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ANTILEUKEMIC IMMUNE RESPONSES IN
CHRONIC MYELOID LEUKEMIA

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<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>7AAD</td>
<td>7-amino-actinomycin D</td>
</tr>
<tr>
<td>ABL1</td>
<td>Abelson murine leukemia viral oncogene homolog 1 gene</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute lymphoblastic leukemia</td>
</tr>
<tr>
<td>AlloHSCT</td>
<td>Allogeneic hematopoietic stem cell transplantation</td>
</tr>
<tr>
<td>AP</td>
<td>Accelerated phase</td>
</tr>
<tr>
<td>APC</td>
<td>Allophycocyanin</td>
</tr>
<tr>
<td>ASO</td>
<td>Allele-specific oligonucleotide</td>
</tr>
<tr>
<td>BC</td>
<td>Blast crisis</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region gene</td>
</tr>
<tr>
<td>BCR</td>
<td>Breakpoint cluster region</td>
</tr>
<tr>
<td>BLAST</td>
<td>The Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>c-Kit</td>
<td>CD117, a receptor tyrosine kinase</td>
</tr>
<tr>
<td>CCgR</td>
<td>Complete cytogenetic response</td>
</tr>
<tr>
<td>CHR</td>
<td>Complete hematological response</td>
</tr>
<tr>
<td>CFSE</td>
<td>Carboxyfluorescein diacetate succinimidyl ester</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic myeloid leukemia</td>
</tr>
<tr>
<td>CML AP</td>
<td>CML acceleration phase</td>
</tr>
<tr>
<td>CML BC</td>
<td>CML blast crisis</td>
</tr>
<tr>
<td>CML CP</td>
<td>CML chronic phase</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CMR</td>
<td>Complete molecular response</td>
</tr>
<tr>
<td>CP</td>
<td>Chronic phase</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T lymphocyte-associated antigen 4</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated cell sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FISH</td>
<td>Fluorescence in situ hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte colony stimulating factor</td>
</tr>
<tr>
<td>GrB</td>
<td>Granzyme B</td>
</tr>
<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IFN-α</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IFN-ON</td>
<td>CML patient on IFN-α monotherapy</td>
</tr>
<tr>
<td>IFN-OFF</td>
<td>CML patient who have successfully discontinued IFN-α monotherapy</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMGT</td>
<td>The international ImMunoGeneTics information system</td>
</tr>
<tr>
<td>IP-10</td>
<td>IFN-inducible protein 10 (CXCL10)</td>
</tr>
<tr>
<td>KIR</td>
<td>Killer immunoglobulin-like receptor</td>
</tr>
<tr>
<td>LGL</td>
<td>Large granular lymphocyte</td>
</tr>
<tr>
<td>LGLhigh</td>
<td>A dasatinib-treated patient with high LGL count</td>
</tr>
<tr>
<td>LGLlow</td>
<td>A dasatinib-treated patient with low LGL count</td>
</tr>
<tr>
<td>LGLneg</td>
<td>A dasatinib-treated patient with no LGL lymphocytosis during therapy</td>
</tr>
<tr>
<td>LGLpos</td>
<td>A dasatinib-treated patient with LGL lymphocytosis during therapy</td>
</tr>
<tr>
<td>MCGR</td>
<td>Major cytogenetic response</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein 1 (CCL2)</td>
</tr>
<tr>
<td>MGG</td>
<td>May-Grünewald-Giemsa</td>
</tr>
<tr>
<td>MIG</td>
<td>Monokine induced by interferon gamma (CXCL9)</td>
</tr>
<tr>
<td>MIP-1a</td>
<td>Macrophage inflammatory protein 1a (CCL3)</td>
</tr>
<tr>
<td>MIP-1b</td>
<td>Macrophage inflammatory protein 1b (CCL4)</td>
</tr>
<tr>
<td>MMR</td>
<td>Major Molecular Response</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NK-LGL</td>
<td>LGL with NK phenotype</td>
</tr>
<tr>
<td>NKT</td>
<td>Natural killer T cell</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCgR</td>
<td>Partial cytogenetic response</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed death 1</td>
</tr>
<tr>
<td>PDGFR</td>
<td>Platelet-derived growth factor receptor</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>peg-IFN-α</td>
<td>Pegylated interferon alpha</td>
</tr>
<tr>
<td>PerCP</td>
<td>Peridinin chlorophyll protein</td>
</tr>
<tr>
<td>Ph</td>
<td>Philadelphia (chromosome)</td>
</tr>
<tr>
<td>RQ-PCR</td>
<td>Real time quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>PR1</td>
<td>Leukemia-associated peptide derived from proteinase 3</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>PB MNC</td>
<td>Peripheral blood mononuclear cell</td>
</tr>
<tr>
<td>SCT</td>
<td>Stem cell transplantation</td>
</tr>
<tr>
<td>SRC</td>
<td>Rous sarcoma oncogene cellular homolog</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
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List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

“Mono/oligoclonal T and NK cells are common in chronic myeloid leukemia patients at diagnosis and expand during dasatinib therapy”

“Expansion of highly differentiated CD8+ T-cells or NK-cells in patients treated with dasatinib is associated with cytomegalovirus reactivation”
Leukemia 2011; 25:1587-1597.

III. Kreutzman A, Vakkila J, Koskela H, Porkka K, Mustjoki S.
“Dasatinib promotes Th1-type responses in CD4+ large granular lymphocytes and enhances the cytotoxicity of natural killer cells in vivo”
Submitted.


*The authors contributed equally to this work.

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Tyrosine kinase inhibitors (TKIs), which include imatinib, dasatinib and nilotinib, have dramatically improved the outcome of chronic myeloid leukemia (CML) patients. Besides inhibiting the actual target kinase in leukemic cells, TKIs also influence several off-target kinases, which affect healthy cells. Moreover, dasatinib has a unique kinase-inhibition profile that is the broadest of these TKIs. Dasatinib treatment can thus affect various off-targets including immune effector cells, which in turn can cause unusual immune responses. Indeed, several in vitro studies confirm that the TKIs are immunosuppressive, but the short-term and long-term effects of in vivo TKI treatment on immune system function are mostly unknown. One such in vivo effect of dasatinib therapy is the induction of an oligoclonal expansion of large granular lymphocytes (LGLs) in the peripheral blood. Importantly, this phenomenon correlates with improved therapy responses. The purpose of this PhD project was to study the evolution and function of such clonal LGLs in detail.

First, the prevalence and molecular background of the clonal lymphocytes were examined. The results showed that dasatinib therapy does not induce the clonality as previously thought. Instead the clonal lymphocytes were already present in the blood before the start of treatment in the majority of CML patients. The clones persisted at low levels during imatinib therapy, and only increased when the patients were put on dasatinib therapy, which eventually lead to absolute lymphocytosis in the blood. Since lymphocytosis was previously shown to associate with enhanced therapy responses, the hypothesis was that these clones are initially anergic and dysfunctional anti-leukemic clones, which subsequently recover and expand during dasatinib therapy.

Next, the detailed phenotype and function of the expanded LGLs were studied. The T and NK cells in patients with LGL lymphocytosis expressed a late differentiated phenotype. Accordingly, T cells were prone to apoptosis, and NK cells had an impaired cytotoxic function, which indicated that LGL lymphocytosis is not due to enhanced survival of the lymphocytes. In addition, all patients who had LGL expansion during dasatinib therapy were cytomegalovirus (CMV) seropositive. Consequently, most of the CMV positive patients also had an elevated number of CMV-specific CD8⁺ T cells. Importantly, a proportion of patients with lymphocytosis also suffered from symptomatic CMV reactivation during treatment and therefore it is likely that CMV has a role in the process. However, a causative role for CMV in LGL lymphocytosis has to be proven. Nevertheless, it is possible that the CMV-specific T cells are cross-reactive and target both leukemic and virally infected cell types.

The impact of long-term dasatinib therapy on LGL evolution and function was subsequently studied in more detail. It was observed that long-term dasatinib in vivo increased the proportion of CD8- and NK-LGLs, but surprisingly also differentiated CD4⁺ T cells into potentially cytotoxic LGLs. The unique CD4-LGL population

Abstract
secreted increased amounts of interferon gamma upon stimulation, which indicated a sensitized role of these cells in controlling and eliminating leukemic cells. Furthermore, dasatinib therapy also enhanced the cytotoxicity of the NK cells. These results further support the immunostimulatory role of dasatinib therapy.

