Studies on therapeutic vaccination and immune evasion in chronic lymphocytic leukemia

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Cover illustration: Chronic lymphocytic leukemia cells © Katarina Junevik

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En droppe droppad i Livets älv,

har ingen kraft att flyta själv.

Det ställs ett krav på varenda droppe:

Hjälp till att hålla de andra oppe!

Tage Danielsson

ABSTRACT

Chronic lymphocytic leukemia (CLL) is characterized by an accumulation of malignant B cells in blood, bone marrow and secondary lymphoid organs and is considered incurable. Our main aims were to investigate different aspects of the cellular immunology in order to develop immunotherapeutic strategies. First, the function of cytotoxic T cells (CTL) in CLL seems to be negatively related to disease stage. In **paper I**, we investigated if natural killer (NK) cell inhibitory receptors on CTL were differentially expressed and found an increased expression of these receptors on CTL in patients with advanced disease. This dysregulation could potentially contribute to immune evasion and disease progression. In **paper IV**, a co-operative study of global methylation profiles of stereotyped subsets in CLL, it was found that genes involved in immune response had higher methylation levels in the poor-prognostic subset #1 than the good-prognostic subset #4. High methylation status of those genes correlated with low expression of CD80 and CD86, two co-stimulatory receptors important for T cell immune response. We performed co-culture experiments, where CLL cell lines expressing CD80/86 induced T cell activation. Thus, one explanation for the poor prognosis in subset #1, could be that low CD80/86 expression on CLL cells leads to immune evasion.

It is believed that CLL could be a good candidate for dendritic cell (DC) anti-tumor vaccination. Yet, in earlier clinic trials there has been minimal response. Optimal DC activation requires specific cytokine production and expression of co-stimulatory receptors. Apart from CD80/86, another prominent co-stimulatory receptor is CD70, which plays an important role by promoting T cell survival and effector functions. In **paper II and III**, we studied two types of DCs matured with different cocktails, the standard PGE_2DC and the alternative αDC1, to investigate if effective DCs could be generated from CLL patients. We found that αDC1s produced a NK, NKT and CTL-attracting cytokine profile, which may favor priming of CTL. Also, αDC1s expressed CD70 in a time-dependent manner and their IL-12p70 production, with a subsequent desirable T helper cell type 1 response, appeared to be CD70-associated. Together, these data imply that the αDC1-cocktail induce a more efficient DC activation and function.

In conclusion, our findings describe new mechanisms of immune evasion in CLL and also give further support to the idea that an αDC1-based vaccine has higher immunotherapeutic potential in CLL patients.

SAMMANFATTNING PÅ SVENSKA

Kronisk lymfatisk leukemi (KLL) är en blodsjukdom som drabbar ca 500 svenskar per år. Vid KLL ansamlas stora mängder sjuka B-celler i blod, benmärg och lymfkörtlar. KLL är en sjukdom där förloppet kan vara mycket olikartat, vissa patienter kan leva symptomfria i decennier, medan andra behöver behandling inom bara några år. De vanligaste behandlingsformerna som finns idag är cytostatika och antikroppar riktade mot tumörcellerna. Även om man i vissa fall efter en benmärgstransplantation kan bli friskförklarad, anses sjukdomen vara obotlig. De flesta som drabbas är äldre personer (hälften är över 70 år) som kan ha svårt att klara av intensiv behandling. Det är känt att patienter med KLL har nedsatt immunförsvar, både när det gäller normala B-celler (lägre produktion av immunoglobuliner) och T-celler (sämre förmåga att attackera KLL-celler). Under årens lopp har man försökt hitta markörer på både KLL-celler och T-celler som kan ge upphov till nya behandlingar. Försök har också gjorts med vaccin-celler för att stimulera det egna immunförsvaret till att attackera tumörcellerna.

I denna avhandling ingår fyra delarbeten: arbete I och IV inriktar sig på hämmande/aktiverande receptorer på cellytan medan II och III studerar alternativa vaccinceller som ska försöka stimulera immunförsvaret att döda KLL-celler. I **delarbete I** visade vi att en subpopulation av T-celler med hämmande receptorer ökar med avancerat sjukdomsstadium, vilket kan leda till sämre förmåga att döda KLL-celler. I **arbete IV** sågs att immun-stimulerande receptorer finns på KLL-celler hos en population patienter med god prognos, medan samma receptorer saknades i en population med dålig prognos. Hos patienterna med dålig prognos, kan KLL-cellen undvika immunförsvaret med hjälp av denna brist på immunstimulerade receptorer. Alternativa vaccinceller som skall stimulera immunförsvaret att producera T-celler som dödar tumörceller studerades i **delarbete II och III**. Tidigare har kliniska försök med vaccinceller inte visat någon egentlig effekt på sjukdomen, men det förefaller dock vara en säker behandling med få och endast lindriga biverkningar. Med den alternativa vaccincell som vi undersökt finns det förhoppning om att den kan ge bättre kliniska resultat eftersom den verkar ha en mycket bättre förmåga än tidigare vaccinceller att kunna aktivera patientens eget immunsystem till att angripa sjukdomen.

Sammanfattningsvis har delarbetena i denna avhandling bidragit med ytterligare information om hur KLL-celler undviker immunförsvaret och hur man med hjälp av rätt vaccincell bättre kan aktivera ett nedsatt immunförsvar till att angripa KLL-sjukdomen.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **Junevik K**, Werlenius O, Hasselblom S, Jacobsson S, Nilsson-Ehle H, Andersson PO.
The expression of NK cell inhibitory receptors on cytotoxic T cells in B-cell chronic lymphocytic leukaemia (B-CLL)
Annals of Hematology 2007;86(2):89-94

II. Gustafsson K, **Junevik K**, Werlenius O, Holmgren S, Karlsson-Parra A, Andersson PO.
Tumour-loaded α-type 1-polarized dendritic cells from patients with chronic lymphocytic leukaemia produce a superior NK-, NKT- and CD8+ T cell-attracting chemokine profile.
Scandinavian Journal of Immunology 2011;74(3):318-26

III. **Junevik K**, Werlenius O, Fogelstrand L, Karlsson-Parra A and Andersson PO.
High functional CD70 expression on α-type 1-polarized dendritic cells from patients with chronic lymphocytic leukemia
In manuscript

IV. Kanduri M, Marincevic M, Halldórsdóttir AM, Mansouri L, **Junevik K**, Ntoufa S, Kultima HG, Isaksson A, Juliusson G, Andersson PO, Ehrencrona H, Stamatopoulos K, Rosenquist R.
Distinct transcriptional control in major immunogenetic subsets of chronic lymphocytic leukemia exhibiting subset-biased global DNA methylation profiles
Epigenetics 2012;7(12):1435-42

CONTENT

ABBREVIATIONS

APC	antigen presenting cell
BCR	B cell receptor
CLL	chronic lymphocytic leukemia
CTL	cytotoxic T lymphocyte
CD	clusters of differentiation
DC	dendritic cell
GM-CSF	granulocyte-macrophage colony stimulating factor
IFN	interferon
IGHV	immunoglobulin heavy chain variable
IL	interleukin
Ig	immunoglobulin
KIR	killer cell immunoglobulin-like receptor
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex
MLR	mixed leukocyte reaction
NK cell	natural killer cell
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cells
PGE_2	prostaglandin E_2
Poly I:C	polyinosinic:polycytidylic acid
PRR	pattern recognition receptor
TA	tumor antigen
TCR	T cell receptor
Th cell	T helper cell
TGF	transforming growth factor
TLR	Toll-like receptor
TNF	tumor necrosis factor

1 INTRODUCTION

1.1 Chronic lymphocytic leukemia

Chronic lymphocytic leukemia (CLL) is characterized by progressive accumulation of mature clonal B lymphocytes in peripheral blood. In Sweden ~500 people are diagnosed CLL per year, two thirds are over 60 years old and approximately 60% are men (Swedish Lymphoma Registry, 2000-2006). CLL is considered as an incurable disease (Hallek, Cheson et al. 2008) even if long-term disease free survival can be seen after allogeneic stem cell transplantation (allo-SCT) (Sorror, Storer et al. 2008).

1.1.1 Pathogenesis

The hypothesis of the origin of CLL cell has changed over the years depending on available techniques. Still, there is no absolute knowledge about the raising of CLL cells. However, an antigen-experienced B lymphocyte appears to be required based on surface membrane phenotype and gene expression profiles (Chiorazzi and Ferrarini 2011). Whether there is a single or dual/multiple normal precursors and where these cells are stimulated to evolve to CLL cells are unknown. Chiorazzi et al presents a stepwise transformation process where the final transformation occurs in a marginal zone B cell. Recently, extensive evidence show that signaling through B cell receptor (BCR) is a key factor in tumor cell survival and proliferation in CLL. The BCR signaling pathway is highly activated in CLL and biological variables that correlate with prognosis in CLL are related to BCR function (Ramsay and Rodriguez-Justo 2013). It is known that healthy individuals (~3 % of the population) can have a small CLL-like cell clone, monoclonal B cell lymphocytosis (MBL) but only 1 %/year of these individuals will progress to CLL or another lymphoma (Chiorazzi and Ferrarini 2011).

1.1.2 Diagnosis

The criterias for diagnosis of CLL are *i*) >5 x 10^9 B lymphocytes/L in peripheral blood, *ii*) immunophenotype with expression on the cell surface of CD5, CD19, CD23 and weak to very dim monoclonal immunoglobulins (sIg κ or λ) and *iii*) a morphology with infiltrating small mature lymphocytes in blood or bone marrow (Hallek, Cheson et al. 2008). A CLL cell should have a narrow border of cytoplasm and a dense nucleus lacking discernible nucleoli. A bone marrow examination is not required to establish CLL.

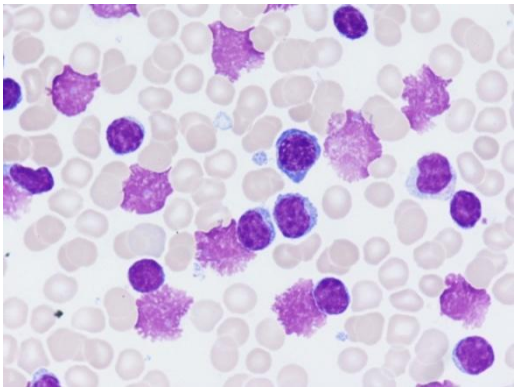

Figure 1. CLL cells in peripheral blood. © H. Johansson 2013

1.1.3 Prognosis

CLL is a very heterogeneous disease where one third can live for decades with no or minor symptoms and no progression. The remaining patients will have a progression of the disease and approximately 5% can even transform into the more aggressive lymphoma, the so called Richters syndrome. (Jain and O'Brien 2012). It is therefore important to use staging systems and prognostic markers.

Clinical staging systems

Binet and Rai are the most commonly used staging systems in CLL (Rai, Sawitsky et al. 1975, Binet, Auquier et al. 1981). Rai use a five step staging list; 0-I (low risk), II-III (medium risk) and IV (high risk) while Binet use a tree step staging; A (low risk), B (medium risk) and C (high risk). Both systems use robust markers such as hemoglobin and platelets levels and numbers of enlarged lymph node areas involved.

