DENDRITIC CELL RESPONSES TO APOPTOTIC CELLS - IS THERE LIFE AFTER DEATH?

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Stockholm 2010
To Lois and Joni
“Utan tvivel är man inte klok”

Tage Danielsson
ABSTRACT

Dendritic cells (DC) are antigen-presenting cells that are crucial for the induction of immune responses to pathogens and for the maintenance of self-tolerance. DCs efficiently capture and process material from their local environment, including invading pathogens and dying cells of the host, and present the material to the adaptive immune response. Both in disease and during steady-state, cells die by apoptosis which lead to engulfment by surrounding DCs. Clearance of apoptotic cells by DCs has generally been considered to be an immunological silent event or to be involved in peripheral tolerance mechanisms. However recent discoveries suggest that apoptosis, under certain circumstances, can be immunogenic.

In this work we find that the activation state of the cells undergoing apoptosis may determine whether exposed DCs will become activated and efficiently present antigen from these apoptotic cells to T cells. We demonstrated that activated, but not resting, apoptotic PBMCs induced increased expression of co-stimulatory molecules as well as release of pro-inflammatory cytokines in human DCs. Additionally, we showed that DCs exposed to allogeneic, activated, apoptotic PBMCs were able to induce proliferation and IFNγ production in autologous T cells.

We have also examined the ability of DCs to produce the Th1 promoting cytokine IL-12p70 upon apoptotic cell uptake. We demonstrated that IL-12p70 production in DCs after uptake of apoptotic cells and subsequent CD40-ligation or IFNγ/LPS stimulation was influenced by the activation state of the engulfed apoptotic cells. A CD40-ligand transfected cell-line induced IL-12p70 in DCs regardless of previous apoptotic cell uptake. However, IL-12p70 production by DCs in co-culture with allo-responsive autologous T cells required previous exposure to activated apoptotic cells. Resting, but not activated apoptotic cells reduced ongoing IL-12p70 production in DCs. This suggests that apoptotic cells may either “license” DCs for IL-12p70 production or dampen this ability depending on the activation state of the apoptotic cells.

In addition, we have shown that syngeneic, activated apoptotic mouse lymphocytes were able to provide adjuvant activity in an HIV-1 DNA vaccine. Immunization of mice with seven different plasmids (3 env, 2 gag, 1 rev, 1 RT) combined with activated apoptotic cells lead to increased systemic and mucosal B cell responses as well as T cell responses in a magnitude comparable to immunization with plasmids and the cytokine adjuvant GM-CSF. Resting apoptotic mouse lymphocytes did not provide this adjuvant activity.

Furthermore, we demonstrated that exposure to activated apoptotic CD4+ T cells promoted expression of co-stimulatory molecules, cytokine and chemokine release and reduced HIV-1 production in DCs. These effects were detected also with activated, newly infected apoptotic CD4+ T cells and remained in the presence of free HIV-1Bal. Blocking of TNFα decreased CD86 expression and partially restored HIV-1 production in DCs. In addition, up-regulation of HIV-1 interfering APOBEC3G was found in DCs exposed to activated apoptotic CD4+ T cells, which could contribute to the reduced HIV-1 production.

Conclusively, our work demonstrates that the activation state of apoptotic cells influences DC activation and subsequent adaptive immune responses. We suggest that this could play a role in HIV-1 infection, where recurrent apoptosis is a distinctive feature, and also propose that the effects of activated apoptotic cells could be employed in design of vaccines.
LIST OF PUBLICATIONS

This thesis is based on three publications and one manuscript. The individual papers are referred to by their Roman numerals.

I. **Ulrika Johansson**, Lilian Walther-Jallow, Anna Smed-Sörensen, Anna-Lena Spetz. Triggering of dendritic cell responses after exposure to activated, but not resting, apoptotic PBMCs
   *Journal of Immunology*, 2007, 179: 1711-1720

II. **Ulrika Johansson**, Lilian Walther-Jallow, Anette Hofmann, Anna-Lena Spetz. Dendritic cells are able to produce IL-12p70 after uptake of apoptotic cells
   *Immunobiology*, 2010, April, in press

III. Andreas Bråve, **Ulrika Johansson**, David Hallengärd, Shirin Heidari, Hanna Gullberg, Britta Wahren, Jorma Hinkula, Anna-Lena Spetz. Induction of HIV-1-specific cellular and humoral immune responses following immunization with HIV-DNA adjuvanted with activated apoptotic lymphocytes
   *Vaccine*, 2010, 28: 2080-2087

IV. Lilian Walther-Jallow, **Ulrika Johansson**, Venkatramanan Mohanram, Annette Sköld, Joshua Fink, Barbro Mäkitalo, Anna-Lena Spetz. Exposure to activated CD4\(^+\) T cells induces TNF-\(\alpha\) mediated CD86 expression and inhibition of HIV-1 infection in dendritic cells
   *Manuscript*
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<th>Description</th>
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<tr>
<td>AICD</td>
<td>Activation Induced Cell Death</td>
</tr>
<tr>
<td>AIDS</td>
<td>Acquired Immunodeficiency Syndrome</td>
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<td>APC</td>
<td>Antigen Presenting Cell</td>
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<tr>
<td>ART</td>
<td>Anti-Retroviral Therapy</td>
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<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) Ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>CC Chemokine Receptor</td>
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<tr>
<td>CD</td>
<td>Cluster of Differentiation</td>
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<tr>
<td>cDC</td>
<td>Conventional Dendritic Cell</td>
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<tr>
<td>CD40L</td>
<td>CD40-ligand</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T Lymphocyte</td>
</tr>
<tr>
<td>CTLA</td>
<td>Cytotoxic T Lymphocyte-Associated Antigen</td>
</tr>
<tr>
<td>CXCR</td>
<td>CXC Chemokine Receptor</td>
</tr>
<tr>
<td>DAMP</td>
<td>Danger-Associated Molecular Pattern</td>
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<tr>
<td>DC</td>
<td>Dendritic Cell</td>
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<tr>
<td>DC-SIGN</td>
<td>Dendritic Cell Specific ICAM-3 Grabbing Nonintegrin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr Virus</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular Matrix</td>
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<tr>
<td>FACS</td>
<td>Fluorescence-Activated Cell Sorting</td>
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<tr>
<td>FeR</td>
<td>Fc-Receptor</td>
</tr>
<tr>
<td>Gas6</td>
<td>Growth Arrest-Specific factor 6</td>
</tr>
<tr>
<td>HEV</td>
<td>High Endothelial Venule</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>HMGB</td>
<td>High Mobility Group Box</td>
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<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>LC</td>
<td>Langerhans Cell</td>
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<tr>
<td>LPC</td>
<td>Lysophosphatidylcholine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>LOX</td>
<td>Lectin-like Oxidized Low-Density Lipoprotein Receptor</td>
</tr>
<tr>
<td>MBL</td>
<td>Mannose-Binding Lectin</td>
</tr>
<tr>
<td>MCP</td>
<td>Monocyte Chemotactic Protein</td>
</tr>
<tr>
<td>MDA</td>
<td>Melanoma Differentiation-Associated gene</td>
</tr>
<tr>
<td>MDC</td>
<td>Myeloid Dendritic Cell</td>
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<tr>
<td>MDDC</td>
<td>Monocyte-Derived Dendritic Cell</td>
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<tr>
<td>Mertk</td>
<td>Mer receptor tyrosin kinase</td>
</tr>
<tr>
<td>MFGE8</td>
<td>Milk Fat Globule EGF factor 8 protein</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage Inflammatory Protein</td>
</tr>
<tr>
<td>MMR</td>
<td>Macrophage Mannose Receptor</td>
</tr>
<tr>
<td>MSU</td>
<td>Monosodium Urate</td>
</tr>
<tr>
<td>MuLV</td>
<td>Murine Leukemia Virus</td>
</tr>
<tr>
<td>NLR</td>
<td>Nucleotide-binding Oligomerization Domain-Like Receptor</td>
</tr>
<tr>
<td>NKT cell</td>
<td>Natural Killer T cell</td>
</tr>
<tr>
<td>oxLDL</td>
<td>Oxidized Low-Density Lipo-protein</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
</tr>
<tr>
<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cell</td>
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<tr>
<td>PD</td>
<td>Prephenate Dehydratase</td>
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<tr>
<td>pDC</td>
<td>Plasmacytoid Dendritic Cell</td>
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<tr>
<td>PHA</td>
<td>Phytohaemagglutinin</td>
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<tr>
<td>PI</td>
<td>Propidium Iodide</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
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<tr>
<td>RAGE</td>
<td>Receptor for Advanced Glycation Endproducts</td>
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<tr>
<td>RIG</td>
<td>Retinoic acid-Inducible Gene</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>SIV</td>
<td>Simian Immunodeficiency Virus</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TIM</td>
<td>T cell Immunoglobulin domain and Mucin domain protein</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor Necrosis Factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>Tumor necrosis factor-Related Apoptosis-Inducing Ligand</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>TSLP</td>
<td>Thymic Stromal Lymphopoietin</td>
</tr>
<tr>
<td>TSP</td>
<td>Thrombospondin</td>
</tr>
<tr>
<td>UTP</td>
<td>Uridine Triphosphate</td>
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1 INTRODUCTION

The evolution of the immune system has supplied us with immensely complex procedures for the elimination of dangerous pathogens and potentially harmful cells. Not only does it function as a protective force against foreign or defective entities, it also has built-in mechanisms for the active tolerance of normal cells and proteins that constitute our bodies and non-harmful substances coming from the outside world. Evolution does not only apply to us but also to the pathogens that surround us. This may sometimes favour our wellbeing but may also subject our immune system to major strain. In modern times we have found ways to facilitate the eradication of pathogens, for example through the usage of antibiotics. We have also learnt to employ processes engaged in immune defence to prevent or eliminate infections through the invention of different vaccines. In many diseases we are however still faced with problems concerning how to induce, sometimes reduce, to render more effective or to fine tune immune responses. The immense number of factors that have been identified as mediators in an immune response is continuously growing but include for example certain cells, such as antigen-presenting cells (APCs), cell-surface molecules, such as Toll-like receptors (TLRs) or the major histocompatibility complexes (MHCs), soluble molecules such as cytokines, chemokines, antibodies or anti-microbial peptides. Different combinations of these factors can tip the balance towards an immunogenic signal but whether an immune response will occur upon a given signal will also be determined by the timing, strength, location and duration of that signal.

What will be the most efficient tools in trying to induce, increase or reduce an immune response? There are many paths to choose in the search for those tools. The aim of this thesis has been to explore the ability of DCs to respond to, and transmit immunogenic signals from dying cells to other immune cells. We believe that understanding of this process could facilitate the design of potential vaccines directed towards demanding pathogens such as HIV-1. Equally, studies on the immunogenic or tolerogenic potential may provide clues for induction of tolerance. In this thesis I will discuss whether dead cells carry information that can trigger an immune response. The question emerges whether the end is in fact the beginning of something new?

1.1 Dendritic cells

Dendritic cells (DCs) have a powerful role in the initiation of immune responses. They are cells specialized in capturing, processing and presenting antigen. The processed antigen is then exposed as peptides on major histocompatibility complexes (MHC) on their surface. When our bodies encounter an invading pathogen, DCs are among the first cells to respond. The pathogen triggers up-regulation of molecules on the dendritic cell surface that are essential for eliciting effective immunity. Upon pathogen-recognition, phagocytosis is transiently increased and secretion of immunostimulatory cytokines as well as migration to lymphoid organs is induced. Upon arrival to lymphoid tissue, DCs are able to present antigen to peptide specific T cells that can further build an immune response to eliminate the pathogen.
The origin of the antigen engulfed by DCs is not always pathogenic but can also be derived from harmless substances or from host self-antigens. Presentation of these antigens by DCs does not generally result in immune activation but rather in dampening or suppression of responses by induction of T cell regulation, suppression, tolerization or anergy. However, defective responses are sometimes elicited against these antigens and then result in allergy or autoimmunity. DCs are central in induction of immune responses, wanted or unwanted. Therefore they are key tools in finding ways to build, but also reduce or eliminate, immune responses.

### 1.1.1 DC origin

DCs constitute a sparsely distributed group of hematopoietic cells. Paul Langerhans made the first observation of DCs in human skin and assumed, based on the morphology of the cells, that they were nerve cells. These were named Langerhans cells (LCs) (1). The identification of Birbeck granules, a cytoplasmic organelle specific for LCs, further characterized these cells (2, 3). Steinman and Cohn observed these cells in mouse spleen and named them dendritic cells (4-6). It was then proven that LCs and DCs in skin and thymus are not nerve cells but originate from hematopoietic progenitors of the bone marrow (7).

DCs, as all blood derived cells, originate from hematopoietic stem cells. Subsequently these cells develop into precursors of different cell lineages. An early event in development of DCs is the commitment to a myeloid or a lymphoid precursor. Both of these precursors have been shown to be capable of differentiating into various DC subtypes in vitro (8-12).

### 1.1.2 Subsets and distribution

Multiple DC subtypes with different immune functions have been identified since the discovery of DCs. They differ in location, migratory pathways and specialized immunological function. An overview of human DC subset localization and phenotype is found in Table 1.

One way to categorize DC subtypes found during steady state conditions in both mice and human beings is to divide them into pre-DCs and conventional DCs (cDCs). Pre-DCs generally need further stimuli, such as pathogen recognition or inflammation, to develop into fully functional DCs. Plasmacytoid DCs (pDCs) can be found in this category (13). pDCs do not express myeloid markers and are in humans defined by expression of CD123 and lack of CD11c (14-16). They can also be distinguished by their high production of type I Interferons (IFNs) upon microbial stimulation (16, 17). They are low in numbers in peripheral tissue during steady-state but increase in inflammatory sites (18).