As it was shown in the first study that clonal lymphocytes are common at CML diagnosis, the clonality of these cells was studied in a unique group of CML patients treated with interferon-alpha (IFN-α) monotherapy. IFN-α was the drug of choice to treat CML before the era of TKIs, but only a small proportion of the patients responded to the treatment. However, some of these patients were able to discontinue therapy and they have since been without treatment for years and have stayed in remission. This exceptional situation could be caused by an immune mediated mechanism. Therefore, the frequency of different lymphocyte subpopulations in these patients was also investigated. Patients, who were or had been treated with IFN-α monotherapy, had an increased number of CD8+ T cells and a larger proportion of T cells that expressed CD45RO. Furthermore, these patients had unique clonal γ/δ T cells, which were not observed in TKI treated patients. Intriguingly, the patients who were able to stop treatment without relapsing had significantly more NK cells than those patients who were still on treatment or in healthy volunteers. These observations might indicate some of the essential changes in the immune system required for prolonged therapy responses and cure.

Taken together, the results in this PhD project provide proof for a unique, previously unrecognized dual mode of action of dasatinib. In addition to its cytotoxic effects on leukemic cells, dasatinib enhances anti-host and anti-leukemia immune responses that are potentially relevant for the long-term control of CML. This creates the incentive for developing similar rationally multitargeted agents that are applicable not only for leukemia treatment but for treating other cancers as well.
Introduction

Chronic myeloid leukemia (CML) and some cases of acute lymphoblastic leukemia (ALL) are caused by an oncogenic translocation between chromosomes 9 and 22 (9;22). This translocation forms the so-called Philadelphia chromosome (Ph) and the resulting fusion gene encodes for an oncoprotein (BCR-ABL1) with constant tyrosine kinase activity. This in turn leads to an uncontrolled cell proliferation, reduced apoptosis, impaired cell adhesion, and eventually cause a Ph+ leukemia.

Before the era of tyrosine kinase inhibitors (TKIs), interferon-alpha (IFN-α) was the treatment of choice in CML and it prolonged the survival of responding patients. Significantly, a small proportion of IFN-α-treated patients have been able to discontinue treatment without relapse into the disease. However, the majority of CML patients did not receive optimal response to IFN-α, and nowadays TKI therapy has for the most part replaced IFN-α. Imatinib, the current first-line treatment for CML, is well tolerated but does not eliminate all BCR-ABL1-expressing leukemic stem cells. In addition, mutations in the ABL1 kinase domain that result in a resistance to imatinib treatment may evolve. To overcome this resistance, second generation TKIs such as dasatinib and nilotinib were developed. Furthermore, trials investigating the effectiveness of a third generation TKI, ponatinib, are currently ongoing. These new TKIs are relatively non-toxic and are more potent against leukemia cells in vitro than imatinib. Dasatinib is an especially multitargeted kinase inhibitor, which in addition to BCR-ABL1, also inhibits Src family kinases, ephrin receptor kinases, platelet-derived growth factor receptor, and c-Kit. The broader kinase inhibition profile of dasatinib may be useful in overcoming resistant mutated leukemic clones, but unexpected side-effects may also emerge.

One side-effect of dasatinib therapy is a massive clonal expansion of circulating large granular lymphocytes (LGLs) in a proportion of BCR-ABL1-positive leukemia patients. In healthy subjects, the LGLs account for between 10% and 15% of peripheral blood mononuclear cells, whereas the percentage of circulating LGLs is over 50% in dasatinib-treated patients with LGL lymphocytosis. LGLs can be divided into two main lineages: T cell LGLs and NK cell LGLs. Both LGLs are well-known cytotoxic cell types that are able to eliminate targets such as leukemic cells. Several advanced, poor-prognosis leukemia patients who had LGL lymphocytosis during treatment achieved extremely rare, long-lasting complete responses. Therefore, it is reasonable to suggest that the expanded LGLs in dasatinib-treated patients posses an anti-leukemic effect.

In this study, the prevalence of the clonal lymphocytes at diagnosis and during different CML treatments (IFN-α, imatinib and dasatinib) was assessed. Furthermore, a comprehensive immunophenotyping was performed and the function and evolution of the expanded LGLs were investigated to reveal the role of these cells in the enhanced therapy responses.
Review of literature

1. History of CML

John Hughes Bennett (1812-1875) published the first case of chronic myeloid leukemia (CML) in 1845. It was entitled “Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood”. The same year, David Craigie (1793-1866) and Rudolph Virchow (1821-1902) reported two similar cases. Both Bennett and Virchow described their patients to have enlarged spleens and livers, whose blood veins were full of “material resembling thick pus”. All three physicians concluded that the patients died due to the abnormalities in the blood. A few years later in 1847, Virchow suggested the name “leukemia” and described different types of leukemia. He was able to distinguish between “lymphatic” (lymphocytic) and “splenic” (granulocytic) types of leukemia. Further, in 1868 a German pathologist Ernst Christian Neumann (1834-1918) proposed the concept that the bone marrow is responsible for producing blood cells and that some cases of splenic leukemia can arise in the bone marrow. Moreover, Paul Ehrlich (1854-1915) developed novel chemical dyes to identify different blood cells which enabled him to identify several types of leukocytes and distinguish between granulocytes and lymphocytes.1 Ten years later, Ehrlich classified leukemia into myeloid and lymphoid subtypes and confirmed the earlier hypothesis of a common stem cell that give rise to different cell lineages as reviewed by Geary (2000), Piller (2001), and Goldman (2010).2-4

Peter Nowell (1928- ) serendipitously discovered the real cause of CML in 1960 by accident. When studying individual metaphase chromosomes in Giemsa stained leukemia culture slides, he treated his slides with tap water by mistake, which resulted in visualizing the chromosomes.5 Together with David Hungerford (1927-1993), he discovered an abnormally small chromosome, which looked like a Y chromosome, in two male CML patients.6 Later, Nowell and Hungerford confirmed their findings in an additional seven patients, this time also including two female patients. Further, as they noticed that the abnormally small chromosome in CML was not constitutional, they concluded that it might be related to the disease.7 In 1960, at the first International Conference on Chromosomal Nomenclature, the abnormal chromosome got its name, the Philadelphia chromosome (Ph), from the city in which it was discovered. The Ph chromosome was the first disease-specific chromosomal abnormality found in cancer (Fig. 1).

In 1970, the development of chromosome-banding techniques led to the identification of the Ph as being chromosome number 22 with a deletion.8 Three years later, Janet Rowley confirmed the result and added that the Ph chromosome was a reciprocal translocation between chromosomes 9 and 22 t(9;22)(q34;q11).9 The next step was in 1982 when the mapping of the human homolog 1 for the Abelson murine leukemia (ABL) virus, which contains the p160 gag/v-abl oncoprotein, was carried out on chromosome 9.10 Two years later, the chromosome
22 breakpoint was located and named as the “breakpoint cluster region” (BCR). The \textit{ABL1} transcript was defined in CML patients during the same year and subsequently identified as a \textit{BCR-ABL1} fusion transcript that translated into the corresponding p210 fusion protein. Furthermore in 1984, the CML associated \textit{ABL1} was shown to have an enhanced protein tyrosine kinase activity. In 1987 and 1988, the transforming activity of p210 BCR-ABL1 was demonstrated in mouse bone marrow cells and also in other cell lines. The final proof of \textit{BCR-ABL1} being the main causal event for CML came in 1990 when it was shown that retroviral infection of hematopoietic stem cells with p210 BCR-ABL1 induces CML-like disease in mice.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{karyogram.png}
\caption{G-band karyogram results of a CML patient with a standard translocation \textit{t}(9;22)(q34;q11.2) showing the Philadelphia chromosome abnormality 9 and 22 (indicated with arrows). Courtesy of Kirsi Autio.}
\end{figure}

