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# DENDRITIC CELLS IN IMMUNE AND GENE THERAPY AGAINST CANCER

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**DENDRITIC CELLS IN IMMUNE AND GENE THERAPY AGAINST CANCER**

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Doctoral dissertation from Karolinska Institutet, Stockholm, Sweden

Dendritic cells (DC) are extensively used for vaccine therapy due to their superior T cell stimulatory capacity. However, the modest therapeutic effects reported by most clinical trials using DC, together with the rapid developing field of DC biology suggests important considerations for improvement of the clinical outcome. The general aim of this thesis is to develop DC-based cancer vaccines thereby facilitating the translation of such therapeutic approaches to the clinic. An ancillary aim of this study is to better understand the factors that affect the apoptosis of DC particularly during their differentiation from monocyte precursors. Mature DC are effectively resistant to Fas-mediated apoptosis. One of the underlying mechanisms that mediate this resistance is the observed upregulation of Bcl-$X_L$ that accompanies DC maturation. These results provide additional support for the use of mature DC as cellular adjuvants since they not only represent a more potent T cell stimulatory population, but also may mediate a more protracted effect due to their resistance to Fas induced apoptosis.

The mode of antigen delivery is pivotal to the generation of specific immunity. In this study, a comparison of viral and non-viral gene delivery techniques to human DC derived from peripheral blood precursors or $CD34^+$ stem cells were performed. DC differentiated from both types progenitor cells were receptive to non-viral transfection using mRNA and electroporation. In addition, the number of transfected cells was comparable to what could be achieved by adeno- and retro- viral transduction. These findings encourage the use of non-viral methods for transfection of DC as problem of neutralizing antibodies and prohibitively expensive safety tests associated with viral delivery may limit their use in the clinic. Certain viruses can lead to inhibition or maturation of DC or even induce their apoptosis. Furthermore, the effect of viral transduction on cell physiology and APC activity has not been satisfactorily elucidated. Transduction of monocyte derived-DC using adenovirus (AdV) results in generation of DC that are phenotypically and functionally "activated". Taken together, the transfer of antigenic epitopes at high efficiency to human monocyte-derived DC as well as the DC-activating effects are strong arguments for their clinical application of AdV-transduced DC-based vaccines.

The final aspect of the thesis utilizes prostate specific antigen (PSA) as a model tumor antigen for generation of specific T cell response, using DC transfected with different delivery methods. Stimulation with AdV transduced TNF$\alpha$ treated DC resulted in the generation of PSA-specific T cells that predominantly produced IL-10. However, stimulation with untreated, lipopolysaccharide, or anti-CD40 treated AdV transduced DC resulted in generation of IFN$\gamma$, but not IL-10, producing PSA-specific T cells indicating that the predominant population was Th1 T cells. In conclusion, conventional antigen delivery and DC maturation methods may influence the functional characteristics of the generated DC and may produce T cell populations with aberrant functions, that in certain instances may be counter productive to the anti-tumor immune response desired with vaccination strategies.

**Keywords:** dendritic cells, immunotherapy, gene therapy

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## LIST OF PUBLICATIONS

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II. A. Lundqvist, G. Noffz, M. Pavlenko, S. Sæbøe-Larssen, T. Fong, N. Maitland and P. Pisa. Non-viral and viral gene transfer into different subsets of human dendritic cells yield comparable efficiency of transfection. Journal of Immunotherapy, 2002, 25 (6): 445-54.

III. A. Lundqvist, A. Choudhury, T. Nagata, T. Andersson, G. Quinn, T. Fong, N. Maitland, S. Pettersson, S. Pauli, P. Pisa. Recombinant adenovirus vector activates and protects human monocyte-derived dendritic cells from apoptosis. Human Gene Therapy, 2002, 13 (13): 1541-49.

IV. A. Lundqvist, A. Palmborg, M. Pavlenko, J. Levitskaya and P. Pisa. Generation of tumor specific T lymphocytes with regulatory properties by stimulation with human dendritic cells subjected to a distinct combination of activation signals. [manuscript].

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| AdV | Adenovirus |
| ADCC | Antibody dependent cellular cytotoxicity |
| APC | Antigen presenting cell |
| CAR | Coxsackie-adenovirus receptor |
| CCR7 | C-C β-chemokine receptor 7 |
| CTL | Cytotoxic T Lymphocyte |
| DC | Dendritic cell |
| FLIP | FLICE [Fas-associated death-domain-like IL-1β-converting enzyme] inhibitory protein |
| GM-CSF | Granulocyte macrophage colony stimulating factor |
| HLA | Human leukocyte antigen |
| HSC | Hematopoietic stem cell |
| HSP | Heat shock protein |
| IFN | Interferon |
| IL | Interleukin |
| IPC | Interferon producing cell |
| LC | Langerhans cells |
| LPS | Lipopolysaccharide |
| MHC | Major histocompatibility complex |
| MOI | Multiplicity of infection |
| NK | Natural killer |
| PBMC | Peripheral blood mononuclear cell |
| PSA | Prostate specific antigen |
| RV | Retrovirus |
| SMAC | Supramolecular activation clusters |
| TAA | Tumor associated antigen |
| TAP | Transporter associated with antigen processing |
| Th | T helper |
| TLR | Toll like receptor |
| Tr1 | T regulatory type 1 cells |

# INTRODUCTION

The cellular compartments of the body originate from the same bone marrow progenitor - the pluripotent hematopoietic stem cell (HSC). This cell gives rise to precursors of red blood cells, platelets, and the two main types of white blood cells, the common lymphoid progenitor (CLP) and the common myeloid progenitor (CMP).

The CMP is the precursor of macrophages, neutrophils and dendritic cells (DC). Macrophages and neutrophils of the innate immune system provide a first line defense against microorganisms by production of cytokines that will lead to inflammation[1]. These cytokines are not only important for the maintenance of innate immunity, but also crucial for further development of adaptive immunity. Upon encounter with a pathogen, immature DC become activated/mature and travel to lymph nodes where they interact with naïve lymphocytes[2]. This is the initiation of an adaptive immune response. Activated DC also secrete cytokines that are important for both the innate and adaptive immune responses[3].

The CLP gives rise to the two major types of lymphocyte, the B and T cells. B cells differentiate into antibody secreting plasma cells upon activation. T cells exist in two main classes, cytotoxic T cells (CTL) capable of killing cognate targets and helper T (Th) cells that activate other cells of the immune system such as other T and B cells and macrophages. Every single lymphocyte has a unique variant of an antigen binding receptor resulting in a pool of T and B cells with practically infinite repertoire of receptors that differ in their antigen-binding site, rendering them able to mount a specific immune response against virtually any foreign antigen. A third lineage of lymphoid cells, the natural killer (NK) cells, lack antigen specific receptors and are part of the innate immune system. They are able to recognize and kill some abnormal cells, for example certain tumor cells and virus-infected cells. They are thought to be important in the innate immune defense against intracellular pathogens.

Immunological approaches to the treatment of cancer have been explored for over a century. Monoclonal antibodies have been used for tumor immunotherapy by themselves or conjugated to toxins, cytotoxic drugs or radionuclides, which target the delivery at high dose specifically to the tumor cells[4]. Advances in the understanding of the molecular process of antigen presentation and the molecules involved in T cell activation have provided new immunotherapeutic strategies. Attempts have been made to develop vaccines based on tumor cells taken from patients and rendered immunogenic by the addition of adjuvants[5], or by pulsing autologous DC with tumor-

cell extracts or synthesized tumor antigens[6]. However, tumor cells and bacteria have evolved mechanisms that escape the immune system. In particular, tumor cells can down regulate MHC class I molecules[7], or produce immunosuppressive cytokines[8]. Thus, there is an imperative need for novel vaccination strategies that overcome these escape mechanisms.

## ANTIGEN PROCESSING AND PRESENTATION

Since the first description by Steinman and Cohn in 1973 of the lymphoid DC as a trace cell type isolated from lymphoid tissues of mice, it has become clear, that cells of this lineage are widely distributed throughout lymphoid and most non-lymphoid tissues of all mammalian species studied[9]. DC are able to engulf a wide variety of antigens and further present them in the context of major histocompatibility complex (MHC) molecules to induce an antigen-specific immune response[2; 10; 11]. These responses are critical for defense to infections and even tumors. In some instances, DC can also generate regulatory T cells[12-14] that suppress T cell function and are important for the control of immunopathological phenomena and transplant rejection[15-19] (this concept is discussed later).

DC exist in two major functionally and phenotypically distinct stages, immature and mature[10] (Figure 1). Immature DC are highly endocytic by three distinct mechanisms: 1) macropinocytosis, a process in which large amounts of extracellular fluid are taken up nonspecifically in single vesicles; 2) endocytosis mediated by binding to clathrin-coated pits and 3) phagocytosis, a process leading to ingestion of particles by attachment to receptors and subsequent engulfment;[20-27]. Receptors for antigen uptake include the Fc$\gamma$Rs CD32 and CD64[28]; the high- and low- affinity IgE receptors Fc$\varepsilon$RI and Fc$\varepsilon$RII (CD23), respectively[29]; the complement receptors CD11b and CD11c[30]; a C type lectin of mannan binding receptor[27], DEC205[31] and the scavenger receptor pair for apoptotic cells $\alpha v\beta 5$ and CD36[32].

Upon infection, pathogens may stimulate DC through the release of a variety of soluble factors, including chemokines, which promote recruitment of DC precursors, and cytokines, which promote DC activation. Proinflammatory cytokines, such as TNF$\alpha$ and IL-1$\alpha$, trigger maturation[33-36]. While immature DC have high endocytosis capacity and express low levels of MHC and costimulatory molecules, mature DC have reduced capacity for antigen uptake but exhibit a superior capacity for T cell stimulation[10].