Monocytes circulate in the blood and can also be categorized as pre-DCs. These CD14+ cells can generate DCs during inflammatory conditions but have also been shown to contribute to the steady-state generation of DC subsets such as LCs (19) and DCs in respiratory and intestinal mucosa (20, 21).
The cDCs can be divided into migratory and lymphoid tissue-resident DCs. The migratory cDCs reside in peripheral tissue where they collect antigen. Upon exposure to danger signals they migrate via the lymphatics to the lymph nodes and develop into a mature, immunostimulatory phenotype (13). These cells also migrate during steady state though at a slower rate and display a less mature phenotype as compared with DCs exposed to pathogens or inflammatory mediators (22). DC subsets in mice differ somewhat from human subsets and the cDCs in mice are divided into a CD8α- and a CD8α+ subpopulation where the CD8α+ population does not seem to use the lymphatic-to-lymph node route of migration but rather enter lymph nodes from the blood (18).

Langerhans cells (LCs) are typically migratory cDCs. LCs are found in the epidermis and in epithelia of intestine and in respiratory and reproductive tract (13). They can be distinguished by their expression of Langerin and Birbeck granules and also express CD1a, CD1c, CD11b and CD33 (23). Other migratory cDCs found in non-lymphoid peripheral tissue are dermal and interstitial DCs, expressing CD1a, CD1d, CD11b, CD11c, CD36 and DC specific ICAM-3 grabbing nonintegrin (DC-SIGN)(24-26).

Human blood contains a subset of immature myeloid cDCs. These are believed to be naïve cells migrating from bone marrow to peripheral tissue to become resting interstitial DCs in secondary lymphoid organs and peripheral tissue (27-29). These can be distinguished from blood pDCs by expression of CD1c, CD11c and CD33 (29).

The lymphoid tissue-resident cDCs are found in the thymus, spleen and lymph nodes. These cells do not travel from the periphery but rather sample and present antigen within the lymphoid organ and they display an immature phenotype (30).

1.1.3 In vitro derived DCs

DCs are rare cells and difficult to isolate ex vivo especially without altering their activation status during the isolation procedure. It was therefore a major step in DC research when methods for expanding and differentiating these cells ex-vivo were developed (31-35). The easiest DC precursors to isolate are CD14+ monocytes in human blood. These differentiate into CD14-, CD1a+, CD11c+, MHCIIbright cells when cultured with GM-CSF and IL-4 (33, 34). Isolated human CD14+ monocytes can also be cultured in other cytokines leading to differentiation of DCs resembling activated LCs, dermal DCs or interstitial DCs (36-39). The in vitro experiments included in this thesis are based on human blood monocyte derived DCs (MDDCs) differentiated through culture in GM-CSF and IL-4.

1.2 Dendritic cell activation

As described earlier, DCs reside in peripheral and lymphoid tissue and also circulate in the blood. They act as messengers between the innate and the adaptive immune system. Signals from potentially dangerous microbes, endogenous danger signals and signals from innate immune cells are translated into a message that the adaptive immune cells can read and react to. To elicit immunity the immature, antigen-capturing DC has to
transform into a mature antigen-presenting cell (APC). This activation process includes morphological changes, such as loss of adhesive molecules, reorganization of the cytoskeleton and increased motility, rapidly increased, and then decreased antigen uptake, secretion of chemokines to attract other immune cells, up-regulation of co-stimulatory molecules, translocation of MHC class II compartments to the cell surface, and secretion of cytokines differentiating and polarizing effector T cells (24, 40).

There are several ways in which the DC activation process can be initiated. The pattern recognition theory was put forward by Janeway in 1989 (41). He suggested a general principle of innate immune recognition where the immune system discriminates between non-self pathogen associated molecular patters (PAMPs) and tissue derived self-molecules. This has worked as guiding principles in much of our current understanding of the innate, but also acquired immunity and how they are connected. Although this theory still functions as a framework in immunology research it has been partly modified. An alternative but not mutually exclusive hypothesis, the danger theory, which was built on the idea that the immune system is responding to endogenous danger signals from injured cells, rather than strictly discriminating between non-self and self-antigen, was first put forward by Polly Matzinger (42). This theory suggests that distinct types of cell death induce different types of immune responses. Physiological cell death (apoptosis) does not generate immune activating signals, while pathological cell death (necrosis) gives rise to an adaptive response. These views do not provide satisfying answers as to why immune responses are induced in situations where both PAMPs and injured cells seem to be absent. For example, in a study where chemotherapy-induced apoptosis in tumour cells primed immune responses towards the tumour it was shown that the caspase activation, that is characteristic for apoptosis, was the key event in the immunogenicity(43). The conceptual ideas of Janeway and Matzinger are being further developed and modified as emerging data show immunogenic effects also of certain apoptotic cells in the absence of infection. The final result of an immune response initiated by DCs most likely depends on the initial signals given, be it PAMPs, danger molecules released from necrotic cells, factors present in apoptotic cells or combinations of these, the microenvironment at which these are received and the type of DC receiving them. The different terms and definitions used when describing DC responses to antigen are sometimes indistinct. I will in this thesis define “DC activation” as the process leading to an adaptive response against an antigen. The term “DC maturation” describes a change in phenotype with up-regulation of co-stimulatory molecules that may, but does not necessarily, lead to an adaptive response.

1.2.1 DC recognition of microbes

DC activation initiated by direct microbe contact occurs through recognition of so-called PAMPs (41) by pattern recognition receptors (PRRs) expressed by DCs (44). One group of PRRs is the Toll-like receptors (TLRs) that are expressed by several cell types including DCs. The TLRs recognize different PAMPs and each DC subset expresses a specific set of TLRs (Table 1, Figure 1)(24). As a result this generates a distinct immune response against the specific pathogen. 10 different TLRs have so far been identified in human (13 in mice) and these can be divided into groups depending on their ligands and localization. TLR 1, 2, 4 and 6 recognize lipids or viral
glycoproteins, TLR 5 recognize flagellin, a protein constituting bacterial flagella and TLR 3, 7, 8 and 9 recognize nucleic acids. The ligand of TLR 10 is not known. While TLR 1, 2, 4, 5 and 6 are expressed on the cell surface, TLR 3, 7, 8 and 9 are located in intracellular compartments such as the endosome or lysosome (45).

As for TLRs, different DC subsets have their specific expression of C-type lectins. This group includes for example BDCA-2, expressed on pDCs (46), the macrophage mannose receptor (MMR), expressed by myeloid blood DCs, dermal DCs and MDDCs (47), Langerin/CD207, expressed on LCs (3) and DC-SIGN/CD209, expressed on various myeloid DC subsets (26). C-type lectins have a wide range of microbial affinities covering viruses, bacteria, parasites and fungi (24). They do not only bind microbes and initiate their internalization but they also function as adhesion molecules and bind to other immune cells, which can lead to DC activation or enable activation of the interacting cell (26, 48, 49).

Another group of PRRs involved in DC activation are the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). These recognize microbial components in the cytosol. This family comprises 22 members of whom some are constituents of the “inflammasome”, a protein complex, which is important in activation of proinflammatory caspases that act on secretion of IL-1β and IL-18. (50-53). The list of NLR ligands is still incomplete. However, peptidoglycan fragments from the bacterial cell wall, bacterial RNA and endogenous danger signals, such as uric acid and ATP released from dying cells, have been shown to activate NLRs and the NALP3 inflammasome (45, 50-52, 54, 55). RIG-like helicases (RIG-I and MDA-5) also belong to the intracellular PRRs. Recognition of viral RNA by these receptors leads to production of antiviral type-I IFNs (50).

**Table 1.** DC subtype localization, phenotype and expression of pattern-recognition receptors.

<table>
<thead>
<tr>
<th></th>
<th>Plasmacytoid DCs</th>
<th>Myeloid DCs</th>
<th>Langerhans cells</th>
<th>Dermal/Interstitial DCs</th>
<th>Monocyte-derived DCs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Localization</strong></td>
<td>Blood</td>
<td>Blood</td>
<td>Epidermis</td>
<td>Dermis and other tissues</td>
<td>In vitro</td>
</tr>
<tr>
<td><strong>Phenotype</strong></td>
<td>CD11c CD123&lt;sup&gt;+&lt;/sup&gt; CD33&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD1a&lt;sup&gt;+&lt;/sup&gt; CD1c&lt;sup&gt;-&lt;/sup&gt; CD11c&lt;sup&gt;-&lt;/sup&gt; CD3&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD1a&lt;sup&gt;+&lt;/sup&gt; CD1c&lt;sup&gt;-&lt;/sup&gt; CD11b&lt;sup&gt;-&lt;/sup&gt; CD33&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD1a&lt;sup&gt;+&lt;/sup&gt; CD1d&lt;sup&gt;-&lt;/sup&gt; CD11b&lt;sup&gt;-&lt;/sup&gt; CD1c&lt;sup&gt;-&lt;/sup&gt; CD36&lt;sup&gt;-&lt;/sup&gt;</td>
<td>CD1a&lt;sup&gt;-&lt;/sup&gt; CD11b&lt;sup&gt;-&lt;/sup&gt; CD1c&lt;sup&gt;-&lt;/sup&gt;</td>
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<tr>
<td><strong>TLR expression</strong></td>
<td>TLR1, TLR6, TLR7, TLR9, TLR10</td>
<td>TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR8, TLR10</td>
<td>TLR1, TLR2, TLR3, TLR6, TLR7, TLR8</td>
<td>TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8</td>
<td>TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8</td>
</tr>
<tr>
<td><strong>C-type lectin expression</strong></td>
<td>BDCA2</td>
<td>DC-SIGN, MMR</td>
<td>Langerin</td>
<td>DC-SIGN, MMR</td>
<td>DC-SIGN, MMR</td>
</tr>
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</table>
Figure 1. TLR-expression and PAMP recognition in DCs. TLR 1, 2, and 6 recognize entities expressed by gram+ bacteria, gram- bacteria and/or fungi. TLR3 recognizes double-stranded RNA, TLR5 recognizes flagellin, TLR7 and 8 recognizes single-stranded RNA and the ligand of TLR9 is CpG-containing DNA. The activating ligand of TLR10 is not known.

1.2.2 DC recognition of endogenous danger signals

DCs do not only respond to foreign molecules but have also been shown to recognize a variety of endogenously derived molecules. These are usually referred to as danger associated molecular patterns (DAMPs) and contribute to the activation process of DCs. The danger signals can be released either from cells exposed to PAMPs or from cells objected to stress or injury. These endogenous molecules have been shown to induce DC maturation and production of pro-inflammatory cytokines in vitro (56-58) and some have also indicated adjuvant and pro-inflammatory activity in vivo (58-60). The list of endogenous molecules with DC stimulating effects is long and growing. Here I have chosen to describe some of the molecules with documented effects on DC maturation or activation. A more extensive list of DAMPs can be found in a review by Kono, H. in Nature Reviews Immunology 2008(61).

Members of the group β-defensins function as danger signals. These are small antimicrobial peptides, secreted by neutrophils and epithelial cells in response to microbial products or pro-inflammatory cytokines and have been shown to induce DC maturation through binding of TLR4 (62, 63).

Heat shock proteins (HSPs) have been described to induce maturation and production of pro-inflammatory cytokines in DCs in vitro (56), to induce migration and to provide an adjuvant effect to injected antigen in vivo (59, 64). The effect of HSPs on DCs has however been debated since other studies have indicated that the immunogenic properties of HSPs are due to contaminating microbial products such as lipopolysaccharide (LPS) and that LPS free HSP60 and HSP70 used in DC assays fails to induce DC activation (65).
Uric acid is a degradation product of purines and is the source of monosodium urate (MSU) crystals that are formed when uric acid is released into extracellular fluid, which occurs during necrotic cell death. The MSU crystals are the active form of the molecule and have been shown to stimulate DCs \textit{in vitro} and to have an adjuvant effect \textit{in vivo} (57, 60). The activation of DCs by MSU seems to occur in a TLR4 independent fashion (57) but MSU has been shown to bind to the NLR receptor NALP3 and induce pro-inflammatory cytokines, particularly IL-1\(\beta\) (66). Also the detection of extracellular ATP released from dying cells by DCs has been shown to activate the NLRP3-inflammasome leading to IL-1\(\beta\) production and adaptive immunity against tumours (67).

The High mobility group box 1 (HMGB-1) protein is both an intracellular and a secreted protein. In the cell nucleus it has a structural function bending chromosomal DNA and regulating transcription (61, 68-70). Initially the release of HMGB1 was considered a feature of necrotic cells. It has later been shown that cells in late apoptosis also release HMGB1 (71). HMGB1 can also be secreted as a cytokine from activated monocytes, macrophages and DCs (61, 70). It has been shown to induce DC maturation \textit{in vitro} and to have an adjuvant effect when added to immunizations with soluble antigen (58). Three receptors of HMGB1 have so far been identified; receptor for advanced glycation endproducts (RAGE), TLR2 and TLR4. However the inherent inflammatory effects of HMGB-1 may be discussed since it binds to other molecules of microbial and cellular origin that could have an inflammatory effect (72) and one study showed that injection of HMGB-1-deficient dying cells had the same proinflammatory effect as HMGB-1 containing cells (73). As mentioned earlier, dying cells also release ATP which can trigger production of pro-inflammatory cytokines through the interaction with the inflammasome (50, 51, 55).

It has earlier been shown that the antimicrobial peptide LL37 can bind extracellular self-DNA and induce an autoimmune reaction via TLR9 in pDCs (74, 75). In a recent paper by Ganguly et al. it was shown in a psoriatic model that LL37 also can bind self-RNA released by dying cells, protect it from extracellular degradation and transport it into endosomal compartments of mDCs where it binds to TLR8. This interaction leads to production of pro-inflammatory cytokines and maturation of the DCs (76).

Suggested receptors for the various endogenous danger signals released from dying cells include TLRs, RAGE, CD91, CD14, scavenger receptors, integrins, chemokine receptors, CD44 (61) and C-type lectins (77) including the recently described CLEC9A in mice (78). It has been debated whether some of these receptors are truly involved in endogenous danger signalling since contradictory results are found in this area of research. Some of the receptors, as discussed above, bind to molecules of microbial origin. Difficulties in separating the endogenous danger signals from effects of contaminating microbial products warrants caution when interpreting data concerning endogenous molecules and their receptors, for example HSP-binding to TLRs. In one study, injection of dead cells into TLR-deficient mice demonstrated that no single TLR was required for an inflammatory response (73). The map of specific effects and signalling of endogenous danger molecules is still incomplete. Nevertheless, some of
these events may be keys to developing efficient vaccines or therapies for autoimmunity.

