Advances in Experimental Medicine and Biology 762

Li Wu
Olivier Schwartz *Editors*

HIV Interactions with Dendritic Cells

Infection and Immunity

Springer

Advances in Experimental Medicine and Biology

Volume 762

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ISSN 0065-2598
ISBN 978-1-4614-4432-9 ISBN 978-1-4614-4433-6 (eBook)
DOI 10.1007/978-1-4614-4433-6
Springer New York Heidelberg Dordrecht London

Library of Congress Control Number: 2012945591

Printed on acid-free paper

Springer is part of Springer Science+Business Media (www.springer.com)

Preface

We are excited to introduce this book as an addition to a new series, *Topics in HIV and AIDS Research*. We expect that this volume and other important topics of the book series efficiently promote basic and clinical research of HIV and AIDS in a timely fashion. In this book, we aim to provide the latest research summaries and critical analyses of a broad scope in HIV interactions with dendritic cells (DCs).

As a group of professional potent antigen-presenting cells, DCs bridge the innate and adaptive immune responses. Studying the mechanisms of HIV-1 interactions with DCs is critical to understand HIV pathogenesis and develop more effective interventions against HIV infection and AIDS. DCs perform a pivotal role in the induction and regulation of immune responses. However, HIV impairs DC function and exploits these cells to spread viral infection to target $CD4^+$ lymphocytes. Studies of simian immunodeficiency virus in the macaque model demonstrated that DCs are important for initiating viral infection and spread in vivo. Interactions between HIV and DCs are of vital importance because these cells are among the first to encounter the virus at the mucosal surface. HIV-1 can hijack DCs to facilitate initial stages of viral infection and promote further dissemination throughout the host. Moreover, HIV interactions with DCs alter innate and adaptive immune responses and may thus facilitate viral persistence. In contrast, DCs have been targeted for developing more effective AIDS vaccine by enhancing antigen presentation and immune responses.

Given the recent research progress and the advance in the techniques in studying HIV interactions with host cells and factors, there is a critical need for a reference book on HIV interactions with DCs. This book targets a broad readership to facilitate HIV/AIDS research and provides a practical tool for HIV researchers to continuously address novel questions. The chapter contributors of this book attempted to summarize the literature in the field and provide critical analysis and future directions. The authors are internationally recognized scientists who have contributed significantly to the study of HIV and DC interactions and display complementary expertise in cellular, molecular, virological, immunological, preclinical and clinical research.

We would like to highlight some features of the book: (a) The book systematically addresses HIV interactions with DCs with emphasis on HIV infection and

immunity; (b) State-of-the-art progress in studying HIV interactions with DCs using virological and immunological approaches; (c) A combination of basic and clinical aspects in studying HIV interactions with DCs; and (d) Providing future directions for the specific research topic in each chapter.

This book covers the following important and interesting topics: (1) Immunobiology of DCs and the influence of HIV infection; (2) Antiviral immune responses by human Langerhans cells and DCs in HIV-1 infection; (3) Plasmacytoid DCs in HIV infection; (4) Cellular and viral mechanisms of HIV transmission by DCs; (5) Role of glycosphingolipid in DC-mediated HIV-1 *trans*-infection; (6) Simian immunodeficiency virus interactions with macaque DCs; (7) Interactions between HIV-1 and innate immunity in DCs; (8) HIV impairment of immune responses in DCs; (9) HIV-derived vectors for gene therapy targeting DCs; and (10) Targeting DCs for improved HIV-1 vaccines.

The primary audience for this book includes HIV-1 and AIDS basic and clinical researchers, PhD or MD graduate students, and postdoctoral researchers in virology, immunology, infectious diseases, and pathology fields. This book is suitable for use in university classrooms or seminars. The possible courses in which the book could be used include the following: advanced virology, retrovirology, viral immunology, viral pathogenesis, advanced topic in HIV and AIDS research, and progress in microbiology and immunology.

We hope that the readers will appreciate this book as much as we enjoyed preparing it. We thank all the authors for their timely contributions.

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Chapter 1
Immunobiology of Dendritic Cells and the Influence of HIV Infection

Anthony L. Cunningham, Andrew Harman, Min Kim, Najla Nasr, and Joey Lai

Abstract Recent progress in phenotyping of human dendritic cells (DCs) has allowed a closer alignment of the classification and functions of murine and human dendritic cell subsets. Marked differences in the functions of these human DC subsets and their response to HIV infection have become apparent, relevant to HIV pathogenesis and vaccine and microbicide development. Systems biology approaches to studying HIV uptake and infection of dendritic cells has revealed how markedly HIV subverts their functions, especially in relation to the trafficking pathways and viral transfer to T cells. Furthermore the interactions between DCs and other innate immune cells, NK cells, NKT cells and gamma delta T cells are now known to influence DC and T cell function and are also disturbed by HIV infection in vitro and in vivo. Such cellular interactions are potential targets for vaccine adjuvants and immunotherapy.

1.1 Introduction

Dendritic cells (DC) are a family of professional antigen presenting cells (APC) that form an important link between the innate and adaptive immune systems. They are found as specific subsets in tissue and blood and are of either myeloid or plasmacytoid

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_1,

origin. Sessile myeloid DCs located in the peripheral tissues, and to some extent in blood, act as sentinels to detect and bind foreign antigens. In this state they are "immature" and rapidly endocytose and then process them by cathepsins in the endolysosomal pathway or, if infected within the cytoplasm, via proteosomal degradation. This leads to a process of DC maturation and then usually migration to the submucosa or draining lymph nodes to present foreign antigen bound via MHCI or MHCII to activate CD8 or CD4 lymphocytes respectively, thereby eliciting a cellular and aiding a humoral immune response. Plasmacytoid DCs (pDCs) are found mainly in the blood and lymph nodes and function primarily to provide antiviral defence by secretion of very large quantities of interferon (IFN)-α after migration to areas of foreign antigen exposure or inflammation. In this setting they can also present antigen to and activate T-cells. Certain subsets of DCs are also able to "cross-present" exogenous antigens taken up by endocytosis which then enter the MHCI (rather than MHCII) processing pathway to allow peptide presentation to CD8 lymphocytes. DCs also interact with other immune cells, including natural killer (NK), NKT, gamma delta T cells and B lymphocytes. They may interact with two of these cell types simultaneously, e.g. $CD4^+$ T and B lymphocytes to stimulate antibody production.

1.2 Dendritic Cell Classification, Origins and Development

1.2.1 Myeloid Dendritic Cell Classification (Table 1.1)

The accessibility of a variety of murine lymphoid tissues has allowed more rapid progress in defining murine rather than human DC subsets.

Table 1.1 Comparison of classification of murine and human dendritic cells

	Mice	Human
Blood	$CD8^+$ DC	$BDCA3^+$ $CD141^+$ DC
	$CD8^-$ DC	? $CD11c^+$ $CD1c^+$ DCs
	?	$CD16^+$ $MDC8^+$ DCs
	Monocyte derived DC (at inflammatory sites)	Monocyte derived DC (at inflammatory sites)
	Plasmacytoid DC	Plasmacytoid DC
Skin/mucosa	Langerhans cell	Langerhans cell
	$CD103^-$ Dermal DC	CD14 or $CD1a^+$ Dermal DCs?
	$CD103^+$ langerin + dDC	?BDCA3/$CD141^+$ DC

?: human or murine equivalent is unknown

1.2.1.1 Murine Myeloid Dendritic Cell Subsets

CD4 and CD8 (as an αα rather than αβ dimer), the CLRs DEC205 and langerin, the α chain of Mac1, and CD11b are used to distinguish murine DC subsets.

In lymphoid tissue there are five subsets of myeloid DCs found in normal mice: $CD4^-/CD8^+$, $CD4^+/CD8^-$, $CD4^-/CD8^-$ DCs are all found in the spleen and lymph nodes. Additionally, $CD4^-/CD8^-/CD11b^+/DEC205^+$ DCs and mature $langerin^+/CD11b^+/DEC205^+$ Langerhans cells (LC) (which have migrated from epithelia) are found in the lymph nodes only. $CD8^+$ DCs are concentrated in the T cell areas and the $CD8^-$ DCs in the marginal zones of lymph nodes; however, $CD8^-$ DCs migrate into the T cell zones after microbial stimulation.

In skin and mucosa the DC subsets differ between stratified squamous and columnar/cuboidal mucosa. Only LCs are found in the epidermis of stratified squamous epithelium and there are two subsets of dermal DCs, which can be differentiated according to CD103 expression. The $CD103^-$ dermal DC subset is similar to lamina propria DCs beneath columnar/cuboidal epithelium. In inflamed mouse skin these resident populations are augmented by infiltrating monocytes which differentiate in situ into monocyte-derived DCs (Eidsmo et al. 2009; Shortman 2012; Shortman and Liu 2002; Villadangos and Schnorrer 2007; Villadangos and Shortman 2010).

1.2.1.2 Human Myeloid Dendritic Cell Subsets

Human myeloid DCs can be divided into functional subsets based on anatomical distribution and the expression of cell surface markers.

In blood "classical" DCs express CD11c and CD1c (BDCA1) and a CD141 BDCA3 expressing subset equivalent to mouse $CD8^+$ DCs has also been defined (Ziegler-Heitbrock et al. 2010). DC like blood cells that express CD16 and M-DC8 (MacDonald et al. 2002; Schakel et al. 2006) have been recently classified within the monocyte population (Ziegler-Heitbrock et al. 2010), although it is clear that they share many properties with DCs rather than monocytes (Cros et al. 2010). The $CD141^+/BDCA3^+/CLEC9A^+$ subset and the murine equivalent of the $CD8\alpha^+$ DC found in lymph nodes are both capable of cross-presentation of pathogen antigens (Poulin et al. 2010).

In skin and stratified squamous anogenital mucosa there are at least three DC subsets—the well-characterized epidermal LCs expressing CD1a and langerin, and two found within the dermis that express either CD1a or CD14 (BDCA3/CD141 DCs have also been found in dermis recently). LCs characteristically express Birbeck granules, composed of langerin in endosome-like compartments, and often appear tennis racket shaped by electron microscopy. Both dermal subsets express the mannose receptor but only the CD14 expressing population expresses DC-SIGN (Turville et al. 2002). It is likely that in time, these DC subsets will be further divided based on the discovery of new novel expression markers (Ju et al. 2010).

1.2.1.3 In Vitro Derived Model Myeloid DCs

Isolation of ex vivo DC subsets is problematic as they exist in very low numbers (<1% of human skin and blood) and skin DCs are inherently difficult to isolate as immature cells as they are prone to maturation as a result of extraction (Harman et al. 2006). For these reasons model skin DCs are extensively used for studies of DC function and viral infection. The most common model—monocyte-derived DC (MDDC)—can be produced in large numbers of immature cells by culturing $CD14^+$ monocytes in IL-4 and GM-CSF (Romani et al. 1994; Sallusto and Lanzavecchia 1994). Similarly, LC like cells can be generated in vitro either from $CD34^+$ bone marrow or blood precursors cultured in GM-CSF and TNFα (Caux et al. 1992) or from $CD14^+$ monocytes using TGFβ, GM-CSF and IL4 (Geissmann et al. 1998). Although human monocytes have been shown to differentiate into both macrophages and DCs after *trans*-endothelial migration (Randolph et al. 1998) and a TGFβ dependent pathway from $CD14^+$ monocytes to LCs may exist in vivo (Larregina et al. 2001), it is unclear what the in vivo equivalents of these model DCs are. MDDCs have been proposed to most closely resemble $CD14^+$ dermal DCs partly because both express the CLR DC-SIGN (Turville et al. 2002). Similarly LC like cells derived from $CD34^+$ or $CD14^+$ precursors are assumed to mimic LCs due to their high langerin expression but unlike ex vivo derived LCs they also express the CLRs DC-SIGN or MR, similar to MDDCs. More recently a model LC has been proposed which is derived from the leukaemia derived cell line MUTZ3 (Hu et al. 1996). In addition to expressing high levels of langerin, MUTZ3 LCs lack the expression of DC-SIGN and the MR (Hu et al. 1996) and for this reason they are proposed to a better model LC. However, the cytokine manipulation to achieve this results in very low levels of CCR5 and they can be difficult to infect with HIV.

1.2.2 Plasmacytoid DCs

pDCs are characterized as positive for CD4, CXCR4, CCR5, human leukocyte antigen-DR (HLA-DR), BDCA2, BDCA4 and CD123 but do not express CD11c, DC-SIGN or Mannose Receptor (Colonna et al. 2004). These cells are continuously produced from $CD34^+$ haematopoietic stem cells in the bone marrow and migrate in blood to lymph nodes, mucosal-associated lymphoid tissue and spleen under steady state conditions. While absent in normal uninflamed tissue, circulating pDCs preferentially accumulate in lymph nodes upon exposure to an inflammatory stimulus (Siegal et al. 1999; Yoneyama et al. 2004). Human and murine pDCs are very similar in phenotype and function.

1.2.3 Origin and Development of Murine and Human DCs

Although the haemopoietic origin of DCs is clear (either myeloid or plasmacytoid), the precise relationship of circulating precursors to tissue DCs and the ontogeny of tissue and blood DCs is less well defined, particularly in humans. Skin DC subsets have been proposed to originate from both monocyte precursors and/or from committed local DC precursors largely based on the difference in murine skin DCs in wild type and M-CSF receptor deficient mice (Randolph et al. 1999) and on human *trans*-endothelial migration models (Randolph et al. 2002; Cheong et al. 2010). Common myeloid precursors have been identified in mice (Auffray et al. 2009; Liu et al. 2009) and some studies of human and mouse skin have suggested that $CD14^+$ monocytes are the direct precursors of epidermal LCs (Larregina et al. 2001). It has also been suggested that $CD16^+$ blood DCs (classified as monocytes), including those expressing the marker M-DC8 (Schakel et al. 2006), may be immediate precursors of some tissue DCs (Randolph et al. 2002, 1998). However reconstitution of skin DCs after bone marrow transplantation (Bogunovic et al. 2006) has suggested that LCs are replenished from long lived locally proliferating precursors and that $CD1a^+$ dermal DCs arise from precursors distinct from LCs (Ginhoux et al. 2007). The relationship between the human $CD11c^+$/$CD1c^+$ "classical" blood myeloid DCs as precursors of tissue DC sub-populations has been unclear but in murine models the development of committed DC precursors in blood and tissue from common myeloid precursors and differentiation in lymphoid tissue has been demonstrated (Liu and Nussenzweig 2010).

1.3 DC Pattern Recognition Receptors

Like other immune cells dendritic cells recognize pathogens through their unique molecular components (proteins, lipids, nucleic acids) or "pathogen-associated molecular patterns" (PAMPs) via pattern recognition receptors (PRR). Most PRRs are primarily expressed on the cell surface, or intracellularly; however, PRRs such as opsonins, pentraxins, collectins and mannan-binding lectin can also be secreted into the blood where they bind foreign antigens and activate phagocytic cells. Cellular PRRs are found at various locations in the cell, either on the plasma and endosomal membranes or within the cytosol, allowing recognition of a wide range of invading pathogens. The membrane receptors consist of the ubiquitous Toll-like receptors (TLR) and also a unique group of C-type lectin receptors (CLR). The cytosolic PRRs are either the RNA sensing RIGI-like receptors, RIGI and MDA5 and others, or DNA sensing, e.g. NOD-like receptors. Different subsets of DCs express markedly different patterns of TLRs and CLRs linked to downstream signalling pathways which partly define their phenotypes and functions.

Many CLRs are unique in their expression by DCs RLRs and NLRs which are more ubiquitously expressed by most cell types. For this reason and also due to the wealth of literature on the subject of TLRs and RLRs we follow with a detailed account of HIV binding CLRs expressed by DCs and a much shorter account of TLRs and RLRs.

1.3.1 C-Type Lectin Receptors (Fig. 1.1)

CLRs are of particular importance in DC function. They comprise several families the most notable being the CLR domain family members (CLECs) which bind foreign antigen and include CLEC-1 (CLEC1A), DC-SIGN (CELC4L), langerin (CLEC4K), DCIR (CLEC4A), BDCA2 (CELC4C), dectins 1 and 2 (CLEC7A/6A), mannose receptor types 1 and 2 (CLEC13D/E) and DEC205 (CLEC13B). Other CLRs include the collectins and selectins. CLRs can be classified into two groups on the basis of their molecular structure. Type I CLRs such as the mannose receptor types 1 and 2 and DEC205 are characterized by having their N-termini pointing outwards from the cell membrane, contain an N-terminal cysteine-rich domain (Cys-RD), a fibronectin type II domain and multiple carbohydrate recognition domains (CRD). Type II CLRs such as DC-SIGN, Langerin, DCIR, dectin-1 and -2, BDCA2 and CLEC-1 are characterized by having their N-terminal pointing into the cytoplasm and contain a single C-terminal CRD (Figdor et al. 2002). CLECs recognize conserved oligosaccharide motifs which are frequently found on microbial surface glycoproteins but not on mammalian cells. As HIV gp120 is highly glycosylated (approximately 50% of its molecular weight is due to glycosylation) (Allan et al. 1985), high affinity binding to CLRs is facilitated. So far, eight CLECs have been identified to be able to bind gp120, the most notable being DC-SIGN, MR and Langerin. The expression pattern of these CLRs differs on various DC subsets (Table 1.2). For instance, LCs express Langerin without DC-SIGN or MR, $CD1a^+$ dermal DCs express MR but not DC-SIGN or Langerin and $CD14^+$ dermal DCs and MDDCs express both DC-SIGN and MR but not Langerin (Turville et al. 2002). Therefore, the heterogeneous distribution of CLRs on different DC subsets will affect which CLR plays a dominant role in gp120 binding in each of the different subsets. By definition, recognition of gp120 by all CLRs occurs in a calcium-dependent manner via highly conserved carbohydrate recognition domains (CRD).

1.3.1.1 The DC-SIGN Family

DC-SIGN (CLEC4L), DC-SIGNR or L-SIGN (CLEC4M) and LSECtin (CLEC4G) are highly related and bind to a wide variety of pathogens; however, they have distinct ligand-binding properties and different physiological functions (Khoo et al. 2008). By far the most well known is DC-SIGN which has been found to be the

Fig. 1.1 (continued) (**b**) MR consists of an N-terminal cysteine-rich domain, followed by a fibronecting type-II domain, eight CRDs, a transmembrane domain and a short cytoplasmic tail. CRDs 4 and 5 are involved in Ca^{2+}-dependent binding of carbohydrate ligands. (**c**) Similar to DC-SIGN, langerin consists of a single C-terminal CRD and an α-helical coiled coil neck domain. Depicted is a soluble form of langerin which does not express a transmembrane domain or cytoplasmic tail. The DC-SIGN image was reproduced from Wu and KewalRamani (2006). The langerin image was reproduced from Feinberg et al. (2010)

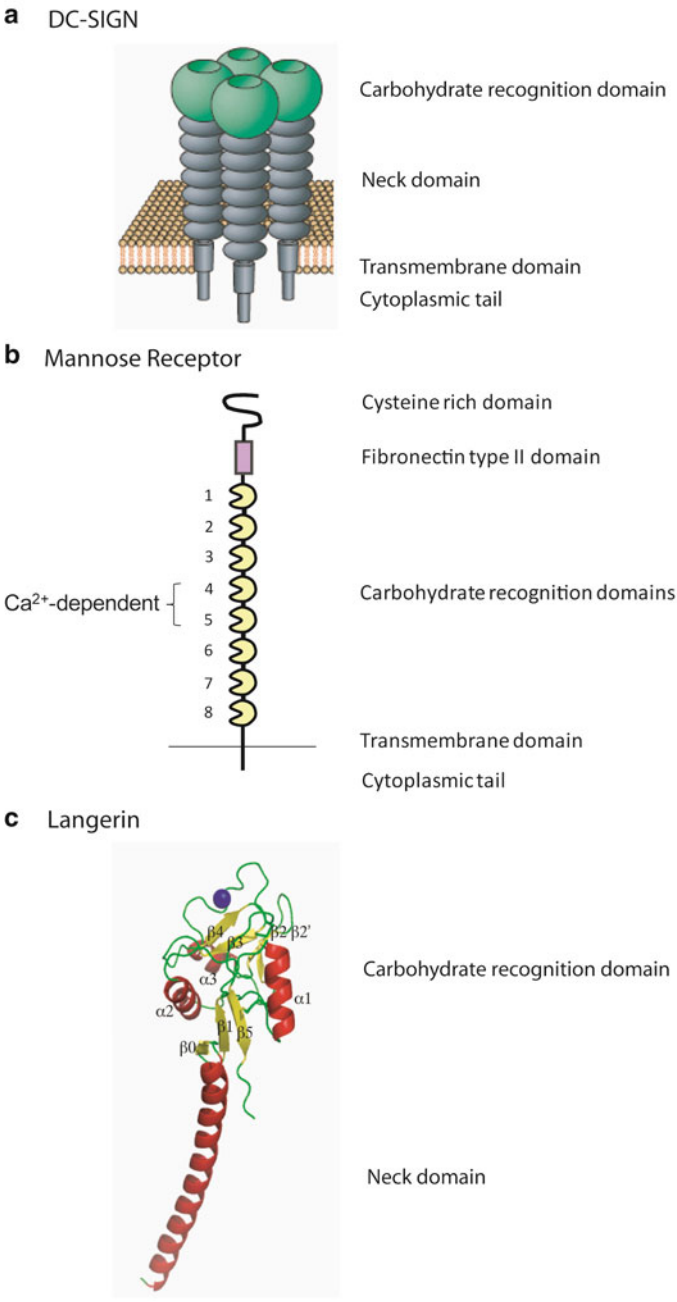

Fig. 1.1 Structure of DC-SIGN, MR and langerin. (**a**) DC-SIGN consists of a single CRD at its C-terminus, followed by an α-helical coiled coil neck domain, a transmembrane domain and a short cytoplasmic tail. DC-SIGN is depicted in its tetrameric form on the cell surface.

main CLR expressed on MDDCs and mediates binding of gp120 from X4, R5 and X4/R5 HIV-1 strains as well as HIV-2 and SIV (Geijtenbeek et al. 2000b). In addition to antigen capture, the biological function of DC-SIGN is to regulate adhesion particularly during DC trafficking and during the formation of DC-T-cell synapses. The natural ligand for DC-SIGN is ICAM-3 expressed on naive $CD4^+$ T-cells, although it can also interact with ICAM-2 (Geijtenbeek et al. 2000a). In vitro, DC-SIGN is expressed highly on immature MDDCs and is partially down-regulated upon HIV infection (Harman et al. 2006). DC-SIGN belongs to the type-II family of CLRs, consisting of a single CRD at its C-terminus followed by a coiled-coil neck domain important for receptor oligomerization, a transmembrane domain and a short cytoplasmic tail mediating interactions with machinery important for ligand internalization. Binding to a wide variety of microbes and viruses has been demonstrated (e.g. *Candida albicans*, hepatitis C virus, dengue virus, Ebola virus and mycobacteria) (Cambi et al. 2003; Pohlmann et al. 2003; Tassaneetrithep et al. 2003; Simmons et al. 2003; Tailleux et al. 2003). High affinity binding of the CRD to tri-mannose glycosylation and Lewis X structures present in N-linked high mannose oligosaccharides found in HIV gp120 is facilitated by tetramerization of the receptor and binding of gp120 has been shown to require DC-SIGN tetramer formation (Bernhard et al. 2004). Similar to DC-SIGN, DC-SIGNR or L-SIGN is involved in binding HIV and its subsequent transmission to T-cells and is associated with increased HIV-RNA loads and HIV sexual transmission (Xu et al. 2010). Little is known about LSECtin; however, it has been shown to mediate antigen capture and pathogen binding by human myeloid DCs (Dominguez-Soto et al. 2007). As well as acting as an attachment factor for pathogens, it also recognizes endogenous activated T cells (Tang et al. 2010).

1.3.1.2 The Mannose Receptor (CD206/CLEC13D)

This is a multi-domain type-I CLR that is expressed on various DC subsets as well as macrophages, astrocytes, and epithelial and liver endothelial cells. It is a recycling receptor present in endocytic compartments and functions as a PRR, recognizing foreign and endogenous ligands bearing mannose residues such as pituitary hormones which are cleared within the liver (Roseman and Baenziger 2000), lysosomal enzymes (Stahl et al. 1980) and tissue plasminogen activator (Otter et al. 1991), which are often released in response to cells during pathological events. Structurally, the extracellular domain of MR consists of an N-terminal Cys-RD involved in binding glycoproteins terminating in four sulphated *N*-acetylgalactosamine residues (GalNAc-4-SO_4), a collagen binding fibronectin type II domain and eight CRDs on a single polypeptide backbone (Taylor et al. 1990; Kornblihtt et al. 1985; Drickamer 1988). Of the eight CRDs, CRD 4 and 5 have been shown to be required for high affinity binding of ligands containing single terminal sugars such as D-mannose, *N*-acetylglucosamine (GlcNAc) and fucose which are present on microbial surfaces including gp120 (Taylor et al. 1992). A short cytoplasmic tail containing tyrosine-based motifs required for endocytic recycling also exists (East and Isacke 2002). Dimerization of MR has also been shown to enhance gp120 binding (Lai et al. 2009).

1.3.1.3 Langerin (CD207/CLEC4K)

This is a type-II CLR which is expressed solely on LCs in vivo and like DC-SIGN, consists of a single C-terminal CRD (Valladeau et al. 1999, 2000) followed by coiled-coils of α-helices in the neck domain that stabilize oligomer formation (Stambach and Taylor 2003). The CRD of langerin contains a Ca^{2+}-dependent sugar binding site which displays mannose specificity and interacts with high mannose structures, fucose, GlcNAc, galactose-6-sulphated oligosaccharides and Lewis Y containing carbohydrate residues (Tateno et al. 2010; Stambach and Taylor 2003; de Jong et al. 2010a) on microbial surfaces. In particular, langerin has been shown to bind yeast mannan (Takahara et al. 2002), β-glucans on the surface of pathogenic fungi (de Jong et al. 2010b; Takahara et al. 2004) and high mannose residues on HIV gp120 (de Witte et al. 2007; Turville et al. 2001). gp120 has been shown to bind to soluble langerin (Stambach and Taylor 2003), langerin expressed on LCs (de Witte et al. 2007; Turville et al. 2002) and Mutz-3-derived LCs (de Jong et al. 2010a). However, unlike DC-SIGN, langerin fails to bind Lewis X containing carbohydrate residues (de Witte et al. 2007; Chatwell et al. 2008), forms trimers rather than tetramers (as soluble recombinant proteins) (Stambach and Taylor 2003; Feinberg et al. 2010), and contains an additional Ca^{2+}-independent binding site in the CRD which binds to maltose residues (Chatwell et al. 2008). Like all other CLRs, the oligomerization of langerin into trimers enhanced its binding affinity towards carbohydrate ligands (Feinberg et al. 2010; Stambach and Taylor 2003).

Other HIV Binding CLRs

Other CLRs which are known to bind HIV include BDCA2 (CLEC4C) (Martinelli et al. 2007) which is a type-II CLR exclusively expressed by pDCs, DCIR (CLEC4A) (Lambert et al. 2008) DEC205 (CLEC13B) (Jiang et al. 1995) and CLEC1B (Mourao-Sa et al. 2011).

1.3.2 Toll-Like Receptors (Fig. 1.2)

There are ten TLRs, TLR1, 2, 4, 5, 6 and 10 which are expressed on the plasma membrane of DCs and TLRs 3, 7/8 and 9 which are expressed on endosomal membranes. The surface TLRs sense bacterial and viral lipids and proteins whereas endosomal TLRs sense nucleic acids; TLR3 double stranded RNA; TLR 7/8 single stranded RNA; TLR9 CpG motifs of pathogen DNA. Different subsets of DCs express markedly different patterns of TLRs linked to downstream signalling pathways which partly define their phenotypes and functions. For example LCs express TLRs 1, 2, 3, 5, 6 and 10 (Siegal et al. 1999) while the TLR repertoire of human dermal DCs probably resembles MDDCs (TLRs 1, 2, 3, 4, 6, 8 and 10) (Kadowaki et al. 2000) (Table 1.2).

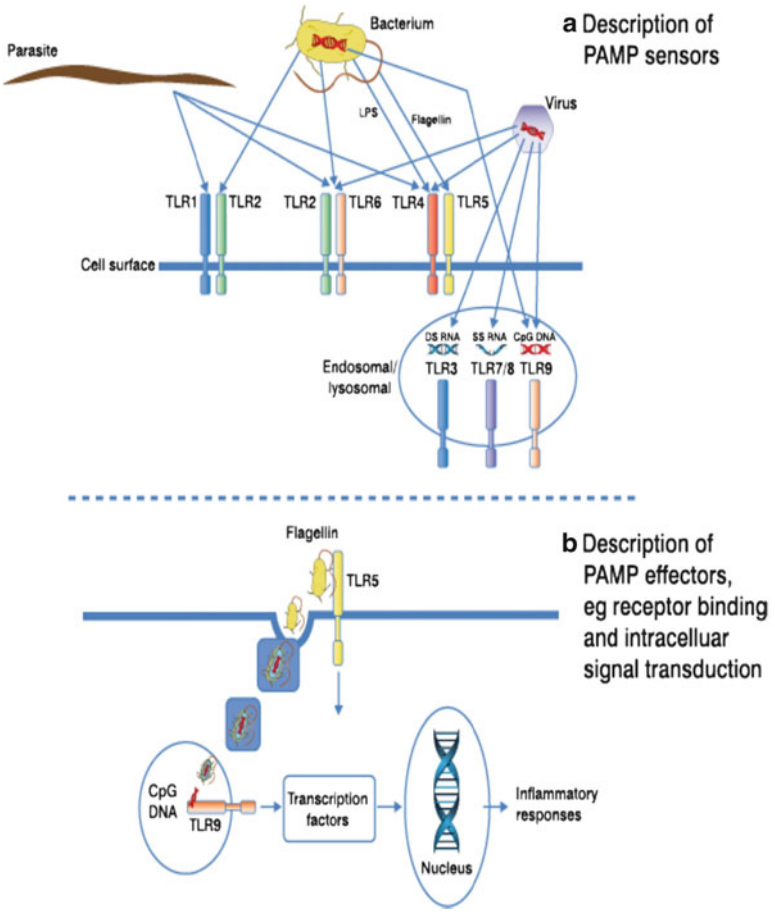

Fig. 1.2 (**a**) Interaction of bacterial and viral molecules (PAMPs) with Toll like receptors expressed on the plasma membrane and in endosomes of DCs (**b**) Simplified signalling pathway following pathogen activation of TLRs. (Reproduced from Garcon N, Stern P, Cunningham AL, Stanberry L (eds) (2011) Understanding modern Vaccines. Elsevier)

1.3.3 Cytoplasmic PRRs

Intracellular pathogen recognition occurs via cytosolic RLRs comprising of the RNA helicases retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation associated gene 5 (MDA5) proteins. In addition another family member known as LGP2 has been identified. However, because LGP2 lacks the caspase-recruitment and activation domains (CARDs), it is postulated that it might exert a negative regulatory role by competing with RIG-I and MDA5 during the detection of viral RNAs (Onoguchi et al. 2007; Yoneyama et al. 2005). Another group of cytoplasmic PRRs are the DNA sensors, including the nucleotide oligomerisation domain (NOD)-like receptors (NLRs) such as the recently discovered z-DNA binding protein 1 (DBP1) (Bonardi et al. 2012).

Table 1.2 TLR and CLR expression by human DC subtypes

		Dermal DCs		Blood DCs		
				Myeloid		
	LCs	$CD14^+$	$CD1a^+$	BDCA1	$BDCA3^+$	Plasmacytoid
C-type lectin receptors[a,b,c]						
CD206 (mannose)	−	+	+++	−	−	−
CD207 (langerin)	+	−	−	−	−	−
CD209 (DC-Sign)	−	+	−	−	−	−
BDCA2		−	−	−	−	+
BDCA3		−	−	−	+	−
BDCA4		−	−	−	−	+
Toll-like receptors[d,e,f,g,h]		Similar to MDDCs?				
TLR1	+	+		−	−	+
TLR2	+	+		+	+	−
TLR3	+	+		+	+	−
TLR4	−	+		+	−	−
TLR5	±	−		−	−	−
TLR6	+	+		−	−	+
TLR7	−	−		+	−	++
TLR8	−	+		+	+	−
TLR9	−	−		−	−	++
TLR10	+	+		−	−	+

[a] Ebner S, Ehammer Z, Holzmann S et al. (2004) Expression of C-type lectin receptors by subsets of dendritic cells in human skin. Int Immunol 16:877–887

[b] Figdor CG, Van Kooyk Y, Adema GJ (2002) C-type lectin receptors on dendritic cells and Langerhans cells (Review). Nat Rev Immunol 2:77–84

[c] Valladeau J, Duvert-Frances V, Pin JJ et al. (1999) The monoclonal antibody DCGM4 recognizes Langerin, a protein specific of Langerhans cells, and is rapidly internalized from the cell surface. Eur J Immunol 29:2695–2704

[d] Flacher V, Bouschbacher M, Verronèse E, Massacrier C, Sisirak V, Berthier-Vergnes O, de Saint-Vis B, Caux C, Dezutter-Dambuyant C, Lebecque S, Valladeau J (2006) Human langerhans cells express a specific TLR profile and differentially respond to viruses and gram-positive bacteria. J Immunol 177:7959–7967

[e] Jarrossay D, Napolitani G, Colonna M, Sallusto F, Lanzavecchia A (2001) Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells. Eur J Immunol 31:3388–3393

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[g] Ito T, Amakawa R, Kaisho T, Hemmi H, Tajima K, Uehira K, Ozaki Y, Tomizawa H, Akira S, Fukuhara S (2002) Interferon-α and interleukin-12 are induced differentially by Toll-like receptor 7 ligands in human blood dendritic cell subsets. J Exp Med 195:1507–1512

[h] Krug A, Towarowski A, Britsch S, Rothenfusser S, Hornung V, Bals R, Giese T, Engelmann H, Endres S, Krieg AM, Hartmann G (2001) Toll-like receptor expression reveals CpG DNA as a unique microbial stimulus for plasmacytoid dendritic cells which synergizes with CD40 ligand to induce high amounts of IL-12. Eur J Immunol 31:3026–3037

1.4 General Dendritic Cell Functions

1.4.1 Dendritic Cell Maturation

Antigen capture by PRRs induces DCs to undergo a process called maturation resulting in the migration of these cells to the draining lymph nodes followed by antigen presentation and activation of T-cells, thus inducing an immune response. Maturation of DCs is marked by changes in surface expression of a variety of proteins, resulting in altered DC function. First, CLR expression is down-regulated resulting in a reduced capacity for antigen binding and endocytic uptake. Second, the expression of molecules involved in chemotaxis are up-regulated, particularly CCR7, which facilitates migration to the lymph nodes along CCL19 and CCL21 chemotactic gradients. Third, various proteins are up-regulated that allow DCs to interact with T-cells upon their arrival at the lymph nodes such as those involved in antigen presentation (MHCII), T-cell activation (CD40, CD80, CD86), and cell adhesion molecules (CD54/ICAM-1). Finally, CD83 is expressed de novo which plays a role in lymphocyte maturation (Breloer and Fleischer 2008). Mature DCs in the lymph nodes form clusters with T-cells resulting in the formation of an immunological synapse (Dustin and Colman 2002; Lee et al. 2002). Key to the formation of these synapses is the remodelling of the actin cytoskeleton and the interaction between CD54 on the DC and LFA1 on the T-cell. Co-stimulatory molecules and those involved in antigen presentation on the DC membrane and their cognate receptors on the T-cell membrane become concentrated at the IS and this contact region facilitates T-cell activation and antigen presentation.

Although we and others have reported that HIV can stimulate partial DC maturation (Fantuzzi et al. 2004; Harman et al. 2006; Wilflingseder et al. 2004) this is controversial with some arguing against (Granelli-Piperno et al. 2004; Muthumani et al. 2005; Smed-Sorensen et al. 2004), although mostly using low HIV inocula. Both vesicular trafficking and de novo infection contribute to the maturation and the latter to up-regulation of the cell adhesion molecule ICAM1, probably contributing to adhesion and clustering with T cells. When HIV infected DCs cluster with T-cells the virus is transferred across a virological synapse at the contact region (Felts et al. 2010; McDonald et al. 2003). Though similar, immunological and virological synapses do differ in their complement of adhesion molecules and other proteins (Fackler et al. 2007).

1.4.2 Endolysosomal Processing

The primary function of CLRs is to mediate rapid endocytosis of foreign antigen though more is being discovered about the signalling pathways that are activated by these PRRs in myeloid cells (Sancho et al. 2012). In particular HIV binding to DC-SIGN has been shown to activate the LARG/Rho signalling pathway which is

important for the formation of virological synapses between DCs and T-cells (Hodges et al. 2007). In addition HIV binding to the mannose receptor can lead to the secretion of lysosomal enzymes, the expression of tumour necrosis factor α, IL-12, matrix metalloproteinases-9 (MMP)-9 and signal regulated protein kinases (Lopez-Herrera et al. 2005). Following CLR mediated endocytosis antigen is taken up into early endosomes. CLRs such as the mannose receptor then enter recycling endosomes and are transported back to the cell surface, whereas foreign antigen progresses through the endolysosomal pathway. This results in degradation of the antigen in the acidic environment of late endosome/lysosome by enzymes, especially the cysteine protease members of the cathepsin family. These are capable of cleaving a wide variety of substrates and play a key role in mediating the loading of antigen fragments onto MHCII which is then transported to the cell surface for presentation to T-cells (Reiser et al. 2010). Recently HIV infection of DCs has been shown to specifically interfere with the function of some of these cathepsins, especially those involved in antigen processing, down-regulating their expression and inhibiting their enzymatic activity (Harman et al. 2009).

1.4.3 Pathogen/Antigen Processing Through MHCI and II Pathways and Cross-presentation in DCs (Fig. 1.3)

Dendritic cells, as the major APCs in the body, can process antigens through either MHCI or MHCII pathways and, in some subsets such as murine $CD8\alpha^+$ and human $BDCA3^+$ DCs and MDDCs, through "cross-presentation" pathway(s).

1.4.3.1 MHCI

MHCI is ubiquitously expressed whereas MHCII is mainly expressed by professional APCs such as DCs, macrophages and B cells, although it can be induced on fibroblasts, epithelial and endothelial cells by IFN-γ. Cytosolic pathogen or endogenous antigen is processed through the MHCI pathway and exogenous pathogens taken up in endosomes or phagosomes through the MHCII pathway, illustrated in Fig. 1.4. Cytosolic viral peptides are cleaved in the proteasome, translocated into the endoplasmic reticulum by transporter associated with antigen presentation (TAP) and assembled with and stabilizing the MHCI (HLA-A, B, C) heterodimer. Prior to this, individual MHCI molecules are stabilized by the endoplasmic reticulum (ER) chaperones, calreticulin, ERp57, protein disulphide isomerase and tapasin. Tapasin links TAP mediated translocation and peptide-MHCI assembly. Fully assembled complexes are transported to the cell membrane to be presented to $CD8^+$ lymphocytes whereas unbound MHCI and viral peptides return to the cytosol for degradation. Thirty to seventy percent of all viral or host cell proteins translated from the ribosome are immediately degraded into so called defective ribosomal products (DRiPs), which are then translocated to the ER to bind MHCI, allowing very rapid recognition of virus infected cells.

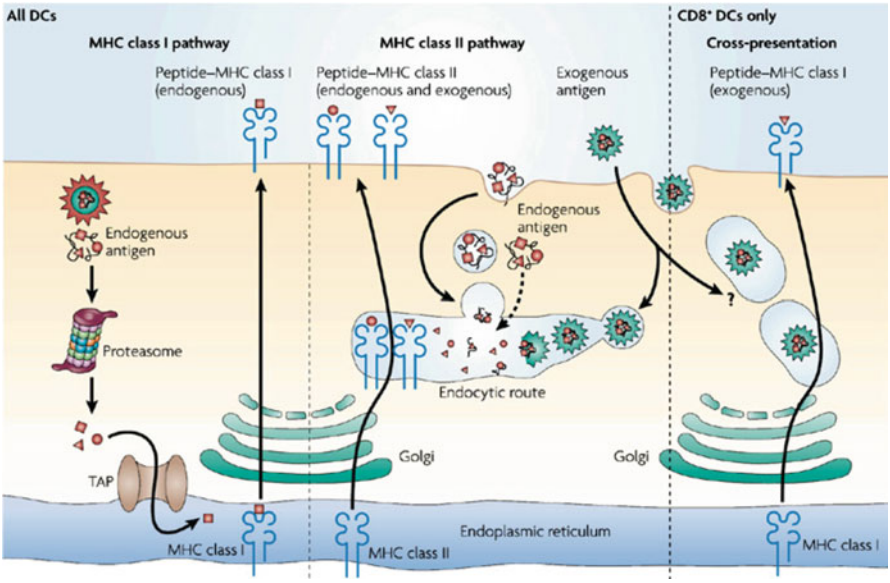

Fig. 1.3 Antigen processing and presentation pathways in dendritic cells. (Reproduced from Villadangos and Schnorrer 2007). *Left panel*: MHC class I molecules present peptides that are derived from proteins degraded mainly in the cytosol, which comprise pathogens (especially viruses) replicating in the cytoplasm or endogenous proteins (synthesized by the cell itself). *Middle panel*: MHC class II molecules acquire peptide cargo generated by proteolytic degradation in endosomal compartments. The precursor proteins of these peptides include exogenous material that is endocytosed from the extracellular environment, and also endogenous molecules, such as plasma membrane proteins, components of the endocytic pathway and cytosolic proteins that gain access to the endosomes by autophagy. *Right panel*: Cross-presentation pathway: Some DCs have a unique ability to deliver exogenous antigens to the MHC class I pathway, although the mechanisms involved in this pathway are still poorly understood. The *bifurcated arrow* indicates that the MHC class II and the MHC class I cross-presentation pathways may "compete" for exogenous antigens in $CD8^+$ DCs, or that the endocytic mechanism involved in internalization of a given antigen may determine whether it is preferentially delivered to the MHC class II pathway or the MHC class I cross-presentation pathway. *TAP* transporter associated with antigen processing

1.4.3.2 MHCII

The MHCII α and β chain heterodimer assembles first in the ER, associates with the invariant chain II and is then transported to the late endosomal MHC class II compartment (MIIC). Invariant chain II is digested here leaving the CLIP peptide in the MHCII peptide binding groove. This peptide is then exchanged for an exogenous (pathogen-derived) endosomal peptide, but only in the presence of HLA-DM. The MHCII-peptide complex is then transported to the plasma membrane to be presented to $CD4^+$ lymphocytes. The minimal requirements for the structurally heterogenous MIIC are MHCII, HLA-DM and the cathepsins (especially L and S) and it may appear in multivesicular and multilamellar bodies. Uniquely, in DCs the

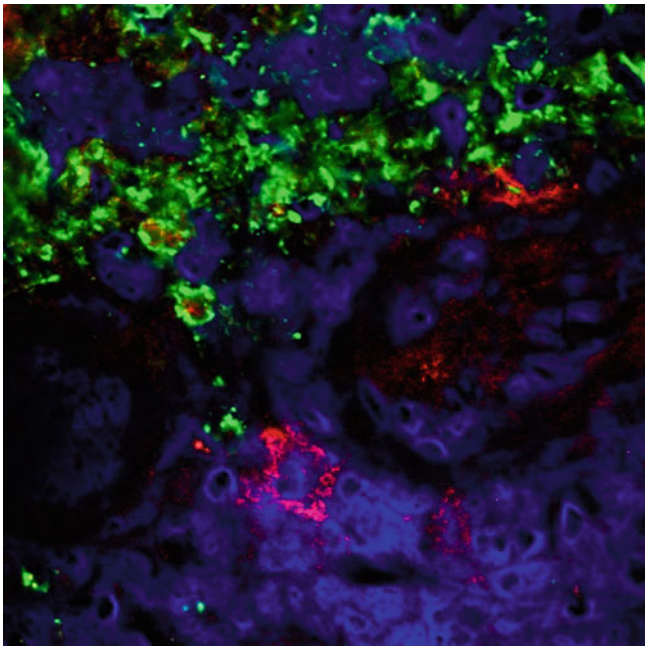

Fig. 1.4 Plasmacytoid dendritic cells (pDCs) in the upper dermis of recurrent herpetic lesions and may be accessible to HIV superinfection. HSV infected epidermal keratinocytes stained green (HSV-GFP). BDCA-2+ pDCs stained red. Nuclei stained blue with TO-PRO-3. Confocal microscopy. Bar 10 μm. (Reproduced from Donaghy et al. 2009)

transport of MHCII containing vesicles to the plasma membrane is enhanced by maturation stimuli which also stabilize the MHCII complex on the membrane (Villadangos and Schnorrer 2007).

1.4.3.3 Cross-presentation

Certain DC subsets can endocytose exogenous antigens and then "cross-present" them on MHCI, transiting the cytosolic pathway. This is of key importance in antiviral immunity. A number of mechanisms have been proposed to explain this pathway, including the exit of antigens from the endosome, and why it is restricted to murine $CD8^+$ DCs or human MDDCs or $BDCA3^+$ DCs: these include specific proteases in the latter cell types, or unique processes such as direct antigen transfer from endosome to the cytosol or via the ER or via fusion of the endosome with the ER. None have been widely accepted (Neefjes et al. 2011; Shen and Rock 2006; Villadangos and Schnorrer 2007).

1.4.3.4 DC Subsets in Antiviral Immunity

Studies of viral infections in murine models have revealed considerable complexity in transport of viral antigens to and presentation to $CD8^+$ lymphocytes in lymph nodes, leading to evidence for two scenarios.

1. Where viruses infecting "migratory" DCs (LCs, dermal/lamina propria DCs) are cytopathic or disable antigen presentation (e.g. HSV, vaccinia; Bosnjak et al. 2005; Villadangos and Schnorrer 2007), the DCs may carry the viral antigens in apoptotic infected DCs and can then transfer them to a second DC, either in the dermis (to $CD103^+$ dermal DCs) or in the lymph nodes (to $CD8^+$ DCs). These DCs then act as the primary APCs to $CD8^+$ lymphocytes by cross-presentation. However, the migratory DCs are the primary APCs for $CD4^+$ lymphocytes.
2. Where the infecting viruses have little cytopathic effect (such as lentiviruses) on the migratory DCs, these DCs act as the primary APCs for both $CD4^+$ and $CD8^+$ lymphocytes in the lymph node, through direct presentation rather than cross-presentation (Villadangos and Schnorrer 2007).

   Parallel studies with human DCs in lymphoid tissues are needed.

1.4.4 Viral Trafficking in Vesicles and Cytosol of DCs

Although HIV can productively infect DCs via CD4/CCR5 mediated neutral fusion with the plasma membrane, this represents a minority route of entry (<5%). Thus, HIV infection of DCs occurs at relatively low levels compared to $CD4^+$ T-cells, with at least a tenfold reduction in infectivity. Whether the remaining 95% of virus is taken up by endocytosis and subsequently traffics through the endolysosomal pathway (as was originally assumed) is currently an area of intense debate and scrutiny as recent advances in microscopy techniques have raised doubts as to the role of this pathway. Recent attempts have been unable to show HIV within early endosomes (Garcia et al. 2005) or lysosomes (Turville et al. 2004) of DCs. In mature DCs HIV is taken up into a single tetraspanin rich $CD81^+$ compartment (Garcia et al. 2005) that is continuous with the plasma membrane (Yu et al. 2008) known as a "vesicular cave". In contrast, immature DCs show a more diffuse pattern of HIV uptake, some of which is co-localized with CD81, but a large proportion is not (Yu et al. 2008). It is likely that the proportion that does not co-localize with CD81 is endocytosed, although the lack of co-localization with early endosomal markers such as EEA1 is intriguing (Garcia et al. 2005). Potentially, virus has already undergone some sort of processing within minutes involving rapid transit through the early endosome, as seen with parvoviruses (Harbison et al. 2009), making detection very difficult. Interestingly, AP3 (a molecule involved in transport of cargo through the endolysosomal pathway) has been shown to accumulate with HIV in $CD81^+$ compartments in productively infected DCs (Garcia et al. 2008). In support of an explicit role for the endolysosomal pathway in HIV trafficking, HIV uptake by clathrin mediated

endocytosis has been shown (Frank et al. 2002; Garcia et al. 2005) and genes associated with endocytosis are up-regulated early after infection (Harman et al. 2009). Furthermore, as discussed above DCs are able to efficiently present HIV antigen to T-cells via MHCII, a process that is integral to the endolysosomal pathway (Fonteneau et al. 2003; Larsson et al. 2002; Moris et al. 2006).

1.4.4.1 Two (or Three) Phase Transfer of HIV from DCs to T-Cells

Corresponding to the two routes of entry of HIV entry into monocyte-derived DCs, the virus can be transferred to T-cells in two distinct phases (Turville et al. 2008, 2004) which is mirrored by two distinct bursts in DC gene expression (Harman et al. 2009). If the infected DC comes into contact with a T-cell within the first few hours of infection (probably in the mucosa) then virus particles present in $CD81^+$ vesicular caves are regurgitated at the virological synapse into the intracellular space where they come into direct contact and infect $CD4^+$ T-cells. By 12–24 h post infection no HIV virions can be detected within DCs presumably because they have all been destroyed by acid proteolysis, most likely in late endosomes, and no transfer of virus to T-cells is observed.

However, by 48 h post infection DCs are again able to transfer HIV to T-cells in a second phase of transfer, with the amount of HIV transferred increasing with time. This second distinct phase of viral transfer occurs as newly synthesized HIV virions bud off from the plasma membrane, which are initially derived from the small percentage of HIV that entered by CD4/CCR5 mediated neutral fusion resulting in low level productive infection. These low levels of HIV infection in DCs, compared to macrophages and T cells are due to most (>90%) of the HIV inoculum being diverted through DC-SIGN/vesicular trafficking pathways (Moris et al. 2006; Laguette et al. 2011) and to recently described constitutive restrictive factors, especially SAMHD1 (Laguette et al. 2011) and also to inducible restriction factors such as ISGs (Harman et al. 2011). Released de novo produced virus can be re-taken up by the same or adjacent DCs, thus generating two pools of virus in the cytosol of some DCs; endocytic/vesicular and de novo replicating (Turville et al. 2008). It is therefore likely that a third phase of viral transfer occurs as these re-taken up virions become regurgitated into the virological synapse. Two phase transfer has also been demonstrated in primary blood myeloid DCs (Lore et al. 2005).

1.4.5 Cytokine and Interferon Induction

DCs secrete pro-inflammatory cytokines and type I IFN in response to foreign pathogens or other "danger" signals at the site of initial exposure. Pro-inflammatory cytokine secretion leads to the recruitment of various immune cells to the site of infection which prevents the pathogen from spreading. The secretion of type I IFNs (consisting of 13 subtypes of IFN-α, and IFN-β) results in the induction of hundreds

to thousands of ISGs which protect cells by impeding viral replication. Cytokine and IFN induction is triggered by the binding of pathogens to PRRs such as TLRs, RLRs and NLRs which leads to the stimulation of signalling pathways that activate NF-kB, MAPK and IFN regulatory factors (IRFs) resulting in the transcription of genes encoding type 1 IFNs, pro-inflammatory cytokines and co-stimulatory molecules (Bonjardim et al. 2009; Cunningham et al. 2010; Pichlmair and Reis e Sousa 2007). Different PRRs found throughout the cell each give rise to the production of a unique set of cytokines as they are specialized in the detection of specific types of foreign antigen. TLR signal transduction occurs via TRIF (TIR domain-containing adaptor protein inducing IFN-β) and MyD88 (myeloid-differentiation primary-response gene 88), which act as adaptor proteins for TLR3 and TLR7–9 respectively leading to the activation of IRF3 and ultimately resulting in IFN-β induction. TLR7–9/MyD88 engagement results in IFN-β gene induction mediated via IRF7 or activation of NF-κB resulting in the production of type I and III IFNs (ω and λ) and pro-inflammatory cytokines (e.g. IL-6 and TNF) mediated by IRF4 and IRF5. Similarly RLR binding to ds-RNA leads to the induction of IFN-α and β which can be mediated either (1) by IRF3 and IRF7 via the activation of TBK1, IKKε and TRAF3 or (2) by IRF3 only via the activation of FADD (FAS-associated death domain), RIP1 (receptor interacting protein 1), TAK1 and IKKα/β, which also results in the production of pro-inflammatory cytokines. Little is known about the function of the viral DNA sensor DBP1. However, it appears to induce the production of type I IFNs via TBK-1 and IRF3. For more recent detailed reviews on PRR signalling pathways see (Bonjardim et al. 2009; Cunningham et al. 2010; Pichlmair and Reis e Sousa 2007). Recently HIV has been shown to block type I IFN induction in DCs via its Vpr protein. Unlike T-cells this is not mediated by targeting IRF3 for degradation by the proteasome (Doehle et al. 2009; Okumura et al. 2008) but rather by blocking its translocation to the nucleus (Harman et al. 2011). It is currently unknown if HIV also interferes with other IRF signalling pathways. However, it is able to directly induce the expression of a small subset of ISGs via activation of IRF1 (Harman et al. 2011). HIV has also been shown to both induce (Granelli-Piperno et al. 2004) and interfere with (Fantuzzi et al. 2004) DC cytokine secretion.

1.5 Functions of DC Subsets

1.5.1 Skin/Mucosal DCs

1.5.1.1 Langerhans Cells

LCs are the sentinel DCs that interact with invading microorganisms in the anus, vagina, ectocervix and male foreskin, usually through CLRs and TLRs. After these interactions they produce effector cytokines and can initiate or restimulate activation

of T and B lymphocytes by antigen presentation. Similar to other myeloid DCs, LCs bridge innate and adaptive immunity.

In their resting state in the epidermis, the immature LCs adhere tightly to keratinocytes via e-cadherin, and are highly endocytic and efficient at antigen processing. However, after inflammation, trauma or pathogen binding to PRRs, they mature and migrate to the draining lymph nodes while endocytic and antigen processing functions are down-regulated. After pathogen binding or inflammation, they become mature, migratory and down-regulate endocytosis and secrete IL1β. Keratinocytes respond by secreting TNFα which induces LC detachment. In mice, the emigration of LCs and dermal DCs from skin towards lymph nodes differs in kinetics. Dermal DCs are more rapid and depend upon CCR7 expression, migrating along CCL19 and 21 chemotactic gradients. LCs are slower showing a multistage complex migration process, first detaching from keratinocytes by down-regulation of e-cadherin, next migrating via CXCL12/CXCR4 interactions, interacting with the epidermal basement membrane via expression of integrin α1β6, traversing it using matrix metalloproteinases (2 and 9) and then finally emigrating from the dermis, via CCR7 expression, like dermal DCs (Villablanca and Mora 2008).

In lymph nodes they present antigens to T cells, resulting in their activation (Mayerova et al. 2004; Zaba et al. 2009). However, recent studies show that in some settings LCs may have an immunosuppressive role in the steady state (Cumberbatch et al. 2006; Jiang et al. 2007; Kaplan et al. 2005; Loser and Beissert 2007) and even lead to a regulatory T-cell response or deletional T-cell tolerance. Dermal DCs may complement the roles of LCs by adopting the immunostimulatory activity previously attributed to LCs (Zhao et al. 2003).

LCs are of key importance in epitheliotropic virus infections as they are situated at the site of entry for viruses such as HIV, the herpesviruses, including herpes simplex virus (HSV) and varicella-zoster virus (VZV), vaccinia and human papillomavirus. However, interactions of these viruses with LCs may have opposite outcomes for the virus–host balance. The host may derive advantage from viral capture and degradation and the induction of innate and adaptive immune responses or the virus may be favoured through infection and DC lysis or through protected transport and transfer to secondary cellular targets (Cunningham et al. 2008).

Studies on HIV infection of primary LCs are difficult because they comprise only 1–2% of cells in stratified squamous epithelium, and keratinocytes are strongly adherent to them and each other, thus resisting dissociation. In vitro models that closely resemble LCs have been difficult to develop and are still being refined as discussed in Sect. 1.1.2 above. Nevertheless, ex vivo LCs can be isolated from human skin by two main methods: emigration from split skin explants or enzymatic (trypsin/collagenase) digestion and flow sorting or magnetic-activated cell sorting bead technology. The latter offers better preservation of the immature phenotype but is labour intensive, technically difficult and care must be taken that the enzymatic treatment does not remove cell-surface proteins such as CD4 or alter the final LC phenotype (Turville et al. 2002).

1.5.1.2 Interactions of HIV with Langerhans Cells

HIV does not infect keratinocytes but must penetrate the superficial layers of the epidermis to gain access to potential resident targets cells, such as LCs and resting T cells. The nature of the interactions of HIV with LCs is noncytopathic and requires LCs for protected transport to T cells. Genital ulcerative diseases such as genital herpes may enhance HIV acquisition three- to fourfold (Wald and Link 2002) not only by facilitating infection through epithelial breaches, but also through provision of the appropriate target cells in the inflammatory infiltrate (Zhu et al. 2009). The mechanisms of HIV entry into the normal uninflamed female genital tract is slowly being clarified but certain aspects remain controversial, including the importance of "microabrasions" and the relative importance of resident LCs, $CD4^+$ lymphocytes and perhaps even macrophages (Gupta et al. 2002; Shattock and Moore 2003; Zhang et al. 1999). More research on the mechanism of HIV-1 transmission and specifically on infection of LCs and lamina propria DCs is needed for development of effective microbicides (Moszynski 2007) and mucosal HIV vaccines (Steinbrook 2007).

The role of LCs in initial HIV/simian immunodeficiency virus acquisition is supported by in vivo and ex vivo data. Vaginal simian immunodeficiency virus infection of Rhesus macaques resulted in infected LCs within the first day of infection and studies with biopsies of human cervical and skin of primate foreskin tissue explants show that LCs can be infected (Miller 1998). LCs and resting T cells were also the major cell types expressing HIV antigen after topical infection of human vaginal epithelial explants. After emigration from the explants often as doublets, both cell types expressed HIV antigen, which was often concentrated at their contact region (Hladik et al. 2007), suggesting transfer of HIV from LCs to CD4 lymphocytes. These LCs may also provide protected intracellular transport of HIV to $CD4^+$ T cells, first in the submucosal lymphoid tissue and then to draining lymph nodes (Cunningham et al. 2008; Hladik et al. 2007; Miller and Hu 1999; Zhang et al. 1999). Immature DCs and LCs express higher levels of CCR5 but on maturation CCR5 is down-regulated and CXCR4 up-regulated, resulting in reduced infectability by R5 (CCR5 using) viral strains, the strains shown to be predominant during sexual transmission (McDonald et al. 2003). In immature LCs and DCs, HIV binding to langerin or DC-SIGN respectively leads to viral endocytosis and then almost complete degradation (de Witte et al. 2007; Turville et al. 2001).

However, the relative effects of surface CLR binding on HIV trafficking and replication in immature DCs and LCs has been reported to differ, although this is still very controversial (Geijtenbeek et al. 2000b; van der Vlist and Geijtenbeek 2010). Although it is clear that DC-SIGN binding of HIV enhances viral-cell membrane fusion and infection of immature DCs (Geijtenbeek et al. 2000b; Turville et al. 2001; Yu et al. 2008) little data is available to suggest the same for langerin. In contrast, one research group reports no role of langerin in LC infection (Kawamura et al. 2008), whereas another reports that Langerin on LCs captures HIV and degrades it in the vesicular organelle Birbeck granules (like MDDC endosomes), but unlike MDDCs, transfer of HIV from the CLR to CD4/CCR5 and subsequent infection does not occur (de Witte et al. 2007). Furthermore, blocking of langerin

enhances LC infection, which is the opposite to the function of DC-SIGN (Felts et al. 2010). However, this study used low HIV MOIs and treated LCs with trypsin (which cleaves CD4). Nevertheless HIV infection in LCs/DCs after CD4/CCR5 binding leads to de novo productive infection, first detectable at 24–48 h with a plateau at 96–120 h in vitro (Turville et al. 2004).

For the above reasons the relative importance of the two (or three) stages of HIV trafficking and transmission in LCs to T cells is still debated. In support, several studies have shown that LC infection with R5-tropic HIV-1 is essential to its role in transmission (Kawamura et al. 2003, 2008). Furthermore, mature $CD34^+$-derived LC-like cells can transmit HIV-1 without infection, and in vaginal LCs, HIV seem to be accumulating in a first phase virological synapse between LCs and T cells in a very similar manner to MDDCs (Fahrbach et al. 2007; Hladik et al. 2007).

LCs seem to be more susceptible to HIV when exposed to various maturation stimuli. For instance, TNF-α and TLR-3 agonists can increase LC infection, (de Jong et al. 2008) whereas Gram-positive bacteria also stimulate HIV replication through TLR1/2 and TLR2/6 (Ogawa et al. 2009). Given the recent observations of maturing LCs becoming more permissive to HIV infection, this may explain, to some extent, the reported discrepancies in the effects of langerin inhibition. For instance, the process of epidermal cell dissociation, followed by LC isolation in vitro may render them more permissive (as this would be a form of "tissue trauma" and thus a primary stimulus to commence LC maturation) to HIV infection after langerin binding.

HIV, VZV and HSV interact with LCs during initial infection of the female genital tract, but the effects are quite different, reflecting differences in biology and pathogenesis; for example HSV infection of LCs induces apoptosis, whereas HIV infection is non-cytopathic and the LCs are used for transfer to CD4 lymphocytes in the submucosa and subsequent dissemination. In addition HIV has been shown to induce Langerhans cells to mature (Harman et al. 2006).

1.5.1.3 Dermal and Lamina Propria/Interstitial DCs

The dermis contains a large variety of cell types, including fibroblasts, macrophages, mast cells, T cells and DCs. Two major subsets of dermal DC are present in the dermis: $CD1a^{high}/CD4^+/CD14^-/CD16^-/CD206^{high}$ and $CD1a^{low}/CD4^+/CD14^+/CD16^-/CD206^{low}$ cells (Geijtenbeek et al. 2000b; Turville et al. 2002). The $CD1a^{low}$ subset shows weaker allogeneic stimulatory capacities compared with the $CD1a^{high}$ subset (Nestle et al. 1993). Expression of CD36, CD209 and the coagulation factor XIII are specific for both populations of dermal DC. Dermal DCs may be hard to distinguish from macrophages as the latter also express CD36 and CD209 (Geijtenbeek et al. 2000b; Lonati et al. 1996; Soilleux et al. 2002). In vitro, dermal DCs can be generated from $CD34^+$ hematopoietic progenitor cells or $CD14^+$ circulating monocytes with cytokine cocktails. All combinations of IL-3, TNF-α, IL-13, or IL-4 together with GM-CSF stimulate the development of dermal DC (Alters et al. 1999; Caux et al. 1999; Piemonti et al. 1995; Sallusto and Lanzavecchia 1994; Harman et al. 2006).

1.5.2 Blood DCs

1.5.2.1 Myeloid DCs

Human $CD11c^+/CD1c^+$ blood myeloid DC constitute about 0.6% of total peripheral blood mononuclear cells and 20% of $HLA\text{-}DR^+$ blood cells. When freshly isolated they express CD86 but not CD80 and are able to stimulate T cell activation unlike their murine counterparts. They express all TLRs except TLR9, but not DC-SIGN or mannose receptor. No evidence has yet been shown for a postulated role as precursors to interstitial DCs in tissue. These blood myeloid DCs express CD4 and CCR5 and are readily infectable with HIV (Cameron et al. 1992). $CD141^+/BDCA3^+$ DCs are very rare in blood (0.04% of PBMCs) and as discussed above are able to cross-present antigen.

1.5.2.2 Plasmacytoid DCs

pDCs are important in both innate and adaptive immune responses. pDCs preferentially express a subset of TLRs including TLR7 and TLR9 and upon stimulation of TLR7 with single stranded RNA or TLR9 with CpG-rich DNA, large amounts of type 1 IFNs (in particular IFN-α) are produced. This cytokine activates NK cell cytolytic activity, restricts viral replication in other potential host cells and facilitates adaptive immunity by promoting Th1 immunity (Parronchi et al. 1996; Rogge et al. 1997). Resting pDCs express intracellular MHCII which is transported to the surface when pDCs are exposed to maturation stimuli such as IL3, bacterial CpG or HSV. Since pDCs specifically express TLRs which sense single stranded RNA viruses such as HIV, influenza and vesiculostomatitis virus and DNA viruses such as HSV types 1 and 2 and cytomegalovirus, pDCs play a key role in antiviral responses (Feldman et al. 2001b; Hume et al. 2010; Tel et al. 2012; Villadangos and Young 2008; Wollenberg et al. 2002).

pDC are not present in normal skin but they infiltrate inflamed skin (Wollenberg et al. 2002), including recurrent herpetic lesions (Donaghy et al. 2009; Lund et al. 2006) and where they may be accessible to HIV superinfection (Fig. 1.4). pDCs have also recently been shown to be cytotoxic for tumour cells after TLR7/9 stimulation via TRAIL (and possibly granzyme B) so they could also kill occasional infected fibroblasts in the upper dermis (Kalb et al. 2012).

Since pDCs express CD4, CCR5 and CXCR4, they are targets for HIV infection as demonstrated by the fact that HIV p24 antigen has been detected in pDCs isolated from infected people (Patterson et al. 2001). Studies have also demonstrated that peripheral blood pDC numbers were decreased in advanced stages of HIV infection and that this loss correlated with high viral load and the occurrence of opportunistic infections (Soumelis et al. 2001; Chehimi et al. 2002; Feldman et al. 2001a). Decreased IFN-α production by pDCs from AIDS patients in response to HSV stimulation has been observed (Lopez et al. 1983) although at

the time immunodeficiency develops activated pDC secreting IFN-α and indoleamine-2,3-deoxygenase have been reported to accumulate in lymph nodes (Boasso et al. 2011). Recently pDCs have been demonstrated to present antigen via MHCI, II and cross-presentation. Following viral interactions pDCs up-regulate co-stimulatory molecules which, coupled with cytokine secretion allows them to present antigen and stimulate different T cell subsets, including naive effector and regulatory T cells (Fonteneau et al. 2004; Kadowaki et al. 2000; Kawamura et al. 2006; Moretta et al. 2006). pDCs can capture antigen through the CLR BDCA2 (CLEC4C), although they endocytose, process and load antigens onto MHC molecules less effectively than myeloid DCs and express lower levels of co-stimulatory molecules and MHCII (Grouard et al. 1997; Asselin-Paturel et al. 2003). Nevertheless, *mature* pDCs are more flexible than myeloid DCs in processing and presenting (MHCI restricted) antigen (Villadangos and Young 2008). Unlike myeloid DCs, they maintain the ability to process antigens after maturation, a useful and flexible accessory function when confronted with pathogens in the mucosa.

1.6 HIV Subverts the Biologic Functions of Myeloid DCs at the Transcriptional Level (Fig. 1.5)

Viruses, including HIV, shape their intracellular environment through alterations in host cell gene transcription, protein translation and post-translational modification, often initiating these changes by signalling through cell surface receptors or at subsequent stages in their replication cycle (Weissman et al. 1997; Sodhi et al. 2004).

We have used DNA microarrays, QPCR and downstream protein analyses and functional assays to determine how HIV shapes the intracellular environment of DCs (and macrophages) either to facilitate the characteristic non-cytopathic persistent infection, HIV trafficking in DCs and transfer to $CD4^+$ T cells and/or evasion of the immune response (Harman et al. 2009, 2006). These studies have used live and Aldrithiol-2 inactivated HIV (and also highly purified recombinant trimeric HIV envelope) as controls and single viral replication cycle kinetics (with high MOIs), the latter being essential in DCs to distinguish the sequential first phase (via caves/vesicles) and de novo replication second phases of trafficking. We showed that $HIV\text{-}1_{BaL}$ induced changes in expression of several distinct gene clusters in two major groups of genes in two transient and sequential phases, one group (~250 genes) corresponding to HIV binding/entry and vesicular uptake over 12 h post infection and the second group (~380 genes) corresponding to the later stages of de novo replication (post reverse transcription) at 24–96 h post infection (Harman et al. 2009). A minor group of genes showed persistent up-regulated expression across both phases. These host cell gene transcription al changes can be clustered functionally and have been followed by functional assays to reveal how HIV alters DC biological functions as summarized in Fig. 1.6.

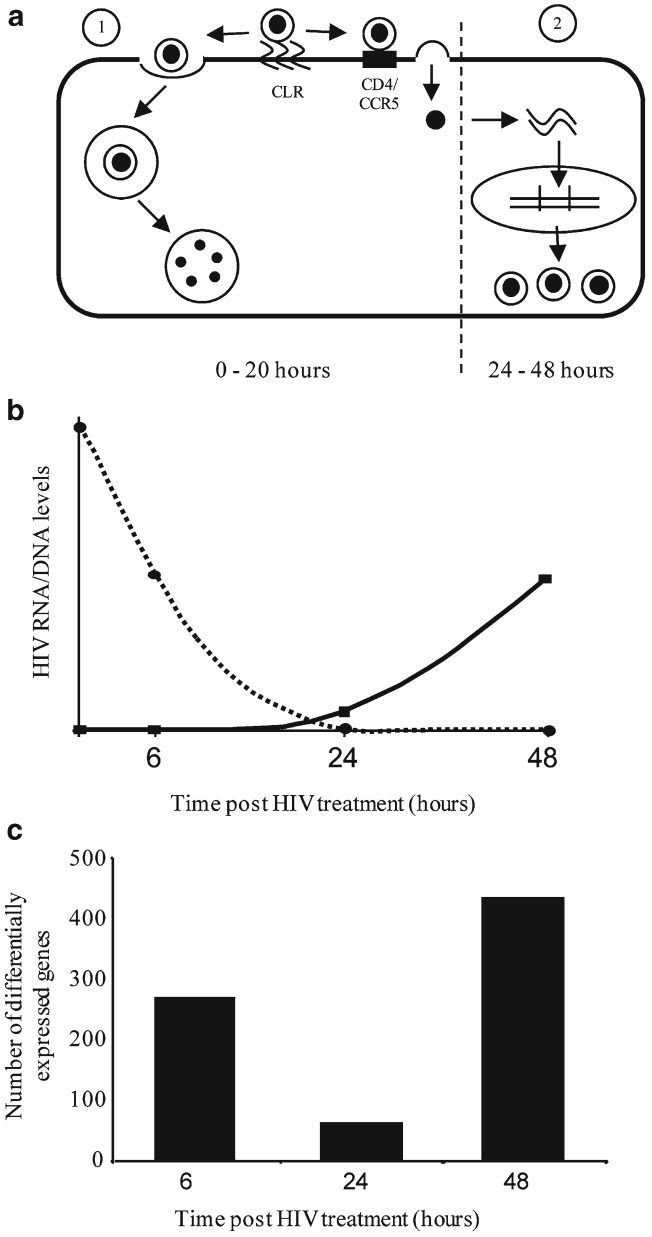

Fig. 1.5 HIV infection of dendritic cells alters the expression of host cell genes in two temporal phases, linked to the two phases of vesicular HIV trafficking and de novo HIV replication. (**a**) HIV traffics through DCs via vesicular (endosome and tetraspanin rich caves) and de novo replication pathways (**b**) Kinetics of HIV RNA in DCs showing initial degradation followed by an increase via de novo replication. (**c**) Numbers of differentially expressed genes in response to HIV-1 infection at 6, 24, and 48 h after inoculation. There was little overlap of individual genes between time points, except for a small subset, including the IFN stimulated genes. (Reproduced from Harman et al. 2009)

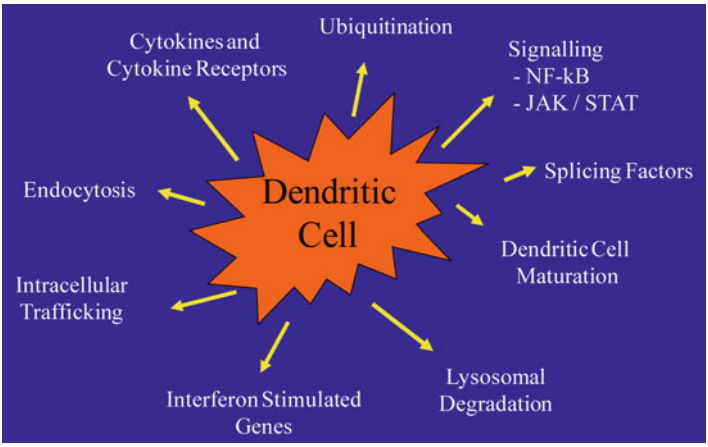

Fig. 1.6 HIV infection of DCs alters the expression of multiple gene clusters of key functional significance. (Harman et al. unpublished)

In the second phase of trafficking HIV induced partial maturation of both the model MDDCs and ex vivo derived LCs which leads to enhanced migration and T-cell stimulation (Harman et al. 2006) and also reduction in lysosomal enzyme expression and function (Harman et al. 2009).

