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Myeloid-Derived Suppressor Cells and Other Immune Escape Mechanisms in Chronic Leukemia

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2013

ISSN 1651-6206
ISBN 978-91-554-8630-3
urn:nbn:se:uu:diva-197604

Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjölds väg 20, Uppsala, Friday, May 17, 2013 at 09:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract
Christiansson, L. 2013. Myeloid-Derived Suppressor Cells and Other Immune Escape Mechanisms in Chronic Leukemia. Acta Universitatis Upsaliensis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 882. 65 pp. Uppsala. ISBN 978-91-554-8630-3.

Chronic myeloid leukemia (CML) is characterized by the Philadelphia chromosome, a minute chromosome that leads to the creation of the fusion gene *BCR/ABL* and the transcription of the fusion protein BCR/ABL in transformed cells. The constitutively active tyrosine kinase BCR/ABL confers enhanced proliferation and survival on leukemic cells. CML has in only a few decades gone from being a disease with very bad prognosis to being a disease that can be effectively treated with oral tyrosine kinase inhibitors (TKIs). TKIs are drugs inhibiting BCR/ABL as well as other tyrosine kinases. In this thesis, the focus has been on the immune system of CML patients, on immune escape mechanisms present in untreated patients and on how these are affected by TKI therapy. We have found that newly diagnosed, untreated CML patients exert different kinds of immune escape mechanisms. Patients belonging to the Sokal high-risk group had higher levels of myeloid-derived suppressor cells (MDSCs) as well as high levels of the programmed death receptor 1 (PD-1)-expressing cytotoxic T cells compared to control subjects. Moreover, CML patients had higher levels of myeloid cells expressing the ligand for PD-1, PD-L1. CML patients as well as patients with B cell malignacies had high levels of soluble CD25 in blood plasma. In B cell malignacies, sCD25 was found to be released from T regulatory cells (Tregs). Treatment with the TKIs imatinib or dasatinib decreased the levels of MDSCs in peripheral blood. Tregs on the other hand increased during TKI therapy. The immunostimulatory molecule CD40 as well as NK cells increased during therapy, indicating an immunostimulatory effect of TKIs. When evaluating immune responses, multiplex techniques for quantification of proteins such as cytokines and chemokines are becoming increasingly popular. With these techniques a lot of information can be gained from a small sample volume and complex networks can be more easily studied than when using for example the singleplex ELISA. When comparing different multiplex platforms we found that the absolute protein concentration measured by one platform rarely correlated with the absolute concentration measured by another platform. However, relative quantification was better correlated.

Keywords: chronic myeloid leukemia, myeloid-derived suppressor cells, sCD25, tyrosine kinase inhibitors, multiplex protein quantification

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ISSN 1651-6206
ISBN 978-91-554-8630-3
urn:nbn:se:uu:diva-197604 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-197604)

List of Papers

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

I Christiansson L, Söderlund S, Svensson E, Mustjoki S, Bengtsson M, Simonsson B, Olsson-Strömberg U, Loskog A S.I. (2013) Increased Level of Myeloid-Derived Suppressor Cells, Programmed Death Receptor Ligand 1/Programmed Death Receptor 1, and Soluble CD25 in Sokal High Risk Chronic Myeloid Leukemia. *PLOS ONE*, 8(1):e55818

II Christiansson L, Söderlund S, Hjorth-Hansen H, Höglund M, Markevärn B, Richter J, Stenke L, Simonsson B, Mustjoki S, Loskog A S.I, Olsson-Strömberg U (2013) Imatinib or dasatinib treatment of chronic myeloid leukemia reduces circulating myeloid-derived suppressor cells but increases their CD40 expression. *Manuscript*

III Christiansson L, Mustjoki S, Loskog A S.I, Mangsbo S (2013) A Comparison of Multiplex Platforms for Absolute and Relative Protein Quantification. *Manuscript*

IV Lindqvist C, Christiansson L, Simonsson B, Enblad G, Olsson-Strömberg U, Loskog A S.I. (2010) T regulatory cells control T-cell proliferation partly by the release of soluble CD25 in patients with B-cell malignacies. *Immunology* 131(3):371-6

Contents

Abbreviations

AML	acute myeloid leukemia
ALL	acute lymphoblastic leukemia
APC	antigen presenting cell
Arg1	arginase 1
ATLL	adult T cell leukemia/lymphoma
ATRA	all-*trans*-retinoic acid
BCR	B cell receptor
BCR/ABL	breakpoint cluster region/Abelson
CCgR	complete cytogenetic response
CCL-2	chemokine (CC-motif) ligand 2
CCR7	C-C chemokine receptor 7
CD	cluster of differentiation
CHR	complete hematologic response
CML	chronic myeloid leukemia
CMR	complete molecular response
CLL	chronic lymphocytic leukemia
COX	cyclooxygenase
CpG ODN	CpG oligodeoxynucleotides
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
DAMP	danger-associated molecular pattern
DC	dendritic cell
ELISA	enzyme-linked immunosorbent assay
FasL	Fas ligand
GM-CSF	granulocyte-macrophage colony-stimulating factor
HRP	horseradish peroxidase
IDO	idoleamine 2,3-dioxygenase
IFN	interferon
IL	interleukin
IL-4Rα	IL-4 receptor α
iNOS	inducible nitric oxide synthase
iTreg	inducible Treg
KML	kronisk myeloisk leukemi
M-CSF	macrophage colony-stimulating factor
MDSC	myeloid-derived suppressor cell
MHC	major histocompatibility complex

MMR	major molecular response
NFkB	nuclear factor kappa-light chain-enhancer of activated B cells
NK	natural killer
NO	nitric oxide
nTreg	naturally occurring Treg
ONOO-	peroxynitrate
PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PD-1	programmed death receptor 1
PDGFR	platelet-derived growth factor receptor
PD-L1	programmed death receptor ligand 1
Ph	Philadelphia chromosome
RCC	renal cell carcinoma
ROS	reactive oxygen species
sCD25	soluble CD25
SCT	stem cell transplantation
STAT	signal transducer and activator of transcription
T(CM)	central memory T
TCR	T cell receptor
T(EM)	effector memory T
Tfh	follicular T helper
TGF	transforming growth factor
Th	helper T
TKI	tyrosine kinase inhibitor
TLR	toll like receptor
Treg	T regulatory cell
VEGF	vascular endothelial growth factor

Introduction

Only a few decades ago, chronic myeloid leukemia (CML) was a disease with a very bad prognosis. CML could be cured by allogeneic stem cell transplantation (SCT), but a lot of patients were old and/or had comorbidities and were therefore not eligible for that harsh treatment. Patients treated with the drugs available at that time eventually progressed to advanced disease and often died within a few years from diagnosis.

Today, CML is a treatable disease and new targeted drugs, tyrosine kinase inhibitors (TKIs), have changed the prognosis completely. Most patients treated with TKIs have a drastic decrease of tumor cells in peripheral blood and bone marrow. However, it is hypothesized that the leukemic stem cells cannot be killed by drugs. Hence, the treatment has been considered to be life-long. Nevertheless, it was recently shown that a proportion of CML patients treated with TKIs could discontinue treatment without relapsing. If this is a consequence of TKIs being able to kill the leukemic stem cell, or a consequence of immune control of the remaining leukemic cells remains to be investigated.

The role of the immune system in CML has not been well characterized. Since CML is a cancer of the immune system it is of interest to define the immune profile of these patients. In this thesis, focus has been to characterize the immune system and potential immune escape mechanisms in both newly diagnosed CML patients, and in patients treated with TKIs. One of the immune escape mechanisms found in CML was further confirmed in patients with B cell malignacies, cancers derived from other cells of the immune system. When investigating the immune system, different single- and multiplex assays for quantification of, for example, cytokines are used. In one of the papers we investigated different single- and multiplex platforms for quantification of proteins and compared their performances using samples from CML patients.

In the era of targeted drugs for CML the significance of the immune system for the outcome of TKI treatment is becoming more and more appreciated. Immune control of leukemia cells as well as the effect of TKIs on the immune system may be important to be able to predict the response to treatment as well as the outcome of patients discontinuing TKI treatment. The work presented in this thesis highlights immune escape mechanisms present in CML patients and suggests cells and mechanisms for further

studies of the immune system before, during and after discontinuation of TKI treatment.

CML

In 1885, the first report describing a patient with a disease similar to that we today call CML was published by John Huges Bennett. The report entitled "Case of Hypertrophy of the Spleen and Liver in which Death Took Place from Suppuration of the Blood" described a patient with symptoms that today would be diagnosed as CML [1]. CML is a proliferative disorder of the blood originating from myeloid progenitor cells in the bone marrow. It accounts for 15% of all adult leukemias and has an incidence of one to two persons per 100 000 per year [2]. In Sweden, an average of about 85 adults are diagnosed with CML per year. The median age at diagnosis is 59 years and only about 17% of the patients are under 40 years of age at diagnosis [3].

Common symptoms in CML are fatigue, weight-loss, malaise and symptoms resulting from splenomegaly such as abdominal fullness, easy satiety and abdominal pain. It is not uncommon that patients are asymptomatic (30-50% in the United States) and that CML is found on a routine blood test or physical examination [4]. The etiology of the disease is unknown, but it has been suggested that ionizing radiation can contribute to disease development, since the incidence of CML increases in persons that have been exposed to high doses of ionizing radiation. As an example, the incidence of CML increased in workers cleaning up after the Chernobyl accident [5]. Moreover, there have been case reports of patients developing CML after treatment with ^{131}I for thyroid carcinomas [6-7].

The Philadelphia Chromosome

In 1960 Nowell and Hungerford presented an abstract in which they described the investigation of chromosomes of seven patients with CML. In all these seven patients, but in non of the patients with other leukemias investigated, a minute chromosome was found [8]. The chromosome was later named the Philadelphia chromosome (Ph) after the town where it was discovered. The discovery of Ph was followed by the recognition by Rowley in 1973, that Ph may be a translocation between the chromosomes 22 and nine [9] (Figure 1). Later it was discovered that the fusion gene created by Ph, breakpoint cluster region/Abelson (*BCR/ABL)*, was enough to induce a CML like disease in mice [10]. Today, Ph and the *BCR/ABL* gene are used for CML diagnostics and treatment follow-up of patients with CML [4].

The fusion protein BCR/ABL, transcribed from the translocated *BCR/ABL* gene, is a constitutively active tyrosine kinase. The activity of this kinase leads to enhanced survival and proliferation as well as to decreased apoptosis in transformed cells. The presence of the fusion protein alone is enough to transform cells into CML cells [11-12]. Ph is thought to arise as a result of genetic instability in the cells induced by for example radiation [13]. BCR/ABL activity leads to production of reactive oxygen species (ROS) in transformed cells which in turn leads to more genetic instability, accumulation of chromosomal aberrations and mutations and eventually to progression to more advanced disease [13].

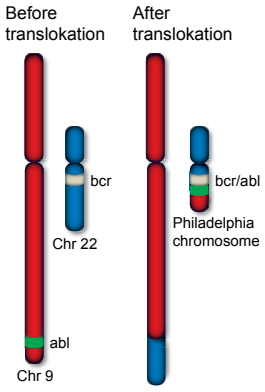

Figure 1. Reciprocal translocation that leads to the formation of the Philadelphia chromosome

Phases of CML

The natural course of CML is divided into three phases. Most patients are diagnosed in the chronic phase. In this phase, patients have an excess of myeloid cells in bone marrow and peripheral blood. The cells have a normal differentiation and function. Without treatment, increased genetic instability and accumulation of chromosomal aberrations and mutations lead to progression into an accelerated phase that can be characterized by increasing levels of blasts or basophils in peripheral blood. The accelerated phase precedes the most advanced phase, the blast crisis. In blast crisis, leukemic cells have lost the ability of terminal differentiation, hence, immature blasts are found in excess in bone marrow and peripheral blood. This phase resembles acute leukemia and is often refractory to treatment. About two-thirds of patients in blast crisis have a disease resembling acute myeloid leukemia (AML) and in one-third of the patients the blast crisis resembles acute lymphoblastic leukemia (ALL). The time from diagnosis to blast crisis is often months up to a couple of years in untreated patients [14-15].