1.2.3 Antigen uptake and maturation

Upon recognition, DCs take up antigen through several mechanisms. One is receptor-mediated endocytosis. This can be triggered by ligation of receptors for the Fc-portion of immunoglobulins, binding of HSPs to HSP-specific receptors, binding of scavenger receptors by a number of different ligands such as chemically modified low-density lipo-proteins (LDL), molecular chaperones, extracellular matrix (ECM) proteins, different PAMPs and moieties on apoptotic bodies (79-81). Also members of the C-type lectin family, such as the macrophage mannose receptor (MMR) and Langerin mediate endocytosis in DCs (3, 47, 82). By macropinocytosis DCs are able to rapidly and non-specifically sample soluble antigen, which occurs continuously in immature DCs. Phagocytosis is generally triggered by the same receptors as for receptor-mediated endocytosis, but involves actin-polymerization and allows uptake of particulate antigens such as for example whole bacteria (83). After engulfment of the antigen by the different mechanisms it is transported through specialized compartments where it is degraded into peptides that are ultimately presented on the major histocompatibility complex (MHC) class I and MHC class II. The intracellular pathways involved in antigen degradation and presentation are outside the scope of this thesis but have been thoroughly reviewed elsewhere (40, 83, 84)

In general, MHC class I present endogenously derived antigen to CD8+ T cells and MHC class II present exogenous antigen to CD4+ T cells. However, the rules for antigen presentation appeared more complex when Bevan in the 1970’s demonstrated that CD8+ cytotoxic T lymphocyte (CTL) responses could be seen after presentation of exogenous antigens by MHC class I molecules. He called this phenomenon cross-priming (85). Several studies have confirmed that internalized antigens may be presented also on MHC class I (86-89). This is referred to as cross-presentation (90). Antigen presentation can also be conducted via CD1 molecules. This group of molecules complexed with lipid or glycolipid antigens stimulates different types of T cells such as CD8+ CTLs, CD4CD8- T cells, γ/δ T cells and NKT cells (91).

In response to pathogens and/or endogenous danger signals DCs will, as a rule, up-regulate and expose molecules that are important in the orchestration of an adaptive immune response. These include MHC class II presenting exogenous antigen, CD80 and CD86, that binds to CD28 or cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) on the T cells and function as co-stimulatory and repressing molecules for T cell activation respectively (92), CD40 that receives signals from CD40 ligand (CD40L) on activated T cells (93, 94), 4-1BB ligand (4-1BBL) and OX40 ligand (OX40L) that both function as co-stimulatory molecules for T cell activation (93) and CCR7 - a chemokine receptor enabling DC migration towards lymphoid compartments (93)(Figure 2). These molecules are important for induction of an adaptive immune response and are commonly used as a measurement of DC maturation. Dying cells or danger molecules derived from dying cells have also been shown to induce expression of maturation markers in DCs (61, 91).
DC maturation can be initiated indirectly by pro-inflammatory cytokines and chemokines secreted by cells surrounding the DC (95). For example type I IFNs produced by virus-infected cells increase viral resistance in neighbouring cells but also act on DC maturation (96, 97). Thymic stromal lymphopoietin (TSLP), TNFα, IL-1, IL-15 and IL-18 are other cytokines that have been shown to promote DC maturation (98-101). As binding of PAMPs to DCs and other cells leads to production of pro-inflammatory cytokines, the maturation of DCs can sometimes be the secondary result of pathogen recognition. It has been argued that this type of DC maturation is insufficient for an effective adaptive response (102) and that this type of DCs are more prone to tolerance induction (103). Although maturation is not the only DC change required for an effective response against a pathogen, it is a necessary step in the activation process.

1.2.4 DC migration

DCs are APCs that are specialized in homing to T cell zones of the lymphoid organs. This distinguishes them from macrophages that are often far more frequent APCs than DCs in peripheral tissues but lack the migratory properties of DCs. The classical way of looking upon DC migration is that peripheral tissue-resident DCs receive pathogen-derived or danger-associated signals that induce their maturation, including up-regulation of the chemokine receptor CCR7. These events initiate chemotaxis and migration via afferent lymphatics towards the chemokine CCR7 ligands CCL19 and CCL21. This guides the DCs towards lymph nodes where immune responses can be initiated. Numerous aspects of DC migration can be added to this scenario where some may be important in determining the final outcome of an immune response.

Migration of DCs occurs also under homeostatic conditions and they do not always travel to lymphoid organs via the lymphatics. Migration of DCs to spleen for example, occurs via a hematogenous route. In mice, these precursors develop into immature CD8α– and CD8α+ conventional DCs (13). DCs in resting spleen do not seem to be monocyte-derived but originate from other precursors (20). Due to the low number of these precursors in circulation these are not very well studied. Rather than using the lymphatics, CD8α+ DCs are believed to enter lymph nodes through high endothelial
venules (HEVs) used also by lymphocytes to enter lymphoid organs. Since the murine
CD8α+ cells are superior in cross-presenting antigen to CD8 T cells but assumingly do
not gain access to antigen in peripheral tissue and carry it through lymph, it has been
discussed whether other DCs donate antigen to the CD8α+ DCs (104, 105).

The HEV route is also used by pDCs that are present in low numbers in peripheral
tissue during homeostatic conditions but increase in numbers in inflamed tissue. In an
animal model, pDCs were not found in afferent lymph, neither during steady state nor
during inflammatory conditions (106). pDCs do acquire antigen but seem to travel to
lymph nodes via the blood. Tissue-to-blood routes of migration have also been
suggested in situations where access to the afferent lymph is constrained, as seen in
atherosclerosis (107). There is also the possibility that DCs relocate from lymphoid
organs to other tissues via either efferent lymph, which means that they fail to be
trapped, or directly migrate across vascular endothelium. This could play a role in for
example skewing of immunity, induction of tolerance and the spread of pathogens from
tissue to tissue. In one study manipulation of injected DCs with vitamin D3 redirected
them from popliteal lymph nodes to mesenteric lymph nodes and Peyer’s patches,
which resulted in induced mucosal immunity (108). It has been proposed that DCs from
peripheral tissue contribute to central tolerance through migration to thymus. One study
documented migration of endogenous peripheral tissue-derived DCs to thymus after
application of FITC on the skin (109), and this migratory route was suggested to favour
central tolerance. The Trojan horse model, where DCs are manipulated to undertake a
migration pattern favourable for microbial spread, has been proposed for different
pathogens such as Mycobacterium tuberculosis, Salmonella and Toxoplasma gondii
(110-112).

The pace at which different DC subsets migrate can also impact immune responses.
Upon contact sensitization dermal DCs migrate with a peak arrival in lymph nodes at 1-
2 days, compared to Langerhans cells where numbers peak at 3-4 days (113-115). This
has generated the idea that even though Langerhans cells have the ability to present
antigen to T cells they may play a more regulatory role. By the time they have reached
a lymphoid compartment an adaptive response should already be initiated. However
upon exposure to exogenous TNFα the density of Langerhans cells in epidermis
decrease in a rapid mode with dramatic differences seen already after one hour (116,
117). The accumulation in lymph nodes and the role of LCs in immune-priming was
however not investigated in these studies.

The pro-inflammatory chemokines direct DCs and some of their precursors towards the
site of inflammation and also guide DCs within the tissue. These molecules and their
receptors include CCL2 binding to CCR2 (118, 119), CCL5 binding CCR5 (120, 121)
and CCL20 binding CCR6 (122). As DCs mature, they down-regulate their
responsiveness to these inflammatory chemokine pathways and up-regulate CCR7,
which responds to the ligands, CCL19 and CCL21 (123-125) that are produced by
peripheral lymphatic endothelial cells and lymph node stroma cells. These chemokines
guide DCs to downstream lymph nodes (126-128).
1.2.5 DC activation of T cells and its consequences

Upon migration to secondary lymphoid tissue DCs interact with antigen-specific T cells. This interaction stimulates T cells to become effector or memory cells but may also induce T cell death or anergy. The outcome depends on the maturation state of the DC, the dose, origin and presentation pathway of the antigen, the duration of the DC-T cell contact and the cytokines secreted during the DC-T cell encounter. The cytokines will also determine the type of effector T cells that is generated (93, 129-131). These things will ultimately skew the T cell responses so that the pathogen or situation that initially triggered the DC will be eliminated or regulated.

Naïve CD4+ T cells are generally destined to one out of four pathways upon initial recognition of presented antigen. The resulting populations are the T helper (Th) 1, Th2, Th17 or induced T regulatory cells (Tregs)(132).

Th1 cells are important both in conquering intracellular infection and in eliciting anti-tumour responses but can also induce autoimmune diseases (133-135). Their main production of cytokines includes IFNγ and IL-2 (132). IFNγ from Th1 cells functions as a positive feedback signal promoting their own differentiation. It also increases the microbicidal activity in macrophages (136), promotes isotype-switching in B cells to IgG2a (137, 138) and regulates local leukocyte-endothelial interactions (139). Th1 cells also support the induction of cytolytic activity in CD8+ T cells, their development into memory cells and the maintenance of the memory pool (140, 141).

The generation of a Th1 response is now generally believed to include three vital steps. Signal 1, being antigen-presentation, signal 2, being co-stimulation but also signal 3 where IL-12 is secreted by DCs (140, 142). IL-12 is central for the differentiation of CD4+ Th1 cells and can be induced either by stimulating a combination of TLRs or by stimulation of a single TLR in presence of type I IFNs, IFNγ, or CD40L signalling (132). In combination with antigen-presentation and co-stimulation IL-12 can also act on naïve CD8+ T cells independently of CD4+ T cell help, inducing CTLs and a memory population (140). Type 1 IFNs (IFNα and IFNβ) have a similar ability to provide CD8+ T cells with the third signal required for clonal expansion and differentiation (143). Additionally IL-12 plays an important role in innate resistance as it induces production of IFNγ in NK and NKT cells in responses against pathogens (135).

Moreover the kinetics of DC activation and subsequent IL-12 production has impact on the resulting T cell response. Activated DCs produce IL-12 within a relatively brief time window (10-18h after stimulation) and will then become unresponsive to further stimulation. Newly stimulated DCs induce strong Th1 responses while at later time points they preferentially prime Th2 cells or a regulatory T cell response (93, 144). This could be due to exhaustion of the DC in their ability to produce IL-12, which has been shown to skew responses towards a Th2 type of response (144), but also to autocrine or paracrine secretion of cytokines, such as IL-10 and TNFα, that have been shown to regulate the IL-12 effect (145-147)
Th2 cells mediate responses against extracellular pathogens but also play a role in induction of allergic diseases such as asthma. Th2 cells produce a number of cytokines that are involved in for example eosinophil recruitment (IL-5, IL-25), mast cell activation (IL-9), epithelial cell activation and proliferation (IL-9, amphiregulin), removal of helminths (IL-13) and amplification of Th2 cytokine production (IL-25)(132). IL-4 is a hallmark of a Th2 type of response. It is both produced by Th2 cells and needed for Th2 differentiation together with IL-2. IL-4 is either provided exogenously or produced in small amounts by the naïve, antigen-recognizing T cell itself leading to a positive feedback loop. It induces IgG1 and IgE class-switching in B cells (132, 137). IL-10 produced by Th2 cells suppresses Th1 proliferation and also has an inhibitory effect on DC function (148, 149).

Th17 cells are involved in immune responses against extracellular bacteria and fungi. They have also been shown to play an important role in induction of organ-specific autoimmunity. The cytokines produced by Th17 cells are IL-17A, IL-17F, IL-21 and IL-22. IL-17A is active in inflammatory responses as it induces other pro-inflammatory cytokines such as IL-6 (150). IL-17A and IL-17F are involved in recruitment and activation of neutrophils during infection with extracellular bacteria and fungi. IL-21 functions as a positive feedback cytokine and can also increase proliferation of T cells, differentiate B cells into memory cells and terminally differentiated plasma cells and promote the activity of NK cells (151). IL-22 is involved in antimicrobial defence, regeneration, and protection against damage by acting on epithelial cells and hepatocytes, where it induces acute phase reactants and some chemokines (152). TGFβ together with IL-6 induce Th17 differentiation, where IL-21 can have an amplifying effect. IL-23 is not active in the initial Th17 differentiation but later promotes survival and maintenance of Th17 function (129, 153).

A critical subset of T cells for regulation of immune responses and for the maintenance of self-tolerance are the Tregs (154). They suppress the function of other effector T cells by secretion of the inhibitory factors TGFβ, IL-10 and IL-35 and by inhibiting cell-cell contact (132). Treg cells share many cell-surface markers with other types of T cells although they have a higher expression of for example CD25 than other T cells. They are therefore generally distinguished by the expression of transcription factor FoxP3 (155). Apart from TGFβ, IL-2 is required for survival and function of Treg cells after their differentiation (155). The regulation of responses seems to occur also in the T cell to DC direction. Treg cells appear to affect DCs to down-modulate maturation (156).

Tolerance can also be achieved by induction of T cell anergy or apoptosis. This may occur if the T cell recognizes only low levels of MHC/peptide complexes, if they have a low affinity for this complex or if co-stimulation by the DCs is lacking (93).

The separation of effector responses into Th1, Th2, Th17 and Treg cells is not absolute. T cells with intermediate or modulated cytokine production have also been found (157, 158). Also within subsets further distinction can be made by function. Polyfunctional T cells, i.e. CD4+ and CD8+ T cells with proliferative capacity, producing IL-2 and IFNγ simultaneously, have been associated with protective anti-viral immunity during chronic virus infection (159). IL-10 can be produced also by Th1 and Th17 cells and
this production is proposed to function as a self-regulatory mechanism (160, 161). The cytokines produced by the different effector types are also involved in cross-regulation where for example IL-4 and IFNγ mutually suppress each other and they both inhibit Th17 differentiation (162).

