (Krug et al., 2004). This may relate to the redundant role of this pathway in type I IFN secretion in response to HSV-1 (Hochrein et al., 2004). Furthermore, despite PDC accumulation in LNs after peripheral infection (Smith et al., 2003b; Yoneyama et al., 2004), PDC are not involved in direct antigen presentation to responding T cells after peripheral infection (Smith et al., 2003b; Zhao et al., 2003; Allan et al., 2003). This may relate to their relatively poor ability to stimulate naïve T cell proliferation (Krug et al., 2003) or their inability to cross-present exogenous antigen from DC draining peripheral infection sites (Salio et al., 2004).

Uninfected bystander DC at the site of infection may migrate towards the lesion. Murine studies of genital mucosa infection show a rapid recruitment of DC within 24 hours of HSV infection. The driving signals are likely to be chemokines, such as CCL3, secreted at the site of infection by keratinocytes (Mikloska et al., 1998) that may recruit MDC (Yoneyama et al., 2004). These DC could have originated either from the migration of tissue resident dermal DC towards the lesion, or the recruitment of monocyte precursors from blood that have differentiated into immature DC (Randolph et al., 1999). Type I IFN secreted at the infection site is likely to promote this differentiation, as discussed in chapter 5 and below.

7.2.3 Role of type I IFNs in resolution of peripheral HSV-1 infection

Type I interferons may be key mediators in terms of the cross-presentation model outlined above, both at the site of infection and in secondary lymphoid organs during the generation of the antiviral response.

7.2.3.1 Type I IFN in the periphery

In the early stages of infection, type I IFN can be secreted by non-DC (Lebre et al., 2003), as well as from infected myeloid DC in tissue (fig. 5.3). In addition to their antiviral activity, type I IFNs may also be an important danger signal detected by bystander uninfected DC, inducing maturation and migration of DC cross-presenting antigens from dying infected cells (chapter 5) (Padovan et al., 2002; Luft et al., 2002b). This initial IFN release may also be important in aiding the differentiation of monocyte precursors of DC (Randolph et al., 1998; Blanco et al., 2001), generating MDC that secrete greater quantities of IFNα following exposure to HSV-1 (Mohty et al., 2003). Concurrent recruitment of PDC to the site of infection (Wollenberg et al., 2002) may amplify the local type I IFN production by responding to HSV DNA (Lund et al., 2003; Lundberg et al., 2003a; Krug et al., 2004).

7.2.3.2 Type I IFN in secondary lymphoid organs

DC may also interact with HSV in peripheral blood, as mucocutaneous HSV infection results in systemic viraemia in a large proportion of individuals (Youssef et al., 2002), and this encounter provides an alternative pathway for the recruitment of both DC subsets to LNs after local infection (Cella et al., 1999a; Yoneyama et al., 2004). Although local type I IFN secretion by MDC may be sufficient in the DC-T cell microenvironment, the large IFN secretion by recruited PDC may be critical to ensure that DC subsets (cross-)presenting viral antigens result in priming of the immune response, as opposed to inducing tolerance (Hawiger et al., 2001; Dalod et al., 2003; Le Bon et al., 2003; Bjorck, 2004). The mechanisms by which this occurs may revolve around increasing IL-12 secretion by the presenting DC (Albert et al., 2001) or increasing the time of contact and interaction between DC and T cells (Benvenuti et al., 2004b).

7.3 HSV-DC interactions: implications from laboratory to clinic

The knowledge gained from studying the relationship between HSV-1 and DC can be applied to a more clinical setting. Not only can HSV-1 infection affect the pathogenesis of other viral infections, but it may also be exploited for the efficient delivery of therapeutic genes into DC, such as in the field of tumour immunotherapy. Furthermore, the central role of DC in the generation of immunity to HSV suggests that detailing the functional consequences of the interaction between virus and the cell may be beneficial in developing rational designs for vaccines to HSV.

7.3.1 Impingement on pathogenesis of HIV-1 infection

The original cohort of HIV infected individuals was identified on the basis of more severe HSV disease. This has subsequently been explained by the immunodeficiency induced by HIV-1 which results in more frequent reactivations from latency and lack of immunological control of peripheral infections (Siegal et al., 1981; Schacker et al., 1998b). However, more recent data has suggested that HSV infection may also accelerate HIV disease progression towards AIDS. HSV reactivation is a significant predictor of onset of AIDS (Hennessey et al., 2000), and plasma HIV RNA levels can also increase during clinical HSV reactivation (Mole et al., 1997), possibly explaining data showing a moderate positive effects on survival of HIV infected individuals treated with acyclovir (Ioannidis et al., 1998).