The virus also subverts the Type I IFN/ISG induction pathway (Harman et al. 2006, 2009, 2011). In MDDCs, macrophages and T cells, we showed that HIV infection (1) fails to induce a type I IFN response and (2) directly induces a small (27) well-characterized antiviral ISG subset (ISG15, Mx1, OAS1–3, PKR, viperin and others) which differ markedly between DCs/macrophages and $CD4^+$ T cells, also reflected in marked differences in key activating and inhibitory IRF levels perhaps contributing to differences in replication levels and cytopathicity. The majority of the ISG subset showed the kinetics of the minor group of up-regulated genes. This ISG subset was regulated by the early and persistent induction of IRF-1/2/7. IRF1 interacted with the IRF1 binding site in the HIV-1 LTR to stimulate HIV-1 replication. The induction of IRF-1 expression early after infection of MDDCs may aid its initial replication as deletion of this binding site from the HIV LTR region resulted in a marked decrease in infectivity. The HIV-1 accessory proteins Vpr and Vif are required for the inhibition of an IFN response in T-cells via targeted degradation of IRF3 (Aerts et al. 2005; Doehle et al. 2009). However, in MDDCs HIV-1 infection had no effect on IRF3 expression. HIV Vpr but not Vif was shown to inhibit the IFN response in MDDCs by inhibiting the translocation of IRF3 to the nucleus. (Okumura et al. 2008) The HIV induced ISGs are likely to be acting as inducible restriction factors for HIV replication, augmenting constitutive factors like SAMHD1 (Laguette et al. 2011). These marked differences between T cells and DCs/Macs are consistent with their very different roles as target cells. Other viruses have been reported to show IFN independent stimulation of individual ISGs but not a whole subset of ISGs as observed with HIV (Harman et al. 2011).

1.7 Biologic Interactions of DCs with Other Innate Immune Cells (Fig. 1.7)

Recently it has become apparent that many of the functions of plasmacytoid and myeloid DCs, including the activation of $CD8^+$ and $CD4^+$ T cells are modulated by interactions with other innate immune cells, especially NK cells, but also NKT cells, and gamma delta T cells. These multilateral cell interactions are referred to as crosstalk. Thus, the outcome of microbial infection may be partly dependent on the collective interaction of their PAMPs with PRRs on the different cell types of the innate immune system. Such interactions may also determine the quality and strength of the subsequent adaptive responses (Ginhoux et al. 2007; Patterson et al. 2001).

1.7.1 DC Interactions with NK Cells

DCs and NKs colocalize and are able to interact at sites of inflammation and in lymph nodes (Moretta 2005; Strowig et al. 2008). In humans, there are two main NK subsets: $CD56^{dim}/CD16^+$ NK cells readily lyse target cells via contact through immunologic synapses, but are low cytokine secretors, but $CD56^{bright}/CD16^-$ NK cells produce large amounts of cytokines, such as IFNγ and TNFα, upon stimulation, and acquire cytotoxicity only after prolonged activation (Lanier et al. 1986). The $CD56^{bright}/CD16^-$ subset can also act as accessory APCs via up-regulated

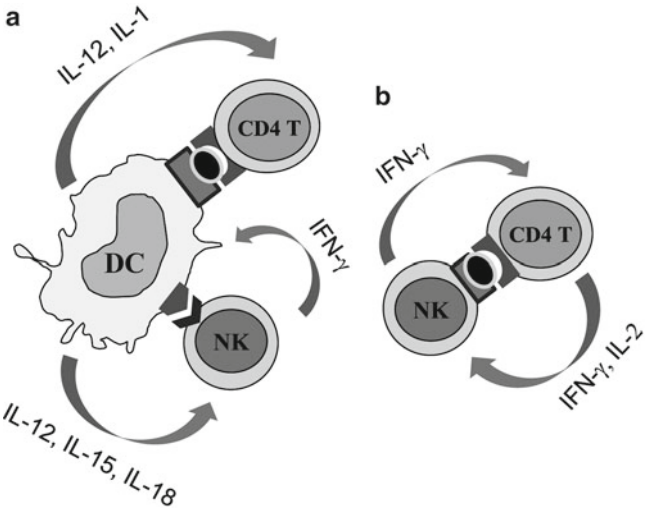

Fig. 1.7 Crosstalk between (**a**) DCs, NK cells and CD4 T cells (**b**) NK cells and CD4 T cells, induced by immunologic stimuli and/or viruses (via cell-to-cell contact or cytokines) (Kim et al. 2012)

HLA-DR and secretion of IFNγ, thus enhancing $CD4^+$ T cell responses (Hanna et al. 2004; Hanna and Mandelboim 2007). Both NK cell subsets are found in lung, liver and skin, and circulate through peripheral blood; however, the $CD56^{bright}/CD16^-$ cells represent the "minor subset" (5%) in blood, but are more abundant in secondary lymphoid organs (SLO), comprising up to 90% of NK cells in lymph nodes and 50% in the spleen (Strowig et al. 2008). The $CD56^{bright}/CD16^-$ population are found in T cell-enriched areas in human lymph nodes and tonsils, and thus can directly contact lymphocytes in their vicinity (Hanna and Mandelboim 2007). After infection with *Leishmania* major, IFN-γ-secreting NK cells were found to contact the same DCs as antigen-specific $CD4^+$ T cells in the lymph node (Bajenoff et al. 2006; Kim et al. 2012).

Circulating human $CD56^{bright}/CD16^-$ NK cells express L-selectin, CCR7 and LFA-1, enabling trafficking and entry into SLOs. In contrast to lymph nodes, both subsets of NK cells migrate to inflammatory sites after sensing cytokines and chemokines such as CCL4, CCL5, CX3CL1, CXCL8 and CXCL10 produced by DCs subsets (and other cells) in those sites. The type of infectious agent triggering inflammation may contribute to the selective enrichment of distinct NK subsets (Yoneyama et al. 2005). Along these lines, the $CD56^{bright}/CD16^-$ subset was found to be enriched in autoimmune inflamed lesions (Pollara et al. 2004), lung cancer lesions (Carrega et al. 2008), renal cell carcinoma (Schleypen et al. 2003, 2006), and possibly with CD4 T cells in recurrent herpetic lesions (Kim et al. 2012).

In inflamed tissues, activated NK cells may encounter immature myeloid DCs and recognize their low level of expression of MHCI molecules using their activating (NKp30, NKp46 and DNAM-1) and inhibitory receptors (killer cell immunoglobulin-like receptors; KIRs). This results in killing immature and tolerogenic DCs through apoptosis induction via the TNF-related apoptosis-inducing ligand (TRAIL)-death receptor 4 (DR4) pathway before their migration into SLOs (Melki et al. 2010). After such migration, NK cells can also kill DCs by perforin-dependent mechanisms (Laffont et al. 2008). The advantages of eliminating immature DCs by NK cells may selectively eliminate such tolerogenic APCs to allow only mature DCs to emigrate from sites of inflammation for efficient induction of immune responses (Barreira da Silva and Munz 2011). This cytolytic DC editing by NK cells has been suggested to reduce graft-versus-host disease in bone marrow transplantation (Arase et al. 2002) and graft rejection in solid organ transplantation (Laffont et al. 2008; Yu et al. 2006). Besides myeloid DC editing, NK cells can mature and differentiate DCs, and promote Th1 priming by DCs. As different DC subsets fulfil complementary and distinct functions to restrict infections and initiate immune response, their interaction with NK cells will be determined accordingly. Furthermore, different subsets of NK cells, different tissue types, and various types of stimulation whether they are tumours or infectious agents such as virus, bacteria, fungi, or parasite, make the crosstalk between DCs and NK cells more complicated.

Upon activation via PRRs, DCs become mature and then activate NK cells through cell-to-cell contact or soluble factors, such as IL-12, IFNα and IL-15 or both. Direct cell-to-cell contacts via immunologic synapses are also essential in promoting full NK activation by DCs (Ferlazzo et al. 2004). Mature myeloid DCs

produce IL-12, IL-15 and IL-18 which are secreted into the contact region with NK cells. When DCs conjugate with NK cells, inhibitory and activating receptors of $CD56^+/CD16^+$ NK cells concurrently engage in the contact site to determine the fate of DCs. If down-regulated MHCI of infected DCs are detected by NK cell receptors, NK cells lyse them facilitating their cell debris to be taken by bystander DCs which will eventually migrate into SLO to prime T cells. On the other hand, mature DCs expressing higher density of MHC I produce cytokines such IL-12, IL-15 or IL-18 which are secreted into the synapse with $CD56^+/CD16^+$ NK cells to activate them. This synapse is called a regulatory synapse and reinforced by filamentous actin, talin and LFA-1 which is located at the periphery of IS stabilizing it. As a result, NK cells become activated by the interaction and become cytotoxic or produce IFN-γ which in turn promotes the Th1 response and probably prolongs the mature state of DCs (Smith et al. 2002). Activated NK cells in turn significantly enhance the cytokine production of mature myeloid DCs in response to subsequent interaction with Th1 cells.

DC-NK cell crosstalk has been shown in EBV (Brilot et al. 2008), Influenza virus (Draghi et al. 2007), MCMV (Brilot et al. 2008) and HSV-1 infection (Kassim et al. 2009). Interestingly, in the co-culture of HIV-infected DCs and NK cells, much less IL-12 was produced by DCs and IFN-γ by NK cells, which in turn led to impairment of Th1 polarization (Saidi et al. 2008). Furthermore, in acute HIV infection there is an early loss of the $CD56^{bright}$ NK cell subset followed by the $CD56^{dim}$ cytolytic NK subset which may reduce immature DC editing. In addition HIV infection of DCs reduces NK cell activation, and together with enhanced IL-10 production in HIV infected subjects this may increase a tolerogenic role for immature DCs, thus contributing to immunodeficiency (Alter and Altfeld 2011).

DC/NK cell crosstalk not only contributes to innate immunity but also shapes adaptive immunity. In addition to these types of crosstalk between NK cells and DCs, a recent study showed that NK cells induced DC differentiation from $CD14^+$ monocyte in the presence of IL-15. Those monocytes co-cultured with NK cells in the presence of IL-15 underwent morphological and phenotypic changes associated with DCs such as down-regulation of CD14, up-regulation of CD40, CD80, CD86, DC-sign, DEC 205 and antigen uptake. The process required cell-to-cell contact as well as soluble factors such as GM-CSF and CD40L secreted by NK cells. $CD56^{bright}$ NK cells induced more efficient DC differentiation than $CD56^{dim}$ NK cells, which implied that the process might occur in LN where majority of NK cells are $CD56^{bright}$ NK subset (Zhang et al. 2007). Triple interactions between DCs, NK cells and CD4 lymphocytes can enhance such responses. IL-12 secreted by TLR-stimulated DC can induce IFNγ production by NK cells (Hart et al. 2005). Crosstalk between HIV-infected DCs and activated NK cells has been shown to be functionally defective, leading to the impaired ability of DCs to induce Th1 polarization of naïve CD4 T cells, due to the defective production of IL-12 and IL-18 by infected DCs. Moreover, these interactions led to a marked increase in viral replication in DCs (shown by increased proviral DNA), which was found to be the effect of high-mobility group protein B1 (HMCB1) produced by NK cells (Saidi et al. 2008). Furthermore,

progressive human HIV infection causes an accumulation of NKp30^{low}-expressing NK cells with aberrant TRAIL activity, resulting in a reduced capacity to eliminated immature DCs (Mavilio et al. 2006; Tasca et al. 2003). There are also several examples of viral immune evasion related to virus-manipulated DC/NK crosstalk resulting in impaired *adaptive* immunity. Chronic lymphocytic choriomeningitis virus, as well as HIV, compromises the quality of adaptive immunity by modulating crosstalk between DCs and NK cells. Both HIV and LCMV induce a high level of IL-10 secretion which results in modified expression of MHC I and NKG2D-ligands on DCs, rendering mature DCs susceptible and immature DCs resistant to killing by NK cells. This favours differentiation of tolerogenic DCs, resulting in poor T cell induction (Fiorentino et al. 1991; de Waal Malefyt et al. 1991; Willems et al. 1994; Steinbrink et al. 1997). Therefore, DC/NK cell crosstalk not only contributes to innate immunity but also shapes adaptive immunity against HIV and other viral infections.

1.7.2 Plasmacytoid DC–NK Cell Interactions

In contrast to immature myeloid DCs, pDCs express high levels of MHCI and low levels of ligands for activating NK receptors, which makes them resistant to lysis by activated NK cells. It has been shown that TLR9-stimulated pDCs induce expression of the early activation marker, CD69, on NK cells, promote a selective proliferation of CD56^{bright}/CD16^{-} NK cells, and enhance NK cell cytotoxicity against tumour cells and immature MDDC. Their interaction requires cell-to-cell contact (Gerosa et al. 2005). NK cells promote pDC maturation regardless of their maturation status. IFNα production by pDC also induces IFNα production from NK cells, which, in turn, contribute to the Th1 adaptive immune response (Morandi et al. 2006).

Moreover, abundant IFNα secretion by pDC was induced by NK cells exposed to IL-12 which implies that IL-12 produced by stimulated myeloid DCs may be an important link in promoting myeloid DC-NK-pDC interactions, i.e. of multidirectional crosstalk between NK cells, MDDC and pDCs (Della Chiesa et al. 2006). Different stimuli to pDCs may affect their interactions with NK cells and subsequent polarization in cytokine production; for example HCMV infected pDC induced activation and elevated production of both TNFα and IFNγ by NK cells, without enhancing cytotoxicity, while CpG-stimulated pDCs induced IFN-γ, rather than IFN-γ production by NK cells (Cederarv et al. 2009).

HIV can infect CD4^{+} pDCs in vitro, impairing pDC production of IFNα which can then inhibit IFN-γ induced NK cell activation. However, in HIV viremic individuals, impaired pDC-NK cell killing activity was largely attributable to an NK cell defect whereas the inhibition of IFN-γ production from pDC-NK cell pairs resulted from both pDC and NK cell defects (Conry et al. 2009). This compromised crosstalk between DCs and NK cells during HIV infections probably contributes to the immune dysfunction.

1.7.3 DC Interaction with NK T Cells

Besides NK cells, NK T cells and gamma deltaT cells are other innate lymphocytes to interact with DCs. NK T cells are involved in a number of immune responses such as autoimmunity and immunity against tumours and infections with viruses, bacteria, fungi and parasites. Human NK T cells express CD56, CD3, CD161, KIR (in human) and invariant TCR (Vα14α28 in mice or Vα24JαQ in humans). Both self and microbial glycolipids presented on non-polymorphic CD1d molecules on DCs can induce NK T cell activation (Morita et al. 2001). Upon activation, NK T cells produce type 1 as well as type 2 cytokines and display cytotoxic activity (Lauwerys et al. 2000). Antigen-specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells could be induced by the interaction between NK T cells and antigen-capturing DCs (Hermans et al. 2003).

1.7.4 DC Interaction with Gamma-Delta T Cells

Another type of innate lymphocyte, γδ T cells differ from the classical αβ T cell subsets by recognizing foreign antigens independently of APCs (Morita et al. 2001). In mice, various γδ T cell subsets based on the variable (V) region of TCR reside in the peripheral tissues including the skin (epidermis and dermis), gut and uterus. However, in humans, tissue-specific γδ T cells are not as prominent and the adult human γδ T cell repertoire is dominated by a polyclonal population bearing the Vγ9Vδ2 TCR. In mice there is a subset of γδ T cells found in epidermis named dendritic epidermal T cells (DETCs) whereas in humans they are located in the dermis rather than epidermis of stratified squamous epithelium. γδ T cells recognize phosphorylated isoprenoid precursors and alkylamines, which are conserved in the metabolic pathways of many species including plants, pathogens and primates (Bukowski et al. 1999). It has been shown that γδ T cells activated by phosphoantigens induce a significant up-regulation of CD86 and MHC I molecules and acquire other functional features typical of mature DCs. DCs not only up-regulate CD69 and CD25 on γδ T cells but also induce IFNγ and TNFα secretion by these cells (Conti et al. 2005). γδ T cells enhance DC maturation induced by TLR-2 and TLR-4 agonists (Leslie et al. 2002; Shrestha et al. 2005). Reciprocal activation of DCs and γδ T cells has been shown with in vitro by mycobacteria (Bacillus Calmette-Guerin). Furthermore, DC-stimulated γδ T cells helped DCs to prime a significantly stronger anti-mycobacterial CD8 T cell response (Schleypen et al. 2003). Both freshly isolated γδ T cells and those activated by phosphoantigens induced maturation of DCs and subsequent IL-12 production (Ismaili et al. 2002).

1.7.5 Conclusion

The outcome of the crosstalk between DCs and innate lymphocytes can have a marked impact on the quality and strength of downstream immune responses through reciprocal activation of interacting cell types and by bridging innate and adaptive immunity. This crosstalk influences both the magnitude and polarization of T cell responses. For example we recently reported that there was greater $CD4^+$ T cell IFNγ response to HSV antigens (and concurrent TLR2 agonist stimulation) when they were co-cultured with DCs and NK cells together than DCs alone or NK cells alone (Kim et al. 2012). Thus, co-stimulation of DCs and NK cells might enhance CD4 lymphocyte responses from HSV and possibly HIV vaccine administration. Similar ideas are being pursued with cancer immunotherapy, in clinical trials of combined NK-DC therapy of melanoma and other cancers (Kalinski et al. 2005). More emphasis on stimulating and manipulating such crosstalk to obtain the required direction and magnitude of the immune response for vaccines and immunotherapy is likely in the future.

1.8 General Conclusion

The influence of HIV on dendritic cell immunobiology and related functions is research field of great potential for the future, particularly with the powerful new tools of systems biology, now including next generation sequencing and phosphoproteomics, and markedly improved imaging techniques. The use of tissue DCs rather than just blood or model DCs for functional studies is increasing. Such investigations will almost certainly help resolve similarities and differences between mouse and human DCs, and allow better use of gene knockout and transgenic murine models and thus help develop new strategies for vaccines, especially new adjuvants, immunotherapy and microbicides.

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Chapter 2
Antiviral Immune Responses by Human Langerhans Cells and Dendritic Cells in HIV-1 Infection

Linda M. van den Berg and Teunis B.H. Geijtenbeek

Abstract The main route of human immunodeficiency virus-1 (HIV-1) infection is via unprotected sexual intercourse, and therefore, vaginal tissues and male foreskin are viral entry sites. Langerhans cells (LCs) and dendritic cells (DCs) are amongst the first immune cells encountering HIV-1 since these cells line these mucosal tissues. Both LCs and DCs are equipped with specific pattern recognition receptors that not only sense pathogens, but induce specific immune responses against these pathogens. LCs express the C-type lectin receptor langerin, which provides protection against HIV-1 infection. In contrast, DCs express the C-type lectin receptor DC-SIGN, which facilitates capture as well as infection of DCs and subsequent transmission to $CD4^+$ T cells. This chapter gives an update on immune responses elicited against viruses and sheds a light on different immune mechanisms that are hijacked by HIV-1 to infect the host. HIV-1 infection ultimately leads to the worldwide pandemic acquired immunodeficiency syndrome (AIDS).

2.1 Introduction

The scientific breakthrough of the year 2011, as announced by *Science*, was the finding that early antiretroviral therapy (ART) reduces the risk of heterosexual transmission of human immunodeficiency virus (HIV-1) (Alberts 2011; Cohen et al. 2011). HIV-1 is the virus causing acquired immunodeficiency syndrome (AIDS) which is a worldwide pandemic. Around 2.7 million people get infected with the virus per year, and at the end of 2010 a total of 34 million people were infected with HIV-1 (WHO 2011). The fact that early treatment can prevent heterosexual

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_2,

transmission of the virus (WHO 2011) gives hope for an AIDS-free generation (Alberts 2011). However, HIV-1 is not always transmitted heterosexually. Moreover, providing patients with ART is expensive and some groups have poorer access to therapy than others (WHO 2011). Therefore, it is important to understand the impact of the virus on the human immune system during acute infection, and to find ways to prevent infection. Therefore, we need to understand how HIV-1 interacts with our immune system, and which immune cells are crucial in the first contact with the virus.

Most HIV-1 transmissions occur via unprotected sexual intercourse (WHO 2011), and mucosal immune cells are the first cells encountering virus. Langerhans cells (LCs) and dendritic cells (DCs) are professional antigen presenting cells (APCs) lining human skin and mucosa (Banchereau and Steinman 1998), which are among the first immune cells encountering HIV-1 (van der Vlist and Geijtenbeek 2010). The major HIV-1 entry receptors are either CCR5 or CXCR4, which are present on $CD4^+$ T cells, macrophages and DCs. HIV-1 infection leads to the progressive loss of $CD4^+$ T cells, and thus loss of acquired immune responses, resulting in development of AIDS (Gorry and Ancuta 2011).

In this chapter we discuss the role of both LCs and DCs in acquiring acute and chronic HIV-1 infection. The first part of this chapter gives a brief overview of the human immune system, which describes efficient antiviral immune responses elicited by innate and adaptive immune cells. The second part of this chapter focuses on acute HIV-1 infection and the immune responses elicited by both LCs and DCs that are not sufficient in clearing HIV-1 from the human body, resulting in chronic HIV-1 infection.

2.2 The Human Immune System

The human immune system is composed of an innate and an adaptive immune system. The innate immune system reacts nonspecific and fast to invading pathogens, while the adaptive immune response develops slowly and reacts highly specific to the particular pathogen, providing long-lasting protection.

2.2.1 The Innate and Adaptive Immune System

The innate system is formed by phagocytes and APCs that phagocytose and eliminate pathogens, and subsequently present antigens to cells from the adaptive immune system to activate specific immune responses and memory. T cells and B cells belong to the adaptive immune system. $CD4^+$ T helper cells help naïve T- and B cells to become effector cells. B cells produce pathogen specific antibodies to clear the pathogens, while $CD8^+$ effector T cells effectively kill infected cells.

DCs are professional antigen presenting cells residing in peripheral tissues that form the bridge between innate and adaptive immunity. DCs sample their surrounding

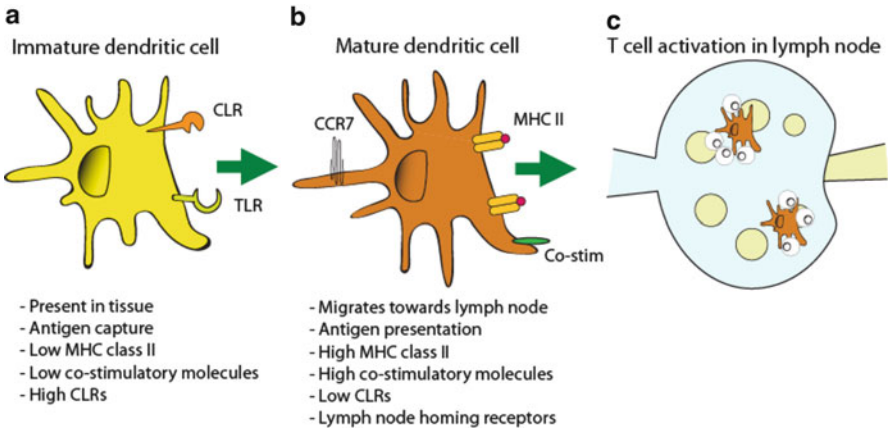

Fig. 2.1 Immature DCs mature upon PRR triggering and migrate towards the lymph nodes. (**a**) Immature DCs reside in peripheral tissues, such as mucosa and skin. The expression of PRRs, such as CLRs and TLRs enables DCs to sense and capture invading pathogens. (**b**) PRR triggering induces maturation of DCs. Mature DCs upregulated MHC class II as well as co-stimulatory molecules and lymph node homing chemokine receptors (e.g. CCR7), while CLRs are downregulated. (**c**) Mature DCs migrate towards the lymph node to interact with T cells. Naïve T cells become activated effector T cells that home to the tissue to clear the infection. *CCR7* C-chemokine receptor 7, *CLR* C-type lectin receptor, *DC* dendritic cell, *MHC* major histocompatibility complex, *PRR* pattern recognition receptor

for pathogen associated molecular patterns (PAMPs) through pattern recognition receptors (PRRs). PRRs sense pathogens and induce signalling leading to DC maturation, but also to cytokine production and migration of DCs towards T cell areas of the lymph nodes (Fig. 2.1). Each pathogen triggers a specific combination of PRRs and it is becoming clear that the combination of PRRs direct naïve $CD4^+$ T cell differentiation into distinct T helper cells (de Jong et al. 2005) (Fig. 2.2). Upon encountering pathogens, DCs process proteins into peptides, which are presented onto major histocompatibility complex (MHC) molecules, for presentation to T cells (Steinman and Banchereau 2007). Recognition of MHC class I molecules by the T cell receptor induces effector $CD8^+$ cytotoxic T cells, while recognition of peptides presented onto MHC class II induces effector $CD4^+$ T cells (Fig. 2.2). Thus, PRRs are crucial in activating immunity as well as shaping pathogen-specific immunity.

2.2.2 T Helper Differentiation by DCs

The specific spectrum of cytokines produced by DCs will tailor the differentiation of $CD4^+$ T cells towards T helper 1 cells (Th1), Th2 or Th-17 cells (Fig. 2.2). Pro-inflammatory cytokines such as interleukin-12 (IL-12) and interferon-γ (IFN-γ; type II IFN) are associated with Th1 differentiation; IL-4 and IL-10 with Th2

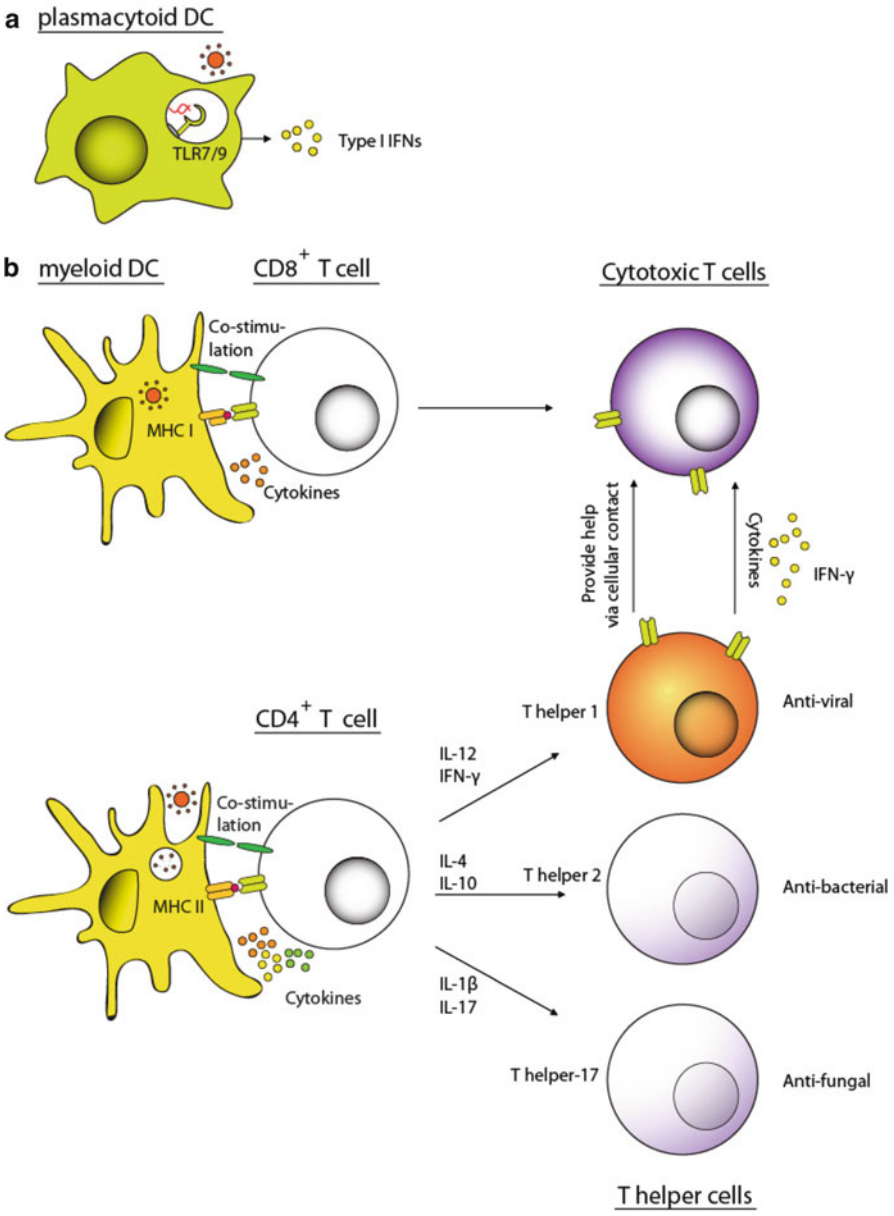

Fig. 2.2 Antiviral immune responses induced by DCs upon pathogen recognition. (**a**) pDCs circulate in blood and recognize viral RNA/DNA via TLR7 and -9. pDCs are potent inducers of type I IFNs-α and -β. Most cells in the body express IFN-receptors that enable them to react to IFNs by upregulating antiviral responses. (**b**) Myeloid DCs, such as LCs and DCs, line mucosal and skin barriers and are efficient in uptake of antigens. Endogenous pathogens are degraded in the cytosol and presented onto MHC class I. Mature DCs present antigens, provide co-stimulation and cytokines to activate $CD8^+$ T cells, which give rise to effector cytotoxic T cells. Exogenous pathogens

differentiation; and IL-17, IL-6 and IL-1β with Th-17 differentiation (de Jong et al. 2005). Adaptive T helper cells are classified according to their function. Th1 cells produce IFN-γ, which activates macrophages and $CD8^+$ cytotoxic T cells to fight viral and intracellular infections. Th2 cells activate B cells and humoral immune responses by secretion of IL-4, IL-5 and IL-13 to fight extracellular pathogens such as helminths and bacteria (Kalinski et al. 2000). The IL-17-secreting Th-17 cells mobilize phagocytes and are required for anti-fungal and antibacterial immunity (Dong 2006). Thus, the information provided by DCs directs the T cell immune response towards a pathogen (Fig. 2.2).

2.2.3 Different Subsets of DCs

Throughout the body, tissues harbour specific DC subsets to survey the environment for invading pathogens. Different subsets have different PRR repertoires and thereby respond differently to pathogens. Plasmacytoid DCs (pDCs; Fig. 2.2) are present in blood, which recognize viral components via Toll-like receptor 7 (TLR7) or TLR9 and rapidly produce high amounts of type I interferons (IFNs) to combat viruses (Palucka et al. 2010; Siegal et al. 1999). pDCs produce 1,000 fold more type I IFNs than myeloid DCs. Almost all nucleated cells express and respond to the type I IFNs, which makes it a very effective alarm system (Tough 2004). Myeloid blood $CD141^+$ DCs (Bachem et al. 2010) and $CD1c^+$ DCs are well capable of cross-presenting antigens to T cells and are thought to boost $CD8^+$ T cell responses (Palucka et al. 2010).

Different DC subsets are present in skin or mucosa: LCs inhabit the epidermis or mucosa, while DCs reside in the underlying dermis or submucosa (Steinman and Banchereau 2007). LCs are distinguished by the expression of langerin (Valladeau et al. 2000) and CD1a and recent data strongly suggest that LCs have antiviral functions and anti-HIV-1 functions (van der Vlist and Geijtenbeek 2010; Cunningham et al. 2008; de Witte et al. 2007). In skin, two different dermal DC subsets have been characterized: $CD1a^+$ DCs and $CD14^+$DC-$SIGN^+$ DCs (Klechevsky et al. 2008). In stark contrast to the antiviral function of LCs, HIV-1 can hijack DC-$SIGN^+$ DCs for its own propagation (Geijtenbeek et al. 2000a; Gringhuis et al. 2010). Although T cells and macrophages are present and infected in mucosa as well, these cells will not migrate to lymph nodes. LCs and DCs are migratory cells that migrate to lymph nodes to induce adaptive immunity, and it is becoming clear that these cells are also

Fig. 2.2 (continued) are endocytosed, degraded and presented onto MHC class II inducing T helper differentiation. Th1 cells provide help to cytotoxic T cells by providing cellular signals and cytokines. Infected cells will present viral peptides onto MHC class I. Cytotoxic T cells survey the body and kill virally infected cells. *DC* dendritic cell, *LC* Langerhans cell, *MHC* major histocompatibility complex, *pDC* plasmacytoid dendritic cell, *Th1* T helper 1 cell

involved in transmitting HIV-1 to T cells in lymph nodes. Therefore, we discuss the role of LCs and DCs in viral infections and HIV-1 transmission.

2.3 Antiviral Immune Response

Pathogens are captured for degradation and pathogen-derived peptides are presented on MHC molecules to T cells by LCs and DCs (Fig. 2.2). $CD8^+$ cytotoxic T cells efficiently kill virally infected cells. To do so, they require help of IFN-γ-producing $CD4^+$ Th1 cells (Constant and Bottomly 1997). Thus, to induce an effective antiviral immune response, DCs need to present antigens to both $CD4^+$ and $CD8^+$ T cells. Efficient antigen presentation requires upregulation of co-stimulatory molecules such as CD40, CD80 and CD86 (Liu and Janeway 1992). DCs differentiate and mature after PRR triggering, which induces maturation directly or indirectly by induction of type I IFNs, IFN-α and IFN-β. Furthermore, PRR triggering is required to induce specific T helper cell differentiation (Fig. 2.2). In order to induce Th1 cells, DCs need to produce pro-inflammatory cytokines and especially IL-12 (Trinchieri 1998). Thus, antigen presentation by DCs, DC maturation and DC-induced T helper cell skewing are prerequisites to induce successful antiviral immune responses.

2.3.1 Antigen Presentation by DCs

Antigens taken up by DCs are processed and subsequently presented to T cells via MHC class I or II molecules (Donaldson and Williams 2009; Hiltbold and Roche 2002). The T cell receptors of $CD8^+$ T cells and $CD4^+$ T cells recognize peptides presented on MHC class I and MHC class II, respectively (Donaldson and Williams 2009; Hiltbold and Roche 2002). MCH class I molecules are expressed by virtually all cells in the body, allowing circulating cytotoxic T cells to survey them, while MHC class II expression is restricted to antigen presenting cells.

2.3.1.1 MHC Class I Presentation

MHC class I molecules present peptides derived from proteins synthesized in the cytosol. Besides presenting self peptides, MHC class I molecules present peptides derived from intracellular pathogens such as intracellular bacteria, parasites or viruses. Cytosolic proteins are cleaved by the proteasome and transported to the endoplasmatic reticulum (ER) (Donaldson and Williams 2009). There, transporter associated with antigen processing (TAP)-1 and TAP-2 transport peptides ranging between 8 and 16 amino acids into the ER to be presented onto MHC class I. TAP-1 and TAP-2 are

inducible by type I IFNs, which are produced after antiviral PRR signalling. Therefore, antiviral IFN responses enhance MHC class I loading by inducing TAP, which further increases MHC class I presentation to, and recognition by, cytotoxic $CD8^+$ T cells. The loaded MHC class I complex is transported from the ER via the secretory pathway to the cell surface (Donaldson and Williams 2009; Gromme and Neefjes 2002). Virus replication in the cytosol increases loading of viral peptides onto MHC class I. MHC class I presentation is a crucial step in recognition and killing of infected cells by cytotoxic T cells. Activation of $CD8^+$ T cells by both LCs and DCs induces specific cytotoxic T cells that home to the infected tissue and kill all infected cells expressing viral peptides on MHC class I (Donaldson and Williams 2009).

2.3.1.2 MHC Class II Presentation

MHC class II presentation is a capacity restricted to APCs, such as LCs and DCs. Exogenous pathogens or antigens, such as bacteria or viral particles, are taken up by LCs and DCs into endosomal vesicles. These vesicles acidify and proteases degrade antigens into polypeptides with variable lengths to optimize MHC class II binding (Lennon-Dumenil et al. 2002). Local cytokines increase endocytic proteolysis: IFN-γ upregulates cathepsin proteases and both IL-6 and IL-1β lower the endosomal pH. In contrast, IL-10 has been shown to raise the pH of endosomes, which attenuates the proteolysis of peptides (Lennon-Dumenil et al. 2002). MHC class II molecules are transported from the ER in vesicles that fuse with endosomes containing antigens, and subsequently MHC class II vesicles will be transported to the cell membrane (Hiltbold and Roche 2002). As with MHC class I, MHC class II molecules in uninfected DC subsets will bind and present self molecules. LCs and DCs present exogenous viral peptides to $CD4^+$ T cells, which will differentiate into Th1 cells that are specialized in helping the $CD8^+$ cytotoxic T cell response (Fig. 2.2).

2.3.1.3 Crosspresentation

DCs are able to crosspresent exogenous endocytosed antigens onto MHC class I molecules without the need for direct infection of the cells (Ackerman and Cresswell 2004; Groothuis and Neefjes 2005). This is different from the classical MHC class I route, which presents antigens on MHC class I molecules derived from endogenously expressed proteins within the cell. Both LCs and DCs are thought to take up antigens from their surrounding derived from infected cells or apoptotic cells and crosspresent them onto MHC class I (Groothuis and Neefjes 2005). There is still a lot of debate on the molecular mechanisms of crosspresentation and whether exogenous antigens are delivered to the cytoplasm, or remain within phagosomes for presentation on MHC class I molecules (Ackerman and Cresswell 2004).

2.3.2 *Pathogen Recognition Receptors on DCs*

DCs are equipped with PRRs recognizing bacterial and viral PAMPs. Both LCs and DCs express a variety of PRRs, including Toll-like receptors (TLRs), NOD-like receptors (NLRs), RNA helicases and C-type lectin receptors (CLRs) that are required for sensing pathogens (Gromme and Neefjes 2002; Lennon-Dumenil et al. 2002; Ackerman et al. 2004; Eisenacher et al. 2007; Mazzoni et al. 2004; van den Berg et al. 2012). Since NLRs are mainly involved in recognition of intracellular bacterial compounds, we focus in the next paragraphs on TLRs, RNA helicases and CLRs (Fig. 2.3) that play an important role in viral recognition.

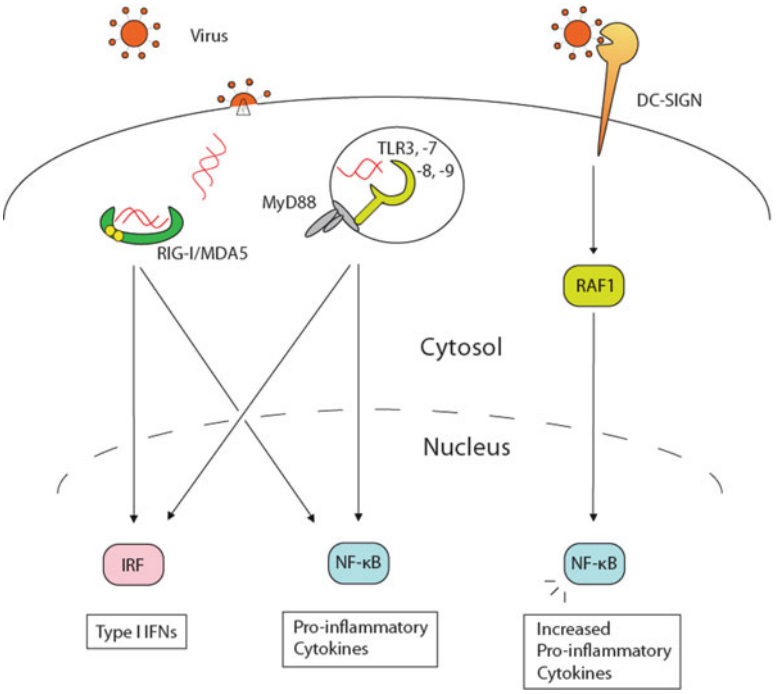

Fig. 2.3 Viral PAMPs trigger PRRs on DCs. Cytosolic viral RNA is sensed by RIG-I and MDA5 that will activate IRF transcription factors, which will lead to the production and secretion of the type I IFNs: IFN-α and IFN-β. Endosomal TLRs are triggered by viral RNA/DNA. TLRs signal via MyD88 and activate the transcription factor NF-κB, which will induce the production of pro-inflammatory cytokines. Extracellular recognition of mannose-containing viruses by DC-SIGN will induce RAF signalling which enhances NF-κB activity and hence enhances the production of pro-inflammatory cytokines. *DC-SIGN* DC-specific ICAM-3 grabbing non-integrin, *IFN* interferon, *IRF* interferon-regulating factor, *MDA5* melanoma differentiation associated gene 5, *MyD88* myeloid differentiation primary response protein 88, *NF-κB* Nuclear Factor-κB, *RIG-I* retinoic acid inducible gene I, *TLR* Toll-like receptor

2.3.2.1 Toll-Like Receptors

TLRs in vertebrates are evolutionary conserved PRRs. The Toll receptors play a role in the development and the defence of infections in *Drosophila melanogaster* (Takeda et al. 2003). In mammals, the homologues proteins were named Toll-*like* receptors and to date, there are ten TLR genes expressed in mice and human (Mazzoni and Segal 2004). TLRs are located in cellular membranes, either on the cell surface (TLR1, -2, -4, -5, -6) or in endosomes (TLR3, -7, -8, -9). TLR1/TLR2 and TLR2/TLR6 form heterodimers recognizing peptidoglycans from Gram-positive bacteria. TLR4 forms homodimers and detects lipopolysaccharides (LPS) originating from Gram-negative bacteria, whereas TLR5 is triggered by flagellin from flagellated bacteria. TLR3 recognizes viral double stranded RNA (dsRNA), while TLR7 and -8 respond to single stranded RNA (ssRNA) and TLR9 responds to unmethylated CpG DNA, derived from viruses or bacteria (Takeda et al. 2003; Mazzoni and Segal 2004; Kawai and Akira 2006b, 2007).

TLRs are triggered by conserved molecular patterns that are not found in vertebrates under healthy conditions. Most TLRs signal by recruiting the adaptor molecule myeloid differentiation primary response protein 88 (MyD88) (Barton and Medzhitov 2003), which contains a Toll/interleukin-1 receptor (TIR) domain (TIR). MyD88 activation results in activation of transcription factors, such as nuclear factor-κB (NF-κB) and interferon-regulatory factors (IRFs), which regulate cytokine transcription and immune responses. TLR2 and -4 signal via TIR containing adaptor protein (TIRAP) to MyD88 (Yamamoto et al. 2002a). Next to the MyD88-dependent pathway, TLR3 also signals via TIR-domain-containing adaptor-inducing IFN-β (TRIF), which is MyD88 independent (Oshiumi et al. 2003; Sato et al. 2003; Yamamoto et al. 2002b). Thus, TLR triggering leads to different transcriptional programs inducing cytokine and type I IFN responses.

Notably, TLR expression profiles are different between LCs and DCs, suggesting division of labour. DCs express TLR1 to TLR8 and -10 (van der Aar et al. 2007; Klechevsky et al. 2009) while LCs have a more restricted TLR expression profile with expression of TLR1, -3, -6, -7, -8 and -10 but no or low expression of TLR2, -4 and -5 (van der Aar et al. 2007; Klechevsky et al. 2009). The finding that LCs lack the specific TLRs that respond to bacteria, suggests an intrinsic property of LCs to tolerate bacterial commensal flora. Both LCs and DCs express TLR3, -7, -8 recognizing viral PAMPs, indicating both cell types are involved in antiviral immune responses.

2.3.2.2 RNA Helicases

RNA helicases are located in the cytosol and recognize specific RNA molecules derived from (replicating) viruses in infected cells (Fig. 2.3) (Yoneyama et al. 2004). Retinoic acid inducible gene I (RIG-I) is able to unwind long dsRNA and signals via its two caspase recruitment domains (CARD) (Yoneyama et al. 2004). Interaction with adaptor proteins delivers a signal to the transcription factors interferon-regulatory

factor 3 (IRF3) and NF-κB, inducing cytokine responses (Yoneyama et al. 2004). A highly homologues protein to RIG-I, is melanoma differentiation associated gene 5 (MDA5) (Yoneyama et al. 2005). MDA5 also binds dsRNA and has CARD domains that recruit adaptor molecules to activate transcription factors IRF3, IRF7 and NF-κB. Thus, in addition to endosomal TLR3, -7, -8 and -9 antiviral signalling, the cytosolic RIG-I and MDA5 also induce interferons and inflammatory cytokines via activation of IRFs and NF-κB in response to viral infection.

2.3.2.3 C-Type Lectin Receptors

CLRs are proteins that bind carbohydrate structures ('lectin') in a calcium (Ca^{2+})-dependent manner ('C-type') (Zelensky and Gready 2005). There are at least 17 subgroups of mammalian CLRs and most family members are adhesion receptors. However, type II, V and IV CLRs are present on myeloid immune cells and function as PRRs to induce signalling and support immune responses (Drickamer 1993; Zelensky and Gready 2005). Type II and V CLRs have one carbohydrate recognition domain (CRD) and usually form receptor oligomers on the cell surface (di-, tri- or tetramers) (Drickamer and Fadden 2002) while type IV CLRs have multiple CRDs but do not form oligomers (Drickamer and Fadden 2002). Langerin and DC-SIGN are type II receptors, dectin-1 is a type V receptor and the mannose receptor belongs to the type IV receptors (van den Berg et al. 2012).

Langerin and DC-specific ICAM3-grabbing non-integrin (DC-SIGN) share a highly homologous CRD (Chatwell et al. 2008) and recognize the monosaccharides mannose, fucose, *N*-acetyl-glucosamine (GlcNAc) and the oligosaccharide mannan (Stambach and Taylor 2003; Figdor et al. 2002). These sugar moieties are generally not found as terminal residues on mammalian glycoproteins but are highly abundant on surface proteins of pathogens such as HIV-1 (Geijtenbeek et al. 2000a), *Mycobacterium* species (Geijtenbeek et al. 2003), *Candida* species (Cambi et al. 2003), proteins from tick saliva (Hovius et al. 2008), *Helicobacter pylori* and helminth structures (Gringhuis et al. 2009). Langerin also recognizes fungal β-glucans (De Jong et al. 2010b; Feinberg et al. 2011). In addition, these CLRs are able to bind self ligands for cell-cell adhesion purposes; DC-SIGN is for example able to interact with ICAM-2 and -3 on T cells and therefore is involved in DC-T cell interactions inducing adaptive immunity (Geijtenbeek et al. 2000b).

Interestingly, langerin and DC-SIGN are present on distinct DC subsets and although they have a broad overlap in ligand recognition they might have distinct functions. Langerin forms trimers on the cell surface of human epidermal or mucosal LCs (Feinberg et al. 2010) while DC-SIGN is expressed as tetramers on DCs present in dermis and submucosa. Typically, Ca^{2+}-dependent CRDs have four Ca^{2+} binding sites, Ca-1 to Ca-4 (Feinberg et al. 2010; Zelensky and Gready 2003). Langerin has one Ca^{2+}-binding site (Ca-2), whereas DC-SIGN has Ca-1 to Ca-3 (Chatwell et al. 2008). Since binding of Ca^{2+} has a large effect on the tertiary structure of the receptor and influences the ligand binding of the receptor (Zelensky and Gready 2005), the Ca^{2+}-binding sites could explain differences in ligand affinity between langerin

and DC-SIGN. CLRs are known to induce signalling pathways. DC-SIGN triggering activates the serine/threonine protein kinase RAF1, which enhances TLR-induced NF-κB activation (Gringhuis et al. 2007). Less is known about langerin signalling pathways.

Dectin-1 belongs to the type V CLRs and binds ligands in a Ca^{2+}-independent manner. Dectin-1 is expressed on both LCs and DCs and is involved in anti-fungal immune responses by recognizing β-glucans (Brown and Gordon 2001). Since dectin-1 is not involved in antiviral immune responses, we will not go into detail about the CLR dectin-1 in this chapter.

The mannose receptor is a type IV CLR that contains eight CRDs, which bind ligands in a Ca^{2+}-dependent manner (Taylor et al. 1990). This CLR is mainly expressed by macrophages; to a lesser extend on DCs and is not expressed by LCs (Mommaas et al. 1999; Taylor et al. 1990). By recognizing mannose, fucose and GlcNAc structures, the mannose receptor binds a broad spectrum of bacteria and viruses, and it mainly functions as phagocytic receptor (Figdor et al. 2002). Dengue virus (Miller et al. 2008), influenza virus (Reading et al. 2000), Hepatitis B virus (den Brouw et al. 2009) and HIV-1 (Lai et al. 2009) are viruses that interact with the mannose receptor. Since the mannose receptor is mainly expressed on macrophages and DCs as a phagocytic receptor (Turville et al. 2003), we will focus on the function of langerin and DC-SIGN in viral infections.

2.3.3 Cytokine Production by DCs

Antigen presentation by MHC class I or II to T cells alone is not sufficient to induce T cell responses. T cells require both co-stimulation by DCs and cytokines to become effector T cells. Co-stimulatory molecules and cytokine production are upregulated upon activation and maturation of a DC by PRR triggering (Fig. 2.1) (Liu and Janeway 1992).

2.3.3.1 Cytokines by TLRs

Viral RNA is sensed by the endosomal located TLR3, -7, -8 and -9 which induces NF-κB and IRF activation (Fig. 2.3). NF-κB is a dimeric transcription factor belonging to the Rel-homology domain-containing protein family (Kawai and Akira 2006a). NF-κB consists of five subunits: p65, RelB, cRel, p50 and p52, which form dimers and translocate to the nucleus upon TLR triggering. NF-κB binds to the *Il10*, *Il6* and *Il12a/b* promoter, inducing Th1 skewing cytokines (Geijtenbeek et al. 2009). Therefore, NF-κB activation is crucial for T helper differentiation. Th1 cells will produce IFN-γ, which is necessary for cytotoxic T cell help and viral clearance. Next to activation of NF-κB, ssRNA will trigger TLR7 and -8, which induces activation of IRF7 and subsequent upregulation of IFN-β (Kawai and Akira 2007). In addition to

the TLR signalling pathways, RIG-I and MDA5 induce both IRF3 and -7, which lead to production of both IFN-α and -β (Kawai and Akira 2007).

2.3.3.2 Interplay Between DC-SIGN and TLRs

Viruses contain glycoproteins that trigger CLRs such as langerin and DC-SIGN. Mannose-induced DC-SIGN triggering activates the serine/threonine protein kinase RAF1 which induces phosphorylation of NF-κB subunit p65 at serine (Ser) residue 276 (Gringhuis et al. 2007). RAF1 activation by DC-SIGN occurs independently of TLR signalling. However, phosphorylation of p65 requires prior activation of NF-κB, which does depend on TLR signalling. Phosphorylation of Ser276 of p65 will recruit histone acetyl transferases CREB-binding protein (CBP) and p300, which subsequently acetylates p65 (Gringhuis et al. 2007). p65 acetylation prolongs and increases transcriptional activity of p65 as well as nuclear localization, which leads to enhanced transcription of *Il6*, *Il10*, *Il12a* and *Il12b*, boosting the Th1 response (Gringhuis et al. 2007, 2009). Thus, TLR triggering is necessary for NF-κB activation and mannose-mediated DC-SIGN activation modulates TLR signalling (Fig. 2.3), thereby shaping the adaptive immune response to the specific pathogen (Gringhuis et al. 2009). This could explain why DC-SIGN recognition of self-ligands, such as adhesion molecules ICAM-2 and ICAM-3 on T cells, does not lead to DC maturation and cytokine production, since there is no simultaneous activation of PRRs that induce NF-κB activation.

2.4 Antiviral Immune Response to HIV-1

Most HIV-1 infections occur by sexual contact, through the genital tract or rectal mucosa. HIV-1 infection can be divided in different stages. The infection starts with the *transmission* phase, where virus is transmitted from one person to the other. It will take up to 10 days before viral RNA becomes detectable in the plasma of the patient, known as the *eclipse* phase. This phase is followed by the *peak viraemia*; the virus replicates rapidly in activated $CD4^+/CCR5^+$ T cells and spreads through the body (McMichael et al. 2010). Viral replication increases and infection is spread to other lymphoid tissues, in particular gut-associated lymphoid tissues (GALT). Most $CD4^+$ T cells that become infected die by apoptosis, resulting in a depletion of almost 80% of $CD4^+$ T cells in the GALT during the first 3 weeks (Brenchley et al. 2004). Interestingly, the majority of apoptotic T cells are nonproductively infected with HIV-1; in 95% of infected T cells reverse transcription of the virus fails, which induces accumulation of abortive viral RNA/DNA in the cytosol. This viral RNA/DNA is recognized in the cytosol and ultimately induces caspase-mediated cell death (Doitsh et al. 2010). The cytosolic component involved could be RIG-I, which recognizes genomic

HIV-1 RNA (Solis et al. 2011). After 3–4 weeks HIV-1-specific antibodies can be detected in the patient, which is called *seroconversion* and coincides with *peak viraemia*. Then, the viral load decreases over 12–20 weeks and reaches a plateau, which is known as the *viral set point* (Ho et al. 1995). Without ART therapy this set point is maintained by a balance between viral escape mutants and the immune responses elicited by specific $CD8^+$ T cells and neutralizing antibody-producing B cells (McMichael et al. 2010) resulting in chronic infection with HIV-1.

LCs and DCs are the first immune cells encountering HIV-1 during sexual transmission, since these cells are located in vaginal mucosa and male foreskin. Both LCs and DCs express the entry receptors CCR5/CXCR4 and CD4. In addition, the HIV-1 envelope glycoprotein gp120 is able to bind both langerin and DC-SIGN (de Witte et al. 2007; Geijtenbeek et al. 2000a; Turville et al. 2002). Although LCs and DCs are crucial in induction of adaptive immunity to HIV-1, it has been shown that both DC subsets are involved in HIV-1 capture and transmission. Infection of LCs and DCs by HIV-1 allows transport of the virus to lymph nodes where these cells efficiently transmit the virus to $CD4^+$ T cells (Geijtenbeek et al. 2000a; Cunningham et al. 2010). The CLRs langerin and DC-SIGN are important viral attachment receptors for HIV-1. These CLRs capture HIV-1 and play a role in infection of the cell. Despite the similarity in gp120 binding, the outcome of interaction of HIV-1 with either langerin or DC-SIGN is remarkably different (de Witte et al. 2007; Geijtenbeek et al. 2000a; Gringhuis et al. 2010). It has been suggested langerin on LCs is involved in HIV-1 degradation, whereas DC-SIGN enhances HIV-1 infection in DCs (de Witte et al. 2007; Gringhuis et al. 2010). Therefore, we focus in the next paragraphs on the role of LCs and DCs in the acute immune response against HIV-1.

2.4.1 HIV-1 Infection and Transmission of LCs

LCs recognize and interact with invading pathogens by extending their dendritic processes to the lumen of the mucosa (Brenchly et al. 2004). Langerin is involved in pathogen uptake, and the expression of langerin on LCs induces the formation of intracellular Birbeck granules (Bachem et al. 2010). The origin and purpose of Birbeck granules are still poorly understood; however, the subcellular compartments are linked with endocytosis (Valladeau et al. 2003).

2.4.1.1 Langerin Has Protective Barrier Function Against HIV-1

Langerin on LCs captures HIV-1 and subsequently internalizes the virus into Birbeck granules, which leads to degradation of the virus and prevents LC infection (de Witte et al. 2007). Thus, LCs have a protective function in HIV-1 infection. Immature human LCs, expressing high levels of langerin, do not transmit HIV-1 to $CD4^+$ T cells, while

blocking langerin by specific antibodies does lead to enhanced infections of $CD4^+$ T cells (de Witte et al. 2007). Upon LC maturation, langerin is downregulated, and in line with this observation, immature LCs are not efficiently infected by HIV-1, while mature LCs are more efficiently infected with HIV-1 (de Witte et al. 2007; Kawamura et al. 2001), suggesting downregulation of langerin plays a role in HIV-1 dissemination (de Witte et al. 2007). The function of langerin in HIV-1 infection in vivo in humans still needs to be elucidated. Studies in a macaque model showed that LCs in the female genital tract could be infected with simian immunodeficiency virus (SIV) (Miller and Hu 1999). However, SIV and HIV-1 share only 40% homology and the difference between human langerin and macaque langerin could also play a role.

2.4.1.2 The Effect of Co-infections on HIV-1 Susceptibility

Co-infection by sexually transmitted diseases (STDs) increases the risk for HIV-1 infection by breaching the mucosal barriers, increasing inflammation and increasing influx of activated $CD4^+$ T cells (Fleming and Wasserheit 1999; De Jong et al. 2008). Moreover, the protective function of langerin against HIV-1 can be abolished by STD co-infections such as herpes simplex virus (HSV) or *Candida* species (De Jong et al. 2008, 2010a). HSV-2 causes genital herpes, inducing ulcerating and inflamed mucosal tissue, while *Candida* fungi cause sexually transmittable genital infections (De Jong et al. 2010a). Both HSV-2 and *Candida* species are able to interact with langerin and thereby occupy the receptor, obstructing langerin function and hence increasing the risk for HIV-1 infection (De Jong et al. 2010a; Turville et al. 2003). Additionally, HSV-2 is able to infect LCs, which decreases langerin expression and therefore decreases its protective function. TNF is locally produced upon HSV-2 or *Candida* infections, which enhances HIV-1 production in LCs and subsequent transmission to T cells. In addition, TLR2 triggering of LCs enhances capture of HIV-1, which subsequently increased *trans*-infection of T cells (De Jong et al. 2008). Thus, co-infections alter the functionality of langerin and abrogate antiviral function of LCs, increasing the risk of acquiring HIV-1 infection and transmission of HIV-1 to T cells (Fleming and Wasserheit 1999; De Jong et al. 2008, 2010a).

2.4.1.3 Antigen Presentation of HIV-1 by LCs

Little is known about the induction of cytokines and T cell responses by LCs after interaction with HIV-1. It is not known whether LCs from infected areas take up HIV-1 particles and present HIV-1-antigens to $CD4^+$ T cells, or crosspresent HIV-1-antigens to $CD8^+$ T cells. For measles virus (MV) infection it has been shown that LCs capture MV via langerin, and subsequently present MV-antigens onto MHC class II molecules to $CD4^+$ T cells (van der Vlist et al. 2011). Notably, in contrast to DCs, LCs do not crosspresent exogenously taken up MV to $CD8^+$ T cells (van der Vlist et al. 2011). Whether this is also the case for HIV-1 remains to be elucidated.

2.4.2 HIV-1 Infection and Transmission of DCs

In a macaque model, it has been shown that SIV enters the mucosa and within 60 min after vaginal inoculation infects local DCs (Hu et al. 2000). Within 18 h the first infected DCs present themselves in the lymph nodes (Hu et al. 2000). Upon HIV-1-infection of DCs, de novo produced virus is transferred to $CD4^+$ T cells, which is named *in cis*-transmission (Dong et al. 2007). Without being productively infected, DCs enhance *in trans*-infection (Geijtenbeek et al. 2000a) (Fig. 2.4). In contrast to langerin-mediated eradication of HIV-1 (de Witte et al. 2007), DC-SIGN-mediated uptake of HIV-1 does not lead to degradation of the virus but promotes transmission and infection of the host (Geijtenbeek et al. 2000a). In HIV-1 patients, elevated levels of DC-$SIGN^+$ DCs were observed in lymph nodes during the acute phase of infection (Lore et al. 2002). DC-SIGN captures HIV-1 and enhances T cell infection *in trans* by transporting HIV-1 from the tissue to the draining lymph nodes where T cells reside (Fig. 2.4) (Geijtenbeek et al. 2000a). Furthermore, the viral HIV-1 protein Nef upregulates DC-SIGN expression, by inhibiting its endocytosis, inducing increased *in trans* infection of $CD4^+$ T cells (Sol-Foulon et al. 2002), indicating HIV-1 has subverted the protective role of DCs in a leading role for viral spreading.

2.4.2.1 HIV-1 Infection in DC-$SIGN^+$ DCs

Mucosal DCs express the HIV-1 entry receptors CD4, CCR5/CXR4 and DC-SIGN. Binding of HIV-1 to the entry receptors results in fusion with the cell membrane, viral uncoating, reverse transcription of HIV-1 ssRNA and integration of the resulting double stranded DNA into the host genome (van der Vlist and Geijtenbeek 2010). It has recently been shown that signalling via DC-SIGN with TLR8 is subverted by HIV-1 to replicate in DCs (Gringhuis et al. 2010).

After DNA integration, HIV-1 is dependent on host as well as viral factors for the initiation and elongation of its transcription. Host transcription factors such as Sp1 and NF-κB are required to initiate HIV-1 transcription by RNA polymerase II (Perkins et al. 1993). However, without the viral factor Tat, RNAPII will detach from the DNA and produce short abortive mRNAs (Gringhuis et al. 2010) resulting in no *de novo* synthesis of viral proteins. Tat is not included in the HIV-1-virion and not present during the first rounds of transcription initiation. Therefore, HIV-1 requires DC-SIGN signalling for the recruitment of host transcription-elongation factors leading to the first Tat transcripts (Gringhuis et al. 2010).

HIV-1 uptake by DCs triggers TLR8, via ssRNA, which results in activation and nuclear translocation of NF-κB (Gringhuis et al. 2010). TLR8 triggering activates NF-κB subunit p65 that induces transcription initiation of the integrated HIV-1 genome. Simultaneously, gp120 triggers DC-SIGN signalling, which induces phosphorylation of p65 at Ser267 through RAF1 signalling, which recruits positive transcription elongation factor-b (pTEFb) to p65 (Gringhuis et al. 2010). pTEFb phosphorylates RNA polymerase II and thereby induces transcription elongation and infection of the DC.

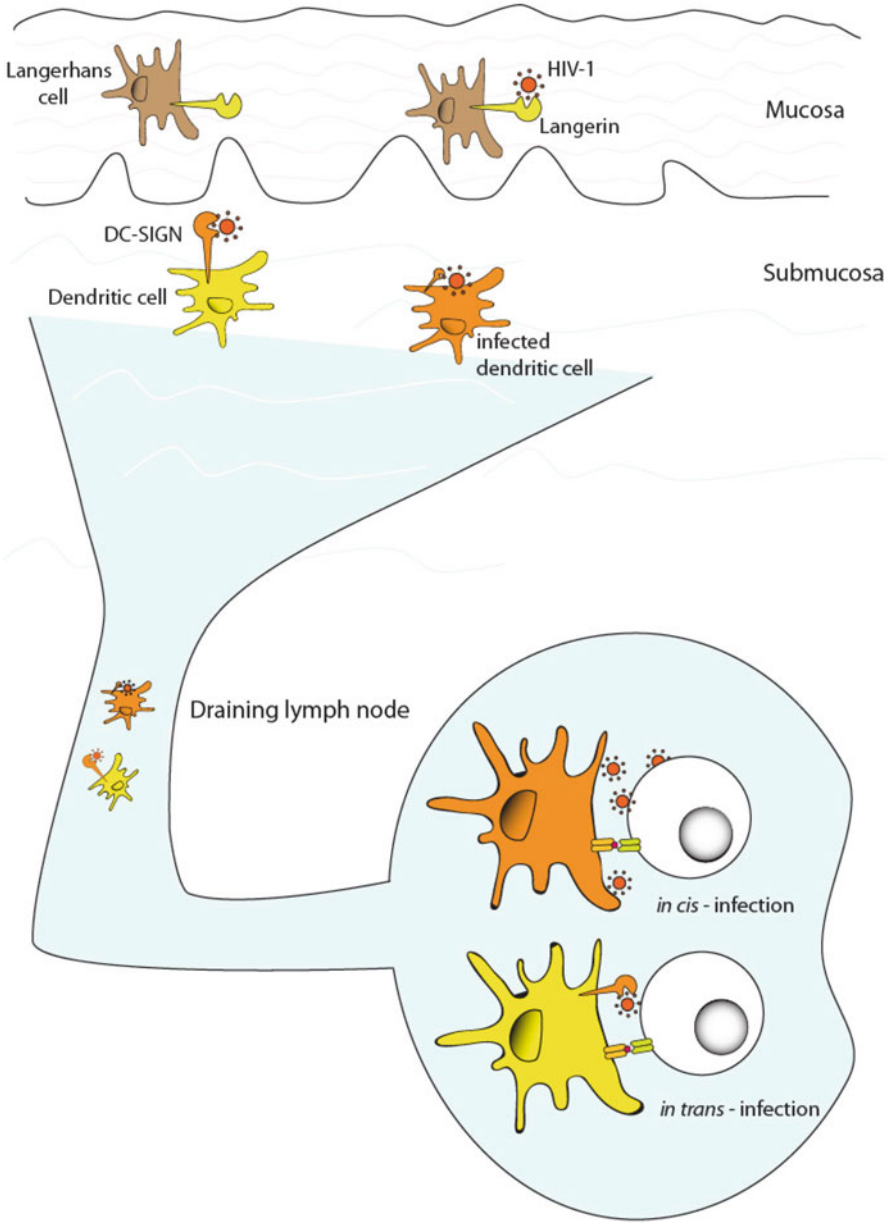

Fig. 2.4 *In cis* and *in trans*-infection of T cells by DCs. LCs reside in the vaginal mucosa and male foreskin, while DCs reside in the underlying submucosa. The HIV-1 glycoprotein gp120 binds langerin on LCs as well as DC-SIGN on DCs. Langerin captures HIV-1 and degrades it in Birbeck granules. DC-SIGN captures HIV-1 and facilitates transport to the lymph node, which is considered as *in trans* transmission to T cells. Infected DCs produce *de novo* virus particles and facilitated *in cis* transmission to T cells

Without DC-SIGN signalling and subsequent p65 phosphorylation, pTEFb is not recruited to the initiation site and RNA polymerase II produces short abortive RNAs (Gringhuis et al. 2010). Therefore, DC-SIGN is indispensable for infection of DC-SIGN$^+$ DCs by HIV-1 and facilitates both *in cis* and *in trans* transmission of HIV-1 to CD4$^+$ T cells (Fig. 2.4) (Gringhuis et al. 2010; Geijtenbeek et al. 2000a).

2.4.3 Cytokine Production Upon HIV-1 Infection

By studying plasma donors who acquired HIV-1 infection, cytokine responses during early HIV-1 infection have been described (Stacey et al. 2009). The acute phase of HIV-1 infection is characterized by a cytokine storm, which is not observed in other acute viral infections, such as hepatitis B and C (Stacey et al. 2009). The cytokine response is accompanied by a rapid increase in plasma viraemia during the *transmission* to *eclipse* phase. Therefore, the intense cytokine response might be responsible for some of the observed immunopathology and might promote viral replication (McMichael et al. 2010). The first detectable plasma cytokines are type I IFNs and are most probably produced by mucosal cells and local immune cells (pDCs, DCs, LCs) that sense ssRNA or RNA:DNA reverse transcription intermediates or products (Stacey et al. 2009). In a humanized mouse model, the production of IFN-α induces upregulation of CCR5 on T cell progenitors, suggesting that IFN-α production is a disadvantage for HIV-1 clearance and even expands R5 tropism, accelerating disease progression (Stoddart et al. 2010). After type I IFNs, levels of IL-15 and CXC-chemokine-ligand 10 (CXCL10) increase rapidly, which activate and attract NK and T cells, respectively. This is followed by expression of the pro-inflammatory cytokines IL-18, IFN-γ, TNF-α and IL-22 (Stacey et al. 2009). Remarkably, in this study no upregulation of IL-12 was detected, which is an important cytokine for antiviral immune responses (Stacey et al. 2009).

The role of DCs in this systemic cytokine storm has not been defined yet. The excessive cytokine production could play a role in the observed reduced number of DCs during acute HIV-1 infection. However, upon HIV-1 infection of DCs, maturation markers were only modestly upregulated, such as CD80, CD86 (Lore et al. 2002), CD83 and HLA-DR (Granelli-Piperno et al. 2004). Coculturing of in vitro infected DCs with autologous T cells generates T cells that produce high amounts of anti-inflammatory cytokine IL-10 (Granelli-Piperno et al. 2004). Thus, a block in DC maturation and release of IL-10 occurring early in HIV-1 infection may render these cells more prone to the induction of regulatory T cells, rather than activated T cells (Fortis and Poli 2005). In conclusion, the cytokine response toward HIV-1 infection is excessive and might promote instead of inhibit viral replication.

2.4.4 T Cell Responses Induced by DCs

DCs are capable of presenting HIV-1 antigens onto both MCH class I and MHC class II molecules to induce HIV-1-specific $CD8^+$ and $CD4^+$ T cells, respectively (Moris et al. 2004, 2006). In the next paragraph we go into more detail about T cell responses mounted upon HIV-1-antigen presentation by DCs.