1.3 DC responses to dying cells

1.3.1 Cell death

Dying cells is a natural part of the living multicellular organism. The death of a cell can be accomplished through complex programmed pathways that are initiated when the cell is no longer required or displays some sort of defect. Cell death can also be the result of more direct damage to the cell. This latter form of cell death is usually referred to as necrosis or primary necrosis and corresponds to a passive type of cell death, which is triggered by noxious stimuli such as toxins, hypoxia or extreme temperatures. It is characterized by cell swelling and loss of membrane integrity leading to leakage of cellular contents and afore-mentioned DAMPs out into the surrounding tissue, which affects neighbouring cells and induces a pro-inflammatory response in most tissues (163).

Kerr, Wylie and Currie were the first to propose the term apoptosis (from ancient Greek “falling, as of leaves from a tree”) for the event of controlled cell deletion that is active in normal cell death as well as in some pathological conditions (164). Morphologically this was characterized by condensation of the nucleus and cytoplasm with disassembly of the cell into membrane-bound vesicles, named apoptotic bodies that were then shed from the cell and taken up by surrounding cells. Based on the morphological observations it was suggested to be an active, inherently programmed phenomenon.

Since then additional features defining apoptosis or programmed cell death have emerged. Brenner, Sulston and Horvitz established the experimental model organism Caenorhabditis elegans (C. elegans) for monitoring programmed cell death of specific cells in a cell lineage during organ development and identified the first mutation of a gene participating in programmed cell death (165-167). Their work also led to the discovery and characterization of the ced genes involved in the initiation of programmed cell death (168). The gene product of ced-3 was later found to be very similar to the mouse and human protein IL-1β-converting-enzyme (ICE) that is also known as caspase-1 (169). The caspases are members of a family of proteases that exist in a pro-form and are activated by cleavage, which can occur either by triggering of an extrinsic pathway or an intrinsic pathway. The extrinsic pathway is activated by ligation of death receptors on the cell surface, such as Fas (CD95), TNF-receptor-1 (TNFR1), TRAIL receptor and DR3, and acts directly on caspases for the execution of cell death. The intrinsic pathway is triggered by intracellular stress, such as cytokine deprivation, exposure to cytotoxic compounds and DNA damage, and activates caspases via the mitochondrial release of cytochrome C (170-173). Further distinctive properties of apoptotic cells were demonstrated by Fadok et al. who identified the exposure of phosphatidylserine (PS) on the outer leaflet of the apoptotic cell and that this mediated removal by macrophages (174, 175). Internucleosomal cleavage of DNA has also been identified as a specific trait of apoptotic cells (176-178).
In vivo, apoptosis is found in scattered cells, as opposed to necrosis, which normally affects sheets of cells. The difficulty in detecting apoptosis in vivo is likely due to the rapid removal of these cells by neighbouring cells (164, 179). The term apoptosis encloses several different cellular manifestations and separately these may not necessarily apply to a dying cell. For example PS can be reversibly exposed on activated T cells (180) and caspase activation can be found also in non-lethal processes and differentiation pathways (181, 182). Also other types of cell death exist (180), however in this thesis I refer mainly to apoptosis, necrosis and secondary necrosis, where secondary necrosis is the dissolution of cells following apoptosis when the apoptotic cells are not removed by other cells. The morphology is similar to necrosis but the responses to secondary necrosis may differ from the ones elicited by primary necrosis (183).

1.3.2 Recognition and uptake of apoptotic cells

Apoptosis and the continuous engulfment of apoptotic cells by professional phagocytes are naturally occurring events in cell homeostasis. This is a complex sequence of events where many of the mediators remain to be defined although some important entities have been recognized. These include molecules exposed on, or released by the apoptotic cells, soluble bridging molecules and receptors on the phagocytes (Figure 3). Some of these molecules function as “find-me” signals that recruit phagocytes to the site of apoptosis (184). Among these mediators are the lipid lysophosphatidylcholine (LPC) (185) and nucleotides such as ATP and UTP (186). “Eat-me” signals are exposed on cells early in the apoptotic process and the most-studied signal is the exposure of PS (174), which has been coupled to immuno-modulatory effects both in vitro and in vivo (187, 188). Other entities have also been shown to be involved in the phagocytic process by mediating uptake or acting as bridging molecules. These include change in surface charge of glycoproteins or lipids at the apoptotic cell surface, the binding of thrombospondin (TSP), mannose-binding lectin (MBL) or the complement component C1q to the apoptotic cell surface and expression of intercellular adhesion molecule 3 (ICAM-3) and oxidized low-density lipoprotein (oxLDL)-like moiety on apoptotic cells (184, 189-191). The milk fat globule EGF factor 8 protein (MFGE8), the glycolipid-anchored protein T cell immunoglobulin domain and mucin domain protein 4 (TIM-4) and growth arrest-specific factor 6 (Gas6) are additional factors that have been identified as molecules binding to PS (192-196).

Not all of these markers need to be displayed in concert to induce uptake and according to the “tethering and tickling” model some are needed to tie the apoptotic cells to the phagocyte but may not directly trigger uptake, while the additional binding of PS to the phagocyte converts tethering into internalization (197, 198). An apoptotic cell also loses the ability to present “don’t eat-me” signals that repel the phagocyte, as represented by the homophilic interaction of CD31 on the live cell and the phagocyte (199).

The molecules involved in uptake of apoptotic cells have mostly been studied in macrophages, however DCs share many of the phagocytosis-mediating receptors recognized on macrophages, such as the scavenger receptors CD36 and CD91, the Mer
receptor tyrosine kinase (Mertk) and the αvβ3 and αvβ5 integrins (200-203). The e-type lectin DEC-205 has also been identified as an apoptotic cell receptor on DCs (204-206). Studies on the direct effects of the phagocytosis-related molecules on DC responses are to this date limited although tolerogenic effects have been demonstrated for some of the molecules (200, 207-210), especially for PS (188, 211-213). Targeting of DEC-205 during steady state has been shown to induce cross-tolerance, however in combination with a DC maturation stimulus, triggering of DEC-205 promotes cross-priming (188, 212-219). Other molecules associated with uptake of apoptotic cells and initiation of immune responses include the human Dectin-1, a member of the C-type lectin family that is expressed mainly by macrophages and DCs and is involved in nonopsonic phagocytosis of yeast. Dectin-1 was additionally shown to be involved in uptake of apoptotic cells and subsequent cross-presentation of cell-derived antigen to CTLs (220). Recently, the Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) was investigated for its role in uptake and presentation of apoptotic cells. The prominent expression of this receptor on IFNα conditioned DCs was associated with phagocytosis of allogeneic apoptotic lymphocytes and induction of T cell immunity against apoptotic cell-derived allo-antigen (221).

Figure 3. Interactions between DCs and dying cells that may result in non-immunogenic, anti-inflammatory responses or immunogenic, pro-inflammatory responses.
Apoptosis is a naturally occurring event and was long considered to be silent, leaving no immunological trace provided that clearance of these cells was not diminished or defective. In support of this view was data showing failure in pro-inflammatory cytokine production by macrophages and other non-professional phagocytes upon ingestion of apoptotic cells (191, 222). It was then demonstrated that apoptotic cells were not only cleared safely and silently by phagocytic cells but rather had an immune-dampening effect shown by apoptotic cell-induced reduction of pro-inflammatory cytokines such as TNFα and IL-1 elicited by LPS stimulation of monocytes (223, 224) and macrophages (187). This was partly mediated through the secretion of anti-inflammatory cytokines such as IL-10 (224), TGFβ, platelet activating factor and prostaglandin E2 (187). TGFβ was additionally shown in vivo to mediate resolution of inflammation upon instillation of apoptotic cells (225).

The immuno-modulatory effects of apoptotic cells were shown to be applicable also in DCs (209, 226-228). The immune dampening effects of apoptotic cells were in these studies however not entirely coupled to anti-inflammatory cytokine secretion in DCs. Antigenic tolerance induced by apoptotic cells engulfed by DCs in vivo have been presented in models of contact hypersensitivity (229), autoimmunity (230-232), and in other types of immune responses (103, 233, 234). The in vivo tolerogenic effect of apoptotic cells has also been addressed in responses to allo-antigen (235, 236).

Work by Blander and Medzhitov suggests that phagocytosis of apoptotic cells and phagocytosis of pathogens is distinguished at a sub cellular level by the presence of TLRs, which elevates presentation of phagocytic cargo to CD4+ T cells (237). Further work by Torchinsky et al. demonstrated that infected apoptotic cells engulfed by DCs generate both pro-inflammatory (IL-6) and anti-inflammatory (TGFβ) signals, which together induce a Th17 response (213). These data argue for the indispensable role of TLRs and recognition of PAMPs in generating an immunogenic signal.

The idea of apoptosis as inherently silent or tolerogenic has however been challenged. Immunostimulatory effects of apoptotic cells on DCs have been presented in a number of studies (220, 221, 238-241). The molecular events governing immunogenicity of apoptotic cells are largely undefined. There are however factors that have been suggested to play a role in the induction of immunity involving apoptotic cells.

HMGB-1 was initially thought to be released only from primary necrotic cells but has in recent years also been associated with immune responses induced by cells succumbing to apoptotic cell death (71, 242-244). It has been demonstrated that HMGB-1 that has been modified by reactive oxygen species (ROS), produced upon caspase activation, induces a tolerogenic response while the non-modified form induces immune responses (244). The modification of HMGB-1 or other DAMPs may therefore be a factor determining whether tolerogenic or immunogenic cell death is induced (183).

The release or exposure of HSPs, such as HSP70 or HSP90, by apoptotic cells have been suggested to be involved in their recognition and facilitated uptake, and also to
enhance antigen-presentation by DCs (245-247). Some chemotherapeutic agents, such as anthracyclins, have been shown to induce the exposure of the chaperone calreticulin that forms complexes with an endoplasmic reticulum-located protein, the disulfide isomerase ERp57, on the surface of dying tumour cells and thereby increases the immunogenicity of these cells compared to cells treated with other agents, such as mitomycin C or etoposide (248, 249).

Caspase activation has also been shown to influence the immunogenicity of cell death. In a study by Castiglioni and colleagues it was shown that a specific and protective CTL response was elicited upon vaccination of mice with apoptotic tumour cells. This was not directed towards immuno-dominant CTL epitopes as these were lost in the tumour cells upon apoptosis induction. This subversion of the epitope hierarchy was dependent on caspase activation (250). The activation of caspases may also lead to exposure of new epitopes facilitating cross-presentation. Rawson et al. demonstrated that during HIV-1 infection, caspase-generated fragments of cellular proteins from apoptotic CD4+ T cells contain a high proportion of distinct T cell epitopes that are recognized by CD8+ T cells. The frequencies of the self-reactive CD8+ T cells correlated with the frequencies of circulating apoptotic CD4+ T cells in infected individuals. This caspase-dependent cleavage of proteins, leading to efficient cross-presentation by DCs, was suggested as contributing to the immune activation seen during chronic HIV-1-infection (251). Inhibition of caspases has been shown to affect exposure of “find-me” signals (185), “eat-me” signals (185), the release of DAMPs from dying cells (242) as well as the calreticulin exposure on anthracyclin-treated tumour cells (252). Inhibition of caspases eradicated the ability of anthracyclin-treated tumour cells to induce anti-tumour responses (43).

Also the location at which the apoptotic cells and the engulfing phagocytes meet appear to be of significance for a pursuing immune response. In several mouse models demonstrating tolerogenic effects of apoptotic cells, the intravenous route of immunization was used (229, 232, 235, 236, 253). In most of the studies showing immunogenic effects of apoptotic cells, the subcutaneous route of immunization was used (43, 64, 254-256). The uptake of cells injected via the subcutaneous route is preferentially mediated by skin-DCs that migrate to lymph nodes (257, 258). The intravenous route directs the apoptotic cells to the spleen where DCs from peripheral tissue are absent (259). The displayed immune responses could be influenced by the type of DCs that are present at the different sites and by their different abilities to present antigen (204, 257, 260). However, a recent study performed in a delayed-type hypersensitivity model showed that resting apoptotic cells promoted tolerance whereas activated apoptotic cells induced immunity, both administered by the intravenous route (261). The maturation state of DCs in conjunction with apoptotic cells also play a role in the induction of immune responses and apoptotic cells have been shown both to prevent (228, 262) and to induce DC maturation (64, 241)(paper I). This would in general represent a tolerogenic and an immunogenic phenotype respectively. However mature DCs have also been shown to be involved in the induction of tolerance (103).

Taken together these things demonstrate the complexity of immune responses to apoptotic cells. The type of cell that dies, the type of apoptotic stimulus, the phenotype of the phagocytic cell, the microenvironment and the presence of pathogens are all
factors that seem to contribute to the final outcome. A signal induced in one setting may generate tolerance while being inoperative or even immunogenic in another setting. Much remains to fully determine the involvement of apoptotic cells in pathological conditions as well as their potential use in therapeutic settings. However controlling apoptotic pathways or using apoptotic and dying cells as therapeutic agents may present opportunities to increase or down-modulate immune responses in order to eradicate or diminish disease.

1.4 HIV

1.4.1 The discovery of HIV-1

Human Immunodeficiency Virus-1 (HIV-1) has caused one of the most severe epidemics in known history. Since the discovery of the virus in 1983 an estimated 25 million people worldwide have died from Acquired Immunodeficiency Syndrome (AIDS), and in 2009, 33 million people were living with HIV (www.unaids.org). Although significant progress has been made in understanding HIV transmission and pathogenesis we are still facing the enormous challenge of finding prevention and cure for this disease.

In 1981 the first reports contributing to the later definition of AIDS were presented. Symptoms of rare diseases, normally seen in immuno-compromised patients, were observed in homosexual young men in California and New York (263-265). Researchers at the Pasteur Institute in Paris and at the National Cancer Institute in the United States then independently isolated and identified the causative agent that later became known as HIV-1 (266, 267).