Active HSV lesions may provide portals for HIV entry and exit (Schacker et al., 1998a), but HSV-1 may induce more direct effects that result in increased HIV replication. One of these is pseudotyping of HIV-1 particles in dually infected cells, subsequently increasing the tropism of the HIV particles (Heng et al., 1994; Calistri et al., 1999). Another possibility is that cytokines secreted after HSV infection induce bystander HIV infected cells to enhance viral replication (Duh et al., 1989). Alternatively, HSV-1 infection per se is sufficient to enhance HIV replication, as supported by evidence of increased HIV replication in cells infected with both HIV-1 and HSV-1 (Heng et al., 1994). The increased replication may result from enhanced

transcription of the HIV LTR in cells that harbour latent HIV, through increased NF-kB activation of the LTR (Mosca et al., 1987; Margolis et al., 1992). Transfection experiments and the use of HSV deletion mutants have proposed important roles for HSV ICP0, ICP4 and ICP27, although the status of HIV-1 infection and the cell type infected may determine their relative contribution (Albrecht et al., 1989; Golden et al., 1992; Margolis et al., 1992; Schafer et al., 1996). It is also possible that HSV promotes post-transcriptional HIV replication through the chaperone functions of US11 (Diaz et al., 1996).

Although the precise details of this dual viral interaction are still to be determined, the implications of this molecular interaction are twofold. Firstly, HSV activation of DC may not only induce the migration of HIV-laden DC to the LN, but also increase viral replication and thus trans infection of CD4+ T cells (Geijtenbeek et al., 2000a). Secondly, it implies that successful immunological control of HSV infection prior to the acquisition of HIV infection, or during the clinical latency stage of infection may reduce the number of HIV latent reservoirs that may be 'restimulated' by HSV infection, and limit or delay the progression to AIDS.

7.3.2 Development of a vaccine to HSV-1

The role of the cellular adaptive immune response in preventing neuronal infection, resolving peripheral infections and controlling the reactivation from latency, justifies the hope that a vaccine can limit the severity, and hence morbidity, of secondary infection (Liu et al., 2000; van Lint et al., 2004). Furthermore, less frequent reactivation may also reduce the number of episodes of asymptomatic viral shedding and therefore, the horizontal and vertical transmission rate in the population. Recent efforts have included the use of a recombinant gB/gD combined subunit vaccine. However, a large clinical trial showed that this offered no protection, despite eliciting strong neutralising antibody responses (Corey et al., 1999). Another recombinant gD vaccine, given with a more Th1-inducing adjuvant, demonstrated protection from HSV-2 disease only in women who were seronegative for HSV-1 and -2 at the time of vaccination (Stanberry et al., 2002). Interestingly, discovery of a recent locus of resistance to HSV in mice was also sex linked, affording protection in females

(Lundberg et al., 2003b). Animal studies demonstrated that the potency of the vaccine was dependent on the use of a Th1 skewing adjuvant (Bourne et al., 2003). Chapter 1 detailed how DC are major determinants of the Th1/Th2 balance in vivo, underlying that modulation of DC function may be important in improving vaccine design.

One approach to target DC in vivo is through DNA vaccination (Larregina et al., 2001). An inherent advantage of DNA over recombinant proteins is that the bacterial DNA from which plasmids are derived possesses intrinsic Th1 inducing adjuvanticity (Hemmi et al., 2000), as they are rich in hypomethylated CpG motifs that activate innate immunity via TLR9 ligation (Harandi et al., 2003). In addition, viral antigens may be administered together with genes coding for molecules which further enhance the function of DC as a "natural adjuvant". The combination of viral and immunological genes may ensure that DC transmit full activation signals to T cells, minimising the induction of regulatory T cells, which could limit the potency of the anti-HSV immune response both qualitatively and quantitatively (Suvas et al., 2003). Vaccination with DNA encoding IL-12 and IL-18 can result in DC transfection and expression of these cytokines at the time of T cell contact, skewing towards a Th1 response (Lee et al., 2003a). Enhanced migration of DC to secondary lymphoid organs has also been achieved, by immunising with DNA encoding the chemokine genes, CCL19 and CCL21 (Lee et al., 2003b), presumably by enhancing the source of these chemokines in the LN (Gunn et al., 1998; Ngo et al., 1998), recruiting further DC and T cells (Forster et al., 1999; Sallusto et al., 1999).