2.4.4.1 Cytotoxic T Cell Activation by DCs

Although HIV-1 infected DCs will present antigens to $CD8^+$ T cells, the lack of immune activation and induction of strong pro-inflammatory cytokines will prevent induction of strong cytotoxic $CD8^+$ T cell responses (Granelli-Piperno et al. 2004; Fortis and Poli 2005). Furthermore, SIV has been shown to induce expression of the co-inhibitory molecule B7-H1 in macaque studies (Xu et al. 2010), supporting dysfunction of infected DCs in $CD8^+$ T cell responses. Therefore, HIV-1 infected DCs are no good inducers of effective anti-HIV-1 $CD8^+$ T cell responses.

Yet, uninfected DCs efficiently take up exogenous HIV-1 particles and crosspresent them to $CD8^+$ T cells in vitro (Buseyne et al. 2001). In addition, the expression of DC-SIGN enhances MHC class I mediated HIV-1 presentation, probably by enhancing uptake (Moris et al. 2004). During acute HIV-1 infection, strong anti-HIV-1 $CD8^+$ responses are observed against the HIV-1 proteins Nef, Tat, Vpr, Gag en Env (Granelli-Piperno et al. 2004; Fortis et al. 2005; Moris et al. 2004). However, under pressure of the adaptive immune response, virus diversification occurs and multiple HIV-1 escape mutants arise that are not longer eradicated by the cytotoxic T cells. Virus diversification induces a balance in effective $CD8^+$ T cell responses and newly arising HIV-1 mutants. Therefore, cytotoxic T cells against HIV-1 infected cells are involved in establishing the *viral set point* (McMichael et al. 2010).

Data from independent cohort studies have identified involvement of human MHC class I HLA-A, -B or -C alleles in the protection or susceptibility to AIDS progression. HLA-B27 and -B57 are associated with a delay in disease progression (Carrington and O'Brien 2003) probably because the epitopes recognized by these T cells are focused on conserved regions of the viral Gag protein (McMichael et al. 2010). In contrast, HLA-B35 and HLA-Cw4 are associated with AIDS progression in the Caucasian population (Carrington and O'Brien 2003), most likely because these alleles do not bind HIV-1 peptides and fail to mediate a protective immune response. Thus, to control HIV-1 infection and progression, effective $CD8^+$ T cell responses are indispensable.

2.4.4.2 T Helper Cell Activation by DCs

HIV-1 infection is associated with the progressive loss of $CD4^+$ T cells, hence leading to acquired immunodeficiency. During the first 3 months of infection, HIV-1 specific

effector $CD4^+$ T cells are induced, which produce IFN-γ and TNF-α (Riou et al. 2012). However, after 12 months, when acute infection has become chronic, IFN-γ specific $CD4^+$ T cell responses are decreased (Riou et al. 2012). In patients with high plasma viraemia compared to patients with low plasma viraemia, HIV-1-specific memory $CD4^+$ T cells display defects in proliferation and IL-2 production (Palmer et al. 2004). Taken together, this is indicating that high HIV-1 viral loads render dysfunctional $CD4^+$ T cell responses to eradicate HIV-1 (Riou et al. 2012; Palmer et al. 2004). Furthermore, HIV-1-specific memory $CD4^+$ T cells from viraemic patients harbour four times more viral RNA compared to other virus-specific memory $CD4^+$ T cells, which suggests that HIV-1-specific $CD4^+$ T cells are more prone to become infected with HIV-1 compared to other memory $CD4^+$ T cells (Douek et al. 2002). At first, it might seem paradoxical that HIV-1 preferentially infects HIV-1-specific T cells; however, HIV-1-specific $CD4^+$ T cells are activated and localize in HIV-1^+ lymph nodes or infected tissues. This will increase the chance of viral transfer from DCs or other infected cells to activated T cells (Moris et al. 2006; Douek et al. 2002).

DCs are the main initiators of T cell activation. Next to the role of DC-SIGN in viral *trans*-infection and inducing effective HIV-1 infection in DCs (Geijtenbeek et al. 2000a; Gringhuis et al. 2010), DC-SIGN also plays a role in MCH class II presentation of HIV-1 peptides to $CD4^+$ T cells. DC-SIGN is involved in viral uptake and subsequent routing to MHC class II loading vesicles. It has been shown that blocking DC-SIGN leads to 50% reduction of specific $CD4^+$ T cell activation (Moris et al. 2006). Other CLRs, such as the mannose receptor, are possibly involved in the remaining capture and MHC class II presentation. Thus, on the one hand specific $CD4^+$ T cells are effective targets for HIV-1 infection, and on the other hand these specific T cells are required to slow down AIDS progression. Furthermore, DCs act as Trojan horses transporting HIV-1 from the infected tissue towards the target $CD4^+$ T cells.

2.4.5 Establishing Latently Infected HIV-1 Reservoirs

One of the largest problems in curing HIV-1 infection is the latent reservoir of cells harbouring the HIV-1 integrated genome. Surviving infected cells show low or absent HIV-1 gene expression, which can be upregulated by different stimuli, for example T cell activation. NF-κB activation will induce HIV-1 mRNA transcripts and produce HIV-1-virions (Siliciano and Greene 2011). The infection will last as long as the lifespan of the cell, which is as long as a patient's life, in case of memory T cells. The latent reservoir is probably already established between day 5 and 25 after HIV-1 infection (McMichael et al. 2010) and includes besides naïve T cells, macrophages, LCs and DCs (Fortis and Poli 2005). Therefore, preventing HIV-1 infection subsequently leading to lifelong chronic HIV-1 infection and AIDS, is the only way to stop the AIDS pandemic.

2.5 Concluding Remarks

The human immune system has evolved innate and adaptive immune systems to efficiently eliminate intruding pathogens. The antiviral immune response contains highly efficient pathogen-sensing pathways to identify and eliminate incoming viruses. pDCs circulate in blood and are effective inducers of type I IFNs, activating antiviral responses in almost all cells in the body (Palucka et al. 2010; Siegal et al. 1999). Epithelial or endothelial cells sense viral RNA by the intracellular located RNA helicases RIG-I and MDA5 and are also able to sound the alarm via type I IFNs (Yoneyama et al. 2004, 2005). Local innate phagocytes immediately phagocytise pathogens and eliminate them in endosomes and lysosomes. Professional APCs, such as LCs and DCs, line the mucosal barrier and express conserved PRRs, such as TLRs and CLRs, recognizing viral PAMPs (Steinman and Banchereau 2007). LCs and DCs migrate towards the lymph node to instruct adaptive T and B cells. By presenting exogenous antigens onto MCH class II, in combination with specific cytokine production and co-stimulation, naïve $CD4^+$ T cells will differentiate into Th1 cells, supporting the cellular immune responses (Constant and Bottomly 1997). By (cross)presenting antigens onto MHC class I, cytotoxic T cells are activated, which will survey the host and kill virally infected cells (Ackerman and Cresswell 2004; Groothuis and Neefjes 2005).

However, HIV-1 has coevolved and adapted to the human immune system by subverting the immune response, using it for its own propagation. First, the type I interferon IFN-α upregulates CCR5 on T cell progenitors, which facilitates HIV-1 entry (Stoddart et al. 2010). Second, the cytokine storm observed in patient upon HIV-1 infection is excessive and rather promotes HIV-1 infection than that it combats HIV-1 infection (Stacey et al. 2009). LCs reside in mucosal tissues and male foreskin and DCs reside in submucosal tissues. Although langerin on LCs has a protective function, the highly homologues DC-SIGN on DCs boosts HIV-1 infection (de Witte et al. 2007, 2008). DC-SIGN binds HIV-1 and infects $CD4^+$ T cells *in trans* by transporting the virus to the lymph nodes (Geijtenbeek et al. 2000a). DC-SIGN is indispensible for DC infection, inducing signalling for transcription elongation (Gringhuis et al. 2010). Furthermore, DC-SIGN is important for MCH class II restricted antigen presentation to $CD4^+$ T cells (Moris et al. 2006).

HIV-1 preferentially infects HIV-1-specific $CD4^+$ T cells that are induced upon antigen presentation by DCs (Buseyne et al. 2001). Thus, the cells that are of utmost importance for HIV-1 eradication are attacked and eradicated by the virus. The immune system and HIV-1 race against each other leading to a balance called *viral set point*: under pressure of the immune responses, HIV-1 mutants arise that escape immune surveillance and infect new targets. On the other hand the immune system continuously induces adaptive immune responses against these mutants, leading to chronic infection. Cells that do not actively transcribe the HIV-1 genome form the latent infected reservoir, including T cells as well as macrophages, LCs and DCs. It is almost impossible to eradicate the latent reservoir, making it impossible to cure from HIV-1 infection. By preventing transmission of HIV-1 from person to person,

and by studying the early immune responses against HIV-1, it should be possible to realize an AIDS-free world.

Acknowledgements We thank the members of the Host Defense group for their valuable input. LMvdB is supported by the Dutch Burns Foundation (grant number 08.109) and TBHG is supported by the Dutch Scientific Research program (grant number NWO VICI 918.10.619).

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Chapter 3
Plasmacytoid Dendritic Cells in HIV Infection

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Abstract Plasmacytoid dendritic cells (pDCs) are innate immune cells that are specialized to produce interferon-alpha (IFNα) and participate in activating adaptive immune responses. Although IFNα inhibits HIV-1 (HIV) replication in vitro, pDCs may act as inflammatory and immunosuppressive dendritic cells (DCs) rather than classical antigen-presenting cells during chronic HIV infection in vivo, contributing more to HIV pathogenesis than to protection. Improved understanding of HIV–pDC interactions may yield potential new avenues of discovery to prevent HIV transmission, to blunt chronic immune activation and exhaustion, and to enhance beneficial adaptive immune responses. In this chapter we discuss pDC biology, including pDC development from progenitors, trafficking and localization of pDCs in the body, and signaling pathways involved in pDC activation. We focus on the role of pDCs in HIV transmission, chronic disease progression and immune activation, and immunosuppression through regulatory T cell development. Lastly, we discuss potential future directions for the field which are needed to strengthen our current understanding of the role of pDCs in HIV transmission and pathogenesis.

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_3,

3.1 Introduction

Dendritic cells (DCs) are innate immune cells that play a critical role in the host response to infection, as they routinely patrol mucosal and lymph tissue and blood, are recruited to inflamed tissues, and are among the first cells to sense and respond to microbes (Steinman and Hemmi 2006). When DCs encounter pathogens, they recognize conserved structures of the microbe termed pathogen-associated molecular patterns (PAMPs). DCs recognize PAMPs by means of germline-encoded pattern-recognition receptors (PRRs). The interaction of microbial PAMPs with DC PRRs, including Toll-like receptors (TLRs) and NOD-like receptors, activates specific intracellular signaling pathways which mediate rapid antimicrobial effector functions at the site of pathogen sensing (Medzhitov 2001; Fritz et al. 2005; Tada et al. 2005). Additionally, DCs process and present microbial antigens to adaptive immune cells to program specific T and B cell responses (Guermonprez et al. 2002; Pulendran et al. 2010). DCs prime expansion of antigen-specific T cells, polarize CD4+ T cells, establish memory, regulate T cell exhaustion, and influence antibody affinity maturation and isotype switching. The specificity of the adaptive immune responses depends on the Major Histocompatability Complex (MHC) class molecule in which the antigen is presented, the concurrent combination of cytokines released, and the co-stimulatory molecules that are expressed by the DCs. Signaling pathways elicited upon PRR sensing by DCs and signals received from the tissue microenvironment ensure tailoring of an immune response to the type of pathogen (extracellular, vacuolar, intracellular) by dictating a cell-mediated vs. humoral immunity. DCs not only dictate the type of immune response acutely, but also help program the type of immune memory and prevent immunopathology through induction of regulatory mechanisms.

The two major subsets of DCs in human blood, myeloid DCs (mDCs—also referred to as conventional DC) and plasmacytoid DCs (pDCs), differ in morphology, phenotype, and function. mDCs and pDCs express different but complementary TLRs, which allow them to respond to different types of pathogens. mDCs recognize diverse pathogens due to their broad TLR expression, and display a flexible program of cytokine secretion influencing Th1, Th2, Th17, or regulatory T cell responses (Treg). While pDCs do not secrete the Th1 skewing cytokine IL-12 in humans, mDCs secrete high amounts of IL-12 in response to some bacterial or viral pathogens. pDCs specifically recognize pathogens containing ssRNA by TLR7 and unmethylated CpG DNA motifs via TLR9 and produce up to 1,000-fold more interferon-alpha (IFNα) than other types of blood cells in response to viruses (McKenna et al. 2005). Like mDCs, pDCs also display a differential response towards different microbes, varying from secretion of type I IFN to maturation and antigen presentation for T helper and T regulatory cell responses.

In this review, we focus on what is known about pDCs in HIV infection. We discuss data gathered from cell biology and immunological experiments, as well as data derived from infected humans and nonhuman primates (NHP), to demonstrate the complexity of pDC functions during acute and chronic HIV infection. In doing

so, we argue that pDCs often effect conflicting functions in antiviral defense and immunopathology. Although much remains to be learned, we propose that pDCs play a crucial role both early during infection and during the chronic phase, contributing to immune activation and eventual disease progression.

Thus, while *lack* of activation of mDCs by HIV impairs the development of adaptive immune responses (Lore et al. 2002; Granelli-Piperno et al. 2004), rapid activation of pDCs by HIV to produce inflammatory cytokines and chemokines at mucosal sites of transmission may enhance initial infection. At the same time, *persistent activation* of pDCs to produce IFNα during chronic infection may contribute to immune activation and inflammation (Fernandez et al. 2011; Jacquelin et al. 2009; Bosinger et al. 2009), which are associated with disease progression to AIDS and with the development of co-morbidities such as cardiovascular disease, kidney and liver disease, and development of non-AIDS malignancies (Baker and Duprez 2010; Lekakis and Ikonomidis 2010; Ho et al. 2010; Lichtenstein et al. 2010; El-Sadr et al. 2006). In general, during acute viral infections, type I IFN produced by pDCs act as immunostimulatory cytokines favoring mDC maturation (Fonteneau et al. 2004), and as antiviral agents, activating intracellular restriction factors, inhibiting proliferation, and inducing apoptosis of infected target cells, such as T cells (Barber 2001). In contrast, chronic and systemic activation of pDCs during persistent infection, such as HIV, can paradoxically lead to deleterious inflammation and perturbation of T cell proliferation, homeostasis, and cell death (Heikenwalder et al. 2004). Additionally, it has been shown that HIV-activated pDCs produce the immunosuppressive enzyme indoleamine (2,3)-dioxygenase (IDO), favoring the development of regulatory T cells (Manches et al. 2008; Fallarino et al. 2007; Munn and Mellor 2004; Honda et al. 2005).

Altogether, pDCs are now implicated in the following aspects of HIV pathogenesis: (1) inciting recruitment of CCR5 + CD4+ T cells to mucosal sites of HIV inoculation during transmission (Haase 2010); (2) inducing apoptosis of CD4+ T cells through their persistent production of type I IFN (Stary et al. 2009; Fonteneau et al. 2004; Boasso et al. 2008; Herbeuval et al. 2005b); (3) increased immune activation of T cell subsets through type I IFN production; and (4) favoring generation of immunosuppressive regulatory T cells over immunostimulatory Th17 cells through their production of IDO.

3.2 Plasmacytoid Dendritic Cell Biology

The discovery and identification of pDCs came from the convergence of studies of rare blood and lymphoid tissue cell populations. Early studies in 1980s identified a specific cell type based on their characteristic plasma cell morphology and location in the T cell areas of reactive human lymphoid tissue (Vollenweider and Lennert 1983). They were later shown to express the T cell-associated marker CD4, but lacked CD3 and B cell markers, while expressing MHC-II and some myeloid markers. Based on this phenotype, the cells were termed “plasmacytoid monocytes” by

Facchetti et al. (1988). In 1991, O'Doherty et al. identified a subset of blood CD4+ CD3− CD11c− immature DC that developed into mature DCs in the presence of monocyte-derived cytokines (O'Doherty et al. 1994, 1993) and in 1993 Grouard et al. also identified a subset of CD4+ CD3− CD11c− cells with a plasmacytoid morphology in the T cell area of human tonsils, which could differentiate into mature DC upon culture with IL-3 or IL-3 and CD40L. These cells were found to display an identical morphology and phenotype to plasmacytoid monocytes and to immature HLA-DR+CD11c− blood DC. Because these cells could differentiate into mature DC, they were termed pDC precursors (Grouard et al. 1997).

The existence of a rare blood cell capable of secreting extremely high amounts of IFNα had been established in the 1970s, when these cells were labeled as "natural IFN-producing cells" (NIPC). Like plasmacytoid monocytes, they did not express markers of T, B, monocyte, or NK cells, expressed MHC-II, and, importantly, secreted massive amounts of type I IFN in response to select viruses. The identical phenotype and morphology led Siegal et al. to finally identify the NIPC with pDCs (Siegal et al. 1999).

Aside from their lack of lineage markers (CD3, CD19, CD14, CD11c) and expression of MHC-II and co-stimulatory molecules (in the activated state), pDCs express several selective markers, such as sialic acid-binding immunoglobulin-like lectin H (SIGLEC-H) and bone marrow stromal antigen 2 (BST2) in mice and blood DC antigen 2 (BDCA2; also known as CLEC4C), BDCA4, leukocyte immunoglobulin-like receptor, subfamily A, member 4 (LILRA4; also known as ILT7) in humans, and high expression of the IL-3 receptor alpha (CD123).

3.2.1 Development

Data regarding pDC development have been garnered from mouse studies, although the recent identification of a genetic DC deficiency in humans has allowed identification of the relevant molecular players and will further help delineate the exact role of pDCs during viral and bacterial infections. Differentiation of DCs from hematopoietic progenitors relies on the activity of cytokines, such as FMS-related tyrosine kinase 3 ligand (Flt3L) and GM-CSF. As such, the Flt3 receptor is expressed in a fraction of hematopoietic stem cells and is maintained in a subpopulation of common myeloid progenitors (CMPs) (D'Amico and Wu 2003). CMPs give rise to macrophage and DC progenitors (MDP), a direct precursor of the Common DC progenitors (CDP). Flt3 and GM-CSFR expression is regulated by the transcription factor PU.1, a member of the ETS family of transcription factors, which regulate the development of monocytes and DCs (Carotta et al. 2010).

As stated above, mDCs and pDCs display complementary roles in immunity, as evidenced by their pattern of PRR, migration, cytokine secretion, and antigen presentation. The development program directing pDC vs. mDC differentiation starts at the CDP stage, through two developmental systems. pDC differentiation requires the expression of the E protein E2-2 (Cisse et al. 2008), in the absence of E protein

antagonist of DNA binding (ID2). The basic helix-loop-helix E2-2 transcription factor directly binds to the promoters of several pDC selective genes such as *BDCA2*, *LILRA4*, *IRF7*, the pre-TCRa chain gene, *IRF8*, and *SPIB* (a relative of PU.1). E proteins are crucial for lymphopoiesis, which may explain the importance of E2-2 for expression of lymphoid-related genes in pDC (*SPIB, RAG1, IL7R, TDT*). Deletion of murine E2-2 blocked the development of pDCs, and haploinsufficiency in humans affected by Pitt–Hopkins syndrome yields an aberrant pDC profile and impaired IFN type I responses (Cisse et al. 2008). ID proteins directly inhibit binding of E proteins, and ID2 expression is extremely low in CDP, pre-pDC, and pDC, in contrast to conventional myeloid DCs which express high levels of ID2.

pDC differentiation also requires a high concentration of IRF8 and low levels of PU.1, and IRF8 and PU.1 may act as a complex on composite DNA elements (Carotta et al. 2010; Schiavoni et al. 2002; Tsujimura et al. 2003). The requirement of PU.1 and IRF8 has been confirmed on PU.1- or IRF8-deficient mice, and in humans deficient in IRF8, resulting in inhibition of pDC differentiation (this may be due to alteration of CDP formation, rather than a specific pDC defect). PU.1 and IRF8 share target genes in pDCs, such as Ciita94, Tlr9, and IFNα. SPIB is the closest homolog of PU.1 in mammals, and inhibiting the expression of either transcription factor in human hematopoietic progenitors inhibits pDC differentiation.

3.2.2 IFN Secretion

One of the primary functions of pDCs is the secretion of large amounts of type I IFN in response to viruses and bacterial DNA. Thus, upon recognition of some viruses, pDCs start transcribing type I IFN genes within a few hours. A major part of the pDC transcriptome is then devoted to type I IFN genes, and pDCs secrete up to 10 pg/cell type I IFN within 24 h, which represents up to 1,000 times the amount produced by any other blood cell type (Liu 2005). Human pDCs can secrete all the subtypes of type I IFNs, IFNα, IFNβ, IFNλ, IFNω, and IFNτ.

Secretion of type I IFN is one functional outcome of recognition of nucleic acids by pDC. Receptors for the sensing of microbial genetic material exist both in the endosomal and cytosolic compartment of the cell, although it seems that pDCs rely on endosomal TLR sensing system for recognition of viruses (Kato et al. 2005). Endosomal TLRs, i.e., TLR7 and TLR9, in pDCs have extracellular domains comprising multiple repeats of Leucine-Rich Repeats which mediate recognition, linked to a cytosolic Toll/IL-1 receptor homology (TIR) domain responsible for initiation of signal transduction (Akira and Takeda 2004).

TLR9 recognizes unmethylated DNA containing Cytosine–Guanosine dinucleotides (CpG), which allows preferential sensing of bacterial genomes, which contain a much higher fraction of unmethylated DNA than mammalian genomes. Based on the functional response of pDCs and B cells to CpG DNA, several classes of CpG motifs have been described. The prototypical type A CpG (CpGA) comprises oligonucleotides

with a phosphodiester backbone and nuclease-resistant phosphorothioate ends. They also contain a poly-G tail, allowing aggregation of oligonucleotides into large complexes. CpGA oligonucleotides induce the secretion of high amounts of type I IFN, but limited phenotypic maturation of pDC (up-regulation of MHC and co-stimulatory molecules). CpG type B (CpGB) oligonucleotides only comprise phosphorothioate links, and do not contain a poly-G tail. CpGB induces limited amounts of Type I IFN, but a strong maturation of pDC accompanied by secretion of TNFα and other inflammatory cytokines (Krieg 2002). TLR7 recognizes guanosine–uridine-rich single-stranded RNA (ssRNA), as well as the potent synthetic antiviral imidazoquinolines. Although Uridine tetramers may be the minimal motifs recognized by TLR7, GU-rich sequences preferentially induce IFNα by pDCs, and there is evidence that CpG motifs in viral RNA can also contribute to RNA recognition (Greenbaum et al. 2009; Jimenez-Baranda et al. 2011). Although guanosine- and uridine-rich regions in the HIV LTR were known to stimulate TLR7 (Heil et al. 2004), the molecular determinant of HIV virion recognition in pDC remained unknown, until it was proven that endosomal delivery of viral RNA, rather than early retrotranscipts, stimulates pDC through TLR7 (Beignon et al. 2005).

The endosomal and lysosomal localization of TLR7 and TLR9 in activated pDCs allow sampling of internalized material while preventing inappropriate activation by self-DNA or -RNA. Further restriction of TLR activation is ensured by processing of TLR in the endo-lysosomal compartments. Delivery of TLR7 and TLR9 to the endosomes is dependent on the RE-resident protein Unc93b1, and activation of TLR9 requires its cleavage by acidic proteases in endo-lysosomal compartments (Barbalat et al. 2011). Asparagine endopeptidase is a key protease allowing N-terminal processing of TLR9 (Sepulveda et al. 2009), in conjunction with other acidic proteases, and only the cleaved form of TLR9 can associate with the signaling adaptor MyD88. The same form of receptor processing likely exists for TLR7. The preprocessing and endosomal localization of TLR7 and TLR9 allows recognition of foreign ligands such as internalized viruses without actual infection of pDC. Many inactivated viruses, including chemically inactivated HIV, can trigger IFNα secretion with the same potency as live viruses (Beignon et al. 2005). Some live cytosolic viruses may still gain access to the endosomal compartments through autophagy-mediated delivery of cytosolic content to the endolysosomes (Lee et al. 2007).

TLR7 and TLR9 both signal through the TIR-containing adaptor MyD88, which leads to activation of IRF7 for type I IFN secretion. The engagement of TLR-MyD88 leads to assembly of a multiprotein complex in the cytoplasmic tail of TLR, comprising IRAK4, Bruton's tyrosine kinase (BTK), and IRF7. IRF7 is ubiquitylated by the ubiquitin E3 ligase activity of TRAF6 and further phosphorylated by IRAK4. Activated IRF7 then interacts with TRAF3, IKKα, IRAK1, and osteopontin, translocating to the nucleus for type I IFN gene transcription. TRAF6 also ubiquitylates the protein kinase transforming growth factor-β (TGFβ)-activated kinase 1 (TAK1) for activation of NF-κB and MAPK, leading to transcription of inflammatory cytokines, chemokines, and co-stimulatory molecules (Akira and Takeda 2004). IRF7 expression in pDCs is constitutively high, in part due to continuous autocrine feedback

by low levels of type I IFN (O'Brien et al. 2011), and by low expression of the translational repressors 4e-BPs (Colina et al. 2008). Upon TLR stimulation, a first wave of type I IFN can also positively feedback to amplify the IFN response, and activation of IRF8 further amplifies IFN secretion by pDCs.

TLR sensing of HIV by pDCs mostly occurs through endosomal recognition of viral RNA by TLR7. Although free virus triggers IFN secretion by pDCs, it is likely that cell-associated virus also contributes to pDC activation, possibly through the uptake of HIV-containing apoptotic cell vesicles (Lepelley et al. 2011), enhancing endosomal delivery of HIV RNA to the TLR-containing endosomes. Uptake of HIV-containing apoptotic vesicles may also potentiate delivery of HIV antigens for cross-presentation (see below). Cytosolic sensing of HIV has not been shown to significantly contribute to IFNα secretion in pDCs. The cytosolic exonuclease TREX1 suppresses cytosolic innate sensing in CD4+ T cells and macrophages (Lepelley et al. 2011), and the HIV Tat gene product can inhibit Protein Kinase R (PKR) (Cai et al. 2000), which may partly account for the predominance of TLR recognition of HIV for IFN secretion in pDCs.

3.2.3 Antigen Presentation

Another major function of pDCs is the presentation of virus antigens to CD4+ and CD8+ T cells. Although pDCs are generally thought to be weaker antigen-presenting cells (APCs) than mDCs, activated pDCs can efficiently activate memory CD4+ and CD8+ responses, and in some instances can prime naïve T cells. Initial characterization of pDCs in terms of T cell stimulation was based on allogeneic mixed lymphocyte reactions. It was shown that pDCs stimulated by influenza virus and CD40L can induce strong allogeneic Th1 cultured with IL-3 or IL-3 + CD40L can prime allogeneic CD4+ T cells to differentiate into IL-4-secreting Th2 cells or IL-10-secreting Tregs (Rissoan et al. 1999). The Th1 polarization induced by Flu-activated pDCs may reflect their high expression of MHC and co-stimulatory molecules, as well as secretion of type I IFN (Cella et al. 2000; Huber and Farrar 2011).

Upon viral infection, pDCs can potently present viral antigens and stimulate expansion of anti-viral CD4+ and CD8+ T cells (Fonteneau et al. 2003). However, in HIV infection, pDCs are poorly infected, and it is unclear to what extent they can present endogenous viral antigens. However, pDCs can acquire exogenous antigens in the form of soluble proteins or cell-derived material to cross-present to CD4+ and CD8+ T cells. Early work suggested that pDCs are not able to cross-present exogenous antigens to CD8+ T cells, even upon activation by CpG, but could present endogenous antigens. These experiments involved loading of pDCs with model antigen with concomitant CpG activation, and injection of antigen-loaded pDC in mice (Salio et al. 2004). However, in other studies, pDC could present exogenous ovalbumin (OVA) to naïve CD8 T cells in vitro, but only when pDCs were activated by CpG. In this study, they could not present OVA to OVA-specific CD4+ T cells,

unless the soluble protein was complexed with specific antibodies (Kool et al. 2011). OVA-immune complexes enhanced antigen uptake and induced its rerouting to acidic MHC-II+ compartments (Benitez-Ribas et al. 2006). More recent work has examined the cell biology of viral antigen cross-presentation in mouse and human pDCs. DiPucchio et al. showed that influenza virus stimulation induces routing of MHC-I molecules to the cell surface within 30 min of exposure, and cross-presentation of Flu antigen to CD8+ T cells within 4 h. Infection of pDCs was probably not required, as cross-presentation was dependent on endocytic recycling and independent of the proteasome. Cross-presentation by pDCs to memory CD8+ T cells was more potent than cross-presentation by mDCs, suggesting that pDCs may have evolved a rapid mechanism of viral antigen presentation for activation of memory responses (Di Pucchio et al. 2008). Other reports have shown that pDCs can cross-prime naïve CD8+ T cells after TLR activation (Mouries et al. 2008). The requirement for TLR activation for cross-presentation may be due to TLR-MyD88 recruitment of TAP molecules to nonacidic early endosomal compartment in some forms of cross-presentation (Burgdorf et al. 2008). Finally, it has been shown that type I IFN can enhance cross-presentation of viral antigens by dendritic cells in vivo, possibly through enhanced delivery of co-stimulatory signals (Le Bon et al. 2003).

Cross-presentation of HIV antigen by pDCs has now been conclusively demonstrated in several in vitro studies. Uptake of HIV lipopeptides (Hoeffel et al. 2007), uptake of noninfectious HIV particles, and exposure to HIV-infected apoptotic cells (Larsson et al. 2002) lead to cross-presentation of HIV antigen to specific CD8+ T cells in a proteasome-dependent manner. These studies highlight the dual role of pDCs during HIV infection, where they can secrete high amounts of type I IFN, but also present viral antigens to CD4+ and CD8+ T cells. Furthermore, cytokines secreted by pDC can also induce maturation of bystander APCs in a TNFα- and IFNα-dependent way (Fonteneau et al. 2004).

3.2.4 Migration

Unlike mDCs, pDCs are found in rare numbers in peripheral tissues under steady-state conditions. pDCs circulate through the body via the bloodstream, and enter secondary lymphoid tissues via High Endothelial venules (HEV) (Cella et al. 1999; Liu 2005). In inflammatory conditions, pDCs leave the bloodstream and accumulate at the site of infection, where they can secrete IFNα, take up antigens, and migrate to draining lymph nodes for antigen presentation. pDC homing to HEV is facilitated by its expression of L-selectin and PSGL1, the counter ligands of E and P selectins, respectively. Furthermore, pDCs express CXCR4, a receptor for the homeostatic chemokine CXCL12 expressed by HEV. Under reactive conditions, pDCs express additional molecules involved in homing to secondary lymphoid organs, such as CCR5 and CXCR3 (Diacovo et al. 2005; Yoneyama et al. 2004). pDCs can also express the chemokine receptor CCR9 and migrate to its ligand CCL25 to home into the small intestine (Wendland et al. 2007). pDCs purified

from blood express an array of chemokine receptors. However, pDCs do not migrate in vitro towards the respective ligands (with the exception of CXCR4), indicating that these chemokine receptors are not functional in the steady state (Penna et al. 2001). Although CXCR3 is unable to induce migration of pDCs, it potentiates the chemotactic response to CXCL12, and mediates adhesion and migration to heparan sulfates expressed by endothelial cells (Krug et al. 2002). In addition, pDCs exposed to IFNα acquire the ability to migrate in response to CCR2, CCR5, and CXCR3 ligands (Cicinnati et al. 2008).

In addition to chemokines, pDCs can respond to two agonists released by damaged tissues at the site of inflammation: Adenosine can engage the Adenosine Receptor A1, and F2L can trigger the formyl peptide receptor FPR3 (Devosse et al. 2009; Schnurr et al. 2004). pDCs express functional receptor for the anaphylatoxins C3a and C5a, and can also migrate in response to IL-18, an inflammasome-generated inflammatory mediator (Gutzmer et al. 2006; Kaser et al. 2004). Thus, in addition to chemotactic chemokines, pDCs can be recruited through signals associated with inflammation and tissue damage (Jimenez-Baranda et al. 2012). We discuss below the crucial role of pDC recruitment at the site of HIV entry. Viruses can activate pDCs and direct their secretion of T and NK cell chemotactic chemokines, such as CXCL9, CXCL10, and CCL4 (Piqueras et al. 2006; Megjugorac et al. 2004; Bendriss-Vermare et al. 2005). Although it is possible that secretion of the CCR5 ligands CCL3 and CCL4 by HIV-activated pDCs could limit viral spreading, the presence of inflammatory pDCs at the site of viral transmission seems to fuel HIV spread rather than limit it.

3.3 HIV–Plasmacytoid Dendritic Cell Interactions

3.3.1 Entry

HIV enters susceptible cells either through direct fusion with the cell membrane or through receptor-mediated endocytosis. Direct fusion of HIV occurs following a series of interactions between the heterotrimeric HIV glycoprotein gp120/gp41 and cell-surface CD4 receptor and a co-receptor, CXCR4 or CCR5. HIV fusion is pH independent and results in insertion of HIV cores into the cytoplasm with subsequent reverse transcription, integration, and productive infection (Stein et al. 1987). HIV enters mDCs either through CD4 receptor-mediated endocytosis and/or through C-type lectin receptors such as DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN) (Geijtenbeek et al. 2000). mDCs do not become activated by HIV to produce cytokines or to mature for antigen presentation (Fonteneau et al. 2004). mDCs are minimally infected by HIV but coculture of HIV-infected mDCs with T cells results in explosive infection (Frank et al. 1999). The block to HIV replication/infection in mDC is likely due to the high expression of the restriction factor SAMHD1 (Laguette et al. 2011; Goldstone et al. 2011; Hrecka et al. 2011), a deoxynucleoside triphosphate triphosphohydrolase. It is thought that SAMHD1, which is

highly expressed in mDCs, restricts HIV replication by hydrolyzing the majority of dNTPs, thus inhibiting reverse transcription and viral complementary DNA (cDNA) synthesis. SAMHD1 has only been analyzed in monocyte-derived dendritic cells and macrophages, but has not yet been investigated in primary mDCs, pDCs, or monocytes; therefore it is unclear whether SAMHD1 function in these cell types contributes to inhibition of HIV replication.

pDCs are also minimally infected by HIV (Fong et al. 2002), but in contrast to mDCs are highly activated to produce IFNα by both live and inactivated but fusion-competent HIV (Beignon et al. 2005; Fonteneau et al. 2004). pDCs express CD4, CXCR4, and CCR5, but not DC-SIGN. HIV entry and activation of pDCs to produce IFNα require HIV envelope glycoprotein (Beignon et al. 2005). When pDCs are incubated with HIV and b12, a human neutralizing anti-gp 120 mAb, or 17b, a human neutralizing anti-gp120 mAb that binds to an epitope induced by binding gp120 to CD4, IFNα production is inhibited. HIV entry and activation of pDCs to produce IFNα also require cell surface CD4. In pDCs incubated with HIV in the presence of an anti-CD4 antibody, IFNα is markedly inhibited. However, co-receptor usage does not seem necessary for pDC activation by HIV (Beignon et al. 2005; Herbeuval et al. 2005a; Schmidt et al. 2005; Haupt et al. 2008). Further support of the necessity of CD4-gp120 binding to pDC to induce IFNα production has been supported by the finding that the degree of IFNα induction is correlated with the affinity of the virus to CD4 (Haupt et al. 2008). pDCs are not activated by non-virion-associated HIV envelope glycoprotein, however, and may actually inhibit pDC activation (Beignon et al. 2005; Martinelli et al. 2007).

3.3.2 Activation

pDCs are activated by the interaction of ssRNA or unmethylated CpG DNA (CpG) with TLRs 7 and 9, respectively, in endosomal compartments of the cell. The source of ssRNA is usually viral and the source of CpG DNA may be bacterial products or self-DNA, as seen in systemic lupus erythematosis (SLE) (Boule et al. 2004). Depending on the activating stimuli, pDCs can be functionally dichotomous. pDCs can either become IFNα-producing cells (IPCs) that minimally upregulate maturation molecules but produce substantial amounts of IFNα (over 1,000× other cell types) (McKenna et al. 2005) *or* become potent APCs that do not produce IFNα but rather produce NF-κB-dependent inflammatory cytokines such as TNFα, IL-6, and IL-8, and upregulate co-stimulatory molecules for potent presentation of antigen to T cells (O'Brien et al. 2011).

Whether pDCs become IPCs or APCs seems related to the endosomal compartments to which the stimulating ligands traffic. Much of the work on understanding how trafficking of TLR ligands determines phenotype and function in pDCs has been done by studying CpGs in pDCs. It is well established that different CpGs predominantly induce IFNα- or NF-κB-dependent responses, based primarily on their sequence and/or secondary structure. For example, it has been shown that

CpGA has poly G sequences at its ends as well as internal palindromic sequences containing CG dinucleotides that form multimolecular complexes. A large fraction of internalized CpGA traffics to and is retained in early endosomal compartments of pDCs, and results in IFNα signaling. In contrast, CpGB, which comprises one or more unmethylated 6mer CpG motifs in a fully phosphorothioated backbone, remains monomeric and traffics rapidly to lysosomes to stimulate NF-κB-dependent responses (Honda et al. 2005).

Compartmental localization of TLR agonists within pDCs determines whether pDCs can act as effective APCs. CpGB and influenza stimulate pDCs to form NF-κB-dependent intracellular pools of MHC II molecules that are persistently neosynthesized and accumulate in antigen-loading compartments. In contrast, CpGA stimulation of pDCs does not lead to the formation of MHC II intracellular clusters (Sadaka et al. 2009). CpGB-stimulated pDCs efficiently process and present CMV antigens and are capable of stimulating CMV-specific effector memory T helper cells. CpGA-stimulated pDCs produce large amounts of type I IFNs, but fail to induce CMV-specific CD4+ effector memory T cells to produce IFN-γ (Jaehn et al. 2008). Interestingly, influenza virus induces IFNα production but also matures pDCs fully, in comparison to CpGB, which stimulates minimal IFNα production. Agonists such as influenza virus or CpGC can both stimulate IFNα production and antigen-presenting capacity in pDCs; however, these pDCs that obtain the mature antigen-presenting phenotype become refractory to IFNα production (Kerkmann et al. 2003). Thus, TLR agonists which strongly activate NF-κB signaling pathways to fully mature pDCs to gain an APC phenotype result in a pDC refractory state to further cytokine production.

Mechanisms by which IFNα signaling pathways are turned off in TLR-activated pDCs remain to be elucidated. Potential mechanisms for inhibited IFNα production after strong maturation include changes at the transcriptional and posttranscriptional level. At the transcriptional level, it has been shown that *IRF7* mRNA is downregulated after a strong maturational stimulus (O'Brien et al. 2011). At the posttranscriptional level, it has previously been shown that strong activation of the NF-κB signaling pathway causes ubiquitination and proteasomal degradation of IRAK1, a necessary component of the transductional transcriptional processor complex necessary for *IRF7* phosphorylation and nuclear translocation (Kubo-Murai et al. 2008). In contrast, *IRF7* is upregulated in human pDCs both in vitro and in vivo, possibly contributing to persistence of IFN signaling in HIV disease (O'Brien et al. 2011; Sabado et al. 2010).

New data also implicate adaptor-protein 3 (AP-3) as a critical endosomal protein complex involved in IFNα signaling in pDCs (Sasai et al. 2010). A newer model of pDC spatiotemporal trafficking is emerging, whereby stimulatory agonists first traffic to a common early endosomal compartment to trigger NF-κB-dependent responses, including IL-6 and TNFα production and expression of co-stimulatory molecules. CpGB, which strongly stimulates NF-κB pathways but does not stimulate IFNα, is rapidly found in lysosomal compartments. Alternatively, CpGA, which weakly stimulates NF-κB pathways but stimulates substantial IFNα production, traffics from the common early endosomal compartment to an AP-3 lysosome-related organelle (LRO) compartment, where IFNα signaling pathways are triggered (Sasai et al. 2010).

The activation of pDCs by HIV has been studied in detail. HIV activates pDCs to produce high levels of IFNα and lower levels of TNFα, and minimally upregulates co-stimulatory molecules, regardless of the HIV CXCR4 or CCR5 strain used. Endocytosis, but not fusion, is required for pDC activation by HIV. C34, a peptide that inhibits post-CD4 binding conformational changes in the transmembrane gp41 that is required for fusion, does not inhibit HIV activation of pDCs. However, inhibitors of endocytosis such as dimethyl amiloride, cytochalasin D, and chlorpromazine inhibit IFNα production by HIV-activated pDCs. Additionally, inhibitors of endosomal acidification, such as chloroquine, quinacrine, ammonium chloride, and bafilomycin, inhibit IFNα production by HIV-activated pDCs. Altogether, CD4-mediated endocytosis and endosomal acidification seem to be required for pDCs to become activated and to produce IFNα in response to HIV (Beignon et al. 2005).

Studies have been performed to understand whether HIV RNA or DNA is responsible for pDC activation. pDCs cultured with recombinant virions deficient in viral RNA do not become stimulated to produce IFNα or TNFα. Additionally, pDCs cultured with plasmid DNA encoding HIV, circular or linear, and formulated in cationic lipids, do not become stimulated to produce IFNα. In contrast to viral DNA, pDCs cultured with purified HIV RNA preincubated with cationic lipids produce high levels of IFNα and TNFα, and RNAse treatment inhibits this effect (Beignon et al. 2005). Additionally, pDCs are activated to produce IFNα by guanosine- and uridine-rich ssRNA motifs derived from the 5′ untranslated region of HIV-1, which have been reported to interact with TLR7 and TLR8 (Heil et al. 2004). HIV pseudotyped with vesicular stomatitis virus envelope (VSV-G), which is pantropic and allows for infection of all cell types tested, activates pDCs to produce IFNα and TNFα but does not activate mDCs (Beignon et al. 2005).

pDCs are likely activated by HIV through TLR7, as TLR7 oligonucleotide inhibitors are much more potent than TLR9 inhibitors in blocking IFNα production by HIV-exposed pDCs in human pDCs (Beignon et al. 2005; Mandl et al. 2008). Furthermore, silencing of TLR7 by siRNA in a pDC line inhibited the majority of IFNα production by HIV-1-infected cells (Lepelley et al. 2011). It remains to be elucidated whether incomplete inhibition of IFNα in HIV-activated pDCs by TLR7 oligonucleotide inhibitors and siRNA occurs because of limited specificity of these reagents, or whether additional and as yet undefined pDC PRRs sense HIV.

Classically, dendritic cells that have been activated by a microbial pathogen become refractory to subsequent activation. This phenomenon, commonly referred to as TLR tolerance, is thought to be a protective mechanism against unopposed and deleterious immune activation and inflammation (Geisel et al. 2007). In pDCs, however, certain viral and synthetic TLR ligands do not conform to this phenomenon and do not make pDCs refractory to subsequent activation. Importantly, both CpGA and HIV have been shown to activate the type I IFN receptor-mediated autocrine feedback loop and to persistently activate pDCs to produce IFNα. pDCs stimulated with CpGA or HIV can be restimulated to produce IFNα whereas CpGB- and influenza-activated pDCs become refractory to stimulation and cannot secrete IFNα. Both HIV and CpGA traffic to "early" EEA-1 and "recycling" transferrin receptor endosomal compartments. This results in limited NF-κB-dependent responses and weak T cell immunity but strong and persistent IFNα secretion.

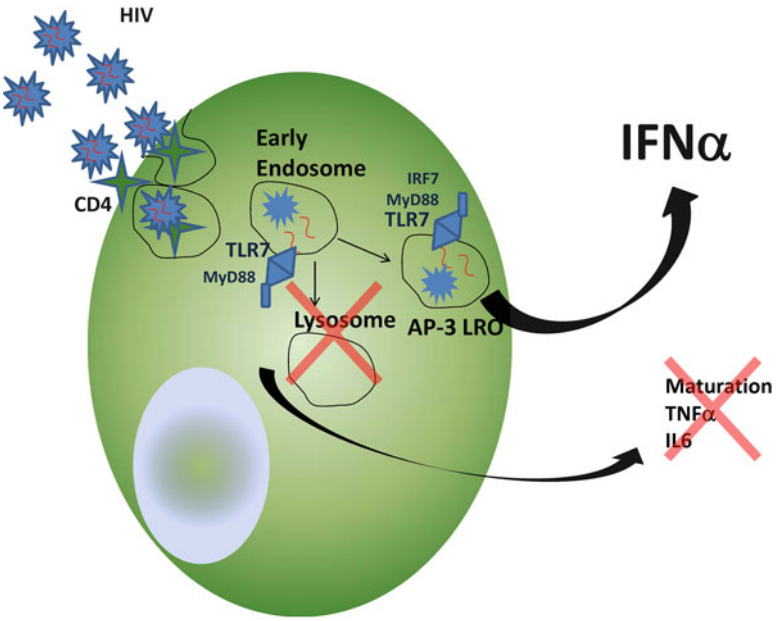

Fig. 3.1 pDC entry and activation by HIV

In contrast, influenza virus strongly activates NF-κB-dependent responses like CpGB to mature pDC for strong antigen presentation to T cells and to inhibit ongoing IFNα production (O'Brien et al. 2011).

Altogether, the data support a model (Fig. 3.1) in which HIV enters pDCs through CD4-mediated endocytosis to traffic predominantly to early and recycling endosomes to stimulate weak NF-κB-dependent responses but strong and persistent IFNα responses. Both NF-κB and IFNα responses are pH dependent, and HIV RNA predominantly stimulates these responses through endosomal TLR7. It remains unclear whether HIV activation of pDCs to become IPCs instead of APCs plays a role in HIV transmission, persistent inflammation and immune activation, or suboptimal adaptive immune response development during HIV infection.

3.4 The Role of Plasmacytoid Dendritic Cells in HIV Transmission and Primary Infection

3.4.1 Transmission

Although lentiviral infections such as HIV are characterized by long incubation periods and a protracted clinical course, the initial transmission event which leads to systemic infection is rapid and explosive (Geisel et al. 2007). Much of the research that informs this knowledge comes from the simian Immunodeficiency Virus (SIV)

rhesus macaque NHP model of mucosal transmission. The rhesus macaque (RM) model of pathogenic SIV closely parallels that of HIV, in that the animals experience high levels of viral replication, progressive CD4+ T cell depletion, chronic immune activation, and mucosal and systemic immunodeficiency leading to AIDS (Paiardini et al. 2009; Brenchley et al. 2010).

The transmission events that transpire during the first 2 weeks of HIV infection are logistically and ethically difficult to study in humans because HIV is asymptomatic during this time period. RM studies have proven invaluable to investigate these critical early time points of infection. From these studies, we now understand that virions cross mucosal epithelia within hours of inoculation and comprise a small homogeneous founder population. Genetic studies of transmitted virus support the concept of a small infected founder population, as infection in humans is acquired from a single virus genotype in 80% of cases (Keele et al. 2008). This small founder population then undergoes a local expansion during the first week of infection to establish an irreversible, systemic, and self-propagating chronic infection. During the second week of infection the virus can be detected in lymph nodes where latent infection is thought to persist. At this time gut-associated lymphoid tissue (GALT) is also highly infected, with resultant massive loss of CD4+ T cells (Li et al. 2009; Brenchley and Douek 2008).

A new model of HIV mucosal transmission events has emerged, based upon detailed immunohistochemistry studies of cervical and vaginal tissues taken from RMs that were intravaginally inoculated with SIV in vivo. Upon exposure to SIV, endocervical epithelial cells produce macrophage inflammatory protein 3 (MIP-3α) which then attracts substantial numbers of pDCs. pDCs are the first predominant cell type to arrive to infected mucosal sites and are activated by HIV to produce MIP1β and other chemokines and cytokines which attract CD4+ T cells. While CD4+ T cells are present in small numbers in normal endocervical mucosal tissue, release of cytokines and chemokines by HIV-activated pDCs in locally HIV-infected tissue recruits high numbers of CD4+ T cells for explosive and sustained infection. Furthermore, it has been shown that topical vaginal application of glycerol monolaurate, which inhibits the production of MIP-3α, prevents local and systemic SIV infection, therefore suggesting that pDC recruitment to mucosal sites of HIV inoculation is critical to transmission and subsequent systemic infection (Li et al. 2009). Altogether, this emerging model of initial SIV transmission events suggests that innate signaling, including pDC activation, during HIV mucosal inoculation paradoxically facilitates rather than restricts viral replication and expansion of infection.

3.5 pDC Function in Primary Infection

In chronic HIV infection, pDC and mDC are lost from the blood and this depletion correlates with high plasma viral load and low CD4+ T cell counts (Barron et al. 2003; Donaghy et al. 2001, 2003). Similarly, blood mDC and pDC numbers are markedly reduced in primary infection and are dysfunctional. Sabado et al. found

that the ability of mDC and pDC to stimulate T cells, as measured by a mixed lymphocyte reaction (MLR), was not deficient (Sabado et al. 2010), whereas Huang et al. found that mDC's ability to stimulate T cells in MLR was diminished (pDC MLRs were not tested) (Huang et al. 2011). Differences in study results were thought due to different techniques used for performing the MLR. Sabado et al. tested MLR using radioactive incorporation of tritium on day 5 of T-cell DC coculture, whereas Huang et al. used a CFSE-based assay that allows for measuring proliferative responses over the entire period of culture. Additionally, MLRs in the Huang study were performed in more patients with earlier acute HIV-1 infection. Notably in the Sabado study, pDC from acutely HIV-infected subjects with the highest HIV RNA viral loads had the lowest ability to stimulate T cells.

In the Sabado study, both mDC and pDC from primary HIV-infected subjects (mostly with later acute HIV infection, as characterized by Fiebig stage III–VI) were low in number but were hyperfunctional as compared to uninfected control subjects. In response to overnight TLR7 agonist stimulation with R848 (resiquimod), mDCs and pDCs from HIV-infected subjects produced more inflammatory cytokines in culture supernatants than normal control DCs. mDC produced significantly more IL-6, TNFα, IP-10, MIP1α, MIP1β, RANTES, and IL-12p70 in response to R848, while pDC produced significantly higher levels of IFNα, IL-6, TNFα, MIP1α, MIP1β, and RANTES in response to R848. pDCs from primary HIV-infected subjects also produced cytokines/chemokines in response to stimulation with HIV. In contrast, in the Huang study, which used intracellular cytokine staining to study cytokine production in response to CLO97, which is a synthetic TLR7/8 agonist very similar to R848, results were mixed, with mDC IL-12p70 or TNF-α production being significantly reduced during acute- and early-stage HIV-1 infection, whereas frequencies of IL-6-secreting mDCs were similar between the patient groups. They showed that IFNα-producing pDCs, as well as the per-cell intensity of IFNα production by pDCs, were substantially decreased in subjects with acute- and early-stage HIV-1 infection. Proportions of IL6- and TNFα-secreting pDCs were similar between patients from primary HIV-1 infection and HIV-1-negative controls, but per-cell intensities of cytokine production tended to be higher in patients with primary HIV-1 infection, specifically with regard to IL-6. Future studies may elucidate differing results between these two studies, but it seems apparent that DC dysfunction during the earliest stages of HIV infection likely contributes to inadequate adaptive immune response development.

In AIDS-susceptible NHP (RMs) that have been infected intravenously with SIV, pDCs are mobilized in the blood within 3 days after infection (Barratt-Boyes et al. 2010) to increase three- to sevenfold in blood and 10- to 20-fold in lymphoid tissue (Brown et al. 2009). Subsequently, by day 14 pDCs are depleted in blood but are highly activated, highly infected, and apoptotic in lymphoid tissue. Nevertheless, remaining pDCs had essentially normal functional responses to stimulation through TLR 7, with half of lymph node pDCs producing both TNFα and IFNα (Brown et al. 2009). These findings reveal that cell migration and death both contribute to pDC depletion in acute SIV infection. Lymph node pDCs during acute SIV are highly activated, infected, and apoptotic, which likely influences CD4+ T cell activation and infection.

3.6 pDC Function in Chronic Infection

HIV-1 disease progression is associated with a decline of circulating pDCs in blood, which correlates with high viral load and reduced CD4 counts. Chronic production of IFNα has been observed in HIV patients and is associated with disease progression. Increased IFNα expression in circulating peripheral blood mononuclear cells (PBMCs) correlates with HIV/SIV disease progression (Barron et al. 2003; Donaghy et al. 2001, 2003). In particular, a specific subtype of IFNα, namely, IFNα2b, is preferentially upregulated in HIV-1 patients throughout the course of the disease (Lehmann et al. 2009). In SIV models, persistent decline of circulating pDCs and chronic over-expression of IFNα are also observed during pathogenic SIV infection of nonnatural hosts, but not during nonpathogenic SIV infection of natural hosts.

Although pDCs are depleted in blood during chronic HIV infection, they accumulate in lymph nodes. The frequency of pDCs in lymph nodes is higher in HIV-infected patients than in HIV-uninfected controls, and highest in patients with the highest viral loads (Lehmann et al. 2010). Additionally, pDCs from lymph nodes of HIV-infected patients secrete higher amounts of IFNα spontaneously (in the absence of exogenous stimuli) but do not express higher levels of co-stimulatory molecules, suggesting that these pDCs are activated in vivo to produce IFNα but are immature IPCs, not APCs. pDCs in lymph nodes of HIV-infected patients, although higher in frequency than in uninfected controls, also exhibit increased apoptosis, as evidenced by increased staining of Annexin V, a marker for cells undergoing apoptosis. Circulating pDCs in the blood of HIV-infected patients express higher levels of lymph-node homing markers CCR7 and CD62L. Additionally, pDCs from HIV-infected patients respond more potently to CCR7 ligands, CCL19 and CCL21, in migration assays, suggesting that pDCs in chronically HIV-infected patients are poised to migrate more readily to lymphoid tissues. Therefore pDC depletion in the blood of HIV-infected subjects is likely due to redistribution of pDCs to lymphoid compartments where they ultimately die. These results have been confirmed in chronically SIV-infected macaques, which evidenced four times more pDC in GALT than uninfected control macaques, and the pDCs in chronically SIV-infected RMs were more stimulatory to produce IFNα than uninfected control pDCs (Reeves et al. 2012). SIV-infected macaques also have depleted pDCs in blood but elevated pDCs in lymphoid compartments during acute infection, which decline in AIDS, likely due to apoptosis. The acute phase of nonpathogenic SIV infection in natural hosts (e.g., sooty mangabeys (SMs) and African green monkeys (AGMs)) also involves a decline of circulating pDCs, which initially relocate to lymph nodes, and then return to the circulation following the onset of the chronic phase, therefore implicating a role for pDC dynamics in pathogenic lentiviral infection in humans and animal models (Diop et al. 2008).

During chronic HIV infection, circulating blood pDCs have diminished functionality in response to TLR7 and TLR9 stimulation (Finke et al. 2004; Tilton et al. 2008; Kamga et al. 2005). This compromised functionality is thought to occur because pDCs are exposed to HIV, CpGs from bacterial translocation from the gut, and inflammatory cytokines in vivo; therefore they are more refractory to stimulation.

Notably, CD4 is expressed at lower levels on the surface of pDCs in viremic HIV patients, but is more highly expressed on the surface of pDCs from uninfected controls and elite controllers. However, the intracellular CD4 is higher in HIV viremic patients than uninfected controls and elite controllers, suggesting that pDC activation in vivo causes CD4 internalization by pDCs. Decreased surface CD4 expression correlated with decreased IFN production in response to stimulation of pDCs with HIV (Machmach et al. 2012). It is unclear why pDCs in lymphoid tissues are hyperstimulatory during chronic HIV or SIV infection (Lehmann et al. 2010) while circulating blood pDCs are hypostimulatory. One possible explanation for these divergent responses between lymphoid and blood pDCs is that pDCs were stimulated with different reagents. In the blood pDC studies, pDCs were stimulated with synthetic TLR7 and TLR9 agonists. In the human lymphoid tissue pDC study, spontaneous activation was measured and in the SIV GALT study, pDCs within PBMCs were stimulated with PMA and/or poly:IC.

3.7 The Role of pDCs in Immune Activation During Chronic HIV Infection

HIV infection is marked by aberrant immune activation, which is a better correlate of disease progression to AIDS than viremia (Hazenberg et al. 2003; Deeks et al. 2004; Papagno et al. 2004; Benito et al. 2005; Giorgi et al. 1999; Bofill et al. 1996). Chronic immune activation and inflammation also persist in HIV infection despite antiretroviral therapy (ART) (Hunt et al. 2003; El-Sadr et al. 2006) and contribute to increased risk of serious non-AIDS conditions such as cardiovascular disease, kidney disease, liver disease, and non-AIDS-defining malignancies (Baker and Duprez 2010; Lekakis and Ikonomidis 2010; Ho et al. 2010; Lichtenstein et al. 2010; El-Sadr et al. 2006). Immune activation is characterized by increased expression of HLADR, CD38, and Ki67 on CD4+ and CD8+ T cells. The cause of immune activation in AIDS is unknown, but stimulation of innate immune cells directly by HIV and indirectly by products of bacterial translocation may be major contributors. Thus chronic injection of TLR7 or TLR9 agonists in mice induces characteristic lymphoid tissue disruption and immune deficiency, a syndrome resembling HIV-induced immunodeficiency (Heikenwalder et al. 2004; Baenziger et al. 2009).

Both human and animal studies support a role for pDCs and IFNα in the pathogenesis of HIV immune activation and inflammation. High plasma titers of IFNα during acute- and late-stage disease have been shown to correlate with disease progression (von Sydow et al. 1991). When matched for blood HIV RNA levels, women progress to AIDS more rapidly than men, express higher markers of immune activation, and produce more IFNα per pDC when challenged with HIV ex vivo, thought related to effects of female sex hormones on pDC functionality (Meier et al. 2009). Lymphoid tissue and circulating PBMCs derived from HIV-infected subjects with progressive disease express much higher levels of IFNα and related inducible genes as compared to uninfected controls (Herbeuval et al. 2006; Lehmann et al. 2008).

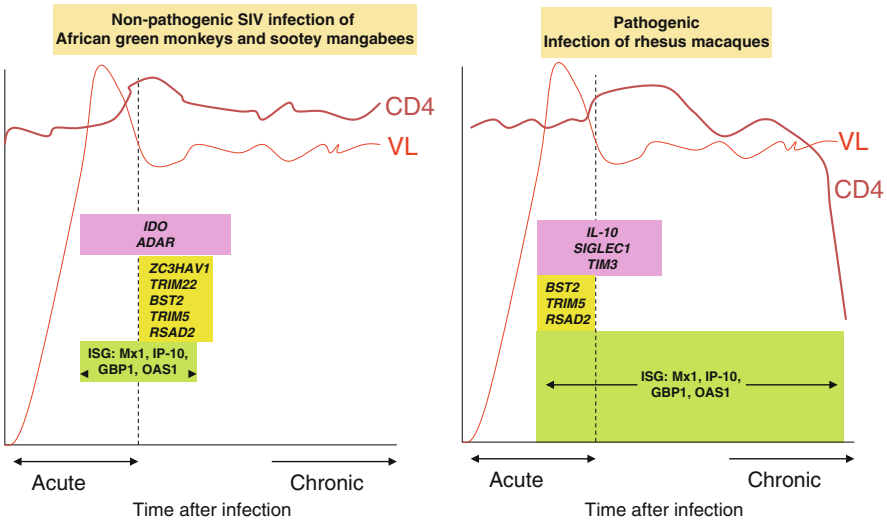

Fig. 3.2 Differential transcriptomes in nonpathogenic vs. pathogenic SIV infection

Primate studies have contributed critical insight into potential mechanisms of immune activation in chronic HIV infection. In contrast to the RM model where SIV infection results in AIDS, nonpathogenic or natural SIV hosts exist, including the SM and the AGM among others. Natural hosts are endemically infected with SIV with high levels of viral replication and yet they have a life span similar to uninfected animals, as they do not develop immunodeficiency, immune activation, or AIDS (Paiardini et al. 2009; Brenchley et al. 2010; Jacquelin et al. 2009). Transcriptional profiling in pathogenic and nonpathogenic SIV-infected primates reveals differences in IFNα responses, where both hosts have strong IFNα response signatures during acute infection, but only the pathogenically infected animals that go on to develop AIDS maintain elevated IFNα response signatures over the course of chronic infection (Jacquelin et al. 2009; Manches and Bhardwaj 2009). Currently, it remains unresolved whether the lack of sustained immune activation in natural SIV infection is due to a general attenuated response to infection, or due to induction of regulatory mechanisms that suppress immune responses generated during the acute infection. While one report claimed that pDCs from natural SIV hosts produced deficient IFNα responses to SIV because of perturbation of IFNα signaling pathways, other groups have not found differences in pDC responsiveness between pathogenic and nonpathogenic (natural) SIV hosts. Studies are underway to closely examine whether there are true differences between pDC antigen-presentation and IFNα responses between pathogenic and nonpathogenic SIV models (Fig. 3.2).

In pathogenic SIV/HIV infection, GALT is a critical site of mucosal CD4 depletion and immune activation-induced tissue pathology (Brenchley and Douek 2008). In AIDS-susceptible RMs, pDCs in the blood upregulate β7-integrin and are rapidly recruited to the colorectum after intravenous SIV infection. In vivo blockade of α4 β7-integrin inhibited pDC recruitment to the colorectum and reduced immune

activation (Ansari et al. 2011). The up-regulation of β7-integrin expression on pDCs in the blood also was observed in HIV-infected humans but not in chronically SIV-infected SMs (Kwa et al. 2011). These studies further implicate a role for activated pDC in the pathophysiology of immune activation in HIV.

Although IFNα is directly antiviral because it programs cell death and reduces viral replication in HIV-infected cells (Karpov 2001), it likely contributes to inhibition of T cell differentiation and death of HIV-uninfected bystander cells in HIV infection (Demoulins et al. 2008). IFNα induces TNF-related apoptosis-inducing ligand (TRAIL) and its death receptor (DR) 5 on CD4+ T lymphocytes in peripheral blood and in secondary lymphatic tissue, leading to the apoptosis of uninfected CD4+ T cells, likely contributing to the characteristic destruction of lymph node architecture in advanced stages of HIV-1 infection (Herbeuval et al. 2005a). Type I IFN can upregulate transcription of p53 and integrates with the p53 pathway to regulate apoptosis of virally infected cells (Takaoka et al. 2003). It has been observed that type I IFN-regulated genes, including cell cycle-associated genes, are upregulated in activated CD4+ T cells of HIV-infected patients (Sedaghat et al. 2008). Additionally, IFNα administration significantly enhances CD8+ T cell activation in chronically HIV-infected subjects (Manion et al. 2012). Besides IFN-induced TRAIL-mediated apoptosis of CD4+ T cells, recent models argue for a dynamic regulation of memory T cell homeostasis by inflammatory cytokines and type I IFN. First, chronic TLR stimulation can induce reversible blockade of thymic output, in a type I IFN-dependent way (Demoulins et al. 2008). Second, type I IFN is known to enhance bystander T cell proliferation (Tough et al. 1996), and constitutive proliferation of memory T cells due to specific or bystander activation could contribute to exhaustion of the memory CD4+ T cell reservoir. In addition, IFNα has been shown to inhibit telomerase activity and contribute to telomere shortening in human T cells (Reed et al. 2004). Depletion of self-renewing central memory CD4+ T cells by chronic stimulation has been shown to be a tipping point in SIV-infected macaques, upon which insufficient central memory cells are available to generate CD4+ effector memory and immunodeficiency ensues (Okoye et al. 2007). The hyperproliferative state modulated by chronic IFNα secretion may drive memory T cell exhaustion in the long term (Sedaghat et al. 2008).

Because stimulation of innate immune cells, including pDCs, by HIV and circulating bacterial products from gut translocation is thought to be a major cause of immune activation and inflammation in HIV infection, clinical trials have tested whether IFNα inhibitors and inhibitors of innate immune TLR signaling in HIV infection blunt immune activation. In older studies, HIV-infected subjects were vaccinated against IFN-alpha-2b in a phase I/II study and then a double-blind placebo-controlled phase II/III clinical trial, respectively (Gringeri et al. 1996, 1999). Although the immunogenicity of the vaccine was low, subjects who responded to vaccination had a lower rate of disease progression, supporting the evidence that IFNα plays a role in driving immune activation and disease progression. Chloroquine has also been studied as an immunomodulatory agent in HIV infection as it has been shown to reduce endosomal TLR signaling through inhibition of acidification in endosomal compartments where TLR ligands bind TLRs. Endosomal acidification

is necessary for cleavage of TLRs for activation of inflammatory signaling pathways. In vitro, chloroquine inhibits IFNα production by HIV-activated pDCs. Chloroquine also decreases CD8+ T cell activation and inhibits indoleamine 2,3 dioxygenase (IDO) and programmed death ligand 1 (PDL-1), two negative regulators of T cell responses (Martinson et al. 2010). When mice are treated with chloroquine, the production of proinflammatory cytokines upon stimulation with LPS is reduced (Hong et al. 2004).

Clinical trials have supported the use of chloroquine to reduce immune activation in HIV-infected patients. When chloroquine was administered to ART-naïve subjects for 2 months, patients showed immunological improvement, as the frequency of CD38+ HLA-DR+CD8+ T cells, proliferation of T cells, and circulating LPS levels were significantly reduced (Murray et al. 2010). Six months of chloroquine administration to HIV-infected clinical nonresponders, who had inadequate reconstitution of CD4+ T cells despite suppressive ART, had decreased frequency of activated T cells, decreased circulating LPS, decreased production of inflammatory cytokines (IL-6, TNF-alpha) in response to ex vivo stimulation of TLR ligands, and evidenced improved CD4+ T cell counts and pDC numbers (Piconi et al. 2011). Larger placebo-controlled trials are under way to fully elucidate whether chloroquine is effective at immunomodulating innate immune signaling to curb immune activation and T cell depletion in HIV infection.

3.8 Contribution of pDCs to Th17/Treg Balance During HIV Infection

In addition to the role of pDCs in inflammation-associated decline of the CD4+ T cell compartment, pDCs can potentially affect the balance of CD4+ T cell subsets in the periphery and in the gastrointestinal tract of infected individuals. Recent studies have focused on specific populations of CD4+ T cells that seem to share a common developmental pathway, and may be important in controlling HIV-associated immune activation: Th17 and Treg. Th17 cells are thought to be important for host defense against microbes, and in particular may be crucial for preservation of the integrity of the gut-associated mucosa and prevention of bacterial translocation (Kanwar et al. 2010). On the other hand, Tregs are potent suppressors of T cells and DC activation, and they may play a dual role in HIV infection, by suppressing HIV-specific T cell responses, but also by dampening the damaging immune activation during chronic disease. Th17 and Treg differentiation is reciprocally regulated in vitro upon stimulation of naïve CD4+ T cells, in the presence of TGFβ and the inflammatory cytokines IL-6, IL-1β, and IL-23. While TGFβ upregulates the expression of the master transcription factors RORγt and Foxp3 for Th17 and Treg development, respectively, signaling through IL-6 skews Treg development towards Th17 commitment (Veldhoen et al. 2006; Bettelli et al. 2006; Zhou et al. 2008). Treg and Th17 cells also share common chemokine receptors (CCR4, CCR6) and homing properties towards CCL20 (Acosta-Rodriguez et al. 2007).

Early studies of Treg dynamics during HIV infection reported decreased numbers of Treg in the blood of chronically infected patients, a concept compatible with the fact that Treg can be infected by HIV (El Hed et al. 2010). The interpretation of those data is complicated by the fact that there is no unambiguous marker of Treg in humans, as Foxp3 can also be expressed by recently activated CD4+ effector cells. However, Tregs can be identified with a good probability using a combination of markers, including high expression of Foxp3, CD25, low expression of the IL-7Rα (CD127), and expression of inhibitory surface molecules, such as CTLA-4 and GITR (Miyara et al. 2011). The emerging picture is that despite a relative disappearance of Tregs in the circulation, both data in humans and in NHP show an increased frequency of Foxp3 CTLA4+ Tregs in the secondary lymphoid tissue of chronically infected individuals (Boasso et al. 2006). Furthermore, SIV infection in pathogenic and nonpathogenic NHP models showed that disease progression is associated with the relative loss of Th17 cells and increase in the frequency of CD4+ Foxp3+ Tregs in blood and lymphoid mucosal tissue (Kanwar et al. 2010). Infection and loss of Th17 in acute SIV infection (Kader et al. 2009) may contribute to altered Treg/Th17 ratios in later phases. In addition, even though massive depletion of CD4+ T cells occurs in the gastrointestinal tract of all SIV-infected monkeys, bacterial translocation is not observed in nonpathogenic infections, suggesting the crucial role of the preservation of a normal Treg/T17 balance in the gut.

As mentioned, the relative expansion of Tregs in infected individuals plays a dual role. In vitro removal of CD4+ CD25+ cells from peripheral leukocytes of HIV-infected patients or SIV-infected macaques enhances HIV- or SIV-specific immune responses (Kinter et al. 2004; Aandahl et al. 2004). Furthermore, the frequency of Tregs inversely correlates with the magnitude of SIV-specific CTL in infected RMs (Estes et al. 2006). On the other hand, decreased frequency of circulating Tregs correlates with hyperactivation in HIV-infected patients, and early induction of TGFβ, Foxp3, and IL-10 in nonpathogenic models of SIV infection is associated with early resolution of inflammation (Bosinger et al. 2009; Jacquelin et al. 2009; Ploquin et al. 2006).