1.4.2 HIV-1 life cycle

HIV-1 is a lentivirus of the family Retroviridae. It is a double-stranded RNA virus carrying a genome consisting of 9 genes that encode 15 different proteins. Three of the genes encode Gag, Pol and Env polyproteins that can be further processed into individual proteins. The Gag and Env proteins constitute the core of the virion and the outer envelope. The three Pol proteins (protease, reverse transcriptase and integrase) are encapsulated within the viral particle and execute indispensable enzymatic functions of the virus. HIV-1 also encodes for six accessory proteins. These are the Vif, Vpr and Nef, that are also contained within the particle, the Tat and Rev, that are essential for gene regulatory functions of the virus, and Vpu that facilitates the assembly of the virus particle (268). The HIV-1 reverse transcriptase lacks proof-reading activity resulting in a high degree of mutation which accounts for the enormous sequence variability of this virus, where the env region of the viral genome is the most variable between isolates (141).

HIV-1 infection of a host cell begins with the binding of CD4 on the cell surface by gp120 on the envelope (269-271). This initiates a conformational change in the gp120, which enables additional binding of co-receptors, mainly CXCR4 or CCR5, which in turn leads to fusion of the virus with the cellular membrane (272-274). The nucleocapsid is then shed inside the cell and the viral RNA is transcribed to DNA by the
reverse transcriptase. The DNA enters the nucleus and is integrated in the host genome by the viral enzyme integrase. The viral genes can either remain in a dormant stage or be transcribed, upon which the viral proteins are transported to the plasma membrane where they are cleaved by the viral protease and assemble to form new viral particles that bud from the cell membrane (268)(Fig. 4).

![Diagram of HIV life cycle](image)

**Figure 4.** The life cycle of HIV-1. HIV-1 binds to the target cell via CD4 and co-receptors and fuses with the cell membrane. The reverse transcriptase (RT) transforms RNA into DNA, which is then integrated into the host-genome. The pro-virus is transcribed into new RNA, which is translated into new viral proteins that assemble at the cell membrane. A successful cycle ends with budding of virus from the cell membrane.

### 1.4.3 HIV-1 transmission and dissemination

In the majority of cases where HIV-1 is transmitted from one host to another, this occurs through the passage of the virus across mucosal surfaces during sexual contact. Other transmission routes include transfusion of HIV-1-infected blood or blood products, mother to child transmission and usage of infected needles during intravenous drug use (275, 276). During sexual exposure various factors associated with mucosal integrity and genetic constitution will influence the susceptibility and resistance to HIV-1 virus (276). The initial events of HIV-1 transmission would not be possible to study in humans *in vivo* but some knowledge is gained through studies of *in vitro* infection of mucosal tissue explants (277-279) and in macaques inoculated with simian immunodeficiency virus SIV (280, 281). It is still uncertain whether HIV-1 is transmitted as free viral particles or in a cell-bound form but it has been proposed to cross the mucosal barrier by transcytosis (the vesicular transport from one side of a cell to the other) or binding to intraepithelial DC dendrites. Also the passage of virions through intercellular spaces in the epithelium has been suggested as a way of reaching
Langerhans cells and CD4+ T cells in the underlying mucosa (282). An established infection appears to originate from a single focus of infected CD4+ T cell in the mucosa (283-285).

Upon transmission the virus is transported from the mucosa to the draining lymph node, where it infects activated CD4+, CCR5+ T cells. DCs present at the site of infection can mediate this transport by the capture of virus on C-type lectins such as DC-SIGN and subsequent migration to draining lymph nodes where the virus is transferred to CD4+ T cells (286). DCs of different subtypes also become productively infected by HIV-1 (287-293). The rate of replication is however low in DCs and their role in establishing infection appear to be mainly as carriers of HIV-1 to sites of more susceptible targets, the CD4+ T cells.

1.4.4 Pathogenic events in HIV-1 disease progression

The course of HIV-1 infection can be divided into three different phases. During the acute or primary infection, which lasts between 6 and 12 weeks, the exposed individual can either be asymptomatic or display symptoms resembling those of influenza. Primary HIV-1 infection is characterized by intense viral replication resulting in high virus titers in plasma and a decrease in CD4+ T cell numbers (294, 295). During the pursuing weeks these numbers start to recover and the viremia declines to reach a steady state level referred to as the viral set point (294, 295). The decline in viremia coincides with an increase in HIV-1 specific CTLs (296, 297). The following chronic phase may persist for years without clinical symptoms, however during this phase there is a gradual decline in CD4+ T cells. CD4+ T cell numbers below a critical level together with the impaired function of the innate and adaptive immune responses result in susceptibility to a number of opportunistic infections and the final phase of AIDS (294, 295, 298).

In HIV-1 infection a relatively small proportion of the CD4+ T cell population appears to be infected (estimated at 1 in 100 to 1 in 1000). Still, the depletion of CD4+ T cells is a hallmark of this infection. The loss of CD4+ T cells probably contributes to the impairment of CTLs and the eventual failure in controlling infection. Lysis of the infected cell due to extensive expression of viral genes is one mechanism of cell death. During the acute phase and throughout the course of the disease the gastro-intestinal (GI) tract displays a major depletion of CD4+ T cells that may be due to direct infection (299). Several of the viral proteins (Env, Tat, Nef, Vpr, Vpu, protease) exhibit pro-apoptotic entities and mediate apoptosis both in infected and non-infected cells either through death receptors or through the mitochondrial pathway (300). T cells from blood of HIV-1 infected patients are more prone to undergo spontaneous apoptosis in vitro than lymphocytes from non-infected subjects (301-304) and T cells from HIV-1 positive individuals are also more prone to activation induced cell death (AICD) (301, 305). These events have been associated with a general state of activation and the down-regulation of Bcl-2 (a negative regulator of apoptosis) (306-308) and the up-regulation of both the death receptor CD95 and its ligand CD95L in CD4+ and CD8+ T cells (309-311). The molecule PD-1 has also been identified as a negative regulator of the T cell function that is associated with apoptosis induction during HIV-infection (312, 313). The increased sensitivity to apoptosis in T cells is, as mentioned, associated
with a general, non-specific, state of activation. The destruction of the mucosal tissue in the GI-tract, originally caused by the virus, appears to be the major contributing factor to the generalized immune activation seen during HIV-1 infection, which leads to augmented viral replication and further tissue destruction. When viral replication is halted by anti-retroviral therapy (ART), the defect immune responses caused by the infection appear to, at least partially, revert back to normal (299).

1.4.5 DCs in HIV-1 infection

DCs are among the first cells targeted by HIV-1 and are also infected and subjected to the immunomodulation exerted by HIV-1, which may contribute to the pathogenesis of this virus. Langerhans cells and myeloid DCs are present within epithelial- and sub epithelial mucosal surfaces respectively and are both targets of HIV-1 upon transmission in mucosa. The migratory capacity of these cells makes them suitable vehicles for viral hijacking and further transmission to CD4+ T cells. Indeed, HIV-1 infection induces expression of CCR7 in DCs (314, 315) and increased numbers of DCs are seen in the lymphoid tissue during primary infection concurrent with a high plasma viral load (316) while DC levels in blood are decreased (317-322).

Animal studies have shown that the transmission of HIV-1 by DCs can occur either by direct infection (cis-transmission) or through binding of virus particles by the DCs without infection (trans-transmission)(323-325). This binding is mediated by C type lectins such as the mannose receptor, Langerin and DC-SIGN (326, 327). In vitro, DC-SIGN is expressed on immature and mature MDDCs (328) but has also been found on blood MDCs, mucosal MDCs and dermal MDCs (26, 329). This molecule has been shown to mediate internalization of the virus into cellular compartments protected from degradation, which enables efficient transmission to recipient CD4+ T cells (286, 330, 331). The CD4 and chemokine receptors (CCR5 and CXR4) required for productive infection are expressed on all DC subsets (327). In addition to HIV-1 receptors, the binding and internalization of HIV-1 appear to be dependent on lipid rafts (332).

During HIV-1 infection DCs exhibit modulated functions such as impaired maturation, altered cytokine profile and defective ability to stimulate T cells. These things may contribute to the increased infection and dissemination of the virus. The DCs found in lymphoid tissue during acute infection exhibit reduced levels of CD80 and CD86 (316). In later stages of infection impaired maturation of myeloid blood DCs has been demonstrated (333). The HIV-proteins Vpr and Nef have been shown to reduce expression of CD86, CD80 and HLA-DR in DCs, which also coincided with a reduced ability to induce CD8+ T cell activation (334-337).

In vitro infection of DCs has been shown to generate an altered cytokine profile in these cells (338, 339). The monitored differences compared to non-infected DCs, included increased IFNα and IL-6 in HIV-1 exposed blood DCs (338) and higher levels of TNFα and IL-1β and reduced IL-1ra in HIV-1 infected MDDCs upon LPS stimulation (339). The levels of IL-10 produced by MDDCs generated from blood of HIV-1 infected patients were higher than for controls upon LPS or CD40L stimulation (340). MDDCs productively infected with HIV-1 are able to mature but exhibit a defect ability to produce IL-12p70 upon CD40-ligation (341). pDCs have been shown to up-
regulate IFNα, TNFα and the chemokines RANTES and MIP-1α upon HIV-1 exposure (288, 342, 343). Although IFNα produced by the pDCs inhibited viral replication the virus was efficiently transmitted to CD4+ T cells, a process that was augmented by CD40 ligation (288). DC expression of co-stimulatory molecules and an accurate secretion of cytokines are required to induce a specific and efficient T cell response. Therefore the reduced maturation and the alterations of cytokine secretion likely contribute to the severe impairment of T cell function seen during HIV-1 infection. In addition decreased levels of MHC class I and CD4 have been reported in infected MDDCs (344) along with up-regulation of DC-SIGN (345), which could favour DC-T cell interactions where virus is transmitted efficiently to CD4+ T cells instead of being cross-presented or directly exposed on MHC class I for immune-recognition by CTLs. HIV-1 specific memory CD4+ T cells are preferentially infected compared to CD4+ T cells with other specificities (346). This is probably due to close interactions with HIV-1 specific CD4+ T cells during antigen presentation. The close interaction with CD4+ T cells has been shown to favour HIV-1 replication in DCs (347) and in a recent paper replication and production of HIV-1 was enhanced in DCs upon co-culture with activated CD4+ T cells but not CD8+ T cells in a cell-cell contact dependent manner (348).

HIV-1 exerts a range of dampening effects on DC function. However, DCs fully capable of initiating effective T cell responses are present in acute and early HIV-1 infection. These DCs are most likely uninfected and prime T cells by cross-presentation or direct presentation of exogenously derived HIV-1 antigen. This antigen may originate from previously infected apoptotic cells, from immune complexes or from non-infectious virus. The cross-presentation of HIV-1 antigen derived from apoptotic cells has been demonstrated to activate both CD4+ and CD8+ T cell memory responses (349, 350) and cross-presentation of infected apoptotic cells has also been shown to be more efficient than direct presentation of non-infectious or infectious virus (351). In disease progression the fully functional DCs may be outnumbered by DCs with impaired function, which could contribute to the decrease of functional T cells, however this remains to be established.

Infection with HIV-1 poses a difficult problem to the immune system. DCs that are crucial in initiating a specific T cell response also transmit the virus to HIV-1 specific CD4+ T cells, either by cis-transmission, where the DCs remain as viral reservoirs or by trans-transmission where viral particles are directly transferred to the T cells. Further insight into the mechanisms involved in DC binding, uptake, production and transmission of HIV-1 and how these things influence DC function will certainly bring us closer to strategies by which infection can be prevented and eliminated.
2 AIMS OF THIS THESIS

The general purpose of this thesis was to study the effects of apoptotic cells on DCs in absence or presence of HIV-1 and to study the adjuvant effect of apoptotic cells in vivo. The more specific aims were:

1. To determine whether the activation state of cells before apoptosis directs DC responses after uptake of the apoptotic cells.

2. To study whether apoptotic cells silence, reduce or are able to trigger DC production of the Th1 promoting cytokine IL-12p70

3. To determine whether apoptotic cells promote adjuvant effects in an HIV-1 DNA vaccine in vivo

4. To investigate whether apoptotic CD4⁺T cells influence HIV-1 production in DCs
3 RESULTS AND DISCUSSION

3.1 DC responses to apoptotic cells

Apoptosis is involved in normal homeostasis and tissue-turover and is therefore generally not considered as immunogenic. The non-immunogenic, immuno-regulatory or tolerogenic effects of apoptotic cells have been demonstrated both in vitro and in vivo (226, 228, 229, 234, 352-355). The silent or immune dampening effects do however not seem to represent the entire plethora of responses induced by apoptotic cells. There is also a growing quantity of data showing immuno-stimulatory effects of apoptotic cells (43, 64, 238-241, 356). The discrepancy of results demonstrating immuno-regulatory or tolerogenic effects of apoptotic cells with data that show immune-activation by apoptotic cells has lately been a matter of investigation. Differences in apoptotic cell entities depending on the type apoptosis induction that is being used have emerged as one determinant of apoptotic cell immunogenicity (43, 248).

The work included in this thesis has been focused on determining whether the activation state of a cell before it dies impacts DC responses and subsequent initiation of T cell responses (paper I and II). Furthermore we have investigated whether activated apoptotic cells can function as an adjuvant in an HIV-1-DNA vaccine in vivo (paper III) and also how apoptotic cell exposure affects HIV-1 infection of DCs (paper IV). These studies were initially performed as part of the pre-clinical program of a therapeutic HIV-1 vaccine, but the results may also be applicable when studying other infectious diseases, autoimmunity or transplantation tolerance.