Treatment

The treatment of CML was revolutionized in 1998 when the first targeted cancer therapy, the TKI imatinib mesylate (Gleevec, Novartis), was introduced [16]. Before imatinib, CML had been treated with the cytotoxic agents busulfan and hydroxyurea which could control CML symptoms, but not prolong the time to onset of more advanced phases of the disease. In the 1980ths two new treatment options for CML were introduced, allogeneic

SCT and IFNα treatment. Both could delay the onset of advanced phase disease and allogeneic SCT was for a long time the only known cure for CML [17]. Even though allogeneic SCT can cure CML, the treatment is also associated with great risks for the patients. Hence, in the era of TKIs, allogeneic SCT is only used for patients that have failed first- and second-line treatment with TKIs as well as for patients in the advanced phases of the disease. Moreover, allogeneic SCT is also used for patients with the T315I mutation, a *BCR/ABL* mutation that confers resistance to most of the TKIs used. Patients in advanced phase commonly have only a brief response to TKI treatment, and to achieve best results, allogeneic SCT is performed after inducing controlled disease in the patient with for example TKIs or cytostatic drugs [18].

Treatment Response

The response to CML treatment is measured by normal blood values (hematologic response), the presence of Ph+ metaphases (cytogenetic response) and the presence of *BCR/ABL* mRNA by PCR (molecular response). A patient with a white blood cell count of less than $10*10^9$ cells per liter as well as low basophil and platelet counts, a nonpalpable spleen and undetectable myeloycytes, promyelocytes and myeloblasts in peripheral blood is in complete hematologic response (CHR). A patient without Ph+ metaphases has achieved complete cytogenetic response (CCgR). Complete molecular response (CMR) was first defined as undetectable *BCR/ABL* mRNA levels by PCR in two consecutive blood samples [19]. However, with more patients achieving this level of response CMR has been redefined as CMR^4, $CMR^{4,5}$ and CMR^5. The superscripted numbers indicate a log reduction of the *BCR/ABL* mRNA levels compared to a defined baseline. As endpoint in many clinical trials evaluating treatment responses of patients with CML, major molecular response (MMR) at a defined time point is used. MMR is defined as a 3 log reduction of *BCR/ABL* mRNA levels as measured by PCR [20].

Prognostic Score

Three different prognostic scores for patients with CML exist. The Sokal score was developed in 1984 as a prognostic score for CML patients treated with chemotherapy. The Sokal score divides the patients into a high-, an intermediate- and a low-risk group dependent on patient age, spleen size, platelet count and percentage blasts in peripheral blood [21]. The Hasford score (also called the Euro score) was developed for patients on interferon (IFN) α treatment. This score takes, besides the parameters of the Sokal score, also eosinophil and basophil counts into account [22]. In 2011 the Eutos score, a new prognostic score for CML patients on imatinib treatment was presented. The Eutos score divides patients into high-risk and low-risk

groups based on spleen size and percentage basophils in peripheral blood [23].

Imatinib

Imatinib is a small molecule TKI that selectively binds and inhibits the tyrosine kinases BCR/ABL, cKit, Abl related gene, c-FMS and platelet-derived growth factor receptor (PDGFR) [24]. By binding and inhibiting BCR/ABL in CML, imatinib reverts the enhanced survival, proliferation and decreased apoptosis of the leukemic cells and restores normal hematopoesis [25]. This is achieved through inhibition of proliferation and induction of apoptosis in BCR/ABL positive cells [26]. CML stem cells, residing in the bone marrow of patients with CML, are thought to be insensitive to imatinib since their growth is independent of BCR/ABL [27]. Imatinib is today used as standard treatment for chronic phase CML. In the first big multinational study comparing imatinib to IFNα in combination with cytarabine treatment, the IRIS–study, overall survival after six years of imatinib treatment was 88% and 95% if deaths unrelated to CML were excluded [28]. A follow-up study of 639 Japanese patients receiving imatinib as first-line treatment for chronic phase CML showed an overall survival rate of 95,1% after seven years [29]. Despite these great treatment results, primary and secondary resistances to imatinib exist and can lead to treatment failure [25]. The most common cause of resistance to imatinib is *BCR/ABL* mutations. More than 90 different mutations in *BCR/ABL* have been described in patients resistant to imatinib [30].

Imatinib is generally well tolerated, but adverse events, some leading to discontinuation of the treatment, occur. The most common grade I and II non-hematological adverse events are edema, muscle cramps, diarrhea, nausea, musculoskeletal pain, skin problems such as rash, abdominal pain, fatigue, joint pain, and headache. These adverse events were reported by 37-60% of the patients in the IRIS-study. Hematological adverse events of grade III-IV, consisting of for example neutropenia, thrombocytopenia, and anemia were also reported but these were less frequent [31].

During imatinib treatment most leukemic cells are targeted and killed, and patients commonly respond well. However, since the leukemic stem cells are resistant to imatinib they will remain in the bone marrow and can cause relapse if imatinib treatment is discontinued [27, 32]. Recently, however, results pointing at a potential cure of CML with imatinib treatment have been published. In a French trial, patients that were in CMR and that had been on imatinib treatment for at least two years discontinued treatment. 41% of the 69 patients with at least 12 months follow-up did not relapse in the absence of imatinib. Importantly, all relapsing patients responded to reintroduction of imatinib [33]. In another smaller study, 28,6% (4/14) of the patients remained in CMR after discontinuation [34]. Currently more

stopping studies are ongoing and they will hopefully show which patients that are suitable and can benefit from imatinib discontinuation.

Second Generation TKIs

Apart from imatinib, that was the first TKI on the market, there are now even more potent, second generation, TKIs (dasatinib, Spyrcel, Bristol-Myers Squibb and nilotinib, Tasigna, Novartis) approved for first-line treatment of CML [4]. In Sweden, only imatinib and nilotinib are, however, subsidized by the state, hence, these two drugs are the ones mostly prescribed as first-line treatment for CML patients outside clinical trials [35].

Nilotinib

Nilotinib inhibits the same kinases as imatinib [36], but it is more potent and unlike imatinib it also inhibits many *BCR/ABL* mutants seen in CML patients [37]. Nilotinib is used as first-line treatment for CML, as second-line treatment for patients failing first-line treatment and for patients in accelerated phase. The ENESTnd study [38] comparing nilotinib with imatinib for first-line CML treatment showed significantly higher numbers of patients achieving MMR when treated with nilotinib. The adverse event profiles differed between the treatments. The frequencies of rash, headache, pruritus, and alopecia was much higher and elevated levels of the liver enzymes alanine aminotransferase and aspartate aminotransferase as well as bilirubin, lipase and amylase were more frequent in nilotinib-treated patients. However, nausea, diarrhea, vomiting, edema, and muscle spasms were more frequent in patients treated with imatinib [39-40].

Dasatinib

Dasatinib is 300 times more potent than imatinib *in vitro* [41]. Besides the inhibition of BCR/ABL, dasatinib also inhibits cKit, Abl related gene, PDGFR, Scr and many other kinases [36]. Dasatinib is used as first-line treatment for CML as well as second-line treatment for CML patients resistant or intolerant to imatinib. Dasatinib is also used for patients in accelerated phase and in blast crisis. A clinical trial comparing dasatinib to imatinib for first-line treatment of CML showed a better and faster response with dasatinib, measured as CCgR [42]. Hematological adverse events like neutropenia, thrombocytopenia and anemia were more common in dasatinib-treated patients while non-hematological adverse events like edema, nausea, muscle inflammation, and rash were more common in imatinib-treated patients. Pleural effusions occurred in 10% of dasatinib-treated patients but in none of the patients treated with imatinib [42].

Other Treatment Options for CML

Ponatinib

Ponatinib is a third generation TKI active against all tested *BCR/ABL* mutations including T315I [43]. In a clinical trial it has been shown that patients with the T315I mutation as well as patients without this mutation but refractory to other TKIs respond to ponatinib treatment. The most common adverse event was rash affecting 32% of the patients. The most common serious adverse event was pancreatitis that occurred in 10% of the patients [44].

Combination Therapy

Several clinical trials have investigated the combination of imatinib and IFNα for CML treatment with mixed results. Some studies show better responses in patients treated with the combination treatment compared to treatment with imatinib alone [45-46], while others show no beneficial effect of IFNα addition [47-48].

Basic Immunology

Innate and Adaptive Immunity

The human immune system consists of innate and adaptive immunity. Innate immunity is the first line of defense that rapidly responds to microbial infection. It consists of physical barriers like epithelial surfaces, soluble proteins like complement proteins and cells such as macrophages, neutrophils and natural killer (NK) cells. These components recognize and respond to patterns on microbes called pathogen-associated molecular patterns (PAMPs) as well as to molecules expressed by stressed cells called danger-associated molecular patterns (DAMPs). Innate immune cells combat the infecting microbes. Moreover, signals from innate immunity recruits and activates the second line of defense, the components of the adaptive immunity.

Adaptive immunity, also called specific immunity, consists of cells and molecules with the capacity to adapt to specific killing of invading microbes as well as to killing of cells infected by microbes and malignant cells. T cells and B cells of the adaptive immune system express receptors on the cell surface that specifically can recognize antigens derived from invading microbes, leading to the creation of a specific immune response. The receptors called T cell receptors (TCR) and B cell receptors (BCR), have the ability to rearrange, creating a wide variety of receptors recognizing different antigens. A cell of the adaptive immune system recognizing an antigen undergoes clonal expansion. Clonal expansion creates a pool of cells that all have the same specificity resulting in efficient clearing of the microbe or

cells infected by microbes. Apart from direct killing of microbes and infected cells, adaptive immunity also functions by secretion of cytokines important for further activation of cells of both innate and adaptive immunity. Moreover, an activated adaptive immune response creates memory cells that can be rapidly reactivated upon reinfection with the same pathogen.

Dendritic cells (DCs) act as an important link between innate and adaptive immunity. DCs engulf cells, cell debris and invading microbes and present antigen in major histocompatibility complex (MHC) molecules on their surface. The antigen can be recognized by cells of the adaptive immunity and a specific immune response can be created. Moreover, DCs also recognize PAMPs and respond to signals such as TNFα and CD40L produced by innate cells. This results in activation/maturation of the DCs and a better induction of the adaptive immune system.

A Selection of Effector Cells of Innate and Adaptive Immunity

NK cells

NK cells are lymphocytes of the innate immune system that function through either killing of target cells or secretion of cytokines resulting in activation of for example DCs. NK cells are defined as cells lacking the T cell-specific molecule cluster of differentiation (CD) 3 but that express the neural cell adhesion molecule CD56. NK cells are derived from hematopoetic stem cells in the bone marrow and they are thought to mature in secondary lymphoid organs such as lymph nodes. NK cells recognize and kill cells lacking MHC I, a molecule that is often downregulated on virally-infected and malignant cells. A fine balance between activating and inhibiting signals directs the activation of NK cells. MHC I molecules bind to inhibiting receptors on the NK cell. The lack of binding of MHC I molecules leads to lack of inhibitory signals transmitted in the NK cell. For NK cell activation, however, also activating signals are needed. These are transmitted after ligand binding of activating receptors on the NK cells. Ligands for NK cell activating receptors are often upregulated by stressed cells such as virally-infected cells.

Apart from killing of virally-infected and malignant cells, NK cells also play an important role in a mechanism called antibody-dependent cellular cytotoxicity. Antibodies recognize and bind pathogens and cell debris. The antibody-coated pathogens are then bound to the Fc-receptor CD16 expressed on NK cells and the pathogens can be killed by perforin released from the NK cells [49].

T cells

T cells are lymphocytes of the adaptive immune system that mature in the thymus. They are important both for direct killing of infected cells and malignant cells as well as for providing help to other immune cells. The T cells are divided into two major subsets defined by the expression of the surface molecules CD4 and CD8.

For activation of T cells, the TCR binds to a MHC molecule with a bound peptide presented on an antigen-presenting cell (APC), for example a DC. The activation also requires cytokine stimulation and engagement of co-stimulatory molecules such as CD80 and CD86 that bind to the CD28 molecule on the T cell.

CD4+ T cells

CD4+ T cells, also called helper T (Th) cells, are important for directing the immune response. Th cells provide help for activation of other cells of innate and adaptive immunity. Moreover, one subset of CD4+ cells, T regulatory cells (Tregs), regulate the immune response by inhibiting T cells and other immune cells. There are different subsets of Th cells and the differentiation of a naïve Th cell into a distinct subset is dependent on the nature of the antigen and the cytokines present in the microenvironment during T cell activation.