The potency of immunity is further enhanced by combining DNA vaccines with attenuated recombinant poxviruses in heterologous prime boost strategies (Toka et al., 2003). The mechanisms responsible for this enhanced efficacy are unclear but may relate to the interaction of DC with poxviruses. Wild type VV does not induce DC maturation (Engelmayer et al., 1999), but DC infected with the attenuated modified vaccinia virus Ankara (MVA) strain or with canarypox are induced to mature (Drillien et al., 2004; Ignatius et al., 2000). This may also explain the protection offered against lethal HSV-1 infection by the adoptive transfer of DC from MVA-treated mice (Franchini et al., 2004).

In addition, the data presented in chapter 6 illustrates that gD itself may possess adjuvant properties. It would be ideal to conceive gD as both adjuvant and antigen, but the data from vaccine trials using recombinant gD suggest that this does not occur to a significant degree in vivo. One possibility is that insufficient gD is delivered to DC in adjuvant form to stimulate them. In this respect, the role of other envelope glycoproteins and their mechanism of interaction with DC receptors that induces maturation will need to be evaluated, as they may synergise in transmitting positive signals to DC (see section 6.3.5).

7.3.3 Use of HSV-1 as a gene delivery vector

HSV-1 has been proposed to be a suitable vector for the delivery of antigens to DC. Its ability to infect DC efficiently and express engineered transgenes to a high degree (as determined by GFP expression in this thesis) are attractive properties as an ex vivo gene delivery vector (Coffin et al., 1998). The relative advantages and disadvantages of HSV-1 as a gene delivery vector relative to other viruses and non-viruses is beyond the scope of this thesis, and has been reviewed elsewhere recently (Lachmann, 2004). Nevertheless, data presented here demonstrate that the virus' ability to disrupt the function of DC requires these pathogenic viral factors to be identified and removed, in order to achieve a successful therapeutic application. Initial attempts have suggested that it is feasible to produce efficient, yet attenuated, HSV-1 vectors for gene delivery to DC (Samady et al., 2003). The deletion of IE genes to attenuate the pathogenicity of HSV-1 is consistent with the data using UV-HSV in chapter 6.

One way in which it has been proposed to exploit HSV infection of DC is to transfect the cells ex vivo, and then reintroduce them subcutaneously. As a result, a critical step in the success of this approach is subsequent emigration of DC from the skin to draining LNs. Recent evidence has shown that this process is highly inefficient in immature DC (de Vries et al., 2003). Maturation of DC induced by HSV-1 (chapter 6) suggests that the initial virus activation may prime DC for increased migration potential, enhancing the number of transfected DC reaching LNs, and stimulating appropriate antiviral, and therefore anti-transgene, immune responses.

7.4 Central role of DC in the pathogenesis of viral infections

The model described above (section 7.2) of a human peripheral herpetic lesion is partly based on data acquired in mice studies. The advantages of mouse models include the evaluation of disease outcomes in the presence of an intact immune system. However, many human viruses are restricted to infect human cells only, requiring the use of other members of the same virus family to infect primates (e.g. SIV infection of macaques), or transgenic mouse models (e.g. CD46 transgenic mice susceptible to MV infection (Marie et al., 2001)). These scenarios are imperfect, as they require extrapolation to humans or interpretation of virus infections in a host the virus has not evolved to replicate in. Where mouse models are available, the virus' life cycle may be different to that in humans. For example, HSV-1 ICP47 binds human TAP to prevent MHC class I loading and expression, but binds and inhibits murine TAP much less efficiently (Tomazin et al., 1998). The murine equivalent of HCMV, murine CMV (MCMV), uses different immunomodulatory genes and mechanisms to HCMV (Tortorella et al., 2000).

The analysis of ex-vivo derived human DC has also been be carried out but its usefulness is limited only to certain scenarios, particularly in systemic infections, such as HCV or HIV, where DC in circulation are infected by the virus (Navas et al., 2002; Goutagny et al., 2003; Donaghy et al., 2003). Changes in the numbers of DC in peripheral blood of these patients can also be used to infer the role of these cells in the control of the viral infection (Pichyangkul et al., 2003). However, this analysis must be interpreted cautiously, as changes in DC numbers could occur equally from suppression of haematopoiesis, or extravasation of DC to infected lesions in the periphery or into secondary lymphoid organs.

