From DEPARTMENT OF MEDICINE, HUDDINGE
Karolinska Institutet, Stockholm, Sweden

STRATEGIES FOR MODULATION OF DENDRITIC CELL RESPONSES

Annette E. Sköld

Karolinska Institutet

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To my loved ones

ABSTRACT

With increased knowledge in dendritic cell (DC) biology, innate immune receptors and their ligands, and the shaping of adaptive responses, refined approaches to modulate our immune system are today emerging as treatment strategies for chronic infections and severe cancers. At the center of attention stand DCs – the innate immune cells that orchestrate the adaptive immune responses. In this thesis, strategies to activate and to inhibit DC activation are described, and the effect of different types of activation of DCs on HIV-1 infection is also investigated.

In **paper I**, we have characterized a novel strategy of TLR3 inhibition in DCs and in other TLR3 expressing cells. The TLR3 ligand poly I:C normally activates DCs to upregulate maturation markers CD80 and CD86 and to secreted pro-inflammatory cytokines. We found that simultaneous addition of oligodeoxynucleotides (ODNs) based on a phosphorothioate (PS) backbone together with poly I:C inhibited the TLR3-mediated DC activation. This inhibition was dependent on the structure of the ODN backbone, since ODNs built on a phosphodiester backbone did not have inhibitory effects, but independent of the sequence, since both CpG and non-CpG containing PS-ODNs had the ability to inhibit the effect of poly I:C. We could repeat the PS-ODN-mediated inhibition on poly I:C activation in three additional non-hematopoietic cell types. Upon investigation of the mechanism behind this observation, we determined that PS-ODNs are preferably taken up into DCs over poly I:C, and are thereby inhibiting the ligand interaction with TLR3. To confirm this finding *in vivo*, we treated cynomolgus macaques intranasally with the ligands, either alone or in combination, and measured the secreted cytokine levels. Significantly reduced levels of IL-12p40 were detected in animals receiving PS-ODNs compared to animals treated with poly I:C alone, and a similar trend was observed also for additional pro-inflammatory cytokines and chemokines measured. Hence, these findings encourage the development of PS-ODNs as a treatment strategy during TLR3-mediated pathology.

Our group has previously reported that irradiated activated PBMCs have the ability to induce DC maturation. In **paper II**, we set out to determine the underlying mechanism for this finding. First, we investigated whether the activated apoptotic cells (ACs) had to be phagocytosed for mediating their effect, but cell-cell contact was shown to be enough for DC maturation when co-cultured with ACs. We then tested if both cellular and supernatant fractions of activated ACs had the ability to mature DCs. Activated ACs were previously shown to release low levels of TNF-α, and we could confirm that the cytokine was a maturing agent in the supernatant fraction. The cellular fraction also matured DCs, and to investigate what molecules could be involved, we neutralized receptors previously shown to be stimulated by endogenous substances. We found that DC-SIGN, TLR4, and β2-integrins all were involved in AC-induced DC maturation, and a plausible ligand for TLR4 was shown to be heat shock protein 60. When investigating the intracellular signaling pathways mediating this effect, we determined that activated ACs induced signaling via Src family of tyrosine kinases, PI3K/Akt, JNK, and p38, and activated the NF-κB and AP-1 transcription factors.

We further investigated the effect of activated apoptotic T cells on DC and HIV-1 infection in **paper III**. These activated ACs, either HIV-1 infected or uninfected, had the ability to mature DCs, and also to reduce HIV-1 infection in DCs. This reduction was partly due to TNF-α produced by stimulated DCs, but mainly due to the increased expression of the HIV-1 host restriction factor APOBEC3G in DCs. In **paper IV**, we continued to investigate the expression of APOBEC3 family members in DCs upon treatment with TNF-α or IFN-α. We could confirm previous reports on expression of APOBEC3A, F, and G in DCs, and we also concluded that TNF-α, despite induction of DC activation, did not induce expression of APOBEC3 molecules, but more probably stimulated additional host restriction factors in DCs.

LIST OF PUBLICATIONS

I. **Annette E. Sköld***, Maroof Hasan*, Leonardo Vargas, Hela Saidi, Nathalie Bosquet, Roger LeGrand, C. I. Edvard Smith, Anna-Lena Spetz. Single-stranded DNA oligonucleotides inhibit TLR3-mediated responses in human monocyte-derived dendritic cells and in vivo in cynomolgus macaques
Manuscript

II. Sushil Kumar Pathak, **Annette E. Sköld**, Venkatramanan Mohanram, Catrine Persson, Ulrika Johansson, Anna-Lena Spetz. Activated Apoptotic Cells Induce Dendritic Cell Maturation via Engagement of Toll-like Receptor 4 (TLR4), Dendritic Cell-specific Intercellular Adhesion Molecule 3 (ICAM-3)-grabbing Nonintegrin (DC-SIGN), and β2 Integrins
The Journal of Biological Chemistry, 2012, 287: 13731–13742

III. Venkatramanan Mohanram*, Ulrika Johansson*, **Annette E. Sköld**, Joshua Fink, Sushil Kumar Pathak, Barbro Mäkitalo, Lilian Walther-Jallow[§], Anna-Lena Spetz[§], Exposure to Apoptotic Activated CD4+ T Cells Induces Maturation and APOBEC3G- Mediated Inhibition of HIV-1 Infection in Dendritic Cells.
PLoS One, 2011, 6: e21171

IV. Venkatramanan Mohanram*, **Annette E. Sköld***, Sushil Kumar Pathak, Anna-Lena Spetz. Low quantities of IFN-α induce Apolipoprotein B mRNA editing enzyme, catalytic-like 3 (APOBEC3) A, F and G without concomitant dendritic cell maturation
Manuscript

*, [§] These authors contributed equally

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LIST OF ABBREVIATIONS

AC	Apoptotic Cell
AIDS	Acquired Immunodeficiency Syndrome
AIM2	Absent In Melanoma 2
AP-1	Activator Protein 1
APC	Antigen Presenting Cell
APOBEC	Apolipoprotein B mRNA-editing Enzyme-Catalytic polypeptide
ATP	Adenosine-5′-Trihosphate
CARD	Caspase Activation and Recruitment Domain
CCR5	CC chemokine Receptor 5
cDC	conventional DC
CDP	Common DC Progenitor
CLEC	C-type Lectin
CLR	C-type Lectin Receptor
CM	Conditioned Medium
CMP	Common Myeloid Progenitor
CMV	Cytomegalovirus
CNS	Central Nervous System
CpG	Cytidine-phosphate-Guanosine
CTL	Cytotoxic T Lymphocyte
CXCR4	CXC chemokine Receptor 4
DAI	DNA-dependent Activator of IFN-regulatory factors
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DC-SIGN	DC-Specific Intercellular adhesion molecule 3-Grabbing Non-integrin
dNTP	deoxynucleoside 5′-Triphosphate
ds	double-stranded
EBV	Epstein-Barr Virus
Flt3L	Fms-like thyrosine kinase 3 Ligand
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
HMGB1	High-Mobility Group Box 1

HSP	Heat Shock Protein
HSV-2	Herpes Simplex Virus type 2
IFN	Interferon
IL	Interleukin
IPS-1	IFN-β Promoter Stimulator 1
IRF	IFN Regulatory Factor
ISG	IFN-Stimulated Gene
ISGF3	IFN-Stimulated Gene Factor 3
ITAM	Immunoreceptor Tyrosine based Activation Motif
ITIM	Immunoreceptor Tyrosine based Inhibition Motif
JNK	Jun-amino-terminal Kinase
LC	Langerhans Cell
LFA-1	Lymphocyte Function-associated Antigen 1
LGP2	Laboratory of Genetics and Physiology 2
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeat
LTR	Long Terminal Repeat
Mac-1	Macrophage-1 antigen
MAPK	Mitogen-Activated Protein Kinase
MDA5	Melanoma Differentiation Associated factor 5
MDP	Macrophage-DC Progenitor
MHC	Major Histocompatibility Complex
MLR	Mixed Lymphoid Reaction
MPLA	Monophosphoryl Lipid A
MSU	Monosodium Urate
MyD88	Myeloid Differentiation primary response gene 88
NBD	Nuclear Binding Domain
NF-κB	Nuclear Factor κ-light-chain enhancer of activated B cells
NK	Natural Killer
NLR	Nucleotide-binding domain LRR-containing protein
ODN	DNA oligonucleotide/Oligodeoxynucleotide
PAMP	Pathogen Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cell
PD	Phosphodiester

pDC	plasmacytoid DC
PHA	Phytohaemagglutinin
PI3K	Phosphatidylinositol 3-Kinase
PRR	Pattern Recognition Receptor
PS	Phosphorothioate
PYD	Pyrin Domain
RAGE	Receptor for Advanced Glycan End products
RIG-I	Retinoic acid-Inducible Gene I
RIP1	Receptor Interacting Protein 1
RLR	RIG-I-Like Receptor
RT	Reverse Transcriptase
SAMHD	SAM domain and HD domain-containing protein
SFK	Src Family of tyrosine Kinases
si	small interfering
SIV	Simian Immunodeficiency Virus
SLE	Systemic Lupus Erythematous
STAT	Signal Transducer and Activator of Transcription
Syk	Spleen tyrosine kinase
ss	single-stranded
TGF	Transforming Growth Factor
Th	T helper
TIR	Toll-IL-1 Receptor
TIRAP	TIR domain-containing Adaptor Protein
TLR	Toll-Like Receptor
TNF	Tumour Necrosis Factor
TRAM	TRIF-Related Adaptor Molecule
Treg	T regulatory
TRIF	TIR domain-containing adapter-inducing IFN-β
TRIM	Tripartite Motif-containing protein
wt	wild-type
ZAP	Zinc-finger Antiviral Protein

1 INTRODUCTION

In the beginning, the protocell was alone in the oceans, and no pathogens existed. However, when life evolved into diversity, the condensed form of nutrition contained in a cell quickly became an attractive source of energy for other cells. Only organisms with mechanisms of protection and recognition of their own species survived. When the organism then went from a single-cellular entity to a multicellular form, the need for protection against microbial colonization increased. Cells specialized in defence developed and what we today call the innate immune system started to take shape. A great variety of mechanisms to prevent infection were created, and the features of the innate immune system were refined by evolution to form the complex multilayer system it is today [1].

As organisms grew larger and more complex, adaption to an ever-changing environment merely on a generation basis was not sufficient. In addition to the diverse repertoire of germline encoded pathogen recognition receptors, cells with adaptive genes, with the ability to rearrange and mutate within the cell, coding for immune receptors were evolved and can now be found in all jaw vertebrate species. Upon differentiation of the cells, these genes are rearranged and a great repertoire of cells with unique immune receptors is created, based on a limited amount of genetic material. In theory, this enables an almost infinite repertoire of pathogen-specific cells, but it is only the ones actually recognizing the encountered pathogens that will expand and take action. After clearance of an infection, a fraction of the pathogen-specific cells remains in the body as distant memories, and if the same pathogen is encountered again, these memory cells will quickly be re-activated and protect from disease [1].

These two branches of defence strategies have co-evolved and are both important for our survival. The inherited innate immune system acts immediately but unspecifically upon infection, while the adaptive immunity is continuously progressing, to specifically target and remember the pathogens we encounter. Despite the distinctive mechanisms of action, cross-talk between the innate and adaptive immune systems is essential for clearance of severe infections. As a translator between the two systems, the dendritic cell (DC) is a crucial bridging component, and the message it carries from the site of infection to the cells of the adaptive immune system determines what kind of response will be initiated against the intruder [2].

This thesis will discuss how DCs can be activated, prevented from activation, and prevented from viral infection, using various strategies. Enhanced knowledge in this area will shed light on how the innate immune system can be facilitated to impact the adaptive immune responses, and in the end, how vaccines and treatment strategies for certain patient groups can be improved and better understood.

1.1 DENDRITIC CELLS AND CONTROL OF THE IMMUNE SYSTEM

The immune system consists of a diversity of cells, collectively called leukocytes, with varying functional properties important in host defence against pathogens. Common to leukocytes is that they all originate from hematopoietic stem cells in the primary lymphoid tissue in the bone marrow. Two distinct developmental pathways have been characterized – the lymphoid and myeloid linage. Myeloid stem cells differentiate into

distinct progenitor cells with varying capacity to further differentiate into myeloid cells, such as monocytes, neutrophils, eosinophils, mast cells, erythrocytes, and DCs. The lymphoid stem cell gives rise to progenitor cells that can further differentiate into cells referred to as lymphocytes, which consist of T cells, B cells, natural killer (NK) cells, and NKT cells. After differentiation, the cells exit the bone marrow and either home to secondary lymphoid organs or peripheral tissue, or they circulate in the blood until they receive signals to migrate into inflamed tissue or grow too old and are cleared from the circulation. T cells go in an undifferentiated state to the thymus, which also is defined as a primary lymphoid organ, where they finalize their maturation process to become specific for self major histocompatibility complex (MHC) molecules in complex with non-self peptides. Cells not fulfilling these criteria are not provided with enough survival stimuli to continue development, or are actively killed if they are auto-reactive and recognize MHC complexes with self-peptides. After this selection, the T cells home to secondary lymphoid tissue, such as the lymph nodes. In addition to the T cells, B cells and lymph node resident macrophages and DCs are also found in lymph nodes and secondary lymphoid tissue. Antigens are transported here, either in a soluble form in the afferent lymph or via migratory DCs, and presented to T and B cells. If the antigen is derived from a foreign entity, it will be recognized as non-self by the lymphocytes, and depending on the instructions accompanied from the innate immune response in the tissue from which it was transported, an appropriate adaptive response will be initiated [2].

1.1.1 Characteristics of dendritic cells

Dendritic cells are the main bridging component between the innate and adaptive immune systems. They have a unique ability to acquire antigens in the periphery and then present them to cells of the adaptive immune system. Although DCs are considered to be part of the innate immune system, their antigen presentation is crucial for activation of specific adaptive immune responses.

1.1.1.1 The discovery of dendritic cells

The first DC to be described was the Langerhans cell (LC) in 1868 [3]. Paul Langerhans discovered a cell type in the epidermis of the skin with long, branching dendrites spreading in the tissue. Due to its morphological appearance and the staining method used, believed to be specific for neurons, Langerhans concluded that the cells he had observed were epidermal nerve endings [4]. The function of LCs long eluded researchers and was not properly determined until a comparison with other subsets of DCs could be performed [5, 6].

In the early 1970s, Ralph Steinman and Zanvil Cohn published a series of articles describing a novel cell type, which they named dendritic cell, in peripheral lymphoid organs of mice [7-9]. Dendritic cells were soon shown to have superior capability to induce proliferation of cells in mixed leukocyte reactions (MLRs) compared with other leukocytes, such as B cells or macrophages [10]. Today, DCs are defined as professional antigen presenting cells (APCs) that take up antigens, either in the peripheral tissue or in lymphoid organs, process them, and present them to adaptive immune cells [11].

1.1.1.2 Dendritic cell functions

Dendritic cells can be found in most tissue, but in particular at the body surface linings, which are highly exposed to microbial intrusions, including the skin, gut, lungs, and vagina. There are several subtypes of DCs and their function varies between location and subtype, but generally they sense the surrounding milieu for threats or abnormalities by responding to non-self structures and dying cells.

If nothing stimulatory is encountered, DCs act to maintain the tissue homeostasis [12]. However, if a pathogen or inflammatory agent is detected by a DC, either by sensing the antigen directly or by signals derived from other innate cells in the tissue, the cell is activated and participates in the inflammatory response. Initially, activated DCs secrete pro-inflammatory chemokines and cytokines to attract additional immune cells and to have them exert their effector functions or to replenish the pool of DCs. The activated DCs briefly enhance their uptake of antigen, and thereafter migrate to the adjacent lymph node while maturing and enhancing their antigen presenting capacity [13]. In the lymph node, DCs either transfer their carried antigen to lymph node resident DCs, or directly present their cargo to T and B cells. An adaptive immune response, custom made for the infection from which the DC migrated, is then initiated [14-17].

1.1.2 Dendritic cell subsets

Since the discovery of DCs, an increasing number of subsets of DCs have been described. This increase can partly be explained by the localization of the cells and influence from the milieu to which they are exposed, but the focus on DC ontogeny has also increased in recent years.