The mechanisms of Treg/Th17 deregulation are not known. However, besides direct infection of Th17 cells, there is evidence that DC-derived cytokines and immunoregulatory enzymes can play an important role in regulating Treg/Th17 balance. Thus, type I IFN constrains the development of Th17 cells (Guo et al. 2008). Type I IFN induces up-regulation of SOCS3 and downregulation of IL-1β and IL-23 in DC, while naïve T cells cultured in Th17 polarizing condition in the presence of type I IFN downregulate RORγt, IL-17A, and IL23R in CD4+ T cells (Ramgolam et al. 2009). Another mechanism controlling peripheral Treg differentiation and affecting Treg/Th17 ratios is expression of immunoregulatory enzymes in dendritic cells. The enzyme IDO that catalyzes the rate-limiting step in tryptophan catabolism has a well-identified role in dampening T cell activation, through depletion of tryptophan, an amino acid essential for proliferation of effector cells. IDO also plays a role in the generation and activation of Treg, through activation of the amino-acid starvation response gene GCN2 in developing CD4+ T cells, but also through the generation of soluble tryptophan catabolites, which can contribute to Foxp3+ Treg differentiation (Munn et al. 2005; Fallarino et al. 2006; Favre et al. 2010). Importantly, IDO activity is upregulated in HIV-infected patients, and is associated with disease

progression and a deregulated Treg/Th17 ratio (Favre et al. 2010). pDCs activated by HIV and other TLR agonists express IDO, and induce the generation of Treg from naïve CD4+ T cells (Manches et al. 2008; Moseman et al. 2004). In addition, IDO expression by pDC has been shown to prevent conversion of Treg into Th17 cells and IDO functioned as a molecular switch to maintain the stability of Treg at the detriment of Th17 differentiation in an inflammatory environment (Baban et al. 2009; Sharma et al. 2009). Inhibition of IDO by 1-methyl tryptophan showed enhancement of HIV-specific CTL responses and clearance of HIV-infected macrophages, in an in vivo model of HIV infection in humanized mice (Potula et al. 2005). Furthermore, Tregs have been shown to induce IDO in DCs through CTLA-4 engagement, and CTLA-4 blockade in RMs inhibits IDO expression, enhances CD8+ T-cell responses, and contributes to lower viral load (Hryniewicz et al. 2006).

New mouse models of conditional pDC knockout have recently been developed, and provide remarkable insights into the role of pDC during acute and chronic viral infections. Thus, conditional knockout of BDCA-2 depletes mouse pDC, which impairs generation of anti-vesicular stomatitis virus CD8+ T cell responses, but had no effect in a model of acute lymphocytic choriomeningitis virus (LCMV) infection (Swieki, Immunol Rev 2010). Similarly, conditional knockout of E2-2 preventing the development and maintenance of pDC did not affect T cell responses and clearance of LCMV Armstrong strain. However, pDC-deficient animals failed to clear LCMV Docile strain, resulting in chronic infection (Cervantes-Barragan et al. 2012). pDC-deficient mice displayed reduced numbers of LCMV-specific CD4+ T cell and impaired CD8+ T cell responses. Enhancement of CD4+ T cell responses was nevertheless independent of antigen presentation by pDC and likely due to decreased IFN in pDC-deficient mice. Interestingly, in yet another conditional knockout, Siglec-H deletion leads to specific pDC depletion, but it was shown that pDC promoted the induction of peripheral tolerance by generating antigen-specific CD4+ Foxp3+ Treg. Importantly, pDC maintained higher number of CD4+ Foxp3+ Treg in the lamina propria of the small intestine, but not in the periphery, while pDC-depleted mice had enhanced number of Th17 cells in the lamina propria, indicating that pDCs are involved in the homeostasis of Th17/Treg ratios in the gastrointestinal tract (Takagi et al. 2011). Thus, although the role of pDC-derived IFN and IDO expression may be part of a complex immunoregulatory network, the high levels of type I IFN and possibly the antigen-presenting capabilities of pDCs likely affect the balance of beneficial and detrimental Th17 and Treg responses in chronically infected individuals.

3.9 Conclusions and Future Directions

The role of pDCs in HIV immunopathogenesis remains incompletely characterized. Much of the evidence linking pDCs and IFNα to HIV transmission and disease progression is correlative, but causative data is emerging. Although pDCs have the

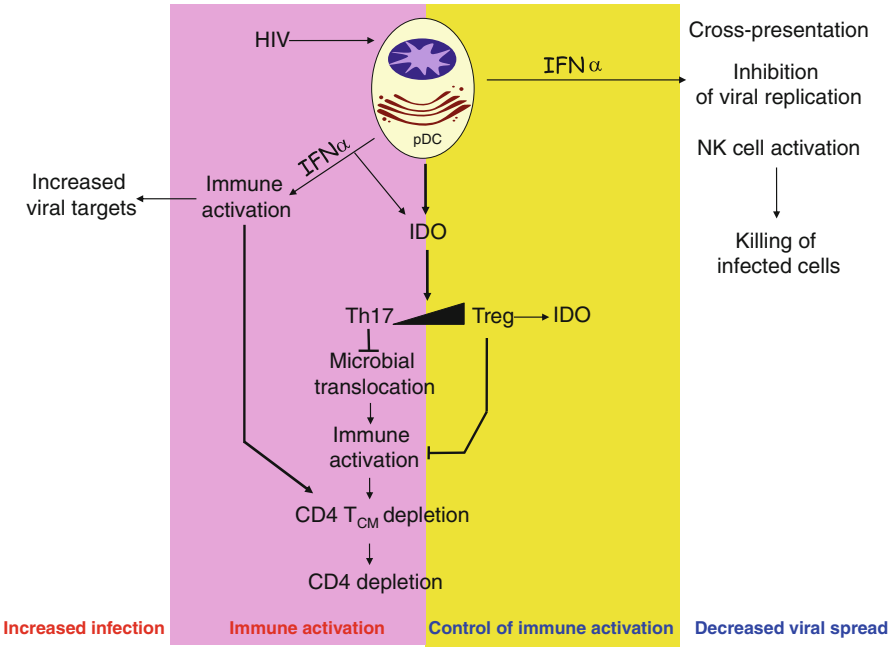

Fig. 3.3 Dual role of pDC in HIV infection

capacity to mature into APCs to stimulate potent adaptive immune responses, in HIV infection pDCs more likely effect a cytokine- and chemokine-producing phenotype, which may paradoxically fuel viral transmission and immune activation. Further research is needed to clarify the role of pDCs in HIV transmission, disease progression, and chronic inflammation (Fig. 3.3).

3.9.1 Future Directions: Transmission

Through careful analysis of mucosal tissue samples from macaques infected in vivo with SIV, it has been shown that pDCs arrive first to produce inflammatory cytokines and T-cell-attracting chemokines (Li et al. 2009). Inhibition of pDC trafficking to mucosal tissues using topical glycerol monolaurate actually prevented SIV transmission and systemic infection. Based on this report it seems that inhibition of innate immune responses and inflammation during HIV mucosal exposure actually prevent transmission, whereas earlier studies which tested immune-activating topical microbicides, like imiquimod and CpG, actually enhanced infection and viremia (Wang et al. 2005). Additionally, it has been shown that exposed, uninfected sex workers actually have depressed mucosal immunity as compared to uninfected controls (Lajoie et al. 2012), supporting an immune quiescence model of protection, whereby

lower T-cell activation/recruitment at the mucosal compartment reduces HIV target cell numbers and is an important component of protection from HIV infection.

Topical and oral ART as pre-exposure prophylaxis has proven to be a highly efficacious strategy to prevent HIV transmission (Grant et al. 2010; Abdool Karim et al. 2010), but this approach has had various limitations. Some of these studies were stopped prematurely due to futility (van der Straten et al. 2012) and others have raised concerns about systemic toxicity like bone loss with oral tenofovir use (Liu et al. 2011). Strategies that employ daily oral ART pre-exposure prophylaxis also may not be cost-effective (Keller and Smith 2011). Therefore, integrating immunomodulating microbicides like glycerol monolaurate into the armamentarium of prevention strategies should be considered. Additionally, specific inhibitors of pDC activation could be explored as topical microbicides. In vaginal and foreskin tissue explants, Phosphorothioate 2′ deoxyribose oligomers, when applied topically, have been shown to both inhibit HIV infection and dampen HIV-associated local inflammation, including IFNα production (Fraietta et al. 2010). Cross-linking CD4, BDCA-2, BDCA-4, or CD-123 on pDCs has been shown to inhibit IFNα production both by inhibiting translocation of IRF7 to the nucleus, therefore inhibiting IFNα signaling pathways, and by maturing the pDCs so that IFN signaling is inhibited (Fanning et al. 2006). Immunoproteasome inhibitors (Ichikawa et al. 2012) have also been used to inhibit pathogenic IFNα signaling in pDCs.

3.9.2 Future Directions: Chronic Immune Activation

The cause of immune activation and inflammation in HIV infection remains unknown, but candidate mechanisms include the stimulation of innate immune cells by replicating and non-replicating HIV and by circulating bacterial products that have translocated from damaged gut mucosa. Critical insight into the pathogenesis of immune activation in HIV infection has been gained by studying pathogenic as compared to nonpathogenic SIV infection. Although both RMs and SMs or AGMs are highly viremic with SIV, only RMs develop AIDS while both SMs and AGMs have a normal life span. Only RMs have heightened immune activation, inflammation, and CD4 T cell decline. A major clue to the etiology of immune activation in chronic pathogenic SIV and HIV is that type I IFN-stimulated genes (ISGs) are highly upregulated in RMs but not so in SMs or AGMs. Although one study suggested that pDC responses in natural SIV infection are blunted due to defects in IFNα signaling pathways (Mandl et al. 2008), subsequent studies have not supported these findings (Harris et al. 2010) but rather showed that ISGs are highly upregulated in RMs, SMs, and AGMs during acute infection, but this transcription signal normalizes in SMs and AGMs after 4–6 weeks of SIV infection (Manches and Bhardwaj 2009). Similarly, a rare group of HIV-infected humans who maintain a significant viremia but do not experience a decline in CD4+ T cells also have transcriptomes which are characterized by low ISGs as compared to HIV-infected progressors (Rotger et al. 2011). These studies support a model of active immune suppression of IFN responses

in nonpathogenic as compared to pathogenic SIV infection, rather than any specific deficit in the ability of pDCs to produce type I IFN in response to SIV.

Understanding the cause of these persistent IFN responses during chronic HIV and pathogenic SIV infection remains an important unanswered question. Additionally, understanding how IFN-responses are resolved despite persistent viral replication in nonpathogenic SIV infection will provide potential targets for immune therapy in HIV-infected humans. Because pDCs are considered to be the main source of type I IFN, more detailed studies are needed to dissect any differences in SIV–pDC interactions between RMs and SMs or AGMs. For example, differential responses to TLR ligands predict disease transmission and progression in HIV-infected subjects (Bochud et al. 2007; Pine et al. 2009; Rotger et al. 2011; Freguja et al. 2012; Ricci et al. 2010; Soriano-Sarabia et al. 2008); therefore there may be functional differences in the response of innate immune cells, including pDCs, to HIV and to circulating bacterial products from gut translocation. It has been shown that HIV stimulates pDCs to produce IFNα instead of maturing to become an APC, while other ssRNA viruses like influenza both stimulate IFNα production and mature the cells, ultimately inhibiting persistent IFNα production. It is unknown whether trafficking and stimulation of TLR7 agonists, including SIV, or TLR9 agonists, including bacterial DNA (CpG), differ between RM and SM or AGM pDCs. If SIV or CpGs mature SM or AGM pDCs but do not mature RM pDCs, this would be suggestive that differential trafficking and/or stimulation of signaling pathways could account for differential systemic responses to SIV in vitro. Another intriguing possibility is that there is inhibited infection of SIV in lymphoid tissues of SMs, such that there would be a lack of stimulatory ligands for pDCs to produce IFNα (Paiardini et al. 2011). Also, it has been shown that there is increased trafficking of pDCs to GALT (Kwa et al. 2011; Reeves et al. 2012) in RM, which produce inflammatory cytokines, including IFNα.

Non-pDC IFN responses may also contribute to chronic immune activation. Monocyte-derived macrophages derived from PBMCs of macaques upregulated ISGs in response to SIV infection, but ISG expression was inhibited after silencing the expression of MDA-5, a cytosolic RNA receptor that activates IFN signaling pathways. However, when chloroquine was added to the experiment, there was further reduction in ISG expression, indicating that both an endosomal TLR pathway and a cytosolic MDA-5 pathway are implicated in ISG expression (Co et al. 2011).

From the transcriptome studies of PBMCs from blood and lymphoid tissues of pathogenic vs. nonpathogenic PMBCs, immune regulatory genes such as IDO and ADAR were more highly expressed in nonpathogenic SIV disease (Jacquelin et al. 2009). It is not known which cell types are responsible for increased expression of these immunoregulatory molecules. The interplay between innate immune cells and adaptive immune cells to achieve this more immunoregulatory state is also unknown. For example, SIV might induce pDCs in SM and AGMs to become predominantly IDO-producing cells instead of IFNα-producing cells in vivo, as compared to RMs. In addition to IDO and ADAR, the differential expression of other immunoregulatory molecules on innate and adaptive immune cells, both in vivo and ex vivo in AGMs and SMs as compared to RMs, would be informative.

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Chapter 4
Cellular and Viral Mechanisms of HIV-1 Transmission Mediated by Dendritic Cells

Christopher M. Coleman, Corine St. Gelais, and Li Wu

Abstract Dendritic cells (DCs) play a key role in the initial infection and cell-to-cell transmission events that occur upon HIV-1 infection. DCs interact closely with $CD4^+$ T cells, the main target of HIV-1 replication. HIV-1 challenged DCs and target $CD4^+$ T cells form a virological synapse that allows highly efficient transmission of HIV-1 to the target $CD4^+$ T cells, in the absence of productive HIV-1 replication in the DCs. Immature and subsets of mature DCs show distinct patterns of HIV-1 replication and cell-to-cell transmission, depending upon the maturation stimulus that is used. The cellular and viral mechanisms that promote formation of the virological synapse have been the subject of intense study and the most recent progress is discussed here. Characterizing the cellular and viral factors that affect DC-mediated cell-to-cell transmission of HIV-1 to $CD4^+$ T cells is vitally important to understanding, and potentially blocking, the initial dissemination of HIV-1 in vivo.

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_4,

4.1 Dendritic Cell-Mediated HIV-1 Transmission

4.1.1 Immature and Mature DCs and Their Roles in HIV-1 Infection

DCs are important cells in the defense against invading pathogens. DCs act as a bridge between the innate and adaptive immune responses. Immature DCs (iDCs) are present at all mucosal surfaces and come into contact with pathogens, including HIV-1. Once pathogen contact with DCs is established, DCs can undergo maturation and migrate to the lymph node, where they present processed antigens to T cells and B cells, triggering an adaptive immune response to the invading pathogen. Many stimuli can induce maturation of DCs and these can be broadly grouped into pathogenic and immunological factors.

Pathogenic factors that induce DC maturation are factors that are expressed by invading pathogens, referred to as pathogen-associated molecular patterns (PAMPs). Due to the wide range of pathogenic bacteria, viruses, and fungi, PAMPs are specific for groups of pathogens. DCs express a range of receptors for these PAMPs, including toll-like receptors (TLRs) (Kawai and Akira 2010, 2011), a family of molecules in which each member recognizes a specific PAMP. For example, lipopolysaccharide (LPS) is a PAMP expressed by gram-negative bacteria. LPS interacts with TLR4, along with the TLR4 co-receptors MD-2 and CD14, on the cell surface and induces a response to the invading bacteria via a complex signaling cascade (Kumar et al. 2011). LPS stimulation causes DC maturation, leading to increased DC migration, decreased DC endocytosis, and increased expression of co-stimulatory molecules required for interactions with $CD4^+$ T cells on the DCs (Iwasaki and Medzhitov 2004). In the study of HIV-1 interactions with DCs, LPS activation of DCs is important because there is an association between gram-negative bacterial translocation and high levels of LPS in the serum and the systemic immune activation observed in chronic HIV-1 infection (Brenchley et al. 2006). In addition, there is a possibility of coinfection with gram-negative bacteria along with HIV-1 infection (Gringhuis et al. 2010; Hernandez et al. 2011), which may facilitate HIV-1 spread by enhancing LPS-stimulated maturation of DC and, therefore, DC-mediated HIV-1 transmission to $CD4^+$ T cells.

DCs and other immune cells respond to pathogens by releasing cytokines, chemokines, and other soluble factors into the extracellular milieu. Release of these immunological factors is important for preventing spread of infection within the host, as these molecules can act on surrounding naïve cells to promote immune cell activation or to protect surrounding cells by upregulating cellular factors that restrict pathogen spread. In the case of DCs, some immunological factors lead to DC maturation. For example, type I interferons (IFN) are antiviral cytokines produced as part of the innate immune response to an infection. The two main types of type I IFN are IFNα and IFNβ, both of which can prevent virus dissemination, trigger adaptive immune responses to clear the virus, and protect against reinfection (Stetson and Medzhitov 2006). IFNα can inhibit the replication of HIV-1 in $CD4^+$ T cells, DCs, and macrophages in vitro (Coleman et al. 2011; Goujon and Malim 2010; Poli et al. 1989). IFNα can also inhibit the cell-to-cell transmission of HIV-1 between $CD4^+$ T

cells and DC-mediated HIV-1 transmission to CD4+ T cells (Coleman et al. 2011; Vendrame et al. 2009). The type I IFN inhibition of HIV-1 replication in DCs can be relieved by factors such as the Vpx proteins from HIV-2 or certain simian immunodeficiency viruses (SIV) (Pertel et al. 2011), which may allow the identification of type I IFN-inducible HIV-1 restriction factors in DCs. Altogether, these data demonstrate the importance of DCs matured by immunological factors in the prevention of replication and spread of HIV-1.

DCs may also act as important HIV-1 reservoirs and maintain a significant pool of HIV-1 during long-term viral infection. Given the low levels of HIV-1 replication and high levels of DC-mediated transmission of HIV-1 observed in some DC subtypes, it is possible that DC subtypes, particularly those in the lymph node, may act as significant pools of HIV-1 during long-term HIV-1 infection (reviewed in Coleman and Wu 2009).

4.1.2 HIV-1 Proteins Have the Potential to Promote Infection and Transmission Processes

Viruses need to ensure efficient replication and transmission within a host, as there is often a delicate balance between viruses and immune response. Viruses, particularly those with RNA genomes, have limited genomic capacity to encode proteins that can promote viral replication and cell-to-cell transmission. Therefore, viral proteins are often multifunctional and able to promote infection and/or cell-to-cell transmission using many mechanisms.

The HIV-1 genome encodes 15 individual proteins, the structural polyproteins Gag (consisting of matrix, capsid, nucleocapsid, and p6 proteins), Pol (consisting of protease, reverse transcriptase, and integrase proteins), and envelope (Env; consisting of gp120 and gp41); two regulatory proteins (Tat and Rev); and four accessory proteins (Vif, Vpr, Vpu, and Nef). The Gag and Pol polyproteins are cleaved into their constitutive parts during maturation of the virion, making them available to carry out their respective functions upon infection of a new cell. The Env polyprotein is cleaved within the cell and a complex is formed by the association of gp41 and gp120. The Env complexes then form trimers in the endoplasmic reticulum, are heavily glycosylated in the *trans*-golgi network, and are subsequently transported to the cell surface for incorporation into virions (Earl et al. 1991). On the cell surface, gp41 forms the *trans*-membrane portion and the gp120 forms the extracellular portion.

HIV-1 structural proteins and some accessory proteins are present in the virion and have the potential to interact with cellular factors and promote HIV-1 transmission during the initial stages of infection. Furthermore, HIV-1 proteins produced during replication may promote cell-to-cell transmission after the initial infection. Viral proteins may also act in both DC-mediated *cis*- and *trans*-infection; for example, Env is present on the surface of the virion and is able to interact directly with HIV-1 receptors and other cell surface molecules to promote cell-to-cell transmission of the virus (van Montfort et al. 2011) and newly synthesized Env can promote CD4+ T cell-mediated cell-to-cell transmission (Jolly et al. 2004).

4.1.3 Capture of HIV-1 by DCs

The first step in the DC-mediated transmission of HIV-1 to $CD4^+$ T cells is the capture of HIV-1 from the extracellular milieu. iDCs express the HIV-1 receptor CD4 and low levels of the HIV-1 co-receptors CCR5 and CXCR4. Expression levels of CD4 are not significantly affected by DC maturation and small differences in expression of CCR5 and CXCR4 do not correlate with HIV-1 binding or DC-mediated HIV-1 transmission (Dong et al. 2007; Sanders et al. 2002). It has been shown that LPS-matured DCs capture significantly more HIV-1 than iDCs, without any significant upregulation of CD4 or CCR5 (Dong et al. 2007; Izquierdo-Useros et al. 2007). Other cellular factors that affect HIV-1 capture and binding to DCs will be discussed later.

LPS-matured DCs capture significantly more HIV-1 than iDCs (Dong et al. 2007; Izquierdo-Useros et al. 2007; Wang et al. 2007b), suggesting that mature DCs (mDCs) can act as a significant pool of infectious virus during the initial stages of infection. HIV-1 capture in LPS-matured mDCs, unlike in iDCs, has been shown to occur independently of HIV-1 gp120 engagement of CD4 and C-type lectin receptors (Hanley et al. 2010; Izquierdo-Useros et al. 2007). Studies using three-dimensional microscopy and real-time imaging of HIV-1 transfer from LPS-matured mDCs to $CD4^+$ T cells analyzed both cell-free and cell-associated capture of HIV-1 virus-like particles (VLPs) by mDCs. Cell-free HIV-1 capture was found to occur in three distinct phases: HIV-1 VLPs randomly bind to the mDC plasma membrane, then "surf" to a polarized region of the cell, and are eventually concentrated into a distinct, invaginated pocket on the DC surface (Izquierdo-Useros et al. 2011). Coculture of mDCs with an HIV-1-producing T-cell line (MOLT-4) or HIV-1-infected primary $CD4^+$ T cells demonstrates that HIV-1 transfer from infected cells to mDCs is more efficient than cell-free HIV-1 capture by mDCs (Izquierdo-Useros et al. 2011). These results suggest that LPS maturation of DCs enhances capture of HIV-1 released from infected $CD4^+$ T cells and that this could promote spread of HIV-1 within the host and subsequently promote viral pathogenesis.

4.1.4 Mechanisms of DC-Mediated Spread of HIV-1: Cis- and Trans-infection

During HIV-1 replication, there are two major mechanisms of viral transmission between cells (reviewed in Wu and KewalRamani 2006). First, HIV-1 can infect target cells, and productively replicate and produce progeny virions that are released to infect new target cells; this is *cis*-infection. Second, the virus is retained at or near the cell surface of a donor cell and transmitted to a different type of target cell via the close contact and formation of a *virological synapse* (*VS*) or via the *exosome secretion pathway*; this is *trans*-infection. The proposed mechanisms of DC-mediated HIV-1 transmission are summarized in Fig. 4.1.

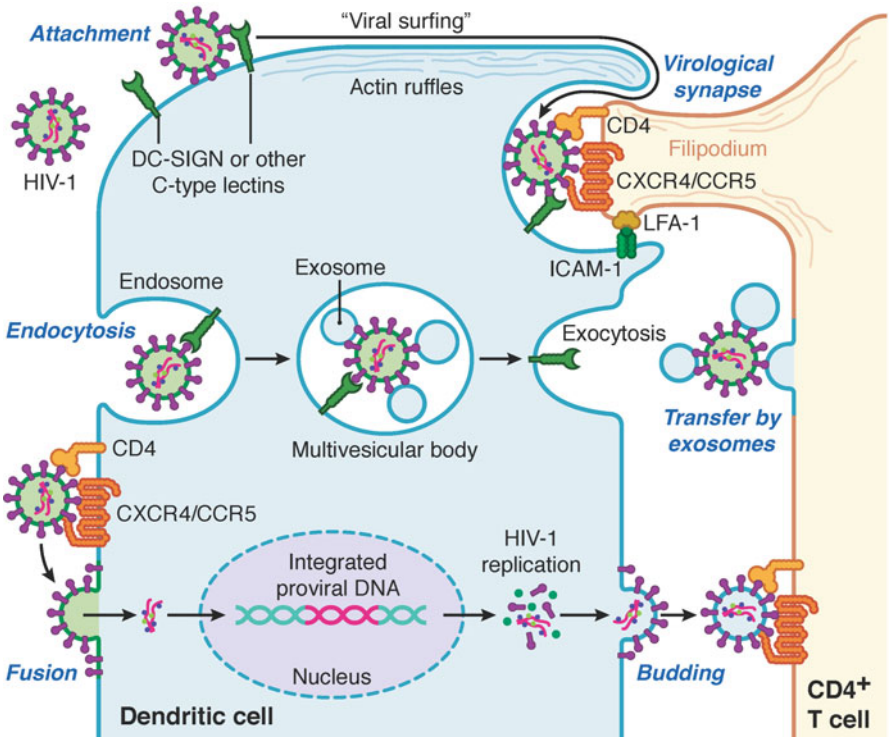

Fig. 4.1 Summary of the mechanisms of DC-mediated HIV-1 transmission to CD4⁺ T cells. Incoming HIV-1 (virions with *green membrane*) can be transmitted from DCs to CD4⁺ T cells by three distinct mechanisms. *First*: *Trans*-infection via the virological synapse: HIV-1 can bind to DC-SIGN on the DC surface, "surf" along actin ruffles, and become polarized to a pocket structure on the cell surface. When DC contact with a CD4⁺ T cell is established, the virological synapse is formed and stabilized through interactions between ICAM-1 and LFA-1. The CD4⁺ T cell extends a filipodium, via actin cytoskeleton rearrangements, into the pocket on the DC surface to capture the HIV-1. *Second*: *Trans*-infection via the exosome secretion pathway: Endocytosed HIV-1 can converge with the exosome secretion pathway in the multi-vesicular bodies and HIV-1 can be released in association with exosomes to infect nearby CD4⁺ T cells. *Third*: *Cis*-infection: HIV-1 can enter the DC by fusion and undergo its complete replication cycle and newly formed infectious HIV-1 (virions with *blue membrane*) bud from the DC and is able to infect nearby CD4⁺ T cells. *DC-SIGN* dendritic cell-specific intercellular adhesion molecule 3-non-grabbing integrin, *ICAM-1* intercellular adhesion molecule-1, *LFA-1* leukocyte function-associated molecule-1

HIV-1 *cis*-infection can result in the transmission of HIV-1 by the completion of all of the steps of the replication cycle within the cell and release of fully infectious new virions to go on and infect new cells. This process is similar in many different cell types, though it can be blocked by the expression of cell type-specific restriction factors and can be affected by expression levels of molecules that are required for productive HIV-1 infection, such as HIV-1 restriction factors, which will be discussed in other chapters.

HIV-1 *trans*-infection is most prominently associated with DCs, as productive HIV-1 replication is relatively inefficient in all DC subtypes compared to more

permissive cell types, such as macrophages and CD4+ T cells (Dong et al. 2007). Phagocytosis of pathogens by DCs usually leads to degradation via the lysosome or proteasome and destruction of the pathogens. By contrast, internalized HIV-1 is rerouted and polarized at the cell surface to escape canonical degradation routes via the lysozyme or proteasome, allowing efficient transmission to CD4+ T-cells (Yu et al. 2008). Once the HIV-1 is concentrated on the cell surface, the infected DC comes into close contact with a target CD4+ T cell and forms the VS, enabling efficient transfer of surface-bound HIV-1 to the target CD4+ T cell (McDonald et al. 2003). The VS is structurally similar to the immunological synapse formed between DCs and CD4+ T cells during an immune response, but does display distinct morphological features and dynamics (reviewed in Vasiliver-Shamis et al. 2010). Formation of the VS involves adhesion molecule interactions (Jolly et al. 2007a; Wang et al. 2009) along with recruitment of HIV-1 receptors and co-receptors (McDonald et al. 2003) to the cellular junction. By being rerouted away from degradation pathways, therefore, HIV-1 is able to avoid destruction and be efficiently transmitted to target CD4+ T-cells.

The 3D architecture of the VS formed between DCs and CD4+ T cells has been visualized using ion abrasion scanning electron microscopy and electron tomography (Felts et al. 2010). These studies reveal that HIV-1 is localized to deep invaginations, or compartments, contiguous with the DC cell surface. Furthermore, two distinct types of contact between the DC and CD4+ T cell at the VS are observed. During the first contact event, sheets of membrane extensions covering the CD4+ T cell provide a secluded region on the cell surface for formation of the VS. The second type of contact occurs within the VS, where filopodial extensions from the CD4+ T cells reach out and penetrate the surface-accessible compartments in which HIV-1 is contained. These observations suggest that transfer of HIV-1 from mDCs to CD4+ T cells occurs, at least in part, via the native antigen capture and presentation capabilities of DCs. These studies also demonstrate that complex interactions between DCs and CD4+ T cells occur at the VS and these cell–cell contacts involve extensive rearrangement of the cellular membranes and cytoskeletal network to allow efficient HIV-1 transmission.

4.1.5 Effects of DC Maturation on Cis- and Trans-infections of HIV-1

Immature DCs and mDCs have differential interactions with HIV-1 with respect to productive virus replication and DC-mediated transmission of HIV-1 to CD4+ T cells (Coleman et al. 2011; Dong et al. 2007; Sanders et al. 2002). It has been proposed that iDCs capture HIV-1 at the mucosal surface and migrate to local lymph nodes, maturing to mDCs in transit, and efficiently transmit HIV-1 to CD4+ T cells in the lymph node, which are the major replication site of HIV-1 (Wu and KewalRamani 2006).

Table 4.1 Relative efficiencies of DC-mediated HIV-1 uptake, viral replication, and transmission of HIV-1 to CD4+ T cells

Maturation stimulus	HIV-1 uptake	Productive HIV-1 infection	Transmission of HIV-1 to target CD4+ T cells	References
None (immature DCs)	+	++	+	Dong et al. (2007) and Wang et al. (2007b)
LPS	++++	–	+++	Dong et al. (2007), Pertel et al. (2011) and Sanders et al. (2002)
PolyI:C	n/a	n/a	++++	Sanders et al. (2002)
IFNα	++	–	+	Coleman et al. (2011) and Pertel et al. (2011)
TNFα	+	++	++	Dong et al. (2007)
CD40L	+	+++	++++	Dong et al. (2007) and Sanders et al. (2002)

In these experiments, immature DCs were treated with the maturation stimuli for 24–48 h and infected with HIV-1 or HIV-1 cell-to-cell transmission assays were performed as described in the noted references. Relative efficiencies of HIV-1 infection and transmission are scored with a "+"; restricted HIV-1 infection is indicated by a "–." n/a = not assessed in current literature. *LPS* lipopolysaccharide, *IFNα* interferon-α, *CD40L* CD40 ligand, *TNF-α* tumor necrosis factor α

Cis- and *trans*-infection mediated by DCs are distinct and dissociable events (Dong et al. 2007). iDCs support productive replication of HIV-1 to a relatively higher level than mDCs (Dong et al. 2007). The effect of DC maturation on the ability of mDCs to support productive HIV-1 replication and to transmit HIV-1 to CD4+ depends upon the maturation stimulus used (Dong et al. 2007; Sanders et al. 2002).

DC-mediated HIV-1 transmission to CD4+ T cells has been modeled in vitro using a range of factors that activate or mature DCs and thereby mimic the normal responses of DCs to PAMPs and cytokines/chemokines. In Table 4.1, a summary of the experiments using a number of different stimuli is presented. LPS is used to model DC stimulation of TLR4 by gram-negative bacteria; Poly I:C is a synthetic RNA analog which mimics the double-stranded RNAs produced during some viral infections and stimulates TLR3; IFNα and tumor necrosis factor α (TNF-α) are innate immune cytokines produced in response to infections; CD40 ligand (CD40L) is a factor released as part of the immune response and binds to CD40 on the cell surface of the DCs to activate the DCs.

4.2 Specific Cellular Factors and Processes That Affect DC-Mediated HIV-1 Transmission

Host cellular molecules have been demonstrated to affect DC-mediated transmission of HIV-1 to CD4+ T cells, including dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN), CD4, and intercellular adhesion

molecules (ICAMs). Viral factors can also affect DC-mediated transmission of HIV-1 to CD4+ T cells, largely by directly interacting with and/or altering the expression of the host cell molecules. As such, the effects of viral factors on DC-mediated transmission of HIV-1 to CD4+ T cells will be described in the context of their effects on the host cell.

HIV-1 replication within DCs, as in any other cell types that are susceptible to HIV-1 infection, can be regulated in a number of ways and therefore affect DC-mediated *cis*-transmission of HIV-1. This chapter does not discuss in detail the range of host factors that are known to promote or restrict HIV-1 replication in DCs; however, some key molecules are presented and are discussed further. Each viral protein expressed as part of the HIV-1 life cycle has a specific role in ensuring productive viral replication and some specific effects of HIV-1 proteins that affect DC-mediated *cis*-transmission will be discussed.

DC-mediated HIV-1 *trans*-infection of CD4+ T cells is largely dependent on cellular factors that are expressed by the DC and interact directly with HIV-1 at initial stages of infection, or during the course of infection.

4.2.1 DC-SIGN

DC-SIGN is a C-type lectin expressed on DCs and functions as an adhesion molecule (van Kooyk and Geijtenbeek 2003). DC-SIGN on DCs is required for the stabilization of the DC:T cell immunological synapse by binding ICAM-3 on the T cells with high affinity (van Gisbergen et al. 2005). DC-SIGN also binds to virion-associated HIV-1 Env (Geijtenbeek et al. 2000a) and DC-SIGN over-expression in DCs can promote HIV-1 entry and infection (Lee et al. 2001). HIV-1 binding to DC-SIGN on the DC surface triggers a signaling cascade that promotes HIV-1 replication in DCs (Gringhuis et al. 2010). These data suggest that DC-SIGN enhances HIV-1 infection of DCs by promoting HIV-1 binding to DCs and through DC-SIGN signaling and promoting *cis*-infection.

DC-SIGN also promotes HIV-1 transmission from DCs to CD4+ T cells (Geijtenbeek et al. 2000a; Gurney et al. 2005) by direct binding of the HIV-1 envelope protein, gp120 (Geijtenbeek et al. 2000a). DC-SIGN also plays a role in the initial internalization of HIV-1 (Kwon et al. 2002), perhaps to protect the virus from degradation or loss into the extracellular milieu prior to VS formation and CD4+ T cell engagement. Taken together, these data suggest that DC-SIGN promotes HIV-1 *trans*-infection by concentrating large amounts of HIV-1 on the cell surface for efficient transmission to CD4+ T cells. Furthermore, DC-SIGN enhancement of HIV-1 transmission is not dependent on the normal interaction of DC-SIGN with ICAM-3 expressed on the CD4+ T cells as DC-mediated HIV-1 transmission cannot be significantly blocked by soluble ICAM-3 or neutralizing antibodies to ICAM-3 (Wu et al. 2002), suggesting that ICAM-3 does not play a role in DC-SIGN-mediated HIV-1 transmission.

DC-SIGN-mediated enhancement of HIV-1 transmission to CD4+ T cells is cell type dependent. In iDCs (Wang et al. 2007b) and in primary activated B lymphocytes (Rappocciolo et al. 2006), blocking DC-SIGN causes a significant impairment of HIV-1 transmission to CD4+ T cells. Furthermore, DC-SIGN over-expression in B cell lines causes significant enhancement of HIV-1 transmission to CD4+ T cells (Wu et al. 2004). Conversely, LPS-matured DCs express lower levels of DC-SIGN than iDCs (Geijtenbeek et al. 2000b) and blocking DC-SIGN on LPS-matured DCs does not significantly affect transmission of HIV-1 to CD4+ T cells (Wang et al. 2007b) and does not promote HIV-1 uptake by mDCs (Izquierdo-Useros et al. 2007). Taken together, these data suggest that the high levels of LPS-matured DC-mediated HIV-1 transmission (Table 4.1) is DC-SIGN independent (Wang et al. 2007b). These data indicate that DC-SIGN plays an important role in iDC-mediated HIV-1 transmission to CD4+ T cells by binding and localizing incoming HIV-1 to a highly concentrated area on the cell surface that can be used for efficient transmission to CD4+ T cells, but does not play a significant role in LPS-matured DC transmission of HIV-1 to CD4+ T cells.

4.2.2 Langerin

Langerhans cells are a specialized DC subset present in mucosal epithelia. In contrast to other DC subtypes, instead of DC-SIGN, langerhans cells express the related C-type lectin receptor, langerin. Langerin efficiently binds HIV-1; however, langerin blocks HIV-1 infection by causing endocytosis of the virion leading to targeted degradation of incoming virions (de Witte et al. 2007). In contrast to DC-SIGN-enhanced HIV-1 transmission, these studies suggest that langerin inhibits DC-mediated HIV-1 transmission.

4.2.3 CD4

CD4 is a cell surface molecule that is highly expressed on a subtype of T cells, on which its main biological function is within the T cell receptor signaling complex. CD4 also acts as the primary receptor of HIV-1 and is expressed at a low level on DCs (Dong et al. 2007; Janas et al. 2008). CD4 expression on DCs inhibits DC-mediated HIV-1 transmission to CD4+ T cells (Wang et al. 2007a). When CD4 is blocked on iDCs using a neutralizing antibody, there is a significant increase in DC-mediated HIV-1 transmission to CD4+ T cells (Wang et al. 2007a). Furthermore, the effect of CD4 can be recapitulated in DC-SIGN expressing Raji B cells. In Raji cells, CD4 over-expression significantly inhibits DC-SIGN-mediated HIV-1 transmission to CD4+ T cells and over-expression of both DC-SIGN and CD4 promotes HIV-1 intracellular retention (Wang et al. 2007a), suggesting that CD4 inhibits DC-SIGN-mediated HIV-1 transmission to CD4+ T cells by causing internalization

of the virion, rather than retention of HIV-1 at the cell surface. Furthermore, DC-SIGN expression significantly increases the affinity of HIV-1 gp140 for CD4 by stabilizing the CD4:gp120 complex (Hijazi et al. 2011), suggesting that, in the presence of high levels of both DC-SIGN and CD4, HIV-1 binding and internalization of HIV-1 are favored over cell-to-cell transmission of HIV-1 to $CD4^+$ T cells. Interestingly, langerin is not able to stabilize the CD4 and gp120 complex (Hijazi et al. 2011), demonstrating that the DC-SIGN:CD4:gp120 complex is specific for DC-SIGN and not a general function of C-type lectins.

4.2.4 ICAMs

ICAMs are cell surface molecules that facilitate contact between cells by binding to integrins. In $CD4^+$ T cells, the major integrin is leukocyte function-associated molecule-1 (LFA-1), which binds to ICAMs expressed on DCs to facilitate cell-to-cell contact during formation of the immunological synapse. A number of ICAM molecules have been investigated for their role in DC-mediated transmission of HIV-1 to $CD4^+$ T cells.

ICAM-1 is significantly upregulated upon maturation of DCs with poly I:C (Sanders et al. 2002) or LPS (Sanders et al. 2002; Wang et al. 2009), correlating with enhanced transmission of HIV-1 to $CD4^+$ T cells (Table 4.1). However, ICAM-1 does not directly enhance HIV-1 infection of DCs (Wang et al. 2009) or $CD4^+$ T cells (Sanders et al. 2002; Wang et al. 2009), indicating that it does not play a role in HIV-1 *cis*-infection of DCs. Blocking of ICAM-1 function on DCs significantly inhibits DC-mediated HIV-1 transmission to $CD4^+$ T cells (Sanders et al. 2002; Wang et al. 2009) and blocking of LFA-1 on $CD4^+$ T cells significantly inhibits LPS-matured DC-mediated HIV-1 transmission to $CD4^+$ T cells (Wang et al. 2009). Conversely, blocking ICAM-1 on $CD4^+$ T cells or LFA-1 on DCs has no significant effect on DC-mediated HIV-1 transmission to $CD4^+$ T cells (Sanders et al. 2002; Wang et al. 2009). Furthermore, the ICAM-1 and LFA-1 interaction that promotes DC-mediated HIV-1 transmission to $CD4^+$ T cells is dependent on cell contact between the DCs and the $CD4^+$ T cells and not on any factor released from the DCs that might indirectly promote HIV-1 replication in the $CD4^+$ T cells (Sanders et al. 2002), suggesting that the ICAM-1 and LFA-1 interaction is vital for stabilization of the VS and promotion of HIV-1 transmission. Finally, ICAM-1 and LFA-1 do not play a significant role in HIV-1 transmission between $CD4^+$ T cells (Puigdomenech et al. 2008), indicating that this interaction is likely specific for DC-mediated transmission of HIV-1 to $CD4^+$ T cells.

ICAM-2 also binds to LFA-1, but ICAM-2 is not highly expressed on monocyte-derived DCs or primary $CD4^+$ T cells and is not upregulated by LPS maturation of DCs (Wang et al. 2009). Blocking ICAM-2 on DCs or $CD4^+$ T cells does not have any significant effect on DC-mediated transmission of HIV-1 to $CD4^+$ T cells (Wang et al. 2009).

ICAM-3 binds strongly to DC-SIGN (Geijtenbeek et al. 2000b) and given the latter's role in DC-mediated transmission of HIV-1 to CD4+ T cells, the role of ICAM-3 has been specifically investigated as a mediator of DC-mediated cell-to-cell transmission of HIV-1. ICAM-3-negative CD4+ T cells show increased replication of HIV-1, suggesting that ICAM-3 plays a role in promoting HIV-1 replication (Biggins et al. 2007) and, therefore, may play a role in HIV-1 *cis*-infection. However, blocking the DC-SIGN interaction with ICAM-3 does not affect HIV-1 cell-to-cell transmission (Wu et al. 2002), nor does blocking of ICAM-3 on DCs and CD4+ T cells have any significant effect on DC-mediated cell-to-cell transmission of HIV-1 (Wang et al. 2009). Taken together with the studies that describe the role of DC-SIGN, these data suggest that the DC-SIGN and ICAM-3 interaction plays no significant role in DC-mediated HIV-1 transmission to CD4+ T cells.

4.2.5 Tetraspanins

Tetraspanins are a broad family of cell-surface proteins, so named because they cross the plasma membrane four times. Tetraspanins facilitate aggregation of proteins into membrane microdomains (Levy and Shoham 2005a) and have multifunctional roles in cell–cell fusion, cell adhesion, cell motility, and formation of immunological signaling complexes (Hemler 2003; Levy and Shoham 2005b).

In LPS-matured DCs, HIV-1 localizes to a surface-accessible (Yu et al. 2008) compartment containing tetraspanins, such as CD9, CD63, CD81, and CD82 (Garcia et al. 2005; Izquierdo-Useros et al. 2007). This compartmentalization of HIV-1 to tetraspanin-rich areas of the membrane is not observed in HIV-1-infected iDCs (Izquierdo-Useros et al. 2007). This suggests a correlation between DC-mediated transmission of HIV-1 to CD4+ T cells and HIV-1 localization to tetraspanin-containing compartments on the cell surface. However, the specific role of each of these molecules in DC-mediated HIV-1 transmission to CD4+ T cells has not been extensively investigated. It appears most likely that the localization of incoming HIV-1 to a tetraspanin-rich area of the host cell membrane is a consequence of DC maturation. Furthermore, tetraspanins may serve as markers for the subcellular localization and trafficking of HIV-1, rather than possessing a functional role in DC-mediated HIV-1 transmission to CD4+ T cells (Garcia et al. 2005; Izquierdo-Useros et al. 2009).

A study using HeLa cells as donor cells to transmit HIV-1 to the CD4+ CEM T cell line has suggested that CD9 and CD63 promote HIV-1 cell-to-cell transmission and CD81 inhibits HIV-1 cell-to-cell transmission without affecting the infectivity of the cell-free virus (Krementsov et al. 2009). However, tetraspanin-mediated HIV-1 transmission to CD4+ T cells has not been demonstrated in DCs. The role of each of these tetraspanins in DC-mediated HIV-1 transmission to CD4+ T cells remains to be investigated.

4.2.6 TLRs

DCs express pathogen-pattern-recognition receptors including TLRs. It has been shown that HIV-1 infection of DCs causes upregulation of TLRs in DCs (Hernandez et al. 2011) and that HIV-1 induces TLR signaling pathways in DCs to promote replication and cell-to-cell transmission (Gringhuis et al. 2010).

HIV-1 infection causes upregulation of TLR2 and TLR4 in DCs and monocytes from individuals co-infected with *Mycobacterium tuberculosis* and HIV-1 (Hernandez et al. 2011). There is a positive correlation between expression of TLR2 and TLR4 and an elevated HIV-1 viral load in these individuals, which suggests that upregulation of these TLRs may enhance HIV-1 replication and/or transmission (Hernandez et al. 2011). However, the direct effects of HIV-1 interactions with TLR2 and TLR4 on HIV-1 replication and DC-mediated cell-to-cell transmission have not been demonstrated. However, LPS-maturation of DCs via TLR4 does promote DC-mediated HIV-1 transmission (Dong et al. 2007; Sanders et al. 2002). It is possible that high levels of TLR4 may render DCs more susceptible to LPS maturation and, therefore, promote DC-mediated HIV-1 transmission to $CD4^+$ T cells. Furthermore, HIV-1 coinfection with *M. tuberculosis* or *Candida albicans*, which stimulate TLR2 homo- or heterodimers, enhances HIV-1 replication in a manner dependent on the cellular kinase raf-1, via phosphorylation of the p65 subunit of NF-κB (Gringhuis et al. 2010), suggesting that TLR2 signaling promotes HIV-1 infection of DCs and DC-mediated transmission of HIV-1. Interestingly, when DCs are treated with the fungus *Penicillium marneffei*, DC-mediated HIV-1 transmission to $CD4^+$ T cells is increased and HIV-1 infection of the DCs is blocked (Qin et al. 2011). Fungal PAMPs are recognized by TLR2 dimers; therefore it is possible that TLR2 stimulation can activate DCs to promote DC-mediated HIV-1 transmission, either by direct signaling or by upregulation of molecules that promote DC-mediated HIV-1 transmission; for example, ICAM-1 upregulation was observed in *P. marneffei*-treated DCs (Qin et al. 2011).

HIV-1 RNA selectively triggers TLR8 signaling, allowing initiation of transcription of HIV-1 genes via NF-κB activation, as knockdown of TLR8 in DCs abrogates HIV-1 transcription and causes abortive transcription of HIV-1 proviral DNA (Gringhuis et al. 2010). For transcriptional elongation to occur, a second signaling pathway is required: binding of HIV-1 gp120 to DC-SIGN activates Raf-1-dependent phosphorylation of the NF-κB p65 subunit. This allows elongation of HIV-1 transcripts, resulting in the production of full-length transcripts and productive HIV-1 infection in DCs and subsequent transmission to $CD4^+$ T cells (Gringhuis et al. 2010). Previous studies suggested that the endocytosis of HIV-1 by DCs and subsequent trafficking through the endosomal pathway target HIV-1 for lysosomal degradation (Yu et al. 2008). By contrast, the study by Gringhuis et al. demonstrated that HIV-1 can use the endosomal route to allow initiation of transcription and ensure active replication, via a mechanism that uses the innate TLR8 and Raf-1 signaling pathways in DCs (Gringhuis et al. 2010). Taken together with the observation that endosomal trafficking does not promote DC-mediated *trans*-infection (Yu et al. 2008),

these data suggest that TLR8 signaling, induced by HIV-1 RNA in the endosome, promotes HIV-1 *cis*-infection, but not *trans*-infection.

4.2.7 Glycosphingolipids

The role of host cell-derived glycosphingolipids (GSLs) incorporated into HIV-1 particles in DC-mediated *trans*-infection has been assessed in both iDCs and mDCs. A novel mechanism has been defined, in which DCs capture HIV-1 independently of the envelope glycoprotein, gp120 (Hatch et al. 2009). Blocking of the GSL biosynthesis pathways using specific inhibitors suggests that host cell-derived GSLs are incorporated into HIV-1 virions and play a key role in HIV-1 interactions with DCs. Furthermore, depletion of the host cell GSLs results in HIV-1 particles that are deficient in GSLs and consequently cannot be efficiently captured or transmitted to $CD4^+$ T cells by the DCs (Hatch et al. 2009). The specific roles of GSL in DC-mediated HIV-1 transmission and interactions will be discussed in more detail in Chap. 6.

4.2.8 The Exosome Secretion Pathway

Exosomes are cellular vesicles that are released from cells for transmission of signaling molecules between cells. In iDCs, there is evidence that the capture of HIV-1 and transmission to $CD4^+$ T cells occur through a cell contact-free transmission event, involving a process called exocytosis (Wiley and Gummuluru 2006). After endocytosis of HIV-1, virus particles are localized to tetraspanin-rich compartments (Garcia et al. 2005; Izquierdo-Useros et al. 2007), postulated to be multi-vesicular bodies (MVBs). From the MVBs, HIV-1 particles are targeted directly to the exosome secretion pathway for release into the extracellular milieu in association with the exosomes (Wiley and Gummuluru 2006). It was hypothesized that HIV-1 uses an internal DC trafficking pathway, wherein fusion between the HIV-1 containing MVB and the intracellular plasma membrane enables HIV-1 particles to escape lysosomal degradation. The fully infectious HIV-1 released from the DCs was confirmed to be a consequence of the release of intact, previously endocytosed HIV-1 virions, and not the result of productive replication within the DCs (Wiley and Gummuluru 2006). But, the infectivity of the HIV-1 released in association with exosomes to $CD4^+$ T cells is lower than that observed in DC:$CD4^+$ T cell cocultures (Wiley and Gummuluru 2006), indicating that the exosome secretion pathway is not as efficient at transmitting HIV-1 from DCs to $CD4^+$ T cells as VS-dependent DC-mediated HIV-1 transmission to $CD4^+$ T cells. Thus, the exosome secretion pathway may play a role as an alternative transmission route that adds to the overall DC-mediated HIV-1 transmission to $CD4^+$ T cells. The transmission of HIV-1 via exosomes is

the first demonstration of a cell contact-free DC-mediated *trans*-infection without de novo viral replication (Wiley and Gummuluru 2006), making exosome-mediated transmission distinct from *cis*-infection of HIV-1.

4.2.9 Cytoskeleton-Dependent Macromolecular Movement

One of the mechanisms that retroviruses have evolved to circumvent host cell obstacles to productive replication, such as lysosomal degradation, is cytoskeleton-dependent macromolecular movement within cells (Fackler and Krausslich 2006; Naghavi and Goff 2007). HIV-1, in particular, is adept at using the host cell cytoskeleton to promote its own replication and cell-to-cell transmission (Lehmann et al. 2011). Numerous studies utilizing a wide variety of techniques have established roles for components of the cytoskeleton in the entry, trafficking, and DC-mediated HIV-1 transmission to $CD4^+$ T-cells, including actin, microtubules, and membrane extensions (Fahrbach et al. 2007; Garcia et al. 2005; Izquierdo-Useros et al. 2007; McDonald et al. 2003; Trumpfheller et al. 2003; Turville et al. 2004; Wang et al. 2007b; Wiley and Gummuluru 2006). Transmission of HIV-1 between $CD4^+$ T cells is an actin-dependent process. Actin allows concentration of HIV-1 at the VS to promote efficient cell-to-cell transmission (Jolly et al. 2007b) and actin-rich membrane extensions confer up to 90% of HIV-1 transmission from iDCs to $CD4^+$ T cells (Nikolic et al. 2011). However, it is unknown if HIV-1 exploits a similar pathway to ensure that the virus is concentrated at the mDC:$CD4^+$ T cell junction during mDC-mediated HIV-1 transmission to $CD4^+$ T cells.

Both HIV-1 and SIV can be endocytosed into DCs through a variety of mechanisms, including clathrin-mediated endocytosis (Frank et al. 2002; Garcia et al. 2005; Wang et al. 2007b) and receptor-mediated endocytosis (de Witte et al. 2007; Kwon et al. 2002). Furthermore, macropinocytosis, an actin-dependent form of nonselective endocytosis, partially contributes to endocytosis of HIV-1 by LPS-matured DCs. Disruption of both macropinocytosis and cytoskeleton, using specific inhibitors, alters HIV-1 trafficking and inhibits DC-mediated HIV-1 transmission to $CD4^+$ T cells (Wang et al. 2008). DC-mediated HIV-1 transmission to $CD4^+$ T cells is inhibited because of impaired formation of the VS (Wang et al. 2008), indicating that HIV-1 exploits macropinocytosis and the cytoskeletal network to promote formation of the VS, allowing efficient HIV-1 transmission and dissemination.

Although studies indicate the importance of the cytoskeletal network in DC-mediated transmission of HIV-1 to $CD4^+$ T cells, it is also evident that some components of the cytoskeletal network function to prevent HIV-1 from successfully trafficking and transmitting to $CD4^+$ T cells. Leukocyte-specific protein (LSP)-1 is a cellular F-actin-binding protein localized on the plasma membrane. LSP-1 specifically interacts with C-type lectins, such as DC-SIGN, and targets HIV-1 particles to the proteasome for degradation. Knockdown of LSP-1 in DCs enhances transmission of HIV-1 to $CD4^+$ T-cells (Smith et al. 2007), indicating that

LSP-1 is responsible for targeting the incoming HIV-1 particles for degradation and, therefore, inhibits the trafficking of the incoming virus towards the VS.

4.2.10 Autophagy

Autophagy is a process of cellular self-digestion in which proteins and organelles are degraded. HIV-1 is able to inhibit autophagy to promote viral replication and DC-mediated *trans*-infection (Blanchet et al. 2010). Autophagy also plays an important role in the regulation of innate and adaptive immune responses to intracellular pathogens (reviewed in Levine and Deretic 2007). It has been shown that HIV-1-mediated inhibition of autophagy impairs the innate and adaptive immune functions of DCs (Blanchet et al. 2010). The details of the role of autophagy in HIV-1 interactions with DCs will be discussed in detail in Chap. 10.

4.3 HIV-1 Proteins That Affect DC-Mediated Cell-to-Cell Transmission of HIV-1

4.3.1 HIV-1 Env Glycosylation Affects DC-SIGN-Meditated HIV-1 Transmission to CD4⁺ T Cells

Glycosylation of Env plays a key role in ensuring efficient DC-mediated HIV-1 transmission to CD4⁺ T cells. HIV-1 Env is heavily glycosylated, with *N*-linked glycans contributing to almost half of the molecular mass of Env (van Montfort et al. 2011). *N*-Linked glycans are a family of carbohydrate moieties that are added to the nitrogen atoms in the side chain of asparagine amino acids in proteins (Schwarz and Aebi 2011). The *N*-linked glycan composition of Env is heterogeneous, with complex carbohydrate moieties present throughout the Env protein (van Montfort et al. 2011). *N*-Linked glycans on Env are important for correct Env folding (Walker et al. 1987), incorporation of Env into virions (Walker et al. 1987), and HIV-1 immune evasion (Reitter et al. 1998; Sanders et al. 2008).

The direct binding of Env to DC-SIGN is dependent on the carbohydrate moieties in Env (Hong et al. 2002) and this interaction has been mapped to specific *N*-glycans within the 2G12 epitope of Env (Hong et al. 2007). Interestingly, the specific *N*-glycan composition of the Env glycoprotein promotes interactions between Env and DC-SIGN and specifically promotes DC-mediated transmission of HIV-1 to CD4⁺ T cells (van Montfort et al. 2011). In fact, the Env protein requires a mix of *N*-glycan residues, as changing the glycans to universal oligomannose *N*-glycan promotes DC-SIGN binding but reduces DC-mediated HIV-1 transmission to CD4⁺ T cells, as the oligomannose residues on Env promote HIV-1 endocytosis and degradation (van Montfort et al. 2011).

4.3.2 HIV-1 Nef Promotes DC-Mediated HIV-1 Transmission to CD4+ T Cells

HIV-1 Nef is a multifunctional accessory protein encoded by HIV-1. It has a range of functions and contributes to HIV-1 pathogenesis in vivo, including modulation of immune evasion (Kirchhoff 2010) and HIV-1 persistence (Arhel and Kirchhoff 2009). Nef interacts with a range of host cell molecules to achieve its diverse effects on HIV-1 pathogenesis (Foster and Garcia 2008).

HIV-1 Nef protein expression causes down-regulation of the main HIV-1 receptors, CD4 and CCR5, on the surface of infected cells (Michel et al. 2005; Wang et al. 2007a). Thus, Nef protein promotes *cis*-infection of HIV-1 by preventing superinfection of the initially infected cell and forcing HIV-1 to infect other cells. Nef also promotes HIV-1 replication in DC and lymphocyte cocultures (Petit et al. 2001) and CD4 inhibits DC-mediated HIV-1 transmission to CD4+ T cells (Wang et al. 2007a); therefore, HIV-1 Nef protein may promote *trans*-infection mediated by DCs by down-regulation of CD4 expression.

HIV-1 Nef expression in iDCs promotes clustering of DCs and CD4+ T cells, DC maturation, and CD4+ T cell activation (Messmer et al. 2002a; Messmer et al. 2002b; Sol-Foulon et al. 2002). HIV-1 Nef causes up-regulation of DC-SIGN in HIV-1-infected DCs (Sol-Foulon et al. 2002) and over-expression of HIV-1 Nef protein into HeLa cells causes up-regulation of surface DC-SIGN expression, by preventing DC-SIGN endocytosis (Sol-Foulon et al. 2002). Nef-upregulated DC-SIGN expression promotes HIV-1 transmission from HeLa cells to cocultured lymphocytes (Sol-Foulon et al. 2002), but DC-SIGN may play only a partial role in DC-mediated HIV-1 transmission to CD4+ T cells (Boggiano et al. 2007; Gurney et al. 2005; Wu et al. 2002). HIV-1 Nef expression in the context of virus infection promotes DC-mediated transmission of HIV-1 to CD4+ T cells, which correlates with decreased CD4 expression and only modest increases in DC-SIGN expression (St Gelais et al. 2012). Therefore, Nef-dependent CD4 downregulation and Nef-dependent clustering of HIV-1-bearing DCs and uninfected target CD4+ T cells via the DC-SIGN:ICAM-3 interaction may both promote highly efficient HIV-1 transmission CD4+ T cells. Furthermore, Nef expression in CD4+ T cells causes increased CD4+ T cell activation and proliferation, which correlates with enhanced HIV-1 replication (St Gelais et al. 2012), suggesting that Nef is important for promoting HIV-1 replication in CD4+ T cells after DC-mediated transmission has occurred.

4.3.3 Viral Factors That Affect the Host Cell Cytoskeletal Network

HIV-1 binding to DC-SIGN on DCs can promote HIV-1 transmission to CD4+ T cells by promoting formation of the VS. It has been suggested that HIV-1 can hijack the cytoskeletal network to enhance its transmission to CD4+ T cells (Arrighi et al.

2004; Wang et al. 2009). Recent studies have demonstrated that binding of DC-SIGN by HIV-1 activates Rho-GTPases and other signaling molecules (Nikolic et al. 2011). It is suggested that HIV-1 induces activation of C-type lectin receptors and affects cytoskeletal remodeling, in particular through activation of Rho-GTPases (Nikolic et al. 2011), which can affect a range of DC functions including cell migration, trafficking, and cell polarity (Heasman and Ridley 2008).

A recent study indicated that HIV-1 is capable of triggering the formation of actin-based protrusions at the surface of iDCs (Nikolic et al. 2011). It was demonstrated that transmission from iDCs to $CD4^+$ T cells occurs in a two-step transfer process in which HIV-1 is bound and concentrated at the VS and then transferred to $CD4^+$ T cells in a Cdc42-dependent manner. Cdc42 is a small GTPase of the Rho-subfamily which acts as a signaling molecule with a range of functions in the cell, including regulation of the cell cycle, cell migration, and endocytosis (Heasman and Ridley 2008). The Cdc42-dependent transmission process is dependent on Env binding to DC-SIGN, causing subsequent activation of Cdc42 (Nikolic et al. 2011). When Cdc42 function is blocked in iDCs, there is a significant decrease in the amount of iDC-mediated HIV-1 transmission to $CD4^+$ T cells (Nikolic et al. 2011). The number of VSs formed and the concentration of HIV-1 at the zone of contact between cells are not affected by blocking Cdc42 activation, indicating that polarization of HIV-1 at the contact point occurs independently of Cdc42 (Nikolic et al. 2011). Furthermore, HIV-1 Env engagement of DC-SIGN at the cell surface leads to Cdc42 activation and other signaling molecules that have been previously implicated in membrane extension formation or DC-SIGN signaling cascades in DCs; Pak1, Wasp, and Src kinases are activated in the presence of HIV-1 Env (Nikolic et al. 2011). These data demonstrate that HIV-1 Env binding to DC-SIGN triggers signaling that results in activation of Cdc42 in iDCs. Efficient DC-mediated HIV-1 transmission to $CD4^+$ T cells occurs in the presence of activated Cdc42, but Cdc42 signaling is not required for the physical formation of the VS between the DCs and $CD4^+$ T cells.

4.4 Conclusions and Future Directions

DCs are, potentially, one of the important cell types in the early transmission of HIV-1 to $CD4^+$ T cells. The cellular and viral factors that affect the process of early-stage HIV-1 transmission have been extensively studied and characterized. Many cell surface molecules affect DC-mediated HIV-1 transmission to $CD4^+$ T cells, but none of these molecules appear to be exclusively responsible for differential transmission between different DC subsets. For example, both DC-SIGN-dependent and independent transmission can occur in DCs.

HIV-1 exploits normal DC cellular process, such as macropinocytosis, and natural interactions with $CD4^+$ T cells, such as ICAM-1 binding to LFA-1, to promote viral infection and cell-to-cell transmission. HIV-1 expresses proteins which promote the process of DC-mediated HIV-1 transmission to $CD4^+$ T cells. In particular,

the multifunctional pathogenic accessory protein Nef plays a key role in promoting DC-mediated HIV-1 transmission to $CD4^+$ T cells by activating $CD4^+$ T cells and modulating DC interactions with T cells. Overall, the cellular and viral factors that promote DC-mediated HIV-1 transmission to $CD4^+$ T cells ensure efficient HIV-1 transmission and spread within the host and establishment of long-term infection.

Future work will involve further characterizations of cellular and viral factors that regulate DC-mediated HIV-1 transmission to $CD4^+$ T cells, particularly those that act specifically in different DC subsets. A major area of study is the characterization of the physical interaction that occurs at the VS that promotes DC-mediated HIV-1 transmission to $CD4^+$ T cells, for example, how $CD4^+$ T cells efficiently retrieve HIV-1 from the deep invagination on the DC cell surface. These studies will help to better understand the mechanisms underlying HIV-1 cell-to-cell transmission.

Cellular and viral factors that affect DC-mediated transmission of HIV-1 to $CD4^+$ T cells are also, potentially, important targets for therapeutic strategies, such as drugs that specifically target the interactions that promote formation of the VS and/or strategies to target the HIV-1 pool associated with DCs. Drugs that target the initial interactions that occur between HIV-1 and DCs have the potential as topical treatments at the mucosal surfaces to prevent the initial DC-mediated HIV-1 transmission events that lead to establishment of persistent infection.

Acknowledgements We thank members of the Wu laboratory for helpful discussions and Tim Vojt for illustration. The research in the Wu laboratory was supported by grants (AI068493, AI078762, and AI098524) to L.W. from the National Institutes of Health and by the program of Public Health Preparedness for Infectious Diseases of The Ohio State University. The authors apologize to all whose work has not been cited as a result of space limitations.

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Chapter 5
Role of Glycosphingolipids in Dendritic Cell-Mediated HIV-1 *Trans*-infection

Wendy Blay Puryear and Suryaram Gummuluru

Abstract Glycosphingolipids (GSLs) are components of the cell membrane that comprise a membrane bound lipid, ceramide, coupled to an extracellular carbohydrate. GSLs impact numerous aspects of membrane biology, including membrane fluidity, curvature, and organization. The role of these molecules in both chronic inflammation and infectious disease and underlying pathogenic mechanisms are just starting to be recognized. As a component of the cell membrane, GSLs are also incorporated into lipid bilayers of diverse enveloped viruses as they bud out from the host cell and can go on to have a significant influence on viral pathogenesis. Dendritic cell (DC) subsets located in the peripheral mucosal tissues are proposed to be one of the earliest cell types that encounter transmitted viruses and help initiate adaptive immune responses against the invading pathogen by interacting with T cells. In turn, viruses, as obligatory intracellular parasites, rely on host cells for completing their replication cycle, and not surprisingly, HIV has evolved to exploit DC biology for the initial transmission event as well as for its dissemination and propagation within the infected host. In this review, we describe the mechanisms by which GSLs impact DC-mediated HIV *trans*-infection by either modulating virus infectivity, serving as a direct virus particle-associated host-derived ligand for specific interactions with DCs, or modulating the T cell membrane in such a way as to impact viral entry and thereby productive infection of $CD4^+$ T cells.

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_5,

5.1 Introduction

Dendritic cells (DCs) play a sentinel role in the peripheral mucosal tissues, patrolling their immediate environment for foreign pathogens. Following pathogen uptake and sensing, DCs become activated, migrate to the draining lymph nodes, and process and present antigen in complex with major histocompatibility complex (MHC) Class I and II molecules to T cells that display cognate receptors specific for the antigen–MHC complexes displayed on the DC surface (Banchereau and Steinman 1998). Thus, DCs are specialized antigen-presenting cells that effectively link the innate and adaptive immune responses (Banchereau and Steinman 1998). Human DCs in the blood can be broadly divided into myeloid DCs (mDCs) or plasmacytoid dendritic cells (pDCs) (Geissmann et al. 2010), and both mDCs and pDCs specialize in the detection of virus infections and can function in innate and adaptive immunity. While pDCs are the primary type I interferon-producing cells upon viral infection (Liu 2005), mDCs have evolved primarily to prime and activate antiviral T cells (Banchereau and Steinman 1998). Because of the ease of availability either from human peripheral blood or generation in vitro from peripheral blood monocyte precursors (Sallusto and Lanzavecchia 1994), most of the work in the literature has involved study of pathogen interactions with mDCs or peripheral blood monocyte-derived DCs. Though peripheral blood monocytes are not the precursors to mDCs, monocytes can exit the blood and enter peripheral mucosal tissues upon pathogen infection, and give rise to inflammatory DCs that share many of the phenotypic and functional features of mDCs (Randolph et al. 1998). In this review, we focus our attentions primarily on HIV-1 interactions with peripheral blood monocyte-derived DCs.

DCs express a wide variety of innate receptors that recognize common pathogen-associated molecular patterns (PAMPs), a recognition that is essential for the induction of proinflammatory genes, DC migration and maturation, and activation of the adaptive immune response (Medzhitov and Janeway 1997). Since viruses are obligate human parasites that require host cell machinery to complete their life cycle, they have evolved diverse immune evasion strategies that interfere with these intrinsic effector mechanisms in DCs. To initiate infection, HIV must traverse the plasma membrane to gain access to the host cell cytoplasm. While HIV entry occurs predominantly at the plasma membrane upon sequential interactions with the receptor, CD4, and coreceptor, CCR5 or CXCR4 (Doms and Trono 2000), some recent studies have argued for receptor-dependent endocytosis of intact virus particles and fusion with endosomal membranes as an alternative entry mechanism (Miyauchi et al. 2009). Endocytosis offers invading virus particles a number of benefits, including a convenient and rapid transport across the plasma membrane and escape from cell-surface expressed pattern-recognition receptors such as the C-type lectin receptors (CLRs) (Figdor et al. 2002). However, the endocytic pathway is also fraught with danger for the incoming virion. Trafficking of virus-containing vesicular compartments to a low-pH late endosome/lysosome might lead to the degradation of

viral capsids, exposure of viral nucleic acids, and initiation of IFN-dependent antiviral responses by the endosomal resident nucleic acid-sensing toll-like receptor (TLR) family members (Pichlmair and Reis e Sousa 2007). Alternatively, fusion of virus with DCs results in the delivery of virus core into the DC cytoplasm, a hostile environment equipped with a battery of intracellular viral nucleic acid-sensing RIG-I like receptors (RLRs), Nod-like receptors (NLRs), and, in the case of HIV, intrinsic cellular restriction factors that determine the course of viral pathogenesis in the infected host. How HIV navigates and exploits DCs for viral dissemination is a question under active study and of significant research interest. In this review, we discuss the role of both cell-associated glycosphingolipids (GSLs) and GSLs incorporated into HIV-1 particle membranes in mediating virus interactions with DCs and facilitating HIV evasion and dissemination via DC-mediated *trans*-infection pathways.

5.2 Glycosphingolipids

5.2.1 Glycosphingolipids Are an Integral Component of the Cellular Membrane

GSLs are part of a larger family of membrane-associated ceramide sphingolipids, defined simply as a sphingosine coupled to a fatty acid. The ceramide backbone is then modified to become sphingomyelin (SM), sulfatides, or glucosylceramide (GlcCer) (reviewed in (Zeidan and Hannun 2007; Rawat et al. 2005)). SM is one of the major membrane phospholipids that, with cholesterol, contributes to the structure and curvature of the membrane (Varki et al. 2008). GlcCer ultimately gives rise to the more complex GSLs, which are further broken down into gangliosides (defined as having a terminal sialic acid), asialo-gangliosides, and globosides. There are a large number of GSL variants, with gangliosides alone having over 40 known variants (Varki et al. 2008). Of the gangliosides, the monosialogangliosides GM3 and GM1 have been frequently studied, while the asialo-ganglioside, asialo-GM1 (the non-sialylated equivalent of GM1), and globoside globotriaosylceramide (Gb3) are frequently noted as examples of their respective subfamilies. Although GSLs only make up approximately 5% of the overall membrane lipid composition, they are highly enriched in membrane rafts (Varki et al. 2008; Bollinger et al. 2005). As HIV is known to bud from membrane rafts (Nguyen and Hildreth 2000; Ono and Freed 2001), this results in an enrichment of GSLs within the HIV particle and can have significant consequences on viral function. Likewise, many of the cellular binding partners that have been described for HIV reside in membrane rafts and are therefore also subjected to the influence of GSLs enriched within these domains. GSLs are therefore poised to make significant contributions to HIV pathogenesis by impacting the virus particle directly, and by impacting the target cell.

5.2.2 *Glycosphingolipid Expression Profiles and Variability in Membrane Distribution Within a Cell*

There is a high degree of complexity and several nuances to the cellular distribution and expression profiles of individual GSLs (Zeidan and Hannun 2007; Alberts et al. 2002). For example, gangliosides are typically expressed at high levels in the brain while globosides are preferentially found on erythrocytes (Varki et al. 2008). Closer analyses of various disease states have elucidated further cellular associations. The ganglioside GM1 is abundant in intestinal epithelial cells, where it can be exploited by cholera toxin B (Alberts et al. 2002), while Gb3 is widely expressed on vascular endothelial cells and can be recognized by *Escherichia coli* verotoxin (Okuda et al. 2006). Burkitt's lymphoma cells have been found to express high levels of the globoside Gb3 (Nudelman et al. 1983) and a number of childhood neurodegenerative diseases are characterized by GSL abnormalities (reviewed in (Xu et al. 2010)). However, it is the cellular distribution of GSLs within leukocytes, or the "immune cell glycomes" (Haslam et al. 2008), that are particularly informative to our ongoing understanding of HIV pathogenesis. Gb3 can serve as a binding partner for HIV glycoprotein but is only found in macrophages and not T cells (Hammache et al. 1999; Ramegowda and Tesh 1996). Although both macrophages and activated $CD4^+$ T cells have high levels of GM3 (Hammache et al. 1999), it is found in higher levels within macrophages than within T cells (Chan et al. 2008). These variations in cellular distribution could potentially impact the tropism and mechanism of action of pathogens such as HIV. It is interesting to note that Gb3, enriched on macrophages, has a strong preference to bind CXCR4 using viruses, while GM3, enriched on T cells, preferentially binds CCR5 using variants (Nehete et al. 2002).