Chromosomal aberrations

During the last decade prognostic markers have been found by genetic techniques. The four most common markers are measured by interphase FISH; **del(17p13)** have a poor prognosis and a short survival, **del(11q23)** is associated with extensive lymphadenopathy and also with a negative impact on both progression-free and overall survival, **trisomy 12** correlates with intermediate prognosis while **del(13q14)** is associated with a good prognosis if there are no other aberrations (Sagatys and Zhang 2012).

IGHV mutational status

Immunoglobulin heavy variable (IGHV) gene mutational status is a strong prognostic factor in patients with CLL (Hamblin, Davis et al. 1999). Patients

with unmutated IGHV have a worse outcome irrespective of clinical stage. Moreover, one third of all CLL cases can be assigned to distinct subsets that express specific IG heavy/light chain genes and carry closely homologous, stereotyped B cell receptors (BCRs). There are over 40 different subsets reported but subset #1 correlate strongly with unmutated IGHV and poor outcome, while subset #4 correlates with mutated IGHV and progress in a more indolent way (Stamatopoulos, Belessi et al. 2007). Lately, Kanduri et al. have shown that methylation pattern, silencing of different promoters, might also have an impact as a prognostic marker (Kanduri, Cahill et al. 2010).

Other prognostic markers

Lymphocyte doubling time (LDT) is shortened with more advanced disease and is used as a criteria of requiring therapy (Molica, Reverter et al. 1990). The serum markers β2-microglobulin and thymidine kinase are elevated in advanced stages of the disease (Hallek, Wanders et al. 1996). CD38 (cell surface marker) and ZAP-70 (an intracellular marker) are associated by more active disease (Sagatys and Zhang 2012, Thompson and Tam 2013), but due to difficulties in cut-off value and standardization their clinical value is not established.

During the last years, analysis of the CLL coding genome has shown there are a number of relevant mutations involved in the disease outcome. One of the most common mutations in CLL (~10%) is NOTCH1. This mutation is an independent poor outcome prognostic marker (Rossi, Rasi et al. 2012). Another mutation revealed by next generation sequencing is SF3B1 mutation (Rossi, Bruscaggin et al. 2011). Even though it is not clearly known how this mutation and CLL pathogenesis are linked, it is thought to have an impact on genomic stability and epigenetic modification which leads to a worse outcome (Wan and Wu 2013). Mutation in BIRC3 gene is a third mutation that is associated with a more progressive disease. Also, BIRC3 has been reported to be associated with fludarabine-refractory CLL and can act as an independent factor for poor prognosis (Rossi, Fangazio et al. 2012).

1.1.4 Treatment

As mentioned earlier, CLL is in most cases an incurable disease with an extremely variable course, where overall survival can differ from months to decades. So far, no study has shown that therapy without specific treatment indications, such as anemia, thrombocytopenia, disease-related B-symptoms, marked lymphadenopathy etc. is of clinical value. Therefore, "watch-and-wait" is still considered as a standard approach for many patients. For

patients with need of treatment there are today several therapy options available, ranging from mild treatment with an aim merely to temporary control the disease, to more intensive regimens leading to prolonged progressions-free (PFS) and overall survival (OS).

Chemotherapy

Chlorambucil, an alkylating agent, is used since the 1950ies and 60-90% of previously untreated patients respond even though complete remissions are rare. Fludarabine, a purine analogue, were introduced during the 1980s, and induce higher response rate and also prolonged progression-free survival (PFS). Today, fludarabine is most commonly used together with cyclophosamide (FC) since this combination improves both remission and PFS. Bendamustin, approved for CLL treatment by FDA in 2008 and EMEA in 2010, are used in patients were fludarabine combinations therapy is considered inappropriate (Hallek and Pflug 2011).

Monoclonal antibodies

Several monoclonal antibodies (MoAbs) can be used for CLL-treatment, either as a single agent or in combination with chemotherapy agents. Alemtuzumab is a humanized anti-CD52, rituximab is a chimeric mouse/human anti-CD20 and ofatumumab is a humanized anti-CD20 (binds to different epitope compared to rituximab) MoAb (Hallek and Pflug 2011, Lu and Wang 2012).

Immunochemotherapy

FC used together with rituximab (FCR) has in clinical trials shown not only better PFS, but also better OS (Hallek and Pflug 2011). FCR is therefore considered first-line option for physically fit patients. Bendamustin in combination with rituximab (BR) was tested in a phase II trial and was found to be an effective and safe treatment for non-treated CLL patients (Fischer, Cramer et al. 2012). A study comparing FCR and FC+alemtuzumab (FCA) had to be stopped due to the extensive toxicity in the FCA arm (Lepetre, Aurran et al. 2009).

Allogeneic stem cell transplantation (allo-SCT)

Allo-SCT is the only potentially curative therapy in CLL, relying on the immune-mediated "graft-versus-leukemia" effect (Dreger, Dohner et al. 2010). Since allo-SCT could be associated with a considerable transplant-related morbidity and mortality, this procedure is reserved for patients with high-risk disease.

Emerging therapies

The immunomodulating drug lenalidomide has shown clinical activity in CLL with overall response rates from 32 % to 47 % (Chanan-Khan, Miller et al. 2006, Ferrajoli, Lee et al. 2008). In these studies, patients with high-risk profiles, such as 17p and 11q deletion, achieved the same response rates. Lenalidomide is currently tested for combinations with chemotherapy or monoclonal antibodies therapy in phase III trials (Lu and Wang 2012).

Since B cell receptor (BCR) signaling has been reported to play a key role in CLL cell survival, inhibitors of BCR associated kinases are considered as new interesting therapies. One such player is BTK, a non-receptor tyrosine kinase, and its inhibitor ibrutinib. Ibrutinib has shown to be very promising in patients with relapsed or refractory CLL where overall response rates up to 71 %, including CLL patients with high-risk disease, are reported (Woyach, Johnson et al. 2012). Idelalisib, also known as CAL-101, is a specific inhibitor of the phosphoinositide-3 kinase (PI3K) pathway and it has been reported that idelalisib as a single salvage therapy induced a response rate of 26 % in patients with relapsed/refractory CLL (Coutre et al, 2011). Both these drugs are now tested in different combination therapies in randomized phase III trials.

1.2 The Immune System

Originally, immunology means "science about exception". If a plague came to a village more than once, people who had been previously exposed did not fall ill again. They were excluded from the illness and the latin word immunis (=except) was used. When talking about immunology today we mean function and interaction of the molecular and cellular components that comprise the immune system.

1.2.1 Innate and adaptive immunity

The immune system is a complicated network of biological processes that act to *i*) protect the body from invading pathogens or *ii*) retain self-protection to avoid autoimmune diseases. The innate or natural immunity is the first line of defense and is present from birth. Cytokines are one of the most important molecular components of the immune system. The word cytokines comes from Greek cyto=cell and kinos=movement. Cytokines are produced by leukocytes and each cytokine has a cell surface-receptor. Cytokines that attract other cells are called chemokines. Cells included in this system are phagocytic cells (monocytes/macrophages, neutrophils and dendritic cells), natural killer (NK) cells, basophils, mast cells, eosinophils and platelets. Receptors of these cells are pattern recognition receptors (PRRs) that are able to recognize broad molecular patterns found on pathogens (pathogen associated molecular patterns, PAMPs) (Medzhitov and Janeway 2000).

Dendritic cells (DCs) are the mediator between the innate and adaptive immune system. They collect antigens in tissues, migrate to lymphoid organs and then identify and activate naïve T cells (Steinman 1991).

The adaptive immune system is mediated by B and T cells. It has specificity for distinct molecules and is able to recognize and remember those specific pathogens. After T cells are being activated by DCs they can interact with other cells, such as B cells for antibody formation, macrophages for cytokine release and target cells for lysing (Banchereau and Steinman 1998). The adaptive immune system seems to be slower compared to the innate system, but upon restimulation it will react quickly as a response of their memory function.

1.2.2 Dendritic cells

In 1973, a novel cell type was first described by Ralph Steinman, found in lymphoid tissues from mouse (Steinman and Cohn 1973). It was called dendritic cell from the Greek word déndron that means tree, due to branch-

like structures on the cell surface. DCs are antigen presenting cells (APCs) which in contrast to other APCs (B cells, macrophages) are able to activate T cells. DCs have four functions that contribute to T cell recognition and responsiveness. *i*) location at body surface and in the T cell areas of lymphoid organs, *ii*) antigen uptake receptors and processing pathways for presentation of the peptid-MHC complex, *iii*) maturation in response to different stimuli and *iv*) subsets with distinct pattern recognition and functions (Steinman and Banchereau 2007)

There are two main subsets of DC generated from hematopoietic stem cell (HSC), plasmacytoid and myeloid DC (pDC or mDC). pDC seems to migrate to inflamed secondary lymphoid tissues, while mDCs migrate to the inflammatory site first and after that migrate to the secondary lymphoid tissues (Ueno, Klechevsky et al. 2007). It is also believed that blood monocytes can differentiate into a mDC-like cell (Hespel and Moser 2012). DCs are found in most peripheral tissues, e.g. lungs, intestines and Langerhans cells in skin.

DC maturation

Immature DCs are located in the peripheral tissue and scan for damage cells or pathogens. They are good at capturing antigens, but poor in T cell stimulation. During antigen capture they will undergo maturation through pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and pathogen-associated danger signals (Ueno, Klechevsky et al. 2007). When DCs start to mature they upregulate co-stimulatory molecules on their cell surface such as CD80, CD86 and CD40 in order to be able to stimulate T cells (Bhargava, Mishra et al. 2012). Also, CD40 ligation is important to induce DC expression of other TNF family members such as CD70 and 4-1BBL. CD70 has a critical role in priming of naïve $CD8^+$ T cells (Glouchkova, Ackermann et al. 2009).

During maturation, they also start to produce immunostimulatory cytokines and chemokines. In addition to migration towards the T cell rich areas, DCs upregulate the lymph node homing receptor CCR7, a ligand to lymphoid chemokines (CCL19 and CCL21) (Banchereau and Steinman 1998). Matured DCs express high levels of major histocompatibility complex (MHC) where the processed peptide is presented.

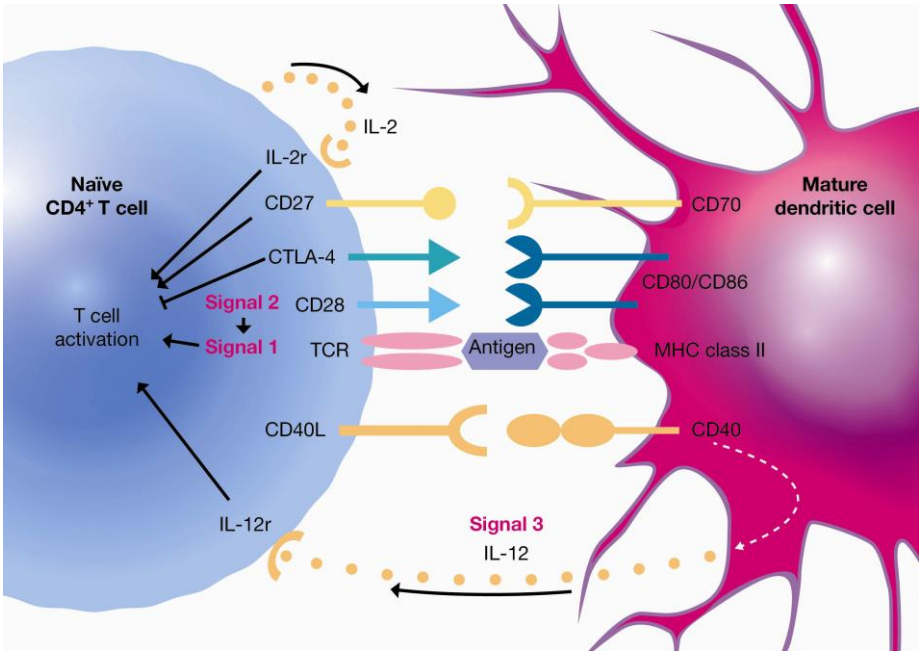

Figure 2. Crosstalk between a dendritic cell and a naïve T cell.