1.1.2.1 Ontogeny

Dendritic cells originate from a common myeloid progenitor (CMP) in the bone marrow. The CMP has been shown to give rise to an intermediate macrophage-dendritic cell progenitor (MDP) [18], which is thereafter differentiated to either monocytes or to a common DC progenitor (CDP). This progenitor finally divides into a pre-DC or plasmacytoid DC (pDC) [19]. When pre-DCs and pDCs are formed, they exit the bone marrow and either home to peripheral or lymphoid tissue, where they become finally differentiated, or circulate the blood and tissue, respectively.

Langerhans cells, the DCs of the epidermis, are however not derived from the bone marrow, but from local stem cells that migrate to the skin during the late embryonic period and replenish the LC population *in situ* [20].

1.1.2.2 Conventional dendritic cells

Commonly, DCs that originate from CMPs but are not pDCs have been described as conventional DCs (cDCs). However, the influence of monocytes on the cDC population is debated. Monocytes have been shown to migrate to inflamed tissue, where they replenish the inflammatory site with DC-like cells during infections, when the resident DCs are activated and migrating to the lymph nodes [21, 22].

Conventional DCs are found in both lymphoid and peripheral tissue. Cells in the lymphoid organs have in mice been divided into $CD8\alpha^+$ and $CD8\alpha^-$ DCs, where the $CD8\alpha^-$ can be further subdivided into $CD4^+$ and $CD4^-$ DCs [23, 24]. $CD8\alpha^+$ DCs are highly efficient in promoting $CD8^+$ T cell responses via cross-presentation and are mostly found in the T cell zones of secondary lymphoid organs [25], while the $CD8\alpha^-$ subsets have been ascribed to have more regulatory functions. If the $CD8\alpha^-CD4^-$ population is stimulated with the proper reagents though, an efficient immune response can be activated by this subset as well [26].

There are several DC subpopulations in peripheral tissue. They are all characteristic of their local environment, but at the same time they resemble each other in function and marker expression, probably due to a common progenitor cell [19]. In addition to the LCs, two DC subsets have in the murine system been characterized in the dermis of the skin. One subset express langerin, a C-type lectin found to be involved in endocytosis, and is positive for the integrin CD103 and negative for the integrin CD11b. Langerhans cells also express high levels of langerin, but the $langerin^+CD103^+CD11b^-$ dermal DC is derived from the CDP progenitor and more closely related with the lymphoid resident $CD8\alpha^+$ DC. They are for example both dependent on the cytokine fms-like tyrosine kinase 3 ligand (Flt3L) and the transcription factors Batf3, interferon regulatory factor (IRF) 8, and Id2 for their development [27-32]. $Langerin^+CD103^+CD11b^-$ dermal DCs and $CD8\alpha^+$ DCs are both specialized in cross-presentation, they have the ability to produce high levels of interleukin-12 (IL-12), and they have the capability to induce strong $CD8^+$ T cell responses [33, 34]. The second DC subset of the skin is defined as $langerin^-CD103^-$ $CD11b^+$ and rather interacts with the $CD4^+$ T cells in the draining lymph node [34]. The origin of this subset has not been determined and the question remains whether it in fact might be a heterogeneous population, consisting of both monocyte-derived cells as well as cells derived from pre-DCs.

The expression of CD103 on DCs can be found on subsets in most peripheral tissue, such as the intestinal tract, lungs, kidneys, and liver, and they seem to have similar functions [30]. There are also $CD103^-CD11b^+$ DCs in this tissue, but their origin is less clear. Most probably they are a heterogeneous population, derived both from pre-DC progenitors and monocytes.

Most research on DC subpopulations has been performed in mice, and less is known about the human system. The dependence of transcription factors during cell development however indicates that similar subsets are present in humans as well, as deficiencies of these factors in humans lead to almost complete abolishment of particular DC subsets *in vivo* [35, 36]. Recently, a subset of DCs in the human system with similar features to the murine $CD8\alpha^+$ and $CD103^+$ cells has been characterized [37-40]. This subset expresses markers such as CD141, also known as blood DC antigen (BDCA) 3, and C-type lectin (CLEC) 9A, and is found mainly in blood, but also in the spleen, lymph nodes and bone marrow. It displays a similar ability as the murine $CD8\alpha^+$ and $CD103^+$ DCs to take up dying cell debris and cross-present antigens to CD8+ T cells, as well as to produce pro-inflammatory cytokines, such as IL-12, and is probably the human counterpart of these subsets. Two other conventional subtypes described in human blood are the $BDCA1^+$ and $CD16^+$ DCs, and a $CD14^+$ and a $CD1a^+$ population have been described in skin, in addition to the LCs [41-43].

1.1.2.3 Plasmacytoid dendritic cells

The DC subsets considered as non-conventional DCs are pDCs and monocyte-derived DCs. Even though pDCs, like the cDCs, are derived from CDPs, they are considered non-conventional due to their non-DC morphology before activation and their specialization in type I interferon (IFN) production. Indeed, before they were characterized as DCs, they were named natural IFN-producing cells [44, 45]. Plasmacytoid DCs can be found both in blood and in inflamed tissue. Sensing viral nucleic acids stimulates the pDCs to produce high quantities of type I IFNs, which put the surrounding tissue in an anti-viral defence mode when cellular activities, such as gene transcription and RNA translation, are down-regulated [46]. Activated pDCs have the ability to present antigens and activate T cells, but not as efficiently as cDCs [47].

1.1.2.4 Monocyte-derived dendritic cells

Monocytes is a heterogeneous myeloid cell population that circulates in the blood. This enables them to monitor all sites of the body and to quickly migrate into inflammatory tissue, where they, depending on stimuli, can act both to enhance the inflammation and to eliminate cellular debris and toxic agents [48, 49]. A subset of monocytes can replenish the macrophage population in the tissue during inflammatory conditions [50], while certain DC subsets in the tissue have been shown to be replaced by a different monocyte subset than the one replacing macrophages [49]. An important step for DC research was when monocytes were shown to acquire a DC-like phenotype *in vitro* if cultured with the cytokines IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) [51]. Human DCs no longer had to be produced from precursor cells derived from bone marrow or cord blood, but could easily be obtained in the lab from normal blood donations. Inflammatory monocyte-derived DCs have been described in several infection models [52, 53] and it has been confirmed that monocytes can give rise to DCs *in vivo* [21, 54], but their contribution to the steady state pool of tissue DCs is still not completely understood.

1.1.3 Induction of adaptive responses

The main function of DCs is to bring a message from the periphery to lymph nodes and the adaptive immune cells and translate it to them. It might be a word of calm, making sure none of the interacting cells are immunoreactive against self-antigens, or it might be instructions on how to attack a harmful intruder. The message the DC delivers has to be very fine-tuned; an erroneous response can be highly detrimental and lead to too weak, too strong, or misdirected immune reactions.

1.1.3.1 Dendritic cell activation

When a DC first migrates into the peripheral tissue, it is considered to be immature. This is characterized by a steady state sampling of components of the surrounding milieu, for example cells undergoing programmed cell death – apoptosis. It is also characterised by a moderate lysosomal degradation efficiency, and low cell-surface expression of MHC-complexes. However, at the sense of danger, the DC initiates a

series of events, which culminates in interaction and activation of T and B cells in secondary lymphoid tissue.

Dendritic cells ingest antigens by a set of mechanisms. Small molecules are taken up via endocytosis, often triggered by engagement of specific high-affinity receptors such as C-type lectins, scavenger receptors, or Fc receptors, whereas larger objects are phagocytosed when bound by specific receptors. In addition, DCs sense the surrounding milieu by engulfing large quantities of extracellular fluids without initial triggering of any specific receptor in a process called macropinocytosis [55, 56]. A captured antigen does not necessarily induce DC activation *per se*, since an interaction with activating receptors expressed by DCs is required. This will be discussed in further detail in chapter 1.2 of this thesis. Certain pro-inflammatory cytokines have also been shown to mature DCs [57-59], even though the functionality of this activation has been questioned [60, 61].

When DCs are activated in the tissue, they first act to alert surrounding cells to the threat and to sample more of the pathogen. By secreting chemokines to attract additional immune cells, such as neutrophils, $CD8^+$ cytotoxic T lymphocytes (CTLs), and NK cells, the local inflammation is boosted by DCs [13]. A brief period of enhanced endocytosis enables the DCs to acquire more of the antigen for processing [62], and an altered phagosomal maturation trims the antigens to be better presented on MHC molecules [63]. Next, activated DCs lose their ability to take up antigens, up-regulate chemokine receptors, and home to secondary lymphoid tissue [64-66]. During the migration, DCs up-regulate expression of MHC-complexes, co-stimulatory molecules, and additional receptors needed for interaction with and stimulation of naïve T cells [67], which are attracted to the DCs by secreted chemokines upon lymph node entry [13].

However, activation is not a prerequisite for DC migration to the lymph node. Dendritic cells only exposed to self structures, such as apoptotic cells, without the presence of any activating agents can also acquire a migratory and antigen-presenting phenotype and home to the lymph node to present self-antigens to T cells [12, 68-70]. This maintains peripheral tolerance by inducing an anergic or regulatory response in T cells specific for the presented self-antigens. Indeed, if DCs would not stimulate tolerance, a lethal autoimmunological response would spontaneously be initiated [71].

1.1.3.2 Antigen presentation

Protein antigens are presented to the immune system as peptides bound by MHC molecules on the cell surface. All nucleated cells express MHC class I, which form complexes with endogenous peptides derived from a fraction of the proteins synthesized within the cell, while only APCs express MHC class II molecules. The MHC class II molecule is mainly loaded with peptides derived from exogenous antigens actively taken up by the APC [72].

Antigen presenting cells, often DCs, present their cargo on MHC class II molecules to $CD4^+$ T cells in secondary lymphoid tissue, such as lymph nodes. An immunological synapse is however only formed between the two cells if the APC presents the antigen for which the T cell receptor is specific, thereby ensuring that only T cells reactive against the particular antigen presented are engaged [73]. If, in addition to the peptide presenting MHC class II complex, co-stimulatory molecules like CD80 and CD86 are expressed by the DC, the $CD4^+$ T cell gets activated and upregulates the ligand for

CD40, which is an activating receptor expressed on DCs. Triggering of CD40 licenses the DC to further activate $CD8^+$ T cells. The activated $CD4^+$ T cell starts producing cytokines to help additional APCs presenting the same antigen as the T cell is primed for, like B cells in the lymph node or macrophages in the peripheral tissue, to exercise their functions. Activated $CD4^+$ T cells are therefore entitled T helper (Th) cells. There are several different classes of Th cells, depending on the cytokines they are instructed to produce. Three common classes of responses are defined as Th1, Th2 and Th17 responses [74].

If a cell is infected by an intracellular pathogen that is hijacking its protein synthesis machinery, or has acquired a genetic mutation resulting in production of proteins with altered sequence and function, these proteins are exposed on MHC class I molecules and recognized by specific CTLs, previously primed and licensed by activated DCs in the secondary lymphoid tissue. They are instructed to kill cells expressing MHC complexes presenting the specific antigen, and thereby eliminating the threat of infection or malignancy [75].

However, DCs do not get infected with all viruses or intracellular bacteria, and they are not producing mutated proteins for presentation on MHC class I molecules. For a long time, it was a mystery how $CD8^+$ T cells were primed for these types of antigens. In 1976, Michael Bevan introduced the concept of cross-priming, when exogenous antigens were cross-presented to $CD8^+$ T cells on MHC class I molecules [76]. Exactly how extracellular antigens are transported onto the MHC class I molecules is still not fully understood, but this pathway has been shown to be highly important for immune defences against intracellular pathogens [77-80]. Dendritic cells commonly also cross-present antigens derived from various malignancies [81], but the induced responses are often not as strong due to the lack of additional activating stimulus when the antigen is taken up [82].

1.1.3.3 Dendritic cell influences on adaptive responses

In secondary lymphoid tissue, DCs present their acquired antigens on MHC-complexes to naïve T cells. This is however not sufficient to induce a strong adaptive immune response against the antigen. In addition to the first direct presentation of the MHC-antigen complex, two extra signals are required [61].

Signal 1 is the specific antigen recognition by the $CD4^+$ T cell, which if not accompanied by additional signals leads to anergy or death of the T cell. Signal 2 is provided from the DC via its co-stimulatory maturation markers, such as CD80, CD86, and additional B7 molecules. These ligands interact with the co-stimulatory receptor CD28 on the T cell, allowing it to respond to the presented antigen. Finally, the type of response that will be induced is determined by signal 3, provided by the DC as expressed ligands or produced cytokines. When instructed, the $CD4^+$ T cell is primed and differentiates to a T helper cell with functions specific for the particular condition [83]. This is also important for $CD8^+$ T cell priming and their ability to differentiate to efficient CTLs [84].

If the interacting DC secretes cytokines such as IL-12, IL-18 and type I IFNs, a Th1 response is initiated, priming $CD8^+$ T cells to differentiate to CTLs and an immune response against intracellular pathogens is induced. Cytokines such as IL-4, IL-5, and IL-13 induce a Th2 phenotype, instructing the immune response to fight extracellular parasites. Extracellular bacteria and fungi are generally fought with a Th17 response,

induced by IL-23, IL-6, and transforming growth factor (TGF)-β secretion from DC. In addition, by producing IL-10, DC can induce regulatory T cells (Tregs), vital for maintaining tissue homeostasis [74, 85, 86].

1.1.4 Therapeutic opportunities

Due to their central part in regulation of immune responses, DCs are attractive targets for immunotherapy. Dendritic cells can be targeted both for stimulation in vaccine strategies or for tolerance induction in transplantations or autoimmunity settings.

During vaccination, the goal is to elicit a specific and qualitative immune response against an antigen. Traditionally, protective vaccines induce high antibody titers, which are mostly efficient against extracellular pathogens, although some intracellular microbes also can be defeated with this strategy. Mostly though, cancers and intracellular pathogens, such as HIV, are difficult to eradicate with a humoral response only. In these settings, a cellular immune response with efficient CTL priming is favorable to eliminate infected or mutated cells. With the increased knowledge about DC subsets and function, more specialized vaccines can be developed. One examined strategy is to culture DCs *ex vivo*, either from monocytes [87-90] or $CD34^+$ progenitor cells [91, 92], and to load them with the desired antigen and stimulus, and thereafter infuse them back to the patient to stimulate an appropriate immune response. However, this is a cumbersome and expensive technique and the efficiency of using primary DC populations is being investigated [93]. Also, an alternative option is to direct the vaccine straight to DCs *in vivo*, using constructs targeting receptors expressed on DCs. This has been tested in several systems, targeting different receptors with varying constructs of antigen and adjuvant [94-98]. There are however many questions that remain to be answered, such as which DC subsets and what receptors are beneficial targets to achieve the desired immune response. Different DC subpopulations express different combinations of activating receptors and the response from the same type of receptor can vary between different cells [99]. Indeed, exploration of pDCs and the newly characterized human $BDCA3^+$ DC subpopulation as a target for vaccines will be very interesting [93, 100, 101].

How DCs acquire their regulatory phenotype is not fully understood and needs to be further investigated before tolerogenic DCs can be induced as a treatment strategy. It has been shown though that targeting an antigen to DCs *in vivo* without the presence of additional stimulus or adjuvant can induce tolerance against the antigen [102, 103], and strategies to use DCs in transplantation settings to prevent graft rejection or to dampen autoimmune responses would truly be very intriguing [104].

Dendritic cells can in certain settings have detrimental effects, by priming too strong or erroneous kind of responses. During HIV infection, DCs are believed to be exploited as Trojan horses, carrying the virus from the mucosal site of infection to the lymph node, highly populated with T cells that HIV can infect [105]. Furthermore, DCs can during inflammatory settings be stimulated by self-antigens without the immediate presence of pathogens, which can lead to immune pathology, autoimmunity, and severe tissue damage [106-109]. Strategies for dampening of these DC functions are therefore needed.