The two first subsets of Th cells described are called Th1 and Th2 cells. The differentiation of a Th1 cell requires beside the TCR-MHC/peptide interaction and co-stimulation also the cytokines interleukin (IL) 12 and IFNγ. These cytokines can be produced by cells of the innate immune system. IFNγ can also be produced by activated CD8+ cells. The cytokines IL-2 and IL-4 are required for the differentiation of Th2 cells. Both these cytokines can be produced by the T cell itself, IL-4 can also be produced by innate immune cells [50].

Differentiation of Th1 cells often follows an infection with an intracellular pathogen or the recognition of tumor cells. Th1 activity leads to activation of macrophages so that they more efficiently can kill phagocytosed pathogens. Moreover, activation of a Th1 response provides help to B cells and to CD8+ T cells leading to antibody production and killing of pathogen-infected cells. The Th2 immune response combats extracellular antigens such as some parasites. Th2 immune responses are also activated in some allergic diseases and in asthma. Activation of Th2 cells leads to secretion of the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13 that promote the production of certain antibodies by B cells and activation of some innate immune cells. Some of these antigens also induce increased secretion from mucosa [51].

The differentiation of a Th17 cell requires the cytokines IL-6 and transforming growth factor (TGF) β as well as IL-21 and IL-23. Infection

with extracellular bacteria or fungi promotes a Th17 response. Activation of Th17 cells leads to production of cytokines that promotes inflammation as well as increased mucosal immune responses [50].

The activation and function of Tregs will be discussed when discussing immune escape mechanisms.

CD8+ T cells

The major function of a CD8+ T cell is to kill pathogen-infected cells and tumor cells, hence, they are called cytotoxic T lymphocytes (CTL). Naïve CD8+ T cells differentiate to CTLs after binding of their TCR to MHC I/peptide complexes presented on APCs. Co-stimulatory molecules and, in most cases, help from Th1 cells are also required for stimulation. Th1 cells provide help in different ways, both by cytokine secretion and by binding to and thereby activating APCs. This activation results in increased expression of co-stimulatory molecules and cytokines needed for T cell activation [52].

An activated CTL circulates in the body until it encounters a cell with a MHC I molecule presenting the antigen that is recognized by the CTLs TCR. Recognizing a virally-infected cell or a tumor cell through the TCR, the CTL performs cytotoxic activities through release of perforin and granzymes that leads to target cell apoptosis. Alternatively, interaction of death receptor ligands such as Fas ligand (FasL) on the T cell with death receptors such as Fas on the target cell can induce apoptosis of the target cell.

Memory T cells

After the activation of T cells and clearance of the pathogen and pathogen-infected cells, the immune response contracts and most T cells undergo apoptosis. Some T cells, however, survive and become long-lived memory cells, ready to be reactivated by a new infection with the same pathogen. These cells are maintained in lymphoid organs and in peripheral tissues, and their survival is dependent on cytokines, but independent on antigen stimulation. Two major classes of memory T cells have been described, central memory (CM) and effector memory (EM) T cells. T(CM) cells express homing receptors such as C-C chemokine receptor 7 (CCR7), making them home to secondary lymphoid organs while the T(EM) cells lack CCR7 and reside in peripheral organs (For selected phenotypes of T(CM) and T(EM) cells investigated in different cancers, see Table 1). After encountering antigen, the memory cells are reactivated and perform effector functions. The T(EM) cells have the ability of extensive proliferation, however, they are also prone to apoptosis. Hence, they do not have the capacity to, by themselves, create a large enough pool of effector cells to generate an effective immune response. T(CM) cells, however, also have proliferative capacities and a pool of cells differentiate into T(EM) cells, resulting in a T(EM) pool large enough to create an immune response [53].

Table 1. *Selection of T memory phenotypes investigated in various human cancers*

Cancer type	T(CM)	T(EM)	Ref.
CML	CD45RO+CD27+CD57-	CD45RO-CD27-CD57+	[54]
AML, CML	CD45RO+CD27+CD57-		[55]
RCC	CD45RA-CCR7+	CD45RA-CCR7-	[56]
Melanoma	CD45RA-CCR7+	CD45RA-CCR7-	[57]
Breast	CD45RA-CD62L+	CD45RA-CD62L-	[58]
CLL	CD45RA-CD62L+	CD45RA-CD62L-	[59]

B cells

B cells are adaptive immune cells maturing in the bone marrow. Mature naïve B cells then travel to the lymph node where they become activated. Activated B cells can differentiate into different subtypes of cells including antibody-producing plasma cells and memory B cells. The activation of B cells requires two signals. The first signal is provided by an antigen binding the BCR and the second signal is provided by a specialized Th cell subset called follicular Th (Tfh) cells. The Tfh cells are present in lymph nodes, they express high levels of CD40L that ligates to the CD40 molecule expressed on B cells and provides activation signals. Moreover, the Tfh cells secrete cytokines like IL-4, IL-10 and IL-21 that promote B cell activation. Th2 cells are also important for providing help to B cells and it was recently shown that the transcription factor signal transducer and activator of transcription (STAT) 3 can induce a Tfh-like differentiation program in Th2 cells in mice [60-62].

Tumor Immunology and Mechanisms of Immune Escape

Apart from protecting us from invading pathogens, the immune system has capacity to recognize and kill tumor cells. Tumor cells can be recognized by T cells because of aberrant or overexpressed proteins, called tumor antigens, or tumor-associated antigens. Moreover, lack of MHC I molecules, normally expressed on endogenous cells, can lead to recognition and killing by NK cells. Despite these mechanisms for tumor recognition, tumors arise. Many lines of evidence suggest that the immune system can control and kill tumor cells at an early stage. Later, however, mechanisms changing the appearance of the tumor cells and/or inhibiting the immune response emerge leading to the formation of a tumor. A hypothesis termed immunoediting describes the immune control of a developing tumor as a process divided into three phases: elimination, equilibrium and escape.

In the first phase, the elimination phase, the immune system can recognize and kill tumor cells and no clinical tumor arises. In the equilibrium phase the tumor cells can still be killed by the immune cells. However, a mechanism called immunoediting, involving for example downregulation of MHC I molecules, also comes into play. Immunoediting leads to creation of tumor cells that cannot be recognized by the immune system, or that are suppressing the immune reactions, resulting in the outgrowth of a tumor and entrance into the last phase, the escape phase. Besides the proliferation of tumor cells that cannot be recognized by the immune system, the escape phase is characterized by recruitment of suppressive cells as well as accumulation of immune suppressive molecules secreted by the tumor cells [63]. Some mechanisms of the escape phase will be discussed below.

Myeloid-Derived Suppressor Cells

In 2007 the term myeloid-derived suppressor cells (MDSCs) was suggested for a heterogeneous population of cells of myeloid origin that had immune suppressive abilities. The population of immature myeloid cells that accumulated in cancer and other diseases had been described by many groups but there had been no consensus of what to call these cells [64]. MDSCs regulate immune responses in various ways. Most studied are the mechanisms by which they regulate T cells, but also NK cells, B cells and DCs have been shown to be regulated by MDSCs in mice models and in human disease [65-67].

Markers and Subgroups of MDSCs

In humans there are no specific markers for MDSCs, however, various sets of markers have been used by different investigators to identify MDSCs. Gabrilovich and Nagaraj defined in 2009 human MDSCs as linage-HLA-DR-CD33+ or CD11b+CD14-CD33+ cells [68]. However, other markers such as CD15, IL-4 receptor α (IL-4Rα) and CD66b have also been used to characterize this heterogeneous group of cells [69-71]. In mice MDSCs are defined as CD11b+Gr1+ cells, and they can be further divided into a granulocytic subgroup expressing Ly6G and a monocytic subgroup expressing Ly6C. Also in humans monocytic and granulocytic subgroups of MDSCs have been defined. Both subgroups express CD11b and CD33 but lack expression of markers such as CD40, CD80, CD83 and HLA-DR that are usually expressed on more mature cells. The human monocytic MDSCs express CD14 while granulocytic MDSCs express CD15 [72].

Expansion and Activation of MDSCs

The development, expansion and activation of MDSCs are dependent on different factors produced by tumor cells, tumor stromal cells, and activated T cells (see Expansion in Figure 2). Cyclooxygenase-2 (COX-2),

prostaglandins, macrophage colony-stimulating factor (M-CSF), granulocyte-macrophage CSF (GM-CSF), vascular endothelial growth factor (VEGF), and IL-6 are all factors that can promote expansion of MDSCs. Release of these factors leads to increased proliferation and survival of myeloid progenitors through activation of signal STAT3, an important transcription factor in MDSC biology [68]. The proinflammatory proteins S100A8/A9 are important for sustained accumulation of MDSCs since they bind to receptors on the MDSC surface and promote migration. These proteins can be produced and released by the MDSCs and function as an autocrine feedback loop [73-74] (see Accumulation in Figure 2). The microRNA 494 has also been shown to be important for MDSC accumulation [75].

Activation of MDSCs is triggered by factors released by activated T cells, tumor cells or tumor stromal cells such as IFNγ, IL-4, IL-23, IL-13, TGFβ and ligands for toll like receptors (TLRs) (see Activation in Figure 2). These factors activate pathways leading to activation of STAT6, STAT1 and nuclear factor kappa-light chain-enhancer of activated B cells (NFkB) in the MDSCs which in turn triggers activation of suppressive functions of the MDSCs [68].

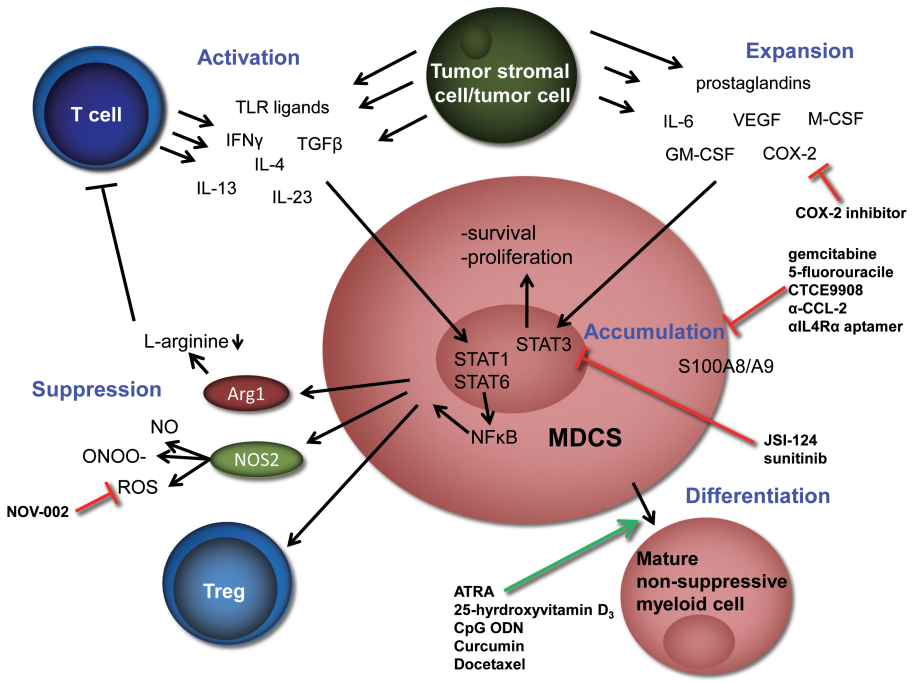

Figure 2. Pathways for activation, expansion, accumulation, differentiation and suppression of MDSCs. Inhibitors of MDSCs are shown in blod.

Suppressive Mechanisms of MDSCs

MDSCs exert their suppressive function in various ways (see Suppression in Figure 2). Arginase 1 (Arg1) and inducible nitric oxide synthase (iNOS, NOS2) are two enzymes important for MDSC T cell suppression. Enzyme activity leads to depletion of L-arginine through conversion of L-arginine to either urea and L-ornithine (Arg1) or nitric oxide and citrulline (NOS2) [76]. Depletion of the conditionally essential amino acid L-arginine arrests T cells in G0-G1 phase of the cell cycle and downregulates the T cell CD3 zeta-chain which leads to inhibition of the T cell [77-78]. Moreover, NOS2 activity generates nitric oxide (NO), ROS and peroxynitrate (ONOO-) that can inhibit T cell activation in various ways. High levels of ONOO- lead to nitration of the TCR on CD8+ cells. A nitrated TCR cannot bind to MHC molecules which results in lack of T cell activation [79]. ONOO- can also nitrate chemokine (C-C motif) ligand 2 (CCL-2) leading to inhibited intratumoral T cell migration [80]. MDSCs further suppress immune responses by induction of Tregs and by preventing cytotoxic T cell homing by shedding of L-selectin from the T cell surface [76, 81]. Interestingly, Nagaraj et al showed that MDSCs only induced inhibition of T cells that were specific to the antigen that was presented by the MDSCs [82].