Dendritic cells also show differences in their glycome profile upon maturation. Maturation of DCs upregulates expression of varied glycosyltransferases, having broad effects on glycan structures, thereby impacting the profile of the DC-associated glycosphingolipidome (Haslam et al. 2008). Expression of ST3Gal1, a sialyltransferase, is upregulated upon DC maturation, resulting in increased expression of globosides and gangliosides. Similarly, bone marrow-derived murine DCs have been shown upon maturation to increase surface expression of globosides, while ganglioside levels are unchanged (Li et al. 2009). These differences in DC GSL composition are particularly interesting to note in light of the differences seen in how HIV interacts with an immature and mature DC (Izquierdo-Useros et al. 2010; Wu and KewalRamani 2006). Though maturation of DCs results in a global decrease in macropinocytosis and fluid-phase uptake (Austyn 1998), there is a dramatic enhancement of HIV-1 capture and enhanced transfer of captured virus particles to T cells, facilitated presumably by a maturation-dependent upregulation of co-stimulatory and adhesion molecules on the DC surface (Dong et al. 2007; Fahrbach et al. 2007; Hatch et al. 2009; Izquierdo-Useros et al. 2007, 2009; McDonald et al. 2003; Wang et al. 2007; Weissman et al. 1995). Similar to the effects observed with mature peripheral blood monocyte-derived DCs, HIV-1 binding and capture by activated Langerhans cells derived from cord blood $CD34^+$ stem cells (Fahrbach et al. 2007), vaginal epithelial sheets (Hladik et al. 2007), or human skin explants (de Jong et al.

2008) were also enhanced upon maturation. Whether differences in GSL composition upon DC maturation, and specifically enhancement in cell surface expression of globosides and gangliosides, can impact the mechanism of HIV-1 capture and *trans*-infection by DCs remains to be determined.

In addition to cell type differences in GSLs, cell-intrinsic GSL expression levels can vary based on cell cycle and cell activation status (Hakomori 1990). For example, control of the cell surface expression level of gangliosides is a finely tuned process, and the Golgi-resident enzyme, GM3 synthase, also named ST3Gal-V or Sial-T1, plays a key regulatory role (Uemura et al. 2009). GM3 synthase catalyzes the transfer of a sialic acid residue to the terminal galactose of lactosylceramide, resulting in the synthesis of the ganglioside, GM3, the common precursor to nearly all of the cellular gangliosides (Kolter et al. 2002). In agreement with early observations that GM3 levels increase upon macrophage-like cell differentiation (Nojiri et al. 1986), the expression of GM3 synthase is dramatically upregulated upon monocyte differentiation into macrophages (Gracheva et al. 2007). TNF-α and other proinflammatory mediators are also associated with increased GM3 synthase gene transcription and expression levels (Tagami et al. 2002; Blander et al. 1999). GSLs are upregulated upon T cell activation, and Gb3 synthesis is induced in PHA/IL2-activated PBMCs (Lund et al. 2006). Interestingly, viral infection has also been demonstrated to impact cellular GSL levels. Both GM1 and asialo-GM1 are upregulated upon infection with respiratory syncytial virus (Moore et al. 2008), while peripheral blood mononuclear cells upregulate GM3 and Gb3 upon HIV infection (Fantini et al. 2000). The inducible modulation of GSL cell content, especially under proinflammatory conditions, has the potential to significantly impact how HIV interacts with the host cell while also influencing the composition of the cellular membrane from which de novo virions will bud.

In addition to intercellular heterogeneity in GSL expression, there is considerable heterogeneity in the intracellular localization of GSLs. Such subtleties in intracellular distribution can potentially impact the nature of the specific GSLs that are ultimately incorporated into a budding virion. Although there is a much higher concentration of GSLs at the plasma membrane than throughout the cell, intracellular membrane compartments do contain GSLs (Schwarz and Futerman 1997). Subcellular compartmentalization of sphingolipid biosynthetic enzymes along the *trans*-Golgi network and directional vesicular transport amongst organelle members of the endomembrane system can result in varied intracytoplasmic membrane concentrations of GSLs (D'Angelo et al. 2007, 2012; Yamaji et al. 2008). Within the cell, there are often higher levels of the ganglioside GM1 in the endoplasmic reticulum, while the *trans*-golgi network possesses higher levels of the ganglioside GM3 (D'Angelo et al. 2012; Yamaji et al. 2008). In some cases, such as motile T cells, distribution of GM1 and GM3 gangliosides can be polarized in response to chemotactic signals (Gomez-Mouton et al. 2001). While a stationary T cell shows relatively uniform distribution of the gangliosides GM1 and GM3, polarization of the cell results in a dramatic segregation of the two molecules, with GM3 redistributing to the leading edge, while GM1 becomes localized to the trailing uropod (Gomez-Mouton et al. 2001).

In the absence of such dramatic polarization, GSLs can also be found segregated into distinct membrane raft domains, sometimes at different perpendicular depths

within an otherwise laterally equivalent region of membrane, although the precise nature of localization remains unclear. In Madin–Darby canine kidney epithelial cells, nanoscale topographic imaging was used to show that GM3 localizes to the peaks of microvillus-like membrane protrusions, while GM1 localizes to the valleys between the protrusions (Chen et al. 2008). However, confocal microscopy of similar membrane structures suggested the opposite distribution, with GM1 located in the microvilli and GM3 residing in the valleys (Janich and Corbeil 2007). Despite these conflicting observations, both lines of evidence demonstrate that GM1 and GM3 reside in separate membrane domains. Though assembly and budding from GSL-enriched lipid rafts is a feature that is well conserved amongst diverse non-segmented RNA virus families, including members of the orthomyxovirus and filovirus families (Suomalainen 2002), this segregation of unique GSL-containing plasma membrane microdomain sites may also help to explain the selective partitioning of virus assembly sites when a single cell is co-infected with two distinct "raftophillic" enveloped viruses, such as HIV-1 and Ebola (Leung et al. 2008) or HIV-1 and influenza (Khurana et al. 2007). In both instances, the HIV-1 glycoprotein is found to localize in membrane domains distinct from those containing Ebola GP or influenza HA glycoproteins.

5.3 The Role of Glycosphingolipids in Cellular Functions and Viral Pathogenesis

A growing body of research is steadily unveiling the requirement of GSLs in a breadth of cellular functions (reviewed in (Xu et al. 2010)). In broad terms, GSLs can act as cellular signaling molecules and contribute to membrane architecture, organization, fluidity, and rearrangements (Hakomori 1990; Hakomori et al. 1998). One mechanism by which GSLs exert these functions is through membrane rafts. Rafts are fluid membrane microdomains, typically 10–50 nm in diameter (Varki et al. 2008), that comprise cholesterol, sphingomyelin, and GSLs. These domains are believed to play a key role in membrane organization and are found to be enriched in specific types of molecules such as tetraspanins and GPI-anchored proteins (Simons and Gerl 2010). Membrane rafts have been shown to play a central role in receptor recruitment, rearrangement, and clustering (Nguyen et al. 2005), and raft-associated GSLs have been well established to act as cell surface signaling molecules that trigger membrane rearrangements (Nguyen et al. 2005; Hakomori and Handa 2002). This is highly relevant to HIV interactions with the target cell and is in part demonstrated by the role of GSLs in the recruitment and clustering of HIV receptors CD4 and CCR5 (Hug et al. 2000). While this review focuses on HIV, it is worth noting that several different viruses have been documented to exploit GSLs to also gain entry into the host cell. A range of viruses including members from orthomyxovirus (Suzuki et al. 1985), rhinovirus (Grassme et al. 2005), paramyxovirus (Cooling et al. 1995; Epand et al. 1995), and polyomavirus (Tsai et al. 2003; Haslam et al. 2008) virus families have all been described to use GSLs as attachment and entry factors.

5.3.1 HIV Buds from Lipid Rafts and Incorporates GM3

In addition to roles in virus entry, membrane rafts play a crucial role in HIV-1 assembly and release (Nguyen and Hildreth 2000; Ono and Freed 2001, 2005). Assembly of HIV-1 particles within lipid rafts involves multiple steps, all mediated by the viral Gag protein, which is sufficient for the assembly and release of virus particles (Adamson and Freed 2007; Ono and Freed 2001, 2005). Stable HIV-1 Gag lipid-raft membrane association is accomplished by a bipartite motif that includes a fatty acid myristate, added co-translationally to the N-terminus of Gag, and the first 31 amino acids of the HIV-1 matrix (MA) protein that form a highly basic patch on the surface of the protein (Spearman et al. 1994; Zhou et al. 1994; Lindwasser and Resh 2002; Ono et al. 2004; Ono and Freed 1999; Bryant and Ratner 1990), and allows for specific contacts with the inner leaflet of phosphatidylinositol (PI) 4,5-bisphosphate [PI(4,5)P_2]-enriched plasma membrane (Ono et al. 2004; Saad et al. 2006).

The general lipid composition of the HIV-1 membrane has been quantitatively analyzed by mass spectrometry and shown to comprise 45.1% (molar percentage) cholesterol, 8.8% phosphatidyl choline (PC), 4.4% Phosphatidylethanolamine (PE), 14.8% plasmalogen-PE (PI-PE), and 8.4% phosphatidylserine (PS) (Brugger et al. 2006; Chan et al. 2008). Although it is clear that HIV particles incorporate host-derived gangliosides, the precise nature and relative proportion of those gangliosides remain unclear, mainly because of lack of robust quantitative mass spectrometry approaches to accurately determine GSL contents of virions. This may also be in part due to the inherent variability of GSL expression within the producer cell, and may further reflect true variations in viral GSL content during the course of natural infection that are acutely dependent upon immune activation status and the cellular source of progeny virions.

Multiple studies have suggested that HIV-1 particles incorporate both GM1 and GM3 gangliosides (Nguyen and Hildreth 2000; Chan et al. 2008; Wubbolts et al. 2003). However a recent report demonstrates that HIV-1 Gag assembly occurs at GM1-deficient lipid rafts, suggesting that GM1 is not incorporated into the virion (Lehmann et al. 2011). Results from our lab support this finding and demonstrate that direct staining of viral particles derived from the macrophage-like THP-1 cells is only able to detect appreciable levels of GM3, but not GM1 (Puryear et al. 2012). Interestingly, within the same cell, GM3 and GM1 seldom colocalize (Freund et al. 2010; Fujita et al. 2007). Thus matrix-dependent HIV-1 Gag targeting to unique plasma membrane microdomains might provide specificity in the types of GSLs incorporated into the budding virus particles. In further agreement for a role of viral incorporation of GM3 in HIV pathogenesis, a functional genomic screen found that knockdown of Gb3 and GM3 in the producer cell led to virions that were deficient in establishing infection (Brass et al. 2008). Interestingly, HIV-1 patients exhibit a significant overexpression of GM3 and Gb3 on their lymphocyte plasma membranes (Sorice et al. 1996) while also

developing antibodies to both of these GSLs (Fantini et al. 1998). This correlative evidence lends further support to the idea that viral incorporation of GM3 is an important component of HIV-1 pathogenesis.

5.4 Mechanisms of HIV-1 Interactions with Dendritic Cells

It is well documented that the HIV-1 Envelope (Env) interacts with DCs via a number of attachment factors. The C-type lectin receptors such as DC-SIGN (expressed in the sub-epithelial rectal and lamina propria DCs) (Geijtenbeek et al. 2000; Jameson et al. 2002), mannose receptor (expressed on dermal DCs) (Turville et al. 2002), langerin (expressed on Langerhans cells in the skin and the genital epithelia (Hladik et al. 2007; Turville et al. 2002)), and DCIR (Lambert et al. 2008) are some of the best-described mechanisms of HIV capture by DCs. These receptors interact with virus particles by binding high-mannose oligosaccharides on the heavily glycosylated HIV Env (Feinberg et al. 2005; Guo et al. 2004; Lin et al. 2003). HIV Env can also bind to DCs by interacting with the charged residues of heparin sulfate proteoglycans (HSPG) (Mondor et al. 1998) or syndecans (Bobardt et al. 2003; de Witte et al. 2007a), as well as the GSL, galactosylceramide (Magerus-Chatinet et al. 2007). In addition, DCs have been shown to express HIV entry receptors, CD4 and CCR5 (Lee et al. 1999; Turville et al. 2002). Hence, the fate of the virus particle within DCs is dependent on the type of receptor(s) that HIV engages.

Productive or *cis*-infection of DCs, though feasible (Burleigh et al. 2006; Turville et al. 2004; Cameron et al. 2007), is ineffective for a myriad of reasons, including low CD4/CCR5 levels on the DC surface (Lee et al. 1999), as well as the presence of potent interferon-dependent (Granelli-Piperno et al. 1997; Neil et al. 2008, 2007; Pion et al. 2006, 2007; Van Damme et al. 2008) and interferon-independent antiviral restriction mechanisms (Granelli-Piperno et al. 1995, 1997). In vivo estimates of HIV-1-infected DCs is 10–100 times lower than that of HIV-1-infected $CD4^+$ T cells (Hosmalin et al. 1995; McIlroy et al. 1995), while in vitro, only 1–3% of peripheral blood mDCs can be productively infected (Smed-Sorensen et al. 2005). Recent studies have defined the molecular details of one such restriction mechanism that blocks HIV-1 infection of DCs at an early step in the viral life cycle (Hrecka et al. 2011; Laguette et al. 2011). SAMHD1, a protein that contains a sterile α-motif and an HD domain, is a deoxynucleotide triphosphohydrolase enzyme expressed in monocytes, DCs, and macrophages that suppresses cellular dNTP levels to inhibit virus life cycle at the reverse transcription step (Goldstone et al. 2011; Lahouassa et al. 2012; Powell et al. 2011). These recent findings provide a mechanistic basis for the previous observations in the literature on the relative paucity of productively infected peripheral blood monocyte-derived and myeloid DCs (Cameron et al. 2007; Smed-Sorensen et al. 2005; Lore et al. 2005).

Interestingly, many primate lentiviruses, including SIV_{mac} and HIV-2, but not HIV-1 or SIV_{cpz}, express the accessory protein, Vpx, to target SAMHD1 for degra-

dation and facilitate productive infection of DCs (Goujon et al. 2006, 2007; Lim et al. 2012). Forced expression of SIV_{mac} vpx gene in HIV-1-exposed DCs can rescue HIV-1 infection (Goujon et al. 2006, 2007), but the ensuing proviral transcription and translation result in the triggering of cryptic cytoplasmic sensors that detect newly synthesized HIV-1 proteins (Manel et al. 2010). Lack of SAMHD1-specific antagonizing function in HIV-1 has led to the provocative hypothesis that HIV-1 actively avoids the productive infection pathway in DCs to avoid activation of viral sensors and induction of antiviral signaling cascades (Manel et al. 2010).

In contrast to *cis*-infection, DCs have long been proposed to capture and internalize HIV-1 particles without initiating fusion. Interactions of HIV-1 with CLRs such as DC-SIGN have been implicated in targeting captured HIV-1 particles in immature DCs to intracellular endosomal compartments and subsequently to sites of DC–T cell infectious synapses (Arrighi et al. 2004a, b; Garcia et al. 2005). Such an HIV trafficking mechanism facilitated by DC-SIGN has been hypothesized to provide an escape mechanism for virus by targeting intra-cytoplasmic compartments that allow for viral persistence and evasion from lysosomal degradation pathways (Engering et al. 2002; Kwon et al. 2002). However, subsequent reports have questioned this hypothesis, and demonstrated DC-SIGN-dependent targeting of HIV to degradative pathways (Smith et al. 2007) and subsequent presentation of viral antigens via Class I MHC molecules (Moris et al. 2004, 2006). In contrast to DC-SIGN, the CLR, langerin, expressed on skin and vaginal epithelial $CD1a^+$ Langerhans cells (LCs), has been proposed to act as a "barrier" to HIV-1 infection. HIV-1 particles captured by $langerin^+$ LCs in vitro were endocytosed within Birbeck granules and targeted for degradation (de Witte et al. 2007b). Furthermore, endocytosis of model antigens by CLRs, such as the macrophage mannose receptor and Dectin-1, has been actively targeted to synergize with TLR-agonists for effective vaccine strategies (Bonifaz et al. 2002; Boscardin et al. 2006; Bozzacco et al. 2007). These studies hence question the prevailing hypothesis that CLRs are targeted by HIV to evade innate host defenses, and suggest that HIV-1 particles utilize alternative pathways to access compartments within DCs for persistence and evasion from host immune defenses.

In contrast to gp120–CLR-dependent interactions of HIV with DCs, our data argues for an existence of CLR-independent, GSL-dependent interactions of virus particles with DCs (Izquierdo-Useros et al. 2009; Hatch et al. 2009; Gummuluru et al. 2003). Interestingly, primary blood mDCs do not express DC-SIGN or other CLRs, and can capture and transfer HIV-1 particle to $CD4^+$ T cells (Izquierdo-Useros et al. 2007; Turville et al. 2001). Furthermore, HIV capture by DCs is dramatically enhanced upon maturation (Izquierdo-Useros et al. 2009). Interestingly, mature DCs downregulate surface DC-SIGN and CLR expression and enhancement of HIV-1 capture is largely independent of Env (Gummuluru et al. 2003; Hatch et al. 2009; Izquierdo-Useros et al. 2007, 2009; Puryear et al. 2012). HIV particles entirely lacking Env are still bound by mature DCs at levels similar to those of fully infectious Env-containing particles. This has been demonstrated with both Env-deficient infectious particles and Gag-EGFP-containing virus-like particles (Hatch

et al. 2009; Izquierdo-Useros et al. 2009). Likewise, pretreatment of mature DCs with agents that block Env-dependent capture, such as mannan (to block CLRs), anti-CD4-neutralizing antibodies (to block gp120–CD4 binding), or oligomeric soluble Env trimers (sgp140 to block all Env-mediated binding), also had minimal impact on viral capture (Gummuluru et al. 2003; Hatch et al. 2009). It is not surprising that HIV-1 gp120-independent attachment mechanisms can exist, considering that there are only 7–14 trimeric gp120 spikes irregularly clustered on the surface of an HIV-1 particle (Zhu et al. 2006), thus leaving open the possibility that host cell-derived determinants incorporated into the virus lipid bilayer can impact virus capture.

5.5 HIV Incorporation of the Host-Derived Ganglioside GM3 Allows the Virion to Bind DCs Independent of the HIV Glycoprotein

Alterations in HIV incorporation of GSLs do however lead to a dramatic decrease in virus capture by mature DCs (Hatch et al. 2009; Izquierdo-Useros et al. 2009). Virions derived from producer cells, rendered deficient in GSL levels, either by targeting GSL biosynthesis pathways by small molecule inhibitors or RNAi targeting GSL biosynthetic enzymes, show a dramatic decline in capture by mature DCs (Hatch et al. 2009; Izquierdo-Useros et al. 2009; Puryear et al. 2012). More specifically, capture of HIV-1 particles with or without Env by mature DCs is competitively inhibited by anti-GM3 antibodies and/or by GM3-containing liposomes while GM3 enrichment of HIV particles leads to enhanced DC capture (Puryear et al. 2012). GM3 enrichment can be artificially achieved by the addition of exogenous lipid to virus-producing cells; however changes in the producer cell can also result in concomitant changes in virion levels of GM3 incorporation. Upon stimulation with a synthetic TLR2/1 ligand Pam3CSK4 or Phorbol 12-myristate 13-acetate (PMA), moncytoid THP-1 cells can be induced to express high levels of surface GM3. HIV particles derived from unstimulated (monoycte-like) or stimulated (macrophage-like) THP-1 cells incorporate low or high levels of GM3, respectively. As compared to untreated THP-1 cells, virus produced from stimulated THP-1 cells displays an enhanced DC capture phenotype. This suggests that viruses derived from activated monocytes or T cells in vivo would also result in enhanced incorporation of GM3 within progeny virions with concomitant enhancements in DC capture and *trans*-infection.

Although the in vitro model of HIV-1 capture by DCs highlights the role of ganglioside GM3, it is possible that the related ganglioside GM1 could also play a similar role in vivo. Similar levels of increased DC capture are observed when virions are exogenously enriched with GM1 (Puryear et al. 2012). However, derivation of HIV-1 particles from Pam3CSK4-stimulated THP1 cells, that display a dramatic increase in cell surface GM1 expression, resulted in no associated increase in virion incorporation of GM1, and negligible enhancement in DC capture of HIV virions

(Puryear et al. 2012). GSL binding interactions typically require a high avidity-binding event to overcome their inherently low-affinity interactions. It is therefore possible that even though GM1 is capable of mediating virion capture by DCs, the concentration of GM1 incorporated into the viral particles does not surpass a minimum threshold necessary to overcome the low-affinity binding interaction. This evidence suggests that in the in vitro model systems under study, HIV particles do not incorporate high enough levels of GM1 to mediate DC capture by the ganglioside-dependent mechanisms. It therefore follows that in vivo differences in cell type-specific GSL expression levels could tip the balance as to which ganglioside is incorporated into a de novo virion at high enough concentrations to mediate DC capture.

5.6 GSLs Impact *Trans*-infection by Influencing Membrane Fluidity on the Cells and on the Viron

DCs play a central role in viral dissemination through the process of *trans*-infection. DCs capture virus and traffic the particles to a DC–T cell junction. It remains unclear if the virus is maintained in an internal compartment (Frank et al. 2002; Garcia et al. 2005; Izquierdo-Useros et al. 2009; Wiley and Gummuluru 2006) or is surface associated (Cavrois et al. 2002; Yu et al. 2008) but regardless of the precise location, virus particles are maintained in an infectious form (Geijtenbeek et al. 2000) and ultimately transferred to $CD4^+$ T cell to establish productive infection by formation of "infectious synapses" between virus-containing DCs and T cells (McDonald et al. 2003; Pope et al. 1994; Frankel et al. 1996; Cameron et al. 1992). Furthermore, mDCs, pDCs, and langerhans cells all use similar mechanisms of HIV-1 *trans*-infection to T cells (Fahrbach et al. 2007; Lore et al. 2005).

A cardinal feature of HIV-1 infections is high level of chronic immune activation, which has been shown to be a strong predictor of disease progression in vivo (reviewed in (Douek et al. 2009)). DCs derived from peripheral blood of HIV-1-infected individuals have invariably been shown to be hyperresponsive to immune activation stimuli, such as TLR7/8 ligands (Sabado et al. 2010), thus triggering secretion of high levels of proinflammatory mediators and prolonged activation of T cells, a condition that is especially suitable for sustenance of high levels of virus replication in the lymph nodes. In fact, some estimates have placed the average daily production of HIV-1 at approximately 1×10^{10} virions, with much of the virus replication occurring in the paracortical regions of the peripheral lymphoid organs that are composed predominantly of DCs and $CD4^+$ T cells (Embretson et al. 1993; Finzi and Silliciano 1998; Pantaleo et al. 1993; Coffin 1996). Intriguingly, immune activation stimuli upregulate cell surface expression of GM3 in $CD4^+$ T cells and macrophages (Blander et al. 1999; Nojiri et al. 1986; Tagami et al. 2002; Gracheva et al. 2007), and hence, enhanced incorporation of GM3 on HIV-1 particles derived from these cells (Puryear et al. 2012). Furthermore, exposure to proinflammatory mediators enhances the ability of DCs to capture and disseminate HIV-1 particles

(Izquierdo-Useros et al. 2007, 2009, 2010; Wang et al. 2007; Wu and KewalRamani 2006) in a GM3-dependent manner (Puryear et al. 2012). These findings highlight the nefarious nature of HIV to use DCs as vehicles for widespread dissemination within the host.

GSLs contribute to this process in a number of ways that have previously been reviewed (Lingwood and Branch 2011; Waheed and Freed 2010), and additional mechanisms are sure to be uncovered as our understanding of intracellular viral trafficking continues to expand. The formation of the infectious synapse between DCs and T cell is likely under significant influence from the membrane composition of GSLs both on the DC and the T cell. Synapse formation requires a large degree of membrane rearrangement in order for synaptic junction molecules to assemble at the site of contact. GSLs have an established ability to trigger membrane rearrangements (Hakomori and Handa 2002; Nguyen et al. 2005) and have been shown to play an important role in the recruitment and clustering of HIV receptors CD4 and CCR5 (Hug et al. 2000). Productive infection of the partnering T cell can be influenced by GSL content, not only for the GSL contribution to receptor rearrangement, but also for GSL contributions to membrane fusion that are required for viral entry. GSL depletion has been shown to inhibit viral fusion (Hug et al. 2000; Puri et al. 1998, 2004) and Gb3 and GM3 in particular can impact HIV-1 fusion with primary T cells (Brass et al. 2008). This inhibition is likely attributed to either an impairment of lateral receptor mobility or impairment of fusion pore formation. Reconstitution of the GSL-depleted membrane with Gb3 or GM3 restores the ability of HIV to establish infection (Hug et al. 2000; Puri et al. 1998, 1999). Similarly, the enrichment of target cells with Gb3 or GM3 can enhance viral infection (Hammache et al. 1999; Hug et al. 2000; Nehete et al. 2002). Recent reports further suggest that GSL content, particularly GM3, can impact $CD4^+$ T cell receptor signaling and activation, which could further impact the formation of the immunological synapse (Zhu et al. 2011; Nagafuku et al. 2012).

The GSL composition of the HIV-1 particle itself can also impact how efficiently the virus is transferred from DCs to $CD4^+$ T cells. Our recent work demonstrating a role for gangliosides in DC capture also found that GSL content impacts DC-mediated *trans*-infection. HIV particles produced from cells where GSL synthesis was inhibited produced virions with impaired transfer (Hatch et al. 2009). In contrast, HIV particles that were enriched for GM3 incorporation show enhanced T cell *trans*-infection (Puryear et al. 2012). Upon contact of the viral particle with the T cell, it remains unclear how many Env spikes are required for productive infection to occur (Yang et al. 2005; Magnus et al. 2009). Some studies suggest that two or more spikes may be required (Magnus et al. 2009) and the spatial requirements of binding may require the Env spikes to relocate within the viral membrane. The ability of the glycoprotein cytoplasmic tail, gp41, to move laterally within the virion is influenced by both cholesterol and sphingomyelin, and impairment of this mobility can lead to a potential decrease in the ability of the virus to fuse with the target cell (Saez-Cirion et al. 2002). Since gangliosides are known to impact membrane fluidity it is also within reason that differences in ganglioside composition of the virus also have

a similar effect. This is supported by the evidence from a functional genomic screen showing that virions produced from Gb3- or GM3-deficient cells go on to be impaired for establishing productive infections (Brass et al. 2008).

5.7 Conclusions and Future Directions

Mounting evidence shows that GSLs impact DC capture and *trans*-infection of HIV on multiple levels; however several key questions still remain. One particularly fascinating area of inquiry warranting further investigation is that of immune evasion strategies of HIV in DCs and the ways in which GSLs may contribute to HIV's ability to hijack the DC for efficient dissemination. The ability of HIV to be captured by DCs and to remain in an infectious form that can subsequently be delivered to $CD4^+$ T cells to establish productive infection remains an enigma. Given the data discussed in this review, future efforts would be well spent to address the contribution of cellular GSLs in trafficking the virus through the DC to a non-lysosomal compartment of the cell.

The ability of HIV to interact directly with DCs via GSLs is particularly intriguing in this regard. It is possible that by binding to a GSL-recognizing receptor, the virus triggers a signaling event that results in membrane rearrangements and provides a mechanism by which the virus can efficiently "surf" the DC membrane. Such interactions may permit the virus to access trafficking pathways within the DC that are separate from the endosomal pathways typical of gp120–CLR binding, or the predominantly dead-end productive infection pathway resulting from gp120 engagement of CD4 and coreceptor. It will be interesting to discern the relative contribution of the various DC binding mechanisms to overall pathogenesis and to better understand how each pathway contributes to the fate of the virion in vivo (Fig. 5.1). Future research should address the proportional frequency of each type of binding event and determine which pathway is preferentially utilized in natural infection and how differences in immune activation or disease state affect preferential receptor engagement. Likewise, given the role of GSLs in DC capture and viral infectivity and given the variability in cellular GSL expression based on cell type and activation status, the contribution of GSLs to overall viral fitness is an additional area of research that warrants further exploration. Viral particles that are otherwise genetically identical could show dramatic differences in viral fitness based simply on the GSL composition that is incorporated into their viral envelopes.

The contribution of virally incorporated gangliosides to DC capture and *trans*-infection is, we believe, a particularly provocative one. The identification of the DC receptor that captures HIV-1 particles in a GM3-dependent manner might provide a potentially novel target for early intervention strategies such as microbicides. To date, all such efforts have been directed against the HIV Env. This has been a particularly challenging target due to the inherent variability and rapid evolution of the viral protein. If further studies verify that a substantial proportion of the early interaction between HIV and the sentinel mucosal DCs is actually mediated by a host-derived

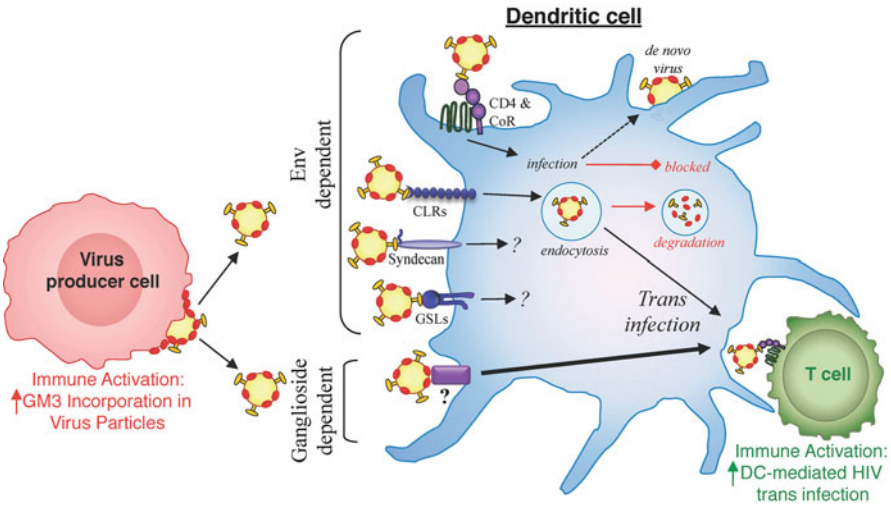

Fig. 5.1 Model of HIV-1 interactions with dendritic cells. HIV-1 gp120-independent virus capture mechanism by DCs is dependent on the ganglioside GM3. Recognition of virus particle-associated GM3 by a yet-to-be identified DC receptor results in trafficking of virus particles to $CD81^+$ compartments, and upon initiation of DC–T cell contact, re-localization of the virus particles to the DC–T cell infectious synapse. Exposure of virus producer cells to proinflammatory mediators, such as microbial TLR ligands, results in enhanced expression of GM3 in the virus producer cell, and hence, enhanced incorporation of GM3 in the budding virus particles. Immune activation and maturation of DCs result in enhanced capture of HIV-1 particles by DCs, establishment of long-lived contacts with $CD4^+$ T cells, and enhanced *trans*-infection of $CD4^+$ T cells. We posit that recognition of HIV-1 gp120 by pathogen recognition receptors such as CLRs is an integral part of the host innate response to the invading virus, while GSL-dependent interactions of HIV with DCs are part of the virus evasion response

ganglioside that is incorporated into the virus membrane, this provides a much less fickle target and one that is potentially much more difficult for the virus to mutate around. Rigorous studies would clearly need to be pursued to ensure that targeting a host ganglioside on HIV does not have detrimental effects on normal functions within the host.

Several studies have shown that exosomes and HIV-1 particles have shared physical properties, including approximate size, composition, and GM3 enrichment (Chan et al. 2008; Wubbolts et al. 2003). Interestingly, DC trafficking characteristics are also very similar between exosomes and HIV. The two types of particles compete for DC capture (Izquierdo-Useros et al. 2009), traffic to a tetraspanin-rich region of the DC to evade lysosomal degradation (Wiley and Gummuluru 2006; Gould et al. 2003), and ultimately arrive at the immunological synapse (Hladik and McElrath 2008; Izquierdo-Useros et al. 2010). This is particularly intriguing given the ways in which the HIV particle mirrors an exosome's GSL composition, and may represent a novel form of molecular mimicry that mediates viral immune evasion.

For the purpose of navigating through the DC, avoiding degradation, and retaining infectivity and competency for transfer at the DC–T cell infectious synapse, exosome mimicry is a fortuitous one for HIV, in that the virus particle presumably appears to the immune system as a "self-antigen." This propensity to hide in plain sight would provide an efficient means of escaping recognition and degradation by the DC. In addition to immune evasion, exosome mimicry provides an efficient means of DC-mediated viral dissemination. Exosomes are part of an intrinsic DC *trans*-dissemination pathway that have been postulated to help sustain an elevated immune response by providing a means to gather and store antigen (Izquierdo-Useros et al. 2009, 2010) while controlling endosomal acidification (Savina et al. 2006). The antigen-containing exosomes avoid fusion (Gould et al. 2003; Izquierdo-Useros et al. 2009) and go on to cross-present antigen to T cells (Savina et al. 2006). Exosomes also serve as a means of intercellular communication, again avoiding fusion while transiting through DCs and providing a mechanism to deliver membrane proteins, signaling proteins, mRNA, and miRNA (Record et al. 2011). Hence, the process of HIV *trans*-infection draws many parallels to the exosome process of *trans*-dissemination, in that upon arrival at the DC–T cell infectious synapse, the virus is then free to infect $CD4^+$ T cells through conventional interactions between HIV Env and CD4/coreceptor.

The burgeoning of our understanding as to how GSLs impact numerous areas of cellular biology and immune function and how these mechanisms impact viral pathogenesis is an exciting one. This is sure to be an active area of research in the coming years and will undoubtedly yield intriguing new insights for both HIV and immune function. It is our hope that such insights will also yield promising new treatment directions as these novel pathways are further defined.

Acknowledgments The authors' work is supported in part by grants from National Institutes of Allergy and Infectious Diseases (AI064099 and AI081596 (S.G.)). W.B.P. is supported by an NRSA F32 AI084558 fellowship and Research Training in Immunology 5T32AI007309-22 fellowship.

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Chapter 6
Simian Immunodeficiency Virus Interactions with Macaque Dendritic Cells

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Abstract This chapter summarizes advances in the following areas: (1) dendritic cell (DC)-mediated simian immunodeficiency virus (SIV) transmission, (2) role of DCs in innate and adaptive immunity against SIV, and (3) approaches to harness DC function to induce anti-SIV responses. The nonhuman primate (NHP) model of human immunodeficiency virus (HIV) infection in rhesus macaques and other Asian NHP species is highly relevant to advance the understanding of virus–host interactions critical for transmission and disease pathogenesis. HIV infection is associated with changes in frequency, phenotype, and function of the two principal subsets of DCs, myeloid DCs and plasmacytoid DCs. DC biology during pathogenic SIV infection is strikingly similar to that observed in HIV-infected patients. The NHP models provide an opportunity to dissect the requirements for DC-driven SIV infection and to understand how SIV distorts the DC system to its advantage. Furthermore, the SIV model of mucosal transmission enables the study of the earliest events of infection at the portal of entry that cannot be studied in humans, and, importantly, the involvement of DCs. Nonpathogenic infection in African NHP hosts allows investigations into the role of DCs in disease control. Understanding how DCs are altered during SIV infection is critical to the design of therapeutic and preventative strategies against HIV.

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_6,

6.1 Introduction

Human immunodeficiency virus (HIV) originated from simian immunodeficiency viruses (SIVs) that naturally infect African nonhuman primates (NHPs), such as the chimpanzee, African green monkeys (AGMs), and sooty mangabeys (SMs) (Heeney et al. 2006). SIVs closely parallel HIV in genomic organization, genetic sequence, and biological properties. SIV infection in natural hosts is generally nonpathogenic despite the high rate of viral replication. In contrast, experimental SIV infection of rhesus macaques (RMs) and other Asian NHP species results in a $CD4^+$ T cell loss and animals typically develop AIDS-like immunodeficiency within 1–2 years (Desrosiers 1990).

As will be discussed herein, macaque DCs exhibit comparable phenotypes, functions, and in vivo distribution to human DCs. Thus, the macaque model of HIV infection is especially useful for examining the roles of DCs in the early events of transmission and pathogenesis. Animals can be challenged with SIV intravenously or mucosally, allowing to (1) dissect the earliest events of transmission and virus dissemination, (2) follow disease progression in treated and untreated settings, and (3) evaluate the efficacy of experimental vaccines or microbicides for their ability to prevent infection and/or disease progression. Infectious SIV–HIV hybrids (SHIVs) can be used to evaluate the activity of HIV-specific inhibitors.

6.2 Macaque DCs

Macaque DCs are found in lymph nodes (LNs), blood, and mucosal tissues (Pope et al. 1997; Hu et al. 1998, 1999; Ignatius et al. 1998, 2001; Coates et al. 2003; Lore 2004; Teleshova et al. 2004a, b; Chung et al. 2005; Brown et al. 2007; Diop et al. 2008; Malleret et al. 2008b; Brown and Barratt-Boyes 2009; Xu et al. 2010; Gujer et al. 2011). Myeloid DCs (mDCs) are defined in blood as $HLA\text{-}DR^+CD11c^+CD123^-$ cells lacking expression of the lineage markers (Lin) CD3, CD14, and CD20, whereas plasmacytoid DCs (pDCs) are identified as $Lin^-HLA\text{-}DR^+CD11c^-CD123^+$ cells. Generation of larger numbers of monocyte-derived DCs (moDCs) (O'Doherty et al. 1997) facilitated the execution of more extensive studies on the macaque DC biology and DC–SIV interplay.

Macaque DCs also require stimulation to differentiate into mature, potent immunostimulatory cells capable of inducing strong adaptive T cell responses (Mehlhop et al. 2002; Frank et al. 2003; Teleshova et al. 2004b). Activation of macaque moDCs or circulating DCs results in (1) up-regulation of CD25, CD40, CD80, CD83, CD86, CD208, CD205, and HLA-DR; (2) reduced endocytic activity; (3) increased production of cytokines and chemokines (e.g., IL-12, IFN-α, TNF-α); and (4) enhanced T cell stimulatory activity (Mehlhop et al. 2002; Coates et al. 2003; Teleshova et al. 2004a, b). Distinct features of mDCs vs. pDCs highlight their unique roles in coordinating these innate and adaptive events (Table 6.1).

Table 6.1 Characteristics of NHP mDCs and pDCs

DC subsets	Co-stimulatory molecules	TLR expression	Functions	SIV-induced maturation
mDCs	$CD40^{mo}$	TLR3	Endocytic	No changes in CD80 and CD86 expression
	$CD80^{low}$	TLR4	Produce IL-12	
	$CD86^{mo}$	TLR7	Stimulate T cells (most potently when mature)	
		TLR8		
pDCs	$CD40^{mo}$	TLR7	Endocytic	Increased CD80 and CD86 expression
	$CD80^{low}$	TLR9	High producers of IFN-α	
	$CD86^{low}$		Produce TNF-α	IFN-α release

Summarized from (Coates et al. 2003; Teleshova et al. 2004b; Ketloy et al. 2008; Brown et al. 2009; Gujer et al. 2011)

6.3 DC-Mediated SIV Transmission

6.3.1 DC–SIV Interactions

Just like HIV–human DC interplay, SIV capture by moDCs requires functional Env glycoproteins (Frank et al. 2002), involving CD4, CCRs, and C-type lectin receptors (CLRs, as well as yet unidentified CLRs) (Turville et al. 2001a, b) (Fig. 6.1). Both immature and mature moDCs are effective in capturing SIV (Mehlhop et al. 2002) and capable of clathrin-coated pit-mediated uptake of the virus (Frank et al. 2002). The cellular distribution of internalized SIV (and HIV) is dramatically different in immature and mature moDCs (Frank et al. 2002, 2003; Morcock et al. 2005). In immature cells, one to two virus particles are retained in vesicles at the cell periphery. This contrasts the perinuclear localization of multiple virions in large vesicular compartments in mature moDCs. Virus captured by moDCs does not colocalize with endolysosomal markers, suggesting that the virus alters classical endolysosomal processing machinery (Frank and Pope 2002; Turville et al. 2004). Entrapped virus (in immature and mature DCs) is mostly degraded within 24 h, coinciding with a decreased ability to transmit captured virus to T cells across DC–T cell synapses, while immature moDCs exhibit a second phase of increased transmissibility as they facilitate R5 HIV replication for transfer to T cells (Frank and Pope 2002; Turville et al. 2004) (Fig. 6.1). Virus transfer from DCs to T cells also involves multiple receptors including CD4, CCR5, and CD209 (Wu et al. 2002; Turville 2001a).

Macaque pDCs express high level of CCR5 (Reeves and Fultz 2007), but the percentage of macaque pDCs that express CXCR4 is highly variable (Reeves and Fultz 2007; Patterson et al. 2001). Comparable to human studies (Donaghy et al. 2003), pDCs were shown to be susceptible to in vivo infection with SIV, although lower levels of SIV *gag* DNA were detected in mDCs (Brown et al. 2009).

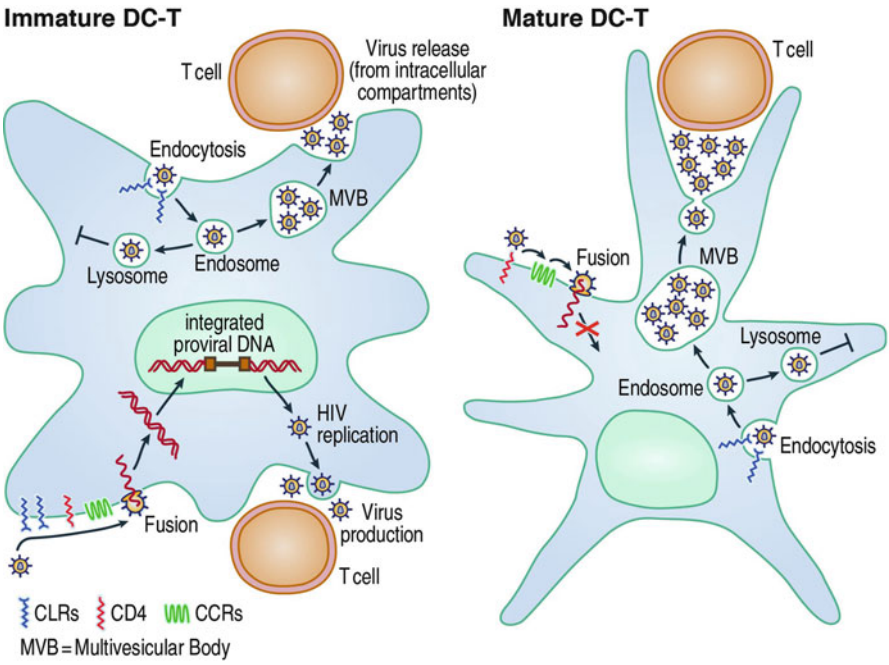


Fig. 6.1 moDC–SIV interactions and possible modes of virus transmission to permissive CD4⁺ T cells. SIV internalized via CLRs (and other possible attachment receptors) is held in the periphery of immature moDCs vs. a deeper perinuclear location in mature moDCs. Captured virus rapidly moves to the contact points between the DC and CD4⁺ T cells and is transferred to the T cells, which amplifies infection. Entrapped virus is degraded in lysosomes within 24 h, ultimately reducing the infectious virus load in the DCs. R5 HIV infection of immature DCs and subsequent spread of newly produced viruses to T cells have also been demonstrated for human moDCs, but this has not been extensively studied with macaque moDCs

6.3.2 Importance of the DC–T Cell Milieu

Macaque DCs emigrating from organ cultures (skin, nasopharyngeal, and vaginal mucosa) form conjugates with T cells that support SIV replication (Pope et al. 1997; Ignatius et al. 1998, 2001; Hu et al. 1999). Blood- and skin-derived DCs from uninfected macaques similarly support high viral replication when mixed with T cells from blood, skin, spleen, or LNs and transmit virus to syngeneic and allogeneic T cells (Ignatius et al. 1998, 2001). In fact, separation of the subsets by cell sorting revealed that SIV replication predominantly occurs in the DC–T cell conjugate fraction (Ignatius et al. 1998). Trypsin treatment of SIV-loaded DCs did not affect their ability to transfer virus to T cells, supporting the idea that the transmitted virus is internalized (Ignatius et al. 1998). SIV replication in these mixtures proceeds in the absence of overt activation of the resting T cells and is augmented in the presence of antigen-responding T cells (Ignatius et al. 1998, 2001; Messmer et al. 2000). Of note,

virus replication is equally robust when DCs or resting T cells introduce the virus to the CD4+ T cell–DC environment (Messmer et al. 2000).

Although SIV replication is comparable when mature macaque moDCs are cocultured with either naïve or memory T cells, immature DCs promote more robust replication in the presence of memory T cells (like the DCs and T cells located at the body surfaces) (Frank and Pope 2002). This supports early observations of vigorous replication of SIV in the DC–memory T cell mixtures obtained from the skin and mucosal surfaces (Pope et al. 1997; Hu et al. 1998; Ignatius et al. 1998, 2001). In agreement, extensive replication of SIV in CCR5+ memory T cells is reported within the gut in the DC–memory T cell locale (Veazey et al. 1998).

moDC–T cell contact triggers SIV movement to the DC–T cell synapse (Turville et al. 2004). Movement of tubular MHC II peptide-bearing compartments to contact points between DCs and peptide-specific T cells was reported (Boes et al. 2002; Chow et al. 2002), suggesting that virus may exploit this machinery to spread from DCs to CD4+ T cells, in particular to virus-specific T cells (Douek et al. 2002; Lore et al. 2005).

6.3.3 DCs and Mucosal SIV Transmission

6.3.3.1 Vaginal SIV Transmission

The first in vivo evidence that DCs may represent targets for SIV was obtained after looking for virus DNA in the mucosa following vaginal challenge of RMs with SIV (Spira et al. 1996). Virus was found to enter the vaginal mucosa within 60 min of exposure, infecting primarily intraepithelial DCs including Langerhans cells (LCs) (Hu et al. 2000). SIV-infected cells are detected in draining LNs within 18 h of intravaginal SIV exposure posing a challenge for the development of preventative strategies. Mucosal site-specific virus-induced events following vaginal SIVmac251 challenge have been reported (Li et al. 2009). The predominant foci of SIV-infected cells were identified in the endocervix and coincided with “outside-in” signaling events that include CCL20 production by endocervical epithelial cells and recruitment of CCR6+ pDCs that produce type I IFNs and chemokines attracting CCR5+ T cells (Li et al. 2009). Subsequent to pDC and T cell recruitment into the endocervix, clusters of infected cells appear in the transformation zone and in the vagina. Infection with SIV induces pro-inflammatory responses in the ectocervical and vaginal mucosa. Importantly, blocking virus-induced inflammation with vaginally administered glycerol monolaurate correlated with protection against SIV infection in vivo (Li et al. 2009). Another cytokine, thymic stromal lymphopoietin (TSLP), was suggested to play a role during mucosal SIV transmission based on its increased expression concurrent with an increase in viral replication in the vaginal mucosa within the first 2 weeks from vaginal SIV exposure (Fontenot et al. 2009). HIV induces human genital epithelial cells to produce TSLP (via NFκB), which activates DCs to promote HIV replication in the DC–T cell milieu (Fontenot et al. 2009).

DCs are sparse in the mucosa and the primary virus amplification occurs rather within resting memory T cells, the predominant available target cells (90%) (Miller et al. 2005; Zhang et al. 2004). These data support the theory that the delivery of virus by the few infected/virus-carrying DCs may represent a "quality" rather than "quantity" type of mechanism and that infected/carrying virus DCs may fuel infection locally and, after migrating, in the lymphoid tissue leading to systemic spread (Turville et al. 2006). The timing of DC-mediated transfer of SIV in the lymphoid tissue is supported by in vitro findings in moDCs (Turville et al. 2004). The potential contributions of DCs in vaginal SIV transmission are summarized in Fig. 6.2.

6.3.3.2 Rectal SIV Transmission

Knowledge of the role of DCs during rectal SIV transmission is limited. Like the HIV-binding $CD209^+$ cells found in human rectal tissues (Gurney et al. 2005; Dieu et al. 1998), an abundance of $CD209^+$ cells that efficiently bind SIV was reported in macaque rectal mucosa (Jameson et al. 2002) (Ribeiro Dos Santos et al. 2011). A low frequency of mDCs and pDCs within Lin^-HLADR^+ cells in macaque rectal tissues was also reported (Kwa et al. 2011). Rapid dissemination of SIV to draining LNs and peripheral blood (at 4 h post infection) after rectal challenge and lack of SIV $antigen^+$ $CD209^+$ cells in the mucosa suggest possible dissemination of virus by $CD209^+$ cells (Ribeiro Dos Santos et al. 2011).

6.3.3.3 Oral/Tonsillar SIV Transmission

SIV can be transmitted via the oral route (Baba et al. 1996). Explosive SIV replication in the oral mucosal associated lymphoid tissue (MALT) following atraumatic application of virus on palatine and lingual tonsils suggests that MALT is a potential site for HIV transmission during oral sex, parturition, and breastfeeding (Stahl-Hennig et al. 1999). DCs are identified in the oral MALT in macaques (Pope et al. 1997). Tonsillar DC–T cell mixtures support SIV replication like other DC–T cell mixtures, with virus replication occurring predominantly in DC–T cell syncytia (Pope et al. 1997). Thus, the very different mucosal environments all house DCs and memory T cells, which likely play central roles in the onset and amplification of SIV infection.

6.3.4 DC Dynamics During Early/Acute and Chronic SIV

With DCs participating in the onset and spread of SIV infection, it is not surprising that DC biology is affected in infected individuals (Fig. 6.3). A decrease in pDC and mDC numbers in HIV-infected patients correlates with disease progression, suggesting that DC loss is related to the ability to control disease (Smed-Sorensen and

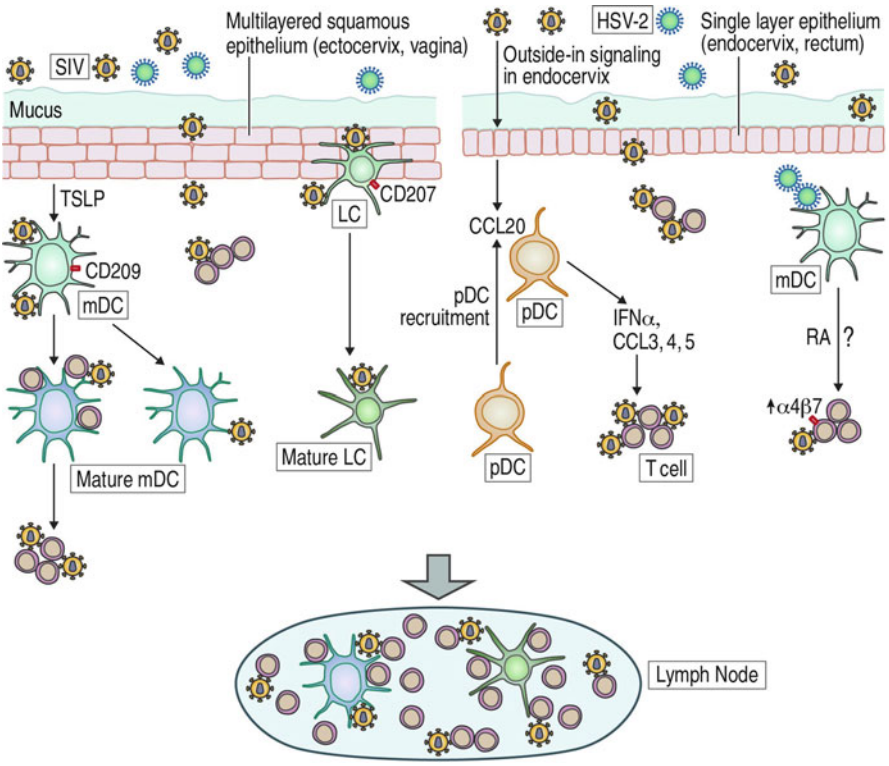
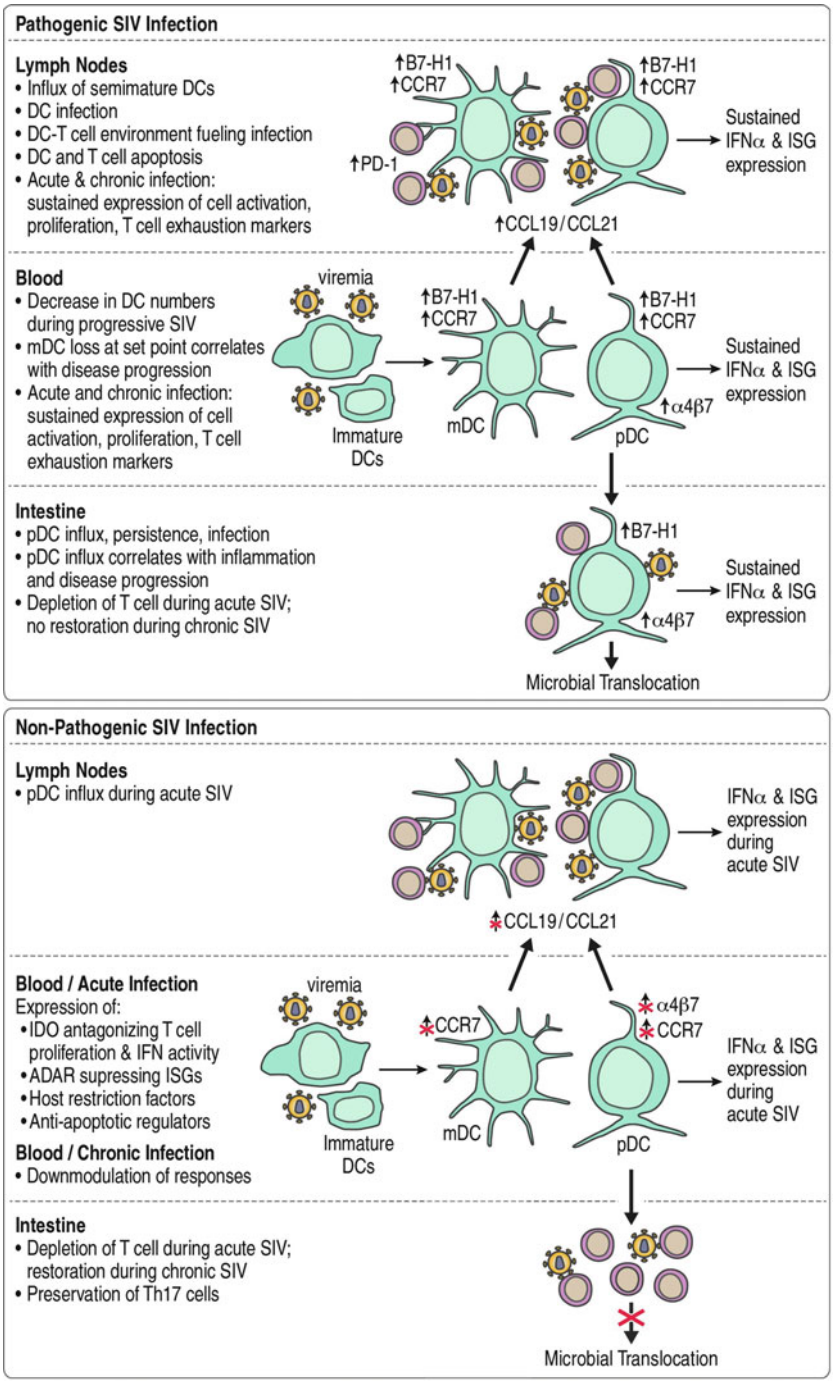


Fig. 6.2 The role of DCs in mucosal SIV transmission. SIV can cross the multilayered squamous epithelium of the ectocervix/vagina and the columnar epithelium of the endocervix and rectum. Cell-free or cell-associated SIV (not shown) is trapped in mucus covering the epithelium. At mucosal surfaces during SIV exposure, DCs are proposed to be among the first target cells to encounter virus. These DCs include LCs in the epithelium and immature mDCs in the submucosa. CLRs (CD207, CD209) or other attachment factors expressed on immature DCs are capable of binding SIV and transferring virus in *trans* to $CD4^+$ T cells locally in the mucosa or in the LNs. SIV carrying DCs can mature as the result of infection or cytokines/chemokines present in the mucosa during infection. Virus crosses the mucosal barrier to establish small founder population that expands using outside-in signaling identified in the endocervix and resulting in endocervical CCL20 production, subsequent recruitment of $CCR6^+$ pDCs, $CCR5^+$ target cell expressing chemokines, and production of IFN-α. SIV also induces the production of TSLP by vaginal epithelial cells that leads to DC maturation and fueling of DC-mediated transmission. In the rectal mucosa $CD209^+$ cells and $CD4^+$ T cells are among the first targets of SIV. HSV-2 is one of the main cofactors for HIV infection. HSV-2 can infect mDCs and increase the susceptibility of mucosal $CD4^+$ T cells to SIV infection by RA-mediated induction of α4β7 on the T cells, as it was demonstrated in vitro

Lore 2011). However, a substantial depletion of pDCs from blood during HIV-2 infection, which is significantly attenuated relative to HIV-1 infection, was reported (Cavaleiro et al. 2009). DC numbers and distribution are similarly affected during SIV infection (Barratt-Boyes and Wijewardana 2011; Reeves and Fultz 2007; Wijewardana et al. 2010; Malleret et al. 2008a), but the dynamics of these changes


Pathogenic SIV Infection
Lymph Nodes
• Influx of semimature DCs
• DC infection
• DC-T cell environment fueling infection
• DC and T cell apoptosis
• Acute & chronic infection: sustained expression of cell activation, proliferation, T cell exhaustion markers
↑B7-H1
↑CCR7
↑PD-1
↑CCL19/CCL21
Sustained IFNα & ISG expression
Blood
• Decrease in DC numbers during progressive SIV
• mDC loss at set point correlates with disease progression
• Acute and chronic infection: sustained expression of cell activation, proliferation, T cell exhaustion markers
viremia
Immature DCs
mDC
pDC
↑α4β7
Intestine
• pDC influx, persistence, infection
• pDC influx correlates with inflammation and disease progression
• Depletion of T cell during acute SIV; no restoration during chronic SIV
Microbial Translocation
Non-Pathogenic SIV Infection
Lymph Nodes
• pDC influx during acute SIV
IFNα & ISG expression during acute SIV
*CCL19/CCL21
Blood / Acute Infection
Expression of:
• IDO antagonizing T cell proliferation & IFN activity
• ADAR supressing ISGs
• Host restriction factors
• Anti-apoptotic regulators
Blood / Chronic Infection
• Downmodulation of responses
*CCR7
*α4β7
Intestine
• Depletion of T cell during acute SIV; restoration during chronic SIV
• Preservation of Th17 cells

can be affected by the challenge virus (e.g., SIVmac239 vs. SHIV-89.6P) (Reeves and Fultz 2007).

Both mDCs and pDCs are lost from blood and lymphoid tissue in SIV-infected RMs with end-stage disease (Brown et al. 2007). Significant loss of mDCs in blood at virus set point is predictive of rapid progression to AIDS, whereas an increase in blood mDCs predicts long-term absence of disease. In stable (disease-free) and progressor animals, blood mDC numbers decreased within 2 weeks post infection; however, only progressor animals experienced irreversible mDC decrease within a 12-week follow-up, and stable animals had a significant increase during this period (Barratt-Boyes and Wijewardana 2011). mDC recruitment to LNs coincides with increased expression of the LN homing and recruitment molecules CCR7, CCL19, and CCL21. LN recruitment is offset by apoptosis in both acute and chronic infection (Wijewardana et al. 2010; Barratt-Boyes and Wijewardana 2011). Negligible SIV DNA is detected in LN mDCs suggesting that infection is not the major cause of the mDCs loss (Brown et al. 2009).

There is a dynamic pDC response in acute SIV infection with an initial increase in number of pDCs in blood followed by a decrease relative to baseline. pDC mobilization into blood and recruitment into the LNs coincide with peak viremia at 1–2 weeks post infection (Brown et al. 2009). Nevertheless, the absolute number of pDCs in both compartments drops, associated with widespread pDC activation, apoptosis, and infection. This parallels studies in humans showing that pDC loss is a better predictor of disease progression than CD4 counts (Soumelis et al. 2001). SIV DNA is detected in more than 4% of pDCs in LNs during acute infection, possibly resulting in pDC death (Brown et al. 2009). In acute SIV infection, pDCs acquire a mature phenotype, upregulating CD80, α4β7 integrin, and CCR7 (Brown et al. 2009; Kwa et al. 2011; Malleret et al. 2008a) and migrate into the colorectal mucosa and LNs. The migration of pDCs to the colorectal mucosa is α4β7 dependent and coincides with mucosal immune activation (Kwa et al. 2011). The induction of α4β7 during acute SIV infection is unique to pDCs, as mDCs do not upregulate this marker.

Fig. 6.3 DC frequency, distribution, and functionality during pathogenic and nonpathogenic SIV infection. Early and sustained changes in pDC and mDC numbers, phenotype, tissue distribution, and function are attributable to pathogenic SIV infection. SIV infection is associated with an activated/mature pDC phenotype and semi-mature mDC phenotype. SIV infection induces the regulatory molecule B7-H1, CCR7, and α4β7 integrin expression by blood DCs that leads to recruitment of cells to lymphoid tissues in a CCR7-dependent manner and to intestinal mucosa in an α4β7-dependent manner. The changes in DC phenotype and increased migration into lymphoid tissues and mucosa seen in pathogenic SIV infections do not occur in natural SIV hosts. Robust immune activation is evident in pathogenic and nonpathogenic SIV hosts. However, nonpathogenic hosts express factors antagonizing IFN activity and suppressing ISGs (i.e., tryptophan-depleting enzyme indoleamine 2,3 dioxygenase (IDO), an adenosine deaminase that suppresses ISG expression (ADAR)). Only nonpathogenic SIV hosts downmodulate their responses in the chronic phase of disease

6.3.5 DC Interactions with Mucosal Pathogens Leading to Increased SIV Infection

Sexually transmitted infections (STIs), such as HSV-2, increase the risk of HIV acquisition (Freeman et al. 2006). Macaque studies found that HSV-2 pre-exposure increases the frequency of vaginal SHIV-RT infection (Crostarosa et al. 2009). In vitro studies have shown that macaque DCs are susceptible to HSV-2 infection, inducing apoptotic death of DCs; decreasing the expression of HLA-DR, CD40, CD80, CD83, and CD86; increasing the release of IL-6, TNF-α, CCL3, and CCL5 but not IL-12 or IFN-α; and reducing their ability to stimulate SIV-specific T cell responses in vitro (Peretti et al. 2005). Interestingly, rectal HSV-2 infection of macaques resulted in increased frequencies of α4β7-expressing T cells in tissues and blood, paralleling the retinoic acid (RA)-dependent increase in α4β7 expression and HIV replication seen in HSV-2-infected DC–T cell mixtures in vitro (Martinelli et al. 2011). These findings underscore the importance of developing NHP models of STIs to study the role of DCs in SIV transmission and the prevention of SIV in the context of a coinfection.

6.4 Innate and Adaptive Immunity

6.4.1 Dysregulation of DC Recognition

Macaque DCs are responsive to exogenous TLR3-, TLR7-, and TLR9-mediated stimulation (Mehlhop et al. 2002; Barratt-Boyes et al. 2010; Teleshova et al. 2004b). Upon exposure to retroviruses, both mDCs and pDCs can be activated by ligation of TLR7, which recognizes single-stranded RNA (ssRNA), initiating the antiviral type I IFN response and the expression of downstream IFN-stimulated genes (ISGs). Multiple studies during both acute and chronic SIV infection have detected cytokines and chemokines associated with an enhanced IFN response (Co et al. 2011; Sanghavi and Reinhart 2005; Hofmann-Lehmann et al. 2002; Durudas et al. 2009; Abel et al. 2002, 2005; Milush et al. 2007), suggesting the involvement of pDCs (the primary producers of IFN-α). Recent data implicate RM TLR7 polymorphisms in SIV set point viral load, pathogenesis, and survival (Siddiqui et al. 2011). The importance of two cytosolic receptors for viral RNA, RIG-I and MDA5, has been documented in human and in experimental SIV infection of macaques, demonstrating that the IFN response is directed primarily by MDA5 (Co et al. 2011). However, the cellular origin of the MDA5- and RIG-I-derived IFN in macaques has not been shown.

TLR3 recognizes double-stranded RNA (dsRNA) and is expressed by mDCs but not pDCs (Ketloy et al. 2008). Since the complex secondary structures formed by the SIV genome have been shown to mimic dsRNA, it is not surprising that dsRNA is detected during SIV infection (Co et al. 2011) or that increased expression of TLR3 mRNA has been observed in the LNs throughout the course of SIV infection (Sanghavi and Reinhart 2005). Stimulation of TLR3 leads to NFκB activation, which both promotes the transcription of antiviral cytokines and also enhances viral transcription from the LTR. In fact, treatment of susceptible cells with the synthetic dsRNA, poly(I:C), has been shown to block SIV replication while poly(I:C) is simultaneously capable of stimulating the viral LTR (Sanghavi and Reinhart 2005). The timing of poly(I:C) treatment of DCs (relative to HIV exposure) has been shown to have variable impact on DC-driven HIV infection (Trapp et al. 2009; de Jong et al. 2010) (Derby N. and Robbiani M., unpublished).

6.4.2 DC Host Restriction Factors

Many host restriction factors are expressed by DCs to protect against HIV and SIV, including the APOBEC family, SAMHD1, and tetherin. APOBEC3G (A3G) and to a lesser extent A3F protect DCs from immunodeficiency virus infection by deaminating minus-strand viral reverse transcripts, thereby generating defective or noninfectious virus (Mangeat et al. 2003). SIV Vif acts on A3G and A3F, targeting them for degradation (Yu et al. 2004). A3B and A3C restrict SIV (not HIV) by deamination-dependent and -independent mechanisms (Yu et al. 2004). The highest expression of A3G and A3F is found in animals with long-term non-progressing infection (Muszil et al. 2011). A3G mRNA was found predominantly in monocytes and DCs with only a small proportion in $CD4^+$ T cells in the mesenteric LNs of macaques treated intracolorectally with poly(I:C) and IL-15, and this correlated with decreased viral load and improved prognosis following infection with SIVmac251 (Sui et al. 2010). Recent work showed that A3A depletion increases the infectivity of SIV and, moreover, *vpx*-deficient SIV displays a severe infectivity defect in DCs, which is partially rescued by A3A knockdown (Berger et al. 2011). This represents an interesting divergence of HIV and SIV evolution as only SIV expresses *vpx*.

Vpx is also important in the context of another barrier to HIV infection in myeloid cells, SAMHD1, which prevents HIV-1 infection in DCs by interfering with synthesis of viral cDNA (Hrecka et al. 2011; Laguette et al. 2011). It is involved in the regulation of the IFN pathway (Rice et al. 2009). In SIVsm, SIVmac, and HIV-2, SAMHD1 activity is overcome by Vpx, which targets SAMHD1 for proteasomal degradation (Sunseri et al. 2011; Laguette et al. 2011). Notably, there is no viral protein in HIV-1 that overcomes the action of SAMHD1, possibly suggesting a mechanism by which the virus failed to avert innate immunity. However, if *vpx* were present in the genome, a stronger immune response might be generated which could be detrimental to the virus. Thus, the lack of a viral antagonist might be the mechanism to avoid effective immune surveillance (Manel et al. 2010; Lim and Emerman 2011).

The existence of SAMHD1 explains why HIV does not replicate efficiently in DCs, but so far, it has only been studied in moDCs and macrophages. Lower viremia, reduced viral replication, and slower disease progression have all been observed in macaques infected with *vpx*-defective SIVsm or SIVmac (Gibbs et al. 1995; Hirsch et al. 1998). No studies have been published to date on SIV replication in macaque DCs and the implications with respect to SAMHD1. It remains to be seen whether there is an association between SIV in natural hosts and the ability of the virus to replicate in DCs and elicit potentially effective antiviral immunity.

An important late restriction factor involved in the type I IFN response is tetherin (BST-2, CD317). Tetherin prevents viral release, maturation, and spreading of infection by tethering newly synthesized virions to the cell surface. Tetherin is constitutively expressed by B cells, activated T cells, and pDCs and is inducible on other cell types by type I IFNs. Identified by its activity against HIV-1 lacking *vpu* (Van Damme et al. 2008; Neil et al. 2008), tetherin is degraded by Vpu through recruitment of the ubiquitin ligase complex. However, most SIVs do not express *vpu*, and still SIV is capable of tetherin-mediated restriction. SIV Env and Nef can counter RM and SM (but not human) tetherin through non-ubiquitin-related mechanisms by downregulating protein expression from the cell surface into endosomes and possibly rerouting protein trafficking (Gupta et al. 2009; Jia et al. 2009; Zhang et al. 2009). Importantly, tetherin is not only an effector of the IFN response, but also a negative feedback regulator of pDC IFN production during inflammation. Tetherin binds to a receptor, immunoglobulin-like transcript 7 (ILT7), on human pDCs, signaling inhibition of IFN release (Cao et al. 2009). ILT7 is uniquely expressed by human pDCs, but a similar regulatory system may exist in NHPs. Such negative feedback mechanisms may also impact on IFN-related hyperimmune activation, another mechanism by which HIV and SIV overcome innate immune responses and promote viral replication.

6.4.3 SIV Dysregulation of Effective Immune Responses

Impaired DC function is a fundamental component of SIV infection leading to immune activation, which is considered the best independent correlate of disease pathogenesis in experimental pathogenic SIV. Many factors possibly play into sustaining immune activation during chronic infection including aberrant TLR signaling (Sanghavi and Reinhart 2005), microbial translocation, preferential infection of central memory instead of effector memory T cells, depleting the T cell pool (Brenchley et al. 2010), and T cell bystander activation resulting from production of proinflammatory cytokines.

Acute SIV infection increases expression of costimulatory molecules by pDCs but not by mDCs (Kwa et al. 2011). Recruited into the lymphoid tissue and intestinal mucosa, pDCs produce IFN-α and contribute to immune activation (Kwa et al. 2011) as highlighted earlier. Semi-mature mDCs with reduced expression of costimulatory molecules and/or CD83 are detected in the lymphoid tissues of SIV-infected macaques (Soderlund et al. 2004; Zimmer et al. 2002). Semi-mature mDCs potentially

contribute to immune dysregulation since fully mature DCs are more adept at stimulating both $CD4^+$ and $CD8^+$ T cell responses (Frank et al. 2003). However, semi-mature mDCs are detected in vivo in animals with long-term stable infection (Wijewardana et al. 2010).