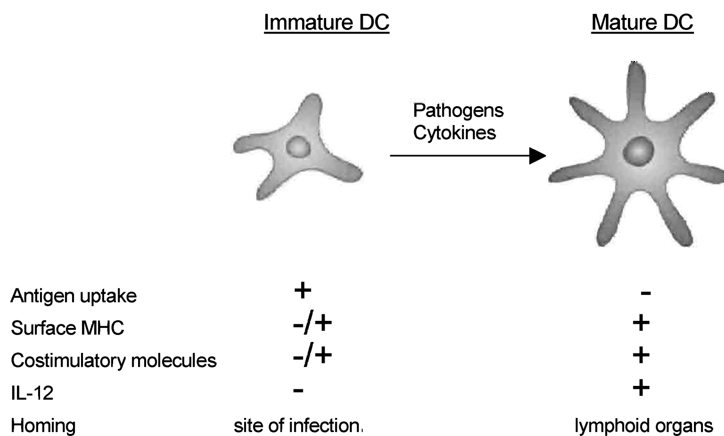


Figure 1 – DC phenotypes and features
Adapted from Banchereau J. et al. Nature, 1998, 392, 245-52

Along with increased MHC expression, DC also modify their profile of chemokine receptors that facilitate homing to lymphoid organs[37]. Furthermore, surface molecules like B7.1 and B7.2 and T cell adhesion molecules like ICAM-1 (CD54) and LFA-3 CD58 are upregulated[38]. MHC class II molecules accumulate in late endosomal compartments and lysosomes in immature DC, while in mature DC; class II molecules accumulate at the cell surface[39; 40]. The increased capacity to generate functional peptide-MHC complexes, may result in these complexes reaching the cell surface partly associated with CD86[41; 42]. In contrast, MHC class I molecules do not accumulate in the lysosomes, but are upregulated upon maturation, possibly reaching the surface in part together with class II molecules[42].

MHC class I molecules consist of two polypeptide chains, the α-chain with its three component domains and the associated β2-microglobulin (β2m), which is smaller, non polymorphic and not encoded in the MHC locus. The complete molecule has four domains, three formed from the MHC-encoded α-chain, and one contributed by β2m. MHC class II molecules, on the other hand, do not associate with β2m. It consists of a complex of two chains, the α and β chains (figure 2). The major differences between class I and II molecules lie at the peptide-binding cleft, which are more open in MHC class II molecules as compared to MHC class I molecules. The main consequence of this is that the ends of a peptide bound to an MHC class I

molecule are more substantially buried within the molecule, whereas the ends of peptides bound to MHC class II molecules are less secured within the molecule. The sites of major polymorphism are located in the peptide-binding cleft, which in the case of an MHC class I molecule are formed by the $\alpha 1$ and $\alpha 2$ domains and for MHC class II by the $\alpha 1$ and $\beta 1$ domains.

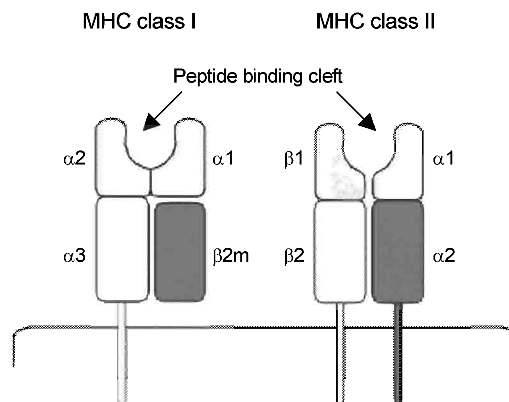


Figure 2 – Structure of MHC class I and II molecules
Adapted from Janeway C. A. et al. Immunobiology, 2001, 5th ed.

Four distinct antigen presentation pathways are described, the exogenous, endogenous, alternative and presentation via CD1 molecules. Endogenous antigens are presented by MHC class I molecules whereas exogenous antigens are generally presented by MHC class II molecules. Recent work has identified several mechanisms, referred to as the alternative pathway, by which DC are able to process exogenous microbial antigens into the MHC class I pathway [43-45] (Figure 3). Two different mechanisms have been identified for the processing of exogenous antigens by the alternative pathway, a TAP-dependent pathway where antigens are selectively transported from a specialized type of endosome called, the proteasome to the cytosol. The current view of the TAP-independent pathway on the other hand is that the antigen is hydrolyzed in endosomes and peptides are directly loaded onto MHC class I molecules.

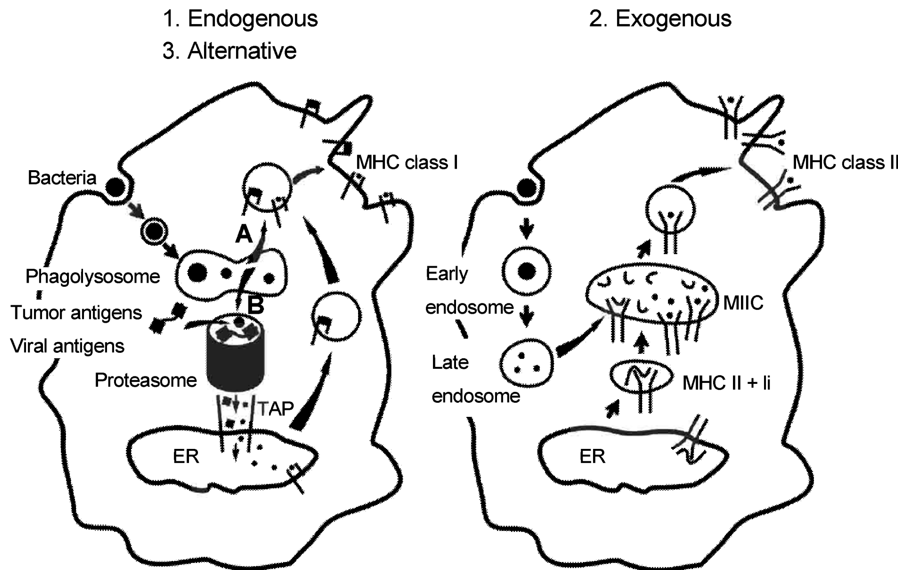


Figure 3 – The three antigen presentation pathways for presentation of proteins. The alternative pathway is divided into TAP-independent (A) and TAP-dependent (B). ER: Endoplasmatic Reticulum; TAP: Transporter Associated with Antigen Processing; li: Invariant Chain; MIIC: MHC class II rich compartments
Adapted from Mary F Lipscomb et al. Physiol. Rev., 2002, 82:97-130

In addition to upregulated MHC complexes and costimulatory molecules, maturation also drives the formation of "immunoproteasomes"[46; 47]. This form of the proteasome influences the production of peptides to be presented on MHC class I. DC are specialized to form MHC class I peptide complexes by what is called the "proteasomal" or endogenous pathway. Peptides and unfolded proteins are hindered from binding to MHC class II molecules in the endoplasmic reticulum (ER) and acidified endocytic vesicles by the invariant chain (li) which is cleaved by proteases, leaving the CLIP peptide bound to the MHC class II molecule. Peptides within acidified endocytic vesicles cannot bind to MHC class II molecules occupied by CLIP. The class II-like molecule, HLA-DM, binds to MHC class II:CLIP complexes, catalyzing the release of CLIP and the binding of antigenic peptides. Antigens presented on MHC class I molecules can activate $CD8^+$ T cells whereas antigens associated with MHC class II molecules are presented to $CD4^+$ T cells.

CD1 molecules are a family of non-polymorphic histocompatibility antigens associated, like MHC class I molecules, with β2m[48]. In fact, there are remarkable similarities between CD1 and MHC class I in their overall configuration[49]. However,

antigen processing and presentation by CD1 is different from that described above for MHC class I and class II. Antigens, presented by CD1 require uptake and intracellular processing by DC, are not peptides but rather lipids and glycolipids of both endogenous and exogenous origin[50]. Although mycobacterial lipids were first identified as CD1 ligands[51; 52], it is now clear that lipids from other microbial pathogens are able to stimulate CD1-restricted T cells[53-55]. CD1-restricted T cells are also distinct from MHC restricted T cells in that they are devoid of both CD4 and CD8, so-called double negative T cells[56]. Still, subsets of CD1-restricted T cells have been found to be either CD4 or CD8 positive[57; 58]. Nevertheless, the role of CD4 or CD8 co-receptors in the recognition of CD1-presented antigens remains unclear. Five CD1 genes (CD1A, B, C, D, E) and their protein products CD1a, b, c and d have been identified in humans. These proteins are further categorized depending on their tissue distribution into group I CD1's (CD1a, b and c) which are expressed by professional antigen presenting cells, such as DC and Langerhans cells, (LC) whereas group II CD1's (CD1d) are expressed on typically B cells, T cells, macrophages, intestinal epithelial cells and liver and also on myeloid DC. Since tumor cells have altered or increased expression of glycolipids[59], development of immunotherapy against tumors may be possible using CD1-restricted T cells.

## CELLULAR AND MICROBIAL INTERACTIONS

### T cells

Antigens that are presented by professional APC stimulate naïve T cells and promote their proliferation and differentiation. In order to recognize antigens, T cells need to establish contact with APC by forming an immunological synapse, where TCR and costimulatory molecules are joined in a central area surrounded by a ring of adhesion molecules[60] (Figure 4). At the synapse, molecules are organized in supramolecular activation clusters (SMAC). Short molecules like TCR-CD3ξ complex, CD2 and CD28 make up the central-SMAC. It is surrounded by the peripheral-SMAC, which consists of lymphocyte function-associate antigen (LFA-1) and CD45[61]. Synapses form within minutes after TCR triggering and are stable in the absence of disturbing events, but can be disrupted by cell division or death of DC. The TCR initiates a tyrosine phosphorylation cascade that triggers multiple signaling pathways within seconds upon engagement with peptide-MHC complexes. However, these early signaling events might be sufficient for triggering of effector functions, such as lysis

of target cells while functions like T cell proliferation require a sustained TCR engagement.