3.1.1 Can apoptotic cells transfer immunogenic information?

Most apoptotic events occur in an apparent silent manner without a subsequent immune response (354). However there are situations where recycling of apoptotic cells and their historical code could have an impact on immune responses. In 1999 it was demonstrated by Holmgren et al. that horizontal gene transfer – a mechanism used by bacteria to develop resistance to antibiotics or to adjust in a new environment – was also detected in somatic cells upon uptake of apoptotic bodies (357). This transfer of genetic material with a pursuing gene expression was suggested to play a role in transfer of Epstein-Barr virus from B-cells to cells lacking the receptor for this virus. A potential role for horizontal gene transfer in treatment of tumours, where massive apoptosis is induced, was also suggested. Horizontal gene transfer through uptake of apoptotic cells was additionally demonstrated for HIV-1 and oncogenes (358, 359). To further speculate, this could be a mechanism by which increased cross-presentation arises upon viral infection. Even though not addressing the horizontal gene transfer mechanism, cross-presentation of antigen from virus infected dying cells have been demonstrated in vitro for several viruses including influenza (360), HIV-1 (349), measles virus (361), human cytomegalovirus (362-364), Epstein-Barr virus (365, 366), canarypox virus (240, 367) and vaccinia virus (368). Also in vivo studies have implicated that DCs mediate cross-presentation of virus-infected cells (369-371). More
direct evidence of DC processing of antigen from virus-infected apoptotic cells in natural infection was presented by Fleeton et al. in a mouse model they demonstrated transfer of viral antigen through uptake of apoptotic reovirus-infected epithelial cells in vivo and subsequent antigen-presentation to CD4+ T cells in vitro (372). It has also been shown in a murine leukaemia/HIV-1 pseudo type virus model that protective immunity can be elicited upon inoculation with infected apoptotic cells (373).

How will the immune system distinguish between apoptotic cell-derived self-peptides that should induce tolerance and pathogen-derived peptides evoking immunity, if cells and pathogenic material are engulfed by the same cell? Charles Janeway was the first to propose mechanisms for the immunological discrimination between non-self and self (41, 207). Later Blander and Medzhitov presented a mechanism for the immune system to discriminate between host apoptotic cells and microbes, when both are engulfed by DCs simultaneously. They demonstrated that MCH class II-presentation of exogenous antigen is dependent on the presence of TLR ligands within the phagocytosed cargo, which induces maturation of the phagosome and transfer of MHC class II to the cell surface (237, 374). This would mean that DCs are able to classify antigens as self or non-self at the sub cellular level and thereby preferentially present microbial peptides in the context of co-stimulation. Whether these mechanisms are engaged also in the cross-presentation pathway was not determined. It is however evident that certain apoptotic tumour cells, in absence of apparent TLR ligands, are able to elicit specific anti-tumour immune responses (43, 64, 238, 239, 356, 375).

3.1.2 Resting versus activated apoptotic cells

In many infections and inflammatory conditions there is a frequent occurrence of cells that enter apoptosis that are then phagocytosed by APCs (300, 376-379). Do these cells die in silence or do they provide information about the infection or inflammatory state that can be passed on to naïve cells? As data presented in this thesis suggest, the activation state of a cell before death will partially determine whether it is able to transfer an immunogenic signal or not. However, not only the state of activation (i.e. resting or activated), but also whether the cell is in an early or a late phase of activation could be important in predicting the immunogenicity of the dying cell. A possible scenario could be that cells that die in a late phase of activation have already performed their task and would therefore lack the ability to deliver a positive feedback signal to the immune system, while newly activated cells that die, that have not yet completed their mission, would be a loss unless they could generate a last message to the immune system which could increase signals initiated by a pathogen. Some of our data indicate that the activation phase of dying cells influence the pursuing responses. We found that recently activated apoptotic cells were more efficient in inducing DC maturation compared to cells that were activated for longer periods of time (several days) (paper I and unpublished observations). The idea that the immunogenicity of a dying cell decreases in later phases of activation can be taken further in the view of data presented by Herndon et al. Their work demonstrated that T cells dying through activation induced cell death after clonal expansion promoted tolerance by stimulating regulatory CD8+ T cells (380). The type of apoptosis induced in this system (Fas-FasL mediated) but also the time-frame during which the T cell death occurred (during a declining response and at a late phase of activation) could be factors determining the tolerogenic
response. Therefore it could be important to consider also the activation phase of cells prior to death when discussing the effects of apoptotic cells on the immune system.

3.1.3  DC maturation in presence of apoptotic cells

When DCs are exposed to PAMPs or to endogenous danger signals they up-regulate co-stimulatory molecules. To determine whether apoptotic cells are able to provide this type of signal we set up an in vitro system where we exposed monocyte-derived, immature DCs to either resting or activated allogeneic PBMCs. Resting cells were first preserved by freezing, then thawed, washed and induced to undergo apoptosis by \( \gamma \)-irradiation. Activated cells from the same donors were prepared in the same way as the resting cells but were activated through PHA-stimulation or CD3- and CD28 stimulation before they were frozen. As a measurement of DC maturation we analyzed the surface expression of CD80, CD83, CD86 and MHC class II. The expression of maturation markers clearly differed between DCs that were exposed to resting apoptotic cells, which resembled medium control DCs, and DCs that were exposed to activated apoptotic cells, which were similar to LPS treated DCs. The apoptotic cells stimulated with PHA were less efficient in maturing DCs than antibody activated cells (paper I).

The induction of apoptosis by \( \gamma \)-irradiation was previously demonstrated by morphological changes, flow-cytometry analysis of PS exposure and DNA-fragmentation on agarose gels (357, 359) and was here assessed by AnnexinV/PI staining. If apoptotic cells are not engulfed by DCs in the culture they will eventually enter a stage of secondary necrosis. This results in leakage of cell contents containing endogenous danger signals, such as ATP, HSPs and uric acid, into the culture medium. Since not all apoptotic cells in our co-cultures were engulfed, leakage of intracellular molecules was one plausible explanation to the observed DC maturation. Another was potential release of pro-inflammatory cytokines from the activated dying cells. We performed a number of experiments examining these possibilities. Necrotic cells were earlier shown to generate immune responses and to release endogenous danger signals (95, 198, 262, 381). We therefore induced necrosis by freeze-thawing in both resting and activated PBMCs to monitor the ability of these cells to induce maturation in DCs compared to activated apoptotic PBMCs. We also collected supernatants from the apoptotic cells up to the time-point where the majority of cells had entered secondary necrosis (as measured by AnnexinV/PI staining) and added these to DCs. Additionally we examined whether the time of incubation after irradiation or the freezing process influenced the ability of the apoptotic cells to induce DC maturation. Although the efficiency of apoptosis induction had been thoroughly documented earlier we also added live PBMCs to the DC cultures to examine their effect on DC maturation. Collectively the results of these experiments showed that if cells in secondary necrosis were present in the co-cultures, it was not the necrosis per se that was responsible for the DC maturation since activated necrotic, but not resting necrotic cells, were able to induce DC maturation. Also, the factors possibly released from the apoptotic cells, either cytokines or intracellular contents, could not alone (although we have not excluded the possibility that they contribute) be responsible for the DC maturation since supernatants collected from activated apoptotic cells had no detectable effect in this respect. It was rather the state of activation of the dead cells that determined the
maturation of DCs and cell-cell contact appeared to be required for the induction of DC maturation. Live, activated cells were also able to mature DCs relative to the medium control. The most efficient inducers of DC maturation however, above both activated necrotic and live cells, were the antibody activated, apoptotic cells. The time of incubation after irradiation (0-24 hours) or the freezing process did not affect their ability to induce DC maturation (paper I). In line with these data, caspase activation has earlier been demonstrated to be important for apoptotic tumour cell immunogenicity (43, 382). The type of apoptosis induction has also been demonstrated to affect the immunogenicity of apoptotic tumour cells. γ-irradiation and certain anti-cancer drugs induce immunogenic cell death while other anti-cancer drugs are less efficient in this respect (382).

As soluble factors released from the apoptotic cells did not induce DC maturation, we hypothesize that certain cell-surface molecules could be accountable for the DC responses. The stimulation of PBMCs with antibodies before apoptosis induction generated the question whether possibly residual antibodies could bind to Fc-receptors (FcR) on the DCs, thereby mediating the observed DC maturation. We therefore added the CD3 and CD28 antibodies directly to DCs or complexed resting PBMCs with CD2 antibody of the same isotype as the CD28 antibody, before apoptosis induction and co-culture with DCs. None of these actions resulted in DC maturation (Paper I), excluding that Fc-receptor interactions were responsible for the DC maturation induced by antibody activated apoptotic cells.

Ligation of CD40 on DCs is another possible factor that could influence DC maturation. The expression of CD40L on resting and antibody activated purified T cells and PBMCs was monitored by surface staining. The positive control, purified activated T cells, displayed a clear up-regulation of CD40L after αCD3αCD28 stimulation, while only a minor expression of CD40L was seen in the activated PBMCs as compared to resting cells (paper I, data not shown). The CD40L exposure seen in activated cells was reduced in both T cells and PBMCs after γ-irradiation (unpublished observations). B cells have earlier been shown to down-modulate CD40L surface expression on T cells (383, 384). The presence of B cells could explain the weak expression of CD40L observed in the activated PBMCs. This together with a very low or undetectable IL-12p70 expression in DC/activated apoptotic cell co-cultures led us to the conclusion that CD40L expression in activated PBMCs was likely not the single factor responsible for the DC maturation. However, blocking of CD40-CD40L signalling was not performed why contribution of this signal cannot be excluded.

3.1.4 DC production of cytokines and chemokines after apoptotic cell exposure

The generation of an efficient adaptive immune response includes the ability of DCs to produce cytokines. Since apoptotic cells have been reported to induce immunoregulatory- rather than pro-inflammatory cytokines in DCs (163, 226, 228, 352) the cytokine production in DC/apoptotic cell co-cultures was analyzed by multiplex analyses. We concluded from these experiments that the pro-inflammatory cytokines IL-6 and TNFα as well as the chemokine MIP-1β were clearly up-regulated in co-cultures of DCs and activated apoptotic cells compared with medium controls. We could not detect these cytokines in cultures where DCs were exposed to resting
apoptotic cells and they were less prominent in co-cultures containing activated, necrotic cells. None of these cytokines were released from the apoptotic cells per se. The cytokine analysis also included IL-2, IL-8 and IFNγ. These cytokines/chemokines could not be attributed entirely to DCs in the DC/activated apoptotic cell co-cultures as they were also present in supernatants from activated apoptotic cells alone.

IL-10 and IL-12p70 were also analyzed but detectable levels were only found in LPS controls. As IL-12p70 production by DCs is important for induction of a Th1 response (135, 385) this prompted us to ask whether the IL-12p70 mediated signal 3 could be generated as a consequence of allo-antigen-presentation to live T cells after uptake and processing of allogeneic activated apoptotic cells. Although no IL-12p70 was detected after DC exposure to activated apoptotic cells, these DCs were able to induce proliferation and IFNγ production in autologous T cells (paper I). In alignment with earlier studies (94, 386, 387) we proposed that IL-12p70 could be produced by DCs in our in vitro system as a response to up-regulated CD40L on allo-responsive, co-stimulated T cells. We therefore choose to further investigate the effect of apoptotic cells on DC ability to produce IL-12p70.

3.1.5 DC expression of IL-12p70 after uptake of apoptotic cells

Apoptotic cells have been shown to specifically exert a silencing effect on IL-12 production in APCs (390). Phagocytosis of apoptotic cells was shown in a study by Kim et al. to suppress transcription of the IL-12p35 subunit through activation of a nuclear factor named GC-binding protein in macrophages (390). As no, or very low levels of IL-12p70 were detected in DC/apoptotic cell co-cultures (paper I, paper II) it was possible that this mechanism was active also in monocyte-derived DCs exposed to apoptotic cells. Since we detected IFNγ in T cells upon encounter with DCs matured by activated apoptotic cells, we hypothesized that if the inhibitory mechanism of apoptotic cells, demonstrated by Kim et al, was present in our system, it could be reversed upon CD40-ligation. Indeed, we could show in our in vitro model that upon the addition of a CD40L transfected cell-line to DC/apoptotic cell co-cultures, DCs produced abundant amounts of IL-12p70, independent of apoptotic cell activation state. However, upon addition of live, autologous T cells to DC/apoptotic cell co-cultures, previous activation of the apoptotic cells was required for detectable IL-12p70 production (paper II). The levels of IL-12p70 were relatively low in these cultures compared to levels induced by CD40L transfected cells. The levels of IL-12p70 produced by DCs in cultures containing responder T cells should however not be regarded as absolute as T cells may bind some of the secreted IL-12p70. Even though the IL-12p70 levels detected in DC/activated apoptotic cell/responder T cell triple co-cultures were relatively low we assumed that DCs exposed to apoptotic cells were not irreversibly impaired in their ability to produce IL-12p70. It was however uncertain whether phagocytic DCs were able to produce IL-12p70 or whether this was attributed to bystander DCs. By FACS analysis we showed that DCs that had engulfed apoptotic cells were capable of producing IL-12p70. Interestingly there was a larger fraction of IL-12p70 producing DCs in the population that had engulfed activated apoptotic cells compared with the population that had taken up resting apoptotic cells. Within the phagocytic population taking up activated apoptotic cells there was also a tendency towards a greater fraction of IL-12p70+ DCs than IL-12p70− DCs. For the population engulfing resting apoptotic
cells this tendency was reversed (fig 4A and B paper II). Collectively these results show that DCs are able to produce IL-12p70 upon engulfment of apoptotic cells and that the activation state of the apoptotic cell will determine whether uptake will prepare the DC for interactions with T cells that lead to IL-12p70 production or if it will reduce the IL-12p70 producing capacity.

The CD40-CD40L signalling and the subsequently generated IL-12p70 would in turn lead to proliferation and IFNγ production in the responding T cells. There are however reports showing IL-12 independent initiation of Th1 responses (388, 389). These mechanisms were not examined in our work and can therefore not be excluded as alternative ways of Th1 induction although we found CD40-CD40L interactions and DC generation of IL-12p70 the most plausible initiator of IFNγ production in the T cells of our in vitro system.

3.1.6 What are the properties of immunogenic apoptotic cells?

In the work presented in this thesis I have suggested that the activation state of a cell before death is a contributing factor in the outcome of an immune response involving apoptotic cells. There are however many additional features of apoptotic cells that may determine their immunogenicity. One example is the way apoptosis is induced. In a paper by Obeid et al. it was demonstrated that the exposure of calreticulin on the surface of apoptotic tumour cells is a key factor in determining anticancer immune responses elicited by apoptotic tumour cells (248). This exposure was seen upon treatment with some cytotoxic agents, such as anthracyclins, but not with others, such as etoposide and mitomycin C. γ-irradiation and UVC light were also found to be good inducers of calreticulin exposure with pursuing anticancer activity (252). It was however stated that calreticulin alone was not sufficient to elicit antitumor immunity and required additional signals from dying tumour cells in order to promote DC maturation and activation (248). It could be speculated that physiologically induced apoptosis, for example by Fas-FasL interaction, do not intrinsically lead to calreticulin exposure but could in addition of other signals reach an immunogenic state. The type of cell that dies, where it dies, what type of phagocyte that engulfs it and in what type of milieu this occurs will likely also determine to what degree an apoptotic cell will elicit immune responses.