1.2.3 T cells

The T cell originates from the bone marrow, but needs to be matured in the thymus. Upon maturation, the T cell receptor (TCR) α and β chains are rearranged in various ways for each T cell. An immature T cell express both CD4 and CD8 on the cell surface, but after being matured only CD4 on T helper cells (Th) (Luckheeram, Zhou et al. 2012), or CD8 on cytotoxic T lymphocytes (CTL), is expressed (Williams and Bevan 2007). $CD4^+$ T cells recognize peptides presented on MHC class II (expressed by APCs) and $CD8^+$ T cells recognize processed peptides presented by MHC class I (expressed on all nucleated cells).

T helper cells

Th cells help other immune cells by releasing T cell cytokines. Importantly, they are essential in B cell antibody class switching and in activation and growth of CTLs (Rodriguez-Pinto 2005, Luckheeram, Zhou et al. 2012). For activation, MCH class II on DCs (APC) has to create a complex with the TCR on the naïve Th cell. This complex is called the signal 1. A second signal is also required before activation is possible; co-stimulatory receptors CD80 and CD86 on the DC bind to CD28 on the Th cell. This is a verification step in order to establish that the peptide presented by MHC is foreign and will decrease the risk of auto-immunity. IL-2 is then produced by the Th cell and act in an autocrine and paracrine manner to induce proliferation of T cells. Depending on the microenvironment the cell will proliferate into a Th1 or a Th2 cell (also called signal 3) (Kalinski 2009). IL-12 and interferon (IFN)-γ is crucial to induce a Th1 cellular immune response, while the humoral Th2 immune response is dependent on IL-4 and IL-5. Other Th subpopulations have been discovered over the years, such as Th17, but are not discussed in this thesis (Moser and Murphy 2000, Luckheeram, Zhou et al. 2012)

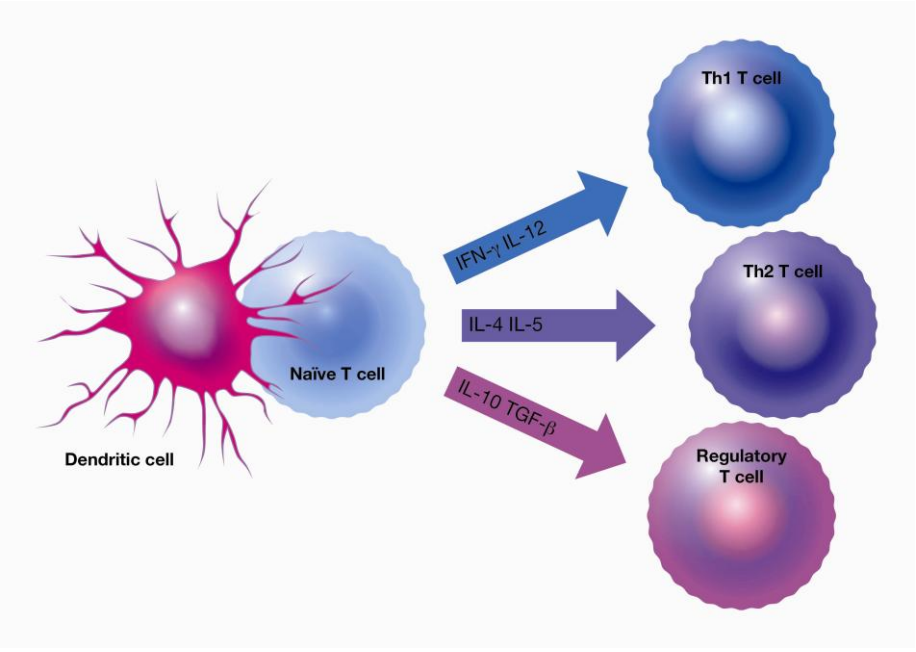

Figure 3. Interaction between naïve T cells and DCs produce different cytokines, creating T helper cell or regulatory T cells.

Cytotoxic T lymphocytes

Like Th cells, CTLs are activated in a similar manner with signal 1 and 2 by DCs. However, important for the activation of CTLs is that DCs first has to interact with an antigen-specific Th cell. After activation, CTLs upregulate the inflammatory cytokine receptor CXCR3 on the cell surface, allowing them to enter peripheral tissues (Zhang and Bevan 2011). CTLs will kill cells that express antigens as determined by their TCR specificity through *i*) release cytotoxic granulae containing perforin and granzyme B (Zhang and Bevan 2011) and/or *ii*) via the FasLigand apoptotic pathway (Varadhachary and Salgame 1998).

Regulatory T cells

Another important T cell subset are regulatory T cells (Tregs), which function is to downregulate the immune response after pathogen elimination and tolerance of self-antigens. They are often identified by cell surface expression of $CD4^+$, $CD25^+$ and intracellular $FOXP3^+$ (Feuerer, Hill et al. 2009).

1.2.4 NK cells

Natural killer (NK) cells are cells involved in the innate immune system and constitute around 10-15% of lymphocytes in peripheral blood. Their primary function is through immune surveillance kill transformed and virally infected cells. Expression of CD16 and CD56 in the absence of CD3 expression is the most common way to define NK cells. They are considered as large granular lymphocytes due to their dense intracellular cytolytic granules containing perforin and granzyme B. Upon detection of an abnormal tumor- or virally infected cell, the NK cell will simultaneously release the granules toward the target cell and kill it. These cells can also produce large amounts of cyto- and chemokines, especially IFN-γ, TNF-α and GM-CSF in order to activate other immune cells (Vivier, Raulet et al. 2011).

NK cells are specialized in killing cells that express low levels of MHC class I, opposite to CTL that instead need MHC class I to be activated. This process has been called "missing-self" recognition (Ljunggren and Karre 1990). However, this "missing self" process is controlled via cell surface activating and inhibitory receptors, such as NKG2A/CD94 and killer immunoglobulin-like receptor (KIR) family, in order to tolerate normal cells (Purdy and Campbell 2009).

Moreover, a stimulatory crosstalk between NK cells and mature DCs has been found in lymphoid organs, where DCs stimulate NK cell cytotoxicity and NK cells aid DC maturation (Gerosa, Baldani-Guerra et al. 2002).

1.2.5 NKT cells

Another specialized lymphocyte subpopulation is natural killer T cells (NKTs). They are αβ-TCR$^+$ (less variable), CD3$^+$ T cells with NK receptors expressed on their cell surface. The TCR on NKT cells do not create a complex with a peptide bearing MHC, instead, they are activated by a glycolipidbased antigen presented by CD1d on APCs (Mattner, Debord et al. 2005).

When activated, NKTs start to produce large amounts of cytokines (IFN-γ, TNF-α, IL-4 and IL-13) 1-2 hours after TCR-ligation (Godfrey and Kronenberg 2004).

1.2.6 B cells

B (bursa) cells are lymphocytes that similar to T cells receive a unique antigen specific B cell receptor (BCR) during maturation through a rearrangement process. They can function as APC through cross-linking of BCR by antigen. BCR ligation induces antigen internalization through the endocytic pathway towards MHC II to create a peptide-MHC II complex. During this activation the B cell will upregulate CD86, an important co-stimulatory molecule (see Th cell). Of utmost importance is the CD40–CD154 ligation between B cell and Th2 cell. CD40 signaling will increase expression of MHC II, stabilize CD86 expression and induce CD80 expression (Rodriguez-Pinto 2005). Activated B cells will then differentiate into antibody producing cells.

1.2.7 Activating and inhibiting receptors on NK and T cells

The cytotoxicity of NK and subsets of T cells is regulated through activating and inhibitory signals. Activating receptors are natural cytotoxicity receptors (NCR), CD16, NKG2D and the HLA class 1 recognizing killer-cell immunoglobulin-like receptor (KIR) family. NKG2D is a homodimer and recognizes a number of stress induced MHC class I-like ligands (Zafirova, Wensveen et al. 2011). The KIR family is also included in inhibitory receptors together with heterodimeric NKG2A/CD94. Signals from activating or inhibitory receptors can be balanced by changes in surface expression levels of ligands on target cell (Purdy and Campbell 2009). Nomenclature of KIRs is depending on the numbers of extracellular domain (2 or 3D) and length of the cytoplasmatic tail (long or short). Inhibitory KIRs has a long

cytoplasmatic tail and activating KIR has short a tail. Due to the clusters of differentiation (CD) nomenclature, the KIR family is also named CD158.

1.3 Immunity and cancer

In the early 1900s, the Nobel Prize winner Paul Ehrlich introduced the theory of immune surveillance (Ehrlich 1909). He hypothesized that one very important function of the immune system was to detect and eliminate tumors in the host (Maruta 2009). Even though this thesis has been challenged, it seems to hold true. Important to understand is how, when and why immune surveillance fails when a clinical disease occurs. First, it was believed that only immune cells were dysfunctional, but later it has become clear that also the microenvironment is of importance. Six hallmarks to define a cell as cancerous was published in 2000 by Hanahan and Weinberg (Hanahan and Weinberg 2000). They were; the capacity to *i*) sustain proliferative signaling, *ii*) resist cell death, *iii*) induce angiogenesis, *iv*) enable replicative immortality, *v*) activate invasion and metastasis and *vi*) avoid growth suppressors. These consensus characteristics were later revised when Hanahan and Weinberg realized that microenvironment also is a key player in tumor progression. Four new hallmarks were proposed and two of them are related to the immune system (Hanahan and Weinberg 2011).

Despite a functional immune system, tumor cells can progress to clinical detectable cancer. A hypothesis were suggested by Schrieber and co-workers regarding the three E´s in immune-editing (Dunn, Old et al. 2004), elimination, equilibrium and escape. Elimination starts up when the immune system is triggered by tumor cells. Normally, the immune system will eliminate an arising tumor, but in less immune competent humans or in case of a less immunogenic tumor, there might not be a complete elimination. Over a period of time, tumor cells will slowly progress accompanied by repeated activation of the immune system (equilibrium) but not as a clinical detectable disease. Finally, changes in the tumor cells that allow them to avoid immune surveillance or changes in the immune system efficiency will lead to tumor escape and the disease will become clinically detectable.

1.3.1 Tumor-induced escape from immunosurveillance

There are several mechanisms that tumor cells can develop in order to escape immune surveillance. Tumor cells can downregulate or even have a complete loss of MHC I in order to become "undetectable". Tumor cells are also able to increase the expression of inhibitory receptors or downregulate activation markers on cell surface to avoid cell death (Ramsay, Clear et al. 2012). In

some cases tumor cells interfere with the perforine/granzyme pathway through inhibiting granzyme B by a serine protease inhibitor (Lovo, Zhang et al. 2012). Apoptosis of tumor cells can also be hindered by blocking the "death receptor pathway" in several steps, such as the CD95/CD95L interaction (Varadhachary and Salgame 1998). High levels of TGF-β within the tumor can inhibit T cell activation, proliferation and differentiation. Tumor cells can also produce cytokines that will attract Tregs and myeloid derived suppressor cells (MDSC) like IL-10, GM-CSF and TGF-β (Topfer, Kempe et al. 2011) in order to tolerate and not kill them.

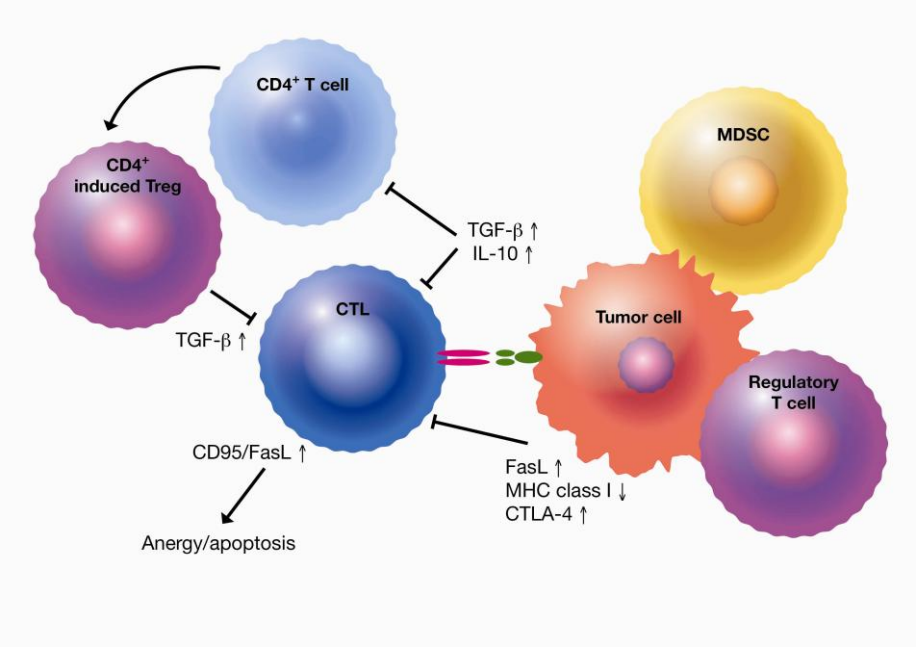

Figure 4. Tumor cells, MDSCs and Tregs create a microenvironment, resulting in tumor evasion.

1.4 Immune dysfunction in CLL

The immune surveillance hypothesis suggests that CLL cells must have created strategies to escape from or to suppress the immune system, especially T cells effect on cancer cells (Dunn, Bruce et al. 2002). Some CLL patients develop hypogammaglobulinemia, which might depend on the ligation of surface CD30L on non-malignant B cells, making them more sensitive to FasL-mediated cell death. Abnormal $CD4^+$ and $CD8^+$ T cells are also found in CLL patients. $CD8^+$ IL-4 producing cells protect CLL cells from apoptosis through upregulation of the anti-apoptotic molecule Bcl-2.

Recently, Riches et al. showed an impaired function of proliferation and cytotoxicity in $CD8^+$ T cells, even though those dysfunctional T cells retained their cytokine production (Riches, Davies et al. 2013). Also, absolute numbers of Tregs are increased and correlate with advanced disease stage (Giannopoulos, Schmitt et al. 2008). Tregs might also secrete the soluble IL-2 receptor, leading to inhibition of Th1 differentiation (Chiorazzi and Ferrarini 2011). Furthermore, CLL cells are capable of creating a microenvironment able to generate "nurse-like cells" from peripheral blood mononuclear cells (PBMC). These "nurse-like cells" protect tumor cells from apoptosis and increase their proliferation. (Riches, Ramsay et al. 2012).

1.5 Immunotherapy in cancer

Most cancer therapies are toxic and might not be suitable for elderly patients. Also, many tumors induce profound immune defects and drug combinations (like e.g. FCR) will create even more immunosuppressive effects. Standard anticancer therapies are effective to eliminate bulk tumor cells, but they could lack the ability to remove residual cancer cells and therefore not having the capacity to cure the patient. The underlying principles of tumor biology and immunology have become clearer during the latest years and new immunotherapeutical strategies might offer a more efficient treatment.

1.5.1 Dendritic cells used as an antitumor vaccine strategy

One way of trying to activate the antitumor immune system is by vaccination. Dendritic cells, the most potent APCs controlling the immune system, can be generated from either autologous or allogeneic monocytes and then used for vaccination. Indeed, DC-based immunotherapeutic strategies have been shown to induce immune responses in both animal models and in humans, but without significant clinical efficiency. The aim of DC vaccination is to induce tumor-specific T cells that can attack the tumor cells and to induce an immunologic memory to prevent tumor relapse (Palucka and Banchereau 2012). To improve the clinical efficacy of DC-based vaccination, it is important to understand the biology of DCs. Essential components for a DC vaccine optimization is the maturation-cocktail, loading with tumor-antigen, and the injection site. A lot of effort in producing effective DC maturation and pulsing strategies has been done, both *in vitro* and *in vivo*, over the years, but still there are problems to overcome. However, some clinical advances have been made and in 2010 the US Food and Drug Administration (FDA) approved the first therapeutic cancer vaccine, Sipuleucel-T, for treatment of advanced prostate cancer. Sipuleucel-T consists of autologous PBMC including APCs, which have been activated *ex vivo* with a recombinant

fusion protein (a combination of prostate antigen PSA fused to GM-CSF). In a randomized phase III trial, treated patients experienced a 4-month prolonged overall survival (Kantoff, Higano et al. 2010).

DCs in blood are rare in patients and they are also dysfunctional due to the microenvironment induced by the tumor cells, e.g. by the secretion of TGF-β and IL-10 (Mami, Mohty et al. 2008). To overcome this problem, vaccine DCs in high yields are generated by *in vitro* cultured monocytes. Thus, there are several factors of great importance to take into account when setting up a DC-based strategy. First, they need a mature DC immunophenotype with co-stimulatory and migratory molecules upregulated. When the first DC vaccine cell experiments were done, immature or partly matured DCs were used (Hsu, Benike et al 1996, Nestle, Alijagic et al. 1998). They were probably good at capturing tumor antigen, but with reduced capacity to upregulate co-stimulatory receptors and to induce the T cell stimulation as needed.

To obtain a more mature DC, Jonuleit and colleagues studied a maturation cocktail, that later has been considered as "golden standard", containing TNF-α, IL-1β, IL-6 and prostaglandin E_2 (PGE_2) (Jonuleit, Kuhn et al. 1997). These cells, PGE_2DCs, were capable to induce IFN-γ producing T cells and showed a mature DC immune phenotype. Also, CCR7 (the receptor for CCL19 and CCL22) was also expressed on the cell surface, resulting in DC migration toward lymphocyte rich regions. Unfortunately, PGE_2DC could not produce IL-12p70 upon ligation with $CD4^+$ T cells, which is crucial for Th1 polarizing. PGE_2DCs are also known to recruit more Tregs, resulting in downregulation of the immune response (Jongmans, Tiemessen et al. 2005). Instead, Kalinski´s group introduced a new Th1 polarizing DC, not using PGE_2 but instead a Toll like receptor 3 (TLR3) ligand, polyinosinic:polycytidylic acid (poly-I:C) and IFN-α (Mailliard, Wankowicz-Kalinska et al. 2004). They showed that this α-type-1 polarized DC (αDC1) had a mature DC immunophenotype and could upon CD40/CD40L interaction both produce high amounts of IL-12p70 and induce long lived CTLs.

Another important aspect of developing a successful DC vaccine is how to load the DCs with a suitable tumor antigen. There are different approaches described, e.g. MHC-restricted synthetic peptides derived from tumor cells (Carrasco, Van Pel et al. 2008), tumor RNA (Kreiter, Selmi et al. 2010), tumor lysate (Ying, Zhen et al. 2009), tumor-derived exosomes (Zhang, Zhang et al. 2010) and apoptotic tumor cells (Brusa, Garetto et al. 2008). However, use of tumor lysates seems to be a better alternative, since multiple antigens reduces the risk of tumor cell escape (Ribas, Camacho et al. 2010).

Moreover, success in antitumor vaccination is also dependent on several tumor- and host-related factors such as age, immune unresponsiveness, and disease stage. Efficient vaccine delivery in order to DCs homing to secondary lymphoid organs is also of importance. Until today, intradermal administration is superior in eliciting an effective antitumor Th1 response (Huck, Tang et al. 2008). DCs are also supposed to retain viability and activity upon freezing and thawing for multiple-dose administrations to patients. Furthermore, validated methods for post-vaccination monitoring of immune response are necessary to estimate vaccine efficiency. So, to develop a clinically more successful DC-based immunotherapy, significant verification of principle preclinical studies and DCs standard criterion should be done before administration.

1.5.2 Dendritic cells in CLL immunotherapy

In the first clinical trial using DC vaccine cells for B cell proliferative disorders performed by Hsu and colleagues, DCs sorted from peripheral blood were pulsed with tumor antigen (Hsu, Benike et al. 1996). In that study all patients showed measurable antitumor cellular immune responses, but a clinical response was seen only in one patient. Similar results were also seen in a study on CLL patients where tumor pulsed DCs matured only with TNF-α were used (Hus, Rolinski et al. 2005, Hus, Schmitt et al. 2008). However, these studies proved that antitumor vaccination appears to be safe and feasible, and no autoimmunity was reported. Later, a group in Stockholm has performed a phase I trial in CLL patients using DCs pulsed with tumor apoptotic bodies and TNF-α as DC-maturating agent. They observed an immune response in 10 of 15 patients, however, without any clinical response (Palma, Hansson et al. 2012).

In a paper from 2008, Lee et al. showed that αDC1 generated from CLL patients are more competent in producing IL-12p70 and inducing an antigen-specific $CD8^+$ T cell response than PGE_2DC, indicating that such αDC1s could be of promising clinical value (Lee, Foon et al. 2008).

1.5.3 Cytokines

IFN-α was the first immunotherapeutical drug approved by FDA for the treatment of melanoma in 1995 (Kirkwood, Strawderman et al. 1996). IL-2 was the second cytokine to be approved by FDA against metastatic melanoma in 1998 (Atkins, Lotze et al. 1999).

1.5.4 Monoclonal antibodies

MoAbs directed against tumor antigen are commonly used in both hematological and solid cancers. They can be administrated alone or in combination with chemo- and/or radiotherapy. MoAbs can also be used to block activation signals required for malignant cell growth or viability. Such antibodies are anti-CTLA-4 and anti-PD-1. They both interfere with CD28-CD80/CD86 co-stimulatory signal, important for Th1 immune response (Engelhardt, Sullivan et al. 2006, Brahmer, Drake et al. 2010). An anti-CTLA-4-antibody (ipilimumab), is one of the latest MoAbs approved by FDA. Blocking of inhibitory receptors on NK and $CD8^+$ T cells to enhance cytotoxicity is also a possible approach, by using an anti-inhibitory KIR MoAb (Vey, Bourhis et al. 2012).

1.5.5 Immunomodulating drugs

Lenalidomide is a drug originally identified for its ability to inhibit TNF-α production from lipopolysaccharide stimulated PBMCs. Moreover, lenalidomide has been demonstrated to have a co-stimulatory effect, showing T cell activation with increased IL-2 and IFN-γ production. Lenalidomide has also showed reduced amount of Tregs (Riches, Ramsay et al. 2012).

It was recently shown that CLL cells express inhibitory ligands inducing an impaired T cell immunologic synapse function (Ramsay, Clear et al. 2012) and that CLL cells induce defects in T cell migration (Ramsay, Evans et al. 2013).The same group have found that lenalidomide prevent and repair these T cell dysfunctions. Lenalidomide is thought to enhance the antitumor immunity and reduce the production of pro-tumoral factors in the microenvironment. Furthermore, it has been suggested that lenalidomide has an effect on the malignant B-cells by upregulation of CD40L, which make them more sensitive to apoptosis (Lapalombella, Andritsos et al. 2010).

1.5.6 Chimeric antigen receptor T cells

A new upcoming therapy is the use of chimeric antigen receptor (CAR) T cells. CAR is an antibody-derived antigen-binding moiety fused with an internal signaling domain such as CD3ζ. CAR T cell responses can be further enhanced with the addition of a co-stimulatory domain, e.g. CD137 (4-1BB). By this CAR technique, MHC restriction is eliminated and the same CAR could be used in different patients. However, it is of great importance to choose the right target antigen to avoid an effect on nonmalignant organs (Kochenderfer, Dudley et al. 2012). In CLL, very promising results have

been reported, when using CAR T cells directed toward CD19 (Porter, Levine 2011)

1.5.7 Allogeneic hematopoietic stem cell transplantation

Allogeneic stem cell transplantation (allo-HSCT), relying on an antitumor immunologic principle, lowers the risk of relapse and has a curative potential in many hematopoietic malignancies. Unfortunately, there is also a high, 20-40%, transplantation related mortality which reduces the potential advantages. Balancing between anti-leukemia effect and the risk of non-relapse mortality depends on the disease risk category and patient variables (Gladstone and Fuchs 2012). Over the last 10 years investigators have used reduction of conditioning intensity, so called non-myeloablative or reduced intensity conditioning stem cell transplantation (RIC-SCT). RIC-SCT induces the curative graft-versus-leukemia (GVL) effect and reduces the transplantation related death incidence (Barrett and Savani 2006).

2 AIM

2.1 Overall aim

To better understand mechanism of immune evasion through inhibitory receptors on T cells in CLL patients and to study a desirable DC antitumor vaccine, generated by autologous monocytes from CLL patients, are the main focuses in this thesis. Our initial hypothesis was that T cells from CLL patients have an upregulation of inhibitory receptors on their cell surface compared to healthy controls and need assistance to activate the adaptive immune system.

2.2 Specific aims

Our specific aims were, in CLL patients

- to study if inhibitory killer immunoglobulin like receptors (KIR) on $CD8^+$ T cells correlate with disease stage (Binet)

- to generate functional autologous DC-based antitumor vaccine with a Th1 polarized immune response

- to evaluate the expression and functional role of the co-stimulatory CD70 molecule in αDC1s

3 PATIENTS AND METHODS

Detailed descriptions of the experimental procedures are given in the individual papers. Only those methods that are of particular importance for this thesis are presented as a general overview.

3.1 CLL patients and healthy controls

CLL patients were all in clinical stage Binet A in paper II and III, whereas in paper I patients in stage B and C were also included. All patients were recruited from the Hematology department at Sahlgrenska University Hospital, Gothenburg, Sweden.

Healthy controls were recruited from the western part of Sweden. In paper I controls were age-matched with CLL patients, but in paper II and III no matching was done.

Informed written consent was provided according to the Declaration of Helsinki and was approved by the Ethics Committee of the Medical Faculty, Gothenburg University, Sweden.

Regarding patients in paper IV, detailed information is given in the article.

3.2 Cell sorting

Peripheral blood from both healthy controls and CLL patients were first separated into peripheral blood mononuclear cells (PBMC) by Fiqoll-Paque gradient centrifugation (GE Healthcare Bio-Sciences AB, Uppsala, Sweden).

To further separate cells into different subpopulations, antibodies conjugated with magnetic beads were used. After incubation time, cells passed through a magnetic field within a column and cells with antibodies attach to the surface, staid in the column (positive separation) while the untouched cells passed through (negative separation). CD19 positive separation was used to obtain CLL cells (paper II-III), CD14 positive separation for monocytes (paper II and III), T cell negative separation to get T cells (paper III) and CD4 negative separation to get $CD4^+$ T cells (paper IV).

When sorting monocytes from CLL patients, a pre-sorting were made first to deplete CLL cells by a CD19 negative sorting. Cell suspension was also

passed through two columns (instead of one) to obtain the best purity of CD14 positive cells.

3.3 Cell culture

DC generation
In all cell cultures generating DCs, serum-free CellGro (CellGenix, Freiburg, Germany) medium were used. It is known that DCs matured in CellGro compared to AIM V (Invitrogen, Paisley, UK) produce lower levels of cytokines, but have a better viability (Lee, Foon et al. 2008).

MLR
In all mixed leukocyte reaction (MLR) experiments RPMI 1640 medium with 10% Fetal Calf Serum (FCS) were used (paper III and IV). DCs generated from CLL patients were cultured with healthy allogenous T cells in a (DC/Tcells) 1:10 ratio (paper III) while CLL cell line cells cultured together with $CD4^+$ T cells in a 1:1 ratio (paper IV).

Migration assay
Lower chambers of 24-(trans)well plates, 8.0 μm pore size filters covered with matrigel matrix, (BD Biosciences, San Jose, CA, USA) were filled with 600 μL culture supernatants collected from 24 hours matured DCs. 600 μL of medium only was used as a control to determine spontaneous fallout. $1x10^6$ PBMCs re-suspended in 100 μL medium were placed in the upper insert. Plates were incubated for 24 hours when cells migrated to the lower well, before recruited cells were counted and analyzed.

All cell cultures were performed without any supplementary agent except for FCS. There were no signs of unwanted growth from virus or bacteria.

3.4 Flow cytometry

Flow cytometry is commonly used within the immunology and hematology field due to the single cell analyzing. The quality of the results relies on accurate instrument setting, daily performance (calibrate beads) and negative control. There are different ways to use negative control i.e. matched isotype control, fluorescence minus one or internal control.

In all papers a three laser flow cytometer (FACSAria, BDBiosciences, San Jose, CA) was used to analyze cells by flow cytometry. Up to eight fluorochromes were used in the same tube. Simultaneously, forward scatter

(FSC) and Side scatter (SSC) are analyzed and representing the morphology of the cell, FCS represents relative size and SSC relative complexity.

Immunophenotyping by flow cytometry was used in all papers. Since different cell types were analyzed, separate instrument settings were done for each cell type (lymphocyte or dendritic cell). Gating strategy is important when analyzing data. First cell identification markers were gated before markers of interest were analyzed.

In co-culture experiments, T cells were labeled with CellTrace™ CFSE cell proliferation kit (Invitrogen, Paisley,UK) to identify lymphocytes from DCs in MLR experiments and to evaluate proliferation status (paper III).

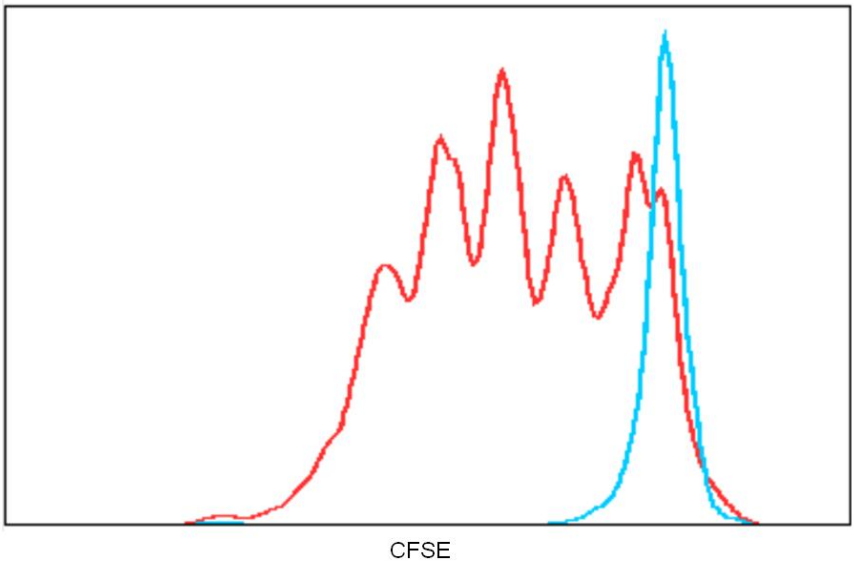

Figure 5. Six day T cell proliferation histogram (blue negative control, red PHA positive control).

To be able to calculate cell number in migration experiment, tubes containing exact number plastic beads (TrueCount, BDBiosciences) were used when analyzing different cell type markers. The following calculation was used:
(# of events in cell region x beads per test)/# of events in bead region = absolute count

Flow cytometry was also used to check purity and viability of sorted cells.

3.5 Cell culture supernatant cytokine analysis

ELISA

The most common method to analyze cytokines is enzyme-linked immunosorbent assay (ELISA). In all cell cultures where DCs were generated, the ELISA method was used. Matured DCs were carefully washed and put back in the incubator for another 24 hours before supernatant were removed and analyzed (paper II and III). To evaluate the ability of matured DCs to secrete $CD8^+$ attracting chemokines (MIP-1α, MIP-1β) and IL-12p70 upon $CD4^+$ cell activation in draining lymph nodes, DCs were stimulated with soluble, histidine-tagged, CD40L protein (R&D systems) followed by the addition of an anti-polyhistidine MoAb (R&D Systems) 20 min later. Cells were incubated 24 hours before supernatant was removed and analyzed (paper II).

Bio-Plex

In paper III where MLR analyzes were done, and small volumes of supernatant were collected, a Bio-Plex® (Bio-Rad, Hercules, CA) method was used instead. Using this method several cytokines can be analyzed in only 50 μL of supernatant. Antibodies conjugated with magnetic beads, labelled with distinct colour code, binds to the biomarker of interest. A biotinylated detection antibody is added and the final complex is formed in addition of streptavidin-phycoerythrin conjugate. This method can allow up to more than 100 different types of molecules in a single well.

4 RESULTS

4.1 Paper I

Immune surveillance of tumors is principally mediated by cytotoxic T cells (CTL) that recognise tumor antigen. Receptors, interacting with MHC class I and MHC class I-like molecules, inhibit and sometimes activate, CTL and NK cell immune responses. They mainly belong to two families: killer cell immunoglobulin-like receptors (KIR) and C-type lectin-like receptors.

Patients with CLL in an advanced stage express higher proportion of CD8$^+$ T cell with inhibitory receptors on surface

In our first paper we wanted to examine if inhibitory KIR (CD158a, b, e) and CD94/NKG2A were related to a more advanced disease stage. We found a significantly higher proportion of CD8$^+$ cells expressing CD158a (3.59±1.62), CD158b (9.87±1.79), CD158e (3.97±1.31) and CD94NKG2 (34.9±4.13) in Binet C patients compared to Binet A patients CD158a (0.87±0.31 p=0.019), CD158b (4.62±0.87, p=0.008), CD158e (1.13±0.26, p=0.0018), CD94/NKG2A (19.9±2.18 p=0.005) So, inhibitory KIR and CD94/NKG2A are upregulated in CLL patients with advanced disease (*Figure 6*).

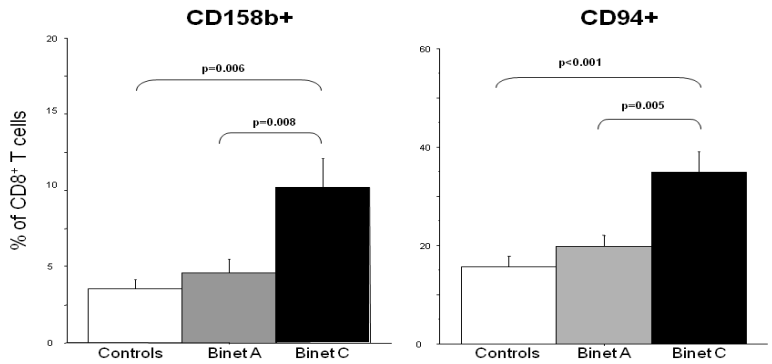

Figure 6. CD8$^+$ T cell expressing CD158b and CD94 in controls, Binet A and Binet C patients.

4.2 Paper II

In this study we wanted to *in vitro* examine the capacity of tumor-loaded αDC1s and PGE_2DCs, to: i) produce a chemokine profile rich in CXCR3-ligands, ii) recruit NK and NKT cells, and iii) to produce CCL3/CCL4 and IL-12p70 upon CD40 ligation.

Tumor-loaded αDC1 mainly produce Th1 attracting chemokines

When creating a tumor vaccine it is important that vaccine cells are able to produce an appropriate immune response. We found that αDC1s produced substantially higher levels of CXCR3 associated cytokines compared to PGE_2DCs *(Figure 7)*.

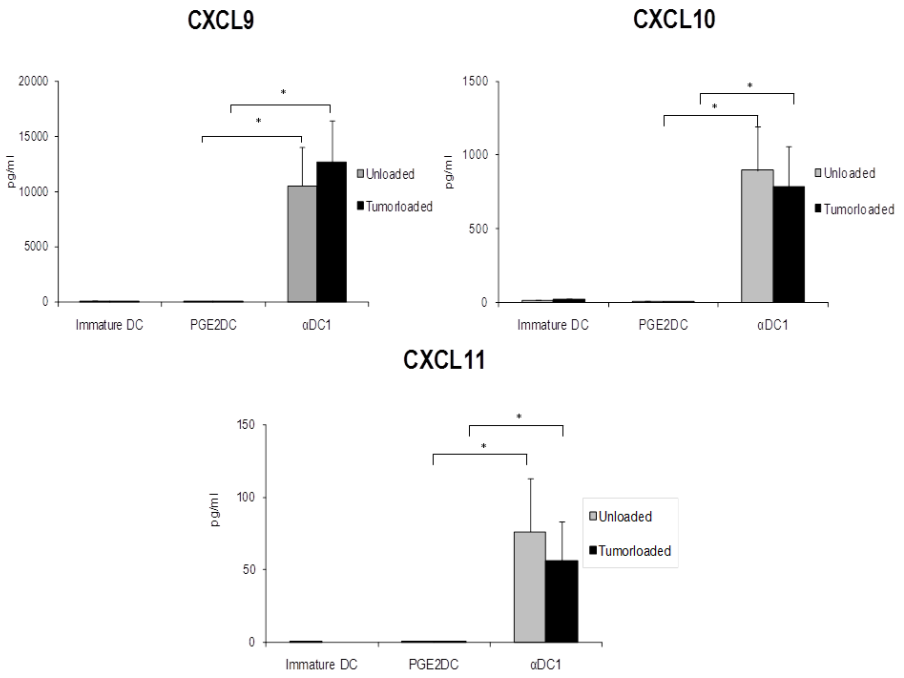

Figure 7. CXCL3-associated chemokines CXCL9-11produced by PGE_2DC and αDC1.

Tumor-loaded αDC1 show an increased NK and NKT cell recruiting capacity compared to PGE_2DC

To examine the recruiting capacity of DC produced supernatant we used a migration technique where supernatant from 24-hour matured αDC1 or PGE_2DC were placed in the bottom well and CD19-depleted PBMC were added in the insert and incubated for 24 hours. All migrated cells in the lower wells were then counted and analyzed by flow cytometry. Supernatants from

αDC1 were able to recruit significantly higher numbers of NK and NKT cells compared to PGE_2DC as expected *(Figure 8)*.

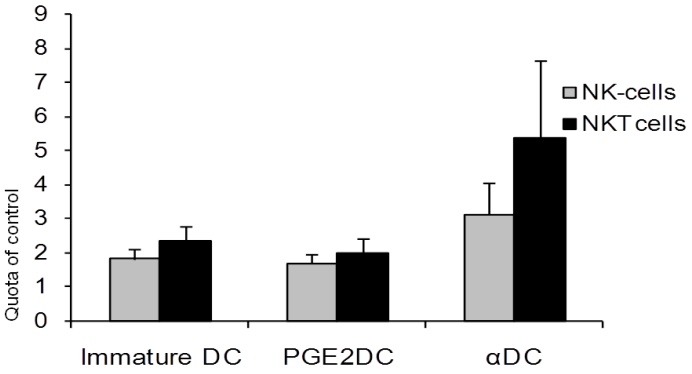

Figure 8. NK and NKT cell migration towards PGE_2DC or αDC1 supernatants.

Tumor-loaded αDC1s are superior producers of CCL3, CCL4 and IL-12p70 compared to PGE_2DC upon CD40 ligation

Effective vaccine DCs should optimally 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 by secretion of CCL3/MIP-1α and CCL4/MIP-1β chemokines. DCs interaction with $CD4^+$ T cells are also supposed to produce IL-12 due to CD40 ligation to create a Th1 immune response.

In our experiments we used a CD40L protein, mimicking DC interaction with $CD4^+$ T cells. αDC1 were able to produce considerable higher amounts of CCL3, CCL4 and IL-12p70 *(Figure 9)*.

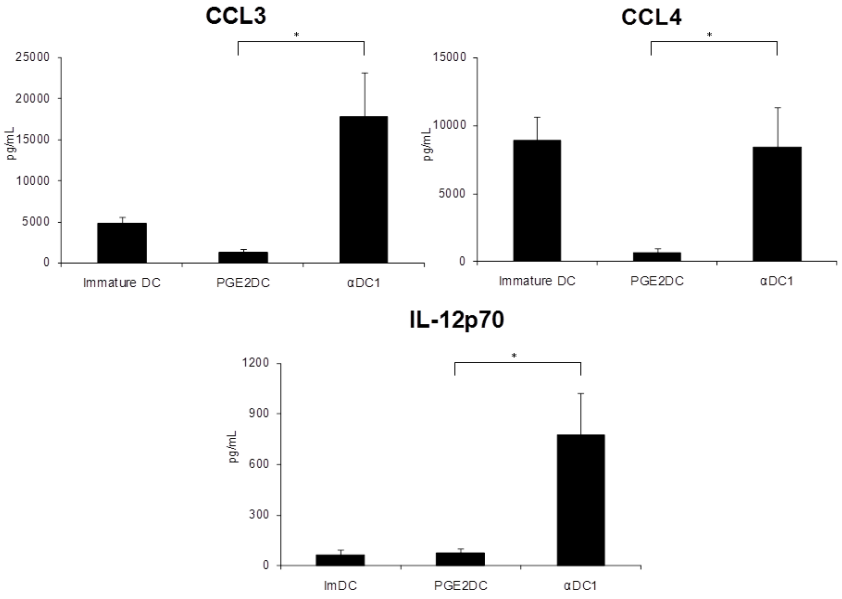

Figure 9. Production of CCL3, CCL4 and IL-12p70 upon CD40-ligation.

4.3 Paper III

CD70, expressed on DCs, and its interaction with the T cell molecule CD27 is reported to be an important signal, for both T cell proliferation and survival (Denoeud and Moser 2011). In paper III we wanted to examine CD70 expression and function on αDC1.

The αDC1 maturation cocktail induces CD70 expression, which is further upregulated by irradiation

After being matured, αDC1s from both controls and CLL were found to induce a significantly higher percentage of cells expressing CD70 both at 24 and 48 hours after maturation/irradiation. Moreover, irradiation with 25 Gy could further upregulate CD70 expression on αDC1s in both controls and patients *(Figure 10)*.

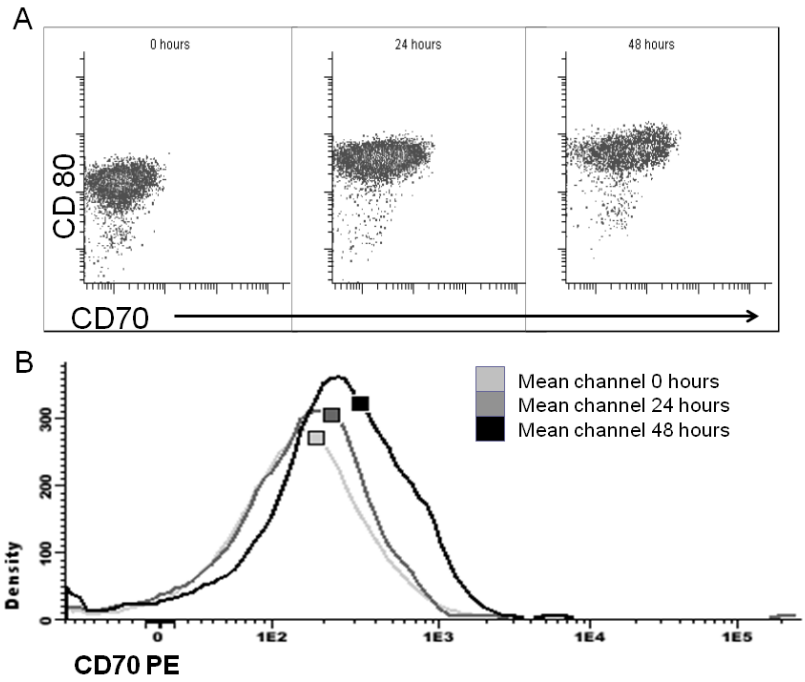

Figure 10. Matured αDC1s expression of CD70 after 0, 24 and 48 hours. A) Dotplots of CD70/CD80 B) Histogram of CD70.

Irradiation had no impact on DC production production of TNF-α or CXCL-10

Irradiation of DCs *in vitro* has been reported to possibly enhance the production of IL-12p70 and IL-23. However, we did not find any difference in cytokine production after 24 hours between irradiated and non-irradiated DCs.

CD70 expression on αDC1 induces effector cell proliferation and promotes a desirable Th1 cytokine profile

In MLR experiments where CD70 receptor on DCs was blocked by a CD70-antibody, we analyzed T cell proliferation and cytokines in the MLR supernatants.

In controls, a significant decrease in PBMC proliferation was observed when blocking CD70. However, no difference in proliferation was seen by blocking CD70 in co-culture with allogeneic PBMCs or T cells and αDC1s from CLL patients.

In the MLR supernatants, we found that blocking of CD70 seemed to decrease the capacity of αDC1s to produce IL-12p70. Furthermore, CD70-

blocking had a negative influence on the production of the Th1 cytokine IFN-γ, while the production of the Th2 cytokine IL-5 appeared to be enhanced. Taken together, blocking of CD70 seems to lead to an undesirable Th2 immune response *(Figure 11)*.

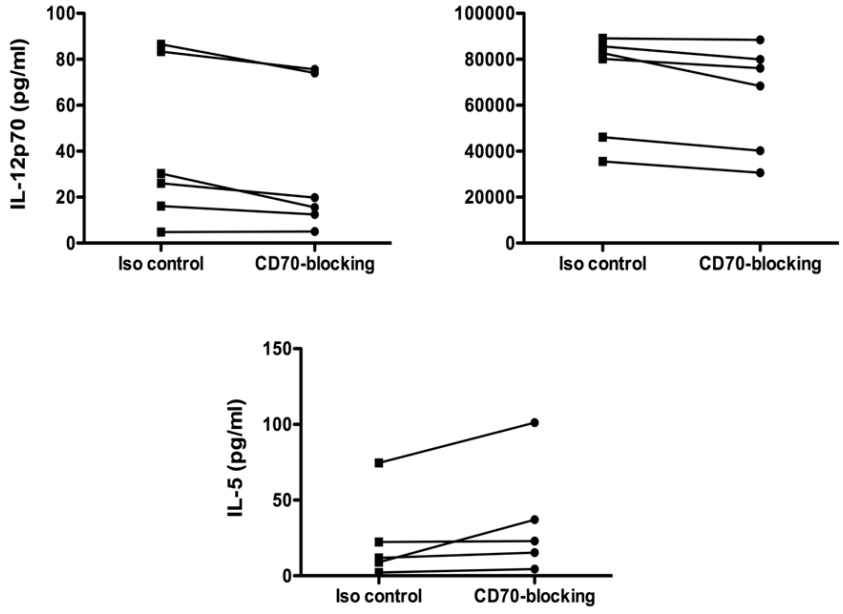

Figure 11. Cytokine production from MLR with T cells and αDC1s from CLL patients.

4.4 Paper IV

In paper IV it was found that in the poor-prognostic subset #1 genes coding for CD80/86 were methylated and thereby silenced, while these genes in the good-prognostic subset #4 were unmethylated. These findings were validated via mRNA expression that showed subset #4 to have higher expression of CD80/86 molecules.

CD80/CD86 expressing CLL cell-lines upregulate activation marker CD69 on $CD4^+$ T cells

In a co-culture experiment, high levels of CD69; a T cell activation marker, were shown on $CD4^+$ T cells cultured with CD80/CD86 expressing CLL cell lines (HG3 & MEC1) compared to T cells cultured alone. Expression of

CD28, receptor to CD80/86, was expressed at high levels in all co-culture wells *(Figure 12)*.

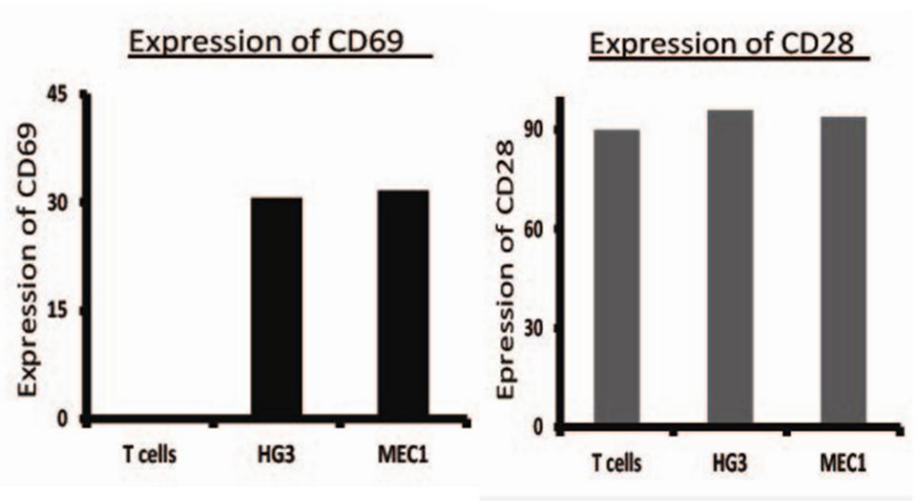

Figure 12. HG3 and Mec1 CLL cell lines expressing CD80/86 upregulate the activating molecule CD69 on allogeneic $CD4^+$ T cells.

5 DISCUSSION

CLL patients are reported to have a higher amount of Tregs (Giannopoulos, Schmitt et al. 2008), reduced expression of co-stimulatory and adhesion molecules on different cells (Dai, Chen et al. 2009), high levels of serum TGF-β and IL-10 (Beyer, Kochanek et al. 2005), all together resulting in immune evasion of tumor cells. T cells in CLL patients are also unable to maintain a sufficient cellular immune response against tumor cells. Moreover, an adequate CTL response requires that tumor antigen must be presented by APCs with sufficient numbers of co-stimulatory receptors and a Th1 derived cytokine production. DCs are the most potent APC in the immune system and it has been showed that even if circulating DCs from CLL patients are defect, functional DCs can be generated from monocytes in peripheral blood (Vuillier, Maloum et al. 2001). However, in patients with active disease the expression of some co-stimulatory molecules on DCs (CD80 and CD40) seemed to be reduced, but functional capacities appeared to be restored in DCs from remission patients (Orsini, Pasquale et al. 2004). All this has to be taken into account when designing a DC-based immunotherapeutic approach.

5.1 The CLL cell escapes immune surveillance

In general, tumor cells are known to develop strategies to escape immune surveillance and to inhibit the antitumor response. In CLL, antitumor responses are limited due to dysfunctional T cells. Surprisingly, T cells in CLL patients are increased in number with an excess of $CD8^+$ T cells, which give a fall in CD4:CD8 ratio. These $CD8^+$ T cells are reported to be abnormal in various ways (Riches, Ramsay et al. 2012) and the low autologous cytotoxic responses against CLL cells may depend on primary or secondary alterations in the CTL population. MHC class I molecules interact with receptors known to control the activation of both T and NK cells. Such inhibitory or activating receptors mainly belong to two families, killer cell immunoglobulin-like receptor (KIR) and C-type lectin-like receptors (e.g NKG2 receptors) (Purdy and Campbell 2009). The activation mediated by engagement of the activating factor NKG2D with its ligands (MIC or ULPB) is a crucial step in the regulation of both innate and adaptive immune responses (Fernandez-Messina, Reyburn et al. 2012). NKG2D ligation in NK cells is sufficient to trigger cell activation, while when expressed in $CD8^+$ T cells, the interaction has a co-stimulatory function similar to CD28. Furthermore, presence of soluble ligands for NKG2D in the sera of cancer patients can also occur, where they weaken the immune response by impaired

NKG2D-mediated cytolytic functions. KIRs recognize specific motifs of HLA class I molecules, where HLA-C plays a key role for KIR-mediated recognition of target cells (Babor, Fischer et al. 2013) but, compared to NKG2D, the regulation of KIR and CD94/NKG2A is not as well known.

So, further studies how to identify and to stimulate the anti-CLL effector cells are needed. We found, in **paper I**, a higher proportion of $CD8^+$ T cells with inhibitory KIR, CD158 a, b, e and CD94/NKG2A in patients with Binet stage C compared to stage A. This could indicate that induced expression of such receptors on CTL may result in the inhibition of their cytolytic function and, thereby, in the reduction of the tumor growth control. Previously, it has been shown that CLL patients with progressive disease have increased expression of the inhibitory CTLA-4 receptor on $CD8^+$ T cells compared to non-progressive patients (Rossmann, Jeddi-Tehrani et al. 2003). Also, cytotoxic functions of T cells in CLL patients seems to be associated with disease stage; patients with high-risk disease had significantly lower expression of the activating receptor NKG2D on their Vδ1 T cells (Poggi, Venturino et al. 2004). Thus, CTL in CLL seem to be associated with alterations in both inhibitory and activating receptors, and their expression is apparently correlated with disease stage. On the other hand, this might imply that CLL patients with low percentage of KIR- and CD94-expressing CTL still have at least a semi-functional tumor surveillance system.

Just recently, clinical phase I studies with the anti-KIR monoclonal antibody (IPH2101; binds to KIR2DL1, 2 and 3) have been done in patients with multiple myeloma and acute myeloid leukemia (Benson, Hofmeister et al. 2012, Vey, Bourhis et al. 2012). They both reached the biologic endpoint of full KIR2D neutralizing occupancy without none or limited dose-related toxicity. Such therapy could be a promising approach for future studies in CLL patients with T and NK cells showing high KIR-expression.

Furthermore, an adequate CTL response also requires that tumor antigen must be presented by APCs with sufficient numbers of co-stimulatory receptors. B cells are poor APCs and CLL cells have even less capacity to act as APCs since they express low levels of co-stimulatory molecules. (Mellstedt and Choudhury 2006). To induce an effective tumor immune response APCs are supposed to interact with their microenvironment, including T cells. The co-stimulatory signaling through the CD80/86-CD28 interaction is important to verify activation of naïve T cells. In CLL, where tumor cells act as both APCs and target cells, CD80/86 expression is low or absent. When co-cultured with activated T cells, CD80/86 expression can be upregulated due to the interaction of CD40/CD40L (Ranheim and Kipps 1993), which makes them more detectable to the immune system. In a phase I

trial, where CD40L transfected autologous CLL cells were infused in patients, induction of pro-apoptotic CLL cells were seen by upregulation of CD95 (Wierda, Castro et al. 2010). In **paper IV** a global methylation array in subsets with stereotyped BCR was studied. In the poor-prognostic subset #1, an overrepresentation of genes involved in immune response was methylated compared to the favorable-prognostic subset #4. Especially CD80 and CD86 came up as methylated in subset #1 and unmethylated in subset #4. Those genes were further validated by mRNA analysis, and higher levels were found in subset #1 compared to subset #4. In our co-culture experiment, CLL cell lines expressing CD80/CD86 could upregulate the activation marker CD69 on $CD4^+$ T cells from healthy donors. Taken together, these results may imply that CLL cells are actively dependent on crosstalk with their microenvironment, including T cells. One explanation for the favorable prognosis in subset #4 could thus be the induction of an antitumor immune response while low CD80/86 expression in subset #1 instead leads to immune evasion and poor prognosis.

5.2 DC vaccination

The strong anti-tumor response seen after alloSCT in CLL implies that other less toxic immunotherapeutic strategies, such as DC vaccination, could be an attractive approach for these patients. In order to induce an effective immune response in CLL patients, some clinical trials have investigated the use of DC vaccination (Hus, Rolinski et al. 2005, Hus, Schmitt et al. 2008, Palma, Hansson et al. 2012). In all studies, monocyte-derived immature DCs from CLL patients were matured with TNF-α solely. A proportion of patients showed immune responses, but few or no clinical responses were shown. Thus, it is tempting to speculate if inadequate maturation conditions of DC vaccines could be one important reason for the previous limited clinical efficacy of DC-based anti-tumor vaccination in CLL patients.

When setting up a DC vaccination strategy, it is very important how the DC is matured, to obtain a desirable immune response. When designing and evaluating such strategy it is also of crucial value to take the expression of co-stimulatory molecules and DC produced chemokine profile into account. In addition, given the importance of NK-DC crosstalk to induce an appropriate antitumor immune response, it is suggested that the development of DC-based vaccination strategies should implement NK cell-stimulating potency both in the preclinical phase and in clinical trials (Lion, Smits et al. 2012). We found, in **paper II**, that αDC1s produce a favorable NK/NKT/$CD8^+$ recruiting chemokines and IL-12p70 upon CD40 ligation,

while the more commonly used PGE_2DC instead produced a Treg/Th2-recruiting immune response. Moreover, NK cells circulates in peripheral blood but are mostly excluded from lymph nodes under normal conditions. By using a mouse model, Sallusto and co-writers demonstrated that NK cells were recruited to lymph node after stimulation by some injected DCs in a CXCR3-dependent manner. Those NK cells could provide an early source of IFN-γ that is important for Th1 polarization (Martin-Fontecha, Thomsen et al. 2004). In line with this, we found αDC1s to produce superior levels of the chemokines CXCL9-11 (CXCR3 ligands) compared to PGE_2DCs. As NKT cells express a similar chemokine receptor profile as NK cells (Thomas, Hou et al. 2003), CD40 ligand could possibly be provided by co-recruited CD1d restricted NKT cells which are known to up-regulate CD40L when recognizing endogenous glycolipids presented on mature DCs (Mattner, Debord et al. 2005).

It has also been suggested that antigen-pulsing of DCs with tumor cells might reduce their ability to produce IL-12 (Lee, Foon et al. 2008). However, we used a heat-shocked CLL cell lysate and could not find that antigen-pulsing had any negative impact on DC function.

Another co-stimulatory signaling through the CD70(DC)/CD27(T cell)-interaction is reported to be able to overcome tumor-induced tolerance (Bak, Barnkob et al. 2012), which is necessary for $CD4^+$ T cell-dependent priming of $CD8^+$ T cells (Taraban, Rowley et al. 2006). This signal also induces long-lasting Th1 responses (Iwamoto, Ishida et al. 2005) while lack of CD70 expression induces a dysfunctional antitumor response and no $CD8^+$ T cell memory (Keller, Xiao et al. 2009). To induce CD70 on monocyte-derived DCs, IFN-γ has been reported as the most important maturation factor (Arimoto-Miyamoto, Kadowaki et al. 2010). In **paper III,** αDC1s were found to express CD70 on their cell surface in a time-dependent manner. Indeed, IFN-γ is included in the αDC1-cocktail while absent in the original PGE_2DC cocktail. It has also been suggested that radiation can upregulate CD70 on DCs (Huang, Wang et al. 2011). Even though irradiation of DCs enhanced their CD70 expression, in line with the study by Huang et al, no differences in DC function were seen. Also, irradiation of DCs *in vitro* at the doses mentioned in their study, even though DCs are rather radioinsensitive, would probably interfere with both their function and ability to migrate (Merrick, Errington et al. 2005, Liu, Lin et al. 2011). Furthermore, PGE_2 has been reported to be one of the most important CD70 upregulating factors in DCs (Krause, Bruckner et al. 2009). Yet, in our study we found no difference in CD 70 expression between PGE_2DCs and αDC1s. In a previous experimental comparison between αDC1s and PGE_2DCs, CD70 and 4-1BBL expression

was low in both DC types (Trepiakas, Pedersen et al. 2008). However, in that study, determination of all cell surface receptors was performed directly after maturation while we analyzed the surface expression after another 24 and 48 hours. One could argue that measurement of co-stimulatory receptors should be performed after at least 16-24 hours, since arrival in the draining lymph node of subcutaneously injected DCs probably need that time to migrate. Therefore, expression of co-stimulatory receptors after DC maturation appears to be a dynamic process and proper timing for the determination of such receptors seems to be crucial, in order to establish an optimal antitumor DC vaccine protocol. Moreover, blocking of CD70-CD27 signaling by an anti-CD70 antibody decreased αDC1 production of IL-12p70 and the Th1 cytokine IFN-γ while the Th2 cytokine IL-5 seemed to be enhanced, thus unfavorable concerning a Th1 immune response. These findings underscores the importance of the CD70-CD27 signaling between αDC1s and effector cells, which probably is necessary for eliciting an adequate antitumor immune response.

5.3 Methodological considerations

Bringing DC-based antitumor vaccination from *in vitro* experiments into clinical trials needs a lot of different efforts. αDC1s in our experiments is cultured in CellGro, a serum-free, Good Manufactory Practice (GMP) media, but the cytokines used for maturation of DCs has not been carrier-free. It is also known that the yield of DCs obtained from monocytes cultured in CellGro is lower compared to DCs obtained in other culture-media (Lee, Foon et al. 2008). Still, CellGro-cultured DCs show less apoptosis and higher expression of maturation receptors and was therefore used in our experiments.

Peripheral blood in CLL patients consists of ~60-95% of CLL cells. When sorting $CD14^+$ monocytes, purity was approximately 70-80% in paper II, but after adding an additional magnetic column step after sorting with anti-CD19 beads in paper III, $CD14^+$ purity was raised to 80-95%.

In paper I and II we did not perform any cytotoxicity assays. It would have been valuable to examine the inhibitory KIR function by blocking with a anti-KIR-antibody in a cytotoxicity assay. However, due to the fact that it was very difficult to obtain sufficient amounts of $CD8^+$ cells from our CLL patients, we did not perform that assay. In addition, Kalinski and collegues already had performed a cytotoxicity assay with IFN-γ producing $CD8^+$ T cells in similar experimental settings (Lee, Foon et al. 2008).

In paper I, we did not confirm $CD8^+$ T cells to be activated cells by e.g. CD45RA and CD45RO. Thus, the $CD8^+$ T cells expressing KIR antigen consisted of both naïve and effector cells.

The co-culture experiments done in paper IV, were also performed with CLL cells from subset #1 and subset #4. However, since CLL cells were frozen and thawed before co-culturing with $CD4^+$ T cells, most of them had died during thawing and therefore only experiments with viable cell line CLL cells were reported in the paper.

6 CONCLUSIONS

The aim of this thesis was to study conditions in the immune system that could explain immune evasion in patients with CLL. Furthermore, we wanted to further study a functional DC-based antitumor vaccine cell that could give promise to clinical responses in these patients. The main conclusions, based on the experimental studies in this thesis, are as follows:

- CLL patients with advanced disease had significantly higher percentage of CTL expressing both KIR and CD94 compared to CLL patients with non-progressive disease. This could indicate that induced expression of such receptors on CTL may result in inhibition of their cytolytic function and thereby reduced tumor growth control.

- Tumor-loaded αDC1s derived from CLL patients produced substantially higher levels of NK/NKT/$CD8^+$ T cell recruiting chemokines and that they were superior to PGE_2DCs in the recruitment of NK and NKT cells.

- αDC1 derived from CLL patients expressed the important co-stimulatory receptor CD70 in a time-dependent manner and IL-12p70 production and a subsequent Th1 response was seemingly CD70-associated. Together with the previous conclusion, this gives further support to the idea that αDC1s should be considered as a suitable candidate for clinical immunotherapeutical strategies in CLL patients.

- Unmethylated and thereby higher expressed immune response genes, such as CD80 and CD86, on CLL cells can activate naïve $CD4^+$ T cells, possibly inducing a prognostically favorable immune response.

7 FUTURE PERSPECTIVES

Further knowledge regarding inhibitory and activating receptors on CTL and how to maximize a DC-based vaccination strategy is necessary to improve an antitumor immune and clinical response.

Clinical trial

To perform a clinical study with tumor antigen-loaded αDC1 vaccine cells in Binet stage A patients would be an interesting next step. However, taking DCs into clinical trials, they must be treated in a GMP way, which includes that all reagents and laboratory protocols are to be verified for clinical trials. Patients that are in remission after the first treatment or having a slow disease progress would also be of great interest to include.

Combination therapy

Since there are several immune defects in CLL, it might not be enough with just one treatment approach. Yet, patients with CLL are mostly elderly and frail and the immunosuppressive chemotherapy available today might be too toxic. In the light of this, there is need for new more safe treatment therapies. Antitumor DC-based vaccination is reported to be safe, but have not shown to be enough to obtain a clinical response. Even though much effort has been done in finding an effective vaccination strategy, earlier clinical trials might not have used the optimal vaccination DC. Instead, new studies are being performed with αDC1 in different diseases, e.g. glioma and multiple myeloma patients (Yang, Kim et al. 2011, Akiyama, Oshita et al. 2012). Also, new immunostimulatory MoAbs, such as ipilimumab (anti-CTLA-4-antibody) or nivolumab or lambrolizumab (anti-PD1-antibodies) have been developed and more are to be discovered. Also, among these promising MoAbs, is an anti-KIR antibody (IPH2101).

Still, those immunotherapies alone might not be enough to achieve an optimal antitumor response. To take full advantage of benefits, combinations with conventional chemotherapies or other immunotherapies will probably be needed. Not only the numbers of therapy combinations are exciting, the numbers of patients that may benefit are arising. Patient groups that earlier were not included in immunotherapies, might now be a target for combination treatments (Eggermont 2012). Also, T cell modulating antibodies in combination with chemotherapy are promising in clinical trials (Drake 2012). Combining agents, with different immune targets, is suggested to give a synergistic effect in tumor response and overall survival by preclinical studies. Indeed, most recently a combination of ipilimumab and

nivolumab in patients with advanced malignant melanoma showed a clinical activity that appeared to be distinct from that previously published on monotherapy (Wolchok, Kluger et al. 2013).

So, combining an antigen-loaded αDC1 vaccine with adjuvant immunotherapeutical antibodies, such as ipilimumab and /or nivolumab, would be a very interesting approach to study in CLL patients. Also, an αDC1-based combination therapy including lenalidomide would be of interest. However, it is of utmost importance to plan and evaluate both timing and dosage of the included therapeutic agents in order to obtain optimal clinical effect and to avoid excessive toxicity.

As an anti-KIR-antibody has been taken into clinical trials, showing limited side effects and able to block KIR for prolonged periods; this could be an alternative for combination therapy for patients in Binet stage C. Of course, an *in vitro* experiment to study the effect of KIR blocking in CLL patients, with a cytotoxic assay would be a first step.

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