In light of the difficulties in interpreting animal and ex-vivo derived DC data, extrapolation of in vitro interaction of DC with viruses can also aid understanding the functional consequence in vivo. Analysis of disease outcomes may allow the result of in vitro infection to predict the role of DC infection in vivo. For example, DV and hantavirus, despite being of distinct viral phylogeny, both result in haemorrhagic fevers in man. Upon DC infection, both viruses induce maturation and elevate the resultant capacity to stimulate T cells (Ho et al., 2001; Raftery et al., 2002), suggesting

that DC infected with these viruses may accelerate the generation of the immunopathology responsible for the infection morbidity. Conversely, it could be that certain viruses have evolved to enhance or dampen the function of DC specifically in order to, increase their own survival and transmission. This would be most clearly evident by considering the topic from within specific virus families, and therefore this approach is used to explore the question below.

7.4.1 Herpesviruses

HSV-1, HSV-2, VZV, HCMV or HHV-6 infection of DC results in an impaired ability to stimulate T cell proliferation (Chapter 4) (Jones et al., 2003; Morrow et al., 2003; Moutaftsi et al., 2002; Kakimoto et al., 2002). Not only would this delay the generation of the antiviral response, but the infection of DC may also be central to the survival and transmission of these viruses in vivo. This is supported by analysing viral effects on various levels of the DC lifecycle.

It has been suggested that trans-infection of T cells from VZV infected DC is a critical initial step required for T cell mediated dissemination of the virus to the skin, resulting in the characteristic skin vesicular rash that may be important for the transmission of the virus (Abendroth et al., 2001; Ku et al., 2004). Although the infection of T cells by other herpesviruses has not yet been documented, the ability of HCMV to bind DC-SIGN suggests that this receptor may aid HCMV infection of T cells in trans, analogously to that seen with HIV-1 (Halary et al., 2002; Geijtenbeek et al., 2000a). Infection of T cells is not seen following co-culture with HSV-1 infected DC in vitro (chapter 4), but infected T cells could have been deleted by fratricide prior to detection (Raftery et al., 1999).

Some herpesviruses also prevent the differentiation of DC from their precursors. Both EBV and HCMV can infect monocytes and prevent their differentiation into immature DC (Li et al., 2002; Gredmark and Soderberg-Naucler, 2003; Guerreiro-Cacais et al., 2004). A decrease in DC numbers may allow sufficient time for EBV and HCMV to establish latency. As TLR ligands can also prevent the differentiation of monocytes into DC (Xie et al., 2003), it is not yet possible to determine whether this effect is

virally driven or secondary to the viral induced activation of monocytes (D'Addario et al., 2000; Simmen et al., 2001).

Herpesviruses may also have modified the expression of molecules important in the DC-T cell interaction. HSV-1 and HCMV infected DC are refractory to increases in co-stimulatory molecule expression after DC maturation (Chapter 4) (Salio et al., 1999; Moutaftsi et al., 2002). It is interesting to note that both HSV-1 and VZV specifically downregulate CD83 upon infection of mature DC (Kruse et al., 2000; Morrow et al., 2003). The functional significance of this event is not yet established but its conservation among these two α-herpesviruses suggests that it may favour their survival. In addition, HCMV infected DC become cytotoxic and kill surrounding T cells by FasL and TRAIL dependent mechanisms. Though not all T cells die in this manner, the ones that escape are anergic, suggesting that regulatory cytokines may also be secreted from HCMV infected DC (Raftery et al., 2001). Recent data demonstrating that HSV-1 also induces upregulation of TRAIL and Fas in infected DC suggests that this virus may utilise similar mechanisms to disrupt the proliferation of T cells (Muller et al., 2004), and that herpesviruses may have evolved to target the DC-T cell interaction specifically.

DC may also be central to herpesvirus establishment of latency, a characteristic of all herpesviruses. While EBV is latent in B cells, one of the HCMV reservoirs are myeloid DC progenitor cells, thus potentially undergoing latency in the precursors of DC themselves (Hahn et al., 1998). The central role of DC (specifically LC) in the establishment of HSV-1 latency was discussed in section 7.2.1.