The immunogenic signal/signals delivered by activated apoptotic cells that induce DC maturation/activation remains to be determined at the molecular level. Cell-cell contact, but not phagocytosis, appears to be required for the generation of DC maturation signals (paper I and unpublished observations). CD40L is one factor that has been discussed as a possible inducer of the immunogenic signal upon DC encounter with activated apoptotic cells as this is expressed on activated- but not on resting T cells. CD40L expression has been shown to conduct immunogenicity in a delayed-type hypersensitivity model in vivo (261). In our in vitro system this could certainly account for some of the DC maturation/activation seen. However the low occurrence of CD40L on activated apoptotic PBMCs (as determined by FACS analysis) and the low or non-detectable expression of IL-12p70 by DCs upon activated apoptotic cell exposure would argue for a requirement of additional signals on the activated apoptotic PBMCs. Some of the DAMPs discussed in previous sections could contribute to the responses
seen in DCs upon exposure of activated PBMCs. However, these are many times attributed to necrotic cells, which we found were less efficient in inducing DC maturation (paper I). Calreticulin exposure is another factor possibly acting in our system. A recently described factor that could influence DC responses to activated apoptotic cells is the human Dectin-1, a member of the C-type lectin family, that in DCs has been shown to be involved in uptake and cross-presentation of cellular antigen (220). Additional work will however be required to determine the contribution of the above-mentioned factors.

A T cell response induced upon DC exposure to apoptotic cells would likely be dependent on the apoptotic cell properties. We showed that uptake of allogeneic activated, but not resting apoptotic PBMCs induced proliferation and IFNγ production in autologous T cells (paper I). In addition to these data it would be interesting to study whether other types of T cell responses can be generated upon recognition of antigen from activated apoptotic cells and whether these responses depend on the type of apoptotic cell, apoptosis stimulus, and surrounding milieu during DC uptake and presentation.

3.2 The adjuvant effect of activated apoptotic cells

DNA vaccination is a promising approach when aiming at a focused immune response towards different pathogens or tumours. DNA vaccines also have manufacturing benefits, as it is stable and relatively easy to produce. DNA vaccination can induce adaptive immune responses and has been effective in animal models, however DNA vaccines have shown low immunogenicity in human clinical trials (391, 392). Enhancing DNA vaccine potency remains a challenge and a solution to the problem could be to identify efficient DNA adjuvants. The nature of the adjuvant will determine the particular pursuing immune response that can be skewed towards cytotoxic T cell responses, T helper responses of different classes or antibody responses of different isotypes (393). The entities of an ideal vaccine depend on the target antigen. Actions of an adjuvant may comprise depot-effects but often appear to engage immune-receptors, such as the TLRs, expressed by APCs, such as DCs. In addition many adjuvants contain lipid components (394).

The development of an efficient HIV-1 vaccine is urgently needed but remains a highly challenging goal. As induction of virus-specific neutralizing antibodies has been a successful approach for many licensed vaccines, the first attempts to generate an HIV vaccine aimed at eliciting neutralizing antibody responses towards the HIV-1 Env protein (395, 396). Induction of a strong CTL response has been another approach (396), as these cells indirectly could limit viral spread by inducing apoptosis in infected cells and in addition produce soluble factors, such as β-chemokines or other less defined factors (397-399), that in different ways can contribute to the reduction of viral replication (400). Viral escape is a caveat both in therapies based on generation of antibodies and CTLs as these exert a selective pressure on HIV-1 (400). So far none of these approaches have been successful in generating sterilizing immunity towards HIV-1 infection. However, passive transfer of neutralizing antibodies has been shown to protect against SHIV infection (401-403).
A study recently conducted in Thailand using a recombinant canarypox vector vaccine for priming and a recombinant gp120 subunit vaccine for boosting, showed a tendency towards a reduced risk in vaccinated subjects (404). These are encouraging data in further search of an HIV-1 vaccine, however it remains to be elucidated exactly what type of responses that should be elicited to mount protective HIV-1 immunity. Presence of neutralizing antibodies at the mucosal level seems like an important piece of the puzzle inhibiting or limiting dissemination of the virus. An efficient HIV-1 vaccine should likely engage also cell-mediated responses but without providing new targets for viral replication. At the time of writing there are around 30 AIDS vaccine candidates in the clinical pipeline and some of these involve DNA-based strategies (www.iavi.org). In a study presented by Spetz et al. it was shown that activated apoptotic cells infected with a HIV-1LAI/murine leukaemia (MuLV) pseudo type virus were able to elicit HIV-1 specific immune responses in vivo. Immunization of mice with the infected apoptotic cells induced CD4⁺ and CD8⁺ T cell proliferation as well as IFNγ production and protected the mice against experimental HIV-challenge. Also systemic and mucosal antibody responses were elicited upon immunization (373).

3.2.1 Activated apoptotic cells provide adjuvant activity in an HIV-1 DNA vaccine

Since we observed pro-inflammatory DC responses upon exposure to activated apoptotic cells in vitro and the HIV-1/MuLV study by Spetz et al. reported immunity in mice after inoculation of activated, infected apoptotic cells, we set up a study to examine whether activated apoptotic cells possess endogenous adjuvant activity in vivo (paper III). In this study a cocktail of seven HIV-1 plasmids was mixed with ConA activated, γ-irradiated splenocytes and the combination was then used for intranasal (i.n.) immunization of mice. The vaccine was administered three times and mice were sacrificed 10-12 days after the last immunization. In view of our previous finding, where activated and resting apoptotic cells differed in their ability to induce DC maturation (paper I), we compared the adjuvant capacity of activated apoptotic splenocytes to responses induced by resting apoptotic splenocytes. The cytokine adjuvant GM-CSF was used as a positive control as it had earlier been shown to promote adjuvant activity in mice (405-407). We also used an approach where we prior to immunization transfected lymphocytes with HIV-1 p37 antigen, activated the cells and then exposed them to γ-irradiation. The adjuvant effect of the activated apoptotic cells was evaluated by measuring induction of systemic p24-specific IgG, mucosa associated HIV-1 p24- and gp160 specific IgA, and by assessing IFNγ production in splenocytes upon re-stimulation with recombinant p24 in vitro as well as by measuring proliferation after p24- and gp160 re-stimulation.

In mice immunized with HIV-1 DNA together with 10⁶ activated apoptotic splenocytes, we found significantly increased levels of systemic p24-specific IgG compared to immunization with DNA alone. These levels were similar to p24-specific IgG induced by DNA with GM-CSF. HIV-1-DNA mixed with either resting apoptotic cells or with a lower concentration of activated apoptotic cells (10⁵) or empty plasmid mixed with activated apoptotic cells did not induce significant levels of anti-p24 IgG.

To determine whether mucosal antibody responses were generated upon immunization, the content of gp160- and p24-specific IgA in faecal pellets from individual mice was analyzed. Compared to the control group immunized with HIV-1-DNA only, mice in
the group immunized with HIV-1-DNA and the higher concentration of activated apoptotic cells had significant induction of both gp160- and p24-specific IgA, which was not seen in any of the other groups, including the group where GM-CSF was used as adjuvant.

The groups immunized with HIV-1 DNA and the high dose of activated apoptotic cells or HIV-DNA and GM-CSF had low but significantly increased numbers of IFNγ producing p24 specific cells compared to the control group immunized with HIV-1-DNA only. We noted that these groups also had a tendency towards a higher IFNγ production background when a control antigen was used for re-stimulation. Increased proliferation of splenocytes upon in vitro re-stimulation with p24 or gp160 was seen in all groups, except for the group immunized with empty plasmid and activated apoptotic cells, as compared to the HIV-DNA control group. The IFNγ production background that we detected upon re-stimulation with control antigen in groups immunized with HIV-1-DNA and activated apoptotic cells or GM-CSF could be due to remaining immunization-induced antigen-presentation by APCs in the spleen when collecting these cells after the last immunization. Increased proliferation was seen also in the group immunized with HIV-1-DNA and resting apoptotic cells. This was somewhat unexpected, as we in our in vitro assays had not detected any pro-inflammatory effects by resting apoptotic cells on DCs (paper I, II). In vivo it is possible that immunization in presence of resting apoptotic cells could induce proliferation of other types of T cells than the IFNγ producing population. This however remains to be established.

Lymphocytes that were transfected with an HIV-p37 encoding plasmid then activated and exposed to γ-irradiation were used for subcutaneous (s.c.) immunization in mice two times with three weeks interval. Two immunizations s.c. were chosen based on earlier published data where s.c. immunization with HIV-1/MuLV infected cells induced immune responses (408). Mice were sacrificed 2 weeks after the last immunization. Significant levels of IgG titers were detected in serum upon immunization with activated apoptotic cells containing HIV-p37 plasmid compared both to serum from mice immunized with HIV-p37 plasmid alone or activated cells transfected with control DNA. A tendency towards a higher level of IgA was also found compared to controls. Cell-mediated responses were not analyzed after immunization with transfected apoptotic cells.

These data collectively show that activated apoptotic cells provide adjuvant activity to DNA comparable to the cytokine adjuvant GM-CSF, as measured by induction of systemic and mucosa-associated antibodies as well as cellular immune responses. In the light of these data, apoptotic cells in an activated state could be an efficient adjuvant in DNA immunization. We used HIV-1-DNA mixed with activated apoptotic cells or transfected activated apoptotic cells as the vaccine formulation. Another approach could be a vaccine inducing the apoptosis in vivo. Different virus vectors have been studied for their potential use as delivery vehicles in vaccines against tumours and infectious diseases (409-413) and some of these vectors have been shown to induce apoptosis and subsequent cross-presentation of the antigen (367, 414, 415), which could contribute to enhance the immunogenicity of these vaccines.
3.2.2  The effect of vaccination route

It is not merely the composition of a vaccine that will determine the pursuing immune response. Also the route of immunization influences the outcome of vaccination. Here we used the traditional s.c. route of immunization with transfected apoptotic cells as infected apoptotic cells earlier were shown to induce immunity by this route (408). For immunization with HIV-1 DNA mixed with apoptotic cells the i.n. route was chosen as this could have advantages in administration and has also been shown to elicit mucosal responses (416-418). The proximity to the brain however warrants for thorough safety- and toxicology studies before application in humans. Another issue arising when discussing apoptotic cells as an adjuvant is autoimmunity. In this vaccine study we used syngeneic activated apoptotic splenocytes as DNA adjuvant. These cells are a source of self-antigens that together with immune-activating properties of the vaccine could elicit autoimmune responses. We have not detected any alarming signs of induction of autoimmunity in mice or macaques (ongoing studies) after immunization with apoptotic cells although it remains to be determined whether autoantibody production is induced in our experimental system. However both preclinical and clinical studies using syngeneic apoptotic cell-based therapies have reported absence of autoimmunity and only non-severe toxicities such as local injection site reactions and flu-like symptoms (375, 419) The risk of inducing autoimmunity could also be overcome by using an allogeneic vaccine platform. The effect of activated apoptotic cells as adjuvant in human vaccines however remains a matter for further investigation.

3.2.3  The role of DCs in immunization

The requirement of DCs for the generation of cell-mediated as well as humoral responses upon mucosal immunization has earlier been shown (420, 421) and the importance of targeting DCs in DNA vaccination has also been demonstrated (391). In our study (paper III) we did not analyze the specific contribution of DCs but rather the general ability of activated apoptotic cells to function as adjuvants in DNA immunization. It is however likely that DCs play a major role in the generation of the cell-mediated and humoral responses to HIV-1-antigen that we detected upon i.n. immunization with DNA and activated apoptotic cells. To sharpen the edge of an adjuvant like the one described in paper III, it could be beneficial to study the direct role of DCs upon immunization. It would be valuable to know what type of DCs that respond to the vaccine and what type of immune receptors these DCs express. Is it DCs initially residing in peripheral tissue that take up the apoptotic cells that migrate to lymph nodes and present antigen to T cells or is it inflammatory DCs recruited upon immunization that are responsible for antigen-presentation? Further it could be important to examine whether other types of T cells are induced, in addition to the IFNγ producing T cells, and whether these will be skewed differently if the entities of the apoptotic cells are altered.

3.3  Effect of activated apoptotic cells on HIV-1 infection in DCs

DCs are present at mucosal sites where they are among the first cells to encounter invading HIV-1 particles. DCs are susceptible to HIV-infection and have been shown to host viral replication (291, 422-425). Thereby they can function as a reservoir of
HIV-1 in vivo. Depletion of CD4+ T cells is detected in HIV-1-infected patients already in primary infection. After the primary infection the CD4+ T cells are reconstituted and can remain at good levels during few or many years, before they ultimately start to decrease as the disease progresses towards AIDS. To a large extent this depletion of CD4+ T cells appears to be the result of apoptosis induction, particularly in bystander T cells (426-429). As DCs survey the tissue of the infected host they are likely to be exposed to HIV-1 induced apoptotic T cells simultaneously with virus. These apoptotic cells may therefore play a role in the initiation and quality of DC responses during HIV-1 infection. DC maturation has earlier been shown to suppress HIV-1-infection through different mechanisms including inhibition of viral fusion (430), blocking of reverse transcription (431, 432) and restriction of replication at a post-integration level (433). In vitro studies have also shown activation of CD4+ and CD8+ T cells after DC uptake of HIV-1-infected apoptotic bodies (349-351).

We found that upon exposure to activated apoptotic PBMCs, DCs matured and expressed the chemokine MIP-1β (paper I). These two events have earlier been shown to exert inhibitory effects on HIV-1-infection (433). We went on to examine the effect of dying CD4+ T cells on HIV-1-infection in DCs. We found that co-stimulatory molecules were up-regulated on DCs exposed to activated apoptotic CD4+ T cells. This occurred also when the apoptotic cells were previously HIV-1-infected. Addition of free HIV-1 virus to the cultures did not interfere with this process (Paper IV).