Cysteine is an essential amino acid for T cells since it cannot be produced by the cells themselves. MDSCs can inhibit T cells by depleting cysteine in the T cell microenvironment [83]. Besides inhibiting T cells, MDSCs promote tumor progression by pro-angiogenic mechanisms. It is has been proposed that MDSCs can secrete VEGF and also that MDSCs can differentiate into endothelial cells, mechanisms that both promote vascularization [76].

MDSCs in Solid Tumors

In tumor-bearing mice MDSCs are increased in spleens and at the tumor site. In humans, data showing elevated levels of MDSCs in peripheral blood of patients with different types of solid tumors are accumulating [84]. Increased levels of circulating MDSCs have been found in for example patients with renal cell carcinoma (RCC), non-small cell lung cancer, breast cancer, prostate cancer, metastatic melanoma, bladder cancer, gastrointestinal malignancies and glioma [70, 85-93]. These cells suppressed T cell functions by different mechanisms like TGFβ secretion, Arg1 activity and induction of Tregs [70, 81, 88-89, 94]. The level of MDSCs in cancer patients have been found to correlate with disease stage [74, 86] and also to be an independent prognostic factor for some cancer forms such as pancreatic, esophageal and gastric cancers [93, 95]. In non-small cell lung cancer, the population of MDSCs decreased in patients responding to chemotherapy [85]. In a clinical trial with a cancer vaccine for patients with premalignant lesions, it was shown that lack of an immunologic response to the vaccine correlated with

high levels of MDSCs before vaccination [96]. Moreover, in another clinical trial, breast cancer patients with lower levels of MDSCs had higher probability of achieving a complete response after treatment [97].

MDSCs in Hematological Malignancies

The knowledge about MDSCs in hematological malignacies is still scarce, with only a few published papers on the subject. Serafini et al showed in 2008 that MDSCs induced T cell tolerance in an A20 B cell mouse model by inducing tumor-specific Tregs [98] and Van Valckenborgh et al recently showed that MDSCs were induced in response to multiple myeloma cells in a mouse model [99]. Moreover, MDSCs were increased in patients with multiple myeloma [100]. Lin et al have identified a population of immunosuppressive monocytes in non-Hodgkin lymphoma patients. This immunosuppressive population was increased in patients with more aggressive disease [101]. Patients with diffuse large B-cell lymphoma also had increased number of MDSCs at diagnosis and the levels returned to normal for patients in remission [102].

Inhibiting MDSCs

Studies trying to inhibit MDSCs in tumor-bearing mice and in cancer patients have been performed, often resulting in decreased levels of MDSCs and/or enhanced tumor immunity. The different agents inhibiting MDSCs can be divided into four groups depending on their mechanism of action: agents inhibiting maturation of MDSCs from precursors, agents reducing MDSC accumulation in peripheral organs, agents promoting maturation of MDSCs and agents affecting the function of the MDSCs (see colored arrows in Figure 2) [103].

Inhibiting Maturation from MDSC Precursors

The expansion and activation of MDSCs is highly dependent on STAT3 activity, hence, inhibitors of STAT3 activation can inhibit MDSC maturation from precursors. Different inhibitors of STAT3 have been described for inhibiting MDSCs. For example, Nefedova et al used the JAK2/STAT3 inhibitor JSI-124 in different mouse tumor models and found that treatment with the inhibitor resulted in better DC function and in increased survival of tumor bearing mice after a combination of JSI-124 and tumor immunotherapy [104]. Another inhibitor of the STAT3 pathway is the TKI sunitinib (see Figure 2). In RCC patients, sunitinib has been shown to significantly reduce the number of MDSCs. This reduction correlated with a reduction in Treg levels. *In vitro,* sunitinib reduced the viability and the suppressive effect of patient MDSCs [105].

Reducing MDSCs in Peripheral Organs

The accumulation of MDSCs in peripheral organs can be reduced by different chemotherapeutic agents such as gemcitabine and 5-fluorouracil [106-107], and also by chemokine inhibitors blocking MDSC chemotaxis (see Figure 2). For example, treatment with the CXCR4 inhibitor CTCE9908 reduced the level of intra-tumoral MDSCs expressing CD11b and VEGF receptor 1 in a prostate cancer model [108]. Blocking the chemokine CCL-2 reduced the accumulation of MDSCs in a glioma model [109]. Moreover, treatment with an aptamer blocking IL-4Rα in a mammary carcinoma mouse model reduced the tumor-infiltrating MDSCs and induced MDSC apoptosis [110].

Promoting MDSC Maturation

The vitamin derivates all-*trans*-retinoic acid (ATRA) and 25-hydroxyvitamin D_3 have been used for maturation of MDSCs resulting in reduced suppressive capacity (see Figure 2). *In vitro* experiments demonstrated that ATRA reduced MDSC immunosuppression by promoting differentiation of the suppressive cells [111]. This differentiation is due to increased glutathione levels in the MDSCs which leads to neutralization of ROS and maturation of the MDSCs [112]. In patients with metastatic RCC the numbers of immature myeloid suppressor cells were reduced after ATRA treatment, but only in patients with a high plasma concentration of ATRA [113]. Patients with head and neck squamous cell carcinoma treated with 25-hydroxyvitamin D_3 had decreased levels of CD34+ suppressive cells after treatment as well as increased levels of HLA-DR, IL-12 and IFNγ expression, although no clinical responses could be seen [114]. CpG oligodeoxynucleotides (CpG ODNs), curcumin and docetaxel have also been used to decrease the level of MDSCs in different tumor models through promotion of differentiation [115-117].

Affecting MDSC Function

Selective inhibitors of Arg1 and NOS2, as well as ROS scavengers have been investigated for inhibition of the function of MDSCs [79, 118]. The inhibitors blocked MDSC immunosuppression *in vitro,* however, the treatment of patients with these inhibitors is not recommended due to risk of adverse events [103]. NOV-002 is a glutathione disulfide mimetic that reduces ROS production by MDSCs in a mouse model where ROS production from MDSCs was induced by cyclophosphamide [119].

In mice, COX-2 inhibitors have been used to inhibit MDSCs resulting in reduced MDSC levels in a glioma model and in a mesothelioma model [120-121]. The phosphodiesterase type 5 inhibitor sidenafil has also been shown to downregulate suppressive pathways of MDSCs and to restore tumor immunity in mice. In peripheral blood mononuclear cells (PBMCs) from

patients with multiple myeloma and head and neck cancer, *in vitro* treatment with sidenafil restored the T cell proliferation [122].

Programmed Death Receptor 1 and Programmed Death Receptor Ligand 1

Programmed death receptor 1 (PD-1, CD279) is a co-stimulatory/inhibitory receptor upregulated on activated T cells as well as on B cells, NK cells and activated monocytes [123]. Binding of the CD28 superfamily member PD-1 to programmed death receptor ligand 1 (PD-L1) leads to inhibition of T cells through inhibition of PI3K and the Akt signaling pathway (see Figure 3) [124-125]. Apart from inhibition of T cells, PD-L1 has also been suggested to induce apoptosis in T cells. This mechanism was suggested to, at least in part, be mediated independently of the PD-1 molecule [126]. PD-L1 (B7-H1, CD274) is expressed by various immune and non-immune cells such as T cells, B cells, DCs, macrophages, vascular endothelial cells, pancreatic islets, astrocytes, and keratinocytes. The expression of PD-L1 is upregulated by IFNγ released during an immune response. The mechanism of T cell inhibition through PD-1/PD-L1 interaction is crucial for shutting down the immune system after clearance of infection as well as for creating peripheral tolerance [125]. PD-1/PD-L1 interaction is also used by tumor cells to evade the immune system [127]. Most reports have been focusing on co-inhibitory effects of PD-1/PD-L1 [123] but also co-stimulatory effects of the interaction have been reported [128-130].

PD-1/PD-L1 Co-Inhibitory Effects in Cancer

PD-L1 is expressed by cancer cells in various human solid cancers and hematological malignacies such as adult T cell leukemia/lymphoma (ATLL), T cell-derived non-Hodgkin lymphoma, lung cancer, ovarian cancer, melanoma, AML, Barret carcinoma, colorectal carcinoma, B cell chronic lymphocytic leukemia (CLL) [126, 131-136] as well as in mouse models and tumor cell lines [137-138]. PD-1 has been shown to be upregulated in patients with CML, ATLL, hepatocellular carcinoma, and in patients relapsing with cancer after allogeneic SCT [131, 137, 139-140]. In many of these cancers, blockade of PD-L1/PD-1 increased activation of T cells, implying co-inhibitory effects of the interaction (see Figure 3) [131, 137, 139-141]. Moreover, in a mouse model of CML, mice in CML blast crisis that where treated with PD-L1 blocking antibody survived longer than mice not treated with the antibody [137]. Further, in clinical trials investigating antibodies blocking PD-1 or PD-L1 for patients with advanced solid tumors and hematological malignancies, clinical benefits were observed for some of the patients [142-145]. Expression of PD-L1 has also been shown to be associated with advanced disease and a poor prognosis in some cancers [134,

139, 146-147]. In different forms of leukemia, Salih et al showed expression of PD-L1 in many patients but no inhibitory effects on cytokine production, proliferation or activation of T cells [148].

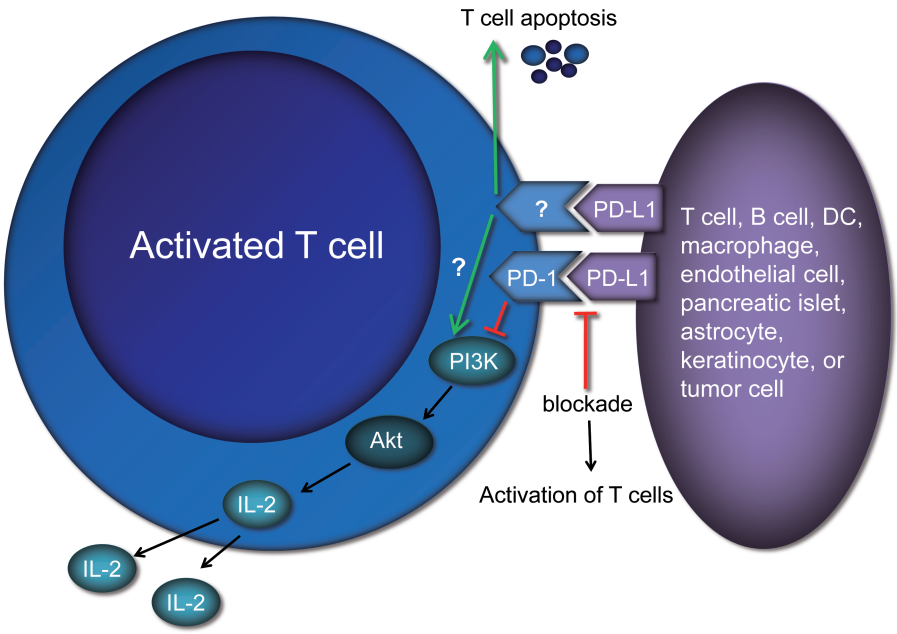

Figure 3. The function of PD-1/PD-L1 interaction on T cells

PD-1/PD-L1 Co-Stimulatory Effects

When the function of PD-L1 was first described in 1999, Dong et al described PD-L1 as a co-stimulatory molecule increasing the proliferation of antigen- or α-CD3 activated T cells. However, they also described increased production of IL-10 after PD-L1 stimulation and therefore proposed that PD-L1 may be involved in negative regulation of cellular immune responses [128]. In a mouse model infected with lethal doses of Listeria monocytogenes, addition of PD-L1 blockade inhibited CD8+ T cells and increased the mortality of the mice [149]. In a diabetic mouse model receiving allogeneic beta cells transgenically expressing PD-L1, accelerated rejection of transplanted islets was seen [129] implicating PD-L1 in co-stimulation. Wang et al proposed in 2003 that PD-L1 could have both a co-inhibitory and a co-stimulatory function but that the co-stimulatory function was separated from PD-1(see Figure 3) [150].

Tregs

Tregs are regulatory immune cells important for maintaining peripheral tolerance, limit inflammation and prevent autoimmune diseases. Tregs are also thought to be important for maintaining pregnancy [151-152]. Apart from regulating the normal immune system, Tregs play an important role in immune evasion of tumors. There are different subgroups of Tregs differing in where they are generated and in suppressive mechanisms. Naturally occurring Tregs (nTregs) are generated in the thymus while inducible Tregs (iTregs) are generated in peripheral lymphoid tissues. Although CD4+ Tregs are the most investigated, also CD8+ Tregs have been described. Hallmarks for most Tregs are high expression of the transcription factor FoxP3, high expression of CD25 and low expression of CD127. Tregs are highly dependent on the cytokine IL-2 produced by other activated T cells [153].

Subsets and Activation of Tregs

Although nTregs and iTregs are defined as different subsets and are generated at different sites, these subsets are often phenotypically indistinguishable. nTregs are developed in the thymus and they suppress cells from both innate and adaptive immunity. While suppression by CD4+ Tregs can be both dependent and independent of cell-cell contact, the suppression through CD8+ Tregs has been described as cell-cell contact dependent. Unlike the nTregs, which have suppressive functions already when they leave the thymus, iTregs develop from naïve T cells and acquire suppressive functions in the lymphoid organs. iTregs are induced by stimulation of the TCR under suppressive conditions that are not optimal for activation of effector T cells. These conditions can be for example low dose of antigen, high levels of cytokines such as IL-2, IL-10 and TGFβ as well as stimulation by immature APCs. [153].

Immune Inhibition by Tregs

Tregs inhibit immune cells in various ways. Secretion of the molecules IL-10 and TGFβ is a suppressive mechanism exerted by some Tregs. IL-10 secretion leads to decreased expression of various pro-inflammatory cytokines as well as alteration of APC function which in turn leads to alterations in T cell activation [154]. TGFβ secretion leads to inhibition of both innate and adaptive immune cells [155]. Moreover, binding of cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) present on many Tregs leads to decreased co-stimulation and thereby decreased effector T cell activation. Further, CTLA-4 binding on DCs induces expression of the enzyme idoleamine 2,3-dioxygenase (IDO) that has immune suppressive functions [153].

Tregs in Cancer

Increased levels of Tregs have been reported in many solid cancers, for example in cancers of the pancreas, breast, ovaria, lung, prostate, liver and skin [153, 156-157]. Moreover, high levels of Tregs have also been found in hematological malignacies such as Hodgkin lymphoma, CLL, AML and CML [158-161]. Tregs are thought to inhibit the anti-tumor immunity and high levels of Tregs are associated with worse prognosis in many solid tumors. In hematological malignacies, the correlation between Tregs and prognosis seem to be more complex since Tregs can inhibit both anti-tumor responses as well as the tumor cells that are derived from the immune system [153].

Soluble CD25

The receptor for IL-2 is expressed on T cells and consists of three chains, the α, β and, γ chains [162]. Activation of T cells results in release of a soluble form of the IL-2 receptor α chain called soluble IL-2 receptor or soluble CD25 (sCD25) [163]. sCD25 binds free IL-2 and is suggested to regulate IL-2 dependent lymphocyte function [164]. Elevated levels of sCD25 have been found in inflammatory and autoimmune diseases as well as in infections and cancer, further implicating a role in immunomodulation. In cancer, sCD25 has been suggested to be released from activated lymphocytes and/or from tumor cells [165].

sCD25 in Hematological Malignancies

In lymphoproliferative malignant disorders, high levels of sCD25 have been found and sCD25 was suggested to be released from activated T cells or from tumor cells [165-166]. In a paper included in this thesis (paper IV) we suggest that sCD25 may be released from Tregs in CLL [167]. The level of sCD25 has been correlated with disease stage and prognosis in different hematologic malignancies [168-171]. In CML, sCD25 is increased in patient serum [172], and plasma [173] (Paper I). In blast crisis CML, patients had even higher sCD25 levels than chronic phase CML and the level of sCD25 was correlated to the blast- and leukocyte count in peripheral blood [174].

The Immune System and Anti-Leukemia Response in CML

Allogeneic SCT was for long the only known cure for CML and the immunological component of this treatment, the graft-versus-leukemia effect, has been thought to play an essential role in treatment outcome. Decreased responses in patients receiving lymphocyte-depleted allogeneic

grafts as well as increased responses with donor lymphocyte infusions underlines the importance of the immune system and in particular the lymphocytes in a favorable response [175]. Moreover, treatment with IFNα induces remission in some patients with CML, an effect that could be partly immune mediated [176]. These results implicate that the immune system in CML may play an important role in the response to treatment.

In both treated and untreated patients with CML as well as in healthy controls, T cells specific for peptides from the BCR/ABL fusion protein have been found [177-178]. The levels of these specific T cells were higher in patients than in healthy controls. Nevertheless, the specific T cells could only be expanded from the healthy controls *in vitro,* implicating a defective function of leukemia-specific T cells in patients with CML [178]. Another study showed that CML-specific T cells were more frequent in patients with low tumor burden and that CML-specific T cells could be expanded from both patients with CML and from healthy controls [177]. The results from these two studies are somewhat contradictory, but both show that CML-specific T cells exist in the patients, implicating that some sort of immune response against the leukemic cells has been initiated. When investigating the total T cells levels as well as CD4+ and CD8+ subsets in patients with CML, these were shown to be comparable to the levels in healthy controls [179-180]. However, both CD4+ and CD8+ cells from patients with CML secreted less cytokines after stimulation compared to cells from healthy controls [181]. Continuing on the path of contradictory results, Kiani et al found normal cytokine levels when stimulating CD4+ T cells sorted from patients with CML [182]. The level of Tregs at diagnosis has been reported to be increased [183] or not different from the levels in healthy controls [184]. Rojas et al reported lower levels of Tregs in patients in CCgR compared to patients not in CCgR [161].

NK cell levels in CML have been shown to be decreased [180] as well as increased or comparable [179, 185] to the levels in healthy controls. Moreover, NK cell levels decreased with progression of the disease [186]. Most reports indicate decreased function of CML NK cells as shown by decreased cytokine production after stimulation, decreased proliferation and cytotoxicity as well as increased apoptosis [180, 185-187]. The cytotoxicity could be restored by culturing the NK cells with IL-2 *in vitro* [188] .

Deficient activation and function of T cells and also NK cells could be a result of deficient function of DCs in CML. There are different reports on DCs and maturation of DCs from patients with CML. Many of these conclude that most DCs in CML carry Ph [189-192], something that could make the DCs potent presenters of antigens derived from the BCR/ABL protein. The BCR/ABL protein, however, may be downregulated as a consequence of DC maturation contradicting increased antigen presentation [193]. Moreover, DCs in CML express low levels of co-stimulatory molecules such as CD80, CD86 and CD40 [181, 190] making them

suboptimal T cell stimulators. CML DCs have also been shown to have defective antigen-uptake and processing, altered F-actin distribution and reduced capacity to migrate, further indicating impaired function [184]. Nevertheless, several studies show that DCs from patients with CML can stimulate T cell proliferation and cytotoxicity after stimulation *in vitro* [181, 190-192].

The Effect of TKIs on the Immune System

The TKIs used for CML treatment inhibit, apart from the BCR/ABL kinase, also other kinases such as PDGFR and cKit, dasatinib also inhibits Scr-family kinases [36]. The cells of the immune system express many tyrosine kinases and can be attributed to, so called, off-target effects of the TKIs. As a result of these off-target effects, increased or decreased functions of immune cells can lead to enhanced or diminished anti-tumor responses in TKI treated patients.

Many *in vitro* studies have investigated the effects of TKIs on cells of the immune system such as T-, B- and NK- cells, DCs and monocytes. In these studies, immune cells from either healthy controls or patients with CML have been cultured with various concentrations of TKIs. Some of these studies show immunostimulatory effects of TKIs [194], but most indicate immune inhibitory effects of TKIs *in vitro* [195-202]. Although TKIs have shown immune inhibitory effects like inhibition of T- [195-198] and NK cells [199-200] and decreased maturation of DCs [201-202] *in vitro,* these results do not seem to translate to the clinical setting.

General immune inhibition in TKI-treated patients would lead to increased susceptibility to infection, something that has not been seen in these patients [203-205]. However, decreased levels of lymphocytes have been seen in imatinib-treated patients, and these lymphocytes were more sensitive to FasL-induced cell death compared to lymphocytes from healthy controls [206]. Moreover, some patients treated with imatinib develop hypogammaglobulinemia [207] indicating an immune inhibitory effect. On the contrary, in some patients treated with dasatinib a lymphocytosis with activated NK- or T cells has been reported. This lymphocytosis was associated with good treatment responses, but also with inflammatory adverse events like pleuritis and colitis [208]. Patients responding to imatinib treatment had increased IgM levels and IgM producing B cells in bone marrow up to nine months after treatment initiation, further indicating immunostimulatory effects. IgM antibodies from these patients induced apoptosis of CML cells *in vitro* [209].

Taken together, there is still no consensus on the effects of different TKIs on the immune system. Dasatinib may induce more immunological off-target effects because of inhibition of Src-family kinases that are expressed in

many immune cells [210]. *In vitro* and *in vivo* effects seem to differ; hence, to unravel the short- and long-term effects of TKIs on the immune system, preferably the immune system of patients on treatment should be studied.

Quantification of the Immune Response

The quantification of an immune response is often made *ex vivo* by measuring the level and function of different immune cells and/or other components of the immune system such as cytokines and chemokines. Phenotypes of immune cells are often measured by flow cytometry while soluble cytokine levels traditionally have been measured by enzyme-linked immunosorbent assay (ELISA). The ELISA measures the concentration of one analyte in a sample. If more analytes should be measured, multiple assays need to be run which results in consumption of large sample volumes. Since signaling in the immune system is dependent on a complex network of cytokines and chemokines, measurement of only one or a few of those is usually not enough to get a full picture of the immune response in an individual. Thus, methods measuring more than one analyte at a time, but consuming less sample volume than multiple ELISAs, have been developed. Many of these multiplex methods are, just like the ELISA, immunoassays with antibodies detecting the analytes of interest, but also other techniques for multiplex quantification are used.

Singleplex ELISA Assay

An ELISA is an immunoassay where soluble antigens can be detected with help of antibodies binding to the antigen of interest. In a sandwich ELISA the Fc-parts of capture antibodies recognizing the antigen are bound to wells in a microtiter plate. Subsequently, antigen is bound to the capture antibody and unbound antigens in the sample are washed off. A detection antibody, recognizing another epitope of the antigen, binds to the bound antigen. The detection antibody is then coupled to an enzyme. After addition of a substrate, the enzyme catalyzes a reaction resulting in color development in the wells. The color intensity can be quantified with an ELISA reader. To quantify the concentration of the antigen in the original samples, calibration samples with known antigen concentrations are run in parallel to the samples and a standard curve is calculated [211].

Multiplex Techniques

Many of the multiplex techniques used for protein quantification are developments of the sandwich ELISA. There are solid phase-based techniques as well as bead-based techniques. In the solid phase-based

techniques antibodies for different antigens are printed in spots on, for example, a chip or in the bottom of a well in a multititer plate. After addition of sample, detection antibodies and reagents, the binding of antigen to each spot can be detected [212]. In the bead-based techniques microbeads with different fluorescence-intensities or sizes are conjugated with capture antibodies. Each capture antibody is conjugated to a bead with specific fluorescence-intensity or size. After binding of the antigen to the capture antibody a detection antibody conjugated with a fluorophore that is different from the bead fluorophore is bound to the antigen. To detect the bead-bound antigens, two different systems are applied. In one system the beads with bound antigens and antibodies are run through a flow cytometer where the fluorescence intensities or sizes of the beads as well as bound detection antibodies are detected by laser excitation. In the other system the fluorescent beads are magnetic and are captured on a magnetic surface. The fluorescence from beads and antibodies is excited by light emitting diods, the emitted light is detected and processed by software [213].

There are also other multiplex techniques not using antibodies as binders of the antigens. One example is an assay using aptamers as binders. Aptamers are single-stranded oligonucleotides with the ability of binding for example proteins with high affinity and specificity. In one platform applying aptamers as binders, the aptamers with different specificities are mixed with the sample. The amounts of different antigens in the sample are then determined by measuring the concentration of different aptamers by DNA microarray [214].