Dysregulated $CD8^+$ T cell responses are another feature of pathogenic SIV infection characterized by the expression of PD-1, a marker of $CD8^+$ T cell exhaustion. PD-1 is upregulated on SIV-specific $CD8^+$ T cells in vitro and in vivo (Velu et al. 2007), and blocking PD-1 in chronically SIV-infected macaques increases the proportion and quality of SIV-specific $CD8^+$ T cells, which is noteworthy considering that polyfunctional $CD8^+$ T cells are correlated with improved control of SIV (Hansen et al. 2009). During acute SIV infection, a ligand for PD-1 (B7-H1) is increased on mDCs (Xu et al. 2010) and pDCs (Barron et al. 2003; Wijewardana et al. 2010), and B7-H1 expression persists and correlates with PD-1 expression on T cells and impaired virus-specific T helper and CTL functions (Xu et al. 2010) (Fig. 6.3). Expression of both B7-H1 and PD-1 is stable in SIV controllers (Xu et al. 2010).

Immunosuppressive regulatory T cells (Tregs, $CD4^+CD25^{hi}FoxP3^+$ T cells) and IL-17-producing Th17 cells are also prominently affected during SIV infection, and their dysregulation is affected by infection-induced DC dysregulation and hyperimmune activation. An increase in the proportion of $CD8^+$ FoxP3-expressing Tregs has also been observed in the colorectal mucosa during pathogenic SIV infection, and this is associated with impaired antiviral responses and loss of viral control (Nigam et al. 2010). Th17 cells are also depleted during SIV infection. Instead of this resulting in suppressed inflammation, Th17 loss interferes with the control of gut pathogens (Raffatellu et al. 2008), particularly important given the increased gut permeability and microbial translocation that results from DC-mediated chronic immune activation. A related subset, the $CD8^+$ Tc17 cells, are depleted in SIV-infected macaques with end-stage disease (Nigam et al. 2011).

6.4.4 Pathogenic vs. Nonpathogenic SIV Infection

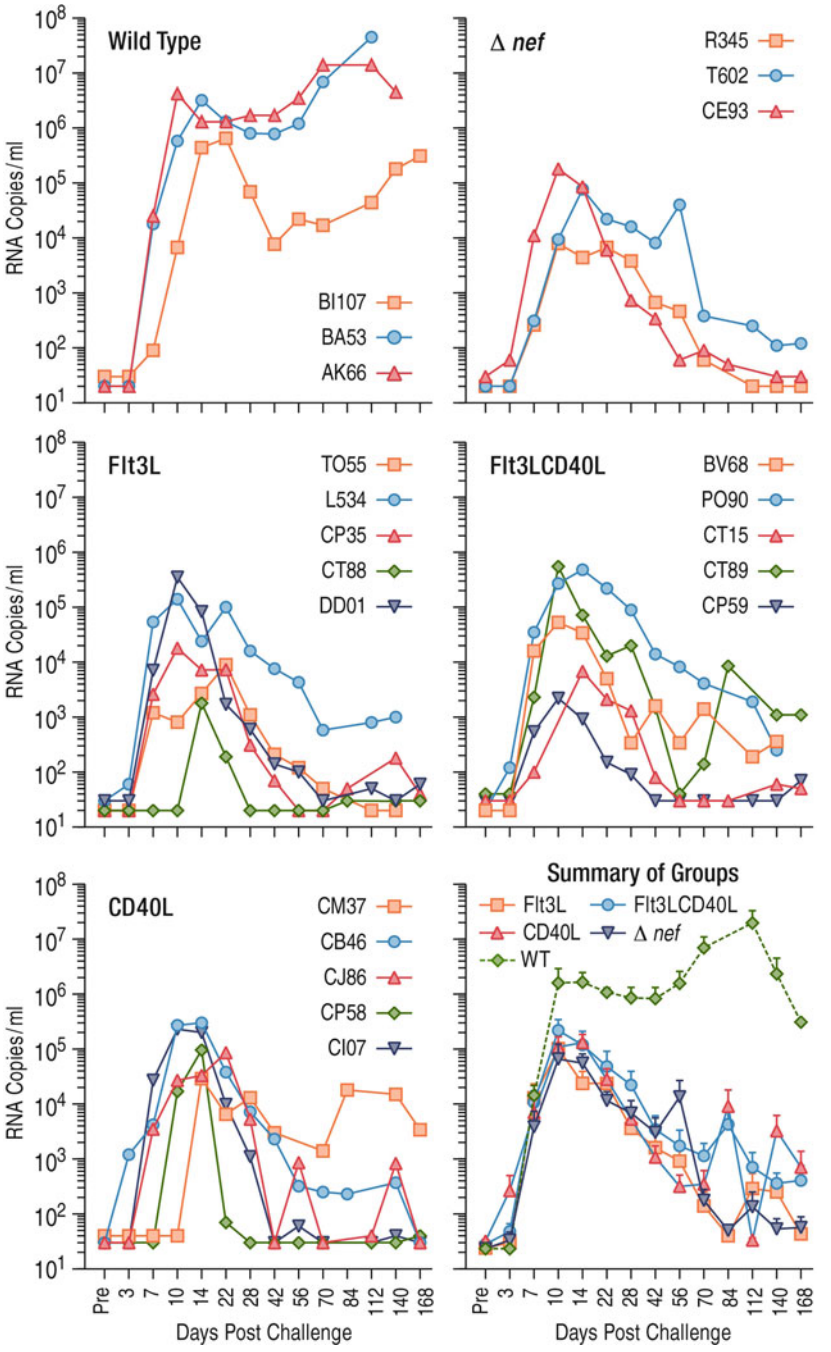
6.4.4.1 Attenuated Virus Infection

In vivo, SIV lacking *nef* (SIVΔ*nef*) results in highly attenuated infection (Kestler et al. 1991) and also provides a "vaccine effect," protecting against wild-type SIV challenge (Daniel et al. 1992). Nef is important for SIV replication in immature DC–T cell mixtures (Messmer et al. 2000, 2002). When immature moDCs or T cells were loaded with SIV prior to coculture, expression of Nef was shown to be critical for SIV replication in the DC–T cell mixtures while mature DCs overcame the need for Nef in this setting (Messmer et al. 2000, 2002). Interestingly, if moDCs were activated after capturing SIVΔ*nef*, the impaired replication in the cocultures was not rescued (Messmer et al. 2002). This indicates that maturing during migration to the LNs DCs that bear SIVΔ*nef* would induce strong immune responses in the absence of overwhelming infection (Messmer et al. 2000). Notably, introduction of *nef*

(via an adenovirus vector) or strong T cell activation (via SEB) rescues SIVΔ*nef* replication in the immature DC–T cell milieu (Messmer et al. 2002). Nef was demonstrated to induce a specific activation program in DCs characterized by cytokine and chemokine production (without changing DC membrane phenotype) (Messmer et al. 2002), suggesting mechanisms underlying the *nef*-dependent replication in the immature DC–T cell environment. The attenuated replication of SIVΔ*nef* was similarly apparent when virus-loaded immature DCs or T cells were injected subcutaneously into macaques and surprisingly resulted in greater macrophage infection compared to cell-free virus (predominantly T cell infections) (Ignatius et al. 2002), underscoring how cell-associated virus is infectious and that it might influence the earliest stages of cell–cell spread.

Exploring whether increased number of circulating (immature vs. mature) DCs would impact SIVΔ*nef* replication in vivo, we exploited the use of Flt3L treatment to mobilize (Coates et al. 2003; Teleshova et al. 2004a; Reeves et al. 2009) and CD40L (Mehlhop et al. 2002; Coates et al. 2003; Teleshova et al. 2004a, b) to activate macaque DCs. Increased number of circulating DCs (>tenfold more $Lin^-HLA\text{-}DR^+$ cells) did not alter the fate of $SIVmac_{239}\Delta nef$ applied to the tonsils (Fig. 6.4; Peretti S. and Robbiani M., unpublished). This likely reflects the predominance

Fig. 6.4 Increased number of circulating immature or partially matured DCs does not change the attenuated course of $SIVmac_{239}\Delta nef$ infection. Naive Indian RMs received Flt3L alone, Flt3L and CD40L, or CD40L alone (five animals per treatment group). Flt3L was administered for 7 days (100 μg/kg/day human Flt3L). To activate the cells, 0.6 mg/kg human CD40L trimer was given daily for 3 days after the Flt3L treatment. Another group of animals received only CD40L treatment. Animals were challenged with 2,000 $TCID_{50}$ of $SIVmac_{239}\Delta nef$ across the palatine/lingual tonsils at the peak of DC mobilization 4 days post FLt3L treatment (Teleshova et al. 2004a) (or 1 day after CD40L). Control groups of untreated animals (three animals each) were challenged with 2,000 $TCID_{50}$ of $SIVmac_{239}\Delta nef$ or wild-type $SIVmac_{239}$ across the tonsils. Individual plasma viral loads (SIV RNA copies/ml of plasma) are shown for each animal in the different groups over time and the mean SIV RNA copies/ml (±SEM) are shown for each group in the summary plot. DC numbers in the blood were measured by four color flow cytometry. In the Flt3L-treated group the percentage of $Lin^-HLA\text{-}DR^+$ cells increased from 3.0% ± 0.5 to 33.1% ± 4.1 while in the Flt3LCD40L-treated group this number increased from 2.2% ± 0.5 to 22.6% ± 6.2 (Mean ± SEM). The percentage of $Lin^-HLA\text{-}DR^+$ cells in the CD40L-treated group did not change after treatment. The total number of $Lin^-HLA\text{-}DR^+$ (DC), $Lin^-HLA\text{-}DR^+CD11c^+CD123^-$ (mDC), $Lin^-HLA\text{-}DR^+CD11c^-CD123^+$ (pDC), and $Lin^-HLA\text{-}DR^+CD11c^-CD123^-$ (double negative) subsets increased by 16.3-, 1.8-, 3.8-, and 29.5-fold, respectively, for the Flt3L-treated group and by 12.3-, 2.8-, 3.1-, and 21.6-fold, respectively, for the Flt3LCD40L-treated group (each number being the fold increase (Post- divided by Pretreatment) of the average of the five animals within each group). The mobilized DCs were functional as evidenced by IL-12 production in response to CD40L stimulation in vitro, which was increased after Flt3L treatment as previously reported (Teleshova et al. 2004a). Of note, CD40L activation in vivo appeared only partially effective at best. In the CD40L-treated animals CD80 and CD86 expression increased by only 1.49- and 2.15-fold on mDCs and 1.34- and 1.98-fold on pDCs (respectively), while the levels of the FLt3L/CD40L-treated animals were basically comparable to those of the Flt3L-treated animals (other than CD86 on pDCs in the Flt3L-treated animals which increased by 1.6-fold). The limited effect of CD40L in the FLt3L-treated animals is likely due to the dose of CD40L being insufficient to activate the increased number of DCs following mobilization and/or the fact that the activated DCs migrated to the CD40L injection site (from the blood)


Wild Type
BI107
BA53
AK66
Δ nef
R345
T602
CE93
Flt3L
TO55
L534
CP35
CT88
DD01
Flt3LCD40L
BV68
PO90
CT15
CT89
CP59
CD40L
CM37
CB46
CJ86
CP58
CI07
Summary of Groups
Flt3L
Flt3LCD40L
CD40L
Δ nef
WT
RNA Copies/ml
Pre
3
7
10
14
22
28
42
56
70
84
112
140
168
Days Post Challenge

of larger number of immature or suboptimally activated DCs that could not rescue the attenuated replication of the SIVmac$_{239}\Delta$*nef* in vivo, in contrast to the in vitro rescue with fully matured DCs.

6.4.4.2 SIV in Natural Hosts

SIV infections of natural hosts result in life-long nonpathogenic infection. More than 40 SIVs naturally infect African NHPs with most studies conducted in SMs, AGMs, and mandrills (Hirsch et al. 1989; Marx et al. 1991). Multiple mechanisms underlying nonpathogenic SIV infections were proposed including lack of chronic immune activation, reduced infection of central memory $CD4^+$ T cells, preserved or enhanced immune regeneration, absence of microbial translocation, and ability to mediate T helper function by $CD3^+CD4^-CD8^-$ T cells (Bosinger et al. 2011). There is no pDC depletion in the blood in nonpathogenic models of SIV infection (Mandl et al. 2008; Campillo-Gimenez et al. 2010).

The possibility that innate responses to SIV infection in natural SIV hosts are less pronounced was suggested based on the observation that SIV-infected pDCs from natural hosts produce less IFN-α in response to TLR stimulation ex vivo than do SIV-infected pDCs from experimental hosts (Mandl et al. 2008; Diop et al. 2008). Recent studies demonstrated that the acute phase of both pathogenic and nonpathogenic infections is associated with a robust innate immune response to the virus (Bosinger et al. 2011). However, a different pattern of innate immune responses and immune activation in SIV infection in natural hosts points to DCs and innate immunity as fundamental players in driving pathogenesis of experimental SIV infection and probably HIV infection of humans as well (Pereira and Ansari 2009). While in natural hosts immune activation is observed during acute infection, particularly production of IFN-α and expression of ISGs (Manches and Bhardwaj 2009; Bosinger et al. 2009; Jacquelin et al. 2009), it does not become chronic leading to loss of mucosal barrier integrity followed by microbial translocation as it does during experimental infection (Brenchley et al. 2006). Instead, immune activation in the periphery and in the mucosa resolves during the acute-to-chronic phase transition, preserving the mucosal barrier and preventing microbial translocation (Manches and Bhardwaj 2009; Bosinger et al. 2009; Jacquelin et al. 2009; Mir et al. 2011; Kaur et al. 2008). During acute infection of natural hosts, an anti-inflammatory cytokine profile emerges characterized by an increased frequency of Tregs and immunosuppressive molecules including TGF-β and IL-10 (Kornfeld et al. 2005) suggesting their involvement in the maintenance of a non-inflamed state. The resolution of immune activation also correlates with induction of PD-1 on $CD8^+$ T cells (Estes et al. 2008), which may serve a protective immunoregulatory role though implicated in pathogenesis in experimental infection. Preservation of the Th17 subset in natural infection may also be important for protection against microbial translocation, consistent with its loss during experimental infection being associated with loss of mucosal integrity. While both SIVmac and SIVsm infections in RMs enhanced α4β7 expression on blood pDCs and recruited pDCs to the intestinal

mucosa, SIVsm infection of SMs did not (Kwa et al. 2011). DC frequency, distribution, and functionality during pathogenic and nonpathogenic SIV infection are summarized in Fig. 6.3.

6.5 Harnessing DC Function to Induce Anti-SIV Responses

6.5.1 DC-Targeted Preventative and Therapeutic Vaccines

Strong virus-specific $CD4^+$ T helper responses and sustained CTL responses are crucial to control infection and DC-targeted approaches are being explored to most effectively induce these responses (Picker et al. 2012). Using Δ*nef*/wild-type infected RMs as a source of primed T cells, mature moDCs pulsed with aldrithiol-2 (AT-2)-inactivated SIV were shown to activate SIV-specific $CD4^+$ and $CD8^+$ T cells in vitro (Mehlhop et al. 2002; Frank et al. 2003). Similarly, DCs transfected with SIV gag mRNA stimulate robust recall $CD8^+$ T cell responses, with greater $CD4^+$ T cell responses when antigen is targeted to the lysosome (Melhem et al. 2007). These data suggested that antigen-loaded moDCs have the potential to activate primed T cells, supporting the strategy to explore their potential as a therapeutic vaccine. Subcutaneous injection of autologous AT-2 SIV-loaded moDCs into infected RMs resulted in the reduction of SIV RNA and DNA, which correlated with SIV-specific humoral and cellular responses (Lu et al. 2003). Similar success was then observed in chronically infected people immunized with AT-2 HIV-loaded DCs (Lu et al. 2004). Thus, inactivated whole virus-pulsed DCs represent a promising strategy in therapeutic vaccination to help control virus replication and disease progression.

There have also been several studies examining the potential of antigen-loaded DCs as preventative vaccines. Intradermal injection of autologous antigen-loaded DCs elicited SIV-specific T and B cell responses and, although animals were not protected from infection upon oral challenge, the set point viremia in the vaccinated animals was at least 1 log lower than that seen in non-vaccinated animals (Wagner et al. 2002). Similarly, animals immunized with DCs loaded with an envelope peptide cocktail vaccine mounted antigen-specific T cell responses and ultimately reduced plasma viremia following virus challenge with SHIV89.6P (Nehete et al. 2005). In an attempt to better target antigens to appropriately activated DCs in order to activate more potent responses, novel strategies involving two types of DC receptors are being explored: receptors mediating antigen uptake (e.g., CD205) and TLRs. Immunization of RMs with CD205-targeted HIV gag with poly(I:C) as an adjuvant resulted in multifunctional Th1 responses and better induction and recall of CD8 immunity, as compared to non-targeted gag immunization (Flynn et al. 2011).

Another approach has been to use recombinant vectors to infect DCs and load them with antigens endogenously. moDCs infected with recombinant canarypox virus or adenovirus (AdV) vectors can stimulate virus-specific T cell responses

in vivo (Villamide-Herrera et al. 2004; Brown et al. 2003). AdV is suggested to be highly efficient in transducing DCs (Brown et al. 2003). AdV-based vaccines administered directly to monkeys induced robust T cell responses and protected against SHIV challenge (Shiver et al. 2002). However, the STEP vaccine trial using three separate replication-defective Ad5 vectors encoding a conserved HIV *gag*, *pol*, or *nef* gene (Merck 023/HVTN 502) was halted as the vaccine was ineffective (Buchbinder et al. 2008). Studies addressing potential reasons for the failure of this vaccine are being done (Benlahrech et al. 2009; Patterson 2011).

Strategies to increase the number of circulating DCs and make them more accessible for vaccine antigens are also being tested. Flt3L treatment of macaques led to mobilization of immature and functional DCs in blood and LNs (Teleshova et al. 2004a). Treatment of SIV- and SHIV-infected macaques with Flt3L resulted in mDC activation and mobilization in the absence of any changes to viral load or $CD4^+$ T cells, suggesting that FLt3L could be added to mDC-targeted vaccines without the risk of creating virus-permissive environment (Reeves et al. 2009). Vaccines would then need to target these mobilized DCs properly (perhaps through CD205 and/or other DC-specific markers) to ensure efficient presentation to the immune system.

6.5.2 Mucosal Vaccination

Since DCs reside in the mucosal surfaces, it is tempting to explore whether they can be harnessed to improve vaccine efficacy. One way to explore this was pursued by exploiting the defective replication of $SIVmac_{239}\Delta nef$ in the tonsillar DC–T cell milieu. Atraumatic application of attenuated $SIVmac_{239}\Delta nef$ vaccine to the tonsils in macaques protects against tonsillar or rectal challenge with infectious $SIVmac_{251}$ (Tenner-Racz et al. 2004; Stahl-Hennig et al. 2007), but this was comparable to the protection afforded by intravenous SIVΔnef infection.

Another strategy is to use inactivated viruses to target mucosal DCs, like that used in the autologous moDC vaccinations (above). AT-2 SIV (that interacts authentically with target cells and activates $CD4^+$ and $CD8^+$ T cells when presented by DCs) (Frank et al. 2003) was used to target the DCs within the tonsillar tissues and tonsillar vaccination of SIV-infected macaques on ART with AT-2 SIV adjuvanted with polyICLC (clinical grade poly(I:C), but not CpG-C ISS-ODNs) resulted in suppressed viremia once ART was removed (Vagenas et al. 2010). The results with respect to CpG-C ISS-ODNs were surprising since prior work indicated that CpG-C ISS-ODN in combination with AT-2 SIV activated pDCs, stimulated IFN-α production, and boosted SIV-specific T cell responses in vitro (Teleshova et al. 2004b). Interestingly, TLR9 expression was shown to decrease during acute SIV infection despite the absence of TLR9 ligands from the virus (Sanghavi and Reinhart 2005) suggesting that noninvasive mucosally applied therapeutic vaccines augmented with polyICLC show promise for controlling HIV/SIV replication.

6.6 Conclusion and Future Directions

Considerable progress has been made to define the role of DCs in SIV transmission and pathogenesis suggesting paradoxical functionality of DCs during infection, with the bulk of the NHP DC–SIV biology mirroring that of the human DC–HIV interplay. It remains unclear how beneficial and detrimental DC responses mounted during SIV are tipped toward the propagation of infection rather than the induction of effective SIV-specific responses. The hallmark of pathogenic SIV infection is aversion and dysregulation of DC-mediated responses that start very early during SIV infection and lead to extensive innate immune responses. In contrast to non-pathogenic SIV infection, these responses are not resolved during the transition from acute to chronic infection. This lack of immune resolution in the pathogenesis of HIV is supported by data in the viremic non-progressors. Although DCs are dysregulated by SIV, proper stimulation may block infection of DCs and transmission to T cells. To this end, there are opportunities to improve anti-SIV/HIV immunity by therapeutic strategies based on providing DCs with appropriate antigen and adjuvants. The best choice of stimulus to induce appropriate DC maturation for induction of immune responses remains to be determined. There are opportunities for successful interventions to prevent transmission in the initial stage of infection where there is the greatest viral vulnerability at the portal of entry. In conclusion, significant progress has been made in recent years, but many important questions on the role of DCs in SIV infection remain to be answered.

Acknowledgements N.T. is supported by the Swedish Ministry of Foreign Affairs, and the USAID Cooperative Agreement GPO-00-04-00019-00. M.R. is supported by NIH grants R37 AI040877, R01s DE018293, and AI084133, the Swedish Ministry of Foreign Affairs, and the USAID Cooperative Agreement GPO-00-04-00019-00. M.R. is a 2002 Elizabeth Glaser Scientist.

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Chapter 7
Interactions Between HIV-1 and Innate Immunity in Dendritic Cells

Aymeric Silvin and Nicolas Manel

Abstract Dendritic cells couple pathogen sensing with induction of innate and adaptive immune responses. Pathogen sensing in dendritic cells relies on interactions between molecular patterns of the pathogens and germline-encoded, also referred to as innate, receptors. In this chapter, we analyze some of the interactions between HIV-1 and the innate immune system in dendritic cells. The HIV-1 replication cycle is constituted by an extracellular and an intracellular phase. The two phases of the cycle provide distinct opportunities for interactions with cell-extrinsic and cell-intrinsic mechanisms in dendritic cells. According to the types of dendritic cells, the mechanisms of innate interactions between dendritic cells and HIV-1 lead to specific responses. These innate interactions may contribute to influencing and shaping the adaptive immune response against the virus.

7.1 Introduction

7.1.1 General Concepts in Innate Immunity

Innate immunity results from processes of germline-encoded interactions with pathogens that lead to effector immune responses but do not require clonal cellular selection (Medzhitov and Janeway 1997). A large number of cell types contribute to innate immunity. In the hematopoietic system, dendritic cells (DCs) play a unique role because of their ability to couple innate immune responses with the stimulation of naïve T cells (Steinman and Hemmi 2006).

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_7,

The principle of innate immunity proposes that the presence of molecular patterns in pathogens but not in the host (pathogen-associated molecular patterns, PAMPs) is sensed by the host, leading to initiation of an immune response (Janeway 1989). This is accompanied by activation of the DCs, often referred to as "maturation" in the case of human DCs. PAMPs are thus initially defined functionally. The best-described sensors of PAMPs are pathogen-recognition receptors (PRRs) that are directly associated with PAMPs. In these cases, the PAMPs are the direct molecular substrate of sensing and thus satisfy a biochemical definition. Innate sensing can also be indirect. For example, in plants, members of the NOD-like receptor (NLRs) proteins family recognize effector-mediated modifications of host proteins (Maekawa et al. 2011). In mammalian cells, the detection of cytosolic DNA can be mediated indirectly through its transcription by RNA polymerase III followed by detection through the RIG-I pathway (Chiu et al. 2009). Thus, the presence of such PAMPs is sensed indirectly in the host, nonetheless leading to initiation of an innate immune response.

In addition, DCs express a number of surface molecules, such as C-type lectins, that can interact with pathogens without inducing the typical DC maturation that corresponds to the classical definition of PRRs (Medzhitov and Janeway 1997). Triggering of these receptors may however modulate signaling and responses during activation of other PRRs.

Innate immunity, whether it is cell-extrinsic or cell-intrinsic (Iwasaki and Medzhitov 2010) (Fig. 7.1), is distinct from intrinsic immunity (Bieniasz 2004). Intrinsic immunity refers to mechanisms, in general to restriction factors, that reduce viral replication in the infected cell, but that do not typically induce an effector response through signaling (Neil et al. 2008; Stremlau et al. 2004; Sheehy et al. 2002). Proteins that play a role in intrinsic immunity can often be induced as part of the effector response of innate immunity (Asaoka et al. 2005; Peng et al. 2006; Amit et al. 2009).

7.1.2 Innate Interactions in the Different Types of DCs

In addition to their unique role in coupling innate and adaptive immunity, DCs uniquely express a great diversity of PRRs in the hematopoietic system. They also constitute a heterogeneous population of cells, and several subsets have been identified in different organs of mice and humans (Geissmann et al. 2010; Vremec et al. 1992). Generally, the different DC subsets express different sets of extrinsic (see for instance, van der Aar et al. 2007; Flacher et al. 2006) and intrinsic (see for instance, Luber et al. 2010; Robbins et al. 2008) PRRs (Iwasaki and Medzhitov 2010). They also express combinations of other types of receptors, which do not satisfy with the functional definition of PRRs (because their triggering does not lead to the expression of costimulatory molecule expression), but that can modulate signaling by PRRs in DCs (e.g., some C-type lectins, scavenger receptors). In addition, activation of a given PRR may induce different types of response in different DC subsets (for example, see below for TLR7 and HIV-1 RNA). Understanding how the

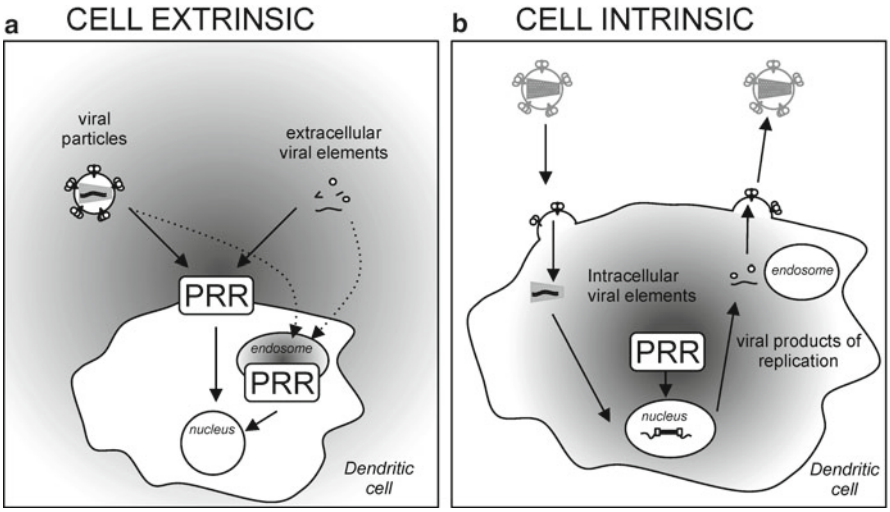

Fig. 7.1 Cell-extrinsic and cell-intrinsic sensing of HIV-1. (**a**) During the extracellular phase of the virus, infectious HIV-1 particles and elements from these particles may interact with cell-extrinsic pattern recognition receptors (PRRs) at the cell surface or in endosomes of DCs, leading to an innate immune response. (**b**) After receptor engagement and membrane fusion, the virus enters an intracellular phase of replication. During this phase, viral elements initially contained in the particle may interact with cell-intrinsic PRRs. In addition, the process of viral replication, including the production of newly synthesized viral elements, may also interact with cell-intrinsic PRRs

pathways of innate interactions between HIV-1 and the different types of DCs operate during pathogenic and nonpathogenic infections may be particularly relevant for HIV-1 vaccination.

7.1.3 Interactions Between Innate Immunity and the HIV-1 Replication Cycle

Lentiviruses have coevolved with several mammalian species (Sharp and Hahn 2011). Thus, it is likely that ancient innate interactions between lentiviruses and mammals are conserved in HIV-1, but also that interactions between HIV-1 and the innate immune system are specific to humans. While innate immunity is most easily appreciated experimentally in antigen-naïve hosts shortly after pathogen exposure, interactions between HIV-1 and immune responses likely contribute to all aspects of HIV-1 infection in vivo, including pathogenesis and immune control. As a retrovirus, HIV-1 encodes only a limited number of proteins and most of its biochemical composition is inherited from the host cell. This does not offer obvious unique molecular patterns for innate immunity comparable to lipopolysaccharides in bacteria, for instance. The two distinct phases in the viral life cycle constitute distinct opportunities for innate interactions (Fig. 7.1). During its extracellular phase as a physical

particle, the components of the particle can act as substrates for cell-extrinsic innate interactions at the cell surface or in endosomal compartments (Iwasaki and Medzhitov 2010). Such cell-extrinsic interactions do not require the DCs to be infected by the virus (Iwasaki and Medzhitov 2010). In the case of retroviruses like HIV-1, this indicates that cell-extrinsic interactions occur before fusion of the cellular and viral membranes. These interactions are sensitive to molecules of the particles, which can be contained within live particles or soluble components such as debris. During its intracellular phase, HIV-1 largely relies on normal cellular processes such as transcription, translation, trafficking, and budding. In this phase, viral components and products can act as substrates for cell-intrinsic innate interactions with PRRs such as cytosolic sensors. Unlike cell-extrinsic interactions, cell-intrinsic interactions require the DCs to be infected by the virus (Iwasaki and Medzhitov 2010). In the case of retroviruses like HIV-1, this indicates that these interactions occur after fusion of the cellular and viral membranes. According to the mechanism involved, cell-intrinsic interactions may or may not require productive replication of HIV-1 in the infected DCs. These interactions can occur for instance when incoming viral components constituting misplaced biomolecules (physically or temporally) trigger innate cytosolic sensors, or when the viral replication process modifies the host cell.

A hallmark of innate immune responses against viruses is the production of type I interferon because of its ability to induce expression of a high number of genes encoding for proteins with antiviral properties (Taniguchi and Takaoka 2002). Importantly, type I interferon induces antiviral activities in *cis* (in the infected cell) and in *trans* (in noninfected cells). In the case of HIV-1, isolation of the virus from infected PBMC initially necessitated the use of a neutralizing antiserum to human interferon-alpha (IFN-α) (Barré-Sinoussi et al. 1983), highlighting the antiviral effect of type I interferon during HIV-1 replication. However, while replication of many viruses in vitro often recapitulated the induction of type I interferon in susceptible cells, the replication of HIV-1 in susceptible primary cells or cell lines did not typically induce detectable levels of type I interferon (Goldfeld et al. 1991).

Recent work in the field of innate immunity has provided mechanistic insights into the diverse interactions between HIV-1 and innate immunity, particularly in DCs (Fig. 7.2). Effector mechanisms of innate interactions between HIV-1 and DCs include the activation of adaptive immunity through antigen presentation and expression of costimulatory molecules, the production of soluble and cell surface mediators of innate immune regulations, and the production of type I interferon that induces intrinsic immunity, including the expression of restriction factors and induction of antiviral states. From the standpoint of viral fitness, innate interactions with HIV-1 may be either antiviral or proviral, and natural selection of HIV-1 at a balance between the two has likely contributed to the epidemic success of the virus.

In the following paragraphs, we select and analyze non-exhaustively some of the reported interactions between HIV-1 and the innate immune system in DCs. In the first part, we analyze cell-extrinsic interactions. In the second part, we analyze cell-intrinsic interactions and we discuss how these interactions may be of particular relevance for antigen presentation and for distinctive response mechanisms between ongoing viral replication and viral debris.

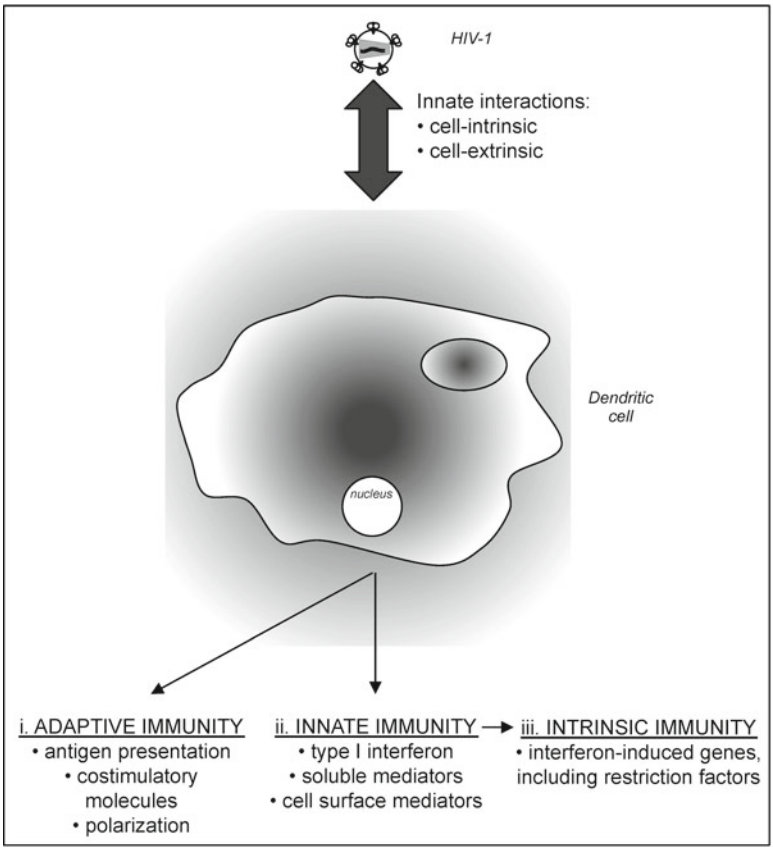

Fig. 7.2 Effector mechanisms of the innate interactions between HIV-1 and dendritic cells. Upon engagement of cell-extrinsic or cell-intrinsic innate interactions with dendritic cells, effector mechanisms are induced. These include (1) the activation of adaptive immunity through antigen presentation, expression of costimulatory molecules, and polarization and (2) the production of type I interferon and soluble and cell surface mediators of innate immune regulations. Effector mechanisms of innate immunity will lead to the induction of (3) intrinsic immunity, including the expression of restriction factors

7.2 Cell-Extrinsic Innate Interactions

During the phase of its replication cycle as an extracellular particle, HIV-1 is susceptible to sensing by cell-extrinsic sensing mechanisms. In this chapter, we expose some of the different pathways engaged by interaction between HIV-1 in DCs, including cell surface PRRs.

7.2.1 TLR7 and TLR8 in Monocyte-Derived DCs

Because the HIV-1 particles contain genomic single-stranded RNAs, the role of TLR7 and TLR8 was investigated. In monocyte-derived DCs (MDDCs), it was found that the viral RNA can engage a TLR8 pathway, leading to modulation of viral transcription (Gringhuis et al. 2010). Silencing of MyD88 did not modify the amount of integrated HIV-1 DNA. However, silencing of MyD88 or TLR8 reduced the amount of mRNA coding for Tat–Rev and Vpu produced, suggesting that TLR8 signaling impacts viral transcription.

Since HIV-1 transcription is regulated by NF-κB (Griffin et al. 1989), its role in this process was investigated. Using chromatin immunoprecipitation, the p65 subunit of NF-κB and RNAPII were found to be associated with the HIV-1 LTR. While p65 recruitment required TLR8, RNAPII recruitment was found to be TLR8 independent. During transcription, the RNAPII C-terminal domain is phosphorylated at the position of Ser5, a modification which is required for initiation. On the HIV-1 LTR, this modification required TLR8 signaling through the recruitment of a CDK7–cyclin H subcomplex.

Engagement of TLR7 and TLR8 by their ligand typically induces production of type I interferon, activation of DCs, and upregulation of costimulatory molecules (Larange et al. 2009). However, HIV-1 infection of MDDCs does not induce type I interferon or DC maturation (Manel et al. 2010; Granelli-Piperno et al. 2004; Harman et al. 2011). Given the process of TLR7 and TLR8 engagement by HIV-1 on DCs shown in the latter work, it suggests that TLR8 engagement does not lead to DC activation. Alternatively, the HIV-1 single-stranded genomic RNA found in the particle may not behave as an agonist for TLR8. Future experiments are needed to distinguish between these possibilities, and to determine for instance how HIV-1 signaling through TLR8 may impact the activity of agonist TLR8 ligands that can induce type I interferon production and DC activation.

7.2.2 DC-SIGN in Monocyte-Derived DCs

DC-SIGN, a C-type lectin, interacts with the glycosylated HIV-1 envelope protein (Geijtenbeek et al. 2000). Using B cells expressing of DC-SIGN, it was initially considered that DC-SIGN is implicated in the phenomenon of *trans*-enhancement of HIV-1 infection (Geijtenbeek et al. 2000; Wu et al. 2004), defined as the ability of DCs to enhance infection of T cells in *trans* in the presence of virus at suboptimal multiplicity of infection (MOI) (Barker et al. 1992). However, later work demonstrated that in DCs derived from primary cells, DC-SIGN is not implicated in *trans*-enhancement of infection (Gummuluru et al. 2003; Granelli-Piperno et al. 2005; Boggiano et al. 2007). Instead, DC-SIGN expression on MDDCs was shown to play a role in the physical "capture" of viral particles, using loading of DCs at high MOI and removal of uncaptured virus (Turville et al. 2004). The nature and functions of

the compartment in which HIV is captured in a DC-SIGN-dependent manner at high MOI are not completely understood (Hanley et al. 2010). Similarly, the process of *trans*-enhancement at suboptimal MOI is still under investigation. DCs that have captured HIV-1 can transmit virus to T cells, but whether this process of transmission after capture at high MOI and the process of trans-enhancement at suboptimal MOI in the presence of virus overlap is not known.

Recent work has investigated the role of HIV-1 and DC-SIGN interactions in the context of innate immunity. Use of an antibody against DC-SIGN, H-200, to stimulate DC-SIGN-mediated signaling (Hodges et al. 2007), followed by a phosphoproteome analysis, revealed that DC-SIGN stimulation induces phosphorylation of LARG. LARG is a leukemia-associated Rho guanine nucleotide exchange factor known to play a role in the activation of guanine nucleotide exchange factor (GEF): accordingly, DC-SIGN stimulation by the H-200 antibody activated Rho-GTPase activity through LARG. Silencing of LARG in DCs reduced the replication of the virus in DC–T-cell cocultures, as measured by reverse transcriptase activity in culture supernatants. Stimulation of DC-SIGN by H200 also led to down-regulation of the expression of pro-inflammatory cytokines. These data suggested that signaling through DC-SIGN possibly reenforces the preservation of an immature DC phenotype. It will be important to determine whether the canonical PRR-mediated DC activation pathways are susceptible or resistant to such effect of preservation of an immature state in DCs.

DC-SIGN triggering by the HIV-1 envelope glycoprotein has also been shown to induce a Raf-1-dependent signaling pathway (Gringhuis et al. 2007, 2010). Treatment of DCs with compounds that inhibit Raf-1 activity led to a decrease in HIV-1 replication. VSV-G-pseudotyped HIV-1 particles that do not bind DC-SIGN did not induce early production of Tat–Rev mRNA, suggesting that DC-SIGN signaling is required for transcription from the HIV-1 LTR. Interestingly, in conditions used for HIV-1 infection in this study, the restriction imposed by SAMHD1 is active and the majority of cells in the culture are not infected. In contrast, all DCs in the culture are presumably sensitive to DC-SIGN and TLR8 signaling, which are events occurring upstream of the SAMHD1 restriction. Whether DC-SIGN and TLR8 signaling in HIV-1-exposed but non-productively infected DCs could modulate the expression of the HIV-1 LTR in *trans* in the minority fraction of productively infected cells remains to be determined.

7.2.3 TLR7 in Plasmacytoid DCs

Plasmacytoid DCs (pDCs) exposed to HIV-1 particles secrete large amounts of IFN-α. This innate sensing mechanism requires TLR7 (Lepelley et al. 2011; Beignon et al. 2005) and, contrary to MDDCs, human pDCs do not express TLR8. The use of antibodies against CD4 and the envelope glycoprotein and of envelope-deficient virus demonstrated that the binding to the HIV-1 envelope to CD4 is required for IFN-α production by pDCs (Beignon et al. 2005). In addition,

acidification of the endocytic compartment of pDC and dynamin is also required for IFN-α production (Pritschet et al. 2012). These data support a role for HIV-1 endocytosis, in an envelope-dependent manner, for induction of interferon production by pDCs. Current data indicates that the main inducer of type I interferon in these conditions is TLR7 (Beignon et al. 2005; Lepelley et al. 2011). It was also observed that a TLR7-independent mechanism is active when an infected $CD4^+$ T cell line is cocultured with pDCs (Lepelley et al. 2011; Schmidt et al. 2005). It will be important to examine interactions between these two pathways.

Interestingly, while TLR7 expression has been reported in MDDC, HIV-1 does not induce type I interferon through TLR7 in MDDC (Gringhuis et al. 2010; Manel et al. 2010). In contrast, activation of TLR7 by the HIV-1 RNA in pDC leads to type I interferon production through IRF7 (Beignon et al. 2005; Lepelley et al. 2011). IRF7 is expressed constitutively in pDCs but not in MDDC (Izaguirre et al. 2003). In MDDCs, IRF7 behaves similarly to type I interferon response genes, possibly in the context of a positive feedback loop (Marié et al. 1998). It will be important to determine whether HIV-1 activates TLR7 in MDDCs when IRF7 is present.

7.2.4 Indirect Innate Interactions with Other Cells

In addition to direct sensing of HIV-1 by cell-extrinsic sensors, the extracellular microenvironment can influence the interactions between HIV-1 and DCs. Neighboring infected cells or infected tissues may produce "danger signals" that contribute to these influences (Matzinger 2002). How DCs process these complex interactions and how this impacts the HIV-1 cycle in these conditions are major questions. We discuss here some studies related to innate interaction between HIV-1, DCs, and other cell types.

7.2.4.1 Innate Interactions Between Epithelial Cells, DCs, and HIV-1

Innate interactions may occur between HIV-1 and DCs in an indirect manner. This occurs when a response to HIV-1 in DCs is observed in the presence or due to the activity of a distinct cell type. Indirect innate interactions with other cells are illustrated for instance by the interaction between HIV-1, DCs, and thymic stromal lymphopoietin (TSLP). In the presence of HIV-1 particles, epithelial cells produced TSLP (Fontenot et al. 2009) which is implicated in DC maturation (Soumelis et al. 2002). However, the molecules involved in the direct interaction with HIV-1 on epithelial cells leading to TSLP production are not known. TSLP acts on DCs to up-regulate the expression of costimulatory molecules such as CD80, CD86, and CD40. Myeloid DCs exposed to HIV-induced TSLP in turn induced a significant expansion of naïve $CD4^+$ T cells after 7 days in a mixed lymphocyte reaction. In cocultures of DCs and $CD4^+$ T cells, infection with HIV-1 was also increased if

the DCs had been treated with TSLP. Overall, this work suggested that in the presence of HIV-1, TSLP secretion by epithelial cells induced a DC maturation state that had in turn a proviral effect on replication in $CD4^+$ T cells. It is not known if the effect of TSLP in this context illustrates the general capacity of mature DCs to better support HIV-1 replication in $CD4^+$ T cells (Izquierdo-Useros et al. 2007), or whether TSLP induces specific proviral factors in DCs. It will also be important to consider the increased maturation of DCs due to the innate interaction between epithelial cells and HIV-1 in the context of the ability of DC-SIGN signaling to preserve an immature state in DCs, as described previously (Hodges et al. 2007).

7.2.4.2 Innate Interaction Between NK Cells, DCs, and HIV-1

NK cells constitute another cell type of the immune system that play an important role in innate immune response. Recent work indicated that MDDCs exposed to HIV-1 have reduced sensitivity to NK cell-mediated killing (Melki et al. 2010). This was associated with induction of the high mobility protein group B1 (HMGB1) in the coculture (Saïdi et al. 2008) and in up-regulation of anti-apoptotic factors c-IAP2 and c-FLIP in DCs. These activities imply a mechanism of sensing of the viral particles or of the viral replication cycle in the NK-DC coculture, which remains uncharacterized.

7.3 Cell-Intrinsic Interactions

In this chapter, we expose some of the pathways engaged by the interactions between HIV-1 and DCs during the intracellular phase of the replication cycle of the virus.

7.3.1 Envelope-Dependent Sensing of Infected Cells

As described previously, a main component of the type I interferon response against HIV-1 in pDCs implicates cell-extrinsic sensing through TLR7 of the RNA contained in the viral particles. However, in Gen2.2 cells, a cell line that maintains a phenotype related to pDCs, depletion of TLR7 protein by RNAi did not completely abolish the production of type I interferon when cocultured with an HIV-1-infected $CD4^+$ T cell line (Lepelley et al. 2011). This suggested that a TLR7-independent mechanism could lead to type I interferon production after coculture with an infected $CD4^+$ T cell line. Accordingly, coculture of 293T cells, which are TLR7 negative, with HIV-1-infected $CD4^+$ T cell line led to activation of the interferon-beta (IFN-β) promoter. This required expression of a functional envelope protein in the infected cell line, suggesting that a cytosolic pathway of sensing of HIV-1-infected cells may be present in 293T cells and in Gen2.2 cells. In 293T cells, the promoter activation

required the transcription factor IRF3. Unlike in pDCs, production of type I interferon protein was not demonstrated in 293T cells. It will be important to investigate if the same envelope-dependent pathway is active in Gen2.2 cells and 293T cells.

7.3.2 Negative Regulation of Cell-Intrinsic Innate Sensing by TREX1

The SET complex is a DNA repair complex associated with the endoplasmic reticulum that contains DNAses. TREX1, an exonuclease active on single-stranded DNA, is part of the SET complex (Chowdhury et al. 2006) and is a susceptibility gene for the Aicardi–Goutières syndrome (Lee-Kirsch et al. 2007; Rice et al. 2007; Crow and Rehwinkel 2009). It was first shown that TREX1 plays a role during HIV-1 infection in limiting auto-integration, leading to increased HIV-1 integration and replication (Yan et al. 2009). In addition to this role in regulating integration, it was later found that TREX1 is a negative regulator of the type I interferon response against HIV-1 (Yan et al. 2010). The role of TREX1 in regulating the IFN response was demonstrated first in mouse embryonic fibroblasts (MEFs). *Trex1*$^{-/-}$ MEFs produced more IFN-β and IL-6 in response to HIV-1 infection compared to wild-type MEFs after approximately 1 day of infection. The role of TREX1 has not yet been addressed in human DCs, but it has been addressed in the related human monocyte-derived macrophages. As in MEFs, reduction of TREX1 protein by RNAi led to induction of type I interferon in an IRF3-dependent manner following HIV-1 infection. Interestingly, unlike the interferon production observed in DCs in the presence of Vpx, production of type I interferon in human macrophages deficient for TREX1 was not apparent after 1 day of infection and increased incrementally at day 4 and day 7 after infection. In *Trex1*-deficient MEFs, production of type I interferon was shown to require TBK1, IRF3, and STING, which are components otherwise implicated in type I interferon production following intracellular sensing of DNA (Ishii et al. 2005; Stetson and Medzhitov 2006; Ishikawa and Barber 2008). However, the cellular sensing mechanism in *Trex1*-deficient cells is not established. Interestingly, MEFs deficient for *Trex1* had a most profound increase in type I interferon following transfection of ssDNA, compared to ssRNA, RNA:DNA duplexes, or dsDNA, suggesting that the response to HIV-1 infection implicated ssDNA molecules which could possibly correspond to the ssDNA intermediate during first-strand synthesis of the revere transcription step. Overall, this work demonstrated the critical role of the TREX1 protein as a negative regulator of the cell-intrinsic sensing of HIV-1.

7.3.3 Cell-Intrinsic Sensing of HIV-1 Capsid by Simian TRIM5

The implication of TRIM5 in innate responses was initially suggested by its ability to potentiate expression from an IFNB1-luciferase reporter system (Pertel et al. 2011). Reduction of TRIM5 protein expression in THP-1 cells differentiated in macrophage-like cells led to a reduced expression of inflammatory response

gene such as CXCL10, CCL8, and IL-6 upon LPS stimulation. The participation of TRIM5 to LPS-induced signaling was shown to implicate the TAK1 kinase and the synthesis of free K6-linked ubiquitin chains (Pertel et al. 2011). Given the role of TRIM5 in restriction of retroviral replication, the interaction between the restrictive function of TRIM5 and this role in signaling was investigated. In human DCs, murine leukemia virus particles were analyzed. Murine leukemia virus particles restricted by TRIM5 (N-tropic) induced expression of pro-inflammatory cytokines including CXCL9, CXCL10, IFIT1, and IFIT2, while unrestricted particles (B-tropic) did not. The low affinity of human TRIM5 for HIV-1 capsid (Li et al. 2006) suggests that this pathway is not active in human DCs upon HIV-1 infection (Pertel et al. 2011). This is consistent with results obtained in human DCs infected with HIV-1 (Manel et al. 2010). Infection of DCs with HIV-1 particles, irrespective of the envelope used, did not induce innate sensing in DCs, demonstrating that the presence of the incoming HIV-1 capsid is not sufficient to trigger an innate response in human DCs.

Owl monkey cells express a TRIM5 ortholog that binds to the HIV-1 capsid and restricts HIV-1 replication, because the TRIM5 protein is fused to a Cyclophilin A domain that targets the TRIMCyp fusion protein to the viral capsid (Sayah et al. 2004). In owl monkey kidney cells, infection with HIV-1 particles containing capsid but devoid of viral genome also induced expression of pro-inflammatory cytokines including PTGS2, IFIT1, and IFIT2 (Pertel et al. 2011). It will be important to determine the response of owl monkey DCs to HIV-1 infection.

7.3.4 Cryptic Innate Sensing of HIV-1 in Human DCs

7.3.4.1 A SAMHD1-Restricted Capsid-Dependent Innate Sensing

A hallmark of innate sensing in DCs is the induction of costimulatory molecule expression. Exposure of DCs to HIV-1 did not lead to costimulatory molecule expression (Manel et al. 2010; Granelli-Piperno et al. 2004) suggesting that there was no sensing mechanism for HIV-1 in human MDDCs, or that the virus either evaded or inhibited this response. Investigation of the potency of different lentiviral vectors to mediate gene transduction in DCs demonstrated that human MDDCs were intrinsically largely resistant to HIV-1 infection (Mangeot et al. 2000; Nègre et al. 2000) owing to a block at the level of reverse transcription that could be alleviated by the Vpx protein found in other lentiviruses such as SIVmac and HIV-2 (Goujon et al. 2006). Based on these results, it was demonstrated that alleviation of the restriction in myeloid cells using the Vpx protein concomitantly to HIV-1 infection leads to upregulation of costimulatory molecule expression, indicative of an active innate sensing mechanism (Manel et al. 2010). The innate response was accompanied by the expression of type I interferon and of an array of interferon-induced genes. This innate sensing required expression of *neo*-synthesized Gag protein of HIV-1. Incoming HIV-1 capsid alone or unintegrated viral cDNA was not sufficient to induce a detectable innate response (Manel et al. 2010). In DCs, cellular

Cyclophilin A and IRF3 were shown to be required. Functionality of these innate sensing mechanism was demonstrated by the ability of the infected and activated DCs to stimulate HIV-1-specific $CD4^+$ and $CD8^+$ T cells and to induce naïve $CD4^+$ T cell proliferation in a costimulatory-dependent assay (Manel et al. 2010). The innate sensing mechanism also led to an overall antiviral state, as shown by inhibition of the infection of $CD4^+$ T cells through the process of *trans*-enhancement in a type I interferon-dependent manner. However, the nature of the sensor and the regulatory mechanisms implicated have not been determined.

These results showed that human DCs are able to induce an effective innate and adaptive immune response against HIV-1 when viral infection and sensing are allowed through Vpx action. Vpx is implicated in the degradation of the restriction factor SAMHD1 (Hrecka et al. 2011; Laguette et al. 2011), an Aicardi–Goutières syndrome gene (Rice et al. 2009; Crow and Rehwinkel 2009). SAMHD1 is a deoxynucleoside triphosphate triphosphohydrolase and it is activated in an allosteric manner by dGTP and cleaves selectively dATP, dCTP, TTP, and dGTP, but not ribonucleoside triphosphates (Goldstone et al. 2011; Powell et al. 2011). Because it reduces dNTP concentration, which are normally required for reverse transcription, SAMHD1 blocks HIV-1 replication at the reverse transcription step (Goujon et al. 2007). Interestingly, several indirect observations suggested that the reverse transcription was inhibited in myeloid cells due to a limiting amount of dNTPs, and that rescue with exogenous dNTPs could partially rescue infectivity (Mangeot et al. 2002; Triques and Stevenson 2004; Ravot et al. 2002). This was confirmed after the identification of SAMHD1 as the Vpx-targeted restriction factor (Lahouassa et al. 2012).

7.3.4.2 Innate Interaction with HIV-1 in DCs and Antigen Presentation

In the case of extracellular antigens, phagocytosis provides a physical platform for coupling innate sensing by TLRs and antigen processing for loading on MHC. Through cross-presentation, MHC-I is loaded with such defined amount of exogenous material (Guermonprez and Amigorena 2005; Guermonprez et al. 2003). Because cell-intrinsic innate sensing of HIV-1 is coupled with productive replication of the virus in DCs upon removal of the SAMHD1 restriction (Manel et al. 2010), a unique environment is generated in the cell, where antigen processing of intracellular proteins is coupled to activation of viral-specific innate responses. Replication of the virus within the DCs produces a specific spectrum of antigens for presentation: the cell produces cytosolic viral antigens in a sustained manner for direct presentation. In addition, presentation of viral proteins, which are not normally present in viral particles, may benefit from this process, such as Tat and Nef. It is also likely that processing of peptides occurs under different rules through cross-presentation as compared to direct presentation (Xu et al. 2010). Finally, re-expression and release of viral particles from the infected DCs may also provide a privileged source of material for cross-presentation as well. To what extent direct presentation and cross-presentation may be beneficial to the host during HIV-1 infection is not known and will require further studies.

7.3.4.3 Innate Interactions May Distinguish Between Replicating Virus and Viral Debris

The major function of the immune system is to protect the host from replicating pathogens, but, an immune response against dead pathogen debris can be considered counterproductive from an evolutionary perspective—a notorious exception being vaccination. In the case of live pathogens such as bacteria, external cues may suffice for discrimination between a replicating and a dead pathogen by the immune system. Indeed, it has been proposed that a class of viability-associated PAMPs ("vita-PAMPS"), such as bacterial mRNA, are detected by the immune system leading to a specific immune response against pathogenic life, as opposed to a response against debris of dead pathogens (Sander et al. 2011). Viruses such as HIV-1 are obligatory parasites, and as such they may not contain similar vita-PAMPs. Instead, the process of viral replication within cells may provide molecular patterns specifically associated with viral "viability," or virulence. In another setting, it has been reported that direct infection of subcapsular sinus macrophages following VSV infection by the natural route, and this process is required to prevent spreading of the virus (Iannacone et al. 2010). In particular, infected macrophages alter the B cell response and provide a type I interferon-dependent innate signals that prevents further viral replication in the lymph node. Functionally, this implies that sentinel cells such as mucosal DCs and subcapsular macrophages are "hypersensitive" to viral infection. Interestingly, hypersensitive response to viral infection in plants contributes to systemic acquired resistance (Ward et al. 1991).

7.4 Conclusions and Future Directions

DCs constitute a unique interface for innate interactions with HIV-1. The two phases of the lentiviral cycle, extracellular and intracellular, contribute to distinct innate sensing mechanisms. Different types of DCs, in different microenvironment, contribute to these innate interactions. Understanding how these mechanisms integrate in DCs and in the host is a challenging problem. It will also be essential to determine how these different innate sensing mechanisms of HIV-1 contribute to proviral or antiviral effects at the level of cellular replication, viral evolution, immune responses against the virus, establishment of reservoirs, and dissemination and transmission of the virus.

Acknowledgements We apologize to the authors and colleagues whose work was not cited. We thank Philippe Benaroch, Vassili Soumelis, Xavier Lahaye, and Takeshi Satoh for critical reading of the manuscript.

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Chapter 8
HIV Impairment of Immune Responses in Dendritic Cells

Zahra Ahmed, Magdalena Czubala, Fabien Blanchet, and Vincent Piguet

Abstract Dendritic cells and their subsets are diverse populations of immune cells in the skin and mucous membranes that possess the ability to sense the presence of microbes and orchestrate an efficient and adapted immune response. Dendritic cells (DC) have the unique ability to act as a bridge between the innate and adaptive immune responses. These cells are composed of a number of subsets behaving with preferential and specific features depending on their location and surrounding environment. Langerhans cells (LC) or dermal DC (dDC) are readily present in mucosal areas. Other DC subsets such as plasmacytoid DC (pDC), myeloid DC (myDC), or monocyte-derived DC (MDDC) are thought to be recruited or differentiated in sites of pathogenic challenge. Upon HIV infection, DC and their subsets are likely among the very first immune cells to encounter incoming pathogens and initiate innate and adaptive immune responses. However, as evidenced during HIV infection, some pathogens have evolved subtle strategies to hijack key cellular machineries essential to generate efficient antiviral responses and subvert immune responses for spread and survival.

In this chapter, we review recent research aimed at investigating the involvement of DC subtypes in HIV transmission at mucosal sites, concentrating on HIV impact on cellular signalling and trafficking pathways in DC leading to DC-mediated immune response alterations and viral immune evasion. We also address some aspects of DC functions during the chronic immune pathogenesis and conclude with an overview of the current and novel therapeutic and prophylactic strategies aimed at improving DC-mediated immune responses, thus to potentially tackle the early events of mucosal HIV infection and spread.

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_8,

8.1 Introduction

Since their discovery almost 40 years ago (Steinman and Cohn 1973), DC were unambiguously shown to play a major role in immune defence against viral infection by generating and regulating innate and adaptive immune responses. Due to their substantial presence at mucosal surfaces, effectiveness at capturing antigens and potent migratory capacity and also due to their privileged interaction with effector T cells in lymphoid tissues, DC are critical intermediates of HIV infection and transmission (Knight and Patterson 1997; Piguet and Steinman 2007; Sewell and Price 2001). These cells, composed of different subsets, are considered as sentinels of the immune system due to their constant antigen sampling activity and their particular fast immune response ignition upon pathogen recognition (Steinman 2012). However it appears that HIV-1 has evolved evasion strategies and is able to exploit the establishment of infection and viral spread in the genital subepithelial region by subverting mucosal resident cells, leading to a delayed initiation of the adaptive immune response. In fact, DC subtypes were suggested to participate in HIV transmission and viral spread to $CD4^+$ target T cells, as evidenced by observations in human samples (Frankel et al. 1996; Howell et al. 1997; Langhoff and Haseltine 1992; Langhoff et al. 1991; Lehner et al. 1991; Niedecken et al. 1987; Pope et al. 1994, 1995b, 1997a, b; Weissman et al. 1995; Zoeteweij and Blauvelt 1998) and further supported by in vivo models of simian immunodeficiency virus (SIV) infection in rhesus macaques (Choi et al. 2003b; Enose et al. 1999; Hu et al. 2000; Li et al. 2009a; Pope 1998; Pope et al. 1997a; Spira et al. 1996). Although DC were shown to be able to bind HIV-1 via cell surface receptors and to internalize virions (Geijtenbeek et al. 2000a; Granelli-Piperno et al. 1996; Turville et al. 2001, 2002), HIV could exploit some of the intracellular signalling and trafficking pathways in order to facilitate its dissemination, while concomitantly impairing an efficient and appropriate DC-mediated immune response (Piguet and Steinman 2007; Wu and KewalRamani 2006). Indeed, along with DC being unable to fully degrade HIV (Garcia et al. 2005; Geijtenbeek et al. 2000a; Kwon et al. 2002), they seemingly also display a relative inability to fully mature (Piguet and Steinman 2007; Steinman et al. 2003), with the exception, however, of very high doses of virus (Harman et al. 2006).

8.2 DC Involvement During Early Events of HIV Infection

Different immature DC subsets are known to widely populate environmentally exposed body surfaces, including the skin and mucosal tissues, or the blood compartment, where they can be discriminated according to specific cellular receptors (Banchereau et al. 2000; Geijtenbeek and van Kooyk 2003; Liu 2001; Pope et al. 1997c; Teleshova et al. 2003; Wu and KewalRamani 2006). In the blood, there are at least two major subsets of DC: $CD11c^+$ myeloid DC (myDC) and $CD11c^-$ $CD123^+$ plasmacytoid DC (pDC). Both are antigen presenting cells (APC) but with, however, some functional differences. While myDC seem more abundant and specifically

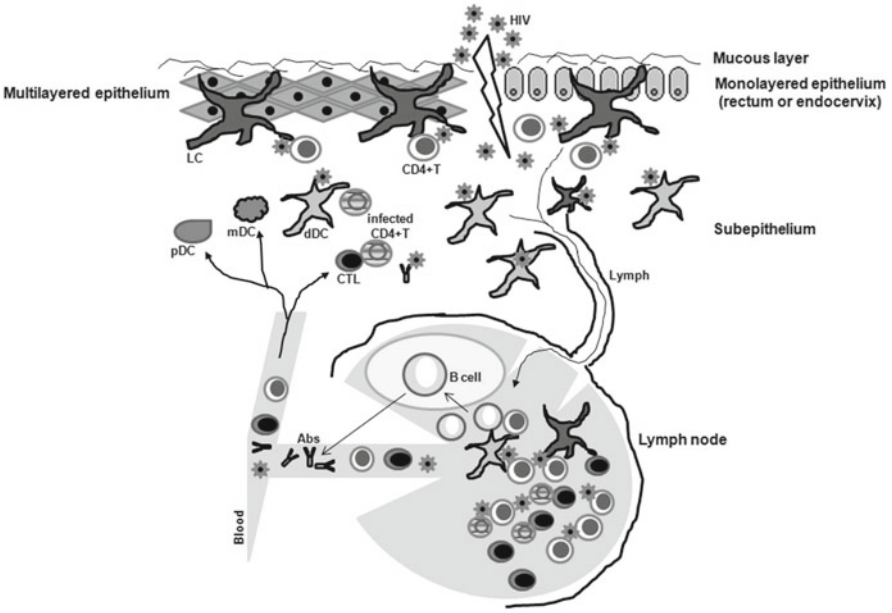

Fig. 8.1 DC subsets and early events of HIV mucosal transmission and viral spread. In the scheme are depicted the cellular subsets and processes involved in HIV transmission upon viral entry through mucosal epithelia. Some mucosal-resident DC (LC and dDC) can intercept incoming virions which also induce chemoattraction of $CD4^+$ T cells. Virus captured by DC can remain infectious during days thus enabling infectious HIV to reach proximal lymphoid tissues upon DC migration. DC-mediated immune responses allow generation of CTL-HIV specific activation as well as $CD4^+$ T helper-mediated humoral responses. Interaction between HIV-containing DC and HIV-specific $CD4^+$ T cells might preferentially promote HIV transfer. HIV-specific CTL and antibodies can migrate to the mucosal site of infection and localized immune response can recruit more HIV target cells thus facilitating infection propagation. Also, some epithelial cells-derived cytokines can recruit other DC subsets, like pDC or myDC, thus also contributing to viral immune evasion

secrete high amounts of IL-12 upon activation (Banchereau et al. 2003; Langrish et al. 2004), pDC appear to produce high amounts of interferon-alpha (IFN-α) in response to foreign antigen and likely play an important role in innate antiviral immunity (Cella et al. 1999; Gilliet et al. 2008; Siegal et al. 1999; Swiecki and Colonna 2010). The skin and mucosal tissues are, on the other hand, populated by other immature DC, which differ by their specific localization and cell surface receptor expression. Langerhans cells (LC), specifically expressing the C-type lectin receptor (CLR) CD207 (Langerin), are mainly residing in the epidermal layer (Langerhans 1868; Valladeau et al. 1999, 2000), while subsets of dermal DC (dDC), some of them expressing CD209 (DC-SIGN), are present throughout the dermis (Lenz et al. 1993; Nestle et al. 1993; Pavli et al. 1993) (see Fig. 8.1). One of the hallmarks of these different DC subsets relies on the specific expression of different sets of CLR, pathogen recognition receptors (PRR) and chemokine receptors enabling them to capture and detect incoming intruders and to migrate to sites of

inflammation where production of chemokine gradients occur (Banchereau et al. 2000; Liu 2001; Steinman 2012; Teleshova et al. 2003; van Vliet et al. 2007). In the best case scenario, DC could bind foreign antigen and internalize it via several means like receptor-mediated endocytosis, phagocytosis and macropinocytosis which ultimately leads to antigen degradation and processing for major histocompatibility complex (MHC)-mediated presentation (Lanzavecchia 1996; Mellman 1990; Sallusto et al. 1995; Watts 1997). The subsequent uptake and degradation of antigen favour also favour exposure of double-stranded RNA, bacteria, LPS thus inducing concomitant maturation and migration of DC to proximal lymph nodes (Cella et al. 1997; Larsen et al. 1990; Steinman et al. 1995). This maturation process is mainly characterized by upregulation of MHC molecules, costimulatory molecules, such as CD80, CD86, CD40 and chemokine receptors, like CXCR4 and CCR7 (Steinman 2001). These particular genetic and phenotypic changes during maturation allow DC to efficiently process and present antigen to $CD4^+$ and $CD8^+$ T cells thus initiating specific adaptive immune responses. However, HIV-1 has developed sophisticated strategies to alter DC immune functions and to exploit cellular processes which could even facilitate infection and virus propagation. Indeed, populations of cells in mucosal surfaces such as Langerhans cells, dendritic cells, macrophages and $CD4^+$ T cells are known to be early targets for HIV. Although it has been difficult to study early events of HIV transmission in humans for a multitude of reasons (timelines from infection, patient tissue sampling or ethical issues related to access to infected samples (Blanchet et al. 2011; Haase 2005)), some data evidenced the presence of HIV in blood- or mucosal-derived DC from HIV-infected patients (Dusserre et al. 1992; Frankel et al. 1996; Langhoff and Haseltine 1992; Schmitt and Dezutter-Dambuyant 1994; Tschachler et al. 1987; Patterson et al. 1994; Rappersberger et al. 1988; Bhoopat et al. 2001) and HIV infection of different DC subtypes was recapitulated ex vivo and in vitro with clinical or laboratory viral strains (Blauvelt et al. 1997; Canque et al. 1999; Granelli-Piperno et al. 1998, 1996; Zaitseva et al. 1997; Ayehunie et al. 1997; Soto-Ramirez et al. 1996; Patterson and Knight 1987). Although HIV replication in DC was not optimal, it appeared that these cells could be early targets for HIV and critically responsible for HIV transmission to $CD4^+$ target T cells and subsequent boost of infection (Cameron et al. 1992, 1994; Granelli-Piperno et al. 1998, 1999; Pope et al. 1994, 1995a, b; Tsunetsugu-Yokota et al. 1995, 1997; Weissman et al. 1995; Macatonia et al. 1989; Hladik et al. 1999; Gummuluru et al. 2002). Importantly, it appeared that increase of viral productive infection upon DC-mediated HIV transfer to $CD4^+$ T cells preferentially occurred during antigen presentation (Lore et al. 2005; Macatonia et al. 1989; Moris et al. 2006; Tsunetsugu-Yokota et al. 1995; Weissman et al. 1996; Lyerly et al. 1987). The suggested involvement of DC during the very first events of HIV infection and transmission was strongly supported in simian models of lentiviral infection, known to closely mimic the steps of HIV transmission at the genital mucosa. These models have clearly shown that, although $CD4^+$ cells are amongst the majority of the infected population, DCs may also be infected and possibly act as early carriers of virus for transmission (Haase 2005; Hu et al. 2000; Ignatius et al. 1998; Miller and Hu 1999; Pope 1998; Pope et al. 1997a; Spira et al. 1996; Turville et al. 2006; Barratt-Boyes et al. 2002).

Upon HIV infection, the appearance of acute phase proteins and a considerable change of chemokines and inflammatory cytokines expression levels in the blood or the mucosal microenvironment were observed (Choi et al. 2003a; McMichael et al. 2010; Olsson et al. 2000; Stacey et al. 2009). This would particularly affect DC phenotype and behaviour. Indeed, an increase in CCL20, potentially derived from endocervical epithelial cells (Berlier et al. 2006; Cremel et al. 2005), could induce CCR6-dependent pDC recruitment in the challenged area and their activation subsequently leading to attract $CD4^+$ T cells via secretion of the chemoattractant factors CCL3 and CCL4 (Li et al. 2009a). Another DC subset, myDC, was shown to be highly repolarized and activated upon thymic stromal lymphopoietin (TSLP) secretion via endocervical epithelial cells challenged in vitro with HIV (Fontenot et al. 2009). Interestingly, increased concentrations of both CCL20 and TSLP were observed in vaginal tissues of SIV-infected animals (Fontenot et al. 2009; Li et al. 2009a; Blanchet et al. 2011). These observations support the notion of DC involvement during the early steps of HIV spread toward $CD4^+$ T cells.

8.3 HIV Interaction with DC: The Bases of DC Functions Impairment

More significantly, it has been known for a long time that HIV interaction with DC had deleterious effects on DC behaviour and immune functions mostly affecting antigen presentation and migratory capacities of these cells (Belsito et al. 1984; Chehimi et al. 1993; Eales et al. 1988; Macatonia et al. 1989; Roberts et al. 1994). While HIV replication was shown to be relatively inefficient in DC, HIV can bind all DC subsets and the engagement of viral envelope with different DC receptors was suggested to set up the subsequent immune dysregulation. Most of the DC subsets express HIV receptors, CD4 and the co-receptors CCR5 and CXCR4, although at much lower levels compared to $CD4^+$ T cells. Other reports have shown that M-tropic HIV (using CCR5) could better replicate in DCs compared to T-tropic viruses (using CXCR4) (Ganesh et al. 2004; Granelli-Piperno et al. 1998; Kawamura et al. 2000; Reece et al. 1998).