7.4.2 Paramyxoviruses

MV, parainfluenza virus (PIV) and RSV all impair infected DC ability to activate T cells (Grosjean et al., 1997; Fugier-Vivier et al., 1997; Plotnicky-Gilquin et al., 2001; Bartz et al., 2003). Although the viral strategies used by these viruses are similar to those used by herpesviruses, such as TRAIL mediated induction of T cell apoptosis by MV infected DC (Vidalain et al., 2000a), distinct differences are also apparent. Despite inducing a more mature phenotype than uninfected DC (Servet-Delprat et al., 2000b),

the overall reduced ability of MV to stimulate T cell proliferation can be attributed at least partly to a direct interaction with MV NP, MV HA and MV F on DC or T cells (Klagge et al., 2000; Marie et al., 2001; Dubois et al., 2001). This mechanism can disrupt IL-12 secretion (Fugier-Vivier et al., 1997; Marie et al., 2001), and may also dampen T cell responses to irrelevant antigens non-specifically, possibly explaining post-MV infection immunosuppression (Marie et al., 2001; Klagge et al., 2000). A similar paradox between DC maturation and impaired T cell stimulation is seen following PIV infection of DC. UV-inactivation of PIV does not prevent the functional inhibition, suggesting that envelope glycoprotein-mediated immunosuppression, similar to MV, may also occur with this virus (Plotnicky-Gilquin et al., 2001).

The CD40 signalling pathway may be targeted by paramyxoviruses. CD40 ligation enhances MV replication (Fugier-Vivier et al., 1997) and CD40L stimulated IL-12 secretion by MV-infected DC is inhibited, in contrast to the enhanced secretion with LPS stimulation (Fugier-Vivier et al., 1997; Schnorr et al., 1997). Following RSV infection, DC are able to enhance secretion of IL-12p70 and T cell stimulation in response to poly (I:C) stimulation, but not to CD40L (Bartz et al., 2003). For both MV and RSV, a gene product appears to play a role, as UV-inactivation of the viruses negated the effect by RSV completely and also partially inhibited that of MV. Coupling these observations with data demonstrating enhanced MV replication in DC after CD40 ligation, it suggests that homologous mechanisms may exist within this virus family to target the CD40 signalling pathway in DC, both to suppress the function of the cell and to enhance the virus' own replication.

7.4.3 Retroviruses

HIV-1 results in immunodeficiency predominantly as a result of the long-term depletion of CD4+ T cells. A hypothetical role for DC in the initiation of this immunosuppression has been proposed for many years. The functional consequences of DC exposure to HIV-1 are still controversial, with some studies describing no inhibition in T cell stimulatory capacity (Sapp et al., 1999; Chougnet et al., 1999), and others showing reduced DC function (Donaghy et al., 2003; Granelli-Piperno et al., 2004). HIV-1 replication in DC alone is low compared to that observed in T cells, but

this is increased following ligation with activated T cells (Granelli-Piperno et al., 1999; MacDougall et al., 2002). This increased inoculum of virus could infect neighbouring CD4+ T cells and demonstrates a similar adaptation as seen for MV (Fugier-Vivier et al., 1997). The ability of HIV-1 to bind DC-SIGN and infect T cells in trans has led to a suggestion that HIV-1 has evolved to exploit DC as a vector to LNs (Geijtenbeek et al., 2000a). Recent evidence has suggested that the HIV-1 protein Nef actively promotes the upregulation and polarisation of DC-SIGN towards T cells in a heterotypic cluster (Sol-Foulon et al., 2002). Along with DC-SIGN mediated signals impinging on DC IL-12 secretion (Geijtenbeek et al., 2003), these observations support the importance of DC-SIGN in the pathogenesis of HIV-1 infection, and may explain the protection from HIV-1 infection offered by DC-SIGN variants (Liu et al., 2004).

DC may also be central to the dissemination of another retrovirus, HTLV-1. DC infection in mucosae may transport the virus to lymphoid organs, where DC scanning of a large number of T cells results in the establishment of numerous cell-to-cell contacts that the virus could exploit to infect many T cells (Igakura et al., 2003). This virus has been linked to the development of an aggressive leukaemia, ATL, in 0.05-0.1% of infected individuals (Tajima, 1990). Non-specific viral impairment of DC function may play a role in its pathogenesis, as asymptomatic HTLV-1 infected individuals have reduced T cell responses to EBV and PPD antigens (Katsuki et al., 1987; Tachibana et al., 1988). In addition, CD4+ T cells from ATL patients are deficient in CD40L expression (Makino et al., 2001), a molecule that is induced by interaction with DC (Johnson-Leger et al., 1998; Peng et al., 1998), possibly explaining the opportunistic infections that occur in ATL patients (Shimoyama, 1991). In this respect, it is notable that DC derived from monocytes infected with HTLV-1 in vitro, or derived from monocytes of HTLV-1 patients, are poor stimulators of HTLV-1 specific T cell responses (Makino et al., 2000). In some individuals, a consequential impaired DC stimulation of CD8+ CTL could reduce the ability to control escape mutants that may give rise to ATL (Furukawa et al., 2001).