There are many advantages of using multiplex assays over singleplex assays for quantification of immune responses. Many different cytokines or chemokines can be measured in the same sample although using less sample volume and at a lower cost (as long as the equipment for the analyses are at hand). However, multiplex assays also come with some potential problems. When mixing antibodies or other binders with a lot of different specificities the risk of cross-reactivity and thereby background increases. Moreover, in singleplex assays, samples can be diluted differently for optimal detection of different antigens. In a multiplex assay, all antigens in a sample are measured at the same dilution, creating high demands on the optimization of the dynamic ranges for the different antigens in the assay [212].

Aim

The aim of this thesis has been to identify immune escape mechanisms in leukemia with focus on CML and to investigate how the immune system of CML patients is affected by TKI treatment.

Specific Aims

Paper I

To map the immune status of patients with newly diagnosed CML with focus on immune escape mechanisms directly affecting T cells.

Paper II

To investigate cells and mechanisms of the immune system in patients with CML prior to and during TKI treatment.

Paper III

To compare single- and multiplex platforms for quantification of proteins. For the comparison, plasma samples from patients with CML taken before and during TKI therapy were used.

Paper IV

To investigate the source and the function of increased levels of sCD25 in patients with B-cell malignancies.

Patient Material and Methods

Blood Samples from Patients and Control Subjects

Samples from patients with CML or with B cell malignacies were investigated in all papers included in this thesis. These samples were obtained from Uppsala University Hospital (Paper I and IV) and Helsinki University Central Hospital (Paper III). For Paper II samples from the clinical study NordCML006 were investigated and samples were obtained from multiple centers in the Nordic countries. Samples from age- and gender matched control subjects were obtained from the blood bank at Uppsala University Hospital.

In Paper I, leukapheresis samples from patients with CML at diagnosis were investigated. Leukapheresis is a method where blood is collected from a patient and after leukocytes have been removed the blood is given back. Leukapheresis can be used for CML treatment to relieve circulation problems caused by excess of tumor cells in the blood. Leukapheresis is often performed to transiently lower the tumor burden before drug treatment. As control samples for this study, buffy coats where erythrocytes had been lysed were used.

In Paper II and IV, PBMCs from patients and control subjects were obtained by ficoll separation of peripheral heparinized blood.

Plasma samples for all studies were obtained through centrifugation of peripheral blood. For Papers I, II and IV, plasma from blood collected in heparin tubes was used. For Paper III, plasma from blood collected in citrate acid tubes was used.

Flow Cytometry

Flow cytometry analysis was performed with the LSRII flow cytometer from BD Bioscience. For multicolor flow cytometry experiments, compensation was made using anti-mouse IgG compensation beads stained with the specific antibodies used in the experiment. To exclude unspecific binding from the experiments and to define the negative populations in the staining, isotype controls were used. To exclude bias that could be created from day to day variations in the stainings, patient samples and respective control

samples were prepared and run at the same time. Results were analyzed with the DIVA Software from BD Bioscience or Flow Jo software from Tree star.

T cell Proliferation Assays (Paper I and IV)

In Paper I, T cell proliferation was assessed with two different methods. When measuring proliferation after co-culturing with CML cell lines, thymidine ^{3}H was used. Thymidine ^{3}H incorporates in synthesized DNA in replicating cells and cell proliferation is measured by determination of the amount of incorporated thymidine ^{3}H. This method measures proliferation of all cells in the culture. Thus, to only measure T cell proliferation in our experiments, cell line proliferation was inhibited by irradiation before co-culture.

When co-culturing T cells with CD3- cells from patients with CML (Paper I), another, non-radioactive, method for assessing T cell proliferation was used. EdU, a modified nucleoside that is incorporated into DNA during synthesis, was added to the cultures. The incorporated EdUs were then coupled to fluorophores and proliferation was detected by flow cytometry. Since EdU staining can be combined with flow cytometric staining, proliferating T cells can be assessed by staining with a T cell-specific antibody.

A third method for assessing T cell proliferation was used in Paper IV. In this assay, Alamar blue was added to the cultures. Alamar blue is a reagent that is reduced by enzymes present in living cells. Reduction of Alamar blue leads to color change of the medium which can be quantified. The more the cells are proliferating, the more living cells there are in the culture, the more color change is seen in the medium.

Methods for Protein Quantification

In paper III, different methods for protein quantification were compared. Four of the five methods (ELISA, Meso Scale Discovery, Myriad RBM and BioPlex) are immunoassays where the antigen is “sandwiched” between a capture and a detection antibody. The capture antibody is either spotted on the bottom of a well in a multititer plate or bound to a bead with intrinsic fluorescence. The way to detect the amount of detection antibody bound to the antigen, which corresponds to the amount of antigen in a sample, varies between the different methods. In the ELISA, horseradish peroxidase (HRP) is coupled to the detection antibody. This enzyme catalyzes the conversion of a substrate into a colored product. The color development that corresponds to the amount antigen in the sample can be quantified with an ELISA reader (Figure 4A). In the kit from Meso Scale Discovery, the

amount of antigen is detected by electrochemiluminescence. The detection antibody is coupled to an electrochemiluminescent label. After addition of co-reactant and electric stimulation from the bottom of the microtiter plate, light is emitted. The amount of light emitted corresponds to the amount of antigen in the sample (Figure 4B).

Myriad RBM as well as the BioPlex kit are bead-based methods. The capture antibodies are coupled to beads with intrinsic fluorescence. The detection antibodies are conjugated with a fluorophore.

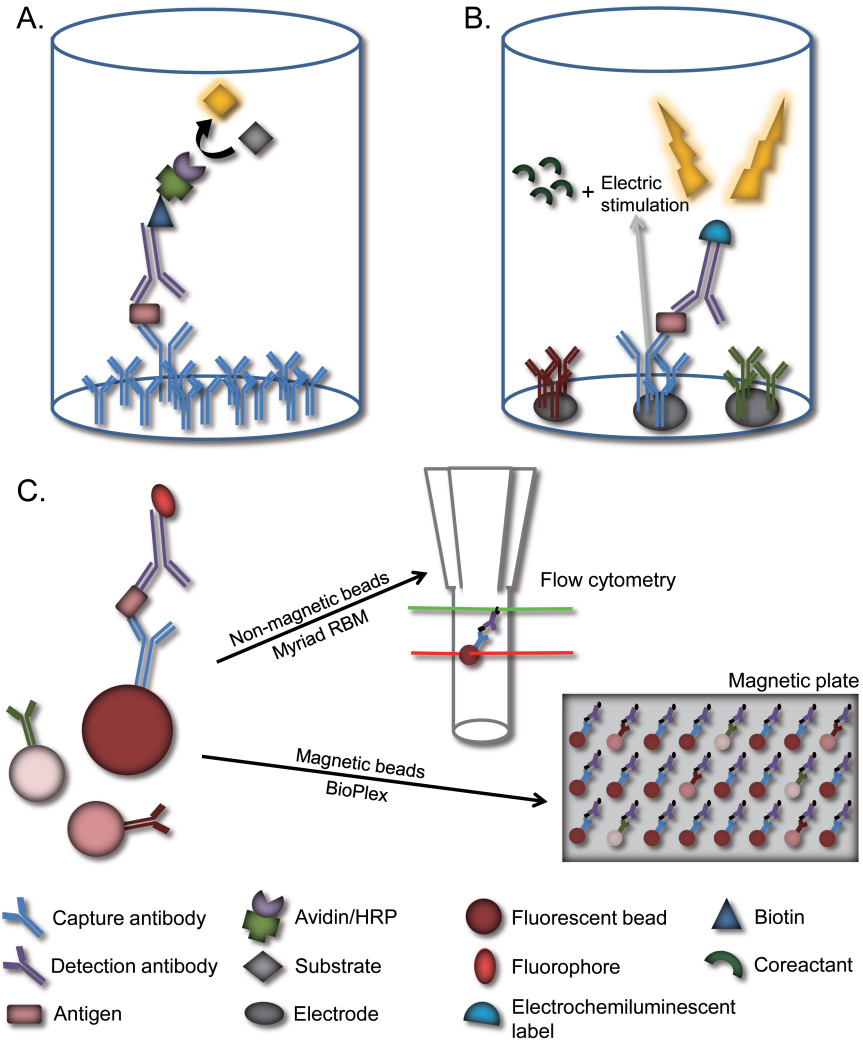

Figure 4. Immunoassays for protein quantification. A. ELISA, B. Technique employed by Meso Scale discovery, C. Bead-based techniques used by Myriad RBM (non-magnetic beads) and BioPlex (magnetic beads).

To detect how much antigen that is bound to the beads Myriad RBM uses flow cytometric quantification. The fluorescence of the beads and the antibodies is excited by two different lasers in a flow cell. The emitted light is captured and the amount of antigen in a sample is determined with help from a software (Figure 4C). The beads in the BioPlex kit are magnetic, hence, instead of using flow cytometry for quantification, a specific reader captures beads on a magnetic plate. The fluorescence of the beads and the conjugated antibodies is exited by light emitting diods, captured and analyzed with the help of a software (Figure 4C).

Somalogic uses modified aptamers, called SOMAmers, as binders of the proteins. Aptamers are oligonucleotides that can recognize and bind proteins. First, sample and biotin-tagged SOMAmers are mixed and the SOMAmers binds both cognate and non-cognate proteins. The biotin-tagged SOMAmers bound to protein are then bound to streptavidin beads. After washing off of unbound antigen, the proteins bound to SOMAmers are biotinylated. Then the SOMAmer/protein complexes are cleaved off from the streptavidin bead with the help of UV-light, the biotin originally placed on the SOMAmer remains on the streptavidin bead. To dissociate complexes of SOMAmers bound to non-cognate proteins, an anionic competitor is added.

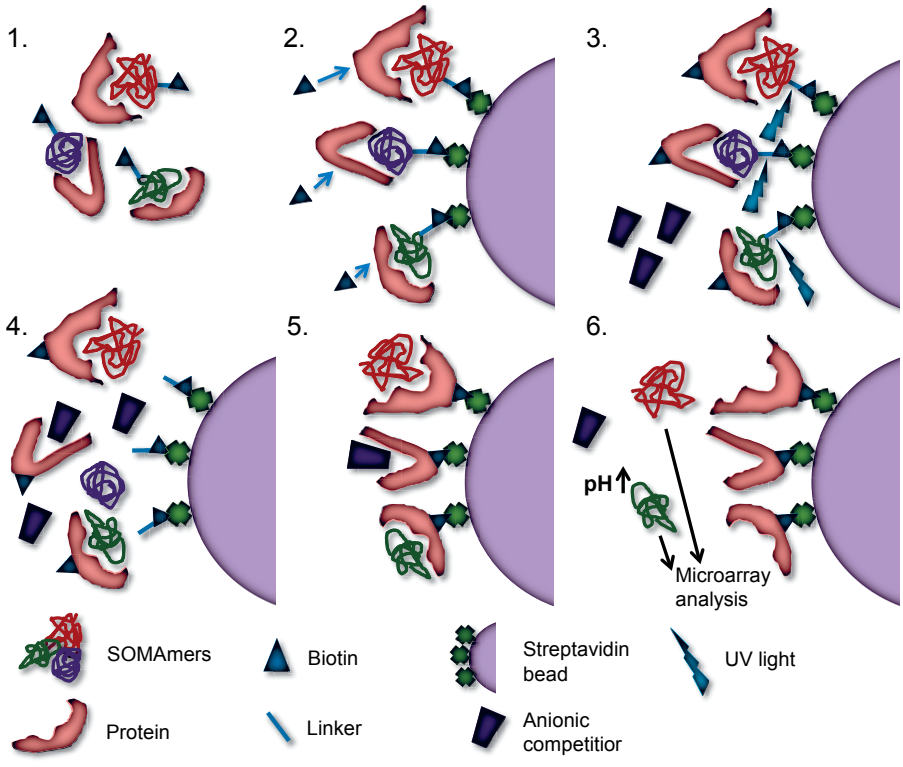

Figure 5. Somalogic assay for protein quantification

Complexes of SOMAmers with cognate proteins are then bound to a new set of streptavidin beads through the biotin on the proteins. Increased buffer pH leads to dissociation of the SOMAmer from the protein bound to the streptavidin bead. The released SOMAmers are denaturated and can be quantified by microarray analysis (Figure 5).

Results and Discussion

Paper I

In this paper samples from patients with chronic phase CML were investigated for immune escape mechanisms. Samples from patients belonging to the Sokal high- and low-risk groups were compared to control subjects. We found that Sokal high-risk patients had higher levels of MDSCs compared to low-risk patients and to control subjects. The MDSC-associated molecule Arg1 was also increased in these patients. Since the phenotype of MDSCs is similar to the phenotype of the CML cell, we hypothesized that the CML cell could qualify as a MDSC. We found that about 95% of the CD34+ cells were Ph+, a characteristic of the CML cell. CD34 was therefore used as a surrogate marker for CML cells. 35% of the MDSCs in high-risk patients expressed CD34, hence, we concluded that MDSCs could be found both in the malignant population and in the population lacking Ph.

Patients with CML had more myeloid cells expressing PD-L1 compared to control subjects. Most of the PD-L1+ cells were found in the CD34- population. Sokal high-risk patients had significantly more cytotoxic T cells expressing PD-1 compared to control subjects. The high level of both PD-L1 and PD-1 in patients with CML implicates a possible immune escape mechanism in CML. To further investigate this mechanism, the proliferation of healthy T cells after stimulation with CML CD3- leukocytes and PD-L1 blocking antibody was assessed. The proliferation of healthy T cells was not altered by addition if the PD-L1 blockade, indicating that the PD-1/PD-L1 pathway may not be an important immune escape mechanism in this setting. There was, however, an increase in IL-2 concentration in the supernatant in some of the cultures with cells from Sokal low-risk patients and control subjects after addition of the PD-L1 blocking antibody, indicating a release of the blockade of IL-2 secretion.

In concordance with others we also found high levels of sCD25 in plasma from patients with Sokal high-risk CML. sCD25 can inhibit T cell proliferation. Hence, we hypothesize that high levels of sCD25 in plasma may be another immune escape mechanism in patients with CML.

Paper II

This study investigated how the cells of the immune system in patients with CML were affected by treatment with the TKIs imatinib or dasatinib. PBMCs as well as blood plasma from diagnosis and from one and six months after treatment initiation were investigated. We found that the level of MDSCs decreased after six months. These results are in line with other results showing decreased levels of MDSCs in patients with RCC after treatment with the TKI sunitinib. The MDSC effector molecule Arg1 was higher in patients with CML at baseline compared to the levels that have been reported for healthy control subjects. TKI treatment decreased the levels of Arg1 in plasma. The levels of Arg1 and MDSCs correlated at diagnosis.

In contrast to MDSCs and to previous *in vitro* data showing inhibited proliferation and function of Tregs after imatinib and dasatinib treatment, we found increased Treg levels after TKI treatment. Tregs can be induced by MDSCs. In our study, however, we found that Treg levels were inversely correlated to MDSC levels at baseline. This indicates that Tregs were not induced by MDSCs in this setting.

CD40 is an immunostimulatory molecule but it has also been suggested to be involved in the induction of Tregs by MDSCs. Hence, the level of CD40 on MDSCs was investigated. CD40 expression on MDSCs was low at diagnosis and increased by TKI treatment. Moreover, the expression of CD40 on the whole PBMC population increased with TKI treatment indicating immune activation. Further signs of immune activation were increased NK cell levels and decreased levels of naïve T cells. The levels of T(CM) and T(EM) cells were not significantly changed with TKI treatment. However, a group of patients had increased levels of T(CM) cells after one month of treatment to which most of these patients responded well.

Paper III

When investigating the immune system of patients and control subjects, cytokines and other soluble proteins in the blood plasma are often studied. The golden standard for measuring soluble proteins is the ELISA. Lately, multiplex platforms measuring more analytes at the same time have become more and more popular. In this study we compared five different platforms (ELISA, BioPlex, Meso Scale Discovery, Myriad RBM, and Somalogic) for single- and multiplex protein measurements. To compare the platforms, identical plasma samples from patients with CML taken at diagnosis and after three months of treatment were run on all platforms.

We found that platform characteristics such as multiplexing abilities, time consumed when performing the assay and assay cost varied a lot between

platforms. Moreover, information about validation of parameters such as specificity was not provided by all manufacturers.

When comparing the ability to measure overlapping proteins in plasma samples we found that the absolute concentration measured varied between the platforms. However, the relative difference in concentration between the two different time points studied had a better correlation for most analytes and platforms. In three of the five platforms studied duplicate samples were run and for these platforms a CV-value was calculated. For most analytes the CV-value was acceptable indicating good intra-assay performance.

The detection of proteins in samples such as blood plasma or serum is complicated by the complex composition of the sample. A lot of different proteins and other molecules are present in the sample and has to be handled by the reagents of the assay without affecting assay performance. In plasma samples from patients with leukemia the composition is probably even more complex because of tumor cells present in the blood. In our study, we found that one platform had problems handling plasma samples from patients with CML taken at diagnosis. The measurements of these samples on that platform, unlike the measurement from the other platforms, created a high background.

Paper IV

sCD25 is increased in plasma from patients with B cell malignancies. In this study, the source of the increased sCD25 was investigated. Moreover, the function of sCD25 on T cells was studied. Tregs express high levels of CD25 and the molecule has been used as a marker for Tregs. Hence, Tregs were investigated as a possible source for the high sCD25 levels. We found that the surface expression level of CD25 on Tregs from patients with B cell malignacies was lower compared to the levels expressed on Tregs from control subjects. In patients with lymphoma, but not in patients with CLL, the level of CD25 on Tregs correlated to the level of sCD25 in plasma. It has previously been shown that the high levels of sCD25 in plasma from patients with B cell malignacies may originate from malignant B cells. In our study this could not be confirmed since sCD25 did not correlate with white blood cell count (mostly malignant B cells) in patients with CLL.

Since sCD25 levels could be correlated with CD25 expression on Tregs but not with the number of malignant B cells we hypothesized that sCD25 was released from Tregs. To further study this, PBMCs from patients and control subjects were sorted into different cell fractions and the release of sCD25 was measured. We found that unstimulated Tregs released sCD25. When cells were stimulated, both Tregs and the whole CD4+ population released sCD25. Malignant B cells did not release sCD25.

After showing that sCD25 could be released from Tregs we investigated the effect of sCD25 on T cells. Healthy T cells were stimulated and cultured together with recombinant sCD25 in a proliferation assay. sCD25 inhibited the proliferation of T cells in a dose dependent manner.

Conclusions

In this thesis it is concluded that patients with CML have increased numbers of immunosuppressive cells and molecules that could confer immune escape. Further, immune escape mechanisms are affected by TKI treatment, something that could affect the immune control of CML for patients receiving TKI therapy.

Specific Conclusions

Paper I

Patients with CML belonging to the Sokal high-risk group have high levels MDSCs and sCD25. Patients from both the Sokal high- and the low-risk groups have high expression on PD-L1. These cells and molecules could promote immune escape in patients with CML.

Paper II

Imatinib or dasatinib therapy of patients with CML leads to modulation of the levels of inhibitory immune cells such as MDSCs and Tregs as well as effector cells such as NK cells. The changed immune balance in TKI-treated patients could contribute to immune control of the leukemic cells.

Paper III

The different multiplex platforms investigated measured various absolute concentrations of the same protein when identical samples were run. However, when a relative ratio in protein concentration before and after treatment was compared among the different platforms these were better correlated.

Paper IV

Patients with B cell malignacies have a high level of sCD25 in peripheral blood. sCD25 is released by Tregs and it inhibits T cell proliferation in *in vitro* cultures.

Future Perspectives

The treatment of CML was revolutionized in 1998 when imatinib was introduced as therapy. Since then, imatinib and other, even more potent, TKIs have continued to decrease the tumor burden in patients with CML to levels hardly detectable by PCR (down to 5logs less than a defined baseline at diagnosis). Moreover, a subpopulation of these patients with great treatment responses can discontinue TKI treatment without relapsing. Despite this, some patients develop resistance to the drugs or have a suboptimal responses. Hence, new therapeutic options are needed for refractory patients.

In this thesis focus has been to investigate the immune system of patients with CML and how it is affected by TKIs. A better understanding of the immune system and immune escape mechanisms in these patients can lead to new therapeutic ideas for refractory CML, for example combining TKIs with immunotherapy. In Paper I, immune escape mechanisms such as MDSCs, high levels of PD-L1 and sCD25 were investigated. The decision to define the MDSC with the markers that we used was based on other studies showing suppressive abilities of cells with these markers. Further experiments investigating the suppressive ability of these cells in patients with CML are needed and should be performed on fresh cells. The notion that the levels of MDSCs were high in patients with Sokal high-risk CML and that the level of MDSCs decreased with TKI treatment, as shown in Paper II, suggest that MDSC blockade could be a treatment option for CML. *In vitro* experiments, where blood cells from patients with CML are cultured and immune activation is measured after MDSCs inhibition, could indicate whether blockade of MDSCs could be a treatment option for CML. In other cancers, the TKI sunitinib has been shown to have MDSC-blocking effects.

In paper I, we show that blockade of PD-1/PD-L1 pathway did not enhance T cell proliferation but IL-2 secretion in some cases. Since antibodies blocking this pathway are available in the clinic it would be interesting to further study this pathway in CML. If PD-1/PD-L1 is shown to be an immune inhibitory mechanism in CML, blocking antibodies could be used to promote an anti-tumor response in the patients.

In Paper II, immunoprofiling of patients with CML before and on TKI treatment was made. To be able to investigate whether the immunoprofile of a patient has an impact on the response to treatment or not, a larger patient cohort is needed. Moreover, to determine if imatinib and dasatinib have

different effects on the immunoprofile of patients, larger treatment groups are warranted. Some of the patients in this study responding well to TKI treatment will go on to TKI discontinuing studies. Mapping the immunoprofile after TKI cessation and correlating this to the effect of cessation (relapse or not) as well as to the immunoprofile on TKI treatment, obtained in our study, could suggest immune markers that could predict whether the patient will relapse or not after TKI discontinuation.

In Paper III, different single- and multiplex platforms for protein quantification were compared. From this study we still have a lot of protein data, obtained from the different platforms, to analyze. Since the patients in this comparison study are the same as some of the patients in Paper II, results from these two studies will be combined to find biomarkers that may predict the response to treatment as well as the response to TKI discontinuation.

Currently, several TKI cessation studies are ongoing and these will hopefully unravel which patients that will benefit from TKI discontinuation. In a sub-study of a large European discontinuation study (Euro-SKI), immunological profiles of patients will be assessed by our Nordic CML study group in an attempt to understand the influence of the immune system on the response. Moreover, except from discontinuation studies, clinical trials combining TKIs with other drugs such as IFNα are ongoing to find treatment combinations that will give a faster and better response to treatment. From an immunological point of view IFNα is an interesting drug because of immune-modulating effects on for example T cells, NK cells, macrophages and DCs. Moreover, IFNα can induce apoptosis and promote the cycling of hematopoetic stem cells and leukemic stem cells. TKIs and IFNα are thought to have different modes of action in CML treatment and the combination of the drugs have shown to add treatment effects compared to treatment with imatinib alone. To investigate if addition of IFNα to TKI treatment further modulates the patient´s immune system in favor of anti-tumor response would be interesting and will be done in our Nordic CML study group. Further, if anti-tumor response is promoted by combination therapy, this may be a good strategy to safely discontinue more patients on TKI treatment.

Populärvetenskaplig sammanfattning

Varför undkommer leukemiceller från patienter med kronisk myeloisk leukemi immunförsvaret?

I Sverige drabbas cirka 85 personer varje år av blodcancerformen kronisk myeloisk leukemi (KML). Det har forskats mycket om sjukdomen genom åren och denna forskning har bland annat lett fram till att KML, som för bara några årtionden sedan hade en väldigt dålig prognos, idag går att behandla effektivt med läkemedel som kallas tyrosinkinashämmare.

Min forskning handlar om hur immunförsvaret hos patienter med KML ser ut och om hur immunförsvaret påverkas av tyrosinkinashämmarna som används för att behandla KML idag.

Immunförsvaret finns till för att skydda oss mot inkräktare så som till exempel bakterier, virus och parasiter. Man tror även att immunförsvaret spelar en roll i att skydda oss mot cancer. Genom evolutionen har immunförsvaret utvecklats till att specifikt känna igen och döda angripare, sjuka kroppsegna celler och cancerceller medan friska celler skonas. Under en individs livstid utbildas immunförsvaret för att kunna känna igen och döda inkräktare som denna person utsätts för. Samtidigt som immunförsvaret tränas och lär sig känna igen olika angripare kan dessa i sin tur utveckla mekanismer som gör dem svårare att känna igen. En del av dessa mekanismer används också av cancerceller för att undvika att bli upptäckta och dödade av immunförsvaret.

I denna avhandling beskriver jag olika mekanismer hos patienter med KML som leder till att deras leukemiceller inte känns igen och dödas av immunförsvaret. Hos patienter med KML som ännu inte har behandlats har vi bland annat funnit höga nivåer av en celltyp som kan hålla tillbaka celler i immunförsvaret och därmed hindra dem att döda leukemicellerna. Dessutom har vi sett att leukemiceller från patienter med KML har molekyler på sin yta som kan förhindra att de dödas av en immuncell. En tredje mekanism som vi fann hos patienter med KML har vi också undersökt hos patienter med andra typer av blodcancer. Vi fann att dessa patienter hade höga nivåer av en löslig molekyl i blodplasman. Denna kan dämpa immunförsvaret genom att binda upp en annan molekyl som är viktig för en viss typ av immuncells tillväxt och överlevnad.

Tyrosinkinashämmarna som används för behandling av KML är effektiva och minskar antalet leukemiceller drastiskt hos de flesta patienter. Om

patienterna helt kan botas med behandlingen är dock fortfarande inte helt säkerställt. I provrörsförsök har man sett att tyrosinkinashämmarna verkar kunna hämma immunförsvaret. Om immunförsvaret hämmas skulle det kunna leda till ett sämre svar på behandlingen eftersom immunförsvaret då inte skulle kunna hjälpa till att bekämpa leukemicellerna. Av denna anledning var vi intresserade av att undersöka hur immunförsvaret ser ut hos KML-patienter som behandlas med tyrosinkinashämmare. Vi fann att andelen av en typ av celler som dämpar immunförsvaret minskade vid behandling, medan en annan typ av immundämpande celler ökade. Vi fann också att en molekyl som är viktigt för aktivering av immunförsvaret ökade vid behandling. Vi kan därför konstatera att även om läkemedlen verkar kunna undertrycka immunförsvaret i provrörsförsök är det inte säkert att de har samma effekt på behandlade patienters immunförsvar.

Man tror att tyrosinkinashämmarna kan döda alla leukemiceller förutom leukemistamcellen, leukemins urmoder som är den cell som hela tiden producerar nya leukemiceller. Så länge patienterna behandlas med tyrosinkinashämmare kan leukemicellerna hållas tillbaka, men om behandlingen avslutas borde leukemin komma tillbaka eftersom leukemistamcellen finns kvar och kan producera nya leukemiceller. Det har dock visat sig att ungefär 40% av de patienter med KML som avslutar flerårig behandling inte får tillbaka sin leukemi. Vad detta beror på är fortfarande oklart. En möjlighet skulle kunna vara att tyrosinkinashämmarna trots allt har dödat leukemistamcellen. En annan skulle kunna vara att patientens eget immunförsvar kan känna igen leukemiceller som produceras av leukemistamcellen och döda dem. Just nu görs många studier där man undersöker om patienter kan avsluta behandling med tyrosinkinashämmare utan att få tillbaka sin leukemi. I en del av dessa studier kommer man bland annat undersöka hur immunförsvaret påverkas av avbruten behandling och om man kan se något samband mellan patienternas immunförsvar och hur de svarar på avbruten behandling. Studierna som presenteras i denna avhandling kan ge idéer om vilka delar av immunförsvaret som kan vara intressanta att undersöka i fortsatta studier. Dessutom kommer en del av de patienter som vi har undersökt så småningom sannolikt avsluta sin behandling. Information om hur dessa patienter svarar på avslutad behandling kan vi sätta ihop med informationen om hur deras immunförsvar såg ut innan och under behandling för att försöka ta reda på vilken roll immunförsvaret spelar i behandling av KML med tyrosinkinashämmare.

Acknowledgements

Arbetet med denna avhandling utfördes vid instutitionen för immunologi, genetik och patologi på Uppsala universitet. Arbetet stöttades av Medicinska fakulteten vid Uppsala Universitet, Vetenskapsrådet, Lions, European LeukemiaNet, Nordic CML study group och Anna-Maria Lundins Stipendiefond.

Jag vill tacka de personer utan vars provskickande, hjälp, stöd och peppning denna avhandling aldrig hade blivit till.

Till att börja med vill jag tacka min huvudhandledare Angelica. Tack för din guidning genom min doktorandtid. Tack för din obotliga optimism, din snabba korrekturläsning och din förmåga att se saker i våra data som jag missar.

Ulla, tack för att du har varit min bihandledare, för alla prover du har skickat, för att du har svarat på alla min KML-frågor och för ditt smittande engagemang för patienterna.

Bengt, tack för att du har delat med av din stora kunskap om KML och din entusiasm för KML-forskning. Ett stort tack för alla kommentarer på och uppmuntrande ord om mina arbeten och min avhandling.

Tack till alla patienter och friska blodgivare som har donerat sitt blod till vår forskning, utan det hade den här avhandlingen aldrig kunnat skrivas.

To all coauthors, thanks for the collection and sending of patient samples and for giving me valuable comments on the manuscripts. A special thanks to the Nordic CML study group, for accepting the Uppsala spin-off project in the 006-study, for sending all samples and for eagerly awaiting the results.

Tack till Maj-Britt, Maj-Britt och Viola på stamcellslab för hjälp med att leta fram alla gamla leukaferesprover till min första artikel.

A big thanks to all former and present colleagues in the GIG group for creating a great working environment. Thanks to Ole, Arian, Angelika and

Fredrik who lead the way and to Chuan, Mohan, Matheus, Matko, Vanessa and Oskar that assure a great future of the GIG group.
Magnus, tack för att du tog emot oss i gruppen när Angelica bestämde sig för att spendera mindre tid på Rudbeck och för alla smarta kommentarer och frågor på olika gruppmöten.
Thomas, tack för kommentarer och frågor på GIG-mötena som får oss att tänka.
Camilla, tack för att du var en så bra handledare på labb när jag kom till klinimm. Tack för att du visar så mycket omtanke om alla och peppar när självförtroendet inte är på topp. Tack för att jag får ha din artikel med i min avhandling!
Lina, med bara en veckas mellanrum kom vi till klinimm och delade då en laptop som tog 15 minuter att starta. Tack för fem år med goda bakverk, trevligt resesällskap och för att du inte valde fönsterplatsen flera gånger om!
Hannah, tack för alla konstiga ljud och härliga skratt som ljuder genom korridoren (och för öronpropparna!). Tack också för att du finns där med din axel när det känns tungt och för att jag får finnas där med min.
Linda, ett av L:en i L-rummet (även kallat Norrland eller Kuvösen) under min första tid på klinimm. Tack för trevligt resesällskap i Italien och för asgarv under luncherna. Tack för att du satte krydda på mitt avhandlingsarbete...
Sara, tack för all din energi och entusiasm för forskning och immunologi som smittar av sig. Tack för att du är en förebild och för gott sammarbete med jämförelsemanuset.
Justyna, tack för att jag fick vara din tolk och för att du peppade mig att tänka själv och ta egna initiativ när vi var rumskamrater.
Joachim, kul att du kom in i gruppen och balanserar cancerforskningen på mötena och X-kromosomerna vi lunchbordet. Tack för att du bjuder på dig själv och får oss att skratta när du delar med dig av 1kg-köttvadslagningar, chokladbröst och mindre lyckad undervisning av sjukgymnaststudenter.
Lotta, tack för att du har gett mig perspektiv på vad som är rimligt ☺!
Di, thanks for having a floppy-disk reader that I can borrow.
Victoria, tack för att du tillsammans med Hannah jobbar hårt för att vårt viktigaste arbetsredskap ska fungera!
Stina, tack för gott sammarbete med de olika manuskripten, för hjälpen i labbet och för att du kommer se till att det finns en fortsättning för våra projekt!
Pella, du är satellitlabbs fasta punkt, har svaren på alla frågor och hanterar allt från invitrogenbeställningar till krånglande inkubatorer. Tack för hjälpen med adenovirusproduktion, luminexkörningar och för att du har dragit det tyngsta lasset med kemikalierna! Tack också för minnesregeln att fluorescens stavas som flugor. En genial regel och mycket välanvänd i denna lilla bok!

Berith, tack för att du har allt man frågar efter och för att du vet var virkonet finns, varje gång jag frågar!
Former project students Sofia, Lisa, Emma and Ying, thanks for helping me in my project and for giving me the opportunity to practice the supervisor role. Emma, tack för laborativ hjälp med min första artikel. Emma och Erika, tack för att ni återinförde (hyfsat) regelbunden fika-time på satellitlabb.
Moa, tack för att du sprider positiv energi omkring dig.
Maryam, tack för att du tog emot oss med öppna armar när vi kom och invaderade ditt skrivrum!
Andrew, tack för att du förgyllde våra lunchstunder med din närvaro och för alla nyttiga engelska ord vi har fått lära oss. Jag kommer ihåg cauliflower och en del ”shit”-kombinationer! I think that we are both humans and dancers, but above all dancers.

Liza, jag är så glad för att du ville ha med mig i ditt projekt. Tyvärr blev det inget manus till avhandlingen, men jag är övertygad om att du kommer fixa det när du kommer tillbaka till Rudbeck. Tack för att du alltid har så mycket energi och för din stora tro på att vi kommer få det att funka, trots många mindre lyckade försök.

Tack till administrativ personal på klinim (Elin, Mona, Anso, Anette och Anna-Maria) och på IGP för hjälp med fakturor, reseräkningar och annat smått och gott. Ett speciellt tack till Christina, för att du med ditt stora hjärta underlättar för och hjälper oss doktorander.

Jag vill också tacka alla er som ger mig ett härligt liv utanför labbet. Tack till alla fina dansvänner som gör mig glad och ger mig ny energi på och utanför dansgolvet och som gör mig lite extra trött på onsdagmornarna!

Tack till nuvarande och före detta medlemmar i MessAround. Jag gillar att träna och stå på scen tillsammans med er. Tack för att ni får mig att koppla bort tankarna från klibbiga celler. Ett speciellt tack till Patrik som har lärt mig nya saker om hur immunförsvaret fungerar när man fyller 25 ☺.

Anna, tack för att du är så mån om att alla ska må bra och vara med och för din asociala inkompetens som resulterar i många trevliga, sociala tillställningar.

Angelica, tack för doktorand- och danssnack, för pepp under långa Stallet-luncher och för trevligt delat boende i sammband med diverse dansläger. Nu bor du långt borta och har säkert skaffat dig en massa nya kompisar, men eftersom du räcker till många hoppas jag att det finns en liten bit kvar till mig!

Gunilla, tack för att du ger mig fler perspektiv på livet (finns mer än bara dans och jobb!). Tack för te och trevliga samtal. I höst blir det 10-årsjubileum, men innan dess kommer vi och hälsar på i Storvreta!

Ett stort tack till min familj som har stöttat och hjälpt till genom åren. Mamma och pappa, tack för att ni alltid har funnits där oavsett om det har gällt glosförhör eller flytthjälp. Snart hoppas jag att ni ska få komma och hälsa på lite oftare. Henrik och Sara, finaste syskonen, tack för att ni har banat vägen och visat minstingen hur man hanterar livet. Tack Sara för att du läste och gjorde den svenska sammanfattningen bättre. Memet och VT, tack för att ni tar så fint hand om mina syskon. Sol, tack för att jag får åka bob, köra bilar och parkera med dig, nu vill jag göra det ännu mer!

Tack till Anna, Pär, Sara, Simon och vovvarna för att ni har släppt in mig i er familj.

Simon, jag beundrar dig för din outtömliga energi och din positiva syn på livet. Du är helt underbar som står ut med mig trots att disputationsångesten alltsom oftast knackar på och solskenshumöret då gömmer sig någonstanns långt inne i en garderob. Tack för att du delar den här tiden med mig, för att du lagar mat, servar och för att du med jämna mellanrum ger mig pauser i skrivandet då jag får testa nya häftiga funktioner i din egenprogrammerade musikapplikation. Puss på dig finaste finingen!

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