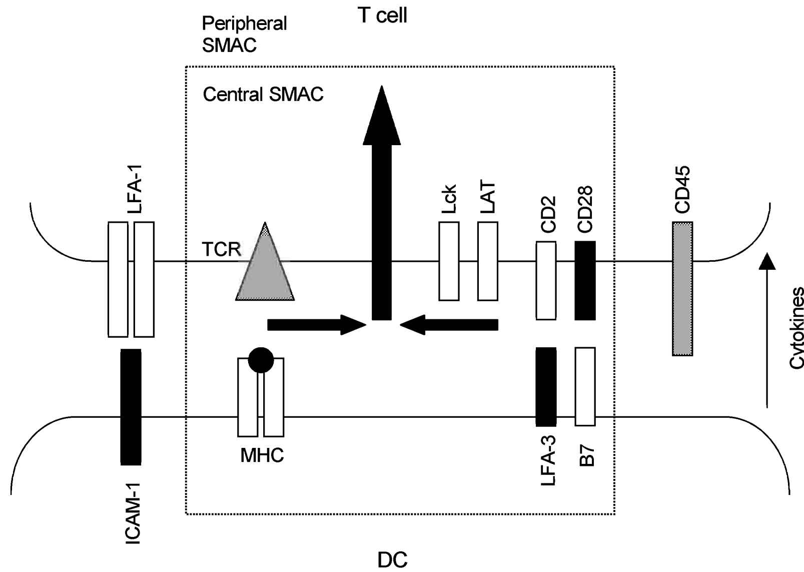


Figure 4 – Immunological synapse
LFA: Lymphocyte Function-associated Antigen; ICAM: Intercellular Adhesion Molecule; LAT: Linker of Activation in T cells
Adapted from Lanzavecchia et al. Nature Immunology, 2001, 2(6):487-92

Two different models have been proposed to explain how T cell become fully activated by sustained TCR triggering, the stable vs the dynamic model. The dynamic or the "serial triggering" model, described by the group of Lanzavecchia, illustrates that a single peptide-MHC complex can engage up to 200 TCRs[62; 63] by triggering them in a serial fashion. The serial triggering model implies that shorter half-life of TCR bound to peptide-MHC complexes are more efficient for T cell activation. The stable or "kinetic proofreading" model describes a quantitative model for T cell activation which involves a set of biochemical steps that must be completed after TCR is bound to peptide-MHC complexes for the TCR to be triggered[64; 65]. This suggests that the half-life of TCR bound to peptide-MHC complexes must be sufficiently long to enable TCR triggering. Thus, these two opposing models imply that there is an optimal half-life for peptide-MHC and TCR binding.

The strength of the immunological synapse is dependent on two factors: 1) the density of peptide-MHC complexes that initiate signals; 2) the density of costimulatory molecules that amplify the signaling process. The stability of the synapse determines how long the signaling process is sustained[61]. The duration of

TCR stimulation, together with the Th1 interleukin-12 (IL-12) or Th2 (IL-4) polarizing cytokines, determines the consequent differentiation of $CD4^+$ T cells[66].

The ability of DC to polarize T helper cells into either Th1 or Th2 phenotype is referred to as the "third signal"[67]. It is generally acknowledged that certain maturation stimuli will differentiate DC into subtypes that will favor the development of either Th1 or Th2 cells *in vitro*[3]. It is also known that certain subsets of DC preferentially secrete either Th1 or Th2 polarizing cytokines, which are discussed in subsequent sections of the thesis. T cells that receive a short TCR stimulation do not differentiate into effector cells. These CD45RA negative CCR7 positive non-polarized, lymph-node-homing cells have been identified *in vivo* as a distinct subset of central memory T cells[68; 69]. Central memory T cells are more responsive to TCR stimulation than naive T cells and they can activate DC to produce IL-12 and efficiently differentiate to effector T cells. In contrast, T cells that receive a prolonged TCR stimulation in the presence of IL-12 differentiate into IFNγ producing Th1 cells while T cells stimulated in the presence of IL-4 differentiate into IL-4, IL-5, and IL-13 producing Th2 cells. These Th1- and Th2 cells migrate to inflamed peripheral tissues where they perform effector functions and persist, in reduced numbers, as a distinct subset of effector memory cells being CCR7 negative and CD45RA negative.

As mentioned above, maturation stimuli upregulate the costimulatory molecules B7.1 and B7.2, which are transported together with MHC class II molecules to the cell surface[42]. The simultaneous upregulation of MHC and B7 molecules synergistically enhances the T cell stimulatory capacity of DC. Activated T cells trigger DC via CD40L or TRANCE, improving their T cell stimulatory capacity, boosting IL-12 production and prolonging their lifespan[70-72]. In contrast, anergic or regulatory T cells may suppress antigen presentation capacity by DC via production of inhibitory cytokines, such as IL-10 and tumor growth factor-β (TGFβ) or direct cell-cell contact via CTLA-4[73-77].

### Other cellular interactions

DC are important not only for T cell priming and regulation, but also for other cell types such as NK cells and B cells that they have an active cross-talk with. DC have been shown to regulate the antibody synthesis by B cells. Naïve B cells incubated with antigen-pulsed DC subsequently secrete IgM and IgG when cultured with an antigen-specific $CD4^+$ T cell line, whereas B cells incubated with antigen without DC, only

IgM is produced[78]. It was also demonstrated that DC provide B cells with isotype-switch signals independent of T cells but that T cell help was essential for antibody production[79]. Furthermore, DC initiate proliferation of B cells independently of CD40, as DC from CD40$^{-}$/- mice induced proliferation of B cells from both wild-type and CD40$^{-}$/- B cells. In addition, wild-type DC improved viability of B cells, but survival was reduced in the absence of CD40 expression[80].

Both immature and mature DC are able to activate resting human NK cells[81]. Immature, but not mature DC are sensitive to granule-dependent NK cell lysis[82]. It was furthermore shown that contact-dependent interactions between activated human NK cells and immature DC provides a "control switch" for the immune system, which results in either DC maturation or cell death. The mechanisms that determine the outcome between death and maturation depend on dynamics between DC and NK cell density and on the DC maturation stage. When immature DC outnumber NK cells, DC become activated and thereby resistant to NK cell lysis, enabling elicitation of T cell mediated immune responses. When NK cells are overwhelming, inhibition of DC functions is the dominant feature due to potent killing by the autologous NK cells.[83]. Similarly, differentiated CD1d-expressing myeloid DC strongly activate CD1d-restricted NK T cells, which secrete multiple cytokines important for the recruitment and differentiation of DC. As myeloid DC are important regulators of Th1/Th2 responses the regulation of myeloid DC by NK T cells controls both the transition from innate to adaptive immunity and the Th-phenotype of subsequent T cell responses[84].

Recent reports suggest that DC may also possess direct cytotoxic effector functions against tumor cells by mechanisms that are either cell-contact dependent or cytokine dependent. Mature DC induced a strong tumor growth inhibitory effect mediated by a TNF$\alpha$, in which soluble FasL and TRAIL were not involved[85]. DC were also found to be cytotoxic for several tumor cell lines through calcium-independent, TNF-, Fas-, or TRAIL-independent pathways but caspase-8-dependent manner[86]. Other studies show that DC-tumor cell contact was not required to effect tumor-cell killing by DC as apoptosis was not mediated by Fas/FasL interactions but partially by the release of nitric oxide[87]. In contrast, IFN$\gamma$ and IFN$\alpha$ treated DC exhibiting an upregulated expression of TRAIL, acquire ability to kill TRAIL-sensitive tumor cell targets but not TRAIL-resistant tumor cells[88]. Conversely, induction of apoptosis in DC by tumor-derived factors involves regulation of Bcl-2 and Bax expression suggesting that a new mechanism of tumor escape from immune recognition exists[89].

### Pathogen interactions

Majority of DC in peripheral tissues exhibit immature phenotype. After contact with microbial products or proinflammatory cytokines, immature DC convert to mature DC that drives their migration first to the lymphatic vessels and then to the draining lymph modes.

Infection by microbial or viral pathogens promotes DC recruitment and activation through a variety of mediators, such as LPS, CpG DNA, or dsRNA[90-95]. DC in the draining lymph nodes are capable of delivering different types of signals depending on the microorganisms that they have encountered. It is generally acknowledged that certain maturation stimuli will differentiate DC into subtypes that favor the development of either Th1 or Th2 cells *in vitro*[3]. In general, DC infected by viruses, intracellular pathogens or yeast, produce IL-12 and skew Th1 T cell differentiation [96-98]. Conversely, DC having encountered extracellular pathogens, including parasites or hyphae, will induce Th2 responses via an unknown mechanism[98; 99]. DC can also release IL-10 and activate regulatory/suppressor T cells after encounter with Bordetella pertussis[100]. Finally, DC can activate B cells in response to viruses and Streptococcus pneumoniae, presumably as a consequence to activation by type I IFNs, and promote immunoglobulin isotype switching[101].

There is emerging evidence that two different classes of receptors are important for the handling of microorganism by DC, one that is involved in the internalization, and the other involving activation of DC. The best ones characterized are the Toll-like receptors (TLR)[102; 103]. They are a family of innate immune-recognition receptors that recognize molecular patterns associated with microbial pathogens (PAMPs), and induce antimicrobial immune responses. TLRs are expressed on multiple cell types including DC, macrophages, and epithelial cells and they cooperate to transduce cellular activation and cytokine production by activating members of NFκB transcription factor family[102]. *In vitro* addition of dsRNA or CpG DNA to DC promotes their activation, via TLR3[104] or TLR9[103], respectively. Alternatively, infection of immature human DC with influenza virus (dsRNA) induces DC activation and stimulates IL-12 production, driving T cell polarization toward the production of Th1-type cytokines[92]. Immune evasion strategies by many pathogens are aimed at impairing recognition of infected cells or providing resistance to immune effector mechanisms[105; 106]. Many viruses are able to infect immature DC and infection by

influenza virus lead to apoptotic DC death[107]. Other viruses can also impair the acquisition and processing of antigens by DC. A more effective mechanism by which pathogens may inhibit the functions of immature DC is via interference with DC maturation, as has been shown in case of herpes simplex virus type 1 (HSV-1), vaccinia virus, T lymphotropic virus type I virus [108-111]. Another strategy is inhibition of migration from sites of antigen capture into the T cell areas of lymphoid tissues. For example, in DC infected with HSV-1, up-regulation of CCR7 is blocked, preventing the responsiveness to chemokines that direct DC migration[111].

## SUBSETS AND LINEAGES OF DC

As described earlier, DC orchestrate the immune network by interactions not only with T cells, but also B and NK cells. All these interactions cannot occur via the same cell, suggesting that there are different sets of DC that perform different functions. Two different models are proposed, the "functional plasticity model" and "the specialized lineage model". The first one describes how specialized DC subtypes might represent different activation states of a single lineage where the functional differences depend entirely on local environmental signals. The second model argues that signals determine the lineage segregation, acting earlier and the immediate precursors of the DC are already separate and functionally committed. Most likely, both these models coexist as many DC subtypes arise from separate developmental pathways and their development and function are modulated by exogenous factors.

It is clear from differences in data reported in the literature that the cellular constitution of human blood DC preparations varies considerably[112-114]. A recent study by MacDonald et.al. demonstrated the presence of five distinct subsets in human blood[115]. These subsets are divided into CD11c$^{-}$ and CD11c$^{+}$ populations where the CD11c$^{+}$ or myeloid blood DC population has been noted to be heterogeneous includes the CD16$^{+}$, CD1b/c$^{+}$ and BDCA3$^{+}$ subpopulations[116-118]. In contrast to the original "myeloid" CD11c$^{+}$ CD123 (low) DC subset, a CD11c$^{-}$ CD123 (high) "lymphoid" DC population was described[112]. Taken together, the five different subsets are: CD123 (high), CD1b/c$^{+}$, CD16$^{+}$, BDCA-3$^{+}$ and CD34$^{+}$ with marked phenotypic differences and culture requirements[115]. The different subsets were ranked by their allo-stimulatory capacity as CD1b/c >CD16 >BDCA-3 >CD123 >CD34. Importantly, CD1a has been reported to distinguish a lin- population that

acquires Langerhans cell features *in vitro*[113] This population has been reinvestigated and redefined as a CD1a$^-$ CD1b/c$^+$ population[119].

Different precursor-cell starting points have been used to generate human DC in culture. The earliest precursor known is the CD34$^+$ cell isolated from bone marrow or umbilical cord blood. During hematopoiesis CD34$^+$ HSC differentiate into common lymphoid progenitors (CLP) or common myeloid progenitors (CMP) in the bone marrow (Figure 5).

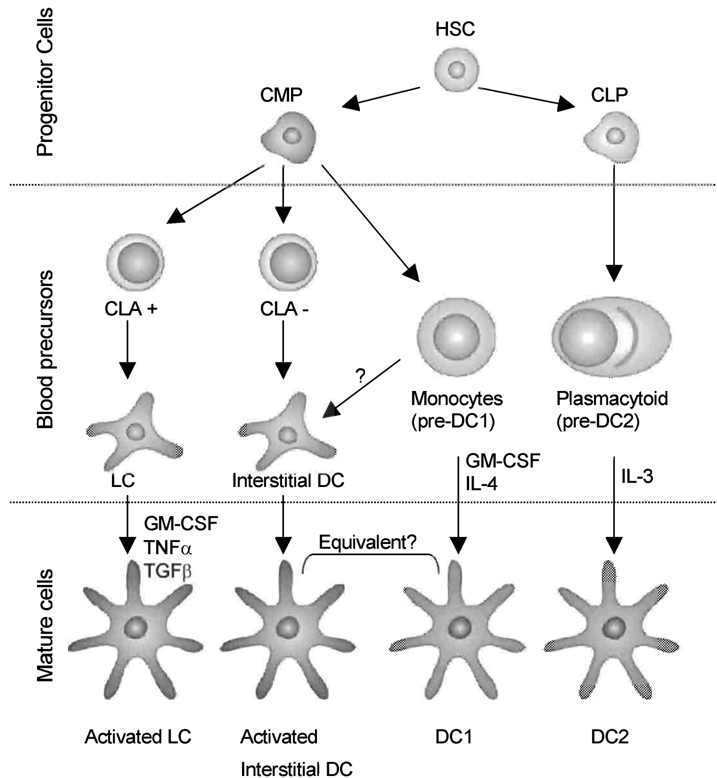


Figure 5 – DC subsets and lineages
CLA: Cutaneous Lymphocyte-associated Antigen; LC: Langerhans Cells; HSC: Hematopoietic Stem Cell; CLP: Common Lymphoid Progenitor; CMP: Common Myeloid Progenitor
Adapted from Shortman K et al. Nature Immunology, 2002, 2(3):151.61

Culturing CMP in the presence of GM-CSF and TNFα leads to two types of intermediate precursor and to two separate pathways of DC development[120; 121]. The intermediates along one of these pathways express the cutaneous lymphocyte-associated antigen (CLA[122]) and CD11c. This pathway in presence of exogenous stimulus, leads to generation of Langerhans cells and is dependent on the presence of

TGFβ[123], which is in agreement with evidence that Langerhans cells are absent in mice lacking TGFβ[124]. The second CMP-pathway leads to DC resembling interstitial DC[113], lacking Langerhans cell specific Birbeck granules and Langerhans-cell-associated antigens (Lag), langerin and E-cadherin. The intermediates along this pathway lack CLA but express the myeloid differentiation antigen CD14 and resemble blood monocytes in many respects. These two types of DC display different phenotypes and functions[121]. Interstitial DC, but not Langerhans cells, have the ability to take up large amounts of antigen by the mannose receptors and to produce IL-10, which may contribute to naïve B cell activation and IgM production[125]. Langerhans cells are specialized antigen-presenting cells that reside in the epidermis with unique migratory ability. They monitor the epidermal microenvironment by taking up antigen and transport it from the epidermis to regional lymph nodes, where they can initiate systemic immune responses.

Besides Langerhans and interstitial DC, CMP and CLP also give rise to two other types of DC precursors, monocytes (pre-DC1) and plasmacytoid cells (pre-DC2)[126], respectively (Figure 5). The pDC1 and pDC2 cells express different sets of pattern-recognition receptors[127] and show corresponding differences in reactivity to different microbial products. Pre-DC1, but not pre-DC2, express mannose receptors and CD1 molecules. Pre-DC1 differentiate into immature myeloid DC1 in culture with GM-CSF and IL-4[128-130] whereas pre-DC2 differentiate into immature DC2 in culture with IL-3. Several lines of evidence suggest that pre-DC2 are of lymphoid origin[126]. DC can be derived from the earliest T cell precursors within the thymus[131] and in humans, pre-DC2 lack expression of the myeloid antigens CD11c, CD13, CD33, mannose receptors, and express the lymphoid markers CD2, CD5, and CD7. Upon stimulation, DC1 produce large amounts of IL-12 and induce strong Th1 and CTL responses[132; 133], while DC2 produce low amounts of IL-12, and induce Th2 responses, or the generation of IL-10-producing $CD8^+$ T suppressor cells[113; 134]. Unlike IL-3 and CD40 ligand-induced DC2, which promote Th2 responses, viral-induced DC2 promote helper T cells to produce both IFNα/β and IL-10[134]. Pre-DC2 represent the key effector cells in the early antiviral innate immune response by producing large amounts of IFNα/β upon viral infection[126]. Thus, pre-DC2 are sometimes referred to as interferon producing cells (IPC).

DC generated from $CD34^+$ $CLA^+$ precursors in the presence of TGFβ resemble Langerhans cells while those derived from $CD34^+CLA^-$ precursors and human

monocytes are most similar to interstitial DC, and those derived from plasmacytoid DC precursors may be equivalent to $CD123^+$ DC found in tissues. As different subsets of DC express different TLR, certain classes of microbes that are specific for particular TLR, activate distinct DC subset. For example, CpG-containing oligonucleotides, signals via TLR9 and expression of TLR9 is restricted to plasmacytoid DC in humans.

### IMMUNITY vs TOLERANCE

As mentioned earlier, DC exist in major functionally and phenotypically distinct stages, first as immature which act as sentinels in diverse tissues. When danger is induced they become mature and move through the lymphatics to the lymph nodes where they interact with T cells, triggering an immune response, which eventually leads to eradication of the invading microbe. It is clear that DC are involved not only in the response to infections but also in the maintenance of tolerance to self-antigens[135]. Central tolerance within the thymus is mediated by thymic (medullary) DC that eliminate any self-antigen reactive T cells that develop in the thymus. Although central tolerance offers a mechanism for the deletion of autoreactive T cells, additional strategies for the peripheral tolerance of T cells specific for tissue-restricted antigen, for example, β cell specific antigens must exist[136]. Peripheral tolerance also may be passive, due to "immunological ignorance" of self-antigens because of presentation below threshold levels of TCR affinity[137; 138].

The original concept was that immature DC by expressing low levels of surface MHC and costimulatory molecules, would tolerize T cells, whereas mature DC by expressing higher amounts of surface MHC and express costimulatory molecules would activate T cells into an immune response. Thus, DC were divided into tolerogenic immature and immunogenic mature differentiation stages[139]. For example, immature DC induce T cell tolerance thereby enabling prolonged acceptance of allogeneic heart transplants[140]. However, recently several publications demonstrated that also mature DC are able to induce T cell tolerance[12; 100; 141]. Albert et al. showed that mature DC expressing costimulatory molecules are required both for inducing immunity and tolerance. In the absence of $CD4^+$ T cell help, DC matured by TNFα and PGE2 stimulate T cell proliferation, but this leads to tolerance induction, as measured by their eventual deletion[142].

These data suggest that the current model for peripheral tolerance in presence of signal 1, but absence of signal 2 needs to be modified. DC maturation seems not

critical, but instead the presence of a third signal, which is active at the DC-$CD4^+$ T cell interface is essential. This would propose that different tolerogenic and immunogenic maturation signals exist or that the tolerogenic DC acquire a maturation stage different from the classical immature and mature ones[143-145]. It has also been suggested that specialized tolerogenic DC are involved in peripheral tolerance[146; 147]. Differences between different maturation stimuli have previously been observed. For instance, stimulation with a cocktail of TNF$\alpha$, IL-1$\beta$, IL-6 and PGE2 does not result in production of IL-12[148; 149]. Other signals involved in the maturation are the CD40–CD40L cross-talk with helper T cells[150]. However, CD40 triggering alone is unable to induce IL-12 production[151]. IL-12 inducing LPS, recognized by TLR4, might be an optimal immunogenic DC activator, while the combination of microbial plus CD40 signaling might be an optimal inducer of DC maturation, at least for Th1 responses. Maturation by proinflammatory cytokines, such as TNF$\alpha$, seems to induce a unique tolerogenic stage for DC. Menges et al. showed that stimulation by TNF$\alpha$ results in expression of high levels of MHC class II and costimulatory molecules, but the production of proinflammatory cytokines remained weak. The incompletely matured DC induced peptide specific IL-10 producing T cells *in vivo* and prevented experimental autoimmune encephalomyelitis (EAE). Such IL-10 producing T cells are also termed T regulatory type 1 cells (Tr1)[141].

Based on these studies Lutz and Schuler proposed to classify such tolerogenic "MHC-high", "costimulation-high" but "cytokine-low" DC as "semi-mature" (Figure 6). Thus, treatment with TNF$\alpha$ leads to generation of semi-mature DC capable of inducing $CD4^+$ regulatory T cells. Mouse experiments indicate that "semi-mature" DC induce $CD4^+$ IL-$10^+$ Tr1 cells *in vivo*[12; 100]. The immunogenic "MHC-high", "costimulation-high" and "cytokine-high" DC are classified as "fully mature". These fully mature DC are producers of large amounts of the proinflammatory cytokines IL-12, TNF$\alpha$, IL-1$\beta$ and IL-6[141]. Regulation of self-reactive immune responses by specialized populations of regulatory T cells constitutes a major mechanism maintaining the tolerant state and avoiding autoimmune diseases.

The existence of a number of regulatory T cell populations has been established. Several populations demonstrate a characteristically low proliferative capacity *in vitro*, including $CD4^+CD25^+$ cells, Tr1 cells and Th3 cells (Table 1). The mechanisms by which regulatory T cells mediate their effect is via cell contact dependent mechanisms, such as interactions via cell surface bound TGF$\beta$ or CTLA-4

and cell contact independent mechanisms, mediated by the release of IL-10, IL-4[152-155].

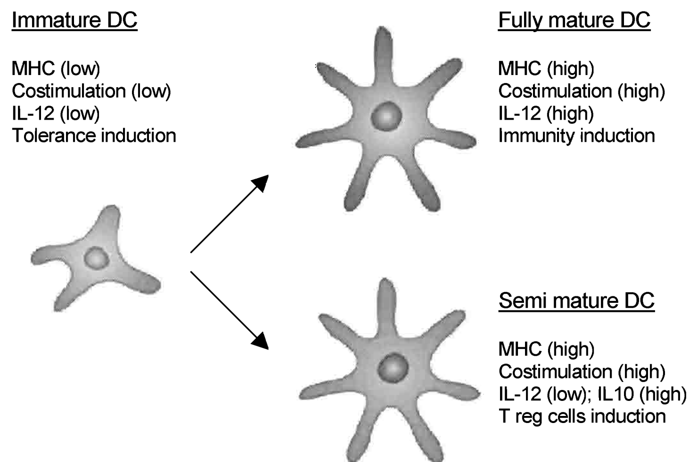


Figure 6 – Immature, fully mature & semi-mature DC

Numerous publications have demonstrated the important role of $CD4^+CD25^+$ regulatory T cells in the control of immune homeostasis and prevention of autoimmune diseases[156-159]. In contrast, tumor immunity has been successfully induced by depletion of $CD4^+CD25^+$ T cells[160-163]. Tr1 cells are characterized by their production of IL-10 and TGFβ. In addition to suppressing other T cells, they are able to suppress DC by downregulation of NFκB and costimulatory molecules, including CD40. This may lead to the production of tolerogenic DC[77; 164]. It was recently shown that human $CD4^+CD25^+$ T cells induce tolerance of $CD4^+CD25^-$ T cells by a cell contact dependent mechanism. These tolerized $CD4^+CD25^-$ T cells in turn suppressed the proliferation of other $CD4^+$ T cells via secretion of IL-10. This phenomenon was not cell contact dependent, as in the case of Tr1 cells[165]. The capability of $CD4^+CD25^+$ T cells to induce Tr1 cells, helps explain their central role for the maintenance of immune homeostasis.

The signaling pathways that determine the ability of DC to induce tolerance are becoming clear. The NFκB and CD40 pathway represents a key mechanism behind many described models of tolerance induction. For example blocking TGFβ or IL-10 enhances DC function through induction of NFκB[166]. B cells in which CD40 is blocked, induce antigen-specific tolerance in recipient animals[167; 168]. Therefore,

blocking either CD40 or NFκB will have similar consequences for the induction of tolerance. It was also shown that expression of OX40L by DC leads to enhanced T cell responses to antigen[169]. Importantly OX40 can break established tolerance, whereas most other costimulatory targets, such as CD28 and CD40, favor induction of immunity or tolerance[170]. Expression of the glucocorticoid-induced TNF receptor (GITR) by $CD4^{+}CD25^{+}$ T cells has been shown to be important to overcome the suppression[171; 172].

Table 1 – Summary of different regulatory T cell types and their mechanisms.

| Cell type | Generation | Function | Production | Mechanism | Refs |
|---|---|---|---|---|---|
| $CD4^{+}CD25^{+}$ | Naturally occurring | Inhibition of autoimmune responses | IL-10 | Contact dependent inhibition, IL-2 inhibition, CTLA-4, membrane TGFβ, Ag non-specific | 155; 160; 173-177 |
| Tr1 | Stimulation in presence of IL-10 | Suppress Th1 & Th2, inhibit colitis | IL-10 & TGFβ | Ag specific, contact independent suppression | 141; 178-181 |
| Th3 | Oral administration | Inhibit autoimmune responses | IL-10 & TGFβ | Ag specific suppression | 182-184 |
| Anergic | Develop in absence of costimulation | Lack of proliferation in response to stimulation or IL-2 | IL-10 | Non-specific IL-10 mediated suppression via APC | 77; 185-189 |

## REGULATION OF APOPTOSIS

Apoptosis is a genetically controlled process, characterized by distinctive morphological and biochemical alterations. The complex apoptotic process can be divided into several stages: 1. Delivery of an apoptosis-inducing signal. 2. Biochemical transduction and amplification of the death signal. 3. Execution of apoptosis, which involves cleavage of different substrates by effector molecules, termed caspases. 4. Resolution phase, when apoptotic cells are engulfed and degraded by phagocytes. Two principle pathways illustrate apoptosis: 1. The death receptor pathway, where ligation of Fas by Fas ligand (CD95L) results in FADD-mediated

recruitment of caspase 8 to the receptor complex is involved. 2. The mitochondrial pathway, which involves triggering of cytochrome C release by various forms of cellular stress. In the death receptor pathway in contrast to CD95L, the TNF-related apoptosis-inducing ligand (TRAIL) may bind to 5 different receptors. Of these, TRAIL-R1 and R2, as well as the CD95 receptor, are characterized by functional cytoplasmic death domains. In contrast, TRAIL-R3 is a membrane-anchored truncated receptor and TRAIL-R4 lacks a functional death domain[190-192]. It was suggested that they might act as decoy receptors by competition for limited amounts of the ligand.[190; 191].

Many of the proteins encoded by the Bcl-2 gene family are predominantly localized to the outer mitochondrial membrane. Two such proteins, Bcl-2 and Bcl-$X_L$ have been shown to be key regulators of apoptosis[193; 194]. They inhibit swelling of the mitochondria and thereby block the cytochrome C leakage[195]. On the other hand, Bax (Bcl-2 associated X protein) has been shown to reside in the cytoplasm and respond to various stimuli by migration to the mitochondria and cause cytochrome C leakage by inhibiting Bcl-2 function[196]. Both Bcl-2 and Bcl-$X_L$ form heterodimers with Bax, thereby inactivating it[197; 198]. Bcl-$X_L$ is not only membrane-associated but also located in the cytosol[199; 200]. Therefore Bcl-$X_L$, seems more flexible and capable of binding Bax more easily than Bcl-2 and thus blocking apoptosis more efficiently. A recently identified molecule able to inhibit death receptor-mediated apoptosis is FLIP (FLICE [Fas-associated death-domain-like IL-1β-converting enzyme]-inhibitory protein), which appears to block death receptor signaling by preventing caspase 8 activation[201] at the death-inducing signaling complex (DISC)[202].

## ANTIGEN LOADING STRATEGIES

Gene therapy strategies have two technical prerequisites: (1) efficient introduction of genetic material into the target cell and (2) expression of the transgene at therapeutic levels. For optimal generation of immune responses against tumors, DC-based vaccines should be applicable to patients with different HLA types, induce both $CD8^+$ and $CD4^+$ T cell responses and target a wide range of tumor antigens. Furthermore, unwanted autoimmune responses should be avoided. The level of gene transfer into immune effector cells has been limited, and partly thought to account for the weak outcome obtained by cancer gene immunotherapy. Therefore, vector design is one of the most critical areas for future research.

### Virus mediated antigen loading

Genetically modified recombinant viruses are highly efficient vectors for delivery of genetic material into DC and a large range of viruses, such as adenovirus[203-205]; retrovirus[205-208]; herpes simplex virus[209]; vaccinia virus[110; 210]; influenza virus[211] and alphavirus[212] has been used as vectors for delivering tumor antigen into DC. For some of these viruses as high as over 90% transduction efficiency can be reached[205; 208; 209; 211; 213].

DC transduced with two of the most commonly used viral vectors for gene therapy: retrovirus (RV) and adenovirus (AdV) have been shown to present both MHC class I and class II antigens and stimulate tumor-specific $CD8^+$ and $CD4^+$ T cells.[214-217]. Recombinant RV are widely used and well characterized vectors for pre-clinical setting and clinical gene therapy trials[218; 219]. A major disadvantage of RV is that they only infect dividing target cells[220]. This creates a problem since non-dividing, easy-to-obtain monocytes are generally preferred as precursors for generation of DC. However, several groups have attempted to bypass this problem by transducing proliferating $CD34^+$ hematopoietic progenitors and then allowing subsequent differentiation along the DC lineage[207; 221; 222]. In contrast, AdV can be used for transduction of monocyte- as well as $CD34^+$ derived DC, resulting in high transgene levels[204; 205; 208; 213; 223]. Yet, there are disadvantages with both RV and AdV mediated gene delivery. RV are limited for clinical use as it is difficult to obtain high titers. This is however not a problem for generation of AdV. In contrast, *in vivo* use of AdV is possibly limited by pre-existing antibodies[224; 225].

For development of virus transduced DC vaccines, safety concerns like vector toxicity or impeded DC function upon transduction limit their use. The biological effect of the transduction on DC varies between viruses. Loss of viability, inhibition of maturation, or impairment in T cell stimulation has been observed for DC transduced with viral vectors derived from vaccinia virus[210; 226] and herpes virus[111; 209]. However, no alterations in DC viability, phenotype, maturation, or function have been reported for DC transduced with other viral vectors derived from retrovirus[206], HIV-1 lentivirus[227], and adenovirus[213; 223]. Moreover, activation, maturation, and enhancement of T cell stimulatory capacity have been observed for DC transduced with influenza[92] and adenoviral vectors[205; 214; 228; 229]. For these reasons, non-viral

gene delivery systems for DC-based vaccines could provide a more attractive approach with clinical perspectives[230].

### Non-virus mediated antigen loading

Non-viral gene transfer techniques offer a safer alternative to virus-mediated gene transfers and have several important advantages; 1. Only the gene of interest is transcribed without immunological interference from viral proteins; 2. There is no associated risk of recombination associated with replication-deficient viral vectors; 3. Insertion of foreign DNA or sequence alterations of genomic DNA is not likely due to the transient nature of gene transfer; 4. DNA can be produced in large quantities and is very stable; 5. This approach is not restricted to proliferating cells or particular cell type. A number of different methods including peptide, protein or loading with whole tumor cells as well as transfection by RNA and DNA have been developed.

Peptides, mostly MHC class I restricted, obtained after acid surface elution of tumor cells, or by affinity purification of the MHC-peptide complex[231; 232] were applied for loading onto DC to induce immune responses[6; 233; 234]. The main advantage of using a peptide for DC loading is to that only epitope-specific immune responses are generated and thus minimizing the possibility of inducing autoimmunity. The clinical usefulness of this approach is the recent demonstration of "epitope spreading", where immunity is generated against related but distinct tumor antigens[235; 236]. Furthermore, there is no requirement for availability of tumor tissue or cells and a more accurate monitoring of immune responses is possible. However, the knowledge of tumor epitopes is a drawback. Moreover, efficient and durable anti-tumor immunity requires adjuvants or MHC class II restricted epitopes for generation of $CD4^+$ T cell help[150; 237; 238]. A further limitation is that peptide-loaded DC vaccines are only applicable to MHC-matched patients. Also, tumor escape from immune recognition is more likely to occur since only a single target needs to be lost[239-241].

Loading with protein instead of peptide in the case where the epitope has not been identified expands the applicability to patients who are not candidates due to the MHC-restriction. Direct loading through co-culture of protein with DC or by liposome-mediated fusion[242] has been shown to be efficient means of delivery for MHC class I presentation of soluble proteins by macropinocytosis[243]. Furthermore, proteins internalized by DC through macropinocytosis may be processed and

presented through both MHC class I and class II pathways[244]. Several studies showed that mouse DC loaded with soluble protein through macropinocytosis stimulated antigen-specific CD8$^+$ T cells *in vitro* and induced the generation of antigen-specific CTL *in vivo*.[45; 203; 245; 246].

FcR-mediated internalization of antigen-IgG complex is yet another method used to deliver proteins into DC[247-249]. Proteins internalized via this FcγRI are processed and presented by MHC class I molecules[247; 248] while delivery of proteins through FcεRI leads to MHC class II processing and presentation[250]. Other receptors by which antigen uptake is highly efficient consists of mannose or DEC-205 receptors and heat shock proteins (hsp). Antigens internalized through this pathway are processed and presented mainly by MHC class II molecules[22; 251; 252]. Some HSP (e.g., gp96 and hsp70) deliver proteins for processing and presentation by MHC class I molecules[253-255].

Recently it has been shown that DC transfected with mRNA amplified from tumor tissue or *in vitro* transcribed from plasmid DNA can stimulate CTL against tumor antigens[256; 257]. RNA transfection of DC is applicable in case of limited availability of tumor material[257; 258]. Drawbacks for the use of RNA for loading may include instability of RNA and greater labor intensity[259; 260]. Although RNA can be transfected directly into DC without any transfection reagent[256; 258], transfection is usually carried out using liposomes such as DOTAP. Electroporation is a well-established technique for *in vitro* gene delivery into a variety of mammalian cell types[261; 262]. Advantages of electroporation include the lack of size constraints on the transgene and minimal need for complex DNA manipulation, high reproducibility and ease of performance[263]. Although electroporation generally achieves only transient transgene expression, stable integration of the genetic material into the host genome of a small proportion of the cell population does occur[264]. Recently, it was shown that human DC can be transfected by RNA electroporation[208; 265; 266].

Loading of DC with DNA has the advantage of expressing the antigen within the cell and therefore the antigen can be processed and presented through the MHC class I pathway. In general, the generation of DC by transfection with tumor antigen DNA remains difficult, mainly due to limitations in DNA delivery techniques. However, two new approaches for delivery of DNA into DC have been developed to improve the transfection efficiency. One approach used a novel 33-amino acid cationic peptide CL22 to condense plasmid DNA carrying the antigen to be

expressed[267]. DC transfected with CL22-DNA complexes stimulated antigen-specific autologous T cell responses including IFNγ secretion and CTL activation *in vitro* and *in vivo* against a model antigen Influenza A virus nucleoprotein. The second approach used particle-mediated bombardment, which involves coating microparticles with plasmid DNA and projecting them on to a target tissue by an electrical discharge or gas pulse device. However, the major hurdle of this technology is the apparent lack of long-term gene expression *in vivo* and the need for repeated injections to achieve long-term immunization.

Since immunogenic tumor antigens have not been identified for most tumor types, several approaches of using antigens from whole tumor cells have been developed. Different sources such as apoptotic, necrotic or killed tumor cells have been used as tumor antigens to load DC. This concept offers several advantages. It is technically simple and, it does not require the use of fresh, live tumor cells or establishment of tumor cell lines, or the identification of antigens. In addition, loading with whole tumor cells or components generates both $CD4^+$ helper T cells and $CD8^+$ CTL. Conflicting data when comparing the immunogenicity of tumor cell lysates, apoptotic, and necrotic tumor cells exists. For example, DC loaded with apoptotic tumor cells were more efficient in cross-priming autologous $CD8^+$ T cells than DC loaded with tumor cell lysates[268]. However, two other studies reported an equally efficient generation of protective antitumor immunity *in vivo*[269; 270]. Furthermore, it was shown that only DC that phagocytosed necrotic tumor cells acquired a mature phenotype as indicated by the upregulation of the maturation-associated markers CD83 and DC-LAMP as well as by the elevated expression levels of CD86, HLA-DR, and CD40[271].

## AIMS OF THE STUDY

The major aim of this work is to better understand DC biology for generation of DC-based cancer vaccines and further translate this knowledge from the laboratory to the clinic. Specific aims of this thesis were:

- To investigate the susceptibility of DC to Fas-mediated apoptosis in the course of maturation.

- To establish viral and non-viral antigen delivery methods for transfection of human DC.

- To compare these different antigen delivery methods in monocyte- and $CD34^+$ derived DC.

- To investigate the alterations in the antigen presenting function of human monocyte- and $CD34^+$ derived DC upon viral transduction.

- To generate and characterize antigen specific T cell responses induced by DC, subjected to the different antigen loading methods.

## RESULTS AND DISCUSSION

This section of the thesis begins with reasoning why to use DC-based vaccination and important parameters to take into consideration when designing a clinical trial. These first two chapters are followed by discussion of our original findings.

### WHY DC-BASED VACCINATION?

Current immunotherapeutic treatment modalities against cancer using cytokines, antibodies, tumor antigens and adoptive T cell transfer are limited due to several reasons. The therapeutic use of IL-2 has demonstrated antitumor effect in at best 20% of patients. However, considerable systemic toxicity affecting practically all organs of the patients was observed[272; 273]. The activity of other cytokines as single agents was tested for IL-4, IL-6, IL-7, IL-12 but the clinical response rate was negligible with the possible exception of IL-12 that is still under investigation[274-276]. Monoclonal antibodies are the most rapidly expanding class of pharmaceuticals for treating cancer with five approved antibodies in US[277]. Antibody therapies at the present appear to have a greater efficacy in diffuse malignancies like leukemias or tumors of limited mass, as penetration into bulky tumors is a problem. More recently, adoptive transfer of cytotoxic T-lymphocytes (CTL) shows clinical promise[278-280]. It was described that treatment with tumor specific T cells following a non-myoablative conditioning regimen lead to regression of patients' metastatic melanoma as well as onset of autoimmune melanocyte destruction[281]. Nevertheless, it is laborious to adapt methods for *in vitro* expansion of large quantities of tumor specific CTL.

Increasing interest has been attracted by vaccination with tumor antigens in the form of a peptide, protein or by naked plasmid DNA. This is performed together with adjuvants, in which play critical roles in determining the quantity and quality of the immune response. These modes of vaccination depend on proper *in vivo* antigen presentation by activated DC. Since cancer patients are often immunocompromised due to several mechanisms[282-289], more attention has lately focused on using *ex vivo* conditioned antigen loaded DC as cancer vaccines. Culture *ex vivo* restores the antigen presenting and costimulatory function of DC from cancer patients. DC are the most potent antigen-presenting cells and the unique cell type capable of activating $CD4^+$ and $CD8^+$ naïve T-lymphocytes leading to induction of primary immune response[10; 290-292]. APC such as B cells and macrophages are typically incapable of inducing such primary responses. DC derive their stimulatory potency from high

constitutive and upregulated expression of MHC class I, MHC class II and accessory molecules as CD40, CD54, CD80, CD86 and T cell activating cytokines, all directly or indirectly involved in the antigen presentation and by providing the essential secondary signals for the initiation of the primary immune response[233]. Additionally, long-lasting tumor immunity, which is a pre-requisite for successful tumor eradication, has been observed in patients vaccinated with antigen loaded DC[148; 293]. These unique characteristics combined with developments of *in vitro* methods for generation of large numbers of DC from $CD34^+$ HSC or PBMC make DC a suitable candidate for immunotherapy to combat infectious and malignant diseases[294-297].

## CONSIDERATIONS IN DC-BASED IMMUNOTHERAPY

Although the results from earlier published clinical trials DC are encouraging, DC vaccination is at an early stage, and several parameters need to be improved (figure 7). Variabilities in antigen loading, administration of DC or the source of DC make it difficult to compare immunological and clinical results. The observation that not all patients have tumor regression, which could be due to inferiority of the vaccine or the immune status of the patient, suggests that there is a place for improvement.

In published clinical trials, DC were usually administered at 2–4 week intervals, and at doses between 1–50 million. *In vitro* studies of T cell activation by DC predict that higher numbers of DC administered more frequently would provide superior and more durable TCR triggering and thus promote T cell priming and polarization[61]. Certain, preliminary results show that some patients who had a partial response to DC therapy after the initial 4 injections experienced further tumor regression after 4 additional vaccinations[298]. However, frequent stimulation may also promote activation-induced death of T cells[299]. One should also be aware that as well as of the risk that repeated immunization with DC poses the theoretical risk of autoimmunity, particularly when targeting shared tumor

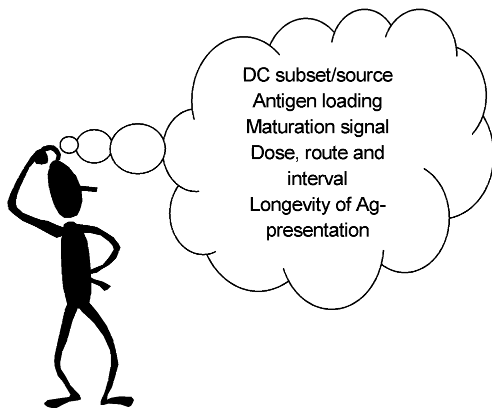


Figure 7 – DC-based vaccination; parameters to improve

antigens[137]. These questions cannot be answered in experimental animal models cannot, but the near future will provide answers to these questions in several clinical trials.

### Culture conditions

FLT-3 ligand and GM-CSF represent two key growth factors that have been harnessed to generate and activate DC both *in vivo* and *ex vivo*[300]. Protocols for generation of DC for clinical use have to circumvent the utilization of xenogeneic serum components in cell cultures. The initial clinical protocols incorporated bovine serum in the culture media[6]. These proteins could interfere with processing and presentation of the antigen of interest. In addition, foreign serum products might function as activators of DC, rendering them less permissive to antigen uptake. To avoid this, and to be compliant with current requirements of regulatory agencies in Sweden, we have avoided the use of foreign serum products in the generation and manipulation of DC. In our studies, this modification does not significantly alter the viability or phenotype of the DC (data not published).

### Source of DC-progenitor

With the progress in development of DC vaccines is and the translation of this strategy into the clinic, generation of large numbers of DC is required. Protocols for isolation of DC precursors and subsequent differentiation into mature DC have been developed. Two such sources of precursors are $CD34^+$ bone marrow or cord blood cells or peripheral blood monocytes. The literature on DC derived from these different precursor cells is confusing. In a recent study it was reported that, the capacity to stimulate allogeneic T cells as well as autologous memory T cell was higher with $CD34^+$ derived DC compared to monocyte derived DC[301]. Other studies show however, that DC from both precursor cells are equally potent with respect to morphology, phenotype, antigen uptake and presentation[302; 303]. Nevertheless, for reasons of logistical convenience and ease for patient, monocytes are the preferential source for clinical use and the majority of clinical studies to date have been carried out with *ex vivo* generated monocyte derived DC[304].

In line with this, we have adapted counter-flow elutriation technique for separation monocytes from cancer patients[305]. With this method, based on total number of monocytes harvested after leukapheresis, a recovery of more than 40% of

DC after 7 day in vitro culture can be obtained. Typically, up to $500x10^6$ DC can be generated. Elutriation technique has a further advantage since it doesn't activate monocytes, as is the case with plastic adherence or bead-selection. These immature DC could furthermore be differentiated into mature DC i.e. TNFα treatment (figure 8).

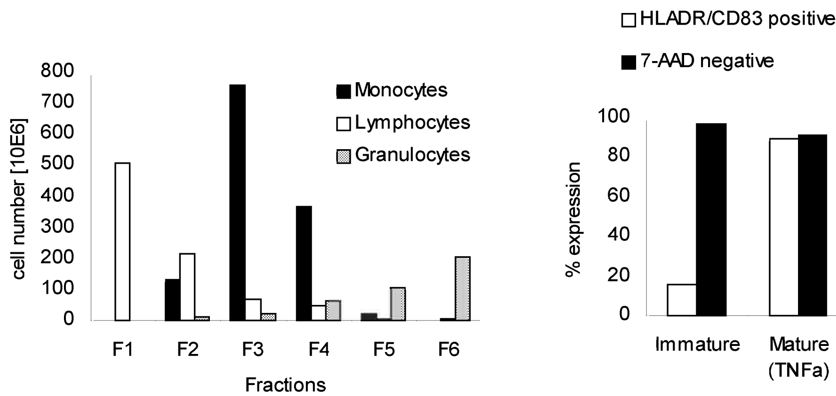


Figure 8 – Monocyte elutriation and DC culture
Adamson et al. 2002

## Peptides vs Whole antigen

Results obtained in animal studies and clinical trials confirm that protein or peptide antigen-pulsed DC are capable of inducing an antigen-specific CTL response resulting in strong protection to viruses and tumors and, in some instances, regression of established tumors[6; 245; 306-315]. Most peptide-based studies used MHC class I restricted epitopes. However, concomitant generation of sufficient and relevant T-helper activity in the form of IFNγ secreting Th1 cells is critical for effective and long-lasting anti-tumor immunity. This requires that the priming antigen contain MHC class II helper epitopes that induce strong tumor-specific Th1 responses. Additional strategies that would provide both MHC class I and class II epitopes leading to a diverse immune response involving many clones of $CD4^+$ T cells and CTL are needed. Antigen delivery systems, such as RNA transfection, viral transduction, loading with tumor lysate or apoptotic bodies or fusion of DC and tumor cells have all been utilized in clinical trials and represent approaches by which polyvalent antigens associated with the tumor may be directed for presentation by professional APC [203; 259; 270; 316].

### Mature vs Immature DC

Generally, in earlier clinical trials of DC cancer vaccines, immature DC were used[316-318]. However, lately the general view is that mature DC are more suitable for generating immunity in the clinical setting. It has been shown that intranodal injection of peptide loaded immature DC does not lead to significant immune response, compared to the intranodal injection of peptide loaded mature DC in the same patient[319]. Normally, the outcome of cross-presentation by immature DC is CTL tolerance[14; 73]. Further advantage of using mature DC relates to our finding that DC treated with either TNFα or LPS are resistant to Fas-mediated lysis **(paper I)**. This might prolong their half-life *in vivo* and in that way their efficacy.

Recent findings suggest that also mature DC, expressing high levels of MHC and costimulatory molecules but low levels of cytokine production, in particular IL-12, are able to induce tolerance[139]. It was reported that lymphocyte stimulation with DC that had been prior maturation stimuli, such as TNFα, lead to induction of peptide-specific IL-10-producing T cells[141]. Our results show that AdV transduction together with TNFα, but not LPS or anti-CD40, treatment results in generation of IL-10 producing antigen specific T cells, extends the view that it is absolutely imperative that proper activation signal is delivered **(paper IV)**.

### RESISTANCE TO APOPTOSIS

The clinical efficacy of DC-based tumor vaccines is very much dependent on the survival potential of the DC *in vivo*. On administration, a fraction of these DC undergo apoptosis due to stress related mechanisms. The remaining cells persist and are responsible for priming an immune response. However, after engagement of T cells, DC probably undergo T cell induced apoptosis. Cytolytic activity of T cells is primarily mediated through the Fas-Fas ligand (CD95-CD95L) and perforin-granzyme pathways[320]. Fas is the most important physiological receptor initiating death through the recruitment of FADD and caspase 8, but other members of the TNF receptor pathway, including TNFR1 and TRAILR, mediate their activity also through FADD[321-324]. In conjunction with the fact that DC express high levels of the human intracellular serine protease inhibitor (PI) 9, which inhibit the activity of granzyme B,[325] the most likely pathway of T cell induced DC apoptosis appears to be via Fas engagement.

Some authors found that maturation of DC facilitates their survival and thus has an anti-apoptotic utility[326; 327]. Conflicting reports exist on this issue describing either immature monocyte-derived DC to be sensitive[328] or resistant[329] to Fas-ligation. However, little is known about the underlying mechanisms. Alterations in culture conditions, mainly the presence of bovine serum, might explain some of the conflicting findings. We investigated effects of TNF$\alpha$ and LPS on the expression of apoptotic molecules during differentiation and maturation of DC under serum-free conditions, and its correlation to the sensitivity to apoptosis by the Fas mediated pathway. Our results show that in serum-free conditions immature DC were more susceptible to Fas-mediated apoptosis compared to mature DC although both populations expressed comparable levels of Fas **(paper I)**.

To investigate the reason for the noted difference, we further examined the expression of several proteins from the Bcl-2 family and FLIP. While Bcl-2 was moderately upregulated, a more pronounced upregulation of Bcl-$X_L$ was observed during maturation. Meanwhile, Bax and FLIP expression remained unchanged. Contradictory observations exist in the literature also on this latter point. It has been reported that CD40L and TRANCE upregulate Bcl-$X_L$ but not Bcl-2[330; 331], and that FLIP is responsible for the increase in DC survival[329; 332-334]. Furthermore it was reported that immature DC harvested on different days of culture exhibited similar levels of FLIP, while they differed in their susceptibility to Fas-induced apoptosis, suggesting that in addition to FLIP, other regulatory mechanisms might also account for the resistance of DC to death ligands, such as proteins from Bcl-2 family[332]. Moreover, many of these studies are performed analyzing the mRNA levels. In our study, protein levels were analyzed since mRNA transcription may not always truly represent the protein quantity. As we have demonstrated, the level of FLIP is strongly upregulated in DC in the presence of serum. Yet, in our study it was noted that Bcl-$X_L$ rather than FLIP, was the key regulatory protein associated with apoptosis of DC cultured in the absence of serum. In line with this, it was demonstrated in a mouse prostate cancer model that DC transduced with Bcl-$X_L$ exhibit higher resistance to apoptosis compared to their untransduced counterparts. Furthermore, mice vaccinated with these DC showed significant inhibition of tumor growth[335]. Our findings are probably more pertinent to DC to be utilized in clinical therapy in clinical therapy, since DC generated in the presence of animal sera may have artifactual traits induced by naturally occurring growth factors in such sera.

## ANTIGEN DELIVERY METHODS IN DIFFERENT DC SUBSETS

### Virus mediated delivery

While morphology, phenotype, antigen uptake and presentation characteristics are largely similar among $CD34^+$ cells and monocyte derived DC, subtle differences in their properties may render one of these populations better suited as cellular adjuvants for immunotherapy[302; 303]. To address this issue, we compared the phenotypic and functional characteristics as well as the transfection efficiency of DC from both precursor sources. Precursor populations were harvested from the same patient, differentiated into DC and compared after transfection with different viral and non-viral delivery systems.

We compared two of the most commonly used viral vectors for gene therapy, RV and AdV, for their ability to transduce DC **(paper II and III)**. Both types of DC were susceptible to AdV transduction. However, $CD34^+$ derived DC were less targeted by the AdV. The Coxsackie-adenovirus receptor (CAR) is thought to be a salient adhesion molecule expressed by cells permissive to adenoviral transfection. Very low levels of CAR are expressed on $CD34^+$ progenitors and monocyte derived DC[336; 337]. An alternative receptor in the form of the integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$, makes them permissive for AdV infection[338-340]. The expression of these integrins is low on $CD34^+$ HSC[341], while monocyte derived DC have significant level of these molecules[336]. Among the patients evaluated, approximately 20% of their $CD34^+$ derived DC were transduced, while ≥95% of the monocyte derived DC were transduced. Nevertheless, the need of high MOI and the associated production of neutralizing antibodies or induction of an immune response against AdV components *in vivo* may limit their clinical use[342]. It has been reported however, that repeated intramuscular administration of AdV vectors is not hindered by the presence of neutralizing antibodies in the serum[343]. Moreover, application of *ex vivo* transduced DC might circumvent the problem of administration of free virus. Alternatively, anti-AdV immune response might act as an adjuvant to prime anti-transgene immunity, as has already been observed for AdV protein in the context of non-DC targeted vaccination strategies[344].

With RV, transduction of $CD34^+$ derived DC resulted in 40% transfection while monocyte derived DC were not susceptible to these vectors. Thus, AdV vectors and monocyte derived DC together represent the most realistic approach to

immunotherapy since abundant numbers of APC obtained relatively easily and readily with AdV vectors would allow administration of cellular vaccines at high dose/kg body weight with the possibility of multiple vaccinations. While retroviral based vectors allow stable, long-term expression of the transgene, in the context of immunotherapy, this is largely superfluous since the APC exerts their effect only transiently. Moreover, the paucity of $CD34^+$ progenitors imply smaller yield of DC in the final product generated *ex vivo* and thus, smaller doses of cells/kg body weight and a limited number of vaccinations that may be achieved. This points overall to less robust immune responses.

### Non-virus mediated delivery

The previous part of the study compared two common viral delivery vectors for antigen delivery. While they offer a feasible and efficacious way of delivering antigen, this system may not be applicable due to safety regulations/testing and production costs. To circumvent clinical problems associated with viral vectors to introduce genes into DC, we next evaluated the possibility to transfect DC using non-viral techniques **(paper II and IV)**. Of all the methods evaluated, mRNA electroporation lead to transfection of DC of both subsets with a significant frequency, while other techniques studied like gene-gun, liposomal transfection resulted in barely detectable transgene expression. In our hands, the percentage of transgene expression after mRNA transfection was comparable to what was achieved after AdV mediated gene transfer **(paper II)**. The advantage of our study, compared to similar published studies preformed, is that comparison between the different gene transfer techniques is performed in DC from both origin isolated from the same patient, excluding the misinterpretations caused by the natural variability between different individuals. We also compared the non-viral methods of tumor antigen loading with PSA as model antigen. With this system, comparable expression was achieved using AdV transduction, mRNA electroporation and also after liposome-mediated protein loading **(paper IV)**.

### Functional consequences of antigen delivery methods

In a comparison of antigen delivery systems, one of the aspects is the level of expression of the transgene and the other is the effect on DC function. Although similar efficiency of transgene expression was achieved using viral and non-viral antigen delivery methods, the functional capacity of the transfected DC could still

differ between these two approaches. Previous studies have reported that vaccination with DC, transfected *ex vivo* to express tumor antigens, could generate potent T cell responses.[203; 207; 223; 258]. However, none of the studies have addressed the question whether the inherent immunogenicity of viral antigens or the physical treatment associated with the various vectors augments or suppresses the effect of transfection with respect to cell physiology and activity. Moreover, some viruses induce cell death, inhibition of maturation and impairment in T cell stimulation[111; 209; 210; 226]. Therefore, we next investigated the ability of antigen loaded to produce cytokines, their stimulatory capacity, survival potential and ability to generate antigen specific T cells.

In **paper III**, we show that transduction of monocyte derived DC using AdV results in generation of DC exhibiting phenotypically and functionally a mature phenotype, with high expression of CD83, IL-12, enhanced allo-stimulatory capacity and ability to resist Fas-induced apoptosis. This enhancement was also correlated with the increase in nuclear levels of NFκB. As discussed earlier, AdV vectors are limited for several reasons. However, adoptive transfer of DC transduced *ex vivo* with AdV vectors can serve to evade difficulties with neutralizing antibodies against AdV surface components or even serve as adjuvants to enhance immune response to the encoded tumor antigens. The combined effect of gene transfer of antigenic epitopes at high efficiency into human monocyte derived DC together with the *in vitro* adjuvant effects, are strong arguments to use AdV-transduced DC-based vaccines. In contrast, AdV transduced $CD34^+$ derived DC did not acquire the same "mature" phenotype as AdV transduced monocyte derived DC. However, a slight increase in stimulatory capacity was detected in AdV transduced $CD34^+$ derived DC. On the contrary, RV transduced $CD34^+$ derived DC showed decreased stimulatory capacity in high stimulator:reponder ratios.

The effect of the physical treatments, such as electroporation and lipofection involved on DC were investigated and compared to virus mediated gene transfer. Although electroporation represents an unrefined and non-specific method of antigen delivery the viability after electroporation of both types of DC were always greater than 80% **(paper II)**. The viability was not affected after liposome-mediated protein delivery. In addition, both electroporation and liposome-mediated protein delivery did not significantly alter the stimulatory capacity or ability to produce the cytokines IL-10 or IL-12 **(paper IV)**. Thus, the "DC priming" effect observed with AdV vectors

was not detected in this system raising the requisite for additional DC maturation factors in conjunction with such non-viral delivery systems.

Although AdV, electroporation and liposome-mediated protein delivery results in comparable transgene expression and none of these methods suppress the DC function the next question was whether these DC stimulated antigen specific T cells. Therefore, we investigated the generation of antigen-specific T cell responses using DC loaded with PSA as a model system **(paper IV)**. Mature DC have a greater ability to recruit naïve T cells compared to immature DC on a per cell basis. Therefore, antigen was loaded onto immature DC that have greater endocytic ability followed by TNFα treatment resulting in an APC with optimal antigen content and T stimulatory capacity. Co-culture of autologous T cells with DC loaded with recombinant protein or mRNA transfection resulted in comparable frequency of PSA-specific IFNγ-producing T cells. It can be conceived that endocytic uptake of the recombinant PSA protein would lead to a predominantly MHC class II associated presentation and RNA transfection depend on intracellular synthesis of the antigen would lead to a predominantly class I associated antigen processing. The comparable frequency of both $CD4^+$ and $CD8^+$ PSA-specific T cells by both of these methods thus raises interesting issues about the mechanisms by which the two pathways of antigen processing cross-talk in a the professional APC. Surprisingly, stimulation with AdV transduced TNFα treated DC resulted in generation of IL-10 producing PSA-specific T cells **(paper IV)**. It is not clear whether this effect resulted from unique properties of the transgene or the AdV backbone. However, if TNFα was excluded or substituted with LPS or anti-CD40 as maturation signal, AdV transduced DC induced IFNγ, but not IL-10, producing PSA-specific T cells were generated.

To analyze the underlying mechanisms of why IFNγ production in these T cells was impaired, we next dissected the differential stimulated DC populations. Transduction with AdV alone resulted in high production of IL-12, which is essential for induction of Th1 responses. These AdV transduced DC became "fully mature" and induced IFNγ producing antigen specific T cells. Additional treatment with TNFα rendered these DC "semi-mature" as the IL-12 production was downregulated and IL-10 production was increased. These DC generated IL-10 producing T cells. These observations reiterate our reasoning that no single strategy for antigen transfer is universally applicable to all malignancies and situations, or can be extrapolated from one scenario to another without serious investigations. It is established that

chronic activation of $CD4^+$ T cells in the milieu of high levels of IL-10 gives rise to T regulatory cells, themselves producers of IL-10 and/or TGFβ[13; 180]. Furthermore, it was shown, that shortly after stimulation DC prime strong Th1 responses due to high production of IL-12, whereas at later time points, the same cells become "exhausted" in their cytokine production and preferentially primed Th2 T cells[345]. Thus, commonly applied techniques of antigen delivery in combination with commonly used maturation stimuli interfere with DC maturation and the distinct combination of serial stimulation with AdV and TNFα, renders DC "exhausted" or tolerogenic. In all cases, antigen-loaded DC populations were comparable with regard to surface phenotype and stimulatory capacity. The results from **paper IV** imply that monitoring of DC-based vaccine preparation with commonly applied methods, such as phenotypic analysis and proliferation stimulatory capacity, to verify the quality of DC is inadequate. Determination of the biological capacity of the DC to produce cytokines, in particular IL-10, is more informative.

## CONCLUDING REMARKS

To complete the overall objective of this thesis two major questions were addressed: 1) the susceptibility to Fas-mediated apoptosis during DC differentiation **(paper I)** and 2) what methods of antigen delivery are applicable to DC and how do they modulate DC function **(paper II-IV)**.

Our results showed that mature DC are more resistant to Fas-mediated apoptosis than immature DC. This resistance correlates with an increase in expression of Bcl-$X_L$. Thus, mature DC should not only be used for the reason that they are superior in stimulating immune responses and home to lymphoid organs, but also because they resist Fas-induced apoptosis. However, it should be noted that our finding is based on the monitoring of apoptosis in immature and mature DC treated with anti-Fas antibody. This should be further evaluated with T cells expressing FasL. These effects are true for treatment with TNFα and LPS. If other stimuli will generate Fas-resistance of DC and whether other mechanisms, such as perforin-granzyme or TRAIL induced apoptosis will have similar effect remains to be elucidated.

The second question was divided into three parts: 1) what methods of antigen delivery are applicable to human $CD34^+$ and monocyte derived DC **(paper II)**, 2) how do such delivery methods modulate DC function **(paper III)** and 3) can DC subjected to these delivery methods be used to generate antigen specific T cells **(paper IV)**.

Results from our studies show that DC of both origins were permissive for virus-mediated gene transfer and mRNA electroporation, but not to any of the DNA delivery methods analyzed. In addition, the level of protein expression after liposome-mediated protein delivery of monocyte derived DC were comparable to AdV and mRNA electroporation.

We further show that none of the different non-viral delivery methods studied impeded DC function. However, AdV transduction of monocyte derived DC resulted in an activation of the DC, as measured by upregulated surface expression of costimulatory molecules, enhanced production of IL-12 and TNFα and upregulated

stimulatory capacity. In contrast, RV transduced $CD34^+$ derived DC exhibited inferior stimulatory capacity when high stimulator:reponder ratios were used.

In our last study we show that stimulation with DC transduced with AdV and treated with TNFα resulted in generation of IL-10 producing antigen specific T cells. However, when TNFα was omitted or replaced with LPS or anti-CD40 generation of IFNγ producing antigen specific T cells were generated. Stimulation with mRNA electroporated or protein loaded TNFα treated DC resulted in generation of IFNγ producing antigen specific T cells. Thus the distinct combination of AdV transduction and TNFα treatment renders DC "exhausted" or tolerogenic.

Taken together, the results presented in this thesis provide better knowledge for generation of DC-based cancer vaccines. We now know how to generate a clinical DC product with improved survival potential, reproducible antigen delivery methods into different DC progenitors and what impact these methods have on DC function. In addition, we show that together with commonly applied methods to fulfill the release criteria of a clinical DC batch, the analysis of cytokine production is absolutely imperative.

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