7.4.4 Other viruses

The filoviruses, Ebola and Marburg viruses, result in fatal haemorrhagic fevers that are a consequence of endothelial cell mediated coagulopathy induced by cytokines of the innate immune system (Geisbert et al., 2003b). However, strong cellular adaptive immune responses correlate with a positive outcome (Jahrling et al., 1996; Baize et al., 1999). In vitro infection of DC with Ebola and Marburg viruses results in impaired stimulation of T cells (Bosio et al., 2003). Recent studies of nonhuman primate models of infection have identified DC as early targets for Ebola virus infection (Geisbert et al., 2003a). Furthermore, Ebola virus infection of DC via DC-SIGN (Alvarez et al., 2002) may facilitate trans infection of T cells, possibly explaining the high level of lymphocyte apoptosis observed in lymphoid organs (Geisbert et al., 2003a). Therefore, specific filovirus targeting of DC in vivo may delay the generation of a rapid and potent antiviral response necessary to clear the infection.

Poxviruses possess a large genome and subsequently a vast array of immunomodulatory molecules, some of which may affect the function of DC specifically. These can modulate MHC class I presentation, and cytokine, chemokine and complement networks (Seet et al., 2003). However, although several aspects of DC physiology could be targeted, VV infection of DC reveals a selective downregulation of CD80 and CD83, with minor effect on MHC and CD86 expression (Jenne et al., 2000). The change in expression of certain molecules expressed by professional antigen presenting cells may be advantageous to the virus' survival and suggests selective viral targeting of DC molecules.

7.5 Future work

This project has generated many questions that would be of interest to follow up through further experiments. Many of the finer details of future directions and of lack of investigation in the literature have been addressed in the relevant chapters. Below are outlined a few broad themes that may be of particular interest.

One important question that remains unanswered are the early effects in vivo on the morphology and migration of DC exposed to HSV-1. Studying chemokine-driven

migration of DC shortly after infection in vitro may be a useful approach, and human skin explants would enable the study of this phenomenon in a more relevant setting. Both these approaches may shed light on whether the morphological changes induced by the virus are critical to prevent migration out of the skin or in the antigen presentation setting in LNs. The added advantage of the skin model would be to demonstrate human DC infected with HSV-1 for the first time, possibly aided by the use of a GFP expressing virus (Larregina et al., 2001).

Simpler multicellular in vitro systems can also be set up to study the cross talk between cytokines/chemokines secreted by infected keratinocytes and DC. It would be interesting to study how the combination of signals emanating from these cells affects the Th skew of the ensuing antiviral T cell response initiated by DC. In a similar manner, the autocrine/paracrine role of type I IFNs in the amplification of immune responses to ligands of the innate immune system has not been documented in detail in humans and deserves further attention.

The interaction between virus and DC should also be examined further. For the use of DC as a vector, work understanding the inhibitory viral genes is ongoing in a collaborating laboratory and may both identify homologous mechanisms used within the herpesvirus family and also highlight novel regulatory components of DC functions. Ultimately, microarray studies of mutant viruses may help identify cellular pathways that are suppressed by the virus. Parallel viral gene expression profiling would both determine the HSV-1 genes that are expressed in DC, and also suggest candidate molecules for the changes in the cellular transcriptome identified.

Future work should also identify the roles of viral envelope glycoproteins and DC receptors responsible for the activation of DC. In particular, this study should be extended to a more detailed analysis of the functional consequence of this interaction. The cytokines secreted and intracellular signalling pathways activated would aid in shaping future vaccine designs. For example, glycoproteins can be designed with greater affinity for one receptor over another (Whitbeck et al., 1997; Krummenacher et al., 1999), shaping their adjuvant properties.

Finally, establishing a role in humans for DC in the regulation of HSV-1 infection needs to be determined. In particular, the role of DC in the establishment of latency after primary infection, and in the recurrence of symptomatic infection after viral reactivation, needs to be investigated. One could envisage that a dual chamber co-culture system of human skin explants and peripheral nerves may provide a useful approach (Mikloska and Cunningham, 2001). However, a central role of DC in the pathogenesis of HSV-1 infection may also be derived from genetic epidemiological approaches involving individuals infected with HSV-1 with varying disease severity and frequency of symptomatic recurrence. A relationship between susceptibility and variation in molecules central to the function of DC would support the hypothesis that this cell type does indeed play a critical role in the control and outcome of this infection in man.

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