A straightforward explanation for the restriction of HIV-X4, and to a lesser extent of HIV-R5, replication in DC could rely on the differential expression of CD4 and chemokine receptors at the cell surface, but all receptors were shown to be functionally present (Pion et al. 2007; Popov et al. 2005) and fusion of HIV-X4 with immature DCs was seemingly restricted irrespective of surface levels of CXCR4 (Pion et al. 2007). Furthermore, it appeared that HIV restriction in DC was mostly taking place at a post-entry step (Goujon et al. 2006; Neil et al. 2001; Pion et al. 2007) and restriction factors, such as members of the APOBEC3 family (A3G, A3F and A3A) and especially the recently identified SAMHD1 account for this restriction in myeloid cells (Hrecka et al. 2011a, b; Laguette et al. 2011; Peng et al. 2007; Pion et al. 2006; Stalder et al. 2010), also explaining the recurrent low activation and maturation levels of DC upon HIV challenge.

8.3.1 C-Type Lectin Receptors

Other than CD4 and chemokine receptors, C-type lectin receptors (CLR) have also been shown to be important in HIV-1 viral capture and transmission (Arrighi et al. 2004a; Geijtenbeek et al. 2000a; Kwon et al. 2002). These receptors are very important component of cell-mediated pathogen recognition and substantially contribute to DC-mediated immune functions. Briefly, lectin and lectin-like receptors are carbohydrate binding receptors abundantly and widely expressed which critically support cell adhesion and migration as well as pathogen binding and internalization in specialized cells. They were extensively studied in the context of glycoproteins clearance by hepatocytes where the asialoglycoprotein receptor was proven to play a major role (Ashwell and Harford 1982). Lectin receptors were then demonstrated to be functionally linked to the endo-lysosomal degradation pathway and were thus suggested to take part in innate and adaptive immune responses (Cambi and Figdor 2003, 2005; Weis et al. 1998). Among members of this receptor family, the CLR are particularly appreciated for their role in pathogens binding, recognition and uptake. Their target binding specificity, however, extends to proteins and lipids on the top of the glycans (Mitchell et al. 2001; Torrelles et al. 2006). CLR-mediated recognition of carbohydrates is specified by carbohydrate recognition domain (CRD) and these receptors can be further subdivided into two different types: type I and type II (Figdor et al. 2002). The representatives of each group, which are believed to have the biggest impact on HIV transmission and infection while also involved in DC-mediated immune responses, are the mannose receptor (MMR; CD206) (Stahl and Ezekowitz 1998) and DEC-205 (CD205) (Bozzacco et al. 2007) belonging to type I and Langerin (CD207) (van der Vlist and Geijtenbeek 2010), Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN or CD209) (Geijtenbeek et al. 2000a; Cunningham et al. 2007) and DC ImmunoReceptor (DCIR also named CLEC4A) (Lambert et al. 2008) belonging to type II. Of note, HIV is only one of many pathogens which exploit lectins for binding and spread. Other viruses, bacteria, fungi, protozoa and parasites also attach to these CLR and these interactions support their spread (Tsegaye and Pohlmann 2010).

Nonetheless, in the case of HIV infection, DC-SIGN appears instead to benefit viral spread. DC-SIGN is a pathogen recognition receptor (PRR) expressed in great quantity on immature DC, in particular on dermal and lymph nodes localized DC, and absent from LC. Its binding partner (ICAM-3) is found on T cells and is required for a formation of transient connection between these cells, which could explain the importance of DC-SIGN for DC-mediated antigen presentation to T cells (Geijtenbeek et al. 2000b). Indeed, while these CLR could serve to internalize virions for degradation, HIV could use them in order to escape from DC-mediated immune recognition while favouring its transmission to $CD4^+$ target T cells (Geijtenbeek et al. 2000a; Geijtenbeek and van Kooyk 2003; Lambert et al. 2008; Arrighi et al. 2004b; Pohlmann et al. 2001). This is rather surprising when considering the contribution of CLR in DC-mediated innate and adaptive immune responses (van Kooyk and Rabinovich 2008). It is difficult to clearly state if HIV has evolved to use these attributes for its

benefits or if simply this protective against some other pathogens is not adapted to HIV encounter. In fact, the amount as well as the preferential binding site of glycosylated antigen on the CRD of the CLR (Gringhuis et al. 2009) could determine and potentially regulate the quality of the induced immune response. It appears that upon ligand binding, DC-SIGN can internalize antigen toward the endo-lysosomal pathway thus supplying MHC intracellular compartments and regulating adaptive immune responses (Dakappagari et al. 2006; Engering et al. 2002), although its role in initiating T cell responses was not fully recapitulated in mixed lymphocyte reaction (MLR) experiments (Granelli-Piperno et al. 2005). In the case of HIV, DC-SIGN was shown to support HIV-derived antigen presentation (Moris et al. 2004; 2006). Surprisingly, however, it has been observed that upon HIV binding and internalization in DC, not all virions were degraded and a substantial fraction was rather exiting the endosomal pathway and escaping lysosomal degradation (Garcia et al. 2005; Turville et al. 2004; Tacchetti et al. 1997). The escape mechanism and viral transmission to $CD4^+$ T cells was suggested to be DC-SIGN-dependent (Kwon et al. 2002; Geijtenbeek et al. 2000a), although DC-SIGN-dependent internalization could be dispensable (Arrighi et al. 2004a; Burleigh et al. 2006; Pohlmann et al. 2001). Furthermore, the same lysosomal degradation escape mechanism was observed for Hepatitis C Virus (HCV) upon DC-SIGN binding (Ludwig et al. 2004). It was suggested that following attachment to DC, HIV could be internalized into compartments lacking typical markers of endolysosomes and retained as infectious particles for a long period of time (Garcia et al. 2005; Hodges et al. 2007; Turville et al. 2004; Trumpfheller et al. 2003; Yu et al. 2008). An explanation for the biased DC-SIGN-mediated trafficking, upon ligand attachment or HIV capture, could rely on the differential DC-SIGN extracellular multivalent binding site engaged and also the type and amount of mannose N-linked carbohydrates recognized on viral pathogens (Cambi and Figdor 2005; Dakappagari et al. 2006; Mitchell et al. 2001; Sierra-Filardi et al. 2010; Geijtenbeek et al. 2002). Indeed, it appeared that depending on the epitopes engaged on the CRD or the extracellular neck region of DC-SIGN, internalization process and/or targeting to endo-lysosomal pathway as well as DC-SIGN-mediated HIV transfer could be largely influenced (Dakappagari et al. 2006; Mitchell et al. 2001; Sierra-Filardi et al. 2010; Chung et al. 2010). When comparing with langerin, another CLR mainly expressed on Langerhans cells, it appears that, although it could bind efficiently HIV-envelope, langerin structural features and potential HIV binding sites are significantly different (Chatwell et al. 2008; Feinberg et al. 2011; Holla and Skerra 2011). These properties could account for the observed antiviral activity of langerin from human skin-derived Langerhans cells (de Witte et al. 2007; van der Vlist and Geijtenbeek 2010) and its contribution to peptide- and nonpeptide-derived ag-presentation to T cells (Girolomoni et al. 1995, 1996; Hunger et al. 2004).

As mentioned above, few possible outcomes of HIV-DC interactions are suggested in the literature: virus degradation, virus endocytosis and/or storage in a tetraspanin-enriched compartment or retention of infectious HIV on the surface of the cells (Cavrois et al. 2007; Garcia et al. 2005; Yu et al. 2008). Most of these consequences largely depend also on the maturation status of the DC. For example,

while immature LC could prevent HIV infection and transmission through the action of langerin, it appeared that, once activated, these cells would gradually loose cell surface langerin expression thus favouring CD4/CCR5-dependent productive infection and facilitating transmission toward $CD4^+$ target T cells (de Jong et al. 2008, 2010; de Jong and Geijtenbeek 2010; de Witte et al. 2007; Fahrbach et al. 2007; Ogawa et al. 2009). DC-SIGN, on the other hand, was shown to promote HIV transmission mainly from immature DC, when expressed naturally or even when exogenously expressed into monocyte or lymphocyte cell lines (Geijtenbeek et al. 2000a; Kwon et al. 2002; Wang et al. 2007a; Wu et al. 2004). While maturation of DC greatly compromised HIV replication, increased intracellular viral retention was seemingly much less DC-SIGN-dependent and transfer to $CD4^+$ T cells was, however, improved, but involvement of DC-SIGN was seemingly not as critical (Boggiano et al. 2007; Izquierdo-Useros et al. 2007; Turville et al. 2004; Wang et al. 2007b; Cavrois et al. 2006), thus adding to the complexity of HIV trafficking upon cellular binding.

Activation and maturation of DC upon pathogen encounter is a critical event allowing them to acquire potent and selective migratory properties and to enhance lysosomal-mediated antigen degradation, processing on MHC molecules and presentation to T cells (Lanzavecchia and Sallusto 2001; Mellman and Steinman 2001; Trombetta et al. 2003). As evidenced in vitro and also in samples from HIV-infected patients, one of the hallmark of HIV-1 interaction with DC is that it profoundly compromises DC maturation process and subsequent DC-mediated innate and adaptive immune responses (Anthony et al. 2004; Chehimi et al. 1993; Eales et al. 1988; Granelli-Piperno et al. 2004; Kawamura et al. 2003; Macatonia et al. 1989; Martinson et al. 2007; Roberts et al. 1994; Shan et al. 2007; Yonkers et al. 2011). Indeed, myDC isolated from blood of HIV-infected patients or MDDC challenged in vitro with clonotypic viral isolates were shown to be less responsive to TLR ligands (Anthony et al. 2004; Martinson et al. 2007; Shan et al. 2007; Yonkers et al. 2011) and were significantly impaired in their ability to stimulate $CD4^+$ T cells (Anthony et al. 2004; Eales et al. 1988; Fantuzzi et al. 2004; Granelli-Piperno et al. 2004; Kawamura et al. 2003; Macatonia et al. 1989; Roberts et al. 1994; Yonkers et al. 2011). Hence, most of these reports demonstrated that HIV-1 was rather inducing a DC-mediated immunosuppressive environment or favouring Th2-mediated immune responses, translated by an increase in IL-10 versus decreased IL-12 cytokines production (Anthony et al. 2004; Granelli-Piperno et al. 2004). Interestingly, these effects were shown to be mainly mediated by HIV-1 envelope upon engagement with DC receptors (Fantuzzi et al. 2004; Kawamura et al. 2003; Shan et al. 2007), which would suggest that HIV-1-gp120-dependent induced signalling would mainly contribute to initiate dysregulation of DC immune functions. Due to their role in HIV-1-gp120 binding and DC-mediated immune responses, CLR could be appropriate candidates to explain the HIV-1 envelope effects. Indeed, HIV-1-gp120-mediated immunosuppression of DC functions was shown to rely on its level and type of glycosylation (Shan et al. 2007), the main feature for CLR binding. The involvement of glycosylated HIV-1-gp120 in dysregulation of DC immune functions is further supported by some reports showing that (1) enzymatic removal of

mannose moieties on HIV-1-gp120 could repolarize the DC-mediated Th2 skewed response (Shan et al. 2007), (2) deglycosylation could increase HIV-1-gp120 immunogenicity and enhance humoral and cellular immune responses (Banerjee et al. 2009), the HIV-2 envelope, known to differ in glycosylation levels compared to HIV-1-gp120 (Shi et al. 2005), did not affect DC differentiation and maturation (Cavaleiro et al. 2009).

Thus interaction between HIV-1-gp120 and CLR seems to critically determine the subsequent immune behaviour of DC. It was shown for example that DC-SIGN engagement could induce phosphoinositol-3-kinase (PI3K) and ERK activation and increase IL-10 production (Caparros et al. 2006). Even if these experiments were performed with specific antibodies, the data could explain the skewed Th2 immune responses usually observed with DC-SIGN-interacting pathogens. DC-SIGN-mediated signalling upon HIV-1-envelope binding was also shown to regulate TLR responses via a pathway involving the kinase Raf-1 (Gringhuis et al. 2007). Furthermore, it appeared that DC-SIGN could shape DC-mediated innate and adaptive immune responses depending on the type of carbohydrate recognized. Indeed, DC-SIGN was shown to bind with high affinity to mannose- or fucose-containing glycolipids (Appelmelk et al. 2003; van Liempt et al. 2006), which regulate a DC-SIGN/LSP1/KSR1/CNK/Raf-1 signalosome to enhance or repress DC-mediated pro-inflammatory cytokine production, respectively (Gringhuis et al. 2009). Activation of DC-SIGN, by HIV-envelope or specific antibodies, was also shown to induce a DC-SIGN/LARG complex-dependent Rho-GTPase activation which could account for the observed immature DC phenotype and favouring HIV immune evasion and spread to target $CD4^+$ T cells via the virological synapse (Hodges et al. 2007; Nikolic et al. 2011). Ultimately, HIV was shown to exploit TLR-8-mediated NF-kB activation and DC-SIGN/Raf-1-dependent NF-kB phosphorylation to initiate viral transcription and recruit the transcription-elongation factor pTEF-b, respectively, thus favouring HIV replication and DC-mediated viral transmission (Gringhuis et al. 2010). Overall, the altered DC phenotype obtained upon HIV binding to DC-SIGN is fully reminiscent of the results observed with other DC-SIGN-binding pathogens. Indeed, mycobacteria species were shown to bind DC-SIGN thus favouring DC infection and altering both innate and adaptive immune responses (Gagliardi et al. 2005; Geijtenbeek et al. 2003). The parasite Schistosoma mansoni-derived egg antigens were shown to bind DC-SIGN, and other CLR, and to suppress DC TLR-mediated activation (van Liempt et al. 2007). So it appeared that most of the glycosylated pathogens would benefit from DC-SIGN binding to escape immune surveillance and enhance their replication. But, it is crucial to realize that CLR are differentially expressed depending on dendritic cell subsets and their maturation status, and DC-SIGN is not the only CLR known to bind HIV-envelope and to regulate DC-mediated immune responses. Indeed, it was shown that DEC-205 could bind HIV and targeting of this CLR in mice with specific antibodies could enhance DC-mediated antigen presentation (Bonifaz et al. 2002; Bozzacco et al. 2007; Jiang et al. 1995). DCIR, another CLR expressed in almost all DC subsets, was also shown to bind HIV and to contribute to DC-mediated HIV transfer (Lambert et al. 2008), and its targeting induced efficient $CD8^+$ T cells cross-priming (Klechevsky et al. 2010).

Interactions between lectin receptors and viral envelope, mainly attributed to extensive glycosylation of the latter and originally identified as protection against antibody recognition, shine a completely new light on HIV-gp120 in terms of immune invasion. The family of CLR expressed on different DC subsets is rapidly growing and their role on HIV capture and DC-mediated immune responses would definitely need further investigations.

8.3.2 Other Pathogen Recognition Receptors and Envelope Independent HIV Uptake

A variety of PRR are expressed on DC that facilitates quick recognition and binding to pathogen-associated molecular patterns (PAMPs) expressed by foreign pathogens at the site of exposure. A consensus is that ligation of PRR leads to the expression of co-stimulatory molecules upon DC maturation and the production of pro-inflammatory cytokines and type-I interferons via activation of NF-kB and Interferon Regulatory Factors (IRF) signalling pathways, respectively. HIV binding to CLR could result in viral immune evasion, mainly due to altered signalling and trafficking pathways, thus avoiding optimal antigen recognition. As mentioned previously, DC are not able to fully mature upon HIV challenge and, while CLR would unwittingly contribute to this, it could be due to a lack of other PRR-mediated response. Indeed, not all DC subsets express the same type of PRR and, as seen before, their susceptibility to HIV infection could be different. Interestingly, it was shown that pDC were highly susceptible to HIV infection (Fong et al. 2002; Lore et al. 2005; Patterson et al. 2001; Smed-Sorensen et al. 2005) and their gradual loss observed in HIV-1-infected patients, along with myDC (Donaghy et al. 2001; Grassi et al. 1999; Pacanowski et al. 2001), correlated well with increased viremia and disease progression (Donaghy et al. 2001; Feldman et al. 2001). Although pDC functions and interferon-alpha (IFN-α) secretion were profoundly altered in the chronic phase of HIV infection (Donaghy et al. 2003; Kamga et al. 2005), these cells were able to recognize and respond to HIV intrusion, in sharp contrast with other DC subsets (Beignon et al. 2005; Fonteneau et al. 2004). In fact, upon CD4-mediated HIV endocytosis, accumulation of viral RNA led to the activation and maturation of pDC via TLR7-mediated recognition (Beignon et al. 2005). However, TLR-7 expression appeared to be confined to this particular DC subset (Kadowaki et al. 2001), and, in accordance with their lymphoid origin, the potential lack of post-entry restriction factors leading to viral RNA accumulation could be sufficient to potently initiate DC-mediated innate immune response. Nonetheless, as reports were clearly suggesting that HIV infection could induce pDC loss and immune function impairment, it would be of importance to better define pDC receptors phenotype and immune function during the early events of viral challenge. For instance, the potential impact of CLR expression on pDC regarding HIV transmission has not been addressed.

The CD4/co-receptor and lectin receptors on DC were, however, not the only possibility for HIV entry and uptake. It was well known that CLR were decreased

upon DC maturation while apparent intracellular HIV retention was increased in mature DC (Arrighi et al. 2004a; Izquierdo-Useros et al. 2007). Although this could be in part explained by the switch of receptor usage, it was strongly suggestive of a parallel CLR-independent HIV uptake pathway which could account for viral immune evasion. A potential mechanism for this receptor-independent uptake and trafficking of HIV was given when scientists unveiled that it was closely mimicking the exosomal dissemination pathway (Izquierdo-Useros et al. 2007) which was reminiscent of the fact that HIV receptor-independent uptake would be cholesterol-dependent (Gummuluru et al. 2003). The mechanism of such uptake is not well defined but it could rely on the involvement of the SYK kinase, previously shown to be activated upon receptor-independent membrane binding (Ng et al. 2008) and involved in DC-mediated HIV transfer to $CD4^+$ T cells (Gilbert et al. 2007). Interestingly, HIV was proposed to converge also with the cholesterol-enriched exosome pathway upon productive infection in DC (Wiley and Gummuluru 2006; Garcia et al. 2008) which was likely also involved during HIV budding in macrophages (Nguyen et al. 2003). Another CLR-independent mean of HIV uptake would be to consider that most of HIV particles are indeed opsonised in vivo. It was suggested that opsonisation of HIV, along with complement, could lead to a different DC-mediated virus uptake (Bouhlal et al. 2007; Pruenster et al. 2005; Wilflingseder et al. 2007), although the mechanisms involved and the consequences on viral spread and DC immune functions are not clear.

Altogether, these results emphasize how important the differential means of viral uptake, viral-mediated signalling and trafficking could be to escape immune recognition, to lower DC immune functions and to favour viral spread.

8.3.3 Other HIV-Envelope Effects and Consequences on DC Immune Responses

As seen before, it is likely that in the early events of viral capture, HIV-1 envelope is critical to determine intracellular viral trafficking and to initiate signalling leading to alter DC immune functions and to favour DC-mediated viral transmission. This was also further supported by the marked inhibition of autophagy in DC upon HIV-1 capture (Blanchet et al. 2010). The word "autophagy" is derived from the Greek language and means to "eat oneself." Autophagy and its related organelles autophagosomes were named and described for the first time by Christian de Duve almost 50 years ago with the observation that dense foci of autolysis could be induced upon glucagon treatment of rat liver and participate in lysosomal-mediated degradation (Deter et al. 1967; Deter and De Duve 1967). Since then, autophagy was demonstrated as a major cellular pathway, conserved in a wide range of eukaryotic organisms and involved in self-digestion to recycle nutrients, remodel and dispose of unwanted cytoplasmic constituents via double-membrane organelles known as autophagosomes [for review see (Levine and Klionsky 2004; Mizushima et al. 2008)]. The appreciation of autophagy as a central player in the immunological control of

intracellular pathogens came only recently when it was shown that it could behave as a cellular defence mechanism against many pathogens (Gutierrez et al. 2004; Nakagawa et al. 2004; Rich et al. 2003). This was rapidly followed by reports showing the critical involvement of autophagy in antiviral innate and adaptive immune responses (Dengjel et al. 2005; Paludan et al. 2005; Lee et al. 2007). It appeared that HIV was able to modulate autophagy in $CD4^+$ T cells upon HIV-envelope binding to CXCR4 thus leading to cell death (Espert et al. 2006). Although this could contribute to the depletion of bystander cells, some reports showed that autophagy was rather downregulated with a marked decrease of autophagosome in HIV-infected $CD4^+$ T cells or macrophages (Espert et al. 2009; Zhou and Spector 2008). In DC, it was demonstrated that HIV-envelope induced a signalling cascade activating mammalian Target Of Rapamycin (mTOR), which is known to block autophagy flux initiation (Blanchet et al. 2010). An increase in cell-associated virus correlated with a reduction in autophagy in DC and with enhanced transfer of HIV infection *in trans* to $CD4^+$ T cells. Reduction of autophagy was shown to impair TLR-mediated innate immune response in DC (Blanchet et al. 2010; Blanchet and Piguet 2010), while strongly affecting antigen processing and MHC-II-mediated antigen presentation to $CD4^+$ T cells in vitro and in vivo (Blanchet et al. 2010; Lee et al. 2010). Interestingly, in productively infected macrophages, HIV was shown to enhance the early steps of autophagosome formation to promote membrane recruitment and enhance viral yields, while inhibiting the late stages of autophagy, in a Nef-dependent manner, in order to avoid intracellular viral degradation (Kyei et al. 2009). Thus the impact of HIV on the autophagic machinery and its role in DC-mediated immune responses upon productive infection needs further investigation.

Upon pathogen encounter, DC immune functions are tightly controlled and correlate with their maturation process. Maturation of DC is usually initiated upon recognition of a danger signal from incoming foreign entities and rapidly upregulate the expression of various receptors involved in migration and chemotaxis. However, in the case of HIV, it appeared that DC were not able to fully mature, which could thus dampen their emigration toward proximal lymph nodes and compromise DC-mediated cellular and humoral immune responses. In fact, some reports suggested that DC chemotaxis was indeed affected upon HIV-envelope binding. First, DC could be attracted toward R5-tropic virus via their chemokine receptor CCR5, which could then enhance DC-mediated HIV capture at the site of infection (Lin et al. 2000). The effect of R5-tropic HIV-gp120 was then shown to increase chemotaxis and migration properties of challenged DC via induction of a signalling cascade involving the kinase PYK2 (Anand et al. 2009). Activation of PYK2 then led to activate p38 mitogen-activated protein kinase (p38MAPK) which resulted in LSP-1 activation, an F-actin binding protein known to regulate actin cytoskeletal events involved in cellular migration (Anand et al. 2009). Thus, even if HIV would impede full DC maturation, it could promote DC migration, in an envelope-dependent manner, in order to reach replication-competent areas and favour its dissemination.

Another subset of DC, pDC, was shown to alter T cell responses upon HIV challenge. Indeed, HIV-envelope interaction with CD4 resulted in an increased expression of indoleamine 2,3-dioxygenase (IDO) (Boasso et al. 2007), a well-described

immunosuppressive enzyme known to catabolize the essential amino acid tryptophan into the kynurenine pathway. Increased IDO expression in pDC of HIV-infected patients was observed and correlated with an impaired T-cell proliferative response which could be rescued upon IDO inhibition (Boasso et al. 2007).

HIV was also shown to alter innate immune functions of DC by subverting the canonical interferon signalling pathway. Indeed, early HIV infection was inducing a significant change of gene expression in DC (Harman et al. 2009), and, particularly, led to an increase in IRF-1 expression which was required for viral replication in DC (Harman et al. 2011). This was, however, paralleled with a failure in IRF-3 activation thus impeding IFN production (Harman et al. 2011). Therefore, the HIV-mediated induction of interferon-stimulated genes (ISG) concomitantly with inhibition of DC innate immune response could enhance noncytopathic viral replication.

8.4 HIV Trafficking in DC: Restriction Factors and Immune Evasion

The ability of HIV to subvert critical cellular signalling pathways and machineries, while remaining infectious in DC, most likely plays a crucial role to ensure its survival and spread. Such mechanisms would potentially allow survival of virus until the time when DC reach lymph nodes and interact with T cells. Upon DC-mediated uptake and infection, HIV was shown to co-localize with cell surface-accessible compartments particularly enriched in cholesterol and tetraspanins and which are reminiscent of the compartments where exosomes were shown to converge during receptor-independent HIV uptake (Garcia et al. 2005; Gummuluru et al. 2003; Izquierdo-Useros et al. 2007, 2009; Turville et al. 2008). Notably, these cholesterol-dependent tetraspanin-enriched microdomains were readily observed in mature DC and correspond to a site of intracellular HIV sequestration (Garcia et al. 2005; Izquierdo-Useros et al. 2007, 2009). Indeed, cholesterol catabolism seemed important for this particular DC-mediated viral capture to occur. It was demonstrated that activation of the nuclear receptors (NR) peroxisome proliferator-activated receptor gamma (PPARγ) and liver X receptor (LXR) could inhibit immature and mature DC-mediated HIV uptake and transmission through a mechanism involving the cholesterol transport protein ATP-binding cassette A1 (ABCA1) and leading to cholesterol efflux (Hanley et al. 2010). Outstandingly, DC-mediated HIV capture and transfer could be rescued upon cholesterol repletion or ABCA1 expression knockdown (Hanley et al. 2010).

Intracellular trafficking and retention in safe compartments allows HIV to escape from DC innate immune surveillance mechanisms, but it could also account for viral spread enhancement by favouring a transient transinfection pathway rather than productive infection (see Fig. 8.2). Indeed, the host also behave with innate antiviral mechanisms through expression of specific restriction factors. To date, four restriction factors have been described which could block HIV-1 replication: *tripartite motif-5α (TRIM-5α), apolipoprotein B mRNA-editing enzyme-catalytic poly-*

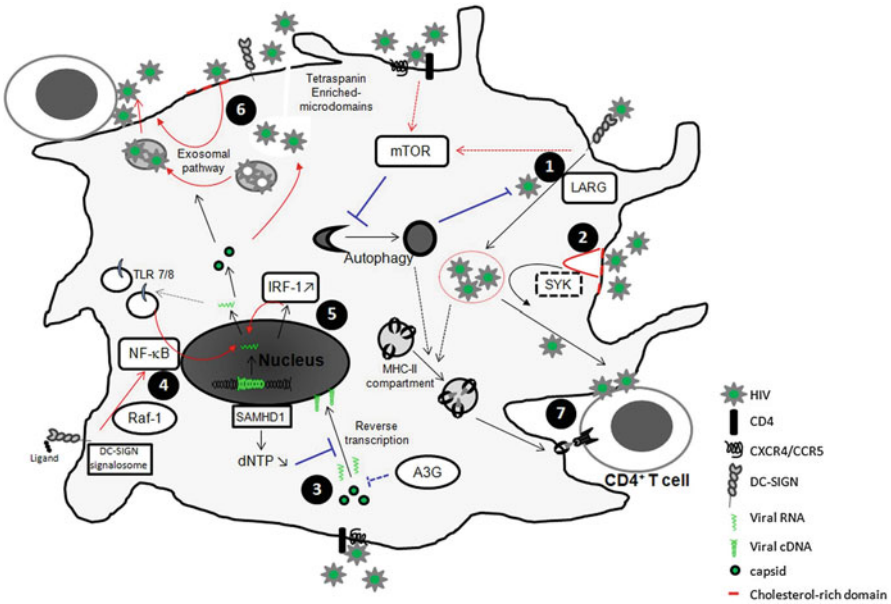

Fig. 8.2 Effects of HIV on DC intracellular machineries and signalling pathways linked to DC-mediated innate and adaptive immune responses. HIV-1 envelope binding to CD4/chemokine receptor or DC-SIGN initiates intracellular signalling pathways leading to mTOR activation (❶) which negatively regulates autophagy induction. Autophagy is required for pathogen clearance and also to feed MHC-II compartments. DC-SIGN-mediated signalling pathway also favours HIV-1 internalization and formation of DC-T cell infectious synapse while dampening DC maturation via the activation of the guanine exchange factor LARG (❶). HIV-1 entry occurs also in a receptor-independent manner requiring cholesterol-rich domains (❷) and potentially involving the tyrosine kinase SYK. These processes would allow HIV-1 to escape immune recognition while favouring DC-mediated HIV transfer to CD4⁺ T cells. Some cellular factors can restrict HIV at a post-entry step in DC. While the mechanism of A3G-mediated HIV post-entry block is not clear, SAMHD1 has been shown to deplete the intracellular dNTP pool thus strongly limiting the viral reverse transcription step (❸). Restriction factors can limit HIV infection in DC therefore also disfavouring antigen load and recognition and limiting DC innate immune response. Upon detection of viral RNA, TLR8-mediated NF-κB activation can synergize with DC-SIGN/Raf-1-mediated pTEF-b recruitment to initiate viral transcription and elongate nascent viral transcripts, respectively (❹). Alternatively, HIV infection regulates cellular gene expression, including upregulation of IRF-1 expression, which sustain viral transcription (❺). Upon viral capture or infection, HIV can benefit from intracellular trafficking compartments, including the exosomal pathway or tetraspanin-enriched compartments, to evade DC-mediated pathogen recognition and spread toward target CD4⁺ T cells (❻). When HIV-derived antigens are delivered in MHC-II compartments and processed on MHC-II molecules, they can be presented to HIV-specific CD4⁺ T cells, which also concomitantly favour DC-mediated HIV transfer to HIV-specific CD4⁺ T cells (❼). In this scheme, the steps known to restrict or enhance HIV infection and transfer are in *blue* or *red*, respectively

peptide-like 3G or 3F (APOBEC3G/3F or A3G/A3F), bone marrow stromal cell antigen-2 (BST-2 also named tetherin, CD317 or HM1.24) and sterile alpha motif and HD domain 1 (SAMHD1) (for review see (Kirchhoff 2010; Laguette and Benkirane 2012)). DC express these four innate antiviral factors. However, BST-2

was seemingly not able to restrict DC-mediated HIV capture and transfer (Coleman et al. 2011), and TRIM-5a, although important for interspecies retroviral replication blockade and recently described as a PRR able to initiate innate immune response upon retroviral capsid lattice recognition (Pertel et al. 2011; Sayah et al. 2004; Stremlau et al. 2004) was seemingly not involved in HIV-1 restriction in human DC (Pion et al. 2006). On the contrary, APOBEC 3G and 3F, which were known to restrict retroviruses during the later steps of viral replication by incorporating into budding virions and promoting guanine to adenine editing of nascent retroviral transcripts in the newly infected cell (Bishop et al. 2004; Harris et al. 2003; Mangeat et al. 2003; Sheehy et al. 2002) appeared to restrict, at least in part, HIV replication and spread in DC from myeloid or plasmacytoid origin (Mohanram et al. 2011; Pion et al. 2006; Stalder et al. 2010; Trapp et al. 2009; Wang et al. 2008a; Peng et al. 2007). HIV-1 could, however, counteract A3G-mediated restriction via the action of the viral infectivity factor (Vif) which could direct A3G toward proteasomal degradation (Sheehy et al. 2003; Stopak et al. 2003). Furthermore, another member of the APOBEC3 family, A3A, was also found to restrict HIV in DC but rather at an early phase of infection (Berger et al. 2011; Peng et al. 2007). Interestingly, A3G-mediated restriction was also proposed to take part at a post-entry level in DC and it could be removed upon pre-treatment with arsenic trioxide (Stalder et al. 2010), although the exact mechanism remains unclear.

The likely most potent block observed in DC and myeloid cells was, however, unveiled recently with the discovery of the antiviral activity owned by the cellular factor SAMHD1 (Hrecka et al. 2011a, b; Laguette et al. 2011). Indeed, the post-entry lentiviral restriction block in DC could be removed in cells previously exposed to SIV-derived lentivectors expressing the viral protein X (Vpx), which is absent from HIV-1 viral genome (Goujon et al. 2003, 2006). In elegant biochemical experiments, it was discovered that Vpx could in fact target SAMHD1 toward proteasome-mediated degradation (Hrecka et al. 2011a, b; Laguette et al. 2011), thus facilitating viral RNA reverse transcription. The mechanism of action relied on the dGTP-regulated deoxynucleoside triphosphate hydrolase enzymatic activity of SAMHD1 (Goldstone et al. 2011; Powell et al. 2011), thus lowering the intracellular pool of deoxynucleotides triphosphates (dNTP) and disfavouring reverse transcription process.

So it could be that the presence of intrinsic immune factors, at first anticipated to block viral dissemination, could, in fact benefit HIV by hiding it from cellular immune surveillance rendering DC much less responsive and ultimately favouring viral spread. This was suggested in a report showing that removal of SAMHD1-mediated HIV-1 restriction could render DC highly responsive to viral infection as evidenced by the enhancement of innate and adaptive immune responses upon HIV-1 challenge (Manel et al. 2010). The question remains, however, on the exact role of A3G and SAMHD1 regarding DC-mediated immune responses. Indeed, while TRIM-5α was reclassified recently as a PRR due to its ability to intercept incoming viral capsids and to initiate innate signalling, it appears that A3G and SAMHD1 do not possess such activity. Interestingly, there are some hints linking these restriction factors not just to innate but also to adaptive immune responses. Accordingly, in addition to its intrinsic antiviral role, A3G enzymatic activity also

resulted in an enhanced adaptive immune response due to the accumulation of abortive viral proteins in A3G-edited HIV-defective infected cells which led to a potent activation of HIV-specific cytotoxic $CD8^+$ T lymphocytes (Casartelli et al. 2010). The link between SAMHD1 and innate immune response was revealed upon the discovery that this protein is often mutated in the type-5 Aicardi-Goutière syndrome (AGS5) (Rice et al. 2009). This syndrome with symptoms similar to congenital viral encephalopathy is, indeed, characterized by a chronic cerebrospinal fluid lymphocytosis and high levels of type-I interferon and thus strongly suggest that SAMHD1 could negatively regulate IFN-α production.

8.5 DC-T Cell Infectious Synapse: The Last Step Before Infection of $CD4^+$ T Cells

Upon contact between HIV-containing DC and $CD4^+$ T cells, infectious virions are rapidly repolarized from the previously described intracellular compartments toward the zone of cell-to-cell contact thus creating a so-called Infectious or Virological Synapse (IS or VS) (McDonald et al. 2003). This mode of cell-to-cell transmission is fully reminiscent of the synapse observed between HTLV-I infected and non-infected T cells (Igakura et al. 2003) and requires an intact cytoskeleton (Wang et al. 2008b). Interestingly, HIV-envelope could also regulate this mode of viral transfer upon binding to DC. Accordingly, it was shown that HIV-envelope in immature DC, upon binding to DC-SIGN, could induce Cdc42 activation required to promote the formation of filopodia-like structures at the cell surface and promoting efficient DC-mediated HIV transfer to the target $CD4^+$ T cells via the IS (Nikolic et al. 2011). In contrast to the situation in immature DC, in mature DC, cell-to-cell transfer of HIV could also occur via direct T cell extensions capable of reaching HIV-containing compartments in DC and gradually picking up virions (Felts et al. 2011). This mode of viral transmission not only allows efficient binding and fusion of HIV to T cells, but it also supports infection with low numbers of viral particles. Finally, it would also create a perfect protecting environment for the virus causing a restricted accessibility to most of the immune mediators like neutralizing antibodies.

8.6 HIV Immunopathogenesis and DC Immune Regulations

Based also on simian models with SIV infection, it is widely accepted that the hallmark of HIV immunopathogenesis is a delayed cellular and humoral response concomitantly with a misled T helper type-2 response which is finally translated in the inability to fully block viral replication leading to a generalized and chronic immune activation (McMichael et al. 2010). In fact, these sequential events could have been set up very early upon HIV intrusion, during a short and transient eclipse phase when the virus face the very first immune sentinels.

A robust and sustained $CD8^+$ T cell-mediated cellular immune response is critically required during the earliest events of HIV and SIV infection. However, this response, while functional, appeared as "too little and too late" and is overall attenuated over time (Li et al. 2009b; Reynolds et al. 2005). While DC subsets critically contribute to the cellular immune response initiation, it appeared that HIV could exploit some of their intracellular pathways in order to promote the clinically observed exhaustion of $CD8^+$ T cell response (Day et al. 2006; Trautmann et al. 2006). Indeed, PD-1 upregulation on $CD4^+$ and $CD8^+$ T cells in HIV-infected patients caused dysfunctional HIV-specific T cell responses, when engaged with its ligand PD-L1, and this correlated with disease progression (Day et al. 2006; Trautmann et al. 2006). Hence, PD-L1 was shown to be highly upregulated on DC and myeloid cells upon HIV infection (Trabattoni et al. 2003; Wang et al. 2008c), seemingly via HIV-induced PI3K/serine threonine kinase signalling pathway activation in DC, and this correlated with an impairment in HIV-specific $CD8^+$ T cell responses (Muthumani et al. 2011). Thus, DC could be involved in the decline of the antiviral cellular immune response.

As explained during this chapter, most of the DC immune defects (see Table 8.1) come from the initial binding of HIV-envelope on various receptors at the surface of DC subsets. Indeed, signalling triggered upon gp120 binding of DC-SIGN involves Erk1 and Erk2 kinases and induces IL-10 production while reducing IL-12 secretion (Blanchet et al. 2010; Shan et al. 2007). This would favour Th2 type immune response while inhibiting a Th1 phenotype. In support of this, investigations on the effect of a plasmid DNA-based vaccine encoding HIV-1-gp120 was shown to stimulate production of innate IL-10 thus favouring a biased Th2 response by the inhibition of DC-mediated Th1 response (Daly et al. 2005). This misled DC-mediated T helper response was indeed observed in HIV-infected patients where a prevalent Th2 switch (Clerici et al. 1993) and even a switch from Th1 to Th0 (T cells expressing both Th1 and Th2 cytokines) phenotype occurred (Maggi et al. 1994). While it could be efficient to clear parasites or some bacteria, the Th1/Th2 switch, along with the subsequent unsustained Th1 phenotype, is rather ineffective against intracellular pathogens such as HIV (McMichael et al. 2010). Another explanation for the HIV-mediated biased Th2 response could rely on the interplay between DC and NK cells. During viral infection and inflammation, DC produce chemokines that recruit innate immune cells such as NK cells. In HIV viraemic individuals, IFN-α production by plasmacytoid DC was altered resulting in defective IFN-α-induced NK cell activity (Conry et al. 2009). Also, it has been shown that co-culture of HIV-infected DC and NK cells results in a lower production of IL-12, IFN-γ and IL-18 leading to the impairment of Th1 polarization of CD4 lymphocytes (Saidi et al. 2008).

Another function of DC is to induce different forms of tolerance, either in the steady state or in the presence of suppressive cytokines such as IL-10 and TGF-β. In addition to the DC-mediated IL-10 production observed, some types of DC could induce the differentiation of $CD4^+/CD25^+$ regulatory T cells (Tregs) that mainly produce IL-10 which would increase and sustain the high levels of this immunosuppressive cytokine. Interestingly, while reports stating the levels of Tregs in HIV-infected patients appeared controversial, two studies demonstrated that these

Table 8.1 Profile of different DC subsets and consequences on their function upon HIV infection

DC subset	TLR	Chemokine receptor expression changes in HIV infection	Characteristic markers and features	Main cytokines produced	HIV infection impairment
LC	TLR1, TLR2, TLR5, TLR6, TLR9	↑CXCR4, ↓CCR5, ↓CCR6	CD207 (langerin), Birbeck granules	IL-1α, IL-1β, IL-10, IL-6, IL-8	Decreased allogeneic T cell stimulation
pDC	TLR7, TLR9	CCR2, CCR4, ↑CCR7, ↓CCR5	CD123, BDCA-2 (CD303)	High levels of INF-α/β, also TNF-α, IL-6, IL-8, IL-12	In chronic stage of infection decrease of pDCs numbers
myDC	TLR1, TLR2, TLR3, TLR4	↓CCR1, ↓CCR2, ↓CCR3, CCR4, ↓CCR5	CD11c, CD141, (BDCA-3), CD1c	Mainly IL-12, also IL-15, IL-18, TNF-α	↑IL-12 at early stage of infection and ↓IL-12 at chronic stage, ↓TNF-α
MDDC	TLR2, TLR3, TLR4	↓CCR1, ↓CCR2, ↑CCR4, ↓CCR5	CD1a, CD11c	IL-12, IL-10, IL-6, TNF-α	↓IL-12, ↑IL-10, impaired maturation

LC Langerhans cells, *pDC* plasmocytoid dendritic cells, *myDC* myeloid dendritic cells, *MDDC* monocyte delivered dendritic cells, *TLR* toll-like receptors

immunosuppressive T cells were indeed highly increased in lymphoid tissues and mucosal areas of HIV-infected patients (Andersson et al. 2005; Epple et al. 2006). This could be of particular importance regarding the dysfunction of specific T cell response and, again, some subsets of DC could be involved in the recruitment and activation of Tregs. In support of this, pDC were shown to induce Tregs via an IDO expression-dependent mechanism which could lead to virus-specific T cell responses impairment and also to the inhibition of bystander conventional DC maturation (Manches et al. 2008). Furthermore, an myDC-mediated IDO increase could also contribute to Tregs increase while reciprocally inducing the loss of Th17 cells in the peripheral blood and rectosigmoid colon area (Favre et al. 2010). This hypothesis would fit with the observed recurrent recruitment of pDC and myDC subsets at sites of infection and inflammation which could drive the induction of immunosuppressive regulatory T cells while dampening the levels of Th17 cells, critically required to maintain the mucosal barrier integrity. A loss of Th17 cells would thus explain the HIV-1-mediated destruction of gut barrier integrity leading to translocation of microbial products into the circulation and promoting the chronic immune activation, a feature hallmark of HIV immunopathogenesis (Brenchley et al. 2006).

Another potentially important aspect of pDC contribution to HIV pathogenesis rely on the hypothesis that their activation threshold and the amount of IFN-α produced would be determinant for immunopathogenesis. Indeed, the level of pDC activation and FN-α production in sooty mangabeys, a natural reservoir host for SIV not progressing to AIDS, appeared markedly lower when compared with HIV infection (Mandl et al. 2008). Thus, uncontrolled and aberrant pDC activation would be a critical set-point for HIV-mediated immunopathogenesis. Furthermore, the two-side coin of the aberrant increased IFN-α production is that it would induce a profound pDC cell death and depletion, via the intrinsic apoptosis pathway, which also an evident feature of viral pathogenesis (Swiecki et al. 2011).

Altogether, these clinical and experimental data strongly suggest that HIV impact on DC and their subsets would critically contribute to the dysregulated antiviral immune responses thus establishing an immunotolerant environment promoting HIV-driven immunopathogenesis.

8.7 Conclusion and Future Directions

Since the first reported case of HIV-1 in an infected human being in 1981, the HIV/AIDS virus has infected over 60 million people worldwide and caused more than 25 million deaths.

Current global estimates suggest that there are greater than 33 million infected individuals with an annual incidence of 3 million cases per year. (www.unaids.org)

The development of HAART (Highly Active Anti-Retroviral Treatment) in the 1990s (highly active anti-retroviral treatment) has led to part control of HIV replication in some patients but failure to eradicate the virus and result in cure. Therefore, the development of a safe and highly effective preventative HIV-1 vaccine has been a global health priority to halt the pandemic (Virgin and Walker 2010).

Early efforts in developing HIV-1 vaccines, concentrated on generating neutralizing antibodies against the envelope protein using gp120. Two of these envelope-based vaccines were tested in phase III clinical trials and offered no protective effect (Flynn et al. 2005; Pitisuttithum et al. 2006). Failure of these types of vaccines was mainly due to the fact that potent neutralizing anti-HIV antibodies were hardly generated. Outstandingly, it was reported recently that a vaccine-induced mucosal humoral immunity could greatly protect against repeated SHIV vaginal challenges. In this study the authors used market-approved virosomes containing gp41-derived peptides targeted to mucosal epithelial cells and DC, via the binding sequence of the galacosyl-ceramide receptor, upon intramuscular and intranasal immunizations (Bomsel et al. 2011).

Hope to develop an effective humoral-based vaccine was also recently reignited with the report of a vector immunoprophylaxis (VIP) vaccine model (Balazs et al. 2012). This methodology could be a promising alternative to immunization consisting of the use of adeno-associated virus (AAV)-based vector to direct expression of potent and well characterized human broadly neutralizing antibodies (Balazs et al. 2012). Although it was shown to fully block HIV replication even at high doses of viral challenge, these experiments were performed in mice, even if humanized, and HIV challenge was done intraperitoneally, thus not mimicking the main route of viral entry via mucosae (Balazs et al. 2012). While being extremely promising to compensate for the lack of naturally occurring potent neutralizing antibodies, this vaccine methodology would need further investigation and would also require to be done in nonhuman primates.

With the knowledge of the importance of early and potent $CD8^+$ T cell-driven cellular immune responses in controlling HIV-1 infection and the difficulty in inducing neutralizing antibodies, growing interest then ensued in developing T cell-based vaccines. From the studies in primates, T cell-based vaccines are unlikely to eradicate infection but rather control HIV-1 replication and so reduce disease progression. Many of these vaccines have been tested clinically but only four reached efficacy (phases III or IIb) trials and the results of two of them were very disappointing (STEP and Phambili). Only the RV144 trial in Thailand showed low-level efficacy (31%) in reducing HIV-1 infection rates (Rerks-Ngarm et al. 2009). Since 1987, over 30 candidates HIV-1 vaccines displaying varying degrees of response in primate models have progressed to clinical trials (Mascola and Montefiori 2010; McMichael 2006), but for now there remains a clear lack of vaccines that work by inducing protective T cells.

However the consensus view is that an effective HIV vaccine will require the coordination and broad repertoire of B cell, $CD4^+$ and $CD8^+$ T cell responses and a vaccine-mediated mucosal immune response could fulfil all these aspects (McElrath 2011).

LC and DC are probably the earliest targets of HIV infection. Despite the fact that HIV replication is quite inefficient in DC, HIV has evolved strategies to exploit these cells in order to promote its dissemination while altering DC maturation events and impairing subsequent DC-mediated immune responses. Understanding the biology of DCs is central to the development of new generation immunotherapies and

the use of DC-based vaccines for cancer or chronic infections. In the case of AIDS, the use of DC-targeted vaccines aimed at improving DC-mediated cellular and humoral immune responses were pioneering ideas over 15 years ago (Steinman 1996a, b). However, many obstacles are still evident in the development of these vaccines for cancer and chronic infection therapies; source of DC preparation and protocols for DC generation, activation and loading of antigens, source of antigens, route of vaccine administration and methods of immunomonitoring. Despite these obstacles there is still promise for these potential vaccines in AIDS. Accordingly, preliminary studies in mice convincingly showed that vaccine delivery to DC, via antibody against DC-specific CLR targeting, could elicit and improve humoral and cellular immune responses (Cheong et al. 2010; Nchinda et al. 2010, 2008; Trumpfheller et al. 2008, 2006). Importantly, a recent study in nonhuman primate using the same approach, clearly demonstrated a suboptimal efficacy of a DC targeted vaccine (+ adjuvant critically required) further improved following a boost with an HIV antigens-expressing recombinant New York vaccinia virus (NYVA) (Flynn et al. 2011).

Another approach to block early events of HIV infection and DC-mediated propagation would rely on the use of microbiocides, such as PRO2000 shown to potently block DC-mediated HIV transfer and infection in the DC-T cell environment (Teleshova et al. 2008), and entry inhibitors targeting the viral envelope at the DC surface which were proven to inhibit HIV infection in DC-T cell co-cultures (Frank et al. 2008).

However, a clearer understanding to delineate the exact interactions between DCs and HIV as well as subsequent consequences on viral transmission and pathogenesis will be required in order to contribute to searching for novel prophylactic, and potentially therapeutic, strategies.

Acknowledgements This book chapter was supported by Cardiff University and by a grant from the Gates Foundation to V.P.

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Chapter 9
HIV-Derived Vectors for Gene Therapy Targeting Dendritic Cells

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Abstract Human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vectors (LV) have the potential to mediate stable therapeutic gene transfer. However, similarly to other viral vectors, their benefit is compromised by the induction of an immune response toward transgene-expressing cells that closely mimics antiviral immunity. LV share with the parental HIV the ability to activate dendritic cells (DC), while lack the peculiar ability of subverting DC functions, which is responsible for HIV immune escape. Understanding the interaction between LV and DC, with plasmacytoid and myeloid DC playing fundamental and distinct roles, has paved the way to novel approaches aimed at regulating transgene-specific immune responses. Thanks to the ability to target either DC subsets LV might be a powerful tool to induce immunity (i.e., gene therapy of cancer), cell death (i.e., in HIV/AIDS infection), or tolerance (i.e., gene therapy strategies for monogenic diseases). In this chapter, similarities and differences between the LV-mediated and HIV-mediated induction of immune responses, with specific focus on their interactions with DC, are discussed.

9.1 Introduction

Gene therapy consists in restoring, modifying, or enhancing cellular functions through the introduction of a functional gene in the target cells. Significant progresses have been achieved in the field, and several new gene transfer systems have

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_9,

been optimized; however, a major challenge is to ensure that vector delivery does not have any adverse effect. Clinical gene therapy protocols for some monogenic diseases, including ADA-SCID and X-linked SCID (Aiuti et al. 2002, 2009), have been developed, but the therapeutic efficacy of gene transfer in immuno-competent patients has been limited by the induction of immune responses against components of the transfer vector and/or against the functional protein, ultimately resulting in the elimination of genetically modified cells (Mendell et al. 2010; Orchard and Wagner 2011). Up to now, immunosuppressive treatments are used to limit undesired immune responses; regrettably, this approach generates nonspecific abrogation of immune functions, does not induce antigen-specific tolerance, and, in most cases, is associated with long-term side effects due to suppression of protective immunity. Therefore, the identification of novel strategies for the induction of transgene-specific tolerance is required to improve the efficacy of therapeutic gene transfer.

Human immunodeficiency virus type 1 (HIV-1)-derived lentiviral vectors (LV) (Naldini et al. 1996) represent an attractive strategy for therapeutic gene transfer (Vigna and Naldini 2000; Follenzi et al. 2002, 2004; Kang et al. 2005) as well as antitumor vaccination (Lizee et al. 2004; Dullaers et al. 2006; Lopes et al. 2006; Rowe et al. 2006; Breckpot et al. 2008; Arce et al. 2009). LV are capable of stable chromosome integration, even in nondividing cells, and have a limited expression of viral antigen, which reduces their immunogenic potential (Naldini et al. 1996). Compared to retroviral vectors (RV), LV have a low genotoxic profile (Montini et al. 2009; Biffi et al. 2011); indeed, while RV preferentially integrate in the 5′ regions of genes, where most of the elements for transcriptional control are located, LV do not, thus perturbing the gene expression profile of the target cells to a lesser extent (Montini et al. 2006). However, LV injection in immuno-competent mice leads to the induction of antigen-specific immune responses, with the final result being the clearance of genetically modified cells (Follenzi et al. 2004). The mechanism underlying the immunogenicity of LV has been extensively investigated, and data from our group show that LV retain features of the parental HIV, which eventually trigger immune activation. In this context, a major role in the induction of transgene-specific immunity has been attributed to dendritic cells (DC). Interestingly, LV-mediated human DC activation closely mimics the one induced by the parental HIV (Rossetti et al. 2011). However, LV do not inherit from their ancestor its peculiar ability of subverting DC functions to weaken effector mechanisms, and to trigger mechanisms of active tolerance, which eventually result in HIV immune escape. Indeed, DC from HIV-infected individuals are not only impaired in their ability to induce effective T cell responses, but are also prone to induce HIV-specific regulatory T cells.

In this chapter, we introduce the basic structure of a typical monocistronic LV and of more complex vectors for gene therapy, and we discuss similarities and differences between the LV-mediated and HIV-mediated induction of immune responses, with specific focus on their interactions with DC. Finally, we introduce how LV themselves can be exploited for the therapy of HIV-infected individuals.

9.2 Lentiviral Vectors for Gene Delivery

LV derived both from primate lentiviruses (HIV-1, HIV-2, and simian immunodeficiency virus, SIV) or non-primate lentiviruses (feline immunodeficiency virus, FIV, and equine infection anemia virus, EIAV) have been developed. In light of the disease caused by the wild-type virus in its natural host, many efforts have been devoted to develop LV compliant with the strictest biosafety standards. The first step has been to separate the *cis*-acting elements required for the transfer of the viral genome to target cells, which are maintained in the transfer vector construct, from the *trans*-acting sequences encoding the viral proteins, which are provided by distinct packaging constructs (Vigna and Naldini 2000). The transfer vector and the packaging constructs are introduced in the same cell to produce functional vector particles. However, since the particles can only encapsidate the transfer vector construct, the infection process (called transduction) is limited to a single round, resulting in the integration of the transfer vector into the genome of the target cell with no further production of viral particles. The extent of the segregation between *cis*- and *trans*-acting elements determines the efficiency and biosafety of a vector. However, multiple recombination events could theoretically generate replication-competent recombinants (RCR). This unlikely event can be minimized in several ways, such as reducing sequence overlap between different constructs, deleting unnecessary and pathogenic sequences, and mismatching the viral source of packaging and transfer constructs (White et al. 1999). On top of that, the use of a heterologous envelope decreases even more the risk of generating a replication-competent virus. The latest (third) generation of HIV-1-derived packaging constructs completely lacks the genes *vif*, *vpr*, *vpu*, and *nef* (Zufferey et al. 1997) to restrict the pathogenic potential of the virus. Moreover, the transcriptional dependence of HIV-1 on TAT has been overcome by fusing strong constitutive promoters with elements of HIV-1 LTR upstream of the transfer vector, thus allowing the deletion of the gene *tat* (Dull et al. 1998; Kim et al. 1998). By contrast, the *rev* gene, which regulates not only nuclear export of un-spliced viral RNA, but also stability and translation of RNA species required for expression of HIV structural proteins and production of genomic RNA (Ellis 2005), is provided as a separate plasmid (Dull et al. 1998) (Fig. 9.1a).

The biosafety of LV has been also improved by generating self-inactivating (SIN) transfer vectors carrying a major deletion in the U3 region of the LTR, which inactivates the LTR transcriptional activity (Miyoshi et al. 1998; Zufferey et al. 1998). SIN vectors reduce the risk of oncogene activation by promoter insertion and the risk of vector mobilization and recombination with wild-type HIV (Bukovsky et al. 1999); moreover, SIN vectors carry internal promoters, which allow tissue-specific or conditional transgene expression. In recent years, non-integrating LV have been developed to further decrease their oncogenic risk and LV-specific immunity (Matrai et al. 2011). Other improvements of the transfer vector include the introduction of the posttranscriptional regulatory sequence from the genome of the woodchuck hepatitis virus (WPRE) in 3′ of the transgene to increase its expression (Zufferey et al. 1999), and insertion of the central polypurine tract (cPPT) in front of the transgene, to improve vector integration rate (Sirven et al. 2000; Zennou et al. 2000) (Fig. 9.1a).

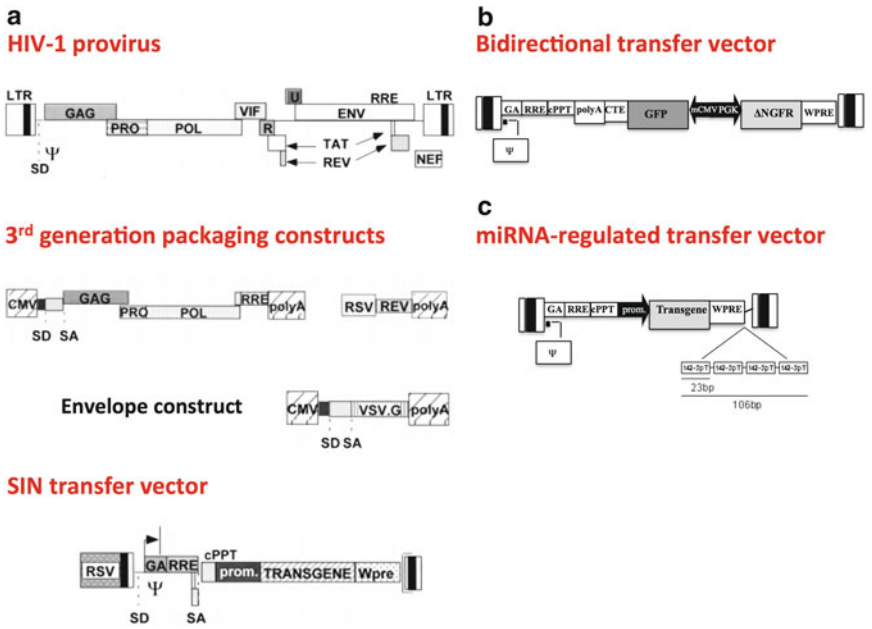

Fig. 9.1 Lentiviral vector structure. (**a**) Schematic representation of the HIV provirus, the 3rd generation packaging constructs, the envelope construct, and the improved SIN transfer vector. The four plasmids are transfected in packaging cell lines to obtain a viral particle packaging only the transfer vector. (**b**) Structure of a bidirectional LV. The presence of the bidirectional promoter (mCMV/PGK in opposite direction) allows co-regulated expression of the two marker transgenes GFP and ΔNGFR. (**c**) Structure of a miRNA-regulated LV incorporating four tandem copies of a sequence completely complementary to a specific miRNA (in this case, mir-142-3p). *LTR* long terminal repeats, *Ψ* packaging signal, *RRE* Rev-responsive element, *cPPT* central poly-purine tract, *polyA* poly-adenylation signal, *CTE* constitutive RNA transport element, *mCMV* minimal core element of the cytomegalovirus promoter, *PGK* human phosphoglycerate kinase promoter, *WPRE* woodchuck posttranscriptional regulatory element, *prom.* internal promoter, *SD* splice donor, *SA* splice acceptor, *GA* a portion of the HIV-1 gag gene with a closed reading frame. Modified from: (Vigna and Naldini 2000; Amendola et al. 2005; Brown et al. 2007a, b)

The co-expression of multiple transgenes within the same target cell is useful or even mandatory for a series of research and clinical applications; these comprise the incorporation of a marker gene allowing for purification and tracking of transgene-expressing cells, and the insertion of drug resistance genes or, vice versa, conditionally cytotoxic genes, in addition to the therapeutic transgenes, to eliminate modified cells for safety reasons. To this aim, several strategies have been developed, including double transduction with two different LV, expression of two transgenes driven by two different promoters within the same LV, use of internal ribosome entry sites (IRES), or production of polyproteins. However, all these strategies have denoted several major limitations hindering their application. A major advance in this field has been the development of bidirectional promoters, formed by a minimal core promoter upstream of an efficient promoter, to allow co-transcription of two transgenes

in opposite direction (Fig. 9.1b) (Amendola et al. 2005). Finally, the incorporation of cell type-specific microRNA downstream a transgene has been used to effectively restrict transgene expression in specific cell types (Fig. 9.1c) (Brown et al. 2007a, b). This technology can be efficiently applied to increase LV safety and reduce unwanted immune responses, by limiting transgene expression only to target cells, as discussed later.

9.3 Immune Responses Against Transgenes and LV-Derived Proteins

The major obstacle for achieving therapeutic efficacy using LV-mediated gene transfer is the development of an immune response toward the transgene and/or LV components, which eventually leads to the clearance of transgene-expressing cells. Long-term transgene expression has been achieved after gene replacement therapy in immuno-compromised mice (Tsui et al. 2002) and humans (Aiuti et al. 2002, 2009). However, systemic administration of LV encoding transgene in immunocompetent mice resulted in the induction of transgene-specific adaptive immune response characterized by infiltration of transgene-specific $CD8^+$ cytotoxic T cells in liver and spleen, and production of transgene-specific antibodies (Follenzi et al. 2004). The direct transgene expression within professional antigen-presenting cells (APC), such as myeloid (my)DC, is one of the mechanisms underlying the induction of an active immune response after LV-mediated gene transfer. In addition, plasmacytoid DC (pDC), due to their unique ability to detect viral infections, are critically involved in promoting immune responses after gene transfer approaches (Follenzi et al. 2007).

9.3.1 LV-Mediated Murine DC Activation In Vivo

A few papers have been published on the role of DC in the response against LV. Brown et al. (2007a, b) demonstrated that systemic LV administration to mice triggers a rapid and transient type I IFN response, which is dependent on functional vector particles; similarly to what happens during viral infections, pDC were proposed to be involved in this response. LV rapidly activate pDC to secrete IFN-α, which induces a generalized antiviral state and reduces cell transduction by LV (Brown et al. 2007a, b). Type I IFN secretion not only strongly inhibited transduction, but also contributed to the immuno-mediated clearance of transduced cells. Indeed, intravenous injection of an LV expressing GFP driven by a ubiquitous promoter in the IFNAR knockout mice led to a dramatic increase in liver transduction. By contrast, Reis e Sousa's group (Pichlmair et al. 2007) showed that both murine bone marrow cells, which include pDC, and isolated pDC secrete high levels of IFN-α when challenged with LV and pseudotyped with the envelope glycoprotein

from vesicular stomatitis virus (VSV-G). In this setting, IFN-α secretion by pDC was not due to the LV itself, but due to specific tubulovesicular structures (TVS) derived from VSV-G-transfected producer cells. These structures, co-purified with LV particles during vector concentration, carried residual amount of plasmid DNA used during LV production, which through TLR9 engagement led to IFN-α secretion by pDC. This problem was overcome when LV were pseudotyped with other viruses than VSV-G (Pichlmair et al. 2007). However, other groups did not report any problem due to the VSV-G envelope, but still emphasized the importance of an efficient LV entry for pDC activation (Brown et al. 2007a, b; Pichlmair et al. 2007). In conclusion, LV activate pDC through the engagement of TLR7/9, like the parental HIV-1 (Beignon et al. 2005); however, blocking TLR7 or TLR9 pathways is not sufficient to prevent LV from inducing a type I IFN response in vivo (Follenzi et al. 2007), thus implying that other mechanisms may be involved.

MyDC activation might be also responsible for the emergence of transgene-specific adaptive immunity, again similarly to viral infections. Indeed, the LV-mediated neo-antigen GFP expression, driven by a ubiquitous promoter, has been shown to become the target of transgene-specific cellular and humoral immune responses, resulting in the clearance of transduced cells (Follenzi et al. 2004; Annoni et al. 2007). In addition, LV pseudotyped with glycoproteins from Ebola Zaire Virus, lymphocytic choriomeningitis virus, Mokola virus, and VSV, and delivered to the mouse lung, resulted in the activation of effector T cells specific for both the transgene and the vector itself (Limberis et al. 2010). LV efficiently transduce myDC in vivo, which in turn induce a potent transgene-specific T cell-mediated response (Esslinger et al. 2003; Arce et al. 2009). However, direct myDC transduction is not required for effective transgene presentation, since myDC can acquire the transgene from transduced cells or apoptotic bodies and present it to $CD4^+$ T cells, or cross-present it to $CD8^+$ T cells (Annoni et al. 2009). In addition, maturation and subsequent immunogenic transgene presentation are critically induced by the inflammation state promoted by the LV injection procedure, and/or by the production of type I IFN and pro-inflammatory cytokines by pDC upon LV recognition.

In conclusion, the induction of an immune response against the transgene or vector-derived proteins after LV-mediated gene transfer results in the direct activation of both innate and adaptive branches of the immune system, represented by pDC and myDC, respectively.

9.3.2 LV-Mediated Human DC Activation Ex Vivo

Little is known about human DC responses to recombinant LV. We recently investigated the effect of LV-mediated transduction on freshly isolated human DC in vitro, demonstrating that myDC are not activated by LV transduction, but are more effectively transduced compared to pDC (Rossetti et al. 2011). Moreover, upon LV exposure, pDC are activated to produce IFN-α and TNF-α, eventually inducing myDC maturation.

LV internalization is required to fully activate pDC, and, by performing studies using inhibitory molecules, we showed that the mechanism responsible for pDC activation involves TLR7 and TLR9. As already reported for HIV, a major role in LV-mediated pDC activation is played by TLR7, since LV genetic material is constituted by single-strand RNA; however, as the inhibitor for TLR7 used in our studies, IRS954 (Barrat et al. 2005), also blocks TLR9 signaling, it cannot be excluded that TLR9 might be involved in pDC activation. Indeed, TLR9 might recognize DNA present in the vector particles as a result of initiation of retro-transcription already in virions or from plasmid contaminants. VSV-G was also involved in pDC activation in vitro, supporting its role in the induction of LV immunity, as already reported in vivo (Marsac et al. 2002; Kuate et al. 2006). Indeed, empty LV particles pseudotyped with VSV-G were still able to induce IFN-α production by pDC, although at significant lower levels compared to fully competent LV. However, it still remains unclear whether pDC activation is mediated by specific VSV-G recognition by an unknown receptor, or by VSV-G-mediated carryover of TLR ligands from producer cells (Pichlmair et al. 2007).

In the early stages of infection, pDC orchestrate innate antiviral responses through the secretion of type I IFN, but upon activation they can differentiate into mature DC, which directly activate T cell functions (Liu 2005). However, in our hands LV induced high levels of IFN-α production but not up-regulation of co-stimulatory molecules on pDC (Rossetti et al. 2011); this data points against a direct role of pDC in the activation of transgene-specific adaptive immune responses, suggesting that they act indirectly, through activation of other cell subsets, such as myDC, which then finely tune the immunological outcome. We therefore propose a mechanism by which LV-activated pDC induce local maturation of myDC, which take up and process the transgene to be presented to T cells in lymphoid organs (Fig. 9.2).

TLR9 ligands with different molecular structure and subcellular localization, such as CpG-A and CpG-B, are known to differentially induce an analogous dichotomic response by pDC, i.e., either IFN-α production or enhancement of antigen presentation (Guiducci et al. 2006). Moreover, while pDC produce significant amount of type I IFN upon recognition of viruses via TLR7, synthetic TLR7 ligands have been shown to induce up-regulation of co-stimulatory molecules and production of pro-inflammatory cytokines by pDC, accompanied by low levels of IFN-α, suggesting a dual signaling pathway downstream TLR7 in pDC. Recently, these divergent pDC responses to TLR7 ligands have been linked to a differential nuclear translocation of IRF-7, which is responsible for the IFN-α release, and NF-κB (Di Domizio et al. 2009), known to regulate the production of pro-inflammatory cytokines and the up-regulation of co-stimulatory molecules (Gilliet et al. 2008). It is therefore possible that LV-mediated pDC activation results in TLR7-driven nuclear translocation of IRF-7 but not NF-κB, once more mimicking the mechanism of pDC activation by viruses. Overall, results obtained in vitro with human pDC are in line and support results obtained in vivo in mice (Brown et al. 2007a, b), and suggest that strategies aimed at using LV in vivo for long-term gene replacement therapy may need to suppress the initial type I IFN response by pDC. On the other hand, enhancing the type I IFN response may be beneficial in the case of cancer immunotherapy.

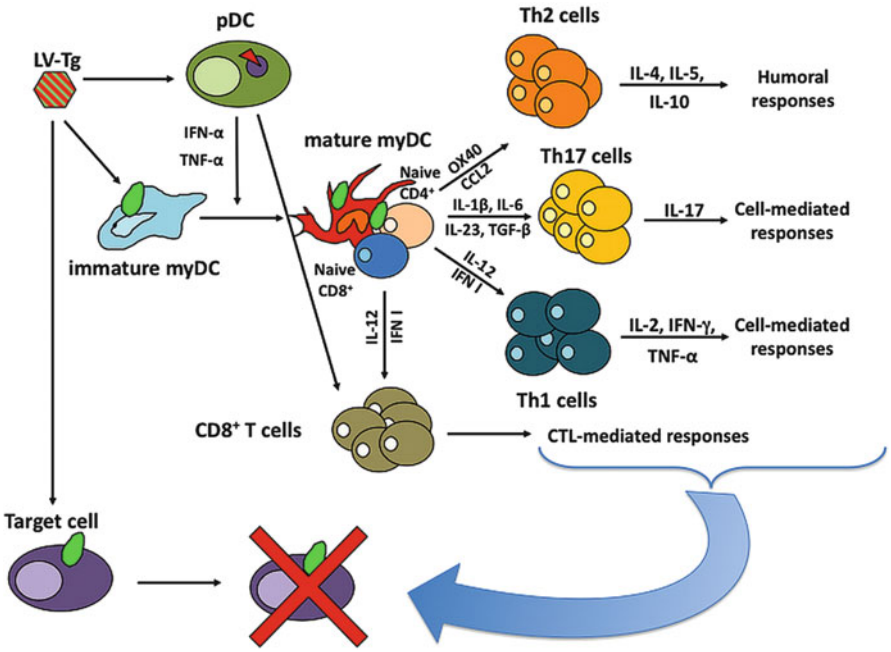

Fig. 9.2 Cross talk between myDC and LV-activated pDC. LV particles trigger intracellular TLR7/9 and induce secretion of type I IFN and TNF-α from pDC. In turn, factors secreted by pDC induce the maturation of bystander myDC, which eventually initiate the antigen-specific immune responses finally leading to the clearance of genetically modified cells

9.3.3 HIV Versus LV-Mediated Activation of Human DC

DC play a major role during HIV infections, and alterations in DC numbers, phenotype, and function have been reported in HIV-1-infected individuals during acute and chronic stage of the infection. Peripheral myDC and pDC are decreased during acute infection (Pacanowski et al. 2001). In chronic HIV infection, pDC levels are inversely correlated with plasma viral load, and the depletion of pDC has been associated with HIV disease progression (Donaghy et al. 2001) and development of opportunistic infections (Soumelis et al. 2001). Evidences indicate that cell redistribution to lymph nodes, possibly due to higher viral replication in these sites, and cell death may contribute to decreased peripheral blood DC populations (Dillon et al. 2008; Lehmann et al. 2010; Meera et al. 2010). HIV-1 induces high levels of CCR7 expression on both pDC and myDC (Grassi et al. 1999; Barron et al. 2003) and this can explain their migration to lymph nodes in primary HIV-1 infection (Lore et al. 2002).

Both pDC and myDC express the HIV receptor CD4 and the co-receptors CCR5 and CXCR4, and both are infected by HIV-1 in vitro; however, myDC appear to be more susceptible to CCR5-using virus than pDC (Smed-Sorensen et al. 2005).

Interestingly, the two DC subsets display differential activation by HIV-1 infection in vitro (Smed-Sorensen et al. 2005): myDC do not mature, unless large amounts of virus are added to the culture (Smed-Sorensen et al. 2005; Harman et al. 2006), whereas pDC are activated and produce IFN-α and TNF-α, which in turn promote myDC maturation (Fonteneau et al. 2004). HIV-1-mediated pDC activation requires the binding of the HIV-1 envelope glycoprotein to CD4, leading to receptor-mediated endocytosis (Beignon et al. 2005). The main viral stimulus for pDC activation is the viral RNA, which induces IFN-α secretion through TLR7 (Beignon et al. 2005). Taken together, these studies suggest that the pathway used by LV and HIV-1 to activate pDC via TLR7 signaling and consequent myDC maturation is overlapping. However, differences may still be identified between the two systems. Several in vitro studies suggest the paradoxical effect of a diminished production of IFN-α by pDC of HIV-1-infected individuals, although high levels of the cytokine are detected in the blood. Tilton et al. (2008), however, showed that this diminished production of IFN-α is due to a feedback inhibition or prior stimulation via type I IFN or HIV in vivo.

Several studies evaluating the function of DC in response to in vitro stimulation to TLR agonists reported diminished responses in infected people (Martinson et al. 2007; Nowroozalizadeh et al. 2009; Yonkers et al. 2011), whilst others have reported an intact TLR responsiveness (Lester et al. 2008; Sabado et al. 2010). Conflicting findings may result from differences in patient cohorts, cell culture conditions, TLR stimulus, and response parameters used. Yonkers et al. (2011) reported that defects in myDC TLR responsiveness appear related to systemic immune activation. Indeed, myDC from HIV-infected subjects show a reduced tendency to up-regulate HLA-DR expression in response to poly I:C, whereas pDC have a reduced tendency to up-regulate CD86 and IFN-α production following TLR7 stimulation. Thus, it is possible that DC become partially attenuated in response to TLR stimulation because of prior activation in vivo, with resulting refractory responsiveness.

While LV do not induce up-regulation of co-stimulatory molecules on pDC, an increase of these molecules as well as markers of maturation was described on these cells when directly studied in HIV-infected individuals (Dillon et al. 2008; Yonkers et al. 2011; Grassi et al. 1999; Barron et al. 2003). Moreover, also myDC have the same phenotypic changes in blood, but have a reduced expression of the co-stimulatory molecules CD80 and CD86 in the lymphoid tissues during acute HIV-1 infection (Lore et al. 2002). These studies suggest that, despite an activation of pDC occurs in vivo during HIV-1 infection, the maturation of myDC is perturbed, which is in contrast to the observation with LV in vitro and in mice (Brown et al. 2007a, b).

The effect of these two DC populations on the activation of $CD4^+$ T cells in HIV-1-infected individuals has shown partially contrasting results. While Yonkers et al. report that myDC- but not pDC-dependent naïve $CD4^+$ T cell activation is impaired in HIV-infected subjects (Yonkers et al. 2011), in a previous study the ability of both myDC and pDC was severely impaired (Donaghy et al. 2003). It still remains to be understood whether the impact of LV on DC-mediated $CD4^+$ T cell activation resembles that of HIV-1.

9.3.4 HIV-Induced Immune Tolerance

DC function as an important bridge between innate and adaptive immunity, either through cellular interactions or through secretion of pro-inflammatory and immunoregulatory cytokines. In adaptive immunity, activated DC are crucial for the presentation of peptides and proteins to T cells and are widely recognized as professional APC, thanks to their ability to prime naïve T cells (Steinman and Nussenzweig 2002). On the other hand, specialized subsets of DC, named tolerogenic DC, mediate suppression of antigen-specific immune responses via the induction of regulatory T cells (Morelli and Thomson 2007). There are several evidences that HIV influences these contrasting outcomes of DC function by blocking the maturation required for effective HIV-specific immunity and at the same time favoring tolerance, even through active immune evasion and induction of regulatory T cells. Distinct stages of DC homeostasis and function may be altered by viral infection, from hematopoietic progenitor differentiation to maturation, or even both (Sevilla et al. 2004). Indeed, HIV-1 selectively infects a subset of BDCA-1$^+$ DC in human blood that fail to mature in culture and exhibit a weak immuno-stimulatory activity, suggesting a mechanism by which HIV evades the direct induction of T cell-mediated immunity (Granelli-Piperno et al. 2006). In another study, it has been reported that peripheral blood BDCA-1$^+$ DC undergo maturation in culture, but this does not take place in the presence of HIV (Patterson et al. 2005). Enriched HIV-1-infected monocyte-derived DC (moDC) express less MHC class I and CD4, and secrete increased levels of IL-10 compared to uninfected cells (Kawamura et al. 2003). The down-regulation of CD4 and MHC class I in infected DC may be due to the expression of HIV-1 nef, vpu, or env. Moreover, MHC class I molecules and CD4 are also down-modulated in HIV-1-infected CD4$^+$ T cells, which may decrease the ability of APC to activate them through TCR engagement (Marodon 2001). Besides leading to immune suppression, these phenotypic changes may protect HIV-1-infected DC and T cells from immune recognition and eradication. In addition, HIV-1 infection correlates with the presence of semi-mature DC in lymph nodes, which promote tolerance rather than immunity (Krathwohl et al. 2006), and an increased frequency of regulatory T cells, due to enhanced DC-mediated conversion of non-regulatory T cells, has been recently described in lymphoid tissues of SIV-infected macaques (Presicce et al. 2011).

HIV-infected immature moDC matured normally by CD40L ligation but had impaired IL-12p70 production compared to uninfected controls (Smed-Sorensen et al. 2004). Furthermore, there was impairment in the IL-12/IL-10 balance in moDC derived from HIV-1-infected individuals, with increased IL-10 and decreased IL-12 production after CD40L activation. The impairment in DC maturation seen in acute and late stages of infection may be a direct effect of HIV-1 infection or due to lower expression of co-stimulatory ligands such as CD40L and CD28 on T cells, essential for the induction of DC with full stimulatory capacity (Vanham et al. 1999; Riley et al. 2000). Moreover, IL-10 was produced in cocultures of infected DC and T cells, leading to immune suppression (Granelli-Piperno et al. 2004). The source

of IL-10 in these cultures was undetermined, but the authors propose that it may derive from HIV-1-specific regulatory T cells induced in the coculture. Accordingly, another study showed an impairment of DC in stimulating $CD4^+$ T cell proliferation due to IL-10 production by DC. Indeed, the T cell stimulatory capacity could be restored when IL-10 was neutralized (Carbonneil et al. 2004). Induction of immune tolerance toward HIV could also be a consequence of a T helper 2 (Th2) phenotype induced by DC. Indeed, triggering of DC-SIGN has been proposed to have a central role in both polarizing the T helper response toward Th2 and viral persistence (Geijtenbeek and van Kooyk 2003; Sevilla et al. 2004). New evidence shows that DC-SIGN binding by HIV on human DC causes activation of Rho guanine nucleotide-exchange factor (LARG), which, in turn, is essential for the formation of the infectious synapses and down-regulation of IL-12p70 secretion (Hodges et al. 2007). Thus, activation of DC-SIGN may favor the production of a Th2-related cytokine profile during infection.

HIV may also act by inducing central tolerance against its own components through deletion in the thymus of HIV-specific T cells. Indeed, a recent study shows that HIV-1 up-regulates MHC class I on infected as well as uninfected thymocytes through IFN-α, which is released by HIV-infected pDC (Keir et al. 2002). In conclusion, several lines of evidence point to a direct role of DC in favoring tolerance rather than immunity against HIV-1.

9.4 LV-Mediated Gene Delivery in Human Monocyte-Derived DC

LV have been used for gene delivery in human DC differentiated in vitro either from peripheral blood $CD14^+$ monocytes (moDC) or from $CD34^+$ hematopoietic stem cells (HSC) (Gruber et al. 2000; Schroers et al. 2000; Dyall et al. 2001; Salmon et al. 2001; Rouas et al. 2002; Breckpot et al. 2004; Chen et al. 2004; Lizee et al. 2004; Tan et al. 2005; Veron et al. 2006), and contrasting results on the efficacy of moDC transduction and their functional response have been obtained. Indeed, the transduction efficacy varied from 10 to more than 80%, with some groups reporting LV-mediated moDC activation, while others, including our, did not detect changes in moDC phenotype and functions. Several factors, including the different LV structure, dose and mode of preparation, time of transduction, protocol used for moDC generation, and use of agents such as polybrene, protamine sulfate, or fibronectin, are likely to influence the outcome of DC transduction. In our hands, LV efficiently transduced moDC precursors (day 0 to day 3 of culture) but not differentiated moDC (day 5–7) without altering their function (Rossetti et al. 2011). This outcome has important safety implications for strategies aimed at ex vivo DC gene transfer, which could be applied for induction of antigen-specific immunity, such as in cancer settings, and tolerance. As moDC represent the easiest tool for ex vivo DC manipulation, these findings pave the way for approaches aimed at inducing transgene-specific tolerance by moDC transduction.

9.4.1 Limits of LV-Mediated Gene Delivery

One important feature of a clinically applicable LV would be the presence of the therapeutic transgene together with a marker/selection gene to purify and follow transduced cells over time in vivo. However, selected types of LV structures or sizes reduce the efficiency of DC transduction. Unpublished data from our group show that LV containing either a bidirectional promoter (Amendola et al. 2005), allowing simultaneous transcription of two transgenes in opposite direction, or hairpin-like structures mimicking pre-microRNA (Amendola et al. 2009) poorly transduce DC, although being quite efficient in other primary cells, such as T cells (Andolfi et al. 2012). The drop in DC transduction correlates with the size of the transfer vector, and cannot be rescued by conventional strategies applied to improve LV transduction, such as multiple-hit or spin transduction, or the use of polybrene. This block is likely at post-entry level, since Measles Virus glycoprotein-pseudotyped LV do not improve DC transduction (Rossetti M., personal communication). Of notice, complex vectors drive a significant lower expression of the GFP marker gene compared to "standard" monocistronic LV; thus, the former might result from nonintegrated LV DNA. Indeed, episomal forms can express proteins, because their genome and transgene remain intact (Stevenson et al. 1990; Brussel and Sonigo 2004); however, the level of transgene expression is highly reduced in nonintegrated compared to integrated LV (Banasik and McCray 2009). As DC do not proliferate, nonintegrated circles are not diluted and persist in transduced cells. Alternatively, it is possible that "long" LV can integrate into DC genome but are more efficiently silenced compared to "short" LV. Although LV silencing is far less frequent than RV silencing, it has been reported in specific cell types, such as embryonic stem cells, HSC, and neural stem cells (Ellis 2005). Further investigation is necessary to dissect this DC-specific post-entry block.

9.5 Possible Approaches to Limit/Overcome Immune Responses to Genetically Modified Cells

Several approaches to limit immune responses against the therapeutic transgene have been investigated, including administration of immunosuppressive drugs, selection of different administration routes, dose of vector, vector engineering, and cellular therapy with regulatory cells (Nayak and Herzog 2009).

9.5.1 Immunosuppressive Drugs

The use of immunosuppressive agents, such as cyclosporine A, FK506, and cyclophosphamide, is a common approach to prevent and suppress adaptive immune responses (Arruda et al. 2009). The regimen and the duration of immunosuppression required in gene therapy approaches are not yet defined; however, due to the

relatively low number of transgene-specific T cells, it might be less intense than in organ transplantation. Regrettably, most immunosuppressive regimens do not allow the induction of antigen-specific tolerance and are associated with life-threatening side effects due to long-term nonselective suppression of protective immunity. In addition to this well-known drawback, the use of immunosuppressive drugs must be carefully evaluated in order to avoid interference with vector stability, distribution, entry, and transduction efficiency in target cells. Another potential caveat is the altered homeostasis of regulatory T cells induced by some immunosuppressive drugs, as regulatory T cells are likely involved in the regulation of transgene-specific immune responses. Indeed, when the immunosuppressive regimen included Mycophenolate mofetil, daclizumab, an anti-IL-2 receptor antibody, and sirolimus, gene transfer to the liver in nonhuman primates induced a drastic reduction in regulatory T cell numbers, together with very strong transgene-specific cellular and humoral immune responses (Mingozzi et al. 2007).

9.5.2 Vector Engineering and miRNA-Regulated Vectors

Depending on the gene delivery method, antigen presentation by professional or nonprofessional APC may be favored. Other strategies to limit transgene-specific immunity thus reside in targeting transgene expression in immuno-privileged sites through different routes of administration and/or vector design. A GP64-pseudotyped FIV drove persistent transgene expression in nasal epithelia when delivered to the nostril (Sinn et al. 2005). Chimeric GP64/Sendai-pseudotyped LV have been shown to reduce macrophage transduction, thereby minimizing LV recognition by the immune system (Markusic et al. 2009). Interestingly, long-term transgene expression has been achieved in three clinical trials for Leber's congenital amaurosis and Parkinson's disease by targeting immuno-privileged sites like the eye and the brain (Kaplitt et al. 2007; Bainbridge et al. 2008; Hauswirth et al. 2008). Tissue-specific promoters are also instrumental to limit the anti-transgene immune response, since they allow targeted gene expression. Moreover, directing transgene expression to the cells where the gene is normally active guarantees a better production and higher biological activity of the therapeutic protein, due to the presence of elements that physiologically regulate the process, and reduces the risk of transgene silencing. Finally, tissue-specific promoters represent another tool to direct transgene expression in immuno-privileged tissues. For instance, systemic delivery of Factor IX (F.IX)-encoding LV under the control of the liver-specific albumin promoter resulted in long-term F.IX expression in immuno-competent mice, while F.IX expression was transient under the control of the ubiquitous CMV promoter (Follenzi et al. 2004). However, tissue-restricted transgene expression was only able to reduce the incidence and the extent of transgene-specific immune responses, which were still observed at lower levels and delayed in time. The improvement is thought to depend on an initial tolerogenic antigen presentation by hepatocytes due to promoter leaking (reviewed in Parker and Picut 2005).

To avoid unwanted off-target expression in APC, Brown et al. developed an LV platform in which transgene expression is regulated by target sequences of mir-142, a microRNA specifically expressed by hematopoietic cells (Brown et al. 2006). The de-targeting of transgene expression from APC by means of mir-142-regulated LV allows stable gene transfer and correction of hemophilia B mice (Brown et al. 2007a, b). Interestingly, the injection of mir-142-regulated LV finally results in induction of transgene-specific immune tolerance via expansion of $CD4^+CD25^+$ regulatory T cells (Annoni et al. 2009). Since in this model DC are not expressing the transgene, the mechanistic hypothesis is that professional APC acquire the antigen from transduced hepatocytes, which provide also the tolerogenic environment (i.e., TGF-β secretion) necessary for the induction of antigen-specific regulatory T cells (Goudy et al. 2011; Matrai et al. 2011). The generation of transgene-specific regulatory T cells in vitro or in vivo could be an interesting approach to modulate transgene-specific immune responses; however, our group demonstrated that adoptive transfer of transgene-specific $CD4^+CD25^+$ regulatory T cells is not sufficient to induce transgene-specific tolerance, but transgene-presenting APC are required (Annoni et al. 2007). These findings suggest that DC modulation could be an attractive strategy to circumvent the immune response to transgene due to their strong antigen-presenting capacity and their key role in the regulation of both immunity and tolerance.

9.6 LV-Mediated Gene Therapy Approaches for HIV

Despite the enormous efforts undertaken to develop new drugs to control the spread of HIV-1 infection, as of today even the most successful of the current therapeutic regimens is incapable of eradicating the virus. In particular, the persistence of viral reservoirs represents an obstacle to the long-term control or eradication of HIV in infected individuals receiving highly antiretroviral therapy. Consequently, alternative strategies to inhibit HIV-1 viral replication are constantly under evaluation. These alternatives contemplate also gene therapy-based approaches aimed at delivering either genes encoding for proteins that promote cytotoxic activity against virus-infected cells, resulting in their elimination from the body, or genes directed against HIV, to render specific cell populations resistant to the virus.

LV are promising for treating HIV-1 infection as they also provide several advantages compared to the extensively used Moloney murine leukemia virus-based vectors (reviewed in Duzgunes et al. 2001). Indeed, LV are capable of stably transducing both dividing and nondividing cells, and specifically those cells involved in HIV-1 replication and immune restoration, such as T cells, hematopoietic cells, macrophages, and DC. LV transduce quiescent cells more efficiently than retroviral vectors and can be produced at high titer in a self-inactivating configuration (Naldini 1998), and may also be safer than retroviral vectors (Montini et al. 2006) due to the differences in their integration profile (Schroder et al. 2002; Wu et al. 2003). Moreover, some of the HIV-based vectors can infect the same cells as the wild-type

HIV, by which they can be mobilized with the advantage of spreading the vector sequences to non-transduced cells, and therefore expanding the range of protection (for a review see Morris and Rossi 2004). Another peculiarity of LV is their capacity to cross-package one another (White et al. 1999; Browning et al. 2001; Goujon et al. 2003), which offers a unique and possibly safer method for delivering antiviral vectors to target cells in HIV-1-infected individuals. It was reported that HIV-1 and HIV-2 vectors being cross-packaged by FIV stably transduced and protected human primary blood mononuclear cells from HIV-1 infection (Morris et al. 2004).

On the other hand, the usage of HIV-1-based LV for HIV/AIDS gene therapy has the disadvantage that the transgenes with anti-HIV activity target also genes of the LV involved in vector production, and consequently can reduce the vector expression (Mautino and Morgan 2000; Bahner et al. 2007). This is generally not a problem for in vitro or mouse studies, where only a limited number of target cells need to be transduced, but may represent a limitation for preclinical or clinical use.

Currently applied gene therapy technologies directed against HIV are designed to render cells refractory to infection or reduce their capacity to produce progeny viruses. LV can be designed to express therapeutic anti-HIV-1 molecules, such as ribozymes, small-interfering RNA (siRNA), and short hairpin RNA (shRNA) that interfere with the function of viral proteins or RNA at different steps of the viral life cycle. These approaches are not exempt from risks. For example, ribozyme-resistant viral variants may emerge due to the high mutation rate of HIV-1. Moreover, the virus may elude siRNA targeting by the evolution of alternative splice variants for the siRNA-targeted transcripts (Westerhout et al. 2005). Despite these limitations, several approaches with ribozymes targeting different HIV-1 genes (U5 region of the LTR, reverse transcriptase, Integrase, Vif, Tat, Rev, Gag, and Pol) have been used to successfully inhibit HIV-1 gene expression (Sioud and Drlica 1991; Dropulic et al. 1992; Leavitt et al. 1994; Zhou et al. 1994; Sun et al. 1995). Analogously, siRNA directed against the HIV-1 genes or the viral receptor CD4 and co-receptor CCR5 reduced infection in $CD4^+$ T cells (Qin et al. 2003) and T cell lines (Novina et al. 2002). In a recent study, HSC genetically modified with an shRNA specific for CCR5 were able to down regulate CCR5 expression in progeny cells in the periphery of humanized mice (Shimizu et al. 2010). The concept of targeting CCR5 with gene therapy approaches is supported by the "German patient" case, an HIV-infected individual who, during treatment for acute myeloid leukemia, received a heterologous transplantation of HSC derived from a homozygous $CCR5^{-/-}$ donor following myeloablation and subsequently controlled virus replication in the absence of antiretroviral therapy (Hutter et al. 2009). In another recent clinical study, HIV/AIDS patients with lymphoma undergoing autologous transplantation were treated with genetically modified peripheral blood-derived hematopoietic progenitor cells expressing three RNA-based anti-HIV moieties, an shRNA targeted to tat/rev transcripts, a TAR decoy, and a CCR5-targeting ribozyme (DiGiusto et al. 2010). The authors demonstrated sustained expression of siRNA and ribozyme for up to 24 months. While antiviral efficacy was not assessed, the safety of this strategy supports the development of an LV-based cell therapy platform for HIV/AIDS treatment.

Recent gene therapy approaches used by several groups aim to improve the targeting of HIV-1-infected cells with an inverse fusion strategy, which consists in incorporating the HIV receptor CD4 and its co-receptor (CCR5 or CXCR4) onto the vector surface (Endres et al. 1997; Mebatsion et al. 1997; Ye et al. 2005; Peretti et al. 2006). Furthermore, modifications of the inverse fusion strategy, called two-molecule method, have been developed to further improve the transduction efficiency and target HIV of different phenotypes with a single vector (Yang et al. 2006; Lee et al. 2011). Indeed, LV co-enveloped with CD4 and a fusogenic protein derived from Sindbis virus glycoprotein, and carrying a suicide gene, were able to successfully transduce and kill cells expressing HIV of different phenotypes.

Alternative gene therapy approaches want to target the virus with neutralizing antibodies by using LV encoding for a broad neutralizing anti-HIV antibody. Recently, HIV infection in humanized mice was inhibited by infusion of HSC transduced with an LV encoding for the anti-HIV-neutralizing antibody 2G12 (Joseph et al. 2010). This study supports the concept that gene therapy using LV encoding a mixture of broadly neutralizing HIV antibodies may represent a new therapeutic approach for the treatment of HIV infection in those patients resistant to multiple antiretroviral drugs.

9.7 Conclusions and Future Directions

Since the development of LV a decade ago, research has firmly established that LV gene therapy has a wide spectrum of therapeutic applications, spanning from primary immunodeficiency to monogenic diseases, cancer, and viral infections, including HIV/AIDS. While a great deal of progress has been made in understanding the mechanisms of DC-mediated activation by LV, a number of questions still remain. First, LV are constantly being engineered to minimize unwanted immune responses. Among others, the use of tissue-specific promoters and the inclusion of miRNA target sequences within the transfer vector are novel approaches to overcome the immune responses against transgene-expressing cells. It still remains to be defined whether agents that specifically suppress the initial type I IFN response, driven by pDC, might be used to overcome the unwanted LV-mediated immune responses after gene therapy. Second, although LV have been exploited to deliver molecules able to increase cell refractoriness to HIV infection, additional research is needed to define if and how LV might target the cells acting as HIV reservoir. Third, the use of ex vivo DC transduction can be successfully used to induce antigen-specific immunity or tolerance; however, it still remains to define which antigens might be used to efficiently reactivate antitumor immune responses or tolerance in vivo. Moreover, efficient methods of DC transduction need to be defined as well as the source of DC precursors to be used. Answering these questions will not only bring us closer to understanding how LV-mediated immune activation is elicited, but also how to exploit or modulate their activity for targeted gene therapy in a wide variety of diseases.

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Chapter 10
Targeting Dendritic Cells for Improved HIV-1 Vaccines

Anna Smed-Sörensen and Karin Loré

Abstract As dendritic cells (DCs) have the unique capacity to activate antigen-naive T cells they likely play a critical role in eliciting immune responses to vaccines. DCs are therefore being explored as attractive targets for vaccines, but understanding the interaction of DCs and clinically relevant vaccine antigens and adjuvants is a prerequisite. The HIV-1/AIDS epidemic continues to be a significant health problem, and despite intense research efforts over the past 30 years a protective vaccine has not yet been developed. A common challenge in vaccine design is to find a vaccine formulation that best shapes the immune response to protect against and/or control the given pathogen. Here, we discuss the importance of understanding the diversity, anatomical location and function of different human DC subsets in order to identify the optimal target cells for an HIV-1 vaccine. We review human DC interactions with some of the HIV-1 vaccine antigen delivery vehicles and adjuvants currently utilized in preclinical and clinical studies. Specifically, the effects of distinctly different vaccine adjuvants in terms of activation of DCs and improving

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6_10,

DC function and vaccine efficacy are discussed. The susceptibility and responses of DCs to recombinant adenovirus vectors are reviewed, as well as the strategy of directly targeting DCs by using DC marker-specific monoclonal antibodies coupled to an antigen.

10.1 Introduction

Dendritic cells (DCs) are professional antigen-presenting cells essential for priming and conditioning virus-specific T cells, which are needed to control viral infections including HIV-1 (Banchereau and Steinman 1998). This process is largely shaped by the immediate innate immune responses mounted by different DC subsets. The responses to vaccines are also elicited based on innate immune functions. Although details of human DC diversity and complexity are only starting to unravel, it is becoming increasingly clear that the DC subsets defined in mice differ in several aspects of composition and function compared to human DC subsets (Palucka et al. 2010; Shortman and Liu 2002). This underlines the importance of investigating human DCs in their interaction with vaccine antigen and adjuvant components to understand how vaccines for humans can be refined (Palucka et al. 2010). Human DC subsets are defined by their phenotype and anatomical location as well as functional capacity. All DC subsets have some level of antigen-presenting capacity, but they differ in their expression of several receptors used for pathogen recognition, antigen uptake, and processing. Understanding the functional patterns of the various human DC subsets and the importance of providing one, some or all of them with antigen and activation signals to optimize vaccine-induced responses is central in vaccine development (Banchereau et al. 2009; Ueno et al. 2010, 2011).

Despite the fact that a significant wealth of knowledge of both virological and immunological aspects of HIV-1 has been accumulated since the virus was identified 30 years ago and that vast efforts have been made to develop a vaccine, advancements towards a protective vaccine have been modest. Major challenges relate to the extremely high mutation rate of HIV-1 leading to a vast pool of virion variants (Rappuoli 2007). As with the majority of existing vaccines against infectious disease agents, stimulation of antigen-specific protective antibody (ab) responses is likely also a central goal for the development of prophylactic vaccines against HIV-1/AIDS (Plotkin 2010). The envelope (Env) glycoprotein gp120 expressed on the surface of HIV-1 represents the sole target for neutralizing abs (Karlsson Hedestam et al. 2008). Env is therefore considered as one key component in an HIV-1 vaccine. Unfortunately, the early Env-based protein subunit vaccine candidate (VaxGen gp120) was unsuccessful at eliciting broadly neutralizing abs to primary HIV-1 strains, which prompted the investigation of vaccines that stimulated cell-mediated immunity (Check 2003; Francis et al. 2003). However, a large clinical vaccine trial (the Step study) using a recombinant viral vector based on adenovirus serotype 5 failed to show efficacy despite the induction of HIV-1 specific T cell responses, suggesting that T cells alone are not sufficient to prevent acquisition

of HIV-1 (Buchbinder et al. 2008). Development of potent neutralizing abs reemerged as a highly desired goal and this is supported by results from a recent clinical trial (RV144) where the VaxGen gp120 vaccine showed some promise when used in a prime-boost regimen together with a modified canarypox vector (ALVAC) (Rerks-Ngarm et al. 2009). This trial resulted in some reduction in susceptibility to HIV-1 and detection of ab responses, albeit non-neutralizing, in some vaccine recipients. Together these data show that heterologous prime-boost immunization with recombinant viral vectors and protein vaccines can confer some level of protection and provide a benchmark for designing more potent HIV-1 vaccine regimens. In addition, several approaches for how to optimally deliver HIV-1 vaccine antigens to cells have been explored, including directed targeting of cells using cell marker specific-abs (Trumpfheller et al. 2012). Significant progress in the understanding of how different vaccine adjuvants work has also been made during recent years. Several of these adjuvants cause activation of DCs. In this chapter, we review and discuss aspects of targeting, functional manipulation of human DCs with different immunostimulatory adjuvants and potential HIV-1 vaccine delivery vehicles.

10.2 The Influence of the Functional and Anatomical Diversity of DC Subsets on Vaccine Responses

The human DC system has evolved into multiple, phenotypically and functionally specialized subsets, optimized to respond appropriately to any given stimuli in different locations around the body. The two described main lineages of DCs in the human immune system are the myeloid DCs (MDCs, also called conventional or classical DCs (cDCs)) and the plasmacytoid DCs (PDCs). MDCs and PDCs are characterized by their expression of CD11c and CD123, respectively. The MDC lineage can be further divided into three separate populations found in blood expressing CD1c (BDCA-1), CD141 (BDCA-3), or CD16 (Piccioli et al. 2007; Mittag et al. 2011) (Figs. 10.1 and 10.2). All MDC subsets have been shown to be superior to PDCs at presenting antigen, inducing naïve CD4+ T cell activation and IL-12 p70 production (Banchereau and Steinman 1998; Piccioli et al. 2007; Mittag et al. 2011; Lore et al. 2003, 2005, 2007). CD1c+ MDCs are good at stimulating CD4+ T cells. The CD141+ MDC subset (also expressing DNGR-1 (Clec9A)) is scarce and has been particularly recognized for its exceptional cross-presenting capacity (Fig. 10.2) (Bachem et al. 2010; Crozat et al. 2010; Jongbloed et al. 2010; Poulin et al. 2010). The CD16+ MDC subset was shown to be the poorest antigen-presenting MDCs and may have a more inflammatory phenotype (Piccioli et al. 2007; Mittag et al. 2011). The MDC population as a whole is more frequent compared to PDCs and distributed throughout the body, being most prevalent at sites of potential antigen exposure such as in the skin (as epidermal CD1a+ Langerhans cells (LCs) and dermal DC subsets) and mucosal tissues. Human epidermal LCs were shown to promote CD8+ T cell responses directly (van der Aar et al. 2011) as

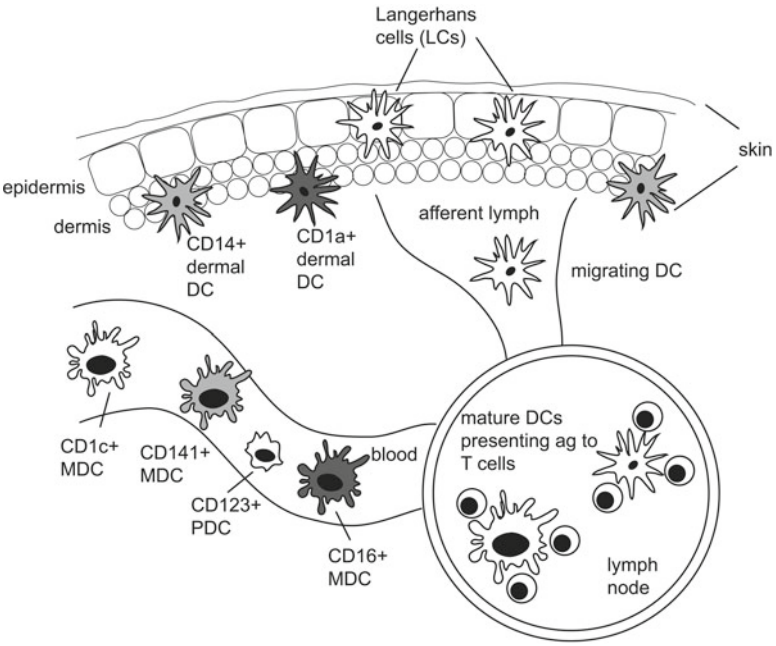

Fig. 10.1 Human DC subset distribution. Immature DCs are spread throughout the human body; in lymphoid tissues as well as lining the body surfaces and circulating in blood. The skin has a dense network of DCs with Langerhans cells (LCs) residing in the epidermis of the skin, while CD14+ and CD1a+ dermal DCs are found in the dermis. Myeloid CD1c+ MDCs, BDCA-3+ MDCs, and CD16+ MDCs, as well as plasmacytoid DCs (PDCs) circulate in peripheral blood. Upon antigen encounter and stimulation, peripheral DCs mature and migrate to draining lymph nodes, where they present the antigen to T cells for the initiation of immune responses

well as prime naïve CD4+ T cells to develop helper functions that support cellular responses, while CD4+ T cells primed by CD14+ dermal DCs predominantly support humoral responses (Klechevsky et al. 2008) (Fig. 10.2). This functional difference was further supported by a clinical study showing that transcutaneous application of an influenza vaccine with the aim of targeting LCs preferentially induced CD8+ T cell responses, whereas intramuscular injection did not (Combadiere et al. 2010).

As mentioned above, MDCs and PDCs differ in several important histological and functional aspects. PDCs are recognized for their unique ability to produce high levels of IFNα (Siegal et al. 1999) in response to foreign antigen structures, particularly viruses, and may therefore play an especially important role in antiviral immunity (Fitzgerald-Bocarsly et al. 2008). PDCs are sparsely distributed and are only found in blood, spleen, lymph nodes, and thymus under normal conditions but can be recruited to sites of antigen exposure and inflammation (Bond et al. 2012). In lymph nodes and spleen PDCs are located in proximity to high endothelial venules (HEVs) and T cell-rich areas through which B cells migrate (Junt et al. 2008; Qi et al. 2006).

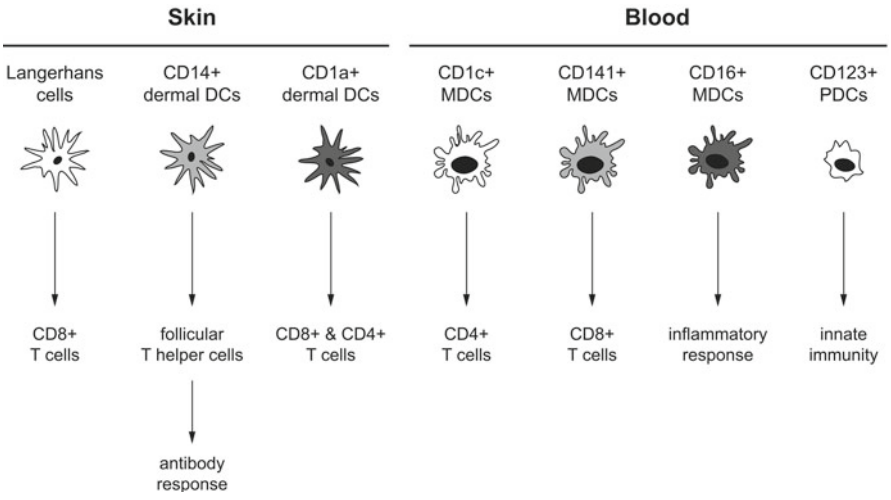

Fig. 10.2 Simplified view of the overlapping but different functions of human DC subsets. Although all human DC subsets have antigen-presenting capacity, emerging data show that they have specialized functions. BDCA3+ DCs and Langerhans cells have shown superior antigen cross-presentation ability, while CD14+ dermal DCs prime CD4+ T cells into T helper follicular cells that promote naïve B cells to switch isotype and become plasma cells. CD1c+ MDCs are potent antigen-presenting cells for stimulation of CD4+ T cells. CD16+ MDCs have a strong pro-inflammatory activity. Plasmacytoid DCs are unique for producing high levels of type I Interferon and can condition the function of other DC subsets in the proximity as well as support B cell differentiation

Notably, while PDCs are generally considered to be poor antigen-presenting cells, they have been shown to enhance B cell proliferation, differentiation and ab-production via their production of IFNα and may therefore have a special role in developing humoral immunity (Jego et al. 2003; Bekeredjian-Ding et al. 2005; Douagi et al. 2009; Gujer et al. 2011a, b).

Due to the seemingly distinct functional specialization of the different DC subsets, it is plausible that the immunological outcome of a vaccine can differ depending on which DC subset is preferentially targeted. Most vaccines are currently delivered by needle and syringe into the muscle. However, the skin may be a more attractive site for vaccination since it, in contrast to muscle, has a dense network of DCs (Romani et al. 2010). It has been shown that delivering influenza and yellow fever vaccines in the skin compared to conventional intramuscular injection results in enhanced responses in healthy individuals and more importantly, also in non- or low vaccine responders such as immunocompromised patients, elderly people, or infants (Roukens et al. 2012). LCs and dermal DCs are ideally positioned to take up vaccine antigens that are delivered in the skin and subsequently migrate and present antigens to T cells in the lymph nodes (Romani et al. 2006; Teunissen et al. 2012). Furthermore, intradermal vaccination has recently been shown to enhance mobilization of LCs (Liard et al. 2011).

Table 10.1 Expression of selected TLRs and CLRs on human blood and skin DC subsets

	Human blood DC subsets				Human skin DC subsets		
	CD1c$^+$ MDC	CD141$^+$ MDC	CD16$^+$ MDC	PDC	LC	CD14$^+$ DC	CD1a$^+$ DC
Surface TLR							
TLR1	+	+	+	+	+/–	+	N.D.
TLR2	++	+	+	+/–	+/–	+	–
TLR4	+	–	+	–	–	+	+
TLR5	+	–	+	–	+/–	+	N.D.
TLR6	+	+	+	+	+/–	+	N.D.
Endosomal TLR							
TLR3	+	+++	–	–	+	+	N.D.
TLR7	+/–	–	+/–	+++	+/–	+	N.D.
TLR8	+	+	+	–	–	+	N.D.
TLR9	–	–	–	+++	–	–	N.D.
CLR							
DCIR	++	++	++	+	+	+	+
DC-SIGN	–	–	N.D.	–	–	+	–
DEC-205	+	++	N.D.	+/–	+	+	++
langerin	–	–	–	–	++	–	–
mannose receptor	+/–	–	–	–	–	+	++

Data is compiled from (van der Aar et al. 2007; Hornung et al. 2002; Ito et al. 2002; Jarrossay et al. 2001; Kadowaki et al. 2001; Iwasaki and Medzhitov 2004; Flacher et al. 2006; Mittag et al. 2011; Piccioli et al. 2007) for TLRs and (Banchereau et al. 2009; Bond et al. 2009; Kato et al. 2006; Turville et al. 2002; Klechevsky et al. 2008, 2010) for CLRs and depicted as expression detected (+), no expression detected (–), contradictory data published (+/–), or data not determined (N.D.)

10.3 Vaccine Targeting of DCs with Receptor Specific Antibodies

While the susceptibility of different DC subsets to HIV-1 infection has been extensively studied (Smed-Sorensen et al. 2005; Turville et al. 2004; Kawamura et al. 2003), the interactions of DCs with soluble HIV-1 Env gp120 used as an immunogen are much less characterized. Env binds CD4 and CCR5/CxCR4 in the viral infection process. In addition, due to its heavy glycosylation, Env binds to multiple C-type lectin receptors (CLRs) expressed on DCs. While DCs are susceptible to HIV-1 infection via CD4 and a co-receptor, there is no evidence that binding of Env to CLRs results in a productive infection of the DCs (Turville et al. 2001, 2002). CLRs known to bind Env include DC-SIGN, Langerin, DCIR and the mannose receptor, all of which are predominantly expressed by specific tissue resident MDCs (Turville et al. 2002) (Table 10.1). The cellular receptor(s) that take up soluble Env, when administered as a vaccine protein in vivo are unknown. Uptake of HIV-1 antigens via CLRs by DCs can lead to antigen presentation (Sabado et al. 2007). However, uptake of antigen via different CLRs leads to different efficiencies and modes of antigen presentation (Delamarre and Mellman 2011; Palucka et al. 2010).

It is not known whether binding of soluble Env to CD4 can result in internalization of Env protein to intracellular compartments and lead to processing and subsequent presentation of Env peptides on MHC molecules (Marsh et al. 1990; Pelchen-Matthews et al. 1995). Also, there are indications that Env has immunomodulatory properties, which could have consequences for how efficiently DCs can present antigen to and stimulate T cells (Shan et al. 2007).

Endosomal localization of an antigen determines the subsequent processing and presentation of the antigen and thereby the immune response. Different receptors used for uptake of antigen will direct the antigen to distinct intracellular compartments (Burgdorf et al. 2007). Therefore, it might be advantageous to target a protein vaccine to a defined receptor pathway in order to optimize and/or modulate the downstream immune response. Furthermore, specifically targeting DCs might allow the use of smaller doses of antigen since the antigen is efficiently administered to the cells most competent at processing and presenting the antigen for stimulation of immune responses. Explicitly targeting distinct DC subsets may also be a strategy to shape the type of immune response induced after vaccination (Fig. 10.2). One approach is to deliver vaccine antigens using monoclonal abs to receptors specifically expressed by DCs. In the past decade, significant efforts have been made towards improving vaccine delivery using this approach with the rationale that this provides a more efficient and directed mode of antigen delivery to DCs (Banchereau et al. 2009). The monoclonal ab of interest is linked or fused, usually via the Fc region, to the vaccine antigen(s). As CLRs facilitate protein antigen uptake and processing, they are being exploited as delivery receptors on DCs to improve immune responses (Tacken et al. 2007). The most advanced in terms of HIV-1 vaccines utilizing this technology is targeting of the DEC-205 receptor using a DEC-205 ab conjugated to the HIV-1 capsid protein Gag (Trumpfheller et al. 2012). Uptake via DEC-205 can deliver antigens to DCs to be processed and loaded onto MHC class I molecules for cross-presentation. This was demonstrated in elegant proof-of-principle studies by the laboratory of the late Ralph Steinman, where DEC-205 ab coupled to ovalbumin (OVA) or HIV-1 Gag p24 proved to induce potent antigen-specific adaptive immune responses using 10- to 100-fold less antigen as compared to soluble protein (Trumpfheller et al. 2006). DEC-205, which in mice is expressed exclusively on the cross-presenting CD8α+ DCs, is currently the best-characterized receptor for DC targeting. However, in humans DEC-205 expression is not exclusively restricted to DCs but is more widely expressed on leukocytes (Kato et al. 2006) (Fig. 10.3). Targeting DEC-205 in humans may therefore have a different result to what has been observed in mice.

To induce optimal adaptive immune responses it is essential to combine antigen targeting to DCs with potent DC activation. The ab targeting rationale therefore includes co-administration of an immune-stimulatory adjuvant to achieve good immunity, or the use of an ab that targets an endocytic receptor known to activate DCs. The only CLRs clearly shown to trigger DC activation upon ligand or ab binding are Dectin-1 and Dectin-2 (Ni et al. 2010; Taylor et al. 2007; Robinson et al. 2009). However, at least Dectin-1 is not expressed on all human DC subsets, including LCs and BDCA-3+ DCs, which would suggest that Dectin-1 is not a good candidate to

induce activation of cytotoxic CD8+ T cells. In contrast, DC immunoreceptor (DCIR) is a CLR expressed by all blood and skin DC subsets (Fig. 10.3). Targeting DCIR on LCs as well as blood MDCs and PDCs with a single low dose of ab-antigen conjugate resulted in expansion of CD8 T cells in vitro (Klechevsky et al. 2010). In PDCs, DCIR is readily internalized upon receptor ligation and was shown to inhibit TLR9 induced IFNα secretion while maintaining presentation to T cells (Meyer-Wentrup et al. 2008). Whether targeting DCIR also modulates the function of other DC subsets is an important aspect of the potential use of DCIR in vaccine development that remains to be determined. Also the CLR Langerin has been explored as a targeting receptor and both mouse and human skin explant models have shown that epidermal LCs rapidly capture antigens that are delivered via Langerin, as well as via DEC-205 (Flacher et al. 2010). In mouse DCs cross-presentation of soluble ovalbumin internalized by mannose receptor (MR) or of a conjugate coupled to transferrin, both presumably targeted to early endosomes, is more efficient than cross-presentation following fluid phase uptake, which also delivers an unspecified portion of soluble ovalbumin to late endosomes and lysosomes (Burgdorf et al. 2007, 2008; Zehner et al. 2011). Most work to date has investigated the ability to target different receptors on DCs for cross-presentation and induction of CD8 T cell responses (Idoyaga et al. 2011; Tsuji et al. 2011; Tacken et al. 2008, 2011; Lahoud et al. 2009). Whether the same receptor targeting abs also induce CD4 T cell and ab responses is important for the development of a broad prophylactic HIV-1 vaccine that will likely rely on elicitation of both humoral and cellular responses.

CD40 is another attractive candidate receptor. Although not a CLR, it is widely expressed on all DC subsets as well as on other antigen-presenting cells including B cells and monocytes but also on endothelial cells. In vitro, CD40 induces DC maturation upon receptor engagement with an agonistic ab and thereby licenses the DCs for T cell priming (Bennett et al. 1998; Ridge et al. 1998; Schoenberger et al. 1998). Whether CD40 signaling alone is sufficient to prime T cell responses in vivo is still unclear. Contradictory data exists with some studies concluding that additional innate immune signaling is required for the induction of cellular immune responses (Ahonen et al. 2004; Schulz et al. 2000), while other studies show that anti-CD40 together with DC-targeted antigen is sufficient (Bonifaz et al. 2004). These discrepancies may be due to differences in the characteristics of the abs used in the various studies, a critical point to consider overall in the field of ab-based vaccine delivery. One possible concern is that due to the wide distribution of CD40 in vivo, CD40 targeting may cause too strong on-target effects via activation of B cells and induction of toxic levels of cytokines. Another potential disadvantage to using an activating ab like CD40 or ab–adjuvant conjugate is if activation of other cells by systemic adjuvant delivery is critical to achieve optimal immune activation.

An important point to note is that ab targeting of DCs almost exclusively induces T cell responses and not ab production. As discussed in the introduction, although elicitation of neutralizing abs is a highly desirable goal with an HIV-1 vaccine, potent cellular responses are also likely to be critical. The rationale behind the choice of HIV-1 Gag over other HIV-1 antigens to be coupled to the DEC-205 ab was that Gag reactive T cells are frequently found in HIV+ individuals and have been associated

with a better clinical outcome (Edwards et al. 2002; Geldmacher et al. 2007). However, to induce ab responses that can neutralize HIV-1, Env is needed as an antigen. Possibly a combination of soluble Env together with Gag coupled to DC specific abs would be superior for inducing both T and B cell responses. Studies to address how much antigen can be linked to each ab are underway and will be important for understanding how to most efficiently use this technology for vaccine delivery.

10.4 Vaccine Adjuvants and Activation of Dendritic Cells

A live attenuated vaccine based on HIV-1 is considered unsafe for obvious reasons. As mentioned earlier, a protein subunit vaccine based on recombinant Env gp120 is a major vaccine candidate, tested in preclinical and clinical studies. Also the strategy of delivering Gag or other HIV-1 antigens via ab targeting to DCs is based on protein. However, protein vaccines are usually poorly immunogenic themselves without the addition of an efficient vaccine adjuvant. Adjuvants are used to enhance the response to a vaccine and ultimately to tailor the immune response to be most effective for protecting against the specific pathogen. Adjuvants generally enable vaccine antigens to elicit protective ab titers while using a lower dose of the antigen and with fewer immunizations. Most adjuvants used today have been developed empirically and their mechanisms of action have only recently started to be revealed. Information of this kind facilitates a more rational selection of the most appropriate adjuvant in order to manipulate the immune response to be most effective against a given antigen. Adjuvants are thought to mainly affect the innate immune system including the induction of pro-inflammatory cytokines and chemokines and recruitment of cells and processes pertaining to initiation of immune responses (Coffman et al. 2010; McKee et al. 2007, 2010). Many adjuvant actions involve ligation and signaling via key receptors used in innate immunity. If there is no direct specificity of the adjuvant itself to a receptor, there are signaling pathways that can be triggered indirectly via constituents released from damaged and dying cells as a result of adjuvant administration. One main action of adjuvants is believed to involve the recruitment, activation and maturation of antigen-presenting DCs (Seubert et al. 2008; Sun et al. 2003; Palucka et al. 2010; Shortman and Liu 2002). As discussed, very few DCs normally reside in the muscle where most vaccines are delivered. The few existing reports on infiltration of DCs to the muscle after vaccine injection are all based on observations in mice in which the injection volume and the antigen/adjuvant doses are often proportionally much larger than the doses that would be given to humans. However, the studies convincingly show that there are no or very few DCs recruited to the muscle after injection of either unadjuvanted DNA plasmid vaccines or protein antigens whereas the addition of an adjuvant leads to a robust infiltration (McKay et al. 2004; Sumida et al. 2004; Calabro et al. 2011; Dupuis et al. 1998, 2001). Also, unless protein antigens are accompanied by an adjuvant, the DCs may not undergo phenotypic maturation and their capacity to present antigen will be inefficient. Phenotypic maturation of DCs is characterized by their conversion from an immature state to a mature

state with an increased capacity to process and present foreign antigen to T cells. Maturation is associated not only with upregulation of multiple co-stimulatory molecules such as CD40, CD80 and CD86 but also by upregulation of chemokine receptors that direct their migration from the site of antigen exposure in the periphery to the spleen or lymph nodes where presentation of antigens occurs (Banchereau and Steinman 1998). There are therefore opportunities to dictate vaccine responses by manipulating DC activation by potent adjuvants.

10.4.1 Licensed Adjuvants and Their Effect on Dendritic Cells

Since prophylactic vaccines are often given to children in the first years of life, adjuvants need to be very safe and well tolerated. To date, there are only a few adjuvants approved for prophylactic vaccines in humans. The most commonly used adjuvant is alum, which consists of precipitates of aluminum phosphate and aluminum hydroxide to which vaccine antigens are adsorbed. Alum was the adjuvant used together with HIV-1 Env gp120 in the clinical studies discussed earlier. Although alum has been used for over 70 years, its mechanisms of action have only started to be determined. The data are still somewhat contradictory but it seems clear that the adjuvant effect by alum is mediated by stimulating the innate immune system (De Gregorio et al. 2008; Lambrecht et al. 2009; Lore and Karlsson Hedestam 2009; Marrack et al. 2009). The immune stimulatory effect has recently been shown to be dependent on the formation of NALP3 inflammasomes (Eisenbarth et al. 2008). In another study, the effect was shown to work indirectly via the induction of cell death that in turn led to the release of the endogenous danger signal uric acid (Kool et al. 2008). Alum was also shown to lead to increased endocytosis, thereby promoting more efficient antigen uptake and monocyte differentiation into mature DCs (Seubert et al. 2008; Mbow et al. 2010).

Other adjuvants for human use are oil in water emulsions based on squalene such as MF59 (manufactured by Novartis) and AS03 (manufactured by GlaxoSmithKline) that are licensed adjuvants for influenza vaccines. They have both demonstrated an extensive clinical safety record and resulted in a significant increase in immune response efficacy to protein vaccine antigens compared to alum as an adjuvant (Stoute et al. 1997; O'Hagan 2007; O'Hagan et al. 2011). Some of the major vaccines tested are the influenza split-virus vaccines that consist mainly of the envelope protein hemagglutinin (HA). There are therefore commonalities in terms of antigen composition with the strategy of using HIV-1 Env gp120 as the source of antigen for an HIV-1 vaccine. These emulsion adjuvants may thus be advantageous for protein based HIV-1 vaccines. The inclusion of MF59 with the influenza vaccine was also shown to increase the breadth of the immune responses leading to protection against heterologous strains of Influenza (O'Hagan 2007; O'Hagan et al. 2011). Induction of broad, cross-protective immune responses is a requisite for an HIV-1 vaccine. The original hypothesis by which emulsion adjuvants work was that a depot of antigen was created at the site of injection (Freund 1956). However, later it was shown

that only a minor fraction of the emulsion and the antigen remained at the injection site as early as 6 h post injection (Ott et al. 1995). Contrary to the establishment of an antigen depot, emerging data indicate that emulsions such as MF59 create an immunocompetent environment in the muscle initiating recruitment and stimulation of critical immune cells including DCs. Injection of different adjuvants including MF59 and alum into the muscle showed that MF59 induced the highest upregulation of genes coding for cytokines, chemokines and their receptors, adhesion molecules as well as antigen presentation related genes (Mosca et al. 2008). Injection of MF59 into the muscle of mice leads to a robust infiltration of DCs, monocytes, and neutrophils to the site (Calabro et al. 2011; Dupuis et al. 2001). Infiltrating neutrophils were found to be the most frequent cell population and had the quickest recruitment kinetics into the muscle after injection but MDCs and macrophages showed the highest uptake of protein antigen (ovalbumin; OVA) (Calabro et al. 2011). DCs and B cells in the draining lymph node were subsequently the most frequent cell types associated with OVA and MF59 content. It is plausible that DCs and B cells had carried antigen and adjuvant from the injection site to the lymph node and that seemingly small numbers of cells are sufficient to initiate an adaptive immune response. The fact that the antigen and MF59 have been observed together in the same DC may mean that MF59 can directly manipulate DC function. In vitro, it was shown that MF59 could facilitate uptake of antigen by DCs and monocytes (Seubert et al. 2008). However, while the activation of DCs directly by MF59 was modest, macrophages and monocytes responded by differentiating into a more DC-like phenotype. Important questions remain addressing whether the augmented immune responses induced with emulsion adjuvants compared to alum are due to enhanced recruitment of antigen-bearing DCs into the lymph nodes, better maturation and antigen-presenting capacity of the DCs or better and/or preferential expansion and differentiation of specific CD4+ T helper cells like T follicular helper cells.

10.4.2 Toll-Like Receptor Ligands as Adjuvants to Target Dendritic Cells

DCs are equipped with a diverse set of Toll-like receptors (TLRs), which are used to sense microbes in the environment (Fig. 10.3). TLRs specifically recognize structurally defined pathogen associated molecular patterns (PAMPs) and play an important role in inducing innate immune responses. Since activation via TLRs leads to robust and rapid cell activation, a range of synthetic TLR ligands has been tested for potency as adjuvants. In contrast to the adjuvants mentioned above, TLR ligands are molecularly defined structures and thus stimulate selected cells based on their cognate TLR expression (Duthie et al. 2011; Hartmann et al. 2005; Lore and Karlsson Hedestam 2009). TLRs are expressed either on the cell surface or on endosomal/lysosomal membranes. TLR1, 2, 4, 5, and 6 are expressed at the cell surface and recognize conserved microbial membrane components such as lipids, lipoproteins, and flagellin proteins. In contrast, TLR3 and TLR7–9 are exclusively expressed in

intracellular endosomes and recognize nucleic acids (Kawai and Akira 2010). All TLRs except TLR3 signal via the adapter molecule myeloid differentiation factor 88 (MyD88) to activate the NF-κB pathway. Instead of MyD88, TLR3 uses the adaptor TRIF, which can also be bound by TLR4 (Kawai and Akira 2006). These adaptors propagate signaling by recruiting kinases that mediate activation of transcription factors including NF-κB, interferon regulatory factors (IRF) 3 and 7, which are all required for the production of inflammatory cytokines including type I interferons (IFNs).

Human DC subsets have different TLR expression patterns and therefore respond accordingly to TLR ligands (Fig. 10.3). Of the candidate TLRs most explored for adjuvant targeting, TLR7 and 9 are expressed on PDCs, while TLR3–6 and 8 are expressed on MDCs (Hornung et al. 2002; Ito et al. 2002; Iwasaki and Medzhitov 2004; Jarrossay et al. 2001; Kadowaki et al. 2001; Poulin et al. 2010). There is considerable divergence in the level of gene expression of different TLRs on human MDC subsets reported. Dermal DCs seem to have a wider range of TLRs than LCs (van der Aar et al. 2007). Some studies show that the MDC subsets CD141+ DCs and epidermal LCs mainly express TLR3 (Poulin et al. 2010; Flacher et al. 2006) while the CD16+ subset mainly expresses TLR4 (Piccioli et al. 2007; Mittag et al. 2011). TLR ligands efficiently induce cytokine production and phenotypic differentiation of DCs which collectively augment their ability to present antigens on MHC I and MHC II to stimulate antigen-specific T cell responses (Lore et al. 2003; Wille-Reece et al. 2005a, 2006; Lahiri et al. 2008). TLR signaling also enhances migration of DCs to lymph nodes (Lahiri et al. 2008). The highly potent yellow fever vaccine activates multiple TLRs, which is a testament to the benefits of TLR targeting as a strategy for stimulating strong vaccine responses (Querec et al. 2006). Importantly, in contrast to the Th2 biased alum and emulsion adjuvants that are clinically approved, TLR3, 4, 7/8 and 9 ligands induce a polarized Th1 cellular response (Coffman et al. 2010). Since an HIV-1 vaccine may need to induce both humoral and cellular responses, TLR ligands could be a critical addition to an HIV-1 protein vaccine formulation. Although certain TLR ligands can also directly activate B cells, stimulation of DCs has been shown to be more important for ab production than direct activation of B cells (Hou et al. 2008). On a related note, TLR stimulated human PDCs have shown a unique ability to enhance B cell activation (Bekeredjian-Ding et al. 2005; Jego et al. 2003; Douagi et al. 2009; Gujer et al. 2011a).

TLR ligand adjuvants have already begun to make an appearance in vaccine adjuvants. AS04 is an adjuvant composed of the TLR4 ligand monophosphoryl lipid A (MPL) adsorbed to alum that is approved for hepatitis B virus and human papilloma virus vaccines in Europe and the USA (Casella and Mitchell 2008). Other selected TLR ligands are under evaluation for their adjuvant effect in different human trials with promising results (Cooper et al. 2004a, b; Halperin et al. 2003; Senti et al. 2009; Coffman et al. 2010). The ligands in clinical trials are mainly compounds that bind TLR3, 7, 8, and 9. The synthetic double-stranded RNA, polyriboinosinic-polyribocytoidylic acid (poly I:C), and the stabilized analogues poly ICLC and poly $IC_{12}U$ are all recognized by TLR3 and the cytoplasmic RNA helicase MDA5. TLR3 ligation of human MDCs including the CD141+ subset

induces IL-12 p70, phenotypic maturation and enhanced antigen presentation (Kadowaki et al. 2001; Lore et al. 2003; Jongbloed et al. 2010; Poulin et al. 2010). Studies have shown that TLR3 ligands are superior as adjuvants over other TLR ligands (Longhi et al. 2009; Stahl-Hennig et al. 2009; Trumpfheller et al. 2012). Poly ICLC has been the most extensively tested adjuvant with the anti-DEC-205-HIV-1 Gag targeting platform with encouraging results (Longhi et al. 2009; Trumpfheller et al. 2012). Using mouse models it was shown that IFNα production, not necessarily from the DCs, was required to obtain a beneficial effect from the poly ICLC (Longhi et al. 2009).

The natural ligand for TLR7 and 8 is ssRNA. However, since ssRNA is rapidly degraded, it would not make a potent adjuvant. Therefore, synthetic small molecules such as imidazoquinolines that specifically bind to TLR7 (e.g., imiquimod or R837) or TLR7 and 8 (e.g., resiquimod or R848) have been developed and tested as immune-stimulatory agents (Hemmi et al. 2002; Gorden et al. 2005). Imiquimod is licensed for use in the form of the cream Aldara (3M Pharmaceuticals) that is topically applied for antiviral and antitumor treatments. TLR7/8 ligands also have the potential advantage of binding to and inducing maturation in both MDCs and PDCs, in addition to their ability to promote IL-12 p70 and IFNα production, respectively (Jarrossay et al. 2001; Kadowaki et al. 2001; Lore et al. 2003; Douagi et al. 2009; Ito et al. 2002). Since TLR7/8 ligands trigger a TLR pathway that is naturally used by DCs to sense internalization of HIV-1 (Beignon et al. 2005; O'Brien et al. 2011), vaccine adjuvants using these ligands may guide a particularly relevant polarized antiviral immune response. TLR7/8 ligands have shown potency in animal models when administered as adjuvants together with recombinant HIV-1 Gag protein, especially when covalently coupled to the antigen (Wille-Reece et al. 2005a, b).

CpG motifs on bacterial DNA bind to TLR9 (Hemmi et al. 2000). Three main CpG classes have been described; CpG ODN class A, B, and C differ substantially in structure and function (Abel et al. 2005). CpG A has a palindromic CpG phosphodiester sequence with phosphorothioate G-rich ends and induces high amounts of IFNα in PDCs (Krug et al. 2001). In contrast, CpG B contains a phosphorothioate backbone and is unable to induce high levels of IFNα in PDCs but is very potent at stimulating B cells. The third class, CpG C, combines the effects of CpG A and CpG B (Vollmer et al. 2004), which makes it an especially attractive adjuvant component. Numerous studies have evaluated the effects of CpG ODN as adjuvants in preclinical and clinical studies (Wille-Reece et al. 2006; Cooper et al. 2004a, b; Halperin et al. 2003; Senti et al. 2009; Sogaard et al. 2010). In humans, TLR9 is only expressed on B cells and PDCs. Rodents, in contrast, have a wider cellular expression of TLR9, which likely has considerable impact on TLR9 ligand-induced effects. The difference in what cell types that express TLR9 in humans and rodents likely limits the use of rodent models for the evaluation of CpG adjuvants for use in humans. The use of CpG as an adjuvant in nonhuman primates, whose DC subsets and TLR repertoire resemble those in humans, has shown that MDCs are essential for antigen presentation, despite their lack of TLR9 expression (Teleshova et al. 2006). PDCs that are strongly activated by CpG may instead have an important conditioning effect on MDC function.

10.5 Adenovirus-Based Vaccine Vectors for Antigen Delivery and Activation of Dendritic Cells

10.5.1 Human Dendritic Cells have Low Susceptibility to rAd5

Several recombinant viral vectors are being intensively investigated as antigen delivery vehicles for an HIV-1 vaccine. Here, we only focus on replication-incompetent recombinant adenoviruses (rAds), which have emerged as one leading antigen delivery system following promising results in preclinical and clinical trials (Barouch and Nabel 2005; Liu 2010). It is not clear whether viral vaccine vectors that efficiently infect and cause maturation of DCs induce superior vaccine responses (Jenne et al. 2001). It is also largely unknown which cells become infected with rAds after administration. Infection of many cell types by rAds leads to transcription of the inserted transgene and high production of its encoded protein, especially when the insert is under the control of an optimized promoter element like a CMV promoter (McVey et al. 2010). This type of promoter has been found to be active in human DCs (Papagatsias et al. 2008). Understanding how human DCs respond to rAd exposure and whether DCs present the vector-encoded protein would help in the design and use of these gene delivery vehicles as vaccine vectors. The genome organization and capsid structure are relatively conserved amongst Ad species, but receptor usage, cellular and tissue tropism, and activation of immune cells differ. Ads use a variety of cellular attachment receptors which can differ substantially depending on the host species, e.g., between humans and mice (Arnberg 2009). This is again highlighting the importance of studying the interactions between human DCs and potential vaccine components. Vaccines based upon a serotype 5 adenovirus (Ad5) have been most extensively explored. The recent major phase II HIV-1 vaccine trial (the Step study) that was based on rAd5 showed well detectable CD8+ T cell responses to the vector encoded HIV-1 proteins Gag, Pol, and Nef, although no benefit of protection against infection was found (Buchbinder et al. 2008). rAd5 is relatively inefficient to infect in vitro-differentiated human DCs (Maguire et al. 2006; Offringa et al. 2005; Rea et al. 1999, 2001; Zhong et al. 1999; Ophorst et al. 2004) and primary blood MDCs and PDCs (Lore et al. 2007). This may be due to the lack of coxsackie-adenovirus receptor (CAR) expression, the primary receptor for Ad5 (Rea et al. 1999; Adams et al. 2011; Lore et al. 2007). However, considering that rAd5 infection of these DCs occurs in the absence of CAR expression plus the fact that neutralizing anti-CAR mAbs have no effect on infection, a CAR-independent infection pathway likely exists in at least some human DCs (Lore et al. 2007; Adams et al. 2009). In contrast, skin DCs (e.g., epidermal LCs and dermal DCs) do express some level of CAR, most likely because they are tissue resident cells and CAR is a tight junction associated molecule (de Gruijl et al. 2006; Adams et al. 2009; Cohen et al. 2001). Blocking CAR was shown to result in a noticeable but incomplete reduction of rAd5 infection of skin DCs (Adams et al. 2009).

Although rAd5 infection of DCs is often detectable, rAd5 infection induces no or very little phenotypic maturation of DCs (Lore et al. 2007; Adams et al. 2009, 2011) or IFNα production in PDCs (Lore et al. 2007). Despite the low infectivity

and capacity for activation of DCs, rAd5 has been shown to be highly immunogenic in vivo in multiple animal models and human trials (Liu 2010). However, high seroprevalence of Ad5 within human populations may limit the clinical utility of rAd5.

10.5.2 Enhanced Infection and Activation of Human Dendritic Cells by rAd35

Alternative Ad serotypes are being studied as vaccine vectors to circumvent the deficiencies with rAd5. Adenovirus serotype 35 (Ad35) is one such vector that has been extensively tested (Abbink et al. 2007). Ad35 binds to the complement binding receptor CD46 (Gaggar et al. 2003). In contrast to CAR, CD46 is ubiquitously expressed on all DC subsets including circulating blood DCs and skin DCs (Lore et al. 2007; Adams et al. 2009). This is likely a reason why DCs are much more easily infected with rAd35 than with rAd5 (Adams et al. 2009; Lore et al. 2007). Using anti-CD46 mAb directed against the known binding sites for rAd35, infection of DCs can be abolished (Lore et al. 2007). Exposure by rAd35 leads to the induction of strong phenotypic differentiation in DCs and high levels of IFNα in PDCs, comparable to that induced by strong maturation stimuli such as the TLR7/8-binding imidazoquinolines. In fact, while very high doses of rAd5 have shown not to induce differentiation, a dose of only a few rAd35 particles per DC induced strong maturation (Lore et al. 2007). It is likely that viral nucleic acids of Ads released after the viral entry process could signal through endosomal or cytosolically expressed pattern recognition receptors (PRRs) and thereby initiate DC maturation. Species C Ads, like Ad35, may differ from species B like rAd5 and have endosomal retention and escape to the cytosol following receptor mediated endocytosis, which would affect this PRR recognition and consequently phenotypic maturation (Miyazawa et al. 2001). IFNα/β production induced by rAd35 binding through CD46 was shown to be dependent on endosomal TLR9 signaling (Iacobelli-Martinez and Nemerow 2007). IFNα production in response to Ad vaccination may have beneficial effects for vaccination on driving adaptive immunity, but potent insert-specific CD8+ T cell responses are mounted even in the absence of intact type-1 IFN signaling (Hensley et al. 2005). Furthermore, even though rAd5, which does not use CD46, infects DCs to a lesser extent than rAd35, rAd5 is superior over rAd35 at generating insert-specific T cell responses in vivo. It has been recently demonstrated that CD11c+ DCs were indispensable for generating strong insert-specific CD8+ T cell responses after rAd5 immunization in mice (Lindsay et al. 2010). In vitro, CD1c+ MDCs were shown to efficiently utilize antigens expressed as inserts by either rAd5 or rAd35 vectors to activate high frequencies of antigen-specific CD4+ and CD8+ memory T cell responses (Lore et al. 2007). While infected DCs likely display rAd-derived peptide on MHC class I to activate CD8+ T cells, the mechanisms for MHC class II presentation to activate CD4+ T cells are less clear. Nevertheless, these studies indicate that rAd5- and rAd35-exposed DCs are capable of presenting Ad-encoded antigen and stimulate antigen-specific T cells. Due to its role as a complement regulatory protein, CD46 regulates immune cell function through putative signaling domains

within its cytoplasmic tails (Kemper and Atkinson 2007; Wang et al. 2000). Thus, CD46-using rAd vectors, such as rAd35, may affect the immune responses independent of DC infection or function (Adams et al. 2011). In conclusion, rAd vectors may be manipulated through genetic modification to more efficiently infect or activate DCs, but it remains to be determined how effective such strategies are in vivo. Expression of the receptors used by many recombinant Ad vectors differs between human and rodent DCs which makes such investigations more challenging.

10.6 Conclusions and Future Directions

DCs are important targets for any vaccine candidate, including a future prophylactic HIV-1 vaccine, due to their critical role in the initiation and shaping of adaptive immune responses. In this chapter, we have reviewed recent advances in selected aspects of HIV-1 vaccinology and adjuvant use with a focus on how to target DCs more specifically with the aim of achieving a broad, cross-reactive immune response (Fig. 10.3). While significant efforts have already been made, a protective HIV-1

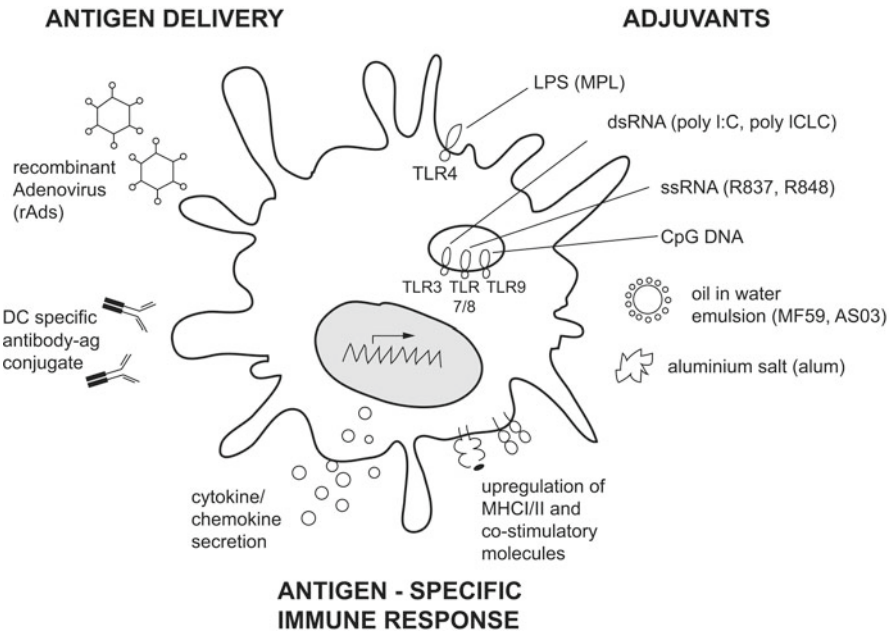

Fig. 10.3 Delivery of antigen and activation of human DCs for the induction of specific immunity. Schematic overview of selected antigen delivery systems and adjuvants that may be potential components of an HIV-1 vaccine. Administration of antigen via viral vectors like recombinant Adenoviruses or via monoclonal antibodies that specifically target DCs, together with a potent adjuvant can facilitate antigen processing and presentation as well as DC maturation (upregulation of MHC, co-stimulatory molecules and secretion of cytokines) and thereby interaction with and activation of antigen-specific T cells

vaccine has yet to be developed. In future studies, it will be important to pinpoint which human DC subset(s) are key in antigen uptake and induction of immunity using a given HIV-1 vaccine delivery strategy. With respect to the strategy of antigen delivery using DC targeting abs, work remains to verify the relevance of important proof-of-principle studies performed in mice using human or nonhuman primate DCs since the receptor expression pattern differs between species. In general, a more detailed understanding of human DC subsets and how they respond to stimulation with different vaccine adjuvants merits further investigation. In addition, a deeper understanding of how different human DC subsets handle endocytosed antigen depending on the mode of delivery and how that impacts subsequent antigen presentation to T cells is an important area of research that may prove to be useful for improved vaccine design or therapeutic approaches.

Acknowledgments The authors would like to thank Robert A. Seder for helpful discussions during the preparation of this chapter, Jessica Ma and Kerrie J. Sandgren for proofreading, and Sara Smed Sörensen for help with the illustrations.

This work was supported by grants from the Swedish International Development Agency (Sida)/Department of Research Cooperation (SAREC) to AS-S (Dnr SWE-2009-086) and KL (Dnr SWE-2009-063), the Swedish Governmental Agency for Innovation Systems (Vinnova) to AS-S (Dnr 2009-04074) and KL (Dnr 2010-00999), the Swedish Research Council to KL (Dnr 521-2009-4809), and the Swedish Physicians against AIDS Foundation to AS-S (Dnr FO2009-0017) and KL (Dnr FOb 2010-0013).

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L. Wu and O. Schwartz (eds.), *HIV Interactions with Dendritic Cells: Infection and Immunity*, Advances in Experimental Medicine and Biology 762,
DOI 10.1007/978-1-4614-4433-6, © Springer Science+Business Media New York 2013