\section{Clinical characteristics of CML}

CML is a malignant blood disorder of the hematopoietic system that is diagnosed by detecting the Ph chromosome. It originates from an aberrant pluripotent hematopoietic stem cell and presents several types of Ph\textsuperscript{+} hematopoietic cells including B cells, erythroid, megakaryocytic, and granulopoietic cells. A diagnosis of CML is in most cases easy, and can be made by characteristic blood differential count showing excess granulocytosis, followed by the detection of the Ph chromosome or \textit{BCR-ABL1} transcripts. Photographs of a peripheral blood sample (left panel) and bone marrow sample (right panel) of a patient with chronic phase CML are presented in Fig. 2.
2.1 Epidemiology
CML is one of the most common chronic myeloproliferative disorders (15% of all leukemias) but still rare, with an annual incidence of 1-3 cases per 100,000 individuals. The disease is more common in elderly persons with a median age of 65 years at diagnosis. No clear ethnic or geographical differences have been observed. However, men are slightly overrepresented. The epidemiology has been reviewed by Hehlmann et al. (2007).25,26

2.2 Clinical course of CML
CML is divided into three stages; the chronic phase (CP), accelerated phase (AP), and blast crisis (BC), based on the clinical characteristics and findings in laboratory analysis. As many as 90% of the newly diagnosed CML patients are in CP, which eventually leads to AP in 80% of the patients if left untreated. The final phase is BC, also called terminal phase, which resembles acute leukemia.26

The WHO criteria for AP and BC have been specified. AP is diagnosed if one of the following criteria is met: 10-19% of blasts in the blood or bone marrow, or over 20% of basophils in the blood, or persistent thrombocytopenia unrelated to therapy or persistent thrombocytosis unresponsive to therapy, or increasing spleen size and increasing white blood cell count unresponsive to therapy, and/or cytogenetic evidence of clonal evolution. BC is diagnosed when over 20% of the peripheral blood white cells or bone marrow cells are blasts, or in case of extramedullary blast proliferation, or/and if large foci or clusters of blasts can be found in bone marrow biopsy.27

2.3 Other Ph+ leukemia
The Philadelphia chromosome is not only found in CML patients. The second most common Ph+ leukemia after CML is acute lymphoblastic leukemia (ALL), which had an extremely poor prognosis before the discovery of tyrosine kinase inhibitors. About 5% of the children diagnosed with ALL (< 20 years) are Ph+, and the incidence increases with age (33% < 40 years, 49% > 40 years, and 35% > 60 years).28
The first recorded attempt to treat CML was in 1882, when a patient whose “leucocytes were enormously increased in number giving a proportion of white blood cells to red cells one to seven” (normal ratio is one to 600) was given iron and quinine. This treatment did not give any beneficial results and lead to a new attempt with arsenic “in large doses, in combination with the iodide and chlorate of potash”. The “new” treatment led to an improvement; the spleen shrank, leukocyte counts dropped and the anemia improved, but the response only lasted for a few months. Other physicians soon confirmed the effectiveness of the therapy, and CML was treated with arsenic until the invention of radiotherapy in 1903.3

Radiotherapy cause a rapid decrease in the size of the spleen and leukocyte counts. It was soon proven to be an effective treatment for CML. Radiotherapy restored the health of patients for weeks, months, or in rare cases for years. However, it soon became apparent that even though radiotherapy led to some remissions, the disease eventually became refractory. Nevertheless, the life expectancy did not significantly increase with radiotherapy, although the period of “efficient life” increased by about 30%.29

The next attempt to treat CML was by the use of nitrogen mustard. During the First World War the use of mustard gas was observed to have an effect on the hematopoietic cells. Exposure to nitrogen mustards led to a profound depression of the leukocyte count. After the war ended, the effect of these compounds was tested in eight CML patients. Despite the clinical improvements noted in the majority of patients, the overall survival remained low. As nitrogen mustards also caused significant toxic effects, the search for a treatment for CML continued and the next drug to be tested was urethane. Urethane has a more or less selective action on hematopoietic tissue and especially on the granulolytic series. Its use has been reviewed by Wintrobe (1947), Marlow (1953), and Scott (1970).30-32

After a large series of trials, busulphan was proven to control the manifestations of CML efficiently and more conveniently than treatment by radiotherapy.33,34 Further, the trials showed that busulphan treatment was relatively safe and slightly improved the survival of CML patients compared to that of radiotherapy. Therefore, busulphan was the treatment of choice for the next 35 years until the less toxic hydroxyurea and interferon-alpha (IFN-α) became available (Fig. 3). In this time, as reviewed by Garcia-Manero (2002), hydroxyurea was an excellent drug, which controlled the blood counts and induced hematological responses in 50-80% of the patients. However, cytogenetic responses were rare and the majority of patients progressed within five years, and hydroxyurea did not, therefore, improve overall survival.26,35
3.2 Interferon-alpha

The intensive studies on interferon (IFN) began after McNeill et al. reported that injection of Poly I-Poly C (a synthetic double-stranded polyribonucleotide) into mice led to the inhibition of hematopoietic colony-forming cells. Poly I-Poly C was previously known to induce IFN, which led to the suggestion that it is IFN that inhibits the forming of colonies. Soon afterwards, several studies showed that IFN reduced the number of colonies that developed from the bone marrow and which caused leukopenia by blocking the differentiation of marrow myeloid precursors. The next in vitro studies showed that IFNs had inhibitory effects on the granulocytic progenitor cells in both hematologically normal cancer patients and in patients with chronic myelogenous leukemia. In addition, a more recent paper has suggested that IFN-α can activate dormant hematopoietic stem cells in vivo thus exposing these to other drugs such as chemotherapeutic agents.

The usage of IFN-α in the treatment of CML began in the 1980s. In contrast to radiotherapy and busulphan, in some cases of CML IFN-α prolonged the survival by inducing Ph negativity. The first trial with IFN-α was on seven CML patients of whom five achieved hematologic remission. IFN-α was the first drug able to re-establish Ph negative hematopoiesis in a minority of cases. It soon became obvious, that patients who achieved such a complete cytogenetic (CCgR) response also survived significantly longer. As summarized by Bonifazi et al., 13% of CML patients treated with IFN-α achieved CCgR (284 of 2227). These studies confirmed that CCgR correlated with survival: after the patients achieved CCgR they were also likely to stay in remission. In addition to the relatively low rate of responding patients, nearly all IFN-α-treated patients experienced side effects during the
treatment. IFN-α treatment has been associated with chronic complications including gastrointestinal disorders (diarrhea, nausea, vomiting), psychiatric symptoms, hair loss, mucositis, autoimmune disorders (thyreoiditis, hemolysis, hepatitis, arthritis). Consequently, the first-generation TKI, imatinib, has gradually replaced IFN-α in treating CML after it entered the clinics in 2000.

3.3 Stem cell transplantation
CML was almost uniformly a fatal disease until the 1970s. Treatment with busulphan seldom resulted in prolonged survivals and the drug had no effect on acute leukemia. This depressing situation began to improve along with the introduction of allogeneic hematopoietic stem cell transplantation (alloHSCT) in the treatment of acute leukemia. The encouraging experiences in treating acute leukemia with alloHSCT with this protocol led to the treatment of also CML. Today, alloHSCT is still performed in advanced cases of CML (AP and BC), in patients who have relapsed several times, and in cases in which the patients developed drug resistant clones such as T315I.

3.4 Tyrosine kinase inhibitors
The development of targeted drugs has emerged rapidly after the discovery of BCR-ABL1 being the main cause of CML. Selective inhibition of BCR-ABL1 prevents the growth of BCR-ABL1 positive cells as shown in 1996. The first trials with a selective inhibition against the BCR-ABL1 tyrosine kinase began the revolution in the treatment of CML. Before the discovery of tyrosine kinase inhibitors (TKI) CML patients died within 5-6 years after diagnosis, whereas today the majority of CML patients can live with a chronic disease (Fig. 4). The improved outcomes have also changed the definitions of the responses (Table 1).

<table>
<thead>
<tr>
<th>Response by type</th>
<th>Definitions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematologic</td>
<td>WBC &lt; 10x10^9/L</td>
</tr>
<tr>
<td>Complete (CHR)</td>
<td>Basophils &lt; 5%</td>
</tr>
<tr>
<td></td>
<td>No myelocytes, promyelocytes, myeloblasts in the differential</td>
</tr>
<tr>
<td></td>
<td>Platelet count &lt; 450 x10^9/L</td>
</tr>
<tr>
<td></td>
<td>Spleen nonpalpable</td>
</tr>
<tr>
<td>Cytogenetic</td>
<td>No Ph^+ metaphases</td>
</tr>
<tr>
<td>Complete (CCgR)</td>
<td>1-35% Ph^+ metaphases</td>
</tr>
<tr>
<td>Partial (PCgR)</td>
<td>36-65% Ph^+ metaphases</td>
</tr>
<tr>
<td>Minor (mCgR)</td>
<td>66-95% Ph^+ metaphases</td>
</tr>
<tr>
<td>Minimal (minCgR)</td>
<td>&gt;95% Ph^+ metaphases</td>
</tr>
<tr>
<td>None (noCgR)</td>
<td>Undetectable BCR-ABL1 mRNA transcripts by real time PCR and/or nested PCR in two consecutive blood samples</td>
</tr>
<tr>
<td>Molecular</td>
<td>Ratio of BCR-ABL1 to a housekeeping gene is ≤ on the international scale</td>
</tr>
<tr>
<td>Complete (CMR)</td>
<td></td>
</tr>
<tr>
<td>Major (MMR)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1. Definitions of hematologic, cytogenetic, and molecular response recommended by the European LeukemiaNet.

Modified from Baccarani et al. 2009.
Figure 4. Relative survival ratios by calendar period of diagnosis in Swedish patients diagnosed with CML between 1973 and 2008. During the time periods 1973-1979 and 1980-1986, the therapy of choice was busulphan. The next five year period (1987-1993), chemotherapy combined with stem cell transplantations increased survival in some patients. In 1990, Swedish physicians began trials on hydroxyurea and IFN-α, which resulted in positive therapy response in a small group of patients. The effect of these therapies can still be seen in the next period (1994-2000). The most substantial increase in survival came after 2001 when imatinib became available. Reprinted with permission. © 2011 American Society of Clinical Oncology. All rights reserved.

3.4.1 Imatinib

After the observation that imatinib therapy results in higher long-term response rates than the previous standard therapies, imatinib quickly took over as the first-line treatment for CML. The first phase II trial with imatinib enrolled 532 IFN-α-resistant or refractory patients. A long-term follow-up of these patients reported that patients treated with imatinib had significantly higher response rates. The following phase III trial, International Randomized Study of Interferon and STI571 (IRIS) study, demonstrated significant advantages of imatinib treatment over that of IFN-α including improved quality of life. This study randomized 1106 newly diagnosed chronic phase CML patients to receive imatinib or IFN-α plus cytarabine (a chemotherapy agent). After 18 months, marked results were achieved: 87% of the patients on imatinib therapy achieved major cytogenetic response (MCgR) compared to only 35% in IFN-α-treated patients. This observation led to the switching of a large proportion of the patients in the IFN-α arm to the imatinib treatment. Despite the superior therapy responses, long-term follow-up showed that a fraction of imatinib-treated patients did not respond well to the therapy or eventually the response declined and progressed to advanced phases of CML. However, this was the first time CML was treated using an effective and safe treatment: approximately 83% of the patients were event-free, and 93% were free of progression to AP or BC after years on imatinib therapy. A retrospective
comparison at the M.D. Anderson Cancer Center confirmed these results. Newly diagnosed CML patients treated with imatinib (years 2000-2004, n=279) achieved significantly more CCgR (87%) compared to CML patients treated with IFN-α monotherapy (28%; years 1982-1997, n=650).61

Even though, imatinib therapy has been a success story, there is still a minority of patients who do not benefit from the drug. Therefore, imatinib resistant or intolerant patients led to the development of second-generation TKIs. In addition to being more potent in inhibiting BCR-ABL1, the second-generation TKIs are more flexible in binding to different BCR-ABL1 conformations and have a broader kinase inhibition spectrum. To date, two second-generation TKIs (dasatinib and nilotinib) have been approved for first-line treatment of CML in Europe.

### 3.4.2 Dasatinib
Dasatinib is approximately 300 times more potent than imatinib against unmutated BCR-ABL1 62 and inhibits platelet-derived growth factor receptors (PDGFRs) and c-KIT more effectively than either imatinib or nilotinib. Furthermore, dasatinib inhibits ephrin receptor tyrosine and the Src family kinases.63 Currently, many countries have approved dasatinib in the treatment of imatinib-resistant CML patients.64-66 In the SRC/ABL Tyrosine Kinase Inhibition Activity Research Trial C (START-C), imatinib resistant or intolerant CML CP patients were treated with dasatinib. After a median time of 15 months on dasatinib therapy, 91% of the patients had CHR, 59% MCgR, and 49% CCgR. Overall survival was 96%.64 Thus, imatinib resistant patients with mutations of the BCR-ABL1 gene will benefit from dasatinib therapy, except for patients with dasatinib resistant mutations: T315I/A, F317L/V/I/C, and V299L.67,68

In the first-line setting of CML CP patients, the rate of both CCgR and MMR after 12 months in dasatinib-treated patients was significantly higher than for those treated with imatinib. In addition, the better responses seen with dasatinib treatment were achieved over a shorter time.69

### 3.4.3 Nilotinib
Nilotinib is a derivative of imatinib and is 30-fold more potent against BCR-ABL1 in vitro than imatinib. Nilotinib is also effective against the most imatinib resistant mutants in both in vitro and murine in vivo models, except for the T315I mutation.70-72 In addition, other nilotinib insensitive mutations (E255K/V, Y253H, F359V/C/I) have been described in CML patients.68 However, phase I and II studies showed beneficial events of nilotinib therapy in all phases of CML 73,74 and that nilotinib is also active in the majority of patients who failed imatinib therapy.75

When a large phase III study randomized CML CP patients to receive either nilotinib or imatinib, results were similar to those shown for dasatinib, in that a significantly higher proportion of nilotinib-treated patients achieved good responses in a shorter time than patients who received imatinib.76
3.4.4 Other second generation tyrosine kinase inhibitors
Another second-generation TKI, which has not yet been approved for treatment of CML, is bosutinib. Bosutinib is highly active against several *BCR-ABL1* mutations, with the exceptions of T315I and V299L.77 One advantage of bosutinib is that it has only a minimal inhibition against c-KIT and PDGFR (platelet-derived growth factor receptors), which are both associated with side effects.78,79

Another new TKI still under investigation is bafetinib (INNO-406). Bafetinib is active against several *BCR-ABL1* mutants, including F317L, but has no effect on T315I. Importantly, bafetinib also has an effect in heavily pretreated patients with *BCR-ABL1* mutations.80,81

3.4.5 Third generation tyrosine kinase inhibitors
Today, the key challenges for treating Ph+ leukemia are the emerging mutations for different TKIs, especially the T315I mutation, which is resistant to all first- and second-generation TKIs. Therefore, new third-generation TKIs are under development. The option for resistant patients is either participation in a clinical trial with new TKIs (if one is available at the appropriate center) or alloHSCT.52,82 Phase I/II trials with new TKI candidate molecules that, also have an effect on T315I, are currently running and include compounds such as XL228, PHA-739358, AP24534, AT9283, and DCC-2036.83

3.4.6 Ponatinib
To date, the third-generation TKI ponatinib (AP24534) will be the next most potent anti-BCR-ABL1 drug to reach the clinics. This inhibitor is active against the T315I mutation, which causes resistance to the first- and second-generation TKIs. In addition, ponatinib showed inhibition of the nilotinib resistant mutations Y253 and F359, and the dasatinib resistant F317 mutant.84 The Ponatinib Ph+ALL and CML Evaluation (PACE) trial began in September 2010. This phase II trial included approximately 400 patients with refractory CML CP, AP, or BC, and Ph+ ALL patients who were either resistant or intolerant to dasatinib or/and nilotinib, or who had the T315I mutation. The preliminary results from the first ongoing clinical trials with ponatinib are encouraging. After only three months, 13/23 CML CP patients with the T315I mutation had achieved MCgR.85

3.5 The comeback of IFN-α
Several trials have combined IFN-α with TKIs in the treatment of CML. Three different approaches have been tested; imatinib after IFN-α failure or intolerance, imatinib combined with IFN-α, or IFN-α therapy after discontinuation of imatinib. As reviewed by Simonsson et al., imatinib was found to be effective in patients who had failed IFN-α treatment, which indicated that the mechanisms that cause resistance to IFN-α and imatinib are different. Further, imatinib was also effective in patients who had achieved good responses with IFN-α.49
Four groups published results from trials in which IFN-α was combined with imatinib; the Italian Gruppo Italiano Malattie EMatologiche dell’Adulto (GIMEMA), the German CML Study group, the French STI571 Prospective Randomized Trial (SPIRIT), and the Nordic CML Study Group. Of these, the Italian, French, and Nordic groups used pegylated IFN-α2b (pegIFN-α; a modified formulation of IFN-α attached to polyethylene glycol), whereas the Germans administrated regular IFN-α. The Italian group published retrospective results, which showed that pegIFN-α combined with imatinib results gave significantly faster responses than imatinib alone. For example, after six months on treatment 13% of patients on combined therapy were in CMR, whereas the corresponding percentage in the imatinib monotherapy patients was 2% (p=0.0002). Similar results have been reported from other groups as well. However, the combination of these two drugs has been also associated with higher probability of side effects.

Several groups have planned trials in which pegIFN-α is combined with either nilotinib (NILOPEG) or dasatinib (NordCML007). Relatively low doses of pegIFN-α (50-90 μg weekly) are recommended when used in combination with TKIs, as higher doses are associated with increased risk of side effects. Moreover, no significant differences in therapy responses has been observed in patients treated with low doses (3 MU/m² 5 times weekly) and high doses (5 MU/m² daily).

Further evidence of the benefits of IFN-α in the treatment of CML, comes from studies in which CML patients were first treated with imatinib and pegIFN-α, and then continued on pegIFN-α monotherapy after imatinib discontinuation. The results indicated that patients who were treated with this combination therapy and who continued on IFN-α maintenance treatment were likely to sustain remission. In addition, some case reports have been published where IFN-α has been successfully used in combination with TKIs or as a monotherapy to treat patients with the highly resistant T315 mutation.

3.6 Discontinuation of CML therapy

Bonifazi et al. summarized overall survival of 317 CML patients (data from nine national study groups) who achieved CCgR with IFN-α monotherapy. The report showed that 24% of these patients had permanently discontinued treatment (n=75). Significantly, 78% of the patients who discontinued therapy for reasons other than loss of response were still alive six years after discontinuation, whereas the median survival of patients who had no response was only 14 months. Another study by Mahon et al. followed 15 CML patients who stopped IFN-α treatment after achieving CCgR. Of these patients, 7 (47%) did not relapse, which shows that a group of IFN-α-treated CML patients are potentially cured by the treatment. They demonstrated that patients who had been in CCgR for at least two years did not relapse after therapy discontinuation. In addition, all these patients belonged to the low Sokal risk group at diagnosis. In contrast, the patients who had been in CCgR for less than two years, relapsed soon after discontinuation of IFN-α therapy.
The prospective, multicenter Stop Imatinib (STIM) trial demonstrated that discontinuation of imatinib therapy can be successful in CML patients who have been in CMR for at least two years. Factors, which could estimate which patients will stay in remission after discontinuation were sex (women have a higher risk of relapse), Sokal score (low score is associated with a higher probability to stay in remission), and a longer period on imatinib treatment (>50 months). Previous treatment with IFN-α did not affect the outcome. Importantly, these results indicate that in contrast to what has been thought earlier, TKIs can cure some CML patients. Encouraged by the results from the STIM trial several additional trials are ongoing or are planned, which investigate the possibility of discontinuing TKI therapy. Among these is a large multicenter trial, EURO-SKI (EUROpe Stop TKI), which includes several European centers and aims to enroll 500 CML CP patients.

4. Immunological background of CML

When transplantations were a popular treatment for CML, the importance of antileukemic cells in achieving favorable responses became evident. Even though the survival rates after sibling transplantations were at first quite disappointing, the hypothesis of antileukemic activity of T cells became stronger, which lead to the suggestion that the key to a cure may be in utilizing immunological approaches. The idea originates from experiences in which T cells in the bone marrow transplantations were depleted to avoid graft-versus-host disease. The depletion of T cells successfully reduced the mortality from graft-versus-host disease, but also increased risk of relapse by 60%. These early experiences implicated T cells in the donor marrow as being a critical part in the success of alloHSCT in CML patients. Data that confirmed the hypothesis emerged later from experiments in which transplanted patients were given donor lymphocyte infusions. The infusions induced complete remission in the absence of chemo- or radiotherapy in patients who had relapsed after allogeneic transplantation.

5. Immunomodulatory effects of tyrosine kinase inhibitors

In addition to the main target BCR-ABL1, TKIs also inhibit other kinases and therefore also affect normal cells involved in immune responses. Indeed, several studies have shown that imatinib, dasatinib and nilotinib have inhibitory effects in vitro on T cell proliferation and activation. In addition, dasatinib and imatinib possess a suppressive function upon the cytotoxicity of NK cells in vitro. However, the effect of TKIs on NK cell function seems controversial, as there are
published reports that imatinib and nilotinib do not impair NK cytotoxicity *in vitro* (Table 2a). 107

Importantly, dasatinib affects a broader array of kinases than do imatinib or nilotinib (Table 2b). 63,111-113 Moreover, in addition to major kinases involved in immune responses, dasatinib seems to inhibit the secretion of immunomodulatory cytokines. 112

**Table 2a. Known immunomodulatory effects of imatinib, nilotinib, and dasatinib.**

<table>
<thead>
<tr>
<th>Effector T cells</th>
<th>Imatinib</th>
<th>Nilotinib</th>
<th>Dasatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proliferation</td>
<td>Inhibition / no effect</td>
<td>Inhibition</td>
<td>Inhibition / no effect</td>
</tr>
<tr>
<td>Activation</td>
<td>Inhibition / no effect</td>
<td>Inhibition</td>
<td>Inhibition / no effect</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>Inhibition / no effect</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Tregs</td>
<td>Inhibition / no effect</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Cytokine production</td>
<td>No effect</td>
<td>Inhibition</td>
<td>Inhibition</td>
</tr>
<tr>
<td>Cytotoxicity</td>
<td>No effect</td>
<td>No effect</td>
<td>Inhibition</td>
</tr>
</tbody>
</table>

*Modified from Krusch and Salih, 2011.* 114

**Table 2b. Targets of imatinib, nilotinib, and dasatinib.**

<table>
<thead>
<tr>
<th>Imatinib</th>
<th>Nilotinib</th>
<th>Dasatinib</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABL</td>
<td>ABL</td>
<td>ABL</td>
</tr>
<tr>
<td>ARG</td>
<td>ARG</td>
<td>ARG</td>
</tr>
<tr>
<td>BCR-ABL1</td>
<td>BCR-ABL1</td>
<td>BCR-ABL1</td>
</tr>
<tr>
<td>c-KIT</td>
<td>c-KIT</td>
<td>c-KIT</td>
</tr>
<tr>
<td>PDGFR</td>
<td>PDGFR</td>
<td>PDGFR</td>
</tr>
<tr>
<td>DDR1</td>
<td>DDR1</td>
<td>DDR1</td>
</tr>
<tr>
<td>NQO2</td>
<td>NQO2</td>
<td>NQO2</td>
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</table>

*Modified from Hantschel et al., 2008.* 63

Several groups have described an *in vivo* side effect of dasatinib. A proportion of dasatinib-treated CML and Ph*+* ALL patients develop an expansion and mobilization of the large granular lymphocytes (LGLs) during therapy. 115-118 Another typical side effect found in dasatinib-treated patients is pleural effusion, which is also associated with lymphocytosis. 119

Further, Rohon et al. performed a detailed *ex vivo* immunophenotyping on bone marrow and peripheral blood from CML patients at diagnosis and during dasatinib or imatinib therapy. The analyses revealed that at diagnosis, the patients had decreased amounts of B cells and dendritic cells, and increased numbers of NKT-like cells in their bone marrow. Imatinib therapy effectively normalized these changes back to resemble those of healthy controls. In contrast, dasatinib patients formed two distinct groups, of which one group was similar to healthy controls and imatinib-treated patients. In contrast, the other group presented an active immunoprofile with significantly increased levels of cytotoxic CD8*+* T cells, NK cells, and NKT-like cells in the blood. The T cells in this group of patients had a late memory cytotoxic phenotype. These results demonstrate that the *in vivo*
situation is the opposite of the previously described immunosuppressive effects of TKIs observed in vitro, especially in a distinct group of dasatinib-treated patients.

6. Large granular lymphocytes

LGLs normally account for approximately 10-15% of circulating mononuclear cells and are characterized by the presence of granules in cytoplasm (Fig. 5). The LGLs can be divided into two subgroups; T (CD3+) and NK-LGLs (CD3negCD16+CD56+) both of which have strong cytotoxic capability. LGLs often express CD57+, a marker used to detect in vivo-activated cytotoxic T cells. LGL-leukemia is an abnormal, long-lasting clonal expansion of LGLs which has an unknown pathogenesis. However, oligoclonal expansions of LGLs can occur in association with several diseases and they can have favorable effects.

![Figure 5. An MGG-stained large granular lymphocyte. Courtesy of Kirsi Latvala.](image)

6.1 Bone marrow transplantation or CMV induced lymphocytosis

Expansion of LGLs of T cell subtype (CD3−CD8+CD56neg) or LGL-leukemia of donor origin has been observed after either autologous or allogeneic bone marrow transplantation. Patients who received either type of transplant often responded well and went to remission, which suggests a graft-versus-leukemia effect. Thus, some of the reported LGL-leukemia cases can instead be LGL lymphocytosis. Furthermore, clonal expansion of LGLs has also been reported to be associated with CMV infection.

6.2 Dasatinib induced lymphocytosis

Clonal expansion of T-LGLs or NK-LGLs occurs in a proportion of dasatinib-treated Ph+ leukemia patients. A characteristic of lymphocytosis is an absolute blood lymphocyte count that is higher than 3.6 x 10^9/L, which typically occurs 3-4 months after initiation of dasatinib treatment. One study reported that
28% (186 of 662) of dasatinib-treated patients developed lymphocytosis during treatment,\textsuperscript{119} which was confirmed by a smaller study.\textsuperscript{117} Importantly, dasatinib induced LGL lymphocytosis has been associated with improved therapy responses in several studies, especially in patients with advanced phases of CML or acute leukemia.\textsuperscript{115-117,119}

As reviewed by Parhman (2005), NK cell and party also CD8\textsuperscript{+} T cell function is regulated by various immunogenetic factors. Examples of such factors are human leukocyte antigen (HLA) and killer immunoglobulin-like receptor (KIR) genes. HLA molecules serve as ligands for KIR and depending on the genotype, the KIRs can act as either stimulatory or inhibitory suppressing or activating the immune response.\textsuperscript{138} Therefore, it is possible that such immunogenetic factors could also play a role in the improved therapy responses seen in dasatinib-treated Ph\textsuperscript{+} leukemia patients who develop T-LGL or NK-LGL lymphocytosis during treatment.

7. Immunosurveillance

F.M. Burnet originally suggested the idea of immunosurveillance in 1970, as he postulated that the immune system is capable of recognizing and destroying transformed cells. However, the hypothesis was rejected for years until new data emerged that supported the concept became available.\textsuperscript{139-141} Today, immunosurveillance is considered as a part of a more general process termed cancer immunoediting and which has been reviewed by Dunn (2004).\textsuperscript{142} Immunoediting is a process by which the immune system protects us from cancer growth and has three phases: elimination, equilibrium, and escape. The elimination process has been reviewed by Dunn (2004) and consists of four events and starts when the cells of the innate immune system recognize the growing tumor and initiate the antitumor immune response. The innate compartment consists of NK cells, NKT cells, γδ T cells, dendritic cells, macrophages, and granulocytes, all of which serve as first-line defense against pathogens such as cancer cells. The growing tumor causes tissue damage, which further induce inflammatory signals. These signals recruit innate cells to the tumor site and tumor-infiltrating lymphocytes begin to produce IFN-γ. IFN-γ in turn, induces apoptosis in tumor cells and stimulates the production of cytokines such as IP-10 (IFN-inducible protein 10; CXCL10) and MIG (monokine induced by interferon gamma; CXCL9). These chemokines attract additional immune cells to the tumor site. For example, IP-10 is known to recruit effector T cells to the leukemic microcompartment.\textsuperscript{143,144} Further, IP-10 can also activate cytotoxic lymphocytes to kill dormant tumor cells.\textsuperscript{145} The eliminated tumor cells are then ingested by dendritic cells, which migrate to the lymph nodes where they present the processed tumor antigens. The presence of antigens in the lymph nodes starts the final event of the elimination process. This step involves naïve T cells that recognize the tumor-antigens and begin to proliferate. The tumor-specific CD4\textsuperscript{+} and
CD8$^+$ T cells will find their way to the tumor with the help of the chemokines, and will finally kill the remaining tumor cells expressing the specific tumor antigen.$^{146}$

Sometimes the elimination process fails and the next phase of immunoediting, the equilibrium phase, is initiated. In the equilibrium phase, immune cells together with cytokines (such as IFN-$\gamma$) will work to eliminate the tumor cells. However, some malignant cells with additional transformations are resistant to the elimination process and thereby receive a growth advantage; these will eventually escape the immune system.$^{146}$ One mechanism by which tumor cells can escape is mediated by causing defects in MHC class I antigen presentation. Tumors can also turn the immune system against itself by attracting for example regulatory T cells (Tregs) and thus create an immunosuppressive environment within the tumor and provide additional protection against the immune system. The details of these mechanisms have been reviewed by Dunn (2002), Dunn (2004) and Poschke (2011).$^{142,146,147}$

Although immunotherapy has been under intensive research, the results have remained too weak or transient to eliminate the complete tumor. Recently, a new promising agent for cancer immunotherapy has been discovered. CTLA4 belongs to a large family of molecules that are involved in the activation or inhibition of T cell immune responses. CTLA4 is expressed on both CD8$^+$ and CD4$^+$ T cells, including Tregs. Antibodies against CTLA4 block inhibitory signals that are normally generated through this receptor, therefore prolonging and sustaining T cell activation and proliferation. One of the new agents blocking CTLA4 is ipilimumab. Ipilimumab sustains an active immune response against tumors by blocking the activity of CTLA4. Ipilimumab has been reported to have durable responses in approximately 10% of patients with metastatic melanoma and trials testing its usefulness in other cancers are ongoing. The role of CTLA4 in immunotherapy has been reviewed by Peggs et al. (2006) and Lesterhuis et al. (2011).$^{148,149}$
Aims of the study

The overall aim of this PhD project was to uncover the cellular and molecular pathogenesis of dasatinib-related LGL expansion, and to examine the immunological effects of IFN-\(\alpha\) therapy. The project includes four parts:

1) The molecular background of LGL lymphocytosis: characterization of clonal lymphocytes

2) The \textit{ex vivo} function of LGL lymphocytes and detailed analysis of the phenotype

3) The anti-leukemia effects of dasatinib-induced LGL expansion

4) The immune activation induced by IFN-\(\alpha\)
Patients and methods

8. Patients

Studies I-IV included a total of 89 Ph+ leukemia patients, of which the majority (n=82) were CML patients. Most of the samples were collected from Finnish patients and healthy volunteers at Helsinki University Central Hospital, Helsinki, Finland. Additional samples were gathered from other centers in Finland, the Czech Republic, Slovakia, Sweden, Norway, Germany, and Portugal.

Study I, in which the clonal properties of lymphocytes in CML patients were characterized included 34 patients, of which 20 were treated with dasatinib at the time of sampling and the remaining 14 with imatinib. A DNA sample was obtained from 18 patients at the time of diagnosis. All patients were diagnosed with CML in CP, with the exception of three of the dasatinib-treated patients of whom two were in BC and one in AP at diagnosis.

Study II investigated the detailed phenotypes and the ex vivo function of the expanded T and NK cells. The study involved 25 dasatinib-treated patients, of whom 18 patients were diagnosed with CML CP, two with CML BC, and four with Ph+ ALL. In addition, one dasatinib-treated patient with ALL (1;9) was included in the study.

In study III, the effects of long-term dasatinib therapy in LGL evolution and function were examined. A total of 28 CML CP patients were included in this study. Of these patients, 12 patients were treated with dasatinib, of which five were second-line dasatinib patients and had been previously treated with imatinib. The rest of the patients were treated as first-line with imatinib (n=8), or nilotinib (n=8).

In study IV, the immunomodulatory effects of IFN-α therapy were investigated. It included 19 CML CP patients treated with IFN-α monotherapy. None of the patients had previous TKI treatment. These patients were further divided into two groups namely: IFN-ON consisted of patients who were still on IFN-α monotherapy (median time for treatment 142 months) and the IFN-OFF, which consisted of patients who had successfully discontinued IFN-α monotherapy (median time without treatment 53 months, minimum 24 months).

In addition to patients, all studies included healthy volunteers (study I n=12, study II n=10, study III n=5, and study IV n=43).

8.1 Grouping of dasatinib-treated patients

In studies I, II and III dasatinib-treated patients were divided into two groups based on their peak absolute lymphocyte counts during treatment. The first group consisted of patients with absolute lymphocytosis with LGL morphology during treatment (LGLpos) and the second group contained patients with normal lymphocyte counts during dasatinib therapy (LGLneg).
In study I, if the patients had absolute lymphocyte counts between 4-20x10⁹/L at any time, they were included in the LGLpos group. In study II, the LGLpos group consisted of patients whose lymphocyte counts exceeded 3.6x10⁹/L at any point during dasatinib therapy. In study III, the dasatinib-treated patients were grouped on the basis of their absolute LGL count. Patients in the LGLhigh group had counts over 1.4x10⁶ LGLs per ml, which was the mean value for the whole group, whereas the patients in the LGLlow group had absolute LGL counts below 0.8x10⁶/ml.

9. Methods

9.1 Routine laboratory tests
Blood cell counts, differential analysis of leukocytes, and CMV serology (i.e. the presence of antibodies against CMV in the blood) were obtained from routine laboratory tests conducted at HUSLAB (Helsinki University Central Hospital, Finland). Molecular genetic analyses of BCR-ABL1 transcripts were performed using real-time quantitative PCR in several laboratories, which have been quality-controlled. In addition, peripheral blood smears stained with May-Grünwald-Giemsa (MGG) were used to calculate the presence of LGLs in study III.

9.2 Dasatinib
In study II, dasatinib was synthesized and dissolved in dimethyl sulfoxide (DMSO) and purity-tested as described. In study III, dasatinib was purchased from Euroasian Chemicals Pvt Ltd (Mumbai, India). DMSO was used in parallel with dasatinib as a solvent control in all experiments conducted in study II. In all experiments, dasatinib was used at clinically relevant doses of 50 nM and 10 nM, which mimics the peak and steady concentrations in the plasma. In study IV, dasatinib was used at 50 nM.

9.3 Sample preparation

9.3.1 Separation of plasma and mononuclear cells
Plasma was first separated from fresh whole blood by centrifugation (300 xg, 10 minutes) and stored as aliquots at -70°C. Peripheral blood mononuclear cells (PB MNCs) were then separated by Ficoll gradient centrifugation (800 xg, 25 minutes; GE Healthcare, Buckinghamshire, UK). PB MNCs were stored as cell pellets in -70°C or as live cells in liquid nitrogen (studies I, II, IV). Alternatively, samples were studied directly ex vivo (studies III-IV).
9.3.2 Selection of lymphocyte subpopulations
In study I, CD8⁺ T cells were selected from PB MNCs by using CD8 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. The actual separation was performed with an AutoMACS cell sorter (Miltenyi BioTec).

In study III, NK cells were purified by negative selection, which sequentially depletes T cells, B cells, monocytes, platelets, dendritic cells, granulocytes, and erythrocytes leaving NK cells untouched. The selection was done using Invitrogen’s Dynabeads® Untouched Human NK cell-kit according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA, USA). The purity of the selected lymphocyte subpopulations in both studies was confirmed by flow cytometry.

9.3.3 Fluorescence-activated cell sorting
In studies I, II and IV, frozen or fresh PB MNCs were stained with cell surface markers for different T cell subsets (CD4⁺ and CD8⁺ T cells, or αβ⁺ and γδ⁺ T cells), NK cells, or B cells (Fig. 6). Each lymphocyte population was gated and sorted with FACSARia or FACSARia II flow cytometers (both BS Biosciences, San Diego, CA, USA). The purities of sorted lymphocyte subpopulations were confirmed to be >95% with flow cytometric analysis.

Figure 6. Strategy of fluorescence-activated cell sorting of different lymphocyte populations in studies I, II and IV. PB MNCs were stained with antibodies raised against CD45, CD3, CD16, CD56, CD8, TCR αβ and TCR γδ. Gates were first set for live cells and then on CD45 positive lymphocytes (A). CD3⁺ T cells were gated to separate from CD45 cells and further divided into TCR αβ⁺ and TCR γδ⁺ T cells (B-C). Alternatively, CD45⁺ lymphocytes were divided into NK and T cells by CD16/CD56 versus CD3 scatter (D). CD45⁺CD3⁰CD16/56⁺ cells were considered most likely to be B cells and CD3⁰CD16/56⁺ as NK cells (D). CD3⁺ T cells were further divided into CD3⁺CD8⁺ T cells and CD3⁺CD4⁺ (gated as CD3⁺CD8⁺) T cells (E).
9.4 Molecular analysis

9.4.1 Fluorescence in situ hybridization
In study I, fluorescence in situ hybridization (FISH) analyses were performed on FACS-sorted T (CD4+ and CD8+ T cells), NK, and B cells. Interphase cells that were prepared on cytopsin slides were analyzed with a locus-specific dual-color, dual-fusion BCR-ABL1 probe mixture (Vysis, le Bretonneux, France). Hybridizations were performed according to the manufacturer’s instructions.

9.4.2 DNA extraction
Genomic DNA samples for projects I, II and IV were isolated from fresh, sorted (CD8+, CD4+, αβ+ and γδ+ T cells, NK cells, or B cells), or frozen PB MNCs using a Blood DNA isolation Kit (MO BIO, Carlsbad, CA, USA) or by using Genomic DNA from Tissue: NucleoSpin Tissue-kit (Machery-Nagel, Düren, Germany) according to the manufacturers´ instructions. The quality and concentration of DNA were measured using NanoDrop (Thermo Scientific, Waltham, MA, USA) and DNA samples were stored at -20°C.

9.4.3 Polymerase chain reaction
To evaluate the presence of clonal lymphocytes in studies I and IV, T cell receptor (TCR) γ- and δ-gene rearrangements were analyzed by polymerase chain reaction (PCR). The primers consisted of a set of 12 different primer pairs for γ-gene and six pairs for δ-gene (Fig. 7 and Table 3) of which all were purchased from Sigma-Aldrich (St Louis, MO, USA). These primer combinations detect most of the known TCR γ- and δ-gene rearrangements. The PCR was done according to the BIOMED-1 protocol.152
Figure 7. Location of primers in TCR δ-gene (A) and TCR γ-gene (B). Dd2 and Dd3 TCR δ-genes are not shown. The patient-specific junctional regions are located between the genes (modified from Pongers-Willemse, 1999). 

Table 3. The primer combinations used to detect clonal lymphocytes. 

<table>
<thead>
<tr>
<th>TCR gene</th>
<th>Sequence for forward primer</th>
<th>TCR gene</th>
<th>Sequence for reverse primer</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vδ1</td>
<td>ACCGAGCGCCAGTCCGAGTCCG</td>
<td>Jδ1</td>
<td>AACCTGCTCCAGGAGTCCTCC</td>
<td>452</td>
</tr>
<tr>
<td>Vδ2</td>
<td>ACCCAAAAGCAGCTGCAGAGAG</td>
<td>Jδ2</td>
<td>GAAATGAGCAGCTTTGCCCTTGAG</td>
<td>501</td>
</tr>
<tr>
<td>Vδ3</td>
<td>GACCGAGCGCTGGGAGATGGC</td>
<td>Jδ3</td>
<td>ACCAGGAGTACGTTCAGTAGATGATATT</td>
<td>608</td>
</tr>
<tr>
<td>Dδ2</td>
<td>ACTCCAGGTCATTTAGATAGATATT</td>
<td>Dδ2</td>
<td>GAGATACGTATGCGAGGACTTATGGAG</td>
<td>550</td>
</tr>
<tr>
<td>Vγ1</td>
<td>CAGGCCGACTGCTGATCTGC</td>
<td>Jγ1</td>
<td>TTACCAACTAAGTTACTATGAGC</td>
<td>329</td>
</tr>
<tr>
<td>Vγ1</td>
<td>Jγ1.2</td>
<td>AAGAAACTAAGCTTGATATAAGC</td>
<td>337</td>
<td></td>
</tr>
<tr>
<td>Vγ1</td>
<td>Jγ1.3/2.3</td>
<td>CGTATATGCGGCAAAGGCAATGC</td>
<td>533</td>
<td></td>
</tr>
<tr>
<td>Vγ2</td>
<td>CAGGCCGACTGCTGATCTGC</td>
<td>Jγ2</td>
<td>TTACCAACTAAGTTACTATGAGC</td>
<td>318</td>
</tr>
<tr>
<td>Vγ2</td>
<td>Jγ2</td>
<td>AAGAAACTAAGCTTGATATAAGC</td>
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<td></td>
</tr>
<tr>
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<td>Jγ3</td>
<td>AAGAAACTAAGCTTGATATAAGC</td>
<td>522</td>
<td></td>
</tr>
<tr>
<td>Vγ3</td>
<td>Jγ3.1/2.1</td>
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<td>318</td>
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<tr>
<td>Vγ3</td>
<td>Jγ3.2</td>
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<td></td>
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<tr>
<td>Vγ3</td>
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<td>522</td>
<td></td>
</tr>
<tr>
<td>Vγ4</td>
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<td>Jγ4</td>
<td>TTACCAACTAAGTTACTATGAGC</td>
<td>354</td>
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<tr>
<td>Vγ4</td>
<td>Jγ4</td>
<td>AAGAAACTAAGCTTGATATAAGC</td>
<td>362</td>
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<tr>
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<td>Jγ4.3/2.3</td>
<td>AAGAAACTAAGCTTGATATAAGC</td>
<td>558</td>
<td></td>
</tr>
</tbody>
</table>

After PCR amplification, the clonality was confirmed by heteroduplex analysis. The amplified DNA was first denatured at 95°C and then renatured by a rapid, random cool down to 4°C for 1 hour. PCR products were separated on a commercial polyacrylamide gel (BioRad, Hercules, CA, USA). After separation, the gels were stained with ethidium bromide for 1 hour, then the clonal PCR products were visualized with ultraviolet illumination. Positive findings of clones were then cut from the gel and sequenced. If necessary, the products were re-amplified with the same pair of primers as was used in the first amplification to gain enough DNA for sequencing.
9.4.4 Sequencing
The pieces of gel containing the clonal PCR products were added directly to the sequencing master mix and sequenced with BigDye Version 1.1 Cycle Sequencing kit (Applied Biosystems, Carlsbad, CA, USA). Sequencing was performed in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) and sequences were analyzed with IMGT database and BLAST search.

9.4.5 Design of allele-specific oligonucleotide primers
For quantitative analysis of the clones, patient-specific allele-specific oligonucleotide (ASO) primers were designed using Primer Express software (Applied Biosystems, version 2.0). If the patient had clonal products in both TCR genes, primer pairs were designed to target both clonal TCR γ- and δ-gene rearrangements. The ASO-primers were designed to target the hypervariable junction regions of the clonal TCR γ- or δ-genes (Fig. 7). In TaqMan RQ-PCR analysis, the forward ASO primers were used in conjunction with a consensus reverse primer. In addition, according to whether a D or a J gene was present in the rearrangement, the appropriate TaqMan probe was used in the reaction.

9.4.6 Quantification of clones
In addition to the samples of interest, each real time quantitative (RQ) PCR reaction included standard curves, a reference sample, and albumin (ALB) as the reference gene. Each reaction was amplified in triplicate in the presence of the specifically designed ASO primer with a consensus primer and a TaqMan probe using a TaqMan Universal PCR Mastermix (Applied Biosystems).

A standard curve for each analysis was prepared by diluting each patient’s DNA sample in polyclonal DNA obtained from healthy donors. As a reference sample, DNA of satisfactory quality and concentration with the sequence-confirmed presence of the clonal rearrangement was used. The proportion of the specifically rearranged DNA relative to albumin DNA as calculated by RQ-PCR was defined to be 1.0 in the reference samples. The dilution series prepared from the reference sample for the standard curve was made by serial 10-fold dilutions. The quantity of DNA with the clonal rearrangement of interest relative to the quantity of albumin DNA could therefore be calculated in all follow-up samples by using the reference sample as a standard. The measurement scales were entirely specific to each clone and to each patient as the purpose was to detect relative variations in serial measurements.

The sensitivity of each allele-specific RQ-PCR analysis obtained from the reference sample dilution series was estimated. Sensitivity was defined to be the lowest dilution still giving specific amplification. When determined this way, the sensitivity estimate depends on 3 factors: (1) quality of DNA in the reference sample, (2) the efficiency of each allele-specific RQ-PCR reaction, and (3) the unknown proportion of clonal cells that represents any specific clone in its respective reference sample. With standard DNA extraction and standard rules for
primer design the factors (1) and (2) remain similar for different allele-specific PCR targets. Therefore, a high sensitivity indicates a high proportion of clonally rearranged lymphocytes in the reference sample, whereas a low sensitivity implies a low proportion of cells with the specific clonal rearrangement.

### 9.4.7 TCR clonotyping

In study II, the clonotypic analysis of CD8$^+$ T cells was done by sorting antigen-specific CD8$^+$ T cells into microtubes that contained RNAlater (Applied Biosystems), then an unbiased amplification of all expressed TRB gene products was done. The amplification was performed by using a template-switch anchored reverse transcription-PCR with a 3′-TRB constant region primer. Amplicons were subcloned, sequenced, and analyzed as described previously using the IMGT database.

### 9.4.8 HLA-A2 analysis

In study IV, 10 patients were analyzed for their HLA-A2 expression using OLERUP SSP™ HLA-kit for A locus (Olerup SSP AB, Saltsjobaden, Sweden) according to the manufacturer’s instructions.

### 9.5 Plasma cytokine and chemokine detection

In studies II and IV, plasma cytokine profiles of patients on different treatments (study I included dasatinib-treated Ph$^+$ leukemia patients and study IV included CML patients treated with IFN-α monotherapy) were examined. For this purpose, a Human Cytokine 25-plex (Invitrogen) was used to measure soluble factors in frozen plasma samples. Plasma samples were thawed the day of analysis and clarified by centrifugation (1000 xg, 10 min). The samples were further diluted in Assay Diluent provided by the manufacturer.

The kit analyzes for the following cytokines: interleukin-1b (IL-1b), IL- 1RA, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, TNF-α (tumor necrosis factor alpha), IFN-α, GM-CSF (granulocyte macrophage colony stimulating factor), MIP-1α (macrophage inflammatory protein 1a/CCL3), MIP-1β (macrophage inflammatory protein 1b/CCL4), IP-10 (IFN-inducible protein 10 /CXCL10), MIG (monokine induced by interferon gamma/CXCL9), Eotaxin (CCL11), Rantes (CCL5), MCP-1 (monocyte chemoattractant protein 1/CCL2), and IFN-γ (interferon gamma). The measurements and analyses were performed using the Bio-Plex™ 200 system (BioRad).

### 9.6 Immunophenotyping

In studies II, III and IV, the immunophenotypes of different patient groups were examined by flow cytometric analysis.
Immunophenotyping in study II was done by a polychromatic flow cytometric analysis that detected 18 parameters simultaneously. The panel consisted of the following antibodies: CD14 and CD19 (both Pacific Blue; Caltag, Invitrogen) were used to exclude monocytes and B cells from the analysis, CD3 (APC-H7; Caltag, Invitrogen), CD4 (Cy5.5PE; Caltag, Invitrogen), CD8 (Cy7-PE; BD Pharmingen, Franklin Lakes, NJ, USA and QDot 705; Molecular Probes, Invitrogen by Life Technologies, Paisley, UK), CD57 (FITC; BD Pharmingen), CD27 (Cy5-PE; Beckman Coulter, Brea, