Dendritic cells in cancer immunotherapy

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2011

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Karin Gustafsson
ISBN 978-91-628-8237-2
Printed by Intellecta Infolog, Göteborg, Sweden 2011

Cover picture: Dendritic cell. Hybrid Medical animation / Science Photo Library / IBL Bildbyrå.

Till min familj

Dendritic cells in cancer immunotherapy

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Abstract

Dendritic cells (DCs) play a central role in the initiation and regulation of innate and adaptive immune responses and have increasingly been applied as vaccines for cancer patients. *Ex vivo* generation and antigen loading of monocyte-derived DCs allows a controlled maturation, with the aim of imprinting different DC functions that are essential for their subsequent induction of a T cell-mediated anti-tumor response. A better understanding of how DCs control T cell immunity is important for the design of novel DC-based cancer vaccines with improved clinical efficiency. The aim of this thesis was to evaluate how different maturation conditions used for generation of clinical grade DC-based cancer vaccines affect their capacity to assist type-1 polarized immune responses, important for elimination of cancer.

Monocyte-derived DCs from healthy blood donors and chronic lymphocytic leukemia (CLL) patients were matured using two different types of cocktails; the "standard" maturation cocktail for human DC-based cancer vaccines consisting of TNF-α, IL-1β, IL-6 and PGE_2 (PGE_2DCs) and the more recently established α-type 1-polarized DC cocktail consisting of TNF-α, IL-1β, IFN-γ, IFN-α, and p-I:C (αDC1s).

Recent data from mouse models indicate that the ability of vaccine DCs to induce a desirable type 1-polarized immune response is strongly dependent on their ability to induce a CXCR3-dependent recruitment of IFN-γ-producing natural killer (NK) cells into vaccine-draining lymph nodes. We found that αDC1s from healthy blood donors secrete substantial amounts of the CXCR3 ligands (CXCL9/CXCL10/CXCL11). In contrast, no measurable production of these chemokines was found in PGE_2DCs. Functional studies revealed that supernatants from mature αDC1s recruited NK cells and further, αDC1s induced IFN-γ production in autologous NK cells, but only if concurrent CD40 ligation was provided.

Despite previous reports of dysfunctional DCs in CLL patients, we found that αDC1s generated from CLL patients also produced substantial amounts of CXCR3-ligands in a sustained fashion. Functional studies demonstrated that αDC1s from CLL patients were superior recruiters of NK cells and potential CD40 ligand-expressing NKT cells compared to PGE_2DCs. Importantly, loading of αDC1s with necrotic CLL cells had no negative impact on chemokine production. It has most recently been shown that autologous DC vaccines indirectly prime naïve T cells *in vivo* by acting as immune adjuvant that transfer antigens to recruited endogenous DC-precursors. In our final study we investigated the ability of allogeneic (foreign) DCs to recruit and differentiate "bystander" monocytes into functional DC-like cells *in vitro*. We found that allogeneic αDC1s efficiently recruited monocytes and Th1-associated lymphocytes from CLL patients. Finally, monocytes primed in such αDC1 but not PGE_2DC-induced environment seem to undergo maturation toward Th1-deviating DCs.

In conclusion, this thesis supports the therapeutic use of αDC1-based vaccines in the traditional autologous setting and further indicates that allogeneic αDC1s could be used as a source of adjuvant and a vehicle for tumor antigen delivery to evoke Th1-polarized immune responses against human cancers.

Keywords: αDC1, PGE_2DC, dendritic cells, natural killer cells, T cells, CLL, vaccines

ISBN 978-91-628-8237-2

POPULÄRVETENSKAPLIG SAMMANFATTNING

Syftet med arbetet som ligger till grund för denna avhandling var att försöka utveckla en alternativ behandlingsmetod mot cancer där man använder sig av patientens eget immunförsvar. Metoden som här presenteras är baserad på ”aktiv immunterapi” där man via vaccination försöker få igång ett specifikt immunsvar mot den befintliga tumören. Vaccinet tillverkas genom att man ur ett blodprov renar fram en viss typ av immunförsvarets vita blodkroppar, så kallade dendritiska celler. Dessa celler odlas sedan i provrör tillsammans med olika inflammatoriska ämnen och ”laddas” med tumörämnen från önskad tumörtyp. I denna konstgjorda inflammatoriska miljö ”lär” man vaccincellen att den aktuella tumörtypen är farlig och bör elimineras. Tanken är sedan att cancerpatientens immunförsvar genom vaccineringen med dessa manipulerade budbärarceller ska omprogrammeras till att uppleva den egna tumören som något kroppsfrämmande som måste elimineras.

I mitt avhandlingsarbete har jag använt vita blodkroppar från friska blodgivare och från patienter med kronisk lymfatisk leukemi (KLL) för att i provrör försöka simulera den immunologiska reaktion som kan tänkas ske i samband med en vaccination. KLL är en vanlig form av blodcancer. Hos ungefär en tredjedel av denna patientgrupp uppträder sjukdomen i en aggressiv form och trots konventionell behandling är prognosen för dessa patienter mycket dålig. Behovet av nya behandlingsmetoder är därför stort. Genom att i olika försöksmodeller odla vita blodkroppar från patientblodprov med potentiella vaccinkandidater har jag kunnat studera olika centrala skeden i den immunologiska kaskadreaktion som krävs för ett effektivt tumördödande immunsvar. Utifrån resultat från dessa försök har jag kommit fram till en mycket lovande vaccinkandidat som tycks besitta flera nyckelegenskaper som sannolikt behövs för att få igång ett effektivt immunsvar mot cancer.

Framtidens behandling av idag obotliga cancerformer kommer troligen att kräva kombinationsbehandlingar. Tänkbart är att ge den typ av vaccinceller som jag studerat i kombination med redan etablerade terapier såsom strålning och cytostatika. Önskvärt är att ta fram skräddarsydda behandlingar för varje enskild patient som effektivt dödar tumörceller men lämnar friska celler opåverkade. Min förhoppning är att resultat som presenteras i denna avhandling kan vara ett bidrag till vår ökade förståelse för hur vi kan använda oss av vårt enormt komplexa immunförsvar i kampen mot cancer.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I. Recruitment and activation of natural killer cells *in vitro* by a human dendritic cell vaccine
Gustafsson K, Ingelsten M, Bergqvist L, Nyström J, Andersson A and Karlsson-Parra A.
Cancer Research, 2008. 68(14):5965-71.

II. Tumor-loaded α-type 1-polarized dendritic cells from patients with chronic lymphocytic leukemia produce a superior NK, NKT and $CD8^+$ T cell attracting chemokine profile

Gustafsson K, Junevik K, Werlenius O, Holmgren S, Karlsson-Parra A and Andersson P,O.
Submitted

III. Allogeneic αDC1s induce recruitment of monocytes from chronic lymphocytic leukemia patients *in vitro* and enhance their phenotypical and functional differentiation towards Th1-deviating DCs
Gustafsson K, Junevik K, Werlenius O, Holmgren S, Kovacka J, Andersson P,O and Karlsson-Parra A.
Manuscript

Additional paper not included in the thesis:

Is indoleamine 2,3-dioxygenase important for graft acceptance in highly sensitized patients after combined auxiliary liver-kidney transplantation?
Ingelsten M, Gustafsson K, Oltean M, Karlsson-Parra A, Olausson M, Haraldsson B and Nyström J.
Transplantation, 2009. 88:911-919.

ABBREVIATIONS

APC	antigen presenting cell
CD40L	CD40 ligand
CLL	chronic lymphocytic leukemia
CTL	cytotoxic T lymphocyte
DC	dendritic cell
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
Ig	immunoglobulin
IGHV	immunoglobulin heavy chain variable
IL	interleukin
IP-10	interferon-inducible protein 10
I-TAC	interferon-inducible T cell alpha chemoattractant
MHC	major histocompatibility complex
MIG	monokine induced by interferon-γ
MLR	mixed leukocyte reaction
NK cell	natural killer cell
NKT cell	natural killer T cell
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PGE_2	prostaglandin E_2
Poly I:C	polyinosinic:polycytidylic acid
PRR	pattern recognition receptor
SEB	staphylococcus enterotoxin B
TGF	transforming growth factor
TCR	T cell receptor
Th cell	T helper cell
Treg	regulatory T cell
TLR	toll-like receptor
TNF	tumor necrosis factor

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INTRODUCTION

GENERAL BIOLOGY OF THE IMMUNE SYSTEM

The immune system is an amazingly complex network of cells, tissues, and organs that work together to protect the body from harmful processes such as microbial infections, cancer and autoimmune disorders.

This system can be divided into the innate immunity and the adaptive immunity. The innate or natural immunity is the first line of defense and a type of general protection, including physical barriers of the body (e.g. skin, mucosa), chemical barriers (e.g. secretions and enzymes), and other soluble factors (e.g. cytokines, chemokines and the complement system). It also includes innate leukocytes such as natural killer (NK) cells, mast cells, and phagocytic cells (e.g. monocytes, dendritic cells (DCs), macrophages and neutrophils (Parkin 2001).

Among the phagocytes, DCs have powerful key functions in the immune system. They capture antigens in peripheral tissues and migrate to secondary lymphoid organs. There, they may provide cells of the adaptive, also called specific, immunity such as T and B lymphocytes with pathogen-related information from the affected tissue and thereby activate suitable antigen-specific immune responses (Steinman 1991; Banchereau 1998).

In contrast to the fast and unspecific innate immunity, the adaptive immune response has specificity for distinct molecules and provides the immune system with the ability to recognize and remember specific pathogens. The memory function of adaptive immunity enables more vigorous responses to repeated exposures to the same microbe. DCs not only provide an important link between the innate and adaptive immunity, they also have a key role in the polarization of adaptive immune responses and in that way contribute to the selection of the most efficient effector mechanisms against a particular pathogen (Abbas 2010).

This thesis is focused on DCs and how they may induce desirable polarized immune responses of importance for fighting tumor cells. However, endogenous DCs in cancer patients are often defective due to tumor-induced immunosuppression, resulting in impaired development of anti-cancer immunity (Yang 2004; Pinzon-Charry 2005). To overcome this obstacle to an effective cancer treatment, immunotherapeutic strategies have been purposed, whereby DCs are loaded with tumor antigens and activated *ex vivo* in a non-suppressive environment. The main focus of this thesis is to *in vitro* evaluate how different maturation conditions used for generation of clinical grade DC-based cancer

vaccines affect their capacity to assist type-1 polarized immune responses, critical for elimination of cancer.

Dendritic cells

DCs are highly specialized antigen-presenting cells (APCs) which in contrast to other APCs, such as macrophages and B cells, are able to activate naïve T cells. Therefore DCs have a unique ability to initiate and regulate immune responses against foreign antigens. Most likely, DCs also play a key role in T cell tolerance to self antigens, thereby avoiding the induction of autoimmune reactions (Steinman 1991; Banchereau 1998; Steinman 2003). There is accumulating evidence for the existence of different subsets of DCs that are responsible for this broad range of responses.

Origin and types of dendritic cells

Various types of DCs with differences in phenotype, function and tissue distribution indicate the coexistence of heterogeneous DC populations (Hart 1997; Ueno 2007). Despite extensive research on DC development, the origin of DCs is still a controversial issue. Briefly, according to current opinion, DCs are generated from either lymphoid or myeloid precursors of hematopoetic origin, and intermediate precursors of these lineages home to different sites of potential antigen entry where they differentiate into DCs.

A subset of circulating DC-precursor cells that express the integrin CD11c and receptor for the cytokine and growth factor granulocyte-macrophage colony-stimulating factor (GM-CSF) may differentiate into conventional myeloid DCs under the influence of GM-CSF and IL-4. Myeloid DCs are thought to be closely related to monocytes, macrophages and granulocytes.

In contrast, $CD11c^-$ DC precursors express low levels of the GM-CSF receptor but high amounts of the IL-3 receptor, mature in response to IL-3 and CD40 ligation, and differentiate into non-conventional plasmacytoid DCs (Grouard 1997; Rissoan 1999). Plasmacytoid DCs were first reported as a cell type resembling plasma cells (Lennert 1958) and seem to have a tight developmental link to lymphocytes (Facchetti 1988; Galy 1995; Facchetti 2003). There is also an alternative hypothesis proposing the existence of a common DC precursor in blood that can give rise to all subsets of DCs (del Hoyo 2002).

The possibility that blood monocytes traffic into tissues and differentiate into DCs upon certain stimuli has become more and more accepted. Local mechanisms that mediate differentiation of such monocyte-derived DCs (MoDCs) are not fully elucidated. However, the process of transendothelial migration itself has *in vitro* been shown to result in differentiation of monocytes into DCs (Randolph 1998). Local production of the DC-differentiating factor

GM-CSF by endothelial cells (Kaushansky 1989) and/or by co-recruited natural killer (NK) and natural killer T (NKT) cells (Hegde 2007; Zhang 2007) are another potential mechanism by which recruited monocytes are induced to differentiate into DCs.

According to several recent studies, MoDCs are a true component of the DC network *in vivo* but they only appear "on demand" at sites of inflammation (Trapani 2002; Tacke 2006). Data from Leon et al (Leon 2007) reveal an important role for this inflammatory MoDC type in mediating an effective immune response at a time when other DC subsets may not be capable of coping with the infection. They observed a massive influx of monocytes at the site of infection and in the draining lymph node after inoculation of the parasite L. major. These monocytes turned into MoDCs in both locations and evidence was presented suggesting that MoDCs from the infected site had migrated to the lymph node. It was convincingly shown that the MoDC subset was the only DC population that presented *L. major* antigens and were able to provide antigen-specific T cells with the desirable type 1-polarizing signals required to generate a protective response against *L. major* infection.

DC maturation and antigen presentation

As immature cells, the heterogeneous subsets of DCs work as immunological sensors and screen the peripheral tissues for damaged cells and pathogens. "Danger signals" are then mediated by pathogen recognition receptors (PRRs), such as Toll-like receptors (TLRs) and C-type lectins that recognize various conserved microbial molecules called pathogen associated molecular patterns (PAMPs) (Pulendran 2004; Akira 2006). Immature DCs are efficient at capturing antigen. When antigen ingestion and processing take place in the presence of pro-inflammatory cytokines such as IL-1β, tumor necrosis factor (TNF)-α, and pathogen-associated danger signals, phenotypic and functional changes are induced. During this process of maturation, DCs up-regulate co-stimulatory molecules such as CD40, CD80 and CD86 on their surface and produce large amounts of immunostimulatory cytokines and chemokines (Sallusto 1999; Langenkamp 2000).

Chemokines are small chemotactic cytokines that guide the migration of immune cells within the body. Each DC population shows a unique spectrum of chemokine responsiveness that change during their development from peripheral immature antigen-capturing DCs to mature migrating DCs able to prime naive T cells. In general, immature DCs migrate in response to chemokines such as MCP chemokines (via CCR2), MIP-1α/β and RANTES (via CCR1, CCR3 and/or CCR5), that are inducible with inflammatory stimuli (Caux 2000). Upon maturation, DCs lose their responsiveness to these inflammatory chemokines and up-regulate the lymph node homing receptor CCR7 whereupon they acquire

responsiveness to lymphoid chemokines such as CCL19 and CCL21 (Steinman 1991; Banchereau 1998; Sallusto 1998; Kellermann 1999). The maturing DCs therefore leave the affected tissues and migrate to T cell rich areas of draining lymphoid organs where, transformed into mature DC, they may present pathogen-derived peptides to antigen-specific naïve T cells and direct their differentiation into effector or memory cells.

During this maturation program DCs also up-regulate the expression of major histocompatibility complex (MHC) molecules. These are key molecules in the DC-mediated induction of adaptive immune responses and are furthermore of central importance for the whole immune system as they provide an ability to distinguish between "self" and "non-self". Every cell in our body displays the same set of unique "self" MHC molecules. Foreign molecules and cells that do not display the same MHC are treated as non-self and therefore attacked. In contrast to MHC Class I molecules, which are expressed on the surface of all nucleated cells, MHC Class II are mainly expressed on antigen-presenting cells such as DCs, macrophages, B cells and monocytes.

T cells express antigen-specific T cell receptors (TCRs) which recognize a specific antigen that is presented as a processed peptide bound to self MHC molecules. TCRs of $CD4^+$ T cells recognize processed peptides that are derived from internalized extracellular proteins and bound to MHC class II whereas TCRs of $CD8^+$ T cells specifically recognize peptides, bound to MHC class I, which binds peptides derived from intracellular proteins. These proteins can be either self-antigens or antigens from intracellular pathogens (Abbas 2010).

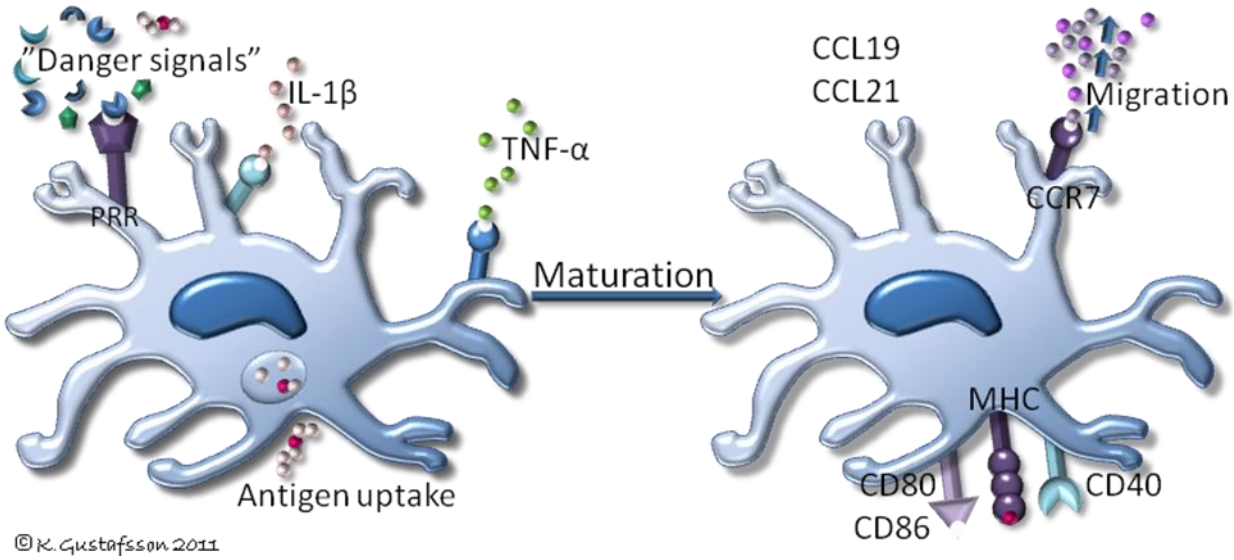

Figure 1. Dendritic cell maturation

Furthermore, pathways of antigenic MHC class I cross-presentation in DCs have been suggested. Phagocytosed antigens that are normally processed and

presented by the phagosome-associated MHC class II pathway may escape to the cytosol and become processed by proteasomes and imported to the MHC class I antigen loading pathway (Albert 1998; Larsson 2001). This enables $CD8^+$ T cells to also recognize exogenous peptides expressed on MHC class I molecules.

DC induced T cell activation and polarization: signal 1, 2, and 3

After maturation DCs lose the antigen-sampling function and migrate to T cell areas of draining lymph nodes where they provide antigen-specific naïve $CD4^+$ T helper (Th) cells and $CD8^+$ T cells with pathogen-related information from the affected tissues. The capacity of DCs to induce immune responses is dependent on their effectiveness at delivering information about the identity and structure of the invading pathogen. This information is provided by the antigen specific "signal l" (Banchereau 1998; Reis e Sousa 2001), which is delivered when antigenic peptide-MHC complexes expressed on DCs are recognized by antigen-specific TCRs on T cells.

DCs may also provide T cells with information about the immunogenic potential of the invader. This type of information is transferred by ligation of co-stimulatory molecules and referred to as "signal 2" (Cella 1997; Salomon 1998). The expression of co-stimulatory molecules on the surface of DCs mirrors the capacity of a certain pathogen to activate DCs. Pathogen-related activation of DCs may be generated by direct recognition of PAMPs (Pulendran 2004; Akira 2006) through pathogen recognition receptors or indirectly by non-specific inflammatory responses induced by the invader. The combination of signal 1 and 2 induces activation and proliferation of naïve T cells.

This tightly regulated activation process is bi-directional whereby recently activated $CD4^+$ T helper (Th) cells rapidly up-regulate CD40 ligand (CD40L) which, via CD40 ligation, protects DCs from apoptosis and stimulates further up-regulation of MHC, co-stimulatory molecules, and increased cytokine production (Cella 1996; Bennett 1998; Ridge 1998; Schoenberger 1998). The expression and secretion of specific co-stimulatory molecules and cytokines represent a DC-delivered third signal, described as a polarizing "signal 3", that drives the development of recently activated, naïve T helper cells towards either a Th1 or a Th2 polarized immune response and thereby regulates the character of the adaptive immune response (Gately 1998; Ohshima 1998; Kalinski 1999; Vieira 2000; Mailliard 2004).

There are an increasing number of reports indicating that the capacity of DCs to polarize T helper cells is imprinted already in the periphery and depends on micro-environmental signals present during DC activation. For example, the

presence of IFN-γ during initial DC maturation is central for their capacity to produce the Th1-polarizing cytokine IL-12p70 upon CD40 ligation (Vieira 2000; Xu 2003; Mailliard 2004; Ten Brinke 2007). The Th1-deviated cellular immunity is the most effective type of response against intracellular pathogens and cancer while Th2-polarized humoral immunity, mediated by antibody producing B lymphocytes, is effective at eliminating pathogens localized outside cells.

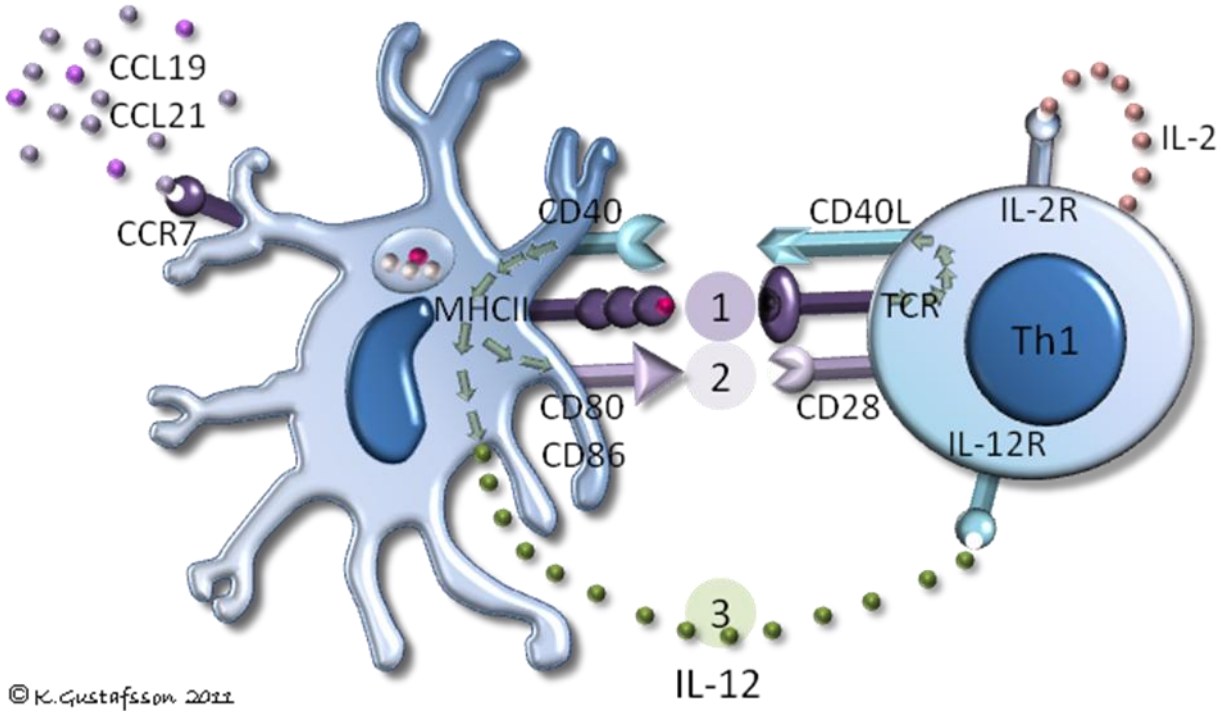

Figure 2. Dendritic cell induced Th1-polarization, signal 1, 2, 3

In addition to their capacity to induce adaptive cellular and humoral effector responses, DCs may also be involved in the induction of peripheral tolerance. During the steady state, in the absence of inflammation or infection, immature DCs migrate from peripheral tissues to lymphoid organs and present antigens to T cells in a tolerogenic way that induces antigen-specific T cell development into regulatory T cells, or alternatively induce T cell anergy or deletion (Steinman 2003). The well-established model for induction of peripheral T cell tolerance is described as an interaction between a naïve $CD4^+$ T cell and immature DC able to deliver signal 1 but not a costimulatory signal 2. However it has been suggested that this type of tolerance induction instead requires DCs providing signal 2 and is further dependent on a third tolerogenic signal (Albert 2001).

It is therefore worth noting that signals which generate phenotypically mature DCs, able to deliver signal 1 and 2, do not always seem to correlate with efficient T cell stimulatory capacity. This issue will be further discussed in later sections of this thesis.

Effector phase of the adaptive immunity

Th1-polarized immunity

The balanced cytokine production of different lymphocyte subsets plays an important role in the regulation of the human immune system. Following the antigen-specific signal 1 and co-stimulatory signal 2 (Schuler 1997; Banchereau 1998; Schuler 2003), naïve $CD4^+$ T cells start to produce the T cell-proliferative cytokine IL-2 acting both in an endocrine and paracrine manner. Depending on DC-mediated instructions, naïve $CD4^+$ T cells develop into various subsets that provide different "helper" functions for other leukocytes of the immune system. This help is provided by cell interactions and/or by cytokine production. Th1 cells produce IL-2 and IFN-γ, and together with IL-12 produced by certain DCs after CD40 ligation (Cella 1996; Bennett 1998; Ridge 1998; Schoenberger 1998), they support the proliferation and differentiation of naïve $CD8^+$ T cells into cytotoxic T lymphocytes (CTL) (Bennett 1998; Ridge 1998; Schoenberger 1998). CTLs home to target cells in peripheral tissue where they induce apoptosis in malignant and virus-infected cells through the release of cytotoxic enzymes, such as granzyme and perforine or by Fas-ligand mediated pathways (Trapani 2002; Voskoboinik 2006). Th1 cells and CTLs thereby play a critical role in cellular immunity.