We also found that DCs exposed to activated, apoptotic CD4+ T cells secreted TNFα, earlier shown to promote DC maturation (434), MIP-1α and MIP-1β, binding to CCR5 and conferring potent anti-HIV-1 activity (435) and MCP-1, which has been shown to enhance mucosal IgA secretion and cytotoxic T cells responses (436). IL-10, IL-12p70 or RANTES were not detected in any of the co-cultures. The presence of free HIV-1 in the cultures did not alter this cytokine/chemokine profile.

When analyzing the p24 expression in DCs upon exposure to virus and activated apoptotic CD4+ T we could see a reduced infection frequency compared to the frequency in DCs exposed to virus alone. This effect was achieved with allogeneic as well as with autologous activated apoptotic cells. This would exclude involvement of an allogeneic effect in the reduction of HIV-1-infection which has previously been suggested to confer anti-HIV-1 activity (437). The prerequisite for maturation, secretion of cytokines and the inhibitory effect on HIV-1 infection was that the CD4+ T cells were activated before apoptosis induction. Resting, apoptotic CD4+ T cells did not promote these activities in DCs.

Conditioned medium from DCs exposed to activated, apoptotic CD4+ T cells also reduced HIV-1 infection in DCs. This effect could be blocked by addition of anti-TNFα antibody. The reduced p24 expression detected in DCs that were exposed to activated apoptotic CD4+ T cells was partially restored upon addition of anti-TNFα but this effect was less prominent than in conditioned medium. Addition of anti-TNFα also reduced the conditioned medium-mediated expression of CD86 in DCs. The reduction of HIV infection in DCs, as measured by p24 expression, could partially be explained by TNFα mediated maturation, as maturation has previously been shown to inhibit viral replication in DCs (430, 433, 438). One factor that is up-regulated upon DC
maturation and has been shown to mediate anti-HIV-1 activity is APOBEC3G (439, 440). In our system increase of APOBEC3G mRNA was detected in DCs upon exposure to activated apoptotic cells but was not promoted by resting apoptotic cells. This suggests that also DC intrinsic factors could play a role in HIV-1-inhibition upon activated apoptotic cell uptake. Functional studies in support of this have however not yet been performed.

3.3.1 Apoptosis in HIV-1 infection

As we have shown by work presented in this thesis, activated apoptotic cells are potent inducers of DC maturation and production of cytokines and chemokines known to reduce HIV-1 activity (435). Activated apoptotic cells, as opposed to resting apoptotic cells, indeed reduced HIV-1 production in DCs and we did not detect any signs of altered DC maturation or ability to produce the analyzed cytokines/chemokines in the presence of free virus (paper IV). Apoptosis, largely attributed to non-infected cells, is a hallmark of HIV-1 pathogenesis (299, 428) and can occur as a direct action of viral proteins on cell-death pathways or as an indirect result of responses to HIV-1 infection. HIV-1 envelope glycoproteins (Env) expressed on infected cells have been shown to indirectly or directly induce apoptosis and autophagy in bystander T cells (441-445). Also the HIV-1 proteins Tat, Vpr, Nef, Vpu, the protease and Vif have been shown to be involved in modulation of CD4+ T cell apoptosis, both as inducers of apoptosis in bystander cells and down-modulators of apoptosis in infected cells (429).

Activation-induced cell death (AICD) is a normal process that primes activated T cells to apoptosis to limit an ongoing immune response. This can be induced through repeated stimulation of CD3/TCR (446), triggering of CD4 alone (447) or by activation in absence of co-stimulation (448). This involves increased susceptibility to Fas-ligation, but also the TNFR1 and TRAIL-receptor can be involved (426, 449). Apoptosis in HIV-1 infection has been suggested to occur by AICD, as HIV-1-infection is associated with an activated T cell phenotype (299, 302, 450, 451). In HIV-1 infection T cells show an increased expression of Fas, increased susceptibility to Fas-mediated killing and also elevated Fas-ligand (FasL) expression, which mediates both autocrine and paracrine apoptosis upon TCR stimulation (310, 311, 452-454). T-cells from HIV-1-infected patients undergo spontaneous apoptosis to a greater extent than T cells obtained from HIV-1 sero-negative subjects and increased susceptibility to apoptosis is seen in ex vivo stimulated CD4+ T cells from HIV-1-infected patients compared to non-infected subjects (302, 310). In addition, administration of Fas-, FasL-, TRAIL/Apo 2-L- or TNF antagonists has been shown to reduce the elevated apoptosis observed in T cells from HIV-1-infected subjects (427). These observations would suggest that AICD, largely mediated by Fas-FasL interactions, play an important role in the extensive apoptosis seen during HIV-1 infection. The expression of PD-1 on CTLs has also emerged as an important determinant of apoptosis sensitivity during HIV-1-infection (313). This molecule was shown to be highly increased on HIV-1-specific CTLs (312, 455, 456) and recently it was demonstrated that PD-1-induced IL-10 production in monocytes impairs the function of CD4+ T cells during HIV-1 infection (457).
Tregs have also been proposed as an important player during HIV-1-infection and could indirectly be influenced by the major occurrence of apoptosis. Increased numbers of Tregs have been reported in tonsils and lymph nodes of HIV-1-infected individuals (458, 459). Also in the gut, which is a primary site of HIV-1 infection where early and prolonged depletion of CD4\(^+\) T cells occurs, increased numbers of Tregs were seen during HIV-1 infection (460). Increased numbers of DCs with a semi-mature phenotype that were able to induce FoxP3 expression \textit{in vitro} were found in lymph nodes of HIV-1-infected subjects (461). Decreased expression of CD80 and CD86 in DCs has also been observed in lymphoid tissue during acute HIV-1 infection (316). Whether apoptosis induced by HIV-1 contributes to the reduced activation of DCs, to the increased number of regulatory T cells seen in different tissues during HIV-1-infection and to increased viral spread however remains unclear.

DCs are important mediators of anti-viral responses and play a complicated role in HIV-1-infection, as they should be the initiators of an adaptive response against the virus but at the same time act as viral reservoirs that transfer virus to T cells. As DCs capture dying cells of the host they are likely to be exposed to virally induced apoptotic cells simultaneously with virus. The apoptosis-induction pathway, the activation state of the cell before death and the time-point and location where death occurs may all determine the immunogenicity of apoptotic cells. Whether apoptosis induced during HIV-1-infection is immunogenic or have a dampening effect on responses against the virus is largely unknown. It is also still uncertain whether activation of DCs by activated apoptotic T cells in parallel with HIV-1 exposure would function protectively or may even enhance further spread of virus. However, we suggest to make a distinction between the different phases of activation before apoptosis, as apoptotic cells in an early phase of activation are more prone to induce DC maturation/activation while apoptotic cells in later phases of activation are less potent and resting apoptotic cells fail to induce DC maturation/activation and lack adjuvant properties (paper I, III and IV). Activated apoptotic cells also reduce HIV-1 infection in DCs while resting apoptotic cells do not possess this ability (paper IV). It could be speculated that the uptake of cells that succumb to apoptosis induced by HIV-1 infection affect the quality of antigen-presentation by DCs and subsequent T cell responses in a way that shifts the focus away from an HIV-1-specific response. DC maturation/activation after exposure to activated apoptotic cells may be of relevance in transmission of cell-associated HIV-1, in the early stages of HIV-1 infection, where a majority of CD4\(^+\) T cells are lost in the GI-tract (462), as well as in chronic stages of infection where high turnover and depletion of T cells involving apoptotic mechanisms continues both in peripheral tissue and in lymph nodes (463, 464).
CONCLUDING REMARKS

Apoptotic cells have long been associated to immune-dampening or tolerogenic effects on the immune system. The view of apoptotic cells as exclusively tolerogenic is now diversifying with the emergence of data demonstrating a role for certain apoptotic cells in the induction of immunity. The immunogenicity of an apoptotic cell will be determined by a variety of factors and events including the type of apoptosis that is induced, the type of cell that is dying, the location at which apoptosis occurs, the surrounding cytokine milieu and the properties of the phagocyte engulfing the apoptotic cell. DCs play a key role both in the induction of immunity towards pathogens and in the generation of tolerance towards self-antigen. Increased knowledge on how DC uptake and presentation of apoptotic cells generates immune responses will possibly bring us closer to efficient vaccines towards challenging pathogens but also to our understanding of pathological conditions originating from infection or autoimmunity.

We have demonstrated that the activation state of a cell before it enters apoptosis is of importance in predicting whether a pro-inflammatory response will be induced in DCs exposed to these cells (paper I). The finding that activated apoptotic cells are able to induce DC activation adds to our knowledge of the DC role in immunity and could explain some of the diverse responses generated towards apoptotic cells in different studies. It could also contribute to increased efficiency of therapies where apoptotic cells may be used such as cancer-therapy involving administration of DCs that have taken up apoptotic tumour cells or induction of apoptosis by chemotherapy. The molecular entities governing the pro-inflammatory signals generated upon DC exposure to- and uptake of activated apoptotic cells are still not fully determined. Experiments are at present being conducted in our lab investigating the main contributing molecules and the intracellular signalling pathways engaged in this process.

We have also investigated the ability of DCs to produce the Th1-promoting cytokine IL-12p70 upon apoptotic cell uptake and exposure to a secondary signal. We demonstrated that activated apoptotic cells “prepare” DCs for IL-12p70 production while resting apoptotic cells conversely dampen the DC ability to produce IL-12p70 (paper II). A Th1 response is important in the defence against intracellular pathogens such as viruses. This finding further supports the view that apoptotic cells are not per se tolerogenic but may under certain circumstances initiate immunogenic signals also in the DCs that bind and engulf the apoptotic cells, which could be of relevance in development of different cell therapies. In these studies the apoptotic cells were applied to MDDCs, which may not directly correlate to DC populations present for example in skin during steady state. For further understanding of the effects induced by apoptotic cells in immunity, DCs could be isolated from tissue, rather than generated from blood, to be used in the analyses of immunogenic signals.

Furthermore we have studied the effects of activated, compared to resting apoptotic cells in vivo where the activated apoptotic cells were shown to exert adjuvant activity in an HIV-1 DNA vaccine (paper III). Whether this effect is mediated by DCs remains to be seen although we find it likely that DCs are important in generation of the responses detected upon immunization. What type of DCs that would be involved in phagocytosis
of apoptotic cells and to what sites these cells migrate would then also be interesting to study. Upon immunization with HIV-1 DNA and activated apoptotic cells we could detect systemic and mucosal antibodies as well as T cell proliferation and IFNγ production in response to HIV-1 peptides. Even though the results in an animal model may not directly correlate to potential results in human, the generation of HIV-1-specific mucosal IgA is encouraging as this isotype could have a protective effect in a human setting. A prerequisite for this is however a broadly neutralizing capacity of generated antibodies. The neutralizing ability of the antibodies generated in this study was not tested due to the low levels of mucosal IgA antibody that was extracted. In addition it would be interesting to study whether this vaccine generates other types of T cells than IFNγ-producing T cells and also whether different types of apoptotic cells would re-direct responses.

Additionally the activated apoptotic cells promoted reduction of HIV-1 production in DCs, which was partially mediated through the induction of TNFα (paper IV). DCs play a complicated role in HIV-1 infection, as they are meant to prime a T cell response against the virus and at the same time transmit viral particles to the T cells. DCs may also play a role in the removal of dead cells in HIV-1 infection where a high percentage of the CD4+ T cell population succumbs to apoptosis. We demonstrated that DCs that are exposed to newly activated, apoptotic CD4+ T cells up-regulate maturation markers and chemokines that have earlier been shown to inhibit HIV-1 infection and display an increased mRNA expression of APOBEC3G, a protein associated with reduced HIV-1 production. DC secretion of TNFα upon apoptotic cell exposure was however the most prominent HIV-1 inhibiting factor although this did not account for 100% of the inhibition. Whether APOBEC3G expression really accounts for some of the inhibition is not known. At what level in the replication cycle the virus is inhibited and whether this inhibition would have a beneficial or even a negative effect on transmission to T cells and T cell priming also remains to be seen. We concluded that activated apoptotic CD4+ T cells induce maturation and reduce HIV-1 production in DCs which distinguishes them from resting apoptotic CD4+ T cells that are unable to exert these effects. This could be of relevance when trying to understand the immuno-pathological condition associated with HIV-1 infection where a major fraction of the CD4+ T cell population is lost by HIV-1 induced apoptosis and could contribute to general activation but fail to promote HIV-1 specific immune responses. These results could also be of relevance in cell-associated transmission of HIV-1 as well as in the design of an efficient vaccine.

Whether there is life after death may still be a question open for discussion. However, in the immunological sense, I believe this view may indeed be applicable. Phagocytosis of apoptotic cells can lead to the phenomenon of cross-presentation where cell-associated, bacterial or viral antigen is presented resulting in new generations of cells through priming of both regulatory and other effector T cells. Uptake of apoptotic cells may even facilitate this process. Signals from apoptotic cells could be employed by the immune system as a feedback mechanism where dying cells would be able to increase an immunogenic signal, if the pathogen or tumour antigen triggering the initial response is not eliminated, and decrease the signal at the end of a successful response. During normal homeostasis, when inflammatory mediators and foreign or tumour antigen are absent, apoptotic cells may contribute to safety by providing self-antigen
for presentation leading to the generation of anergic or regulatory T cells. This balance may be altered during states of autoimmunity. Certain ways of inducing apoptosis during anti-cancer therapy appear to be more prone to generate immunity than others. For example the use of anthracyclins increase the exposure of calreticulin on the surface of apoptotic cells, which has been coupled to elevated anti-cancer immune responses. What trace a cell will leave after its life has ended and how this will impact living cells will probably be influenced by the way of life prior to death (active versus resting) and the cellular environment at the moment of death.

Cell death is a natural part of life and by learning more about the effects of dying cells on the immune system, in particular on DC functions, the mechanisms could be employed and extended to oppose infection, to counteract states of autoimmunity or to improve transplantation tolerance.
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