1.2 DANGER ASSOCIATED MOLECULAR PATTERN

One of the key duties of the innate immune system is to recognize and respond to foreign pathogens that might induce harm to the organism. To do so, a great variety of germline-encoded receptors specific for conserved microbial structures associated with danger have evolved. These so-called pattern recognition receptors (PRRs) recognize danger associated molecular patterns (DAMPs) and are expressed both on hematopoietic and non-hematopoietic cells. Examples of DAMPs are nucleic acids, bacterial wall components, and certain endogenous proteins, such as heat shock proteins (HSPs). A common term in innate immunology is pathogen associated molecular pattern (PAMP), which is a generic term for PRR ligands derived from pathogens. However, since both exogenous and endogenous substances have been shown to engage and activate PRRs, the term DAMP is in this thesis used to describe both types of ligands. Nevertheless, DAMP is also an abbreviation for Damage Associated Molecular Patterns, indicating molecules secreted by the own body in response to tissue damage, for example during infections. In this thesis, DAMPs includes both self and non-self molecules [110].

1.2.1 Pattern recognition receptors

Since the discovery of the first PRR there has been so many additional receptors characterized that they now are divided into families of related types of receptors. The first group to be described was the Toll-like receptors (TLRs). *Toll* is a gene initially described in *Drosophila melanogaster*, where its product plays an important role in establishing the dorsal-ventral axis during embryogenesis [111]. The name "Toll" is said to come from the surprised comment made by the researcher Christiane Nüsslein-Volhard when she first saw the oddly shaped fly larva expressing the mutated gene [112]. A decade later, Jules Hoffmann discovered that *Toll* mediated protection against bacterial and fungal infections [113], introducing the gene into immunology. Soon after, Bruce Beutler assigned the murine *Tlr4* gene to be the long searched for receptor responding to the potent bacterial endotoxin lipopolysaccharide (LPS) [114]. This was the beginning of a new era in innate immunology, and in the last decade, innate detection of DAMPs has grown to a field in it self. In addition to the TLRs, C-type lectin receptors (CLRs), RIG I-like receptors (RLRs), and nucleotide-binding domain LRR-containing proteins (NLRs) have been identified as sensors for pathogens and certain self-structures.

1.2.1.1 Toll-like receptors

There are ten human genes coding for TLRs. The receptors are localized at varying sites in the cell, but all have a type I transmembrane protein structure with leucine-rich repeats (LRRs) recognizing their respective ligands and a cytosolic Toll-IL-1 receptor (TIR) domain to further activate intracellular signaling cascades when the receptor is activated (Figure 1) [115]. Examples of ligands for each receptor, except for TLR10, to which no ligand yet is described, can be found in Table 1. Roughly, the TLRs are divided into two groups, depending on their cellular location. Due to their cell surface expression, TLR1, 2, 4, 5, and 6 recognize extracellular DAMPs, while TLR3, 7, 8, and 9 are found in the endocytic compartments, where they sense nucleic acids [116].

For activation to occur, the TLR binds its ligand, undergoes conformational changes, and forms either a homo- or heterodimer with an additional TLR. This recruits intracellular adaptor proteins to the intracellular TIR domain, such as myeloid differentiation factor 88 (MyD88), TIR domain-containing adapter-inducing interferon-β (TRIF), TIR domain-containing adaptor protein (TIRAP), and TRIF-related adaptor molecule (TRAM), which in turn bind and activate additional signaling molecules [115]. All receptors, except TLR3, engage MyD88, either directly or via TIRAP. Toll-like receptor 3 will be further discussed in chapter 1.2.2 of this thesis. The only receptor that signals both via MyD88 and TRIF is TLR4. Upon ligand binding on the cellular surface, TLR4 recruits TIRAP, which binds MyD88. This mediates initial activation of the transcription factors IRF5, nuclear factor κ-light-chain enhancer of activated B cells (NF-κB), and activator protein 1 (AP-1). Meanwhile, TLR4 is endocytosed and recruits TRAM, which binds to TRIF, and a second path of signals is initiated, also mediating late-phase activation of NF-κB and mitogen-activated protein kinases (MAPKs), which are upstream of AP-1 signaling, and IRF3, a transcription factor important for activation of type I IFNs [117-119]. For TLR4, both MyD88 and TRIF are needed for full activation, but the remaining receptors only use one of the adaptor molecules.

Toll-like receptors are differentially expressed on various cell types. In DCs, different subsets express a specific repertoire of different receptors [120, 121]. In addition to this, the outcome of TLR stimulation also varies depending on which DC subset it is expressed on [99]. Plasmacytoid DCs, for example, express fewer TLRs than other DC subsets, but are highly responsive to single-stranded RNA (ssRNA) and ssDNA via engagement of TLR7 and TLR9, respectively. These receptors signal via MyD88, which forms a multiplex involving numerous kinases and signaling components, among them IRF7 [122]. This transcription factor is constitutively expressed in pDCs [123], and when activated, it is translocated to the nucleus and mediates transcription of IFN-α. The activation complex also mediates activation of additional transcription factors, such as NF-κB, IRF5, and AP-1, which induce maturation and expression of pro-inflammatory cytokines like IL-6 and tumor necrosis factor (TNF) α. In other DCs, TLR7 activation mediates maturation and pro-

Location	Receptor	DAMP	Synthetic ligand
Cell surface	TLR1/2	Triacyl lipopeptides	Synthetic triacylated lipoprotein
	TLR2	Peptidoglycan, Phospholipomannan	Ultrapure peptidoglycan
	TLR4	LPS, MPLA, Mannan	Synthetic MPLA
	TLR5	Flagellin	Recombinant flagellin
	TLR2/6	Diacyl lipopeptides, Lipoteichoic acid,	Synthetic diacylated lipoprotein
Endosomes	TLR3	dsRNA	Poly I:C
	TLR7	ssRNA	Guanosine analog
	TLR8	ssRNA	R848
	TLR9	dsDNA	CpG-ODN

Table 1: Human toll-like receptors examples and their ligands. Adapted from [115].

inflammatory cytokine production, but only induces low levels of type I IFNs compared with levels produced by pDCs.

In humans, pDCs are the only DC subset that expresses TLR9. B cells express the receptor as well, but they are not primed to induce type I IFNs. An interesting observation with TLR9 signaling in pDCs is its dual functions depending on where in the endosomal maturation process the signaling occurs. The ligand for TLR9 is ssDNA oligonucleotides (ODNs). Initially, it was believed that an unmethylated cytidine-phosphate-guanosine (CpG) motifs in the DNA were needed to induce TLR9 activation [124], but it was later shown that the CpG-motif was needed only in ssDNA with the synthetic phosphorothioate (PS) backbone [125]. Natural DNA, based on a phosphodiester (PD) backbone, activates TLR9 independent of sequence, while PS-ODNs can be either inhibitory or stimulatory, depending on sequence [125]. Still, PS-ODNs containing CpG-motifs are the most commonly used TLR9 agonist in experimental settings, due to its higher stability compared to PD ODNs. Two common ODNs are type A and type B ODNs. Type A CpG stimulates a high IFN-α response in pDCs, while type B CpG to a greater extent induces maturation [126]. The reason for this is explained by the ability of type A CpG to retain the endosomal compartment in an immature stage for an extended time and thereby prolonging the IRF7-dependent signaling, which takes place in early endosomes. Type B CpG, on the other hand, rapidly mediates endosomal acidification, and thereby maturation, which leads to proteolytic cleavage of TLR9 and the subsequent induction of pro-inflammatory cytokines [127].

1.2.1.2 C-type lectin receptors

C-type lectins are transmembrane proteins containing a C-type lectin-like domain, initially described in calcium-dependent carbohydrate-binding lectins, but later also found in proteins not binding carbohydrates in a calcium-dependent manner. This is a superfamily consisting of approximately one thousand members with assorted functions, such as adhesion and endocytoses. In mammals, 17 CLR subgroups have been identified, classified after their structure and phylogenetic relationships [128]. Subgroups II, V, and VI are expressed on myeloid cells, and these CLRs are receptors with the ability to bind, and in some cases, respond to DAMPs [129]. Common structures to be recognized are carbohydrates rich in mannose, fucose, and glycan, often found in microbial cell walls, but also in endogenous structures.

For several CLRs, the intracellular signaling pathways is not known, but several receptors have been shown to signal via immunoreceptor tyrosine based activation motif (ITAM), expressed either by the receptor itself or via adaptor molecules associated with the receptor [130]. When activated, ITAM is phosphorylated and spleen tyrosine kinase (Syk) is recruited. Upon binding, Syk mediates activation of down-stream transcription factors, such as NF-κB and AP-1 [131]. Engagement of CLRs often results in Th17 or Th1 responses [132, 133]. Some CLRs express an immunoreceptor tyrosine based inhibition motif (ITIM) with the ability to reduce responses from other PRRs. An example of this is DC immunoreceptor (DCIR), which acts to dampen TLR8-induced IL-12 and TNF-α production [134]. Even though several CLRs cannot initiate cell activation by themselves, they sometimes act in collaboration with additional PRRs. In contrast to DCIR, DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN), which is expressed on dermal and mucosal DCs,

acts to enhance the intracellular NF-κB activation and promote transcription of pro-inflammatory cytokines when activated in parallel with TLR8 during binding of a pathogen, even though it does not induce activation when triggered alone [135, 136].

In addition to activation, CLRs can also induce endocytosis when engaged, making them suitable targets for *in vivo* antigen delivery in vaccine settings [137]. Examples of targeted receptors are DEC-205, Dectin-1, and CLEC9A, which all are expressed on several DC subsets [95, 96, 98, 138] and $BDCA3^+$ DCs in particular [40, 139]. When triggered, DEC-205 has not been shown to have immunostimulatory functions *per se*, while Dectin-1 stimulation can indeed induce DC maturation without additional stimuli [132] and CLEC9A has been shown to mediate cross-presentation of endocytosed antigens, although without induction of DC maturation [100].

1.2.1.3 Cytoplasmic DNA sensors and RIG-I-like receptors

In contrast to TLRs, which selectively are expressed by defined cell types, most cells express RLRs. This is a group of DExD/H-box RNA helicases responding to viral double-stranded RNA (dsRNA) present in the cytosol, and so far three receptors have been described. Retinoic acid-inducible gene I (RIG-I) was the first receptor to be characterized in this group [140], quickly followed by the identification of two additional genes coding for DExD/H-box RNA helicases; melanoma differentiation associated factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [141]. Both RIG-I and MDA5 express a C-terminal domain, a DExD/H-box RNA helicase domain, and at their N-terminus, two caspase activation and recruitment domains (CARDs). The CARD domains are however missing in LGP2. A repressor domain is expressed in the C-terminal domain of RIG-I, which is missing in MDA5. Instead, LGP2 is equipped with one and is hence believed to be a regulator of MDA5.

The RLRs recognize a variety of dsRNA virus intermediates present in the cytosol. Flavi viruses, such as dengue virus and West Nile virus, are detected by both MDA5 and RIG-I [142, 143]. Examples of viruses detected by RIG-I are influenza virus and Epstein-Barr virus (EBV) [142, 144, 145], while picorna viruses are detected by MDA5 [145]. The receptors respond best to dsRNA that have blunt triphosphorylated 5′ ends, which in the absence of 5′ capping is a sign of non-self RNA [146]. Studies using the synthetic dsRNA analogue poly I:C show that MDA5 preferably recognizes high molecular weight poly I:C, while RIG-I responds to shorter sequences [147]. In addition to RNA, DNA can indirectly also be recognized by RLRs. The enzyme RNA polymerase III senses cytosolic DNA that is rich in A and T nucleotides, and subsequently transcribes it to 5′ triphosphate RNA, which is readily detected by RLRs [148, 149].

In its inactive form, RIG-I is found with its repressor domain bound to the CARD domain in a closed conformation [150]. Upon binding to a ligand, the repressor domain releases CARD, which then interacts with the adaptor protein interferon-β promoter stimulator 1 (IPS-1), located in the mitochondrial membrane. A signaling complex is formed, involving members of the NF-κB family and IRF3, which upon activation is translocated to the nucleus, where it initiates transcription of pro-inflammatory genes and type I IFNs, respectively [151].

The RLRs enable most cells and tissue to produce type I IFNs in response to cytosolic RNA, which additionally signals to the surrounding milieu to initiate an antiviral defence. Interferon-β binds to the IFN-α/β receptor in an autocrine or paracrine

manner and initiates the transcription of interferon-stimulated genes (ISGs), such as IFN-α, IRF7, and additional PRRs [142].

Cytosolic DNA is sensed in a similar manner by recently characterized cytosolic DNA sensors. These sensors have previously been described as components in various intracellular type I IFN inducing signaling pathways, but are now shown to bind directly and respond to transfected or viral dsDNA [152]. The two best characterized members in this family are absent in melanoma 2 (AIM2) and DNA-dependent activator of IFN-regulatory factors (DAI) [153-155].

1.2.1.4 Nucleotide-binding domain LRR-containing proteins

A growing family of cytosolic PRRs is the NLRs, with 22 members characterized so far. The NLRs are divided into four subgroups, depending on their structure [156]. The NLRs all express a nucleotide binding domain (NBD) and a LRR in their C-terminus. Additionally, they express various domains at their N-terminus, which divides them into the separate subgroups. The members in the NLRC-group express a CARD domain, which can interact directly with other functional proteins containing CARD domains. The NLRP-group contains a pyrin domain (PYD) that can interact with an adaptor protein consisting of a PYD and a CARD domain, which in turn connects the receptor with additional CARD-expressing effector proteins. The NLRB-group instead has a baculovirus inhibitory domain, and the NLRX group consist of proteins with a variety of N-terminuses that do not fit in the other groups. Among with two members in the dsDNA binding pyrin and HIN200 domain-containing protein (PYHIN) family, several, but not all, NLRs have the ability to form a large, multimeric structure called the inflammasome [157], which has the ability to cleave pro-caspases into their active form. Activation of caspase-1 can mediate inflammatory cell death and cleavage of pro-IL-1β and pro-IL-18 to their active inflammatory forms [158-160].

So far, no actual interaction between NLR and ligand has been demonstrated, and NLRs are not properly classified as receptors. However, several DAMPs have been shown to activate NLRs and inflammasome formation. It is hypothesized that NLRs are sensitive to changes in the cellular milieu [161]. Examples of inflammasome forming NLRs are NLRP3 and NLRC4, which are expressed in myeloid and hematopoietic cells, respectively [162, 163]. Generally, NLRP3 sense self-molecules like adenosine-5′-triphosphate (ATP), cholesterol crystals and monosodium urate (MSU) microcrystals if they are present in an erroneous compartment, such as extracellular ATP [164, 165]. Exogenous crystals and particles, such as asbestos and silica, can also induce NLRP3 activation, as well as the adjuvant Alum [165-167]. Microbial components have also been shown to activate the NLRP3 inflammasome, but often in combination with other NLRs, such as NLRC4. Additional structures that activate NLRC4 are the bacterial protein flagellin [168, 169] and the bacterial type III secretion system [170]. A receptor that mediates inflammasome formation upon recognition of dsDNA is the PYHIN family member AIM2 [153, 171], which is activated in cells infected with vaccinia virus and *Francisella tularensis*, but also in the presence of genomic dsDNA in the cytosol [153, 172].

1.2.2 Toll-like receptor 3

Toll-like receptor 3 recognizes dsRNA and resides in the endosomal compartment of cDCs and macrophages [107, 173-175], but can also be found in epithelial cells and on the surface of fibroblasts [176, 177]. Furthermore, TLR3 expression has also been detected in cells of the central nervous system (CNS) [178-180]. In accordance with the other TLRs, TLR3 is formed by a LRR domain at the N-terminus, a trans-membrane region and a cytoplasmic linker region that for TLR3 directs the protein to the endosomal compartment upon translation, and cytoplasmic TIR domain at the C-terminus. The LRR domain is shaped like a horse shoe and dsRNA binds to TLR3 in the acidic environment of endosomes by interaction with the N- and C-terminus ends of the LRR domain, which induces dimerization of two receptors with the ligand in between and the C-terminal end in the center (Figure 1) [181, 182]. The close interaction enables the two TIR domains to attract and activate TRIF [183-185], which in turn mediates activation of IRF3, NF-κB, and AP-1. This leads to DC maturation and production of pro-inflammatory cytokines and IFN-β. Under certain conditions, dsRNA-induced TRIF activation can additionally facilitate cell death via the activation of receptor interacting protein 1 (RIP1) [186, 187].

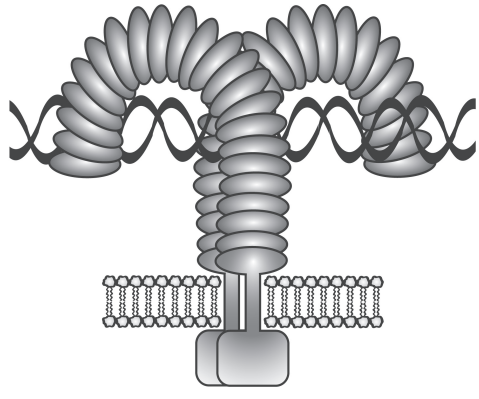

Figure 1: The structure of a TLR3 homodimer bound to its ligand. Adapted from [181].

1.2.2.1 Detrimental effects of TLR3 activation

Toll-like receptor 3 has a role in sensing viral infections. The receptor detects and mediates protective responses to viral genomes or their intermediates during replication, such as during coxsackievirus or murine cytomegalovirus (CMV) infections [188, 189]. Moreover, patients with loss-of-function mutations in *TLR3* or genes involved in TRIF signaling have an increased risk of acquiring herpes simplex encephalitis [190], indicating a protective role of TLR3 in CNS. However, patients with deficiencies in TLR3-mediated responses are surprisingly healthy during other viral infections [191], and in certain infections, a functional *TLR3* gene can actually be detrimental [192]. Indeed, immunopathogenic responses during viral infections have been attributed to TLR3 in several studies [193-198]. Phlebovirus has for example been shown to induce severe inflammation and liver damage in a TLR3-dependent fashion

[193], influenza A virus infection in the lungs mediates increased tissue damage and lethality in wild-type (wt) mice compared to TLR3$^{-/-}$ animals [194], and EBV infection leads to elevated levels of systemic viral dsRNA that mediate immunopathologic disease [195]. In addition, TLR3 detects and responds to RNA released from dying cells during sterile inflammation [107, 199-201], which can mediate pathogenic effect in the lungs following hyperoxia-induced cell death [201], increased inflammation in response to dying cells in rheumatoid arthritis [199], and increased risk for organ rejection during liver transplantations following hepatitis C virus (HCV)-related cirrhosis [200].

This implies that TLR3 might have an alternative function rather than initial sensing of primary infection. Toll-like receptor 3 is highly expressed in cells specialized in cross-presentation [40, 202, 203], and has indeed been shown to mediate antigen-specific responses during exposure to dying cells [39, 204]. Also, type I IFNs, which are induced by TLR3 activation, have been linked to a Th1 type of response and activation of antigen-specific CTLs [205, 206]. Hence, TLR3 might be important in shaping of adaptive immune responses.

1.2.3 Dangerous death

In immunology, it was long believed that immune responses were only initiated upon the recognition of non-self molecules. However, the realization that necrotic cells, dying a dramatic death with ruptured cellular membranes and nucleic DNA and cytosolic content shattered into the extracellular surroundings, mediate activation of DCs and have the ability to initiate adaptive immune responses towards accompanied antigens contradicted this theory [106]. Since then, several mechanisms to explain this have been proposed, and it is today accepted that endogenously produced molecules can have immunostimulatory effects.

1.2.3.1 Cell death

A cell can die in different ways, depending on location and stimuli, but the most common are apoptosis and necrosis [207]. In addition, pyroptosis has recently been described as an inflammatory type of cell death [207, 208].

Apoptosis is a programmed type of cell death involving activation of caspase 3 and typical morphological changes, such as chromatin condensation, nuclear fragmentation, and plasma membrane blebbing [209, 210]. During normal conditions, the apoptotic cell displays so called ‘eat me’ signals, which is recognized by surrounding cells and phagocytes rapidly engulf the dying cell [211]. But if not cleared, the apoptotic cell can not keep its membrane integrity and becomes secondary necrotic and hence immunostimulatory [212, 213]. Apoptosis is a natural phenomenon, constantly occurring and clearing billions of cells in our bodies every day, either due to extrinsic stimulus, like receptor mediated apoptosis, or intrinsic, for example in response to DNA damage. This was long considered to be a silent process, leaving no marks, but emerging evidence indicates that apoptosis is important in maintenance of self-tolerance [214-216]. When occurring in immune privileged sites, apoptosis induces tolerance, even to viral antigens [217], and in correlation to this, DCs phagocytosing antigen-loaded apoptotic cells are shown to induce tolerance, rather than immune activation [103]. Moreover, immunization with apoptotic cells from the donor prior to

organ transplantation increases survival of the graft without addition of any immune suppressants [104]. Apoptosis is not equivalent to tolerogenic responses though. Stressed tumor cells can when undergoing apoptosis induce an immunogenic response [218-222], and activated apoptotic PBMC and T cells have the ability to induce maturation of DCs *in vitro* and anti-viral responses in mice immunized with HIV-1 DNA *in vivo* [223-226]. Also, particular chemotherapeutics have been shown to induce expression of immunogenic find-me signals on the cellular surface of exposed tumors [227], enhancing the phagocytosis and activation of DCs. Furthermore, even though apoptotic cells have been shown to induce anti-inflammatory cytokines in macrophages [228], this response is overcome if the apoptotic cell has been exposed to infection [204, 229, 230].

Necrosis, on the other hand, occurs during tissue trauma and is an unregulated event where the cell membrane integrity is lost, and an influx of fluids due to the higher intracellular salt concentration mediates swelling of the cell and organelles, which culminates in the release of the intracellular content to the extracellular space [207]. This event does indeed induce inflammatory responses and DC maturation in a sterile environment [106, 231, 232], due to the release of various intracellular DAMPs [233].

In contrast to apoptosis, pyroptosis is mediated via caspase-1, but still results in lost membrane integrity and release of DAMPs [159, 207]. Pyroptosis is mediated via activation of the inflammasome and includes secretions of active IL-1β and IL-18 [157, 159, 160].

1.2.3.2 Endogenous DAMPs

Although necrosis has been known to induce inflammation since the signs of inflammation was first documented, its ability to prime adaptive responses has not been investigated until recently [106]. Mediators of inflammation that were characterized early on were HSPs, uric acid, and high-mobility group box 1 (HMGB1) protein. Stressed cells upregulate HSPs, to ensure correct folding of newly translated proteins, which during necrosis or treatment with certain chemotherapeutics are subsequently released [218, 233-235]. A suggested receptor mediating this effect is TLR4 [236]. Uric acid is normally present in high levels in the intracellular compartments, but is also part of the extracellular milieu. Upon necrosis, the intracellular content is released and the elevated concentration in the sodium rich tissue interstitium leads to formation of MSU crystals [237], which for example are found in high levels in patients suffering from gout [238], and have the ability to induce the NLRP3 inflammasome [164]. Additionally, 'find me' molecules secreted by apoptotic cells can also activate the inflammasome, such as ATP [165, 239]. Release of the nuclear chromatin binding protein HMGB1 induces activation of myeloid cells and mediates sterile inflammation [240, 241]. The protein is bound to the receptor for advanced glycan end products (RAGE) and is suggested to induce activation via TLR2 and TLR4 [242-244], but can also complex with self nucleic acids to promote activation via TLR9 [245].

Host-derived nucleic acids also induce signaling via additional nucleotide binding PRRs. Double-stranded DNA can activate the inflammasome via binding of AIM2 [153, 171], mitochondrial DNA has been shown to engage TLR9 [246], and both TLR7 and TLR9 have been implicated in systemic lupus erythematous (SLE), since pDCs are activated to produce IFN-α when cultured with sera from SLE patients [108, 109]. Binding of RNA or DNA by the antimicrobial peptide cathelicidin has also been shown

to activate these receptors [247, 248], while TLR3 in several studies has been shown to sense endogenously derived RNA [107, 199, 201, 249].

1.3 HIV

HIV (human immunodeficiency virus) was discovered in 1983 by Françoise Barré-Sinoussi and Luc Montagnier [250]. This was shown to be the causing agent of acquired immunodeficiency syndrome (AIDS) – an epidemic of opportunistic infections and rare cancers not controlled due to a severely weakened immune system. This syndrome was initially detected in young healthy men who had sex with men, but soon also in other groups [251]. Due to its reverse transcriptase (RT) activity, the virus was characterized as a retrovirus, and later more specifically classified as a lentivirus. Shortly after the discovery of HIV, an additional virus to cause AIDS was identified in two West African patients [252]. It had similar features as HIV, but differed by 55 % in sequence and was antigenically separate. Since these discoveries there are two viruses in the lentiviral group of human retroviruses – HIV-1 and HIV-2.

Today, the viruses are mainly transmitted sexually via heterosexual intercourse. Before a proper HIV screening methodology was set up, a large group of patients receiving infected blood products were also infected. Still, intravenous drug users transmit the infection by sharing infected needles. Approximately 34 million people are living with HIV today, and nearly 30 million have died from the infection by the end of 2010 [253]. The number of intervention strategies for controlling the epidemic has increased over the years. ABC – Abstinence, Being faithful, and Condoms – was the only prevention strategies until the first anti-retroviral drug was released in 1987. However, viral resistance to the drug soon became a problem. Combinational therapy with three or more anti-retroviral drugs was introduced in 1996, and it led to markedly increased life span of infected patients and decreased the transmission to sexual partners. Since then, several prevention strategies have been developed with varying degree of effectiveness, such as treatment as prevention, male circumcision, microbicides, and even an so far unlicensed semi-effective vaccine concept [254].

1.3.1 The life cycle of HIV-1

HIV-1 is a small, enveloped virus containing two ssRNA copies as its genome. It codes for nine genes, which translates to 15 separate proteins with distinct functions (Table 2). The three polyproteins Gag, Pol, and Env are proteolytically cleaved into smaller proteins. Gag and Env encodes for structural proteins, like the viral capsid and gp120, respectively, while Pol encodes for the enzymes necessary for HIV-1 infection [255].

1.3.1.1 The replication cycle

The primary host cells of HIV-1 are the $CD4^+$ expressing cells [259-261], such as T cells, DCs, and macrophages, and they can either be directly pruned to produce large quantities of new viral particles, or act as a latent reservoirs for the virus, to be reactivated much later instead. However, CD4 molecules are not sufficient for infection to occur. The cell membrane-bound CXC chemokine receptor 4 (CXCR4) or CC chemokine receptor 5 (CCR5) is also necessary for viral entry into the cell, and

depending on what co-receptor the virus isolate uses, it is defined as X4 or R5 tropic [262-266]. In addition, there are several CLRs expressed on DCs that have the ability to bind HIV-1, such as DC-SIGN, DCIR, and the mannose receptor. However, interaction of HIV-1 with CLRs is not strong enough to mediate viral penetration of the cell membrane and direct, *cis*-infection [267-269]. Instead, HIV-1 is endocytosed by DCs via binding of these CLRs. The virus has the ability to inhibit endosome maturation, and can in a time span of a few hours also facilitate release from the carrying cell [105, 270]. This is called *trans*-infection, and is considered to be an important route for the virus to get from the site of infection into the lymph nodes.

Upon entry into a host cell, HIV-1 first docks to CD4 with the gp120 part of Env. An interaction with several co-receptors mediates conformational changes in Env, and enables gp41 to penetrate the cell membrane and subsequently deliver the viral capsid into the cytoplasm [271]. In the cytosol, the HIV-1 genome and proteins exit the capsid. While still attached to the core proteins, the viral genome is transcribed into DNA by the viral RT. First, a ssDNA minus-strand is formed, which is used as a template for synthesis of the complementary strand of the viral dsDNA molecule. The dsDNA forms a pre-integration complex with the viral matrix protein, integrase and Vpr and is transported into the nucleus. In the nucleus, the integrase inserts the viral DNA into transcriptionally active regions of the host genome. The inserted viral sequence is referred to as a pro-virus [255, 257, 272].

HIV-1 infects HIV-1-specific $CD4^+$ T cells to a high extent due to their interaction with infected DCs that are presenting endocytosed HIV-1 antigens [273, 274]. This leads to a reduction of HIV-1-specific adaptive responses and further deteriorates the ability of the immune system to control the infection. HIV-1 is dependent on binding of activated NF-κB to its 5´ long terminal repeat (LTR) promoter to initiate transcription of the pro-virus [275-277], and this occurs when the T cell binds to an activated, antigen-specific APC [278]. The infection of DCs is, on the contrary, reduced upon cell

Gene	Protein/Designation	Function
gag	Matrix/p17	Structural protein
	Capsid/p24	Structural protein
	Nucleocapsid/p7	Protection of viral RNA genome
	p6	Viral assembly before budding
pol	Reverse transcriptase p66, p51	Transcription of viral ssRNA to viral dsDNA
	Protease/p10	Posttranslational processing of viral proteins in the immature virion
	Integrase/p32	Integration of viral DNA into the host genome
vif	Vif/p23	Protection of the viral genome from host restriction factors, virion assembly
vpr	Vpr/p15	Transportation of the viral genome to the nucleus
tat	Tat/p14	Pro-virus transcription
rev	Rev/p19	Regulation of viral mRNA processing
vpu	Vpu/p16	CD4 degradation, CD1d inhibition
env	Envelope surface protein/gp120	Binding of target receptors
	Envelope transmembrane protein/gp41	Penetration of cell membrane and mediation of viral entry
nef	Nef/p27	Regulation of host cell activation and survival, down-regulation of CD4 and HLA-A and HLA-B

Table 2: The genes, proteins, and protein functions of HIV-1. Adapted from [255-258].

activation [279, 280]. However, activation of NF-κB still has to occur for pro-viral transcription in immature cells. This is mediated via cooperative signaling from TLR8 and DC-SIGN, but without induction of DC maturation [136, 281]. The initial proteins to be transcribed from the pro-virus are the regulatory genes Tat, Rev, and Nef. These then regulate transcription of additional HIV-1 components and facilitate the assembly of the HIV-1 structural proteins. Finally, new ssRNA HIV-1 strands are formed and incorporated into new virion particles together with essential viral and host proteins. Before the virions bud off the cell, the host cell expression of CD4 is down-regulated, which prevent re-infection of the same cell [255, 257, 271, 282].

1.3.1.2 Routes of transmission

Viral particles and infected cells can be found in blood, breast milk, and genital fluids. Other body fluids, such as tears, urine, and sweat are not infectious. Hence, the main routs of viral transmission are sexual, mother-to-child, and via blood [283].

Sexual transmission is the most common route of infection, and the male-to-female transmission route is modelled in human *ex-vivo* explant models and *in vivo* in macaque studies of the infection with simian immunodeficiency virus (SIV) [284-288]. It is not clear whether HIV-1 is transmitted predominantly via infected cells or free viral particles [284], but most experimental studies have been performed with free virus. Known though is that a single founder virus most often initiates the HIV-1 infection, which successfully replicates and facilitates systemic spread [289]. After infection, the virus rapidly mutates and adapts to the new host [290]. At the mucosal site, transmission can occur either through intact epithelial layers or via small ruptures, which is not uncommon during normal intercourse [291]. The cells to first encounter the virus are likely DCs, in particular LCs, or T cells, and the initial viral replication can occur at the mucosal site, or, if the virus is captured in *trans*, in the adjacent lymph node. HIV-1 does not infect epithelial cells, and it is likely that the virus is transported over intact barriers via grabbing of LCs, which have the ability to stretch out their dendrites in between the epithelial cells [292], or via transcytosis through the epithelial cell layer [293]. The $CD4^+$ T cells, however, outnumber the DCs in mucosal tissue, which make them a more likely target during epithelial layer breakage [294]. Most mucosal $CD4^+$ T cells are in an unactivated state, but HIV-1 has been shown to infect this population as well, although to a lesser extent [295, 296]. Ongoing mucosal immune activation, such as during infection of other sexually transmitted diseases, is correlated with higher risk of HIV-1 transmission [297], suggesting that the first cells to be infected in the mucosa indeed are the activated T cells.

Mother-to-child transmission of HIV-1 either occurs during birth, when the child swallows viral particles present in vaginal fluids and blood, or through breast-feeding, since HIV-1 is secreted into the breast milk. The initial cells to be infected in the new born child are probably located in the tonsils or gingiva, but within the first hours to days after birth, HIV-1 might survive the route through the intestines and infect cells in the small intestine [298]. HIV-1 transmission does not normally occur during oral sex, since anti-viral factors are present in the human saliva [299].

If HIV-1 is introduced directly into the tissue, penetrating the epithelial barriers via needles for example, the infection is more commonly initiated by several founder viruses [300]. This could theoretically be both detrimental and beneficial for the host. A greater genetic variation of transmitted viruses would enhance the risk for immune

escape, while the founder viruses at the same time might be less resistant to the host antiviral restriction strategies than the clones managing to penetrate mucosal barriers and thereby less potent to initiate a swift immune escape.

1.3.2 Host restriction factors

Physical barriers, such as mucus and epithelial layers, are the first obstacles HIV-1 needs to overcome during sexual transmission. In addition to this, anti-viral substances and chemokines blocking the co-receptors for HIV-1 are secreted, which further prevent infection from occurring [298]. However, if cellular infection does take place, the outcome of the infection is highly dependent on the initial restriction that limits the viral spread. Indeed, if a cell is infected with a virus unable to escape the host restriction mechanisms, it is likely that the infection will not disseminate.

Cells have evolved several defence strategies to cope with retroviral infections. Factors that were early characterized to restrict HIV-1 infection are the ISGs apolipoprotein B mRNA-editing enzyme-catalytic polypeptide-like (APOBEC) 3 molecules, tripartite motif (TRIM) 5α, and tetherin [301-303]. Recently, several additional factors involved in restricting the replication of HIV-1 have been identified, such as SAM domain and HD domain-containing protein (SAMHD) 1 and zinc-finger antiviral protein (ZAP) [304-306]. Furthermore, large, genomic screenings utilizing small interfering RNA (siRNA) techniques have identified a great number of additional factors involved in HIV-1 restriction [307]. Further evaluation of these factors will provide extensive understanding on how HIV-1 replication is inhibited, and perhaps overcome, *in vivo*.

The underlying antiviral mechanism of TRIM5α is its ability to mediate pre-mature un-coating of the viral capsid when HIV-1 enters a cell, which interferes with the RT function and inhibits viral replication. It seems not to be highly significant in the restriction of HIV-1 in humans, even though a certain mutation in the *TRIM5* gene has been proposed to be protective against HIV-1 acquisition [308, 309]. In non-human primates though, TRIM5α plays an important role in restricting simian immunodeficiency virus [310]. The newly identified restriction factor SAMHD1 also inhibits HIV-1 infection on a pre-integration level. A recent study shows that SAMDH1 regulates the intracellular pool of deoxynucleoside 5′-triphosphates (dNTPs) [311]. By reducing the levels of dNTP, SAMHD1 prevents RT to properly replicate the viral genome. Acting to restrict HIV-1 on a post-integration level, ZAP rather detects transcribed viral RNA and marks it for degradation. Tetherin acts late in the viral life cycle and restricts the release of HIV-1 and other enveloped viruses by tethering them to the cellular surface and preventing the budding of new viral particles [312, 313]. The viral protein Vpu however marks tetherin for intracellular degradation, thereby counteracting its effect.

1.3.2.1 The APOBEC3 family

One of the first restriction factors for HIV-1 to be characterized was APOBEC3G [301]. It belongs to a family of DNA deaminating enzymes shown to be important restriction factors for the second round of infection by HIV-1. During the assembly of new virus particles in the host cell, APOBEC3G molecules are incorporated and subsequently accompany the virions to the next cell to be infected. There, APOBEC3G

induces hypermutations in the viral genome by deaminating cytosines to become uraciles during RT synthesis of the viral DNA minus-strand. This facilitates incorporation of adenine on the DNA plus-strand and consequently introduction of G-to-A mutations in a GG-context in the incorporated provirus [314-317]. Mutated viral DNA containing uraciles, which normally only are found in RNA, is recognized and degraded by the host cell, preventing integration to occur [318]. If integration still does take place, the mutated sequence gives rise to truncated transcripts and translated proteins with a high degree of errors [319]. It has been speculated that this increased mutation rate could possibly contribute to the high genetic variability utilized by HIV-1 to escape adaptive immune recognition, but a recent study concludes that the APOBEC3G-induced mutations are too prejudicial for the virus and likely result in a dysfunctional pro-virus [320]. Another proposed mechanism of APOBEC3G is an early inhibition of reverse transcription by reducing the presence of the primer needed for initiation of the transcription [321]. The HIV-1 protein Vif counteracts these events by targeting APOBEC3G for degradation via hijacking of the host's ubiquitin-proteasome pathway, and thereby preventing the enzyme from being incorporated into new virions [322].

In addition to APOBEC3G, other APOBEC3 molecules also have been ascribed antiviral activities. For instance, APOBEC3B and F have strong antiviral effects [323-325], while APOBEC3C and D/E are weaker in their ability to insert mutations, but do affect HIV-1 transcription [326, 327]. For long, APOBEC3A was considered only to display weak antiviral properties, but this molecule has recently been shown to be highly active in myeloid cells, such as DCs [328], and it is also less sensitive to Vif-mediated degradation [329], thus making it an important player in the host restriction against HIV-1.

1.3.3 The type I interferon response

In 1957, Alick Isaacs and Jean Lindenmann identified the causing factor behind a phenomenon called viral interference, which had been observed in both animals and plants. It dates back to Edward Jenners observation in 1804 that formation of vaccinia virus lesions was prevented in patients with active herpes infection. The factor was hence called interferon [330], and is today known as the type I IFN family [331]. The type I IFN family consists of 17 members, of which 13 are partially homologous variants of IFN-α, while separate genes encode IFN-β, -ε, -κ, and -ω. They all bind to the IFNα/β receptor, but with varying affinity. The IFNα/β receptor can be found on virtually all cell types, but the response following engagement varies.

1.3.3.1 Induction of type I IFNs

Recognition of viral and microbial, but also self, nucleic acids by TLRs, RLRs, NLRs, and cytoplasmic DNA sensors initiates an interferon response [118, 122, 151, 155, 183, 199, 332-334]. Toll-like receptor 7 and 9 have in pDCs the ability to induce high levels of IFN-α release via the MyD88 pathway due to the constitutive expression of IRF7 [123]. However, remaining type I IFN response-inducing PRRs are dependent on the activation of IRF3, which facilitates production of IFN-β and IFN-α4 [335]. When released, these cytokines bind to the IFN-α/β receptor and stimulate an auto- and paracrine production of additional type I IFNs.

Triggering of the IFN-α/β receptor mediates activation of members of the signal transducer and activator of transcription (STAT) family [336]. Activated STAT1 and STAT2 form a heterodimer that subsequently binds IRF9 and forms a complex called IFN-stimulated gene factor 3 (ISGF3). This complex translocates into the nucleus, where it initiates transcription of various ISGs [337]. Furthermore, STATs can form homodimers and activate additional genes, which are mediating both pro- and anti-inflammatory responses [331].

1.3.3.2 Effector mechanisms for type I IFNs

The interferon response is most prominent during viral infections. In response to infection, the targeted cell secretes type I IFNs, which instruct surrounding cells to prevent production of viral proteins by shutting down RNA translation. Interferon-stimulation also facilitates detection and degradation of viral genomes or their intermediates by activated intracellular RNases, and a hostile intracellular milieu is created by elevated production of nitric oxide [338]. Moreover, antigen processing and presentation is enhanced, with elevated MHC class I expression and enhanced peptide loading [339], and cells are sensitized to receptor-mediated apoptosis [340]. HIV-1 host restriction factors APOBEC3, TRIM5α, and tetherin are other examples of ISGs upregulated by interferon signaling [301-303]. An additional effect of type I IFNs is activation of NK cells, which have the ability to recognize and kill cancer cells or virally infected cells [341, 342].

Type I IFNs can also be beneficial against intracellular bacterial infections, for example during *Chlamydia* infection [343, 344], but more common is that type I IFNs obstruct the immune defence during extracellular infections. Type I IFNs have been shown to be detrimental during *Francisella tularensis* and *Listeria monocytogenes* infections [345-347], and it has been shown that virulent bacterial strains induce higher type I IFN responses than less virulent strains [348]. A type I IFN response can during bacterial infection increase susceptibility to apoptosis [347], or facilitates recruitment of inadequate type of immune cells to the site of infection. This can be seen following influenza infection, when the recruited monocyte-derived DCs mediate immune pathology and the lack of neutrophil infiltration enables elevated bacterial growth [349, 350]. In addition, type I IFNs favor Th1 responses, but suppress IL-17 secretion and the formation of adaptive Th17 responses [346, 351].

The main regulator of Th1 responses is IL-12 and its activation of STAT4 followed by the subsequent activation of the transcription factor T-bet [86, 352]. In addition, type I IFNs also induce STAT4 activation, hence shifting the adaptive response in the Th1 direction, but not as strongly as IL-12 [353]. However, in the combination with additional Th1 promoting cytokines, such as IL-18, type I IFNs are able to induce sufficient STAT4 activation for a robust Th1 response [354]. Type I IFNs can also inhibit IL-4-driven Th2 responses, possibly via STAT2-mediated retention of STAT6 in the cytosol after activation [355, 356], and reduce Th17 development [346, 351, 357]. In addition, cross-presentation of antigens to $CD8^+$ T cells are enhanced by type I IFNs [339, 358], and a central memory phenotype of CTL development is enhanced by direct type I IFN-stimulation of the $CD8^+$ T cells [359, 360].

1.3.3.3 Type I IFNs during HIV-1 infection

The role of type I IFNs in HIV-1 infection is contradictory. High levels of IFNs are detected early in acute HIV-1 infection, indicating that the virus is readily recognized by the innate immune mechanisms [294, 361]. However, even though type I IFNs have anti-viral effects on most non-hematopoietic cells, they also act to activate immune cells, like DCs [59]. In the case of HIV-1, which facilitates activated immune cells to disseminate, a strong type I IFN response can in this setting rather become detrimental to the host. High systemic levels of type I IFNs produced by pDCs sensitizes $CD4^+$ T cells for activation-induced cell death, which contributes to a decrease also in the number of bystander, non-infected cells [340]. Furthermore, a difference in type I IFN-response is observed between sooty mangabeys, a natural host of SIV with high viral levels but low type I IFN levels and virus-induced pathogenesis, and rhesus macaques, which are not a natural reservoir for the virus and do develop AIDS [362]. Nonetheless, IFN-treatment of HCV patients co-infected with HIV-1 results in a reduced viral load [363], and a similar approach in HIV-1 patients indicates that IFN-α treatment indeed reduces viral load in HIV-1 patients [364]. In addition, elevated levels of IFN-α in the vaginal mucosa of uninfected commercial sex workers seems to be protective from acquiring HIV-1 infection [365]. The production of type I IFNs is reduced during established HIV-1 infection [366], denoting that HIV-1 acts to reduce release of the cytokine. Indeed, IFN-α treatment upregulates the expression of the endogenous restriction factors APOBEC3G and F and tetherin in HCV/HIV-1 co-infected patients [367], and the disease progression would most likely be much swifter without the protective effect of type I IFNs.

2 AIMS OF THE THESIS

The general aim of this thesis was to investigate the monocyte-derived DC responses to DAMPs, such as TLR ligands, apoptotic cells, and IFN-α, and to characterize how this can modulate HIV-1 infection. More precisely, the aims were:

- To investigate the effect of combination of different TLR ligands on DCs
- To determine the mechanism behind the DC-stimulatory effect of activated apoptotic PBMC
- To determine the mechanism behind activated apoptotic cell-induced HIV-1-resistance in DCs
- To investigate the effect of IFN-α on DCs in relation to HIV-1 infection

3 RESULTS AND DISCUSSION

3.1 MODULATING DENDRITIC CELL RESPONSES

Since the discovery of DCs and PRRs, the field of immunotherapy has shifted from empirical to strategic. The knowledge about DC function and PRR expression has enabled specific vaccine adjuvants to be developed [368, 369], and novel routes of antigen delivery to be explored [89-91, 95, 100]. By this day, a therapeutic cancer vaccine based on the patients own APCs loaded with tumor antigen *ex vivo* is licensed for treatment of prostatic cancer [370], and other projects are in development [371]. However, the requirements for therapeutic induction of effective adaptive immune responses by activated DCs are still not fully understood, and further research is needed to achieve better fine-tuning of immunomodulatory approaches.

Our immune system clears and protects us from harmful threats every day, elegantly balancing between immunopathology and immune escape. But occasionally, a pathogen evades the immune defence and mediates destructive damage, or the immune system breaks tolerance and turns on self-structures in a chronic inflammatory responses. Will it be possible for us to effectively direct the immune system towards the pathogen, or repair the broken tolerance against self? And where do we start? With a central role in cross-talk between the innate and adaptive immune systems, the dendritic cell is a promising target.

3.1.1 Inhibiting dendritic cell activation (Paper I)

To fight an infection, the innate immune system most often inflicts harm on cells of the host as well. This is partly to contain the infection and prevent it from disseminating systemically, by facilitating the scorched earth policy and the killing of all potential nearby targets [372], partly to clear infected or transformed cells from transferring the disease. We normally identify inflammation as red, swollen, warm, painful, and distressed local tissue or as a systemic fever response. In severe infections, the damage to the tissue can be so advanced that its function is attenuated after clearance of the infection. This kind of response can however also occur in low-pathogenic infections, where the immune response itself is the damage-causing agent. In addition, responses to harmless non-self antigens, such as allergens or the normal flora, are misdirected immune reactions that can inflict tissue damage. In autoimmunity, the immune system attacks self-structures without the presence of a triggering pathogen, although the primary initiation of such responses might be pathogen-driven.

Treatments of allergy and autoimmunity are often focused on dampening of mediators that induce the symptoms, such as the release of histamine or pro-inflammatory cytokines, but not on the initial triggering of the immune response. When studying immune regulation, several innate mechanisms for inhibition of immune activation have been described. Engagement of TLR2 has been shown to inhibit TLR3-mediated responses to dying cells in wounded skin, and NLR, TLR2, and TLR4 signaling is associated with decreased inflammation and increased tissue regeneration following damage to the epithelia in the gut [373-375]. Indeed, by identifying the pathways involved in immune pathogenesis, they can be directly targeted to prevent the

release of pro-inflammatory mediators in the first place. An example of this strategy is the development of inhibitors targeting TLR7 to prevent type I IFN-driven immune exhaustion during HIV-1 infection [376]. Also, in autoimmune settings like SLE or psoriasis, the pathogenesis is partly mediated by recognition and responses to self-DNA and RNA and an enhanced IFN-α response, and specific inhibitors for TLR7 and 9 are being developed to dampen this pathway [108, 109, 377, 378]. Similarly, in conditions such as rheumatoid arthritis, necrotic cells act to stimulate TLR3 on fibroblasts, which leads to the release of inflammatory mediators and enhanced inflammation [199], and in lupus nephritis, expression of TLR3 is increased on mesangial cells and APCs and is believed to aggravate the condition [379].

3.1.1.1 Inhibition of TLR3-mediated cell activation

Toll-like receptor 3 is, as discussed in chapter 1.2.2.1 of this thesis, involved in several immunopathogenic settings, both in sterile inflammation [199-201, 249, 379, 380], and during infections [192-198]. Inhibition of TLR3 could possibly have an ameliorating effect in these conditions and several approaches to block TLR3 signaling have been attempted [381-384]. However, the difference between TLR7/9 inhibitors and TLR3 inhibitors is that while TLR7 and 9 are sequence-specific in their binding of synthetic ligands, TLR3 seems to respond to dsRNA regardless of sequence. Approaches to find specific RNA aptamers with high binding affinity to the TLR3 RNA binding sites in the LRR domain of the receptor have identified sequences with strong affinity to purified TLR3 ectodomains, but with no effect on TLR3 expressing cells [383]. In addition, screenings of small molecule inhibitors have identified compounds with the ability to reduce TLR3 activity, but they have so far only been tested on murine cell lines *in vitro* and their effect in the human system is not known [382]. A blocking antibody targeting murine TLR3 has also been developed [381], but the efficiency of this approach has not yet been tested on human TLR3. In addition, the ability to inhibit endosomally located TLR3 with an antibody might be troublesome

In **paper I** of this thesis, we have characterized ssDNA-ODNs as a potential inhibitor of TLR3-mediated monocyte-derived DC activation. **Paper I** started as an unexpected finding when the combined effect of ligands to different nucleic acid-sensing TLRs on DCs was tested. Although most studies on TLR expression in humans conclude that TLR9 is mainly expressed on B cells and pDCs, there are some reports on functional TLR9 expression in monocyte-derived DCs [385], which made us investigate the effect of a TLR9 ligand in our monocyte-derived DC cultures. Ligands used were for TLR3 the synthetic dsRNA analogue poly I:C, for TLR7/8 the chemical imidazoquinoline compound R848, and for TLR9 a synthetic type B ssCpG-ODN. As a positive control for DC activation, the TLR4 ligand lipopolysaccharide (LPS) was used, while untreated DCs in cell culture medium were used as negative control. Upon activation, DCs upregulate the maturation markers CD80 and CD86, which are important in transmitting signal 2 to the T cell during antigen presentation [83]. On LPS-treated DCs, close to 100% of $CD1a^+$ DCs expressed these markers, as measured by flow cytometry, and a combination with additional TLR ligands did not enhance this expression. However, the combination of R848 and poly I:C had an additive effect on the CD80 and CD86 expression, while ssCpG-ODN did not affect the response to R848 and did not induce any upregulation of maturation markers *per se*. Surprisingly, the increased expression of maturation markers following poly I:C treatment was

diminished in the presence of ssCpG-ODN, as exemplified in Figure 2. The ssCpG-ODN-mediated inhibition could be titrated down in a dose-response manner. Furthermore, the release of all cytokines and chemokines secreted upon poly I:C stimulation of DCs was inhibited by ssCpG-ODNs, and poly I:C-mediated activation of IRF3 was lost in the presence of ssCpG-ODN.

To confirm that poly I:C mediated its effect via TLR3, and not cytosolic RLRs, we pre-treated DCs with chloroquine before the addition of poly I:C. Chloroquine reduces the acidification of the endosomal compartment, and thereby inhibits signaling from endosomally located TLR3, which needs an acidic environment to properly bind its ligand and initiate down-stream signaling [182, 386]. Cytosolic RNA sensors, such as RLRs, are however not affected by chloroquine. Poly I:C-mediated DC activation was significantly reduced in the presence of chloroquine, which indicates that poly I:C acts via TLR3 and that ssCpG-ODNs have the ability to inhibit TLR3-mediated DC activation.

Single-stranded CpG-ODNs have previously been shown to inhibit spontaneous secretion of IL-8 in *in vitro* cultures of human skin keratinocytes [387]. Moreover, while addition of ssCpG-ODNs to CMV-infected human fibroblasts boosted the viral replication, simultaneous addition of the ligand and the virus actually inhibited infection of the cells [388]. Infection with CMV induces upregulation of TLR9 in human fibroblasts, and addition of a TLR9 ligand activates intracellular signaling routes also facilitated by the virus, which could possibly explain the enhanced viral replication. Similarly, infection of HeLa cells with herpes simplex virus type 2 (HSV-2) has been shown to be reduced in the presence of ODNs [389]. We therefore investigated the inhibitory effect of ssCpG-ODNs also on human keratinocytes, fibroblasts, and epithelial cells. The reported expression of TLR3 and 9 on these cell types varies, but in a resting state, we detected expression of TLR3, but not of TLR9, mRNA in all cell types. Hence, poly I:C-stimulated production of IL-8 was inhibited by ssCpG-ODNs in both keratinocytes and fibroblasts. In the epithelial cells, however, the background levels of IL-8 were too high to detect a significant effect of poly I:C, and

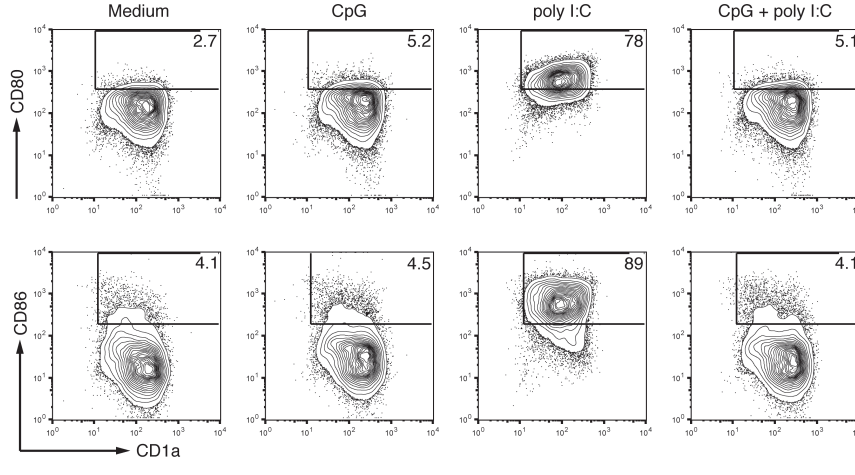

Figure 2: The poly I:C-induced expression of maturation markers CD80 and CD86 on monocyte-derived DCs is inhibited in the presence of ssCpG-ODNs.

ssCpG-ODN did not reduce the IL-8 levels. In these cultures, TNF-α secretion was instead measured, which indeed was upregulated by poly I:C and inhibited by ssCpG-ODNs.

In previous studies, the inhibitory effect of ODNs was shown to be independent of the CpG-motif [387, 389], which is in line with our observations. The inhibitory effect was equally efficient when culturing DCs with poly I:C and a non-CpG-ODN as with the ssCpG-ODN, indicating that the CpG-motif was not necessary for inhibition of poly I:C-mediated cell activation. In extension to this, the importance of the ODN backbone was investigated. Oligodeoxynucleotides based on a synthetic PS backbone were compared with PD-ODNs. The inhibitory effect of ODNs was reduced with the construct based on a PD backbone. In relation to that, when comparing different classes of CpG molecules, the PD-based type A ssCpG-ODN did not inhibit poly I:C-mediated DC activation, while the PS-based type C ssCpG-ODN did. This again is consistent with previous observation of ODN-mediated inhibition of IL-8 release and HSV-2 infection [387, 389].

3.1.1.2 The underlying mechanism of the inhibitory effect of PS-ODNs

The mechanism of these observations is not fully elucidated, and several hypotheses on where in the TLR3 signaling process the inhibition occurs can be made. First, we examined the possibility of complex formation between the two ligands. Poly I:C and ssCpG-ODNs differ greatly in length, and pre-mixing and subsequent separation of the substances via polyacrylamide gel electrophoresis did not reveal any obvious ligand-ligand interaction. As a confirmation of this, the effect of ssCpG-ODNs on TLR9-expressing B cells was investigated. As expected, ssCpG-ODN-treated B cells responded to the stimulation via proliferation, and addition of poly I:C did not affect this response. Poly I:C itself, on the other hand, did not induce any proliferative response in B cells, which was expected since expression of TLR3 was not detected in these cells.

Alternatively, the intracellular signaling pathway activated by TLR3 might be inhibited by ssCpG-ODN engagement of an unidentified inhibitory receptor. In addition to TLR3, TLR4 also has the capacity to signal via the TRIF-mediated pathway, and the TLR4 ligand monophosphoryl lipid A (MPLA) has been shown to preferentially activate TRIF over MyD88 when engaging the receptor [119]. However, ssCpG-ODNs did not have any inhibitory effect on MPLA-treated DCs, indicating that the TRIF signaling pathway was not affected by PS-ODNs.

Next, inhibition of the TLR3 receptor engagement was investigated. We observed that addition of PS-ODNs simultaneously or prior to poly I:C was required for full inhibition to occur, and since the TRIF signaling pathway was not affected directly, this indicates that the inhibition occurs on an upstream receptor level. To investigate whether PS-ODNs reduced the transiently enhanced endocytic capacity that a stimulatory reagent normally induces in DCs [62], the uptake of dextran beads was measured in the presence of poly I:C, ssCpG-ODN, or a combination of the two. Surprisingly, rather the opposite effect was observed. Even though ssCpG-ODNs do not induce activation of DCs, they still stimulated dextran bead uptake in levels comparable with poly I:C-induced endocytosis. Also, a combination of the two ligands did not alter the level of endocytosis as compared to cells treated with either poly I:C or ssCpG-ODNs alone. This implies that it is not primarily the stimulation via an

endosomally expressed TLR that enhances initial endocytosis, but rather the engagement of endocytic receptors. It is not fully characterized how poly I:C and ssCpG-ODNs are taken up into cells, but they are all endocytosed via a clathrin-dependent mechanism and poly I:C and type B and C, but not type A, ssCpG-ODNs are dependent on the cytoplasmic lipid raft protein Raftlin [390-393]. Since increasing the concentration of poly I:C up to ten times higher than previously tested in this paper still did not induce any upregulation of CD80 and CD86 on DCs in the presence of ssCpG-ODN, the uptake of fluorescently labeled poly I:C was investigated in DCs. Indeed, in combination with ssCpG-ODNs, the uptake of poly I:C was markedly reduced as compared to cells treated with poly I:C only (Figure 3). Hence, PS-ODNs act inhibitory on poly I:C by preventing the ligand from being taken up and thereby from coming in contact with its receptor TLR3.

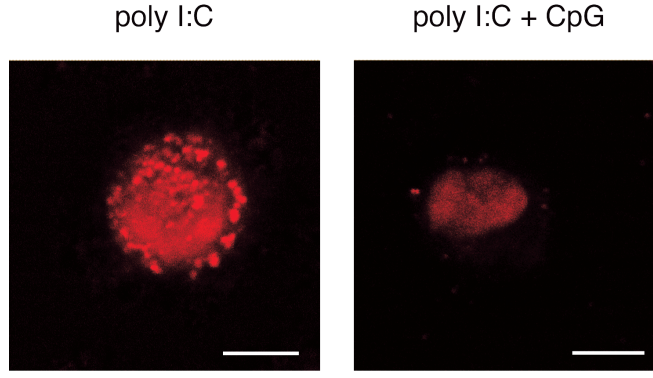

Figure 3: The DC uptake of fluorescently labelled poly I:C in the presence and absence of ssCpG-ODN is detected with confocal microscopy.

Still, the precise receptors mediating the uptake of ODNs and poly I:C are not yet known, but one could speculate that this route is also facilitated by certain viruses, such as previously discussed CMV or HSV-2. Conformably with our conclusion, the PS-ODN-mediated inhibition of infection is indeed occurring on the entry level [394, 395], and this would also explain why later addition of ssCpG-ODNs did not rescue fibroblasts from productive CMV infection [388]. Further exploration of the uptake mechanism in both virus infection and TLR ligand uptake will provide valuable information on how viruses uses uptake routes of the host and possibly the development of novel strategies to prevent infection.

3.1.1.3 Non-CpG-ODNs have an inhibitory effect on poly I:C in vivo

To further evaluate the significance of PS-ODN-mediated poly I:C inhibition, we treated C57Bl6 mice intraperitoneally with poly I:C in the presence or absence of non-CpG-ODNs, and then assessed the levels of pro-inflammatory cytokines in the serum. Surprisingly, we could not detect any inhibitory effects of the PS-ODNs in this model. Similar results were obtained from bronchialveolar lavages after intranasal administration of the ligands. Although not in line with our human *in vitro* observations, the results coincides with several reports on synergistic effects of ssCpG-

ODNs and poly I:C in the murine system [396, 397]. Though, while the uptake of poly I:C in human cells are dependent on raftlin, the homologue raftlin-2 is expressed in the murine system and can mediate poly I:C uptake even in the absence of raftlin [393]. Whether the uptake of PS-ODNs also can be mediated via raftlin-2 or not is not known, but our results indicate that the protein at least not preferentially binds to PS-ODNs, since poly I:C retains its ability to stimulate a response in mice.

Instead, the response to intranasal exposure of poly I:C and non-CpG-ODNs was tested in non-human primates. Local production of cytokines and chemokines was measured in nasal secretions from cynomolgus macaques treated with poly I:C, non-CpG-ODNs, or the combination of the two. Compared with the effects of non-CpG-ODN-treatment, a pro-inflammatory response was indeed induced following poly I:C administration. Furthermore, a reduced response was detected in the animals receiving the combination of poly I:C and non-CpG-ODNs, indicating that PS-ODNs have an inhibitory effect *in vivo* in non-human primates.

These results describe a novel mechanism for inhibition of innate immune responses to dsRNA *in vivo*. We have not confirmed that it in fact is TLR3 that mediates the *in vivo* responses to poly I:C, but considering the *in vitro* experiments involving DCs, fibroblasts and epithelial cells, it is a plausible route. In addition, other dsRNA recognition receptors are located in the cytosol and are primarily engaged by viral intermediates or poly I:C that has to be transfected into the cell to have an effect [142, 146]. Type B ssCpG-ODNs have been developed in clinic-grade settings, and are used as adjuvants in a number of clinical studies [369]. The production of clinic-grade non-CpG-ODNs would therefore be feasible and development of the compound as an immunomodulatory substance would indeed be possible.

3.1.2 Activated apoptotic cell-induced dendritic cell maturation (Paper II – III)

In several reports regarding endogenous activation of DCs, the causing agent is dying cells. As discussed in the previous chapter, the effect of extensive cell death can be detrimental to the host [194, 201, 212], and defect clearance of apoptotic cells has been implicated in several autoimmune diseases [199, 398, 399]. However, cell death also takes part in reducing the dissemination of infectious agents and to signal danger to surrounding tissue when the homeostasis is disturbed [372], as well as maintaining self tolerance [104, 215, 216]. In addition, dying cells can act as endogenous adjuvants, both in inducing immunity against foreign antigens [106, 231], infectious antigens [223, 229, 230, 400-402], as well as to dying tumor cells [218-220]. During necrosis, immunostimulatory DAMPs are released and facilitate activation of nearby cells, whereas apoptosis in the absence of exogenous stimulus is not considered to be immunogenic. Previous studies from our lab have however suggested that activation of peripheral blood mononuclear cells (PBMCs) prior to apoptosis induction enables DCs to mature upon apoptotic cell (AC) co-culture [224, 225]. However, the underlying mechanism for this was not described, and in **paper II**, we have further characterized the receptors involved and intracellular signaling events in DCs following exposure to activated ACs. Moreover, we have in **paper III** investigated the effect of infected and uninfected activated apoptotic $CD4^+$ T cells on DCs and further studied the antiviral response induced.

3.1.2.1 The role of secreted factors in activated AC-induced DC maturation

The ability of activated apoptotic PBMCs to induce monocyte-derived DC maturation has been shown previously [224, 225]. Even though we in present studies do not always investigate all parameters needed for the definition of DC maturation [83], we refer to this initial characterization of activated AC-induced DC maturation and from here on describe DCs with upregulated CD80- or CD86-expression as mature in order to prevent unnecessary confusion during the discussions involving both activated ACs and activated DCs.

The methods used to induce PBMC activation are either activating antibodies against CD3 and CD28, which is the T cell receptor and its co-receptor, respectively, or phytohaemagglutinin (PHA) treatment. Both methods resulted in upregulation of activation markers CD25 and CD69 on T cells with subsequent maturation of DCs when co-cultured. In the presence of activated ACs, DCs matured and secreted pro-inflammatory cytokines and chemokines, and also had the ability to induce strong proliferation *in vitro* in autologous T cells in a system involving presentation of alloantigens. To induce apoptosis in PBMCs or T cells, they were subjected to 150 Gy γ-radiation, which rendered approximately 90% of the cells to be apoptotic or late necrotic 24 h post irradiation. Resting cells from the same donors, treated exactly the same way with exception from the activation step, did not induce any DC maturation. Also, when comparing the effect of ACs with freeze-thawed necrotic cells, activated ACs were more efficient in inducing DC maturation than activated necrotic cells. Neither necrotic nor apoptotic resting cells induced upregulation of maturation markers on DCs [224].

Both strategies used to activate PBMC were directed against the T cells, either via direct targeting of the T cell receptor complex or using the T cell mitogen PHA. In **paper III**, we investigated the effect of activated apoptotic T cells on DCs. First, $CD4^+$ T cells were enriched and activated with antibody stimulation of CD3 and CD28. T cell activation and subsequent DC maturation following T cell irradiation and co-culture was thereafter measured. The activation of the purified T cells, as determined by expression of activation markers CD25 and CD69, was generally slightly lower for enriched T cells than in the PBMC cultures, and we observed that the level of activation seemed to be important for subsequent DC maturation (unpublished observations). However, sufficiently activated apoptotic $CD4^+$ T cells induced similar levels of DC maturation as activated apoptotic PBMCs.

To further characterize the mechanism behind activated AC-induced DC maturation, we cultured activated and irradiated PBMCs for 24 h and then separated the culture into a cellular and supernatant fraction and treated DCs with these entities (**paper II**). To avoid losing some effect due to potential degradation of secreted products, we in additional experiments cultured newly irradiated ACs in the top chamber of a trans-well system. This prevented DCs in the lower chamber from having physical contact with the dying cells, but enabled exposure of factors released from the ACs. Both fractions indeed induced upregulation of maturation markers on DCs, although not as prominently as unseparated ACs. The cellular fraction induced a stronger response than the supernatant fraction. However, only the cellular fraction from activated apoptotic $CD4^+$ T cells induced DC maturation in this setting (unpublished observations).

Activated apoptotic PBMCs have previously been shown to release low amounts of TNF-α [224], but this could not be detected in cultures with activated apoptotic $CD4^+$ T

cells (**paper III**). Since DCs can be activated by TNF-α [57, 66, 403], we investigated the role of this cytokine in the supernatant fraction of the activated ACs (**paper II**). Prior addition to DCs, the supernatant fraction from activated AC was treated with a neutralizing antibody against TNF-α. This almost completely abrogated the supernatant maturation stimuli, indicating that activated apoptotic PBMCs partly mediate its maturing effect on DCs via secretion of TNF-α. The lack of TNF-α secretion from activated apoptotic $CD4^+$ T cell cultures could perhaps also explain why these ACs were sometimes less potent in their DC maturing ability. We have not further investigated the cellular source of TNF-α, but the monocytes present in the PBMC population are likely to produce this cytokine. These cells adhere to the plastic in the cell culture flask during activation, and this has previously been shown to stimulate release of TNF-α, IL-1, and other pro-inflammatory factors [404, 405]. However, DCs treated with either activated apoptotic PBMC or $CD4^+$ T cells were also shown to produce high levels of TNF-α. To investigate whether this production had any paracrine effect on DC maturation, we collected conditioned medium (CM) from DCs co-cultured with activated ACs for 24 h. The CM was thereafter pre-incubated either with a control antibody or the TNF-α neutralizing antibody and then administered to new DC cultures. Indeed, CM had a maturing effect on immature DCs and pre-treatment with the TNF-α neutralizing antibody significantly reduced this effect, revealing that TNF-α acts in a positive feedback-loop to enhance the DC maturation response in the presence of activated apoptotic PBMCs or $CD4^+$ T cells. It has previously been shown that many, but not all, DCs in the AC-DC co-cultures interact with or take up apoptotic material [224]. The induced TNF-α production in DCs encountering activated ACs could hence be a danger signal to further stimulate nearby cells, and activate a pro-inflammatory response in the surrounding environment.

3.1.2.2 Cellular factors involved in activated AC-induced DC maturation

Previous reports on immunostimulatory effects of apoptotic cells have often been in a context of infection [230, 400, 402], and we set out to investigate how HIV-1 infection in activated $CD4^+$ T cells affected their ability to induce DC maturation (**paper III**). Activated $CD4^+$ T cells were readily infected with the R5 laboratory HIV-1 strain HIV_{BaL} for 3-4 days before irradiation and co-culture with DCs. Similar to uninfected controls, infected activated ACs significantly upregulated maturation markers CD83 and CD86 on DCs. Also, the induced secretion of pro-inflammatory cytokines and chemokines did not differ from uninfected activated ACs. If the infection process of the $CD4^+$ T cells instead was prolonged to one week, an increased cell death was observed in the cultures, likely due to the HIV-1 infection, and these cells did not mature DCs to the same level when irradiated (unpublished observations). This implies that virus-induced cell death might not be as efficient at inducing DC maturation. It might also indicate that prolonged activation of the cells reduces their ability to mature DCs, since longer kinetics of cell activation previously has been shown to have less effect on DCs [224].

We further investigated if the uptake of activated ACs was necessary to induce DC maturation by blocking phagocytosis (**paper II**). However, no reduction of DC maturation was detected, neither with the usage of Cytochalasin D, a chemical inhibitor of actin polymerization and thereby the ability of the cell to form phagocytic cups, nor with a blocking antibody against αvβ5, a phagocytic integrin receptor. Hence, the

initiation of activated AC-induced DC maturation is mediated via cell surface receptors and not endocytic danger sensors.

To investigate which receptors on DCs could be involved in the AC-induced maturation, we pre-treated the cells with neutralizing antibodies against receptors known to mediate cell-cell adhesion as well as cell activation (**paper II**). Integrins are important receptors in cell adhesion. They consist of a α and β subunit, which both are products of individual genes [406, 407]. So far, 18 α subunits and 8 β subunits have been identified in vertebrates, and they can be combined in 24 different structures. Best known for mediating cell-cell adhesion are the β2 integrins, consisting of the β2 subunit, also named CD18, and either the αL (CD11a), αM (CD11b), αX (CD11c), or the αD (CD11d) subunit. The β2αL integrin is also known as lymphocyte function-associated antigen 1 (LFA-1), while the β2αM integrin is known as macrophage-1 antigen (Mac-1). They are both expressed on lymphocytes, macrophages, monocytes, and neutrophils [407-409]. Indeed, blockage of CD18 on DCs significantly reduced the AC-induced maturation. In addition, blocking either CD11a or CD11b also reduced the expression of DC maturation markers, but not as efficiently as the CD18 neutralization did. There are many structures known to interact with β2 integrins [407], and we have so far not identified the specific ligand or receptor mediating the integrin-dependent DC maturation.

The CLR DC-SIGN interacts with neutrophils via Mac-1 due to a neutrophil-specific glycosylation pattern on the integrin, and DCs have also been shown to engulf apoptotic neutrophils [229, 410]. In addition, DC-SIGN has the ability to bind several pathogens and shape the intracellular signaling of TLRs [135, 136, 411]. Although our PBMCs did not contain any neutrophils, we investigated the involvement of DC-SIGN in our co-cultures. Dendritic cell maturation was reduced in the presence of two separate neutralizing antibodies, indicating an involvement of the CLR. Since DC-SIGN acts in collaboration with a number of TLRs, we also treated the AC-DC co-cultures with a TLR4 neutralizing antibody. Toll-like receptor 4 is expressed on the cell surface of DCs and interacts with several endogenous DAMPs [236, 244, 412, 413], making it a conceivable receptor for the activated ACs. This as well reduced the DC maturation following activated AC co-culture. To further confirm this observation, we silenced TLR4 in DC, using siRNA. The knockdown strategy partly reduced the DC response to the TLR4 ligand MPLA, and similarly reduced AC-induced DC maturation. We also investigated the adjuvant effect of activated apoptotic splenocytes in $TLR4^{-/-}$ and wt mice *in vivo*. The antibody titer against human serum albumin, the antigen co-administered with activated apoptotic splenocytes in mice, was, in line with above results, significantly reduced in $TLR4^{-/-}$ mice, as compared to wt animals. These results strongly suggest that TLR4 is involved in activated AC-induced DC maturation.

Several DAMPs in necrotic cells are shown to mediate immune activation via TLR4, but this is less investigated for apoptotic cells. One secreted DAMP reported to interact with TLR4 is HMGB1 [244]. However, we could not detect any impact on DC maturation with either neutralizing antibodies or an HMGB1 antagonistic protein. Instead, we investigated the impact of HSPs. Heat shock protein 60 was upregulated in activated PBMCs and has previously been shown to engage TLR4 [236, 413], and addition of a neutralizing antibody to the AC-DC co-culture indeed significantly reduced the DC maturation. From this, we concluded that activated apoptotic PBMCs mediate the DC-maturing effect partly via released TNF-α, partly via engagement of β2 integrins, DC-SIGN, and HSP60-stimulated TLR4.

3.1.2.3 Intracellular signaling induced in DCs by activated ACs

Next, we investigated the intracellular signaling pathways induced in DCs by activated ACs (**paper II**). Activation of the MAPK and the phosphatidylinositol 3-kinases (PI3K)/Akt signaling pathways was detected at various time points in DCs co-cultured with activated ACs. The response to resting ACs was either absent or less prominent compared with activated ACs. Phosphorylation of MAPKs p44/42 and p38 was detected after 10 minutes, while prominent phosphorylation of Jun-amino-terminal kinase (JNK) was not detected until after 40 minutes. Akt was activated within 10 minutes, and remained activated at all time-points investigated. Furthermore, broad inhibition of Src family of tyrosine kinases (SFK), which are important for TLR4-stimulated cytokine production and integrin function [414, 415], in DCs prior to AC addition reduced the maturation response, which indicates an involvement of this pathway as well. However, no activation of Raf-1, which is reported to be down-stream of DC-SIGN activation [135], could be detected. In accordance with activation of the signaling pathways mentioned above, an activation of NF-κB and AP-1 transcription factors could be detected. Only the AP-1 family members c-Jun and c-Fos were present in nuclear extracts from AC-DC co-cultures, indicating that the AP-1 dimer was formed of these two members.

To investigate the dependence of the individual components in the signaling pathways, we blocked the different pathways using specific chemical inhibitors and thereafter measured DC maturation. Blocking of the MAPKs p44/42, p38, or JNK, resulted in significantly decreased DC maturation in the absence of p38 or JNK. However, the activation of p44/42 did not reduce DC maturation and seems to be indispensable for upregulation of CD80 and CD86 on DCs. As with SFK, p38, and JNK, inhibition of PI3K activation, which is upstream of Akt activation, also reduced the maturation of DCs. Hence, these signaling pathways are essential for DC maturation in response to activated ACs. Inhibition of IKK kinase, which is an upstream kinase required for NF-κB activation, also reduced the DC maturation response, confirming the involvement of the transcription factor.

The next step was to connect the intracellular signaling pathways with the receptors engaged by activated ACs (Figure 4). Previously used neutralizing antibodies against CD18, DC-SIGN, and TLR4 were used to block their respective receptor on DCs prior to the addition of activated ACs. Blockage of DC-SIGN reduced the activation of JNK, but none of the other signaling pathways investigated here were affected. When neutralizing CD18, both JNK and p38 activation was completely diminished, and activation of Akt was also strongly reduced. Inhibition of TLR4 also diminished the activation of Akt and p38, and reduced JNK activation. To further investigate the influence of SFK on these intracellular signaling pathways, the impact of SFK inhibition was measured. There are nine members in the SFK family, but we did not investigate the role of each member in this project. A reduced activation of p38 and complete block of JNK could be detected when inhibiting SFK activity. From this, we concluded that JNK was activated by DC-SIGN, CD18, TLR4, and SFK, p38 was activated by CD18, TLR4 and SFK, and PI3K/Akt was activated by CD18 and TLR4. The receptors responsible for SFK activation were not investigated, since the individual activation of the family members was not determined. Next, the impact of PI3K/Akt on p38 and JNK activation was measured. Inhibiting PI3K reduced the activation of p38, but not JNK, indicating that JNK is activated via a PI3K/Akt independent pathway.

Finally, factors needed for activation of NF-κB and AP-1 transcription factors were investigated. Activation of NF-κB was facilitated both upon CD18 and TLR4 engagement. The importance of the different signaling pathways upon AP-1 activation was thereafter examined, and JNK was shown to be important for c-Jun translocation to the nucleus, while p38 was needed for c-Fos.

Taken into account that we could not confirm whether CD18-mediated DC activation was due to interaction with a ligand expressed on ACs or an integrin-activated receptor on DCs, a direct connection between this interaction and down-stream signaling in DCs could not be determined. Hence, the CD18-dependent activation of PI3K might not be directly linked in DCs and this interaction is therefore put as hypothetical in Figure 4. In addition, the pathways mediating SFK activation are not known, but since DC-SIGN did not induce any other intracellular activation than JNK, which was independent of PI3K activation but activated by SFK, it is plausible that DC-SIGN activate SFK. Also, the murine DC-SIGN paralogue DC-SIGNR1 is reported to mediate JNK activation via SFK [416]. However, since this could not be confirmed in our system, this interaction is also put as hypothetical. Both CD18 and TLR4 have previously been shown to signal via SFK [414, 415], so these interactions are also indicated in Figure 4.

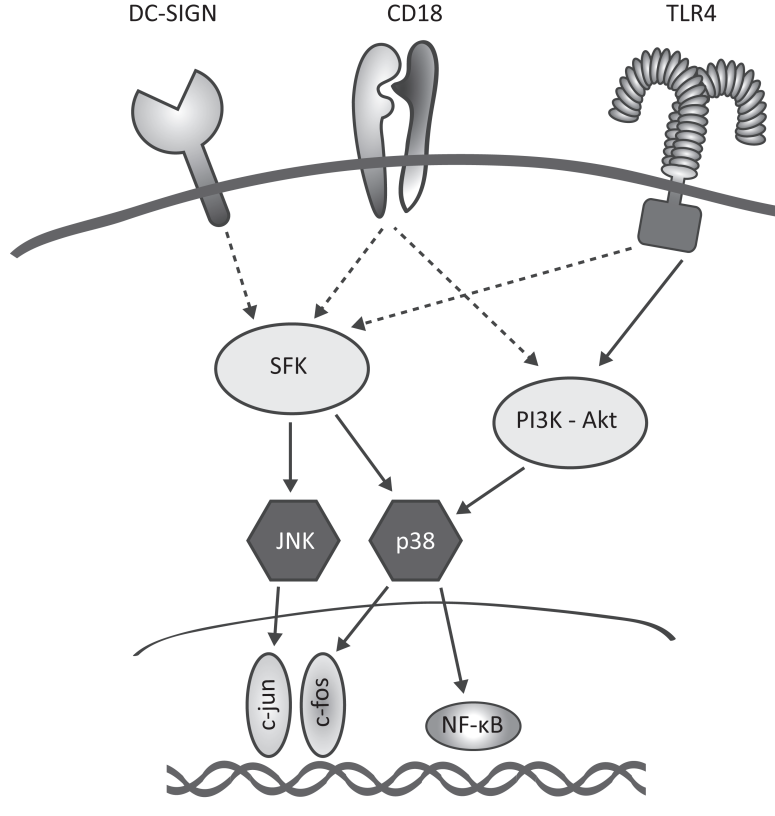

Figure 4: The receptors engaged and signaling pathways induced by activated ACs cultured with DCs.

3.2 PREVENTING HIV-1 INFECTION IN DENDRITIC CELLS

It is still not clear which cells are the most common to be infected by the founder virus in HIV-1 sexual transmission. The most prominent HIV-1 target cell in the vaginal mucosa are the $CD4^+$ T cells, but the vast majority of this population is in a resting state and therefore not an optimal target for the virus [294, 296]. Dendritic cells conversely are more sensitive to HIV-1 infection in an immature state, and in this state they also have the ability to penetrate intact epithelial layers and reach the lumen in their search for potential external threats in healthy mucosa [279, 280, 292, 417]. Since DCs in peripheral tissue predominantly are immature, this would make them a more likely target for HIV-1 during sexual transmission. Elevated immune activation in the vaginal mucosa, as during the presence of additional sexually transmitted diseases, however increases the risk of HIV-1 infection [297], again pointing to activated T cells as the prime target cell during HIV-1 transmission. Whether the virus is transmitted in a free form or cell associated is not fully elucidated either. In intact mucosa, cell-associated HIV-1 does not penetrate the epithelial barrier [284], but it might be taken up by DCs present in the outer layers of the epithelia.

As with the primary infection is the route of dissemination of HIV-1 is not fully understood either. Shortly after infection, the virus spreads to the lymphoid tissue in the gut, where it eradicates the majority of $CD4^+$ T cells [418, 419], and the systemic spread facilitates latent infection in a multiplicity of $CD4^+$ cells [283]. The window of prevention opportunities lies mainly in the primary infection – to prevent cells from being productively infected – but also in limiting the dissemination and the T cell wipe out. By reducing the loss of $CD4^+$ T cells, the adaptive immune system will function better and more efficiently restrict the virus throughout the infection and delay the onset of AIDS. In addition, by reducing the levels of systemic spread, the latent reservoir will probably be less extensive and re-activation of the virus will not be as prominent later during the infection. This will both prolong the healthy period of the patient, and reduce the risk for further transmission [283, 298]. Approaches for preventing not only primary infection, but also establishment of the infection and systemic spread will be greatly beneficial for both the single patient and for the efforts to contain the epidemic. Novel intervention strategies that could act both on the primary infection and systemic spread are therefore desirable.

3.2.1 Upregulating antiviral restriction factors in dendritic cells (paper III – IV)

The first restriction factor during HIV-1 transmission is the mucosal barrier. It consists of a multilayer of epithelial cells covered with viscous mucus highly concentrated with antimicrobial factors and chemokines [298]. However, it is not uncommon with microtrauma in the mucosal barrier during sexual intercourse [291], and this enables HIV-1 to gain direct access to the submucosal tissue, which is highly populated with attractive target cells. Likely, several viral particles infect target cells with various degrees of success, but most of these events results in poor viral replication due to low fit or defect functionality of the virus [283]. A strategy to prevent or to limit the initial infection would be to harness the local tissue against retroviral infection.

3.2.1.1 Activated ACs restricts HIV-1 infection in DCs

Despite in lower frequencies compared to activated $CD4^+$ T cells, immature DCs are readily infected with HIV-1. Initially, there were contradictory reports on whether DCs actually could replicate the virus or not, and it was later discovered that the activation state of the DCs was correlating with infectability [420-423]. The inhibition of DC infection was reported to be due to reduced membrane fusion, blocked RT function, and reduced post-integration transcription [279, 280, 424]. However, no precise mechanism of this reduced HIV-1 infection was provided. The more recently characterized host restriction factors are likely to contribute to these observations [301, 302, 306], as shown for the APOBEC3 molecules [425].

Since activated apoptotic $CD4^+$ T cells efficiently mature human monocyte-derived DCs, we also investigated the effect of this maturation on HIV-1 infection in DCs (**paper III**). As productively infected cells express the viral protein p24, the frequency of HIV-1-infected human monocyte-derived DCs was determined by flow cytometry [426-428]. Although the HIV-1 infection frequency of DCs varied substantially between donors, we found that the percentage of infected, $p24^+$ cells was reduced in the presence of activated ACs in all donors investigated (Figure 5). A similar trend was observed for activated, infected ACs, as they, like the uninfected activated ACs, induced high levels of DC maturation. No reduction of HIV-1 infection was however detected in DCs co-cultured with resting ACs, which indicates that the maturation stimuli of the DCs was the key event for reduced infection, not the exposure of ACs. This was further confirmed by quantitatively assessing the number of HIV-1 DNA copies/cell in DCs cultured with medium only, activated ACs, or resting ACs. Dendritic cells co-cultured with activated ACs also displayed a reduced HIV-1 integration compared to the same control groups. Addition of activated ACs up to 16 h after HIV-1 addition still protected the DCs from high infection rate. *In vitro* HIV-1 infection in DCs is normally not detected by flow cytometry until after 72 h of culture, when several rounds of infection have occurred [427]. Hence, the maturation of DCs probably has inhibitory effect first on the second round of HIV-1 infection.

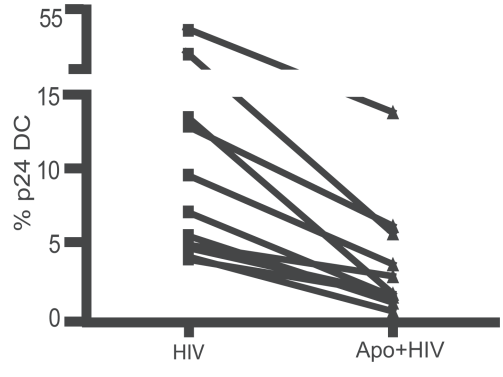

Figure 5: The frequency of HIV-1 infected DCs in the presence or absence of activated AC. Paired results from eleven donor is shown.

3.2.1.2 Strategies to upregulate APOBEC3 molecules in DCs

The best studied host restriction factor to have an impact on the second round of infection is APOBEC3G [301, 320]. We therefore investigated if the expression of APOBEC3G was upregulated upon AC-induced DC maturation (**paper III**). No significant upregulation of ABOBEC3G mRNA as compared to untreated and uninfected DCs was detected 2 h after addition of stimulus, but an increased expression was readily detected in DCs co-cultured with activated ACs for 4-48 h, both in the presence and absence of HIV-1 in the culture. Neither HIV-1 alone nor resting ACs induced significant expression of APOBEC3G. To confirm the importance of APOBEC3G in our system, we knocked down the expression in DCs with siRNA and then treated them with ACs and HIV-1, whereafter the infection rate was measured. Indeed, after silencing of APOBEC3G in DCs, the protective effect of activated ACs was lost. As a mechanism of restriction, APOBEC3G has the ability to induce G-to-A hypermutations in the HIV-1 genome during RT synthesis of the viral DNA minus-strand [314-317]. We subsequently cloned the *Env* gene of HIV_{BaL} derived from DCs co-cultured with either activated or resting ACs and compared the sequence from 26 and 35 clones, respectively, with a clone derived from infected but untreated DCs. The frequency of G-to-A mutations in the virus derived from activated AC-DC co-cultures was significantly elevated, as compared to resting AC co-cultures. Hence, the mechanism of action in reduced HIV-1 infection in activated AC-matured DCs is an upregulated APOBEC3G expression that reduces the second round of infection.

Since APOBEC3G induce hypermutations in a GG-context [324], our sequencing results displaying G-to-A mutations both in GG and GA contexts in the *Env* clones indicate the involvement of additional deaminases in our system. Two other members in the APOBEC3 family have been shown to affect HIV-1 infection in DCs. Both APOBEC3A and APOBEC3F are expressed in DCs [323, 324], whereof APOBEC3A recently has been shown to be highly efficient at inducing hypermutations in this cell type specifically [328]. Furthermore, both induce G-to-A mutations in a GA-context [324, 429]. Since TNF-α was produced by DCs upon AC co-culture and induced paracrine DC maturation, we used this cytokine instead of ACs to stimulate DCs (**paper IV**). The APOBEC3 family is ISGs, and treatment with recombinant IFN-α2b reduces viral load and upregulates the expression of APOBEC3G and APOBEC3F in HIV-1-infected patients [363, 364, 367]. To further investigate the response to type I IFNs, we treated DCs with various types of IFN-α, even though no release of this cytokine could be detected in our previous AC-DC cultures. Indeed, the expression of APOBEC3A, F, and G was upregulated in HIV-1-infected DCs treated with LPS or IFN-α. However, TNF-α did not induce any significant APOBEC3 expression, although treated DCs both upregulated maturation markers and secreted IL-12p40, while only the highest concentration of IFN-α2b used induced expression of maturation markers and no increased IL-12p40 levels. This shows that DC maturation *per se* does not induce expression of APOBEC3 molecules, but the presence of type I IFNs, even in levels that do not induce DC maturation, is most likely required. Upon engagement with TLR4, LPS mediates signaling both via MyD88 and TRIF [117, 118], and TRIF signaling mediates transcription of IFN-α4 and IFN-β [335], which when released have paracrine effect on surrounding cells to initiate an antiviral defence mode.

When investigating the effect of the cytokines on HIV-1 infection in DCs, all tested concentrations of IFN-α2b significantly reduced the infection levels. For TNF-α, the

highest dose used was the only concentration to significantly reduce infection in DCs. We thereafter investigated the effect of the lowest dose IFN-α2b used on G-to-A mutations in HIV-1-infected DCs. On average, 4.84 mutations/100 base pairs were detected, whereof 30% were in a GG-context and 48% in a GA-context. This was higher than our previous results, where also the mutations more preferably occurred in a GG context. This indicates that IFN-α2b treatment more effectively induces APOBEC3 molecules, and perhaps most effectively, APOBEC3A, than activated ACs in DCs. It also demonstrates that maturation of DCs is not a prerequisite for induction of host restriction factors. Elevated expression of IFN-α is found in the cervix in HIV-1 uninfected commercial sex workers in communities with high HIV-1 prevalence, and the longer the woman had been active as a sex worker and stayed uninfected, the higher the levels tended to be [365]. This suggests that local treatment with low doses of IFN-α might have anti-viral effects in a microbicide concept.

These results also shed a light on the effect of TNF-α and activated ACs on HIV-1 infection in DCs. Indeed, TNF-α secreted into the CM upon AC-DC co-culture had the ability to reduce HIV-1 infection in DCs (**paper III**), but only if the cytokine was added simultaneously as the virus. Moreover, addition of a TNF-α neutralizing antibody to the AC-DC co-culture made a fraction of the cells more sensitive to HIV-1 infection. Hence, since TNF-α does not protect DCs from HIV-1 infection via stimulation of the APOBEC3 family, but still protects the cells from elevated infection if added before or simultaneously as the infection, it is likely that TNF-α mediates the induction of additional early host restriction factors, thereby preventing productive infection of DCs. This would also explain why neutralization of TNF-α partly increases the HIV-1 infection in DCs co-cultured with activated ACs, since not all cells physically interact with the dying cells [224], and the secretion of TNF-α into the culture medium probably act to protect these cells. Hence, in the absence of both elevated APOBEC3 expression and TNF-α-induced host restriction, the increased infection of these unprotected cells will result in higher levels of infectious HIV-1 virions, which will further infect additional DCs. Although no type I IFNs were detected in the AC-DC co-cultures, LPS has the ability to induce expression of APOBEC3 molecules [425]. Our finding that activated ACs mediate DC activation via TLR4 (**paper II**) makes this a likely route of APOBEC3 induction in the present system. Since the effect of APOBEC3G is significantly protecting activated AC-exposed DCs from HIV-1 infection, and the frequency of hypermutations in a GG-context is elevated after activated AC co-culture as compared to after IFN-α treatment, these results also suggest that different stimuli might induce different members of the APOBEC3 family, but this has to be further investigated before any conclusions can be drawn.

4 CONCLUDING REMARKS

The dendritic cell relays messages from the peripheral tissue to the lymph nodes and plays a key part in instructing the adaptive immune response. Depending on what signals the DC has retrieved in the tissue, it can induce tolerance, humoral responses, cytotoxic responses, or any adaptive response appropriate for the initial triggering of DC activation. This makes the DC an optimal target for immune therapy strategies to enhance, dampen, or specifically direct the immune response in varying conditions. However, to be able to program the DC-mediated instructions to the adaptive immune system, enhanced knowledge regarding which signals are essential for the specific responses is required. In this thesis, the combination of two PRR ligands is investigated, the mechanism behind activated apoptotic cell-induced DC maturation is characterized and the impact of this maturation on HIV-1 infection in DCs is described, and the resistance to HIV-1 infection of DCs matured with two different cytokines is further characterized.

In vaccine settings, a prominent DC response is often desired. This will induce a powerful adaptive immune response against the antigen of choice, which will protect the vaccinee from disease. Since the discovery of PRRs, the understanding of how DCs are primed has increased substantially. Toll-like receptors are PRRs expressed preferably on immune competent cells and they have emerged as targets in the next generation of vaccine adjuvants. However, their elevated expression in immune cells can also make them mediators of immune pathology. Hence, specific antagonistic substances are being developed for TLR7 and TLR9, which both recognize and respond to self-nucleic acids in several autoimmune diseases with elevated cell death responses. An additional TLR implemented in detrimental responses during necrotic cell death is TLR3, which responds to dsRNA. This receptor does not sense nucleic acids in a sequence-specific manner, and only a few specific inhibitors have been developed and none has so far been tested in humans or non-human primates. We have in **paper I** described a novel mechanism for TLR3 inhibition. Addition of ODNs with a synthetic phosphorothioate backbone in combination with the synthetic dsRNA analogue poly I:C effectively reduced the uptake of dsRNA into the endosomes of treated cells, and thereby inhibited TLR3 engagement. Similar effects have previously been described for PS-ODNs in viral infections, which indicates that PS-ODNs could be used as inhibitors of uptake of larger particles as well. Prevention of TLR3 signaling would be beneficial during conditions of massive cell death, when released dsRNA is taken up and sensed by phagocytic cells, but also in chronic inflammation, autoimmunity and in certain viral infections. The usage of the PS based immunostimulatory TLR9 ligand CpG-ODN has been approved in humans, and this suggests that the development of clinic-proved non-stimulatory PS-ODNs is conceivable. Characterization of the shared uptake mechanism between PS-ODNs, poly I:C and certain viruses will increase our understanding on how these substances are delivered into cells. Also, investigating the impact of PS-ODNs on endocytic uptake of additional substances and particles will further provide understanding of how endocytosis of these structures is initiated. This knowledge could possibly be facilitated in future vaccine delivery systems, or in anti-viral treatments.

Sterile necrotic cell death is seldom beneficial for the host and inhibition of its detrimental effects is important. Apoptotic cell death, on the other hand, is often well

regulated and takes part in maintaining the tolerance for self-structures. But also this path of death can in particular settings induce immune activation. In **paper II**, we have characterized the mechanisms of how activated apoptotic PBMCs stimulate DC maturation. Both released and cellular DAMPs were shown to be important for this response, and they engaged several receptors on DCs. Apoptotic cancer cells have been used in therapeutic vaccination strategies, and the characterization of DAMPs relevant for immune activation have provided an explanation to why some cancer cells have the ability to evoke immune responses while others do not. Apoptotic cells are taken up by DCs *in vivo*, and this makes them an intriguing vehicle for delivery of antigens and adjuvant to relevant immune cells. Our characterization of the receptors engaged on DCs upon co-culture with activated apoptotic cells have revealed the mechanisms of how the DC maturation is induced, and this knowledge will enable a better prediction of the responses induced by activated apoptotic cells in future studies and treatment settings.

Activated apoptotic T cells also have the ability to induce DC maturation. This induction of maturation was in **paper III** shown to reduce HIV-1 infection in DCs, even if the apoptotic cells themselves were HIV-1 infected, and the activation status of the apoptotic cells was also in this setting an important factor. The mechanism behind this reduced infection was induction of the anti-retroviral enzyme APOBEC3G in DCs. In addition to inducing DC maturation, activated apoptotic cells could hence also be used to mediate anti-viral responses in settings for therapeutic vaccination or prevention. The activation status of dying cells is in present settings crucial for immune activation to occur, and it is relevant to investigate the immunogenic quality of the induced cell death when developing viral vaccine vectors with lytic effects.

In **paper IV**, we additionally investigated how the expression of APOBEC3 molecules in DCs was affected by treatment with TNF-α and IFN-α. Both cytokines used in high concentrations had the ability to activate DCs, while only IFN-α induced APOBEC3 molecules, even at concentrations that did not induce DC activation. Hence, IFN-α protects immature DCs from high levels of HIV-1 infection by inducing APOBEC3 restriction factors. Treatment of HCV-HIV-1 co-infected patients with IFN-α is already clinically used, and it would be interesting to investigate the effects of low levels of IFN-α-treatment in a microbicide setting. The mutations induced in the viral genome by APOBEC3 prevent a second round of infection. Hence, elevated levels of the restriction factor would obstruct the transmitted viruses to disseminate and possibly further alarm the immune system of the presence of the intruder and enable additional resistance against infection.

In summary, this thesis provides suggestions of how of detrimental TLR3 activation or high levels of HIV-1 infection in DCs can be prevented, and explains the mechanism of how activated apoptotic cells induce maturation and upregulation of anti-viral restriction factors in DCs. Dendritic cells can be activated and modulated by a vast number of exogenous molecules, but in this thesis, responses to endogenously produced substances is in focus. Detrimental stimulation of TLR3 by self-RNA can be inhibited by PS-ODNs, activation of DCs can be achieved by activated apoptotic cells, and prevention of HIV-1 infection can be mediated both via activated apoptotic cells or endogenous cytokines. Hopefully, the enhanced knowledge of how structures produced by the host itself can affect DCs will contribute to the development of novel strategies for modulation of dendritic cell responses.

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