Th2-polarized immunity

Th2 cells are predominantly involved in humoral immunity against extracellular pathogens but also in the pathogenesis of allergy. They produce the B cell-stimulatory cytokines IL-4 and IL-5, promote B cell proliferation, cytokine production and immunoglobulin (Ig) class switch and stimulate their transformation into antibody-producing plasma cells. Th2 cells also recruit and activate eosinophils and mast cells (Romagnani 1991; Mosmann 1996).

Regulatory T cells and immune suppression

Even though lymphocytes play a central role in inflammatory adaptive immunity, it is clear that they are also important for immune suppression and the maintenance of immune balance and tolerance to self-antigens. Different $CD4^+$ T cell subsets with regulatory functions have been described. The thymus-derived naturally occurring $FOXP3^+CD4^+CD25^{high}$ Tregs have been shown to suppress the activation, proliferation and effector function of both innate and adaptive lymphocytes as well as APCs. As dysfunction in $FOXP3^+$ Tregs causes fatal autoimmune disease, allergy and immunopathology it has been concluded that this cell subset is central in the prevention of such conditions (Bennett 2001; Gambineri 2003; Hori 2003; Wohlfert 2008; Sakaguchi 2010).

Additional subsets of suppressive T cells have also been described. Periphery-induced T regulatory type 1 (Tr1) cells may develop upon antigen stimulation via IL-10-dependent mechanisms. Tr1 mainly produce IL-10 and may thereby suppress antigen-specific effector T-cell responses. These regulatory cells are thought to regulate adaptive immune responses and have been suggested to be involved in the protection against autoimmunity (Groux 1997; O'Garra 2004; Roncarolo 2006)

There is also a population of suppressive Th3 cells that like Tr1 cells, exert their regulatory function by cytokine production. Th3 cells are thought to be involved in the regulation of mucosal immunity and protect the gut mucosa to non-self antigens of non-pathogenic nature. Th3 cells mainly produce transforming growth factor-β (TGF-β) (Fukaura 1996), and may suppress the action of both Th1 and Th2 cells. (O'Garra 2004).

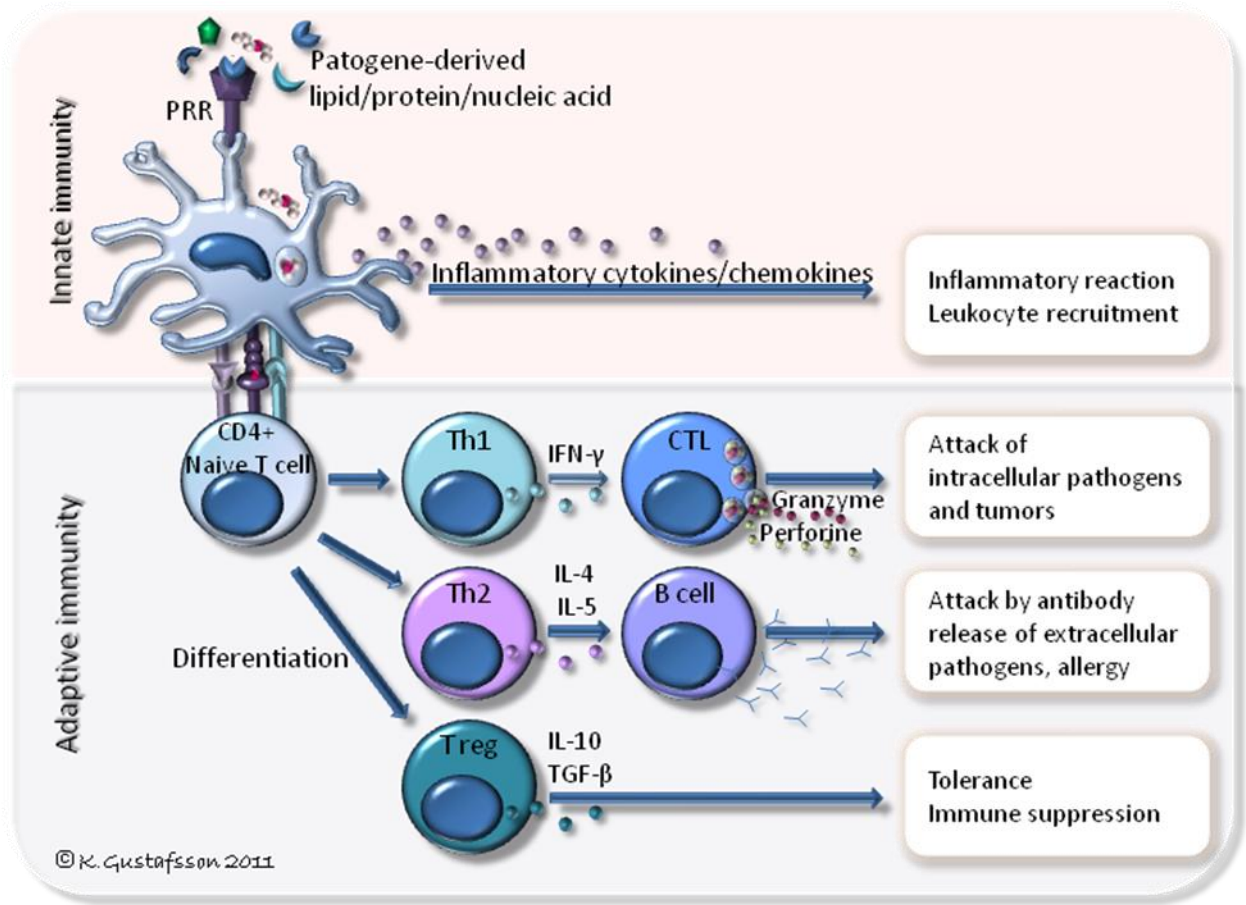

Figure 3. Dendritic cells bridging innate and adaptive immunity

Innate lymphocytes

Two important lymphocyte populations that work in close relation to DCs and T cells are the natural killer (NK) and NKT cells. These cells have been shown to express chemokine receptors such as CCR5 and CXCR3 similar to subsets of effector and memory lymphocytes of adaptive cellular immunity that home to peripheral sites of inflammation (Qin 1998; Sallusto 1998; Tensen 1999; Thomas 2003). Both NK and NKT cells express the NK cell markers CD56 and CD16. NKT cells also express the T cell marker CD3 and an invariant TCR (Lantz 1994) that recognize foreign and self-glycolipids presented by the non-classical MHC class I molecule CD1d (Kawano 1997; Mattner 2005).

NK cells are classically described as natural killers belonging to the innate immune system, due to their ability to kill tumor and virus infected cells without prior sensitization (Herberman 1979). Their activation is regulated by the balance between inhibitory and activating receptors (Bryceson 2006). Similar to CTLs, NK cells screen cells for presence of MHC class I molecules, but in contrast to CTLs that become licensed to kill by recognizing specific antigens presented on self-MHC I molecules, NK cells identify and kill cells with no or low levels of these molecules (Ljunggren 1990). As an attempt to circumvent T cell detection, tumor cells and virus infected cells have been shown to down-regulate their MHC I expression (Lanier 2005). Consequently, by this adaption they instead become possible targets for NK cell mediated killing. NK cells also express inhibitory MHC I receptors which, upon ligation, block NK cytotoxicity (Karlhofer 1992; Moretta 1993).

CD1d-restricted NKT cells are classically defined as innate-like lymphocytes that similar to NK cells exert cytotoxic activity and may kill target cells without prior sensitization. Both exogenous and endogenous glycolipid antigens may be presented on CD1d molecules and have been shown to activate NKT cells during microbial infections (Mattner 2005).

Innate lymphocytes as accessory cells during DC maturation

NK and NKT cells sense infections and cellular transformation via receptors other than TLRs. These recognition mechanisms are now being placed into the context of DC biology and it has been proposed that the interaction of DCs with these innate lymphocytes represents a major control mechanism for immunity that is independent of TLR ligands (Munz 2005). DC maturation has been documented *in vitro* and *in vivo* after NK cell recognition of MHC class I^{low} tumor cells (Mocikat 2003) and NKT cell stimulation by the synthetic invertebrate glycolipid α-galactosylceramide (α-GalCer) presented on DC CD1d molecules (Fujii 2002; Hermans 2003). In line with this data, the reciprocal

interaction of CD1d-restricted NKT cells and DCs has been shown to induce a cellular activation cascade involving elements of innate and adaptive immunity that may lead to anti-tumor immunity (Carnaud 1999).

After activation, NK as well as NKT cells are able to induce DC maturation as evidenced by increased expression of CD86, IL-12 production and priming of T cell responses. TNF has been identified as a crucial inducer of DC maturation in these studies (Fernandez 1999; Gerosa 2002; Fujii 2004). In addition, CD40–CD40L interactions induced by NKT cells allowed for priming of adaptive immune responses by DCs (Fujii 2004). Thus, NK and NKT cells are able to induce DC maturation by a combination of cytokine- and cell contact–dependent signals.

Recent data, from mouse models demonstrate that NK cells are recruited to lymph nodes in a CXCR3-dependent manner after infection or immunization. These NK cells exert helper functions by providing an early source of IFN-γ that is central for Th1 polarization (Martin-Fontecha 2004; Bajenoff 2006).

The interaction of NKT cells with antigen-capturing DCs likewise allows for the induction of antigen-specific, Th1-polarized T cell responses (Fujii 2004). Thus, both NK and NKT cells may act as helper cells that assist DC-induced Th1-deviated adaptive immune response.

Finally, NK and NKT cells can also provide antigenic material for DCs. Fragments of infected cells or tumor cells, generated during the destruction of target cells by these innate lymphocytes, are taken up by DCs and displayed on MHC molecules, thus eliciting an adaptive T cell response *in vivo* (Mocikat 2003).

IMMUNITY AND CANCER

Tumor cells express unique, potentially immunogenic antigens that can be processed and presented by DCs as tumor-specific peptide-MHC complexes and thereby be recognized by the T cell repertoire. The question is, how can potentially immunogenic tumors develop in the presence of an intact immune system?

For this purpose, tumor cells have been shown to develop different mechanisms to escape host immunity. Tumor-specific antigens are in general not strongly immunogenic, and in combination with tumor-produced immunosuppressive factors, such as prostaglandins that are known to reduce MHC II and co-stimulatory molecule expression on DCs, this generates DCs that show impaired ability to deliver signal 1 and 2 (Gabrilovich 2004; Melief 2008). Insufficient

antigen presentation and co-stimulation may induce anergy and apoptosis in tumor-specific T cell populations (Steinman 2003)

Prostaglandins can also suppress NK cell activity, suppress the functions of Th1-deviated immunity (Snijdewint 1993; Hilkens 1995; Kalinski 1997; Kalinski 2001) and prime DCs for elevated Treg and Th2 cell recruitment (Lebre 2005; McIlroy 2006; Muthuswamy 2008). Other ways in which tumor cells may escape the attention of sensitized tumor-specific T cells are by down regulating MHC I and producing TGF-β that suppress CTL activation (Gabrilovich 2004; Li 2006). Furthermore, production of TGF-β and IL-10 can together induce the maturation of different Treg subsets (Jarnicki 2006).

Additionally, essential for the development and maintenance of this tumor protective, immunosuppressive microenvironment is the recruitment of tumor-associated macrophages, myeloid-derived suppressor cells (Marigo 2008) and regulatory T cells (Wing 2008). All together this creates a complex network of cells that support tumor expansion both locally at the tumor site and in lymphoid organs where antigen presentation and polarization of adaptive immunity occurs.

CHRONIC LYMPHOCYTIC LEUKEMIA

Chronic lymphocytic leukemia (CLL) is the most common form of leukemia in Western countries and primarily affects the elderly. The median age at diagnosis is 72 years and CLL is rarely seen in people younger than 40 (Jemal 2009). CLL is a clonal malignancy of B lymphocytes and, with regard to the latest WHO classification scheme, considered a mature B cell neoplasm (Swerdlow 2008). The disease is usually indolent and is characterized by a slowly progressive accumulation of long-lived small B cells in blood, bone marrow, lymph nodes or lymphoid tissue. In time bone marrow failure could occur that affects the development and function of all types of blood cells (Zenz 2010). Despite advances in understanding the pathogenesis and therapy development, the reason for the abnormal expansion of malignant cells is still unknown and CLL remains incurable with conventional therapies.

Diagnosis

To diagnose CLL the presence of more than $5x10^9$/L clonal B cells in the peripheral blood is required (Hallek 2008). Furthermore, flow cytometry is used to confirm the clonality and phenotype of the circulating B cells. Each clone of CLL cells expresses either kappa or lambda Ig light chains (Moreau 1997). CLL cells can phenotypically be characterized by the co-expression of the B cell markers CD19, CD20, CD23 and the T cell marker CD5 (Hallek 2008). The

expression of the surface phosphoprotein CD20 is generally low when compared to normal B cells (Moreau 1997).

Prognosis and staging of CLL

CLL is extremely heterogenous with regard to its clinical course and there are patients that live for decades with no or only marginal need for treatment, while others have a rapidly aggressive clinical course and may die within a couple of years despite aggressive therapy with multiple chemotherapy agents. There are two clinical staging systems, Binet and the Rai, used for prognostic purpose. Both classifications are based on simple and robust clinical parameters, but there are still significant differences in the disease course of the individual patient. Instead, in recent years cytogenetic changes and mutation status of the immunoglobulin heavy chain variable (IGHV) genes have been shown to better identify the progression risk of the individual patient (Van Bockstaele 2009).

Treatment of CLL

As CLL is generally considered incurable, the main focus when treating CLL is not to cure, but rather to control the disease and its symptoms. Detailed discussions of all the different treatments and drugs used in CLL are beyond the scope of this thesis, but some are mentioned below and those of importance for this thesis are described in more detail.

The initial treatment of choice is often a combination of chemotherapeutic agents, sometimes in combination with passive immunotherapy, such as monoclonal antibody-targeted therapies (i.e., the anti-CD20-antibody rituximab) (Hallek 2010). Currently, the only curative approach for CLL is allogeneic stem cell transplantation (alloSCT). However, due to the high risk of treatment-related morbidity and mortality associated with alloSCT, for the majority of patients this is not an available option (Michallet 1991; Schetelig 2003; Sorror 2008). Also, the outcome after alloSCT is strongly dependent on a series of risk factors, including patient age and the phase of the disease (Michallet 1991; Schetelig 2003; Sorror 2008).

Irrespective of the negative side effects, immunological observations and the strong anti-tumor response seen after alloSCT suggest a possible immune-mediated cure for CLL. Therefore, alternative and less aggressive immunotherapeutic approaches, including active immunization strategies, may potentially be used. In addition, the absence of a rapidly progressive disease and the generally indolent clinical course of CLL may also facilitate immunization regimens.

DENDRITIC CELLS IN CANCER IMMUNOTHERAPY

The idea of utilizing the unique immune-modulating capacity of DCs in the context of cancer treatment is not new. The discovery that a large number of human DCs easily can be produced from monocytes isolated from peripheral blood formed the basis for the expanding field of therapeutic cancer vaccines (Sallusto 1994).

Ex vivo-generated vaccine DCs do not have a direct tumor-killing activity, which is the case for chemotherapy and adoptive (passive) immunotherapies with antibodies or *ex vivo*-expanded tumor-specific T cells. Instead DC-based vaccines aim to reset a patient's immune system and thereby elicit anti-tumor responses *in vivo*. The general idea with this immunotherapeutic strategy is to optimize antigen presentation and activation of monocyte-derived autologous DCs *ex vivo* in a non-suppressive environment. Hypothetically, assuming optimal maturation conditions and source of tumor antigens, when adoptively transferred back to the patient, these vaccine DCs should traffic to the draining lymph node and induce effective type-1 polarized anti-tumor immune responses (Mempel 2004).

Multiple strategies have been used to induce maturation of DCs and to introduce antigens into potential DC-based cancer vaccines both in animal models and in humans. In murine models, vaccine DCs that have been pulsed with tumor lysates or peptides, transfected with RNA or DNA encoding tumor antigens, or fused to tumor cells, have been shown to induce promising and sometimes protective tumor-specific immunity (Gilboa 2007; Melief 2008). Although DC-based vaccines have been effective in animal models, the immune responses observed after DC-based vaccines in humans are often weak, and clinical responses are rarely complete and long lasting. This insufficient response in humans may be due to various factors, such as administration of DCs with unsuitable maturation state and Th1-deviating capacity, relatively low cell numbers, inappropriate vaccination route or frequency of injections (Melief 2008).

Dendritic cell-based cancer vaccines and delivery of signal 1, 2 and 3

A key challenge when considering active immunotherapy against cancer is to find out how to develop optimally matured vaccine cells with potent immunostimulatory functions, strong enough to overcome the immune suppressive state characteristic for cancer patients. The traditional view of such a favorable vaccine DC is based on what we know about DCs as key players in inducing efficient elimination and long lasting memory to intracellular bacterial and viral infections. The activated DCs must first of all have lymph node

homing potential. On arrival, they have to provide naïve T cells with antigen-specific signal 1, co-stimulatory signal 2 and also to be functionally mature in order to deliver the Th1-polarizing signal 3 (Schuler 1997; Banchereau 1998; Kalinski 1999; Vieira 2000; Schuler 2003).

The first clinical pilot studies involving active immunotherapy were performed in the 1990s in B-cell lymphoma and malignant melanoma and the vaccine cells used in these studies were relatively immature DCs (Hsu 1996; Nestle 1998). Promising results, such as stabilization and cancer regression, were observed but only in a proportion of patients. This first generation of relatively immature DC-based vaccines has in general been attributed to impaired lymph node homing capacity (Dhodapkar 1999; de Vries 2003). Vaccine DCs, solely activated with TNF-α, were recently evaluated in two clinical trials in CLL (Hus 2005; Hus 2008). However, the results were similar to those obtained for immature DCs, showing a relatively modest clinical effect despite a measurable induction of tumor-specific CTLs.

The second generation of DC-based vaccines, matured with a "gold standard" maturation cocktail, consisting of TNF-α, IL-1β, IL-6 and prostaglandin-E_2 (PGE_2) (Jonuleit 1997), have been the most frequently used vaccine DCs to date. These PGE_2DCs show a fully mature phenotype and are able to present tumor antigen as well as appropriate co-stimulatory molecules (signal 1 and 2). Signals provided by PGE_2 were further shown to induce high expression of CCR7 that made them superior at migrating towards lymphoid chemokines when compared to first generation DC vaccines (Luft 2002; Scandella 2004).

As with first generation DC vaccines, there is clinical data indicating that PGE_2-matured DCs may also be insufficient for cancer treatment: a phase III trial in patients with malignant melanoma failed to show the advantage of PGE_2DCs over standard dacarbazine chemotherapy (Schadendorf 2006). Furthermore, PGE_2DCs were recently shown to be even more effective than immature DCs at inducing Treg expansion *in vitro* and *in vivo* in myeloma patients (Banerjee 2006). These observations underscore the difficulty in generating tumor regression in patients treated with PGE_2-matured DCs. The main reason for the drawback with PGE_2DCs is most likely their impaired IL-12p70 production upon CD40 ligation (lack of a Th1-polarizing signal 3) (Kalinski 1999; Lee 2002). This further emphasizes that other aspects besides phenotypic maturation must be taken into account when designing new DC-maturation protocols.

The main objective with current DC-maturation protocols has been to find a vaccine DC candidate that combines a fully mature phenotype (lymph node homing potential and delivery of signal 1 and 2) with the capacity to produce IL-12p70 and thereby deliver a Th1-polarizing signal 3, known to be important for

anti-tumor immunity. Since IFN-γ has been shown to facilitate the production of IL-12p70 by DCs primed by microbial products or inflammatory cytokines such as TNF-α and IL-1β (Vieira 2000), the addition of IFN-γ to the standard PGE_2-containing maturation cocktail was recently evaluated. However, the addition of IFN-γ was shown to inhibit membrane expression of CCR7 and reduced migration of DCs towards lymph node chemokines (Alder 2006).

A new DC vaccine candidate, first described by Mailliard et. al (Mailliard 2004) seems to express all the key features classically ascribed a Th1-polarizing DC. They reported that the inclusion of IFN-α and the TLR3 ligand polyinosinoine:polycytidylic acid (p-I:C) to the "original" IL-12p70-inducing cytokine cocktail, composed of TNF-α, IL-1β and IFN-γ (Vieira 2000), generated DCs with high migratory function towards lymph node chemokines combined with a strong ability to produce IL-12p70. A recent *in vitro* study demonstrated that such "α-type-1 polarized DCs" (αDC1s) and non-polarized PGE_2DCs induced similar $CD8^+$ T cell expansion, but only αDC1s were able to induce functional CTLs with cytolytic function and tumor relevant homing capacity (Watchmaker 2010).

In vivo activation of endogenous APC in DC-based cancer vaccination

There are still many pieces missing in our understanding of the complex puzzle of sequential events that occurs after injection of *ex vivo* generated vaccine DCs.

Contrary to the classical view, autologous DC-based vaccines were recently shown to have a minor role in the direct priming of antigen-specific T cells *in vivo*. Instead, it was suggested that they indirectly prime naïve $CD8^+$ T cells by acting as an immune adjuvant that transfers antigens to locally recruited endogenous APCs (Yewdall 2010). Similar observations have been reported after injection of allogeneic vaccine DCs. Results from these mouse studies have shown that fully allogeneic vaccine DCs loaded with viral or tumor antigens are able to induce a robust cross-priming of cytotoxic T lymphocytes (CTLs) (Racanelli 2004; Edlich 2010).

These observations were further supported by recent data from migration studies on human monocyte-derived autologous vaccine DCs after intradermal injection (Verdijk 2009). Interestingly, despite the presence of PGE_2 during *ex vivo* maturation, the majority of DCs remained at the injection site, and less than 5 % of the injected DCs reached the draining lymph nodes. Vaccine DCs that were trapped at the injection site rapidly lost their viability and were cleared by recruited antigen-presenting cells within 48 hours.

Taken together, unlike the features classically ascribed an optimal DC-based vaccine, these data indicate that autologous as well as allogeneic vaccine DCs, could play a crucial role as pure immune adjuvants. From this point of view, to be efficient, the tumor-loaded vaccine DCs that are trapped at the injection site must attract and directly or indirectly activate endogenous DC-precursors. Assuming proper activation, these recruited DC-precursors may, after engulfment of relevant antigens from dying vaccine cells and migration to lymph nodes, induce Th1-polarization leading to tumor rejection.

GENERAL AIM

The overall aim of this thesis was to evaluate if different *in vitro* culture conditions, used to generate clinical grade DC-based cancer vaccines, differentially imprint certain functions in DCs that are essential for their ability to induce type-1 polarized, anti-cancer immune responses.

SPECIFIC AIMS:

- to evaluate if the ability of DC-based cancer vaccine to recruit and activate preferable Th1-associated lymphocyte subsets can be differently imprinted during DC maturation by the "standard" cocktail (IL-1β/TNF-α/IL-6/PGE_2) and α-type 1-polarized DC cocktail (IL-1β/TNF-α/IFN-α/IFN-γ/poly-I:C)

- to examine if functional autologous DC-based cancer vaccines with a desirable chemokine/cytokine profile and lymphocyte attracting ability can be generated from CLL patients

- to compare the ability of allogeneic PGE_2DCs and αDC1s to recruit monocytes and different subsets of potential accessory cells from CLL patients, and to investigate if the conditions induced by these allogeneic vaccine DCs support the maturation of recruited monocytes toward desirable Th1-deviating DCs

METHODOLOGICAL CONSIDERATIONS

Detailed descriptions on the experimental procedures are given in the individual papers. The following section contains an overview and general description of some methods of particular importance for this thesis.

PATIENTS AND HEALTHY BLOOD DONORS

After gaining informed consent, peripheral blood was collected from healthy blood donors and CLL patients. The study protocol was approved by the Human Research Ethics Committee at the Sahlgrenska Academy, University of Gothenburg. Patients enrolled to paper II and III were untreated, stable CLL patients, all in Binet stage A, diagnosed at the Section of Hematology and Coagulation at Sahlgrenska University Hospital, Gothenburg, Sweden. The diagnosis of CLL was based on WHO criteria at the time of inclusion (Swerdlow 2008).

CELL SEPARATION AND DENDRITIC CELL MATURATION

The pioneering work of Sallusto and Lanzavecchia in the mid-1990s demonstrated that DCs can be cultured *ex vivo* from human blood monocytes using GM-CSF and IL-4 (Sallusto 1994). These observations initiated the era of *ex vivo* generated human DC-based vaccines and since then this has become the most commonly used protocol for *in vitro* generation on DCs. The procedure used in this thesis for cell isolation and generation of monocyte-derived DC vaccine cells is described below.

Density gradient centrifugation was used to isolate peripheral blood mononuclear cells (PBMC) from heparinised peripheral blood obtained from healthy donors or CLL patients. This is an effective and simple cell separation method that was first described by Dr. Arne Bøyum in 1968 (Boyum 1968). The technique is based on the fact that mononuclear cells (monocytes and lymphocytes) have a lower density than the erythrocytes and the polymorphonuclear leukocytes (granulocytes). The PBMCs can thereby be isolated by centrifugation on a separation medium with a density just above the majority of mononuclear cells. This allows the erythrocytes and the granulocytes to sediment through the medium while the mononuclear cells are concentrated as bands at the sample/medium interface where the cell density matches that of the surrounding solution.

Monocytes were then separated from the PBMC population either by plastic adherence (paper I) or with $CD14^+$ magnetic beads (paper I, II, III). When

plastic adherence was used for monocyte isolation, non-adherent cells were removed after 2 hours. Cell isolation with magnetic beads was frequently used throughout this study. In brief, PBMCs were incubated with magnetically labeled antibodies and labeled cells were subsequently collected or removed using a magnetic field. This technique allows a rapid depletion of unwanted cells or collection of fairly pure populations of desired cell types.

Monocytes from CLL patients were isolated from lymphocytes with a second gradient centrifugation followed by isolation with $CD14^+$ magnetic beads (paper II, III). This was done to get rid of the main proportion of the contaminating malignant B cells that constitute around 70 % of the PBMC population in the untreated, stable CLL patients that were enrolled in this study. Irrespective of the isolation protocol, the remaining population of PBMCs depleted of $CD14^+$ monocytes were often saved for later use as relevant responder cells (circulating blood lymphocytes) during co-culture experiments with vaccine DCs.

Monocytes were cultured in clinical grade serum-free culture medium supplemented with recombinant human GM-CSF and IL-4 (R&D Systems) for 5 days, in order to obtain immature DCs. During the last 24 hours of culture, DC maturation was induced by supplementing the culture media with various maturation stimuli. Immature DCs cultivated without addition of any maturation cocktail were used as controls. In paper II the possible negative impact of loading vaccine DCs with relevant tumor antigens were studied. For this purpose DCs were pulsed with heat stressed, necrotic CLL cells at a ratio of 1:1 at the same time as the maturation-inducing cytokines were added. In all 3 papers two different maturation cocktails were used and compared; the gold standard maturation cocktail for DC-vaccines (TNF-α, IL-1β, IL-6 and PGE_2) (Jonuleit 1997), and the αDC1-maturation cocktail (IFN-α, IFN-γ, TNF-α, IL-1β and poly-I:C) (Mailliard 2004).

In paper I, monocytes were cultured in AIM-V medium (Invitrogen) and in paper II and III AIM-V, was replaced by CellGro DC medium (CellGenix). The medium was substituted as a result of repeated observations of changes in DC behaviors and morphology that, for some unaccountable reason, occurred when new batches of AIM-V medium were used. Changes, such as increased adherence, complicated the collection of cells and resulted in lower rates of cell recovery. This phenomenon was especially pronounced in cultures of αDC1s and therefore recovery rates of this vaccine type was much lower than for loosely adherent PGE_2DCs. This problem was solved by substituting the AIM-V with CellGro DC medium. αDC1s generated in this new medium were smaller and just as loosely adherent as PGE_2DCs, which resulted in similar cell recovery when cells were harvested. Similar observations were recently reported by others. In the study performed by Lee et al. a 30% lower recovery rate of

αDC1s, compared with PGE_2DCs were observed in cultures performed in AIM-V medium (Lee 2008).

CYTOKINE AND CHEMOKINE DETERMINATION BY ELISA

Enzyme-linked immunosorbent assay (ELISA) was used to detect the amount of cytokines and chemokines released into culture supernatants of vaccine DCs alone or in co-culture with various responder cell populations (paper I, II, III). In basic terms, a plate is coated with a capture antibody specific to the analyte of interest. Samples or standards are then added and any analyte present will bind to the immobilized antibody. In the next step, a conjugated detecting antibody (e.g. biotin conjugated), which binds to the analyte, is included. An enzyme-linked detection reagent (e.g. streptavidin-HRP) is then added. This binds to the conjugate. Finally, a substrate solution (e.g. TMB/hydrogen peroxide) is added to activate the enzyme and convert the substrate to a coloured product. The color develops in proportion to the amount of analyte present in the added sample. The quantity of analyte is measured as absorbance.

FLOW CYTOMETRY

Flow cytometry was used to evaluate phenotypic DC maturation induced by different stimuli, and to quantify the migration of different leukocyte subsets in response to chemokine gradients created by vaccine DCs. Flow cytometry was also used for evaluation of the interaction between NK cells with vaccine DCs as determined by expression of CD69 and intracellular IFN-γ.

This technique is also routinely used in the diagnosis of leukemias. Briefly, cells were collected and cell suspensions were incubated with fluorescently labelled antibodies specific for numerous different markers that enabled the identification of cell subsets by flow cytometry.

Flow cytometry is designed for quantification and sorting of microscopic particles, such as cells. By passing the cells one by one in a stream of fluid through a light beam in the apparatus, physical and fluorescent characteristics of individual cells can simultaneously be detected. Every cell that passes through the laser light scatters the beam differently depending on size and granularity, simultaneously the fluorescently-labelled antibodies attached to each cell become excited and emit fluorescent light which is measured using appropriate filters. A number of detectors, specialized at detecting such fluorescent or scattered light, are placed to encircle the site where the stream passes through the light beam. One of the detectors is denoted Forward Scatter (FSC) and placed in line with the laser beam from where it detects the size of the cell as it passes through the beam. Other detectors are arranged vertical to the stream,

such as various numbers of fluorescent detectors and a Side Scatter (SSC) detector that distinguish the granular content of the cells.

The acquired data can be analyzed and plotted in a single dimension, producing a histogram, or in two-dimensional dot plots or density plots. Depending on the fluorescence intensity, correlating with the expression of a specific marker, the cells will cluster into certain regions on these plots. Cell populations can then be further separated by creating a series of electronic "gates" allowing detailed analysis of the cell subsets of interest.

MIGRATION ASSAYS

Migration assays were used to predict the capacity of different DC vaccines to recruit specific subsets of leukocytes of importance for efficient Th1-deviating ability. This method was included in all the three papers and depending on the purpose, slightly different setups were used.

In general terms, migration of monocytes and lymphocytes towards chemokines produced by differently matured vaccine DCs was evaluated in two different transwell assays (chemotaxis and migration assays). In brief, purified monocytes from healthy donors (papers I and III) or CLL patients (paper II) were cultured with GM-CSF and IL-4 for 5 days. At day 4, immature DCs were matured with either the αDC1 or the PGE_2DC maturation cocktail for 24 hours, then washed twice and replaced in fresh medium. Unstimulated DCs were used as immature controls. These different DC subsets or their secreted inflammatory mediators in the culture supernatants were then used in the chemotaxis or migration assays.

In paper I, a transwell assay was used to study chemotaxis of NK cells toward chemokines produced by vaccine DCs generated from healthy blood donors. In this experiment, culture supernatants collected from previously washed and mature or immature DCs were added the lower chambers of transwell plates. Medium only was used as a control to determine spontaneous fallout. PBMCs or purified NK cells were added to the upper chamber and the plate was incubated for 90 min. Cells that migrated to the lower chamber were harvested and stained with fluorescently labelled antibodies specific for CD56 and CD3. $CD3^{-}CD56^{+}$ NK cells were subsequently defined and counted by flow cytometry.

In paper II, a migration assay was used to study migration of NK and NKT cells toward chemokines produced by tumor-loaded vaccine DCs generated from CLL patients. This migration model was aimed to more closely mimic *in vivo* conditions whereby cells in the upper chamber had to actively migrate along gradients of chemokines through an artificial cell layer composed of matrigel marix. Culture supernatants collected from previously washed and mature or

immature DCs were added to the lower chambers of transwell plates. PBMCs isolated from CLL patients were partly depleted of B cells with $CD19^+$ magnetic beads before they were added to the upper chambers. The plate was incubated for 24 hours. Cells that migrated to the lower chamber were harvested and stained with fluorescently labelled antibodies specific for CD5, CD56, HLA-DR, CD3 and CD45. $CD3^-CD56^+$ NK cells and $CD3^+CD56^+$ NKT cells were subsequently defined and counted.

In paper III, a chemotactic transwell assay was used to evaluate the chemotaxis of monocytes and different lymphocyte subsets towards chemokines produced by differently matured tumor-loaded allogeneic vaccine DCs generated from healthy blood donors. Unlike paper I and II, in which supernatants were added to the lower chambers, in paper III the vaccine cells were cultured directly in lower chambers of transwell plates and after 24 hours of maturation, the DCs were washed and replaced in fresh medium for a further 24 hours (a similar procedure to that in paper I and II but in paper III no supernatants were collected). PBMCs isolated from CLL patients were partly depleted of B cells (as in paper II). After 24 hours incubation, PBMCs isolated from CLL patients were added to the upper chamber and the plate was incubated for 90 min. Migrated monocytes were distinguished from vaccine DCs by pre-labeling PBMCs from CLL patients with fluorescent cell linker dyes (PKH2). This technique provides a non-specific cell membrane labeling of live cells over an extended period of time. For quantification of migration, the cells that migrated to the lower chamber were harvested and stained with fluorescently labelled antibodies specific for CD14, CD4, CD8, CD56 and CD3. $CD14^+PKH2^+$ monocytes, $CD3^-CD56^+$ NK cells, $CD3^+CD56^+$ NKT cells, $CD3^+CD8^+$ T cells and $CD3^+CD4^+$ T cells were subsequently defined and counted.

CO-CULTURE SYSTEMS

Various co-culture systems, often referred to as mixed leukocyte reactions (MLRs) were used to study the interaction of vaccine DCs and different subpopulations of leukocytes. The combination of these different experimental systems were intended to give an insight into some key mechanisms in the sequence of events that may occur following injection of vaccine DCs into a cancer patient.

A model to mimic DC-NK cell interactions in lymphoid organs

In paper I, co-culture experiments were performed to study the potential of differentially matured vaccine DCs, generated from healthy blood donors, to induce “helper” NK cell activation, determined by intracellular IFN-γ, which was recently suggested to assist DC-mediated Th1 polarization (Martin-

Fontecha 2004). Therefore, to mimic the *in vivo* situation where subcutaneously injected autologous DCs first have to migrate to draining lymph nodes, previously washed mature vaccine DCs were cultured in fresh medium for a further 24 hours, before co-culturing with non-adherent PBMCs (PBMC/DC ratio, 5:1). Co-cultures were performed in the presence of brefeldin A, a product that inhibits transport of proteins (i.e. IFN-γ) from ER to Golgi and which leads to protein accumulation inside the ER. DCs used in this experiment had previously been stimulated with either the PGE_2DC or the αDC1 maturation cocktail for 24 hours. After 6 hours of subsequent co-culture, the cells were harvested. To allow detection of intracellular IFN-γ, a fixation/permeabilization procedure was performed before fluorescently labelled antibodies specific for IFN-γ, CD56, CD3, and the NK cell activation marker CD69, were added. Activation of $CD3^-CD56^+$ NK cells was analyzed by flow cytometry.

A model to mimic potential vaccine DC-T cell interactions in lymphoid organs

In paper II, an artificial co-culture system was used to study the ability of differentially matured vaccine DCs generated from CLL patients to secrete Th1-polarizing IL-12p70 upon CD40 ligation. The system was also used to evaluate their production of the chemokines CCL3/MIP-1α and CCL4/MIP-1β which have been shown to play a crucial role in recruitment of $CD8^+$ T cells to sites of DC-$CD4^+$ T cell interactions (Castellino 2006). DCs used in this experiment were pulsed with heat stressed, necrotic CLL cells and left immature or stimulated with either the PGE_2DC or the αDC1 maturation cocktail for 24 hours before being washed twice, replaced in the well and cultured in fresh medium for a further 24 hours. Mature DCs were stimulated with soluble, histidine-tagged, CD40 ligand followed by the addition of anti-polyhistidine monoclonal antibody. This procedure was aimed to mimic the interaction of a potential vaccine DC with CD40L-expressing cells, such as $CD4^+$ T cells and NKT cells at lymph node entry. Supernatants were collected after 24 hours and tested for the presence CCL3/MIP-1α, CCL4/MIP-1β and IL-12p70 by ELISA.

Selective allogeneic MLR to mimic immune responses at the injection site

The experimental systems used in paper III were designed to mimic the *in vivo* situation, where allogeneic vaccine DCs must recruit favorable subsets of leukocytes which assist in the generation of a pro-inflammatory milieu at the injection site that is of importance for proper activation of co-recruited endogenous DCs. The chemotaxis transwell assay described earlier was also used to study the production of the proinflammatory cytokine IFN-γ induced in "selective" MLRs of differentially matured allogeneic vaccine DCs and

recruited PBMCs isolated from CLL patients. As previously described monocyte-derived immature DCs from healthy donors were cultured in lower chambers of 24-(trans)well plates, pulsed with heat stressed, necrotic CLL cells and matured with either the αDC1 or PGE_2DC maturation cocktail for 24 hours before being washed and replaced in fresh medium. Unstimulated DCs were used as immature controls. After 24 hours incubation, PBMCs isolated from CLL patients and partly depleted of B cells, were added to the upper chamber. After 90 min incubation, all inserts were removed and the plate was incubated for a further 24h. Thereafter, supernatants from these selective MLRs were collected and the concentration of IFN-γ was measured by ELISA. Supernatants from selective MLRs were also used in functional assays for the maturation of monocytes, representing *in vivo* recruited "bystander" monocytes.

Phenotypic maturation of bystander monocytes

The above described assay was also used to study the phenotypic maturation of recruited bystander monocytes (paper III). Migrated PKH2-labelled monocytes, matured in the microenvironment created by differentially matured allogeneic DCs and recruited lymphocytes for 24h, were analyzed for the presence of DC maturation markers. Cells were stained with fluorescently labelled antibodies specific for CCR7, CD40, CD83, CD86, and analyzed by flow cytometry.

Evaluation of functional maturation in bystander monocytes

The Th1-deviating capacity of bystander DCs was studied in a Staphylococcus Enterotoxin B (SEB)-driven model (paper III). SEB is a superantigen that has been used as a surrogate antigen in different *in vitro* models (Mailliard 2002), as it allows stimulation of a high proportion of autologous $CD4^+$ and $CD8^+$ T cells by cross-linking MHC molecules on DCs and TCRs expressing the appropriate Vβ chains (Fraser 1989). Functional maturation of bystander monocytes was evaluated by culturing monocytes isolated from CLL patients in selective MLR supernatants from differentially matured allogeneic vaccine cells and migrated PBMCs. Monocytes were plated in 24-well plates. After 24 hours stimulation, the cells were washed and replaced in fresh medium for a further 24 hours. This was done in order to mimic the *in vivo* situation, where endogenous DCs that phagocytose dying vaccine cells at the injection site first have to migrate to draining lymph nodes to induce polarized adaptive immune responses. The next step of this system was aimed to simulate cell interactions that may occur when endogenous DCs reach a draining lymph node. Following 24 hours incubation, activated bystander monocytes/DCs were coated with SEB followed by the addition of autologous PBMCs depleted of monocytes and partly depleted of B cells. After 6 days of co-culture, Th1-deviation was estimated by using ELISA to determine the production of the typical Th1 cytokine IFN-γ and the typical

Th2 cytokines IL-4 and IL-5 in these primary autologous MLR supernatants. The Th1-deviating capacity of bystander DCs was further studied by re-stimulating previously washed PBMCs, collected from different primary MLR cultures, with autologous SEB-coated CLL cells. After 24 hours of re-stimulation, supernatants were collected and the production of IFN-γ, IL-4 and IL-5 was evaluated.

RESULTS AND DISCUSSION

PAPER I

Sallusto and colleagues convincingly demonstrated using a mouse model that the chemokine receptor CXCR3 has a central role in lymph node–homing properties of NK cells on stimulation by injected mature DCs or certain adjuvants and further that these recruited NK cells provide an early source of IFN-γ that is necessary for Th1 polarization (Martin-Fontecha 2004; Bajenoff 2006). This unique function demonstrated by certain adjuvants implies that for vaccines that depend on Th1 responses, adjuvants could be selected according to their ability to recruit NK cells into antigen-stimulated lymph nodes. With regard to this important finding, the aim of paper I was to compare the ability of second generation PGE_2DC-vaccines and the new vaccine candidate αDC1s to selectively produce favorable chemokines such as CXCR3 ligands and to recruit and activate human NK cells *in vitro*.

αDC1s but not PGE_2DCs produce desirable chemokines of importance for NK cell recruitment

Our data show that αDC1s generated from healthy blood donors secrete profuse amounts of the CXCR3 ligand CXCL9/MIG and also substantial amounts of CXCL10/IP-10 and CXCL11/I-TAC after withdrawal of maturation stimuli, that is at the point in time when they are ready to be injected. In contrast, no measurable levels of the CXCR3 ligands were produced by PGE_2DCs (paper I figure 2a).

It has become clear that a major challenge in the development of a successful tumor vaccination method is to avoid the recruitment of suppressive Tregs to sites of antigen-specific DC-T cell interactions within vaccine-draining lymph nodes that could hinder optimal activation. In paper I we found that PGE_2DCs generated from healthy blood donors preferentially produced Th2 and Treg-recruiting CCL17/TARC and CCL22/MDC (Bonecchi 1998; Iellem 2001), whereas only marginal levels of these chemokines were produced by αDC1s (paper I figure 2b). These findings are further supported by recent data from Muthuswamy et al. demonstrating *in vitro* that such monocyte-derived PGE_2DCs produced CCL22/MDC that efficiently attracted $FOXP3^+$ Tregs (Muthuswamy 2008). It was concluded that the ability of mature DCs to interact with Treg cells was predetermined at the stage of DC maturation, as the CCL22 producing capacity by PGE_2DCs persisted after the removal of maturation stimuli and was further elevated after secondary stimulation of DCs in a neutral environment.

αDC1s show an increased NK cell recruiting capacity compared to PGE_2DCs

Results from chemotactic transwell experiments show that supernatants collected from previously washed αDC1s, but not from PGE_2DCs, induced a substantial recruitment of NK cells. We evaluated the possibility that the extremely high level of CXCL9 in supernatants from αDC1s was primarily responsible for the observed NK cell recruitment. When anti-CXCL9 antibodies were added to the αDC1 supernatants, a marked reduction of NK cell migration was observed (paper I figure 4). This novel feature of αDC1s, such as high and sustained production of the NK cell–recruiting CXCR3 ligands correlating with functional NK cell recruitment, thus indicates a potential NK cell–recruiting capacity into draining lymph nodes when injected into human patients.

Helper cell requirement for autologous αDC1-induced IFN-γ production by NK cells

In line with recent data from different mouse models (Martin-Fontecha 2004; Bajenoff 2006), our *in vitro* data demonstrate that NK cells became activated on interaction with DCs. However, only αDC1s were able to induce a substantial IFN-γ production, as determined by flow cytometry (paper I figure 5a). Furthermore, when compared with PGE_2DCs, αDC1s proved superior in inducing up-regulation of CD69, which is an inducible cell surface protein that first appears during NK cell activation. The observed activation of NK cells within bulk lymphocyte fractions, induced by co-cultured αDC1s, was dependent on co-factors expressed by lymphocytes within the responding non-adherent PBMC population. One obvious co-factor candidate is CD40L which was recently shown to play a prominent role in DC dependent activation of human NK cells primed with IL-18 (Mailliard 2005). In line with these findings, we found that addition of CD40-mediated stimulation (by crosslinked soluble CD40L) was needed in order to induce substantial IFN-γ production in co-cultures of purified autologous αDC1s and purified NK cells (paper I figure 5b). These ELISA results were thus in line with FACS data on intracellular expression of IFN-γ.

Taken together, these data thus suggest that the observed discrepancy as to IFN-γ production in αDC/NK cell co-cultures using purified NK cells or NK cells contained within a total lymphocytes population depends on the absence or presence of potentially CD40L-expressing lymphocytes that may upregulate CD40L upon stimulation with autologous αDC1s. Since no non-self antigens, such as xenogneic proteins from fetal calf serum, were included in the present culture media, such cells could possibly be CD1d-restricted NKT cells recognizing endogenous glycolipids presented on mature DCs (Mattner 2005) or

autoreactive $CD4^+$ T cells that become activated during an autologous mixed lymphocyte reaction (Scheinecker 1998).

To summarise, these novel findings presented in Paper I indicate that injected human αDC1-based clinical grade vaccines have the potential to recruit and activate NK cells upon arrival at draining lymph nodes and that this feature may be relevant for efficient Th1 polarization by DC based-vaccines.

PAPER II

Despite the previous reports of dysfunctional DCs in patients with CLL, Kalinski and coworkers showed that functional αDC1s, loaded with γ-irradiated autologous tumor cells, could be generated from CLL patients (Lee 2008). Compared with PGE_2DCs, these αDC1s showed higher expression of several co-stimulatory molecules without a significant negative impact on tumor antigen loading. Furthermore, they also produced higher levels of IL-12p70 and were much more effective in inducing functional, tumor-specific CTL responses. However, no information was given regarding their ability to produce CXCR3-ligands or to recruit NK cells and potential CD40 ligand-providing lymphocytes.

Therefore, the initial aim of paper II was to examine *in vitro* the capacity of tumor-loaded αDC1s and PGE_2DCs, derived from CLL patients, to produce a chemokine profile that favors the recruitment of NK and potentially CD40L expressing NKT cells.

Tumor-loaded αDC1s show an increased NK and NKT cell recruiting capacity compared to PGE_2DCs

In paper II we were able to demonstrate that αDC1s generated from CLL patients also produced significantly higher amounts of the CXCR3 ligands CXCL9, 10 and 11 than PGE_2DCs (paper II figure 1a-c). Importantly, loading of αDC1s with necrotic CLL cells had no negative impact on chemokine production. Functional studies further demonstrated that αDC1s from CLL patients were superior recruiters of NK cells (paper II figure 3). Since NKT cells may be potential providers of CD40 ligands when interacting with DCs (Fujii 2004) and further express a similar chemokine receptor pattern as NK cells (Thomas 2003) we also evaluated the NKT-recruiting capacity of vaccine DCs and found that supernatants from αDC1s induced a prominent recruitment of NKT cells (paper II figure 3).

Tumor-loaded αDC1s are superior producers of CCL3/MIP-1α, CCL4/MIP-1β and IL-12p70 compared to PGE_2DCs, upon CD40 ligation

Optimal vaccine DCs should most probably mediate a $CD4^+$ T cell-dependent guiding of rare tumor-specific $CD8^+$ T cells to the site of antigen-dependent DC-$CD4^+$ T cell interactions through secretion of CCL3/MIP-1α and CCL4/MIP-1β chemokines (Castellino 2006). We therefore evaluated whether differentially matured tumor-loaded DCs generated from CLL patients were able to produce these chemokines in response to subsequent CD40-ligation, intended to mimic the interaction with $CD4^+$ T cell. In order to optimally mimic the *in vivo* situation, previously washed mature DCs were cultured in fresh medium for a further 24 hours and subsequently washed before CD40 stimulation by cross-linked soluble CD40L. This was done to compensate for the time required for the DCs to migrate to a draining lymph node. We found that tumor-loaded αDC1s produced higher amounts of $CD8^+$ T cell-recruiting chemokines CCL3/MIP-1α and CCL4/MIP-1β upon CD40 ligation, as compared to PGE_2DCs (paper II figure 4a-b).

Of major importance for efficient induction of tumor specific CTLs is also the production of Th1-polarizing IL-12p70 (Zitvogel 1996; Xu 2003). In accordance with Lee et al (Lee 2008), we could show that tumor-loaded αDC1s were superior in producing IL-12p70 compared to PGE_2DCs after CD40 ligation (paper III figure 4c).

Tumor-loaded PGE_2DCs mainly produce Th2 and Treg attracting chemokines

In line with the observations on PGE_2DCs from normal blood donors in paper I, we found that PGE_2DCs generated from CLL patients produced significantly higher levels of CCL22/MDC after withdrawal of maturation stimuli, as compared to αDC1s (paper II figure 2a).

This tendency towards Treg recruiting properties associated with PGE_2DCs in *in vitro* experiments is further in line with clinical observations on myeloma patients, were injected autologous PGE_2-matured DCs from myeloma patients where shown to expand even more $FOXP3^+$ Treg cells than immature DCs and they therefore concluded that vaccine-mediated induction of Tregs may be an underappreciated effect in clinical trials of human DC vaccination (Banerjee 2006). Taken together, our *in vitro* data and these observations by others underline the importance of developing optimal DC maturation conditions which result in vaccine DCs that avoid interaction with Tregs.

To summarise paper II, we found that tumor-loaded αDC1s derived from CLL patients produced sustained and substantially higher amounts of CXCR3-ligands compared to PGE_2DCs. Instead, PGE_2DCs produced higher levels of Th2 and Treg-attracting chemokines. Functional studies demonstrated that αDC1s from CLL patients were superior recruiters of NK cells and also of potential CD40 ligand-expressing NKT cells and further, upon CD40 ligation, produced higher amounts of IL-12p70 and $CD8^+$ T cell recruiting CCL3/CCL4 compared to PGE_2DCs. Importantly, loading of αDC1s with necrotic CLL cells had no negative impact on chemokine production. Taken together, these findings suggest that functional αDC1s with an imprinted desirable chemokine-producing capacity can be generated from CLL patients, supporting the idea that αDC1-based vaccines have a higher immunotherapeutic potential than PGE_2DCs in this disease.

PAPER III

In an allogeneic DC vaccination setting, priming of the adaptive immune system most likely has to be mediated by endogenous APCs that are recruited to the injection site in response to inflammatory and chemotactic signals. Tumor antigens may be released after NK-cell/CTL-induced apoptosis of the antigen-loaded allogeneic vaccine cells (Laffont 2006; Laffont 2008), and subsequently become ingested**,** processed and presented by endogenous APCs, including monocyte-derived DCs. By trafficking antigens from the site of injection to draining lymph nodes, these endogenous DCs may then present antigen to naïve and self-MHC-restricted T cells.

The aim of paper III was therefore to compare the ability of allogeneic PGE_2DCs and αDC1s derived from healthy blood donors to recruit potential endogenous DC precursors such as monocytes and different subsets of potentially accessory cells from CLL patients, and further to investigate if the recruited monocytes underwent phentotypic and/or functional maturation towards desirable Th1-deviating DCs.

αDC1s provide desirable factors that enable monocyte and accessory cell recruitment

Fundamental for the local recruitment of immune cells is the activation of the endothelial cells at the vaccine site that provide signals for adhesion, arrest and transmigration of these cells. Two of the most potent inducers of endothelial cell adhesion molecules are TNF-α and IL-1β (Langer 2009). In paper III we therefore evaluated whether these pro-inflammatory cytokines were produced by mature vaccine DCs from time of withdrawal of maturation stimuli, a time point when mature vaccine DCs are proposed to be subcutaneously injected. We

found that αDC1s produced sustained and higher levels of TNF-α than PGE_2DCs (paper III figure 1a), while there was a comparable production of IL-1β (paper III figure 1b).

Chemokine gradients selectively stimulate the adhered leukocytes to move in between endothelial cells and pass into the tissues. Therefore, vaccine DCs should produce a desirable chemokine profile in order to facilitate recruitment to the injection site of DC-precursors like monocytes and subsets of potential accessory cells, including NK, NKT cells and possibly also alloreactive T cells (Wallgren 2005), to the injection site. The chemokines CCL2/MCP-1, CCL3/MIP-1α and RANTES play an active role in recruiting DC precursors and Th1-associated lymphocytes into inflammatory sites. We found that previously washed αDC1s produced considerably higher amounts of MCP-1, MIP-1α and RANTES, as compared to PGE_2DCs (paper III figure 2a-c). In functional chemotactic transwell experiments, αDC1s were found to recruit a substantial amount of monocytes. However, contrary to the chemokine data, no evident difference in monocyte recruitment between αDC1s and PGE_2DCs was observed (paper III figure 3). In paper I, we also showed that αDC1s from healthy donors, in contrast to PGE_2DCs, secrete large amounts of CXCR3-ligands after withdrawal of maturation stimuli. In accordance with their chemokine profile, αDC1s were found to induce a substantially higher recruitment of Th1-associated lymphocyte subpopulations, compared to PGE_2DCs (paper III figure 4)

Together, this indicates that injected αDC1s produce factors that would most likely support the recruitment of endogenous monocytes and desirable lymphocyte subsets to the injection site *in vivo*.

Bystander monocytes, matured in an allogeneic αDC1-induced environment become phenotypically mature, DC-like cells, within 24 hours

When monocytes are recruited from the blood into inflammatory sites they may undergo a coordinately regulated process of maturation into DCs. The first step in this process is the differentiation of recruited monocytes into immature dendritic cells, which may be triggered by transendothelial migration itself (Randolph 1998), and by factors such as GM-CSF produced by endothelial cells (Kaushansky 1989) and co-recruited NK and NKT cells (Hegde 2007; Zhang 2007). We therefore investigated the ability of allogeneic mature DCs to induce phenotypic maturation in monocytes within the PBMC population from CLL patients that had been recruited to a micro-environment created by allogeneic vaccine DC and co-recruited lymphocytes. We found that monocytes recruited by both αDC1s and PGE_2DCs up-regulated the DC-associated phenotypic

markers CD40, CD83 and CCR7 within 24 hours of co-culture with mature allogeneic vaccine DCs and recruited lymphocytes (paper III figure 5).

Bystander monocytes primed in an αDC1-induced environment show enhanced Th1-deviating capacity

Further, we examined whether monocytes matured in a αDC1-induced environment became functionally mature Th1-deviating DCs. Potential Th1-deviation was determined by evaluating the typical Th1 cytokine IFN-γ and the Th2 cytokines IL-4 and IL-5. Preliminary data indicated that SEB-coated monocytes from CLL patients that had been exposed to supernatants from co-cultures of allogeneic αDC1s and recruited PBMCs from CLL patients, may activate autologous SEB-reactive T cells into Th1-deviated T cells (paper III figure 6a and b). Importantly, SEB-reactive T cells from these primary cultures retained their Th1-profile upon subsequent restimulation with SEB-coated autologous CLL tumor cells (paper III figure 6c and d). Such prominent and sustained Th1-deviating ability was not seen in monocytes that had been exposed to supernatants from co-cultures of PGE_2DCs and recruited PBMCs (paper III figure 6 a-d).

αDC1s induce IFN-γ production in co-cultures with recruited leukocytes from CLL patients

Although both αDC1s and PGE_2DCs induced a phenotypic maturation in recruited bystander monocytes, results in paper III indicate that only monocytes primed in a αDC1-induced environment seem to favor Th1-deviation. The similar capacity of αDC1s and PGE_2DCs to induce phenotypic maturation is well correlated with our data demonstrating that both αDC1s and PGE_2DCs passively produce the pro-inflammatory cytokines TNF-α and IL-1β which are both known to promote DC maturation (Sallusto 1994; Sallusto 1995). However, in order to induce efficient Th1-deviation by DCs, additional instructions by the pro-inflammatory cytokine IFN-γ during DC maturation have been shown to be required (Vieira 2000). We therefore looked for possible differences in IFN-γ production in supernatants from co-cultures of allogeneic vaccine DCs and recruited PBMCs that were used for bystander monocyte maturation. IFN-γ production was only detected in co-cultures of allogeneic αDC1s and recruited PBMCs (paper III figure 7a and b). These findings highlight the importance of recruiting desirable subsets of Th1-associated lymphocytes that contribute to a favorable immunogenic microenvironment at the vaccine site, where recruited DC-precursors are supposed to be primed.

To summarise, in paper III we found that tumor-loaded αDC1s derived from healthy blood donors recruit monocytes and Th1-associated lymphocytes from

CLL patients. Our data indicate that monocytes that become activated in a pro-inflammatory environment created by allogeneic αDC1s and recruited subsets of PBMCs, may undergo phentotypic and functional maturation toward Th1-deviating DCs. Such Th1-deviating ability was not seen in monocytes that had been primed in a PGE_2DC-induced environment. However these are still preliminary results and further evaluation is needed to confirm these observations.

CONCLUDING REMARKS

From the data presented in this thesis, I conclude that the *ex vivo* conditions present during maturation of DC-based cancer vaccines imprint several functions, such as stable chemokine profiles, that enable a selective interaction between the DCs and different leukocyte subsets, and further that such features are of central importance for their potential to induce type-1 polarized immune responses.

SPECIFIC CONCLUSIONS TO GIVEN AIMS:

- αDC1s but not standard PGE_2DCs generated from healthy individuals, present a stable and desirable Th1-associated chemokine profile that enabled recruitment of NK cells. Only αDC1s were efficient inducers of IFN-γ production in autologous NK cells. However, this was dependent on the presence of a third cell subset providing concurrent CD40 ligation.
- Functional autologous tumor-loaded αDC1s with a desirable Th1-associated cytokine and chemokine profile can also be generated from CLL patients. αDC1s were able to recruit NK cells and potential CD40L providing NKT cells and following CD40 ligation αDC1s were superior producers of factors known to be important for a guided and Th1-deviated priming of $CD8^+$ T cells.
- In the allogeneic setting, αDC1s derived from healthy blood donors were shown to recruit monocytes and Th1-associated lymphocytes from CLL patients. Monocytes primed in such αDC1 but not PGE_2DC-induced environment seem to undergo maturation toward Th1-deviating DCs.

Results in this thesis provide some new pieces to the complex puzzle of DC biology that may help to design future generations of DC-based vaccines. Our findings suggest that to induce efficient Th1-polarized immune responses *in vivo*, DCs must deliver a "selective chemokine-based signal 0" that precedes the classical antigen-specific signal 1 and co-stimulatory signal 2, a guiding signal that we believe is of central importance for the ability of DCs to deliver the Th1-polarizing signal 3 and assist tumor-specific T cells to acquire desirable effector functions. Our findings further indicate that allogeneic αDC1s may be used as an adjuvant in anti-cancer vaccination. By using allogeneic vaccine cells loaded with antigens derived from allogeneic tumor cells it will be possible to develop a fully allogeneic vaccine strategy based on a panel of antigen-loaded vaccine cells that can be prepared in advance and cryopreserved before the initiation of clinical studies.

CLINICAL PERSPECTIVE AND FUTURE DIRECTIONS

Until now the main focus of research in the field of DC-based immunotherapy has been on optimizing the immunostimulatory potential of injected vaccine DCs, aimed at boosting the activation and proliferation of tumor-specific CTLs. Few clinical studies have considered the presence of the often reported abnormal amounts of regulatory T cells or analyzed responses from lymphocyte subsets other than CTL e.g., Th1, NK, and NKT cell from a given patient population. Furthermore, reported "promising" DC-based vaccinations are often defined by success in demonstrating the expansion of circulating tumor-specific $CD8^+$ T cells which does not necessarily correlate with a clinical anti-tumor response (Rosenberg 2005). This emphasizes the need to develop strategies that generate effector cells that efficiently target tumors *in vivo* and further to find out how to "prepare" the cancer-induced suppressive environment to enable effector cell-induced tumor-elimination (Gajewski 2006).

In order to achieve the goal of long lasting cancer eradication and cure, a broader perspective of the cancer field may most probably be required. The increasing knowledge of the molecular pathways involved in the pathogenesis of cancer and the interaction of the immune system with blood cancer cells as well as with different types of solid tumors has provided a rationale for the development of novel treatment strategies.

Perhaps the most unique feature of DC-based cancer vaccines is the fact that the vaccine initiates a dynamic process of host immune responses that may be exploited in other therapies. There are now several clinical studies that have provided evidence of the possibility to boost the effect of different types of cell-based immunotherapy by a rational combination with other therapies that target various mechanisms that are used by tumors to avoid immune-mediated killing.

Pre-treatment with ionizing radiation (IR) and some chemotherapeutic agents, preceding immunotherapy may induce changes in surviving cancer cells, which enhance their antigenicity and immunogenicity that promote adaptive immune responses (Ganss 2002; Chakraborty 2003; Chakraborty 2004; Garnett 2004; Reits 2006; Matsumura 2008; Liu 2010). Dose-dependent effects of the cytostatic drug cyclophosphamide, which frequently is used for the treatment of leukemias, lymphomas, and solid tumors, have been shown to enhance the antitumor efficacy of immunotherapy through the depletion of Tregs (Lutsiak 2005) and induction of desirable cytokine expression (Bracci 2007). Furthermore, combination with antibody therapy or soluble receptors may be used to offset inhibitory signals in effector lymphocytes (Peggs 2009) or for the blockade of tumor-associated suppressive cytokines (Moore 2001; Li 2006).

Further clinical and *in vitro* studies are needed to explore the potential of such immunotherapeutic combination regimens, as well as to provide a better understanding of optimal DC maturation conditions, route of vaccination, dose and schedule. These will all be keys in the development of the most efficient vaccination strategies, with the ultimate goal to improve the cancer patients' survival time with best possible quality of life.

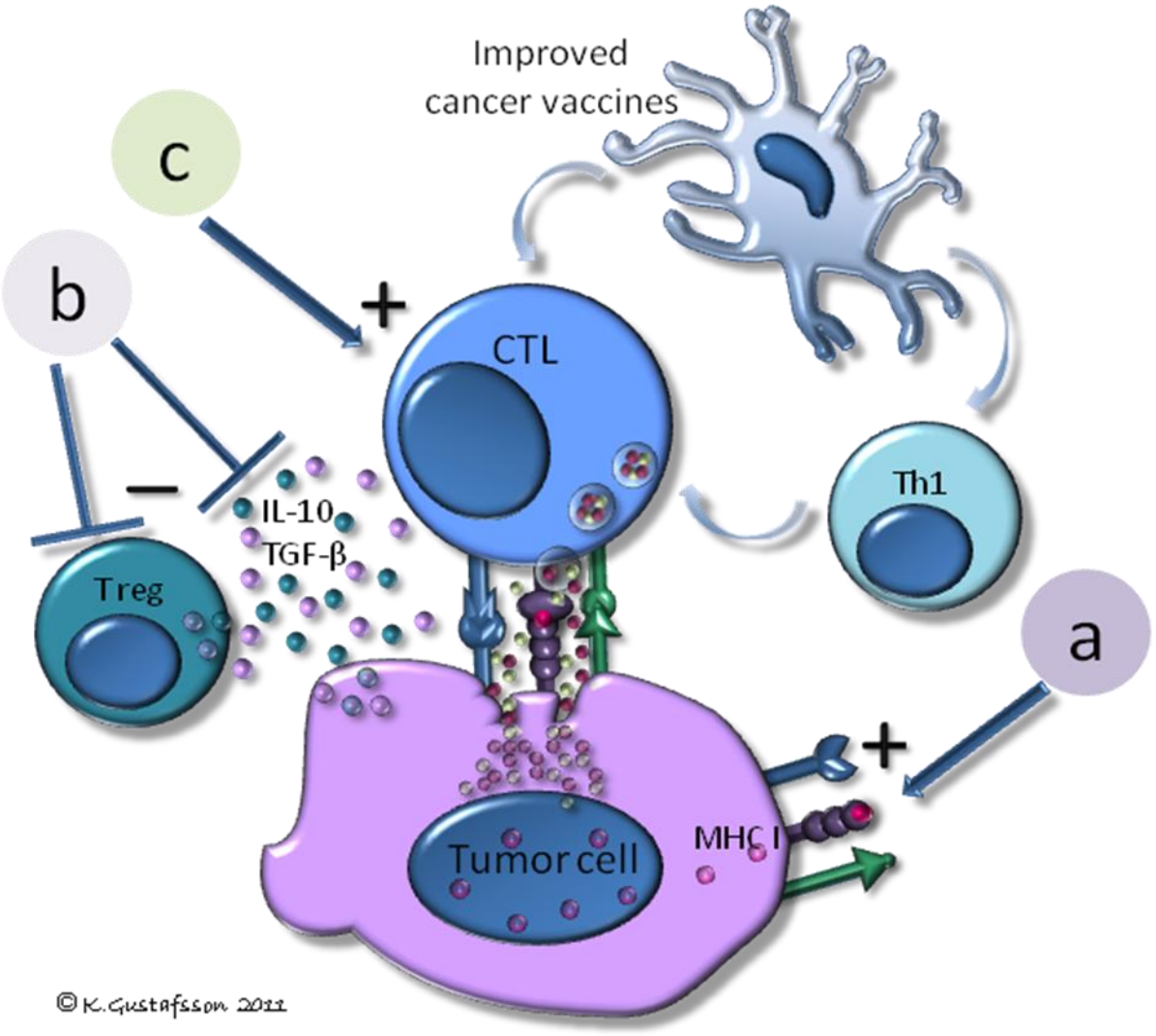

Figure 4. DC-based vaccines in combination therapy

Combination therapy has been shown to improve the clinical efficacy of cell-based immunotherapy. This may be variously attributed to several factors including: a) enhanced presentation of tumor-antigens and other alterations in phenotype that facilitate immune-mediated killing b) reduction of Tregs or suppressive cytokines and c) boosting of tumor-specific effector cells.

ACKNOWLEDGEMENT

Finally, I would like to express my sincere gratitude to all those who have directly or indirectly contributed to the completion of this work. Especially, I wish to thank:

Peter Eriksson, min första huvudhandledare. Tack för att du gav mig möjligheten att få börja forska i din grupp. Har saknat dig. Så även ditt fantastiska sätt och din enorma förmåga att uppmuntra och inspirera. Har dig att tacka för mycket, inte minst för att du öppnade mitt hjärta för Italien och gav mig förtroendet att styra Italienskeppet till stor del på egen hand, en resa som berikat mitt liv och lärt mig massor. Tack för många roliga minnen därifrån. Sörjer och saknar dig oerhört..… Tack!

Alex Karlsson-Parra, min första bihandledare och andra huvudhandledare. Tack för att du ställde upp och tog på dig huvudhandledarskapet när det overkliga plötsligt var ett faktum och tillvaron vändes upp och ner. Tack för att du alltid givmilt delar med dig av din ofantliga kunskap och för alla nya spännande idéer jag fått ta del av. Tack för alla långa samtal om immunologins förunderliga värld. Har verkligen lärt mig ”tänka” immunologi tack vare dig. Tusen tack för allt!

Milos Pekny, min andra bihandledare, tack för att du varit min bihandledare under de sista åren av mitt doktorerande.

Per-Ola Andersson, för ditt engagemang, för att du alltid ställt upp och tagit dig tid, trots att kalendern varit full. Oändligt tacksam för att du gick in och agerade ”bihandledare” under den tuffa slutspurten av mitt doktorerande. Din kunskap och klarsynthet har verkligen varit en tillgång för våra gemensamma projekt, likaså för mitt avhandlingsskrivande. Tusen tack! Tack till **Olle Werlenius** för medförfattarskap, databearbetning och för givande diskussioner.

Katarina Junevik, har varit så fantastiskt kul att jobba tillsamman med dig. Du är en helt underbar kollega och vän, tack för alla intressanta samtal om livet, relationer och annat som känns. Tack även för alla insatser när det gäller våra gemensamma projekt. Du är en klippa på alla sätt och dessutom en fena på FACS, vilket gjort mitt liv som forskare betydligt lättare än det annars skulle ha varit. Kommer sakna dig, tusen tack!

Till mina fina samarbetspartners i njurgruppen, för allt stöd och uppmuntran under de här åren. Tack **Börje Haraldsson**, för din omtanke, värme och support, utan ditt stöd hade den här resan inte varit möjlig att slutföra, är dig innerligt tacksam för allt. Tack **Jenny Nyström** för peppande ord och positiv energi, var verkligen roligt att åka till Bryssel tillsammans med er. **Madeleine Ingelsten** min fina vän, medförfattare, reskamrat och jourhavande doktorandsupport, tack för alla trevliga stunder, intressanta diskussioner, och för att du alltid ställt upp när jag behövt lite stöd och råd. Glad att jag fått lära känna dig, tusen tack! Tack även till övriga medarbetare i njurgruppen för att ni med ert öppna, sprudlande och glada sätt fick mig att känna mig som en i gänget från första stund, även om ”stunden” inte blev så lång.

Kollegor i Italien, stort tack till alla mina medarbetare i San Giovanni Rotondo, tack för alla roliga dagar på labbet och trevliga middagar, fick mig verkligen att känna mig

som hemma. Tusen tack även till min kollega, tolk och vän **Luigi Pellettieri.** Tack för alla fina stunder och roliga minnen från våra gemensamma Italienresor, tack för allt du lärt mig både när det gäller vetenskap och livet, är verkligen glad för att jag fått lära känna dig. Tusen tack för allt!

Alla vänner, nutida och forna kollegor på CBR, tack **Ann-Marie Alborn, Birgit Linder, Linda Paulsson, Niklas Karlsson, Marie Kalm, Ina Nordin, Nina Hellström, Maurice Curtis, Åsa Persson, Cecilia Bull, Helene Andersson, Jonas Faijersson, Jenny Nyberg, Charlotta Lindwall, Mathilda Zetterström Axell, Jenny Zhang, Mikael Nilsson, Georg Kuhn, Christi Kuhn, Fredrik Blomstrand, Axel Jansson, Karin Hultman, Sheila Engdahl, Andrew Naylor, Maria Åberg, Johan Lind, Klas Blomgren, Åsa Widestrand, Lizhen Li, Rita Grandér, Henrik Landgren, Olle Lindberg, Malin Jonsson, Johanna Zandén,** tack till er alla för stöd, kloka råd, många trevliga fester, och annat kul.

Bertil Lindblom, tack för stöd och hjälp under de här åren.

Gunnel Nordström, Anki Nyberg, Ingrid Worth, Ulla Ohlsson, Kirsten Toftered, Carina Mallard, Marianne Arnstein Olsson, Mari Klaesson stort tack till er alla för all hjälpsamhet med allt praktiskt och administrativt av olika slag.

Patrik Johansson och Oskar Bergström, tack för all hjälp med datorer och IT, hade inte kommit långt med mitt skrivande utan er.

Britt-Marie Essman, du var verkligen ovärderlig när jag började som stipendiat en gång för länge sedan, så otroligt tacksam för allt du lärde mig och hjälpte mig med under den tid jag var grön och ovan i labbrocken. Dina finurliga tips och trix har verkligen underlättat mitt arbete. Tusen tack även för fantastiskt trevliga privata pratstunder och mysiga middagar tillsammans med dig och **Arne**. Dessutom, tack för sylektionerna, det fina lapptäcket var helt din förtjänst, tack!

Mirjana, tusen tack för allt stöd genom åren, tack för alla givande och spännande samtal om livet och framtiden. Du är en fantastiskt fin människa, otroligt glad att jag fått lära känna dig.

Erika Lindberg, visserligen en av mina nytillkomna vänner, men ack så betydelsefull du varit under den här processen! Tack för allt stöd, uppmuntrande ord, hjälp med korrekturläsning av texter, energihöjande praliner och behövliga pratstunder även om allt annat som inte rör forskning och avhandlingsskrivande. Så fantastiskt glad att jag hann lära känna dig innan jag lämnade stället, önskar bara att vi fått chansen att jobba ihop på riktigt, då jag känner att vi har liknande syn på mycket och definitivt hade haft många roliga dagar tillsammans. Tusen tack för allt!

Alla på IE som alltid ställt upp och hjälpt mig att köra FACS och ELISA på otaliga prover genom åren. TACK!!

Damerna på allergi, tack för att ni kom och gav lite liv till korridoren igen, alltid kul att komma förbi och träffa er alla!

Tack till alla mina underbara **Vänner** och **min Familj** som har funnits vid min sida under mina år på det här klotet.

Sandra Holmgren, Julia Kovacka, Linda Bergqvist, John Alder och Tanya Karlsson, vet att ni egentligen hör till kategorin kollegor men kändes fel att nämna er där då ni har varit som en familj för mig under min tid som doktorand och lite utav en livlina under de här stundtals tuffa åren. Tackar er verkligen utan någon som helst inbördes ordning. Hittar inte ord för att uttrycka hur mycket ni betyder för mig och hur viktiga ni har varit för färdigställandet av den här boken. Utan er hade det aldrig gått vägen. Tack **Sandra** för fantastisk vänskap, allt stöd och för alla underbara skrattanfall, tänk vi måste båda bli en bra bit över hundra, med tanke på alla de stunder vi dubbelvikta skrattat så tårarna sprutat. Tack **Julia** min fina vän, så otroligt glad för att jag fått lära känna dig, tack för alla långa och intressanta samtal, var alltid lika glad när jag gick hem efter ett av alla sådana. **Linda**, tusen tack för alla roliga stunder på jobbet och privat. Tack även för roligt sällskap på vår trevliga Bambergresa, glömmer aldrig de oväntat tokiga, men mycket trevliga kvällarna! Tack **John** för allt, vänskap, din sköna humor, för allt du lärde mig när jag var ny på labbet och inte visste hur man räknade celler eller separerade blod och inte minst, tack för all ovärderlig hjälp med engelskan. **Tanya**, fina vännen min, tack för alla härliga stunder, för din värme, omtanke och innerlighet. **TACK!!!!**

Susanna Johnson, Marcus Larsson och lilla **Vilma**, även om allt som nämns ovan även inkluderar dig Susanna så kände jag att jag även ville tacka övriga medlemmar i din lilla fina familj. Utöver all ovärderlig hjälp under avhandlingsskrivandet vill jag även tacka för alla fantastiska och minnesvärda stunder vi haft tillsammans med våra små prinsessor. Tusen tack för alla härliga och roliga dagar, kvällar, utflykter och goda middagar. Ni är underbara! Kommer sakna er något oerhört!

Fia Lundgren, vet att du heter Sofia men för mig är du ”min Fia”, känns svårt att på några få rader skriva ner vad du betytt och betyder för mig. Vill hur som verkligen tacka dig för allt, inte minst för alla givande och spännande samtal om livet, relationer och kärlek. Kan inte komma på något vi inte avhandlat under alla våra roliga, allvarliga, nedstämda och sprudlande telefonsamtal och sushiträffar. Tusen tack för alla kloka råd och stöd genom åren. Helt enkelt: Tack för att du finns!

Emelie Wallerstedt, för att du alltid ställer upp, lyssnar, peppar och bara finns där. Tusen tack för allt ditt stöd under den här stundtals tuffa tiden, varit så fantastiskt skönt att ha dig där och som dessutom vet precis vad man går igenom. Du är en fantastisk vän, så otroligt glad att jag fått lära känna dig. Tack för att du finns!

Jenny Nilsson för alla mysiga tjejmiddagar och annat kul under forskarskoleåret och fram till idag, kul att du flyttat hit igen!

Biologivännerna, tack **Johanna Särefjord** tusen tack för fin vänskap, stöd och värme. Tack för alla fantastiska stunder vi haft tillsammans under alla härliga vandringar, mysiga hemmakvällar, svettiga löprundor, mumsiga middagar, och alla

galet roliga partykvällar/nätter i Götet och på Marstrand. Ser verkligen fram emot att få träffa dig betydligt mer igen. Snart på plats i Stockholm! **Jessica Ångström**, min finfina vän, forna pluggkompis och Afrikaresekamrat, tack för allt jag fått uppleva tillsammans med dig, allt roligt vi hittade på när vi båda var nya i stan, alla givande samtal om allt och inget och alla underbara och oförglömliga upplevelser från vår Afrikaresa, så glad att jag har dig att dela alla Afrikaminnen med. Ska bli så fantastiskt kul att få träffa dig och de dina betydligt mer igen! Snart går flyttlasset till er stad. **Linda Hallberg**, tack till dig och din lilla fina familj för mysiga och GODA stunder under de här åren, alltid lika roligt att träffa er, önskar bara att det blivit än mer av den varan. Vem vet vi kanske alla sammanstrålar i staden österut inom kort.

Mariestadtjejerna, Caroline Karlsson, Lisa Lann, tack för alla fantastiska minnen, vår oförglömliga jordenruntresa, alla roliga fester, midsomrar och vänskapligt stöd genom åren. Tänker på er ofta även om det blir långt mellan att vi hörs och ses. Tack **Johanna Hermond,** min fina underbara vän, önskar så att vi hunnit träffas mer än vi gör, saknar dig ofta, du är verkligen en helt underbar vän på alla sätt och vis. Så otroligt glad för att du finns. Tack till er alla!

Jan-Åke, Linda, Emma med familj, min fantastiska svärfamilj, lyckligt lottad som fått en så fin bonusfamilj. Tack för allt!

Mamma, Pappa, Hanna med familj, tusen tack för att ni alltid finns där, vill passa på att tala om hur mycket jag älskar er alla. Tack för att ni alltid trott på min förmåga, även alla de stunder när jag själv har tvivlat. Tack för ert ständiga stöd och er uppmuntran i med och motgång. Ni är ovärderliga, **TACK!**

Släkt och vänner, lilla Farmor, Faster Irene men familj, mostrar, morbröder, kusiner, tack till er alla för fantastiska stunder och roliga minnen.

Sist men definitivt inte minst, snarare allra mest! Min älskade, underbara **Pontus** och vår sagolika lilla solstråle **Ingrid**, **TACK** för att ni finns i mitt liv, ni är det bästa som någonsin hänt mig. Pontus, tack för alla fantastiska ögonblick jag fått uppleva tillsammans med dig, tack även för ditt ovärderliga stöd under den här processen. Utan er hade den här resan inte varit möjlig, ni är det viktigaste jag har. Älskar er mer än jag med ord kan beskriva! TACK 🖤🖤🖤

Blir helt varm i hjärtat när jag inser hur otroligt många underbara människor jag har mött under åren och har i min omgivning, om jag ändå i stressen och förvirringen skulle glömt att tacka någon av er så hoppas jag att ni ändå vet hur glad jag är över att ni finns, stort tack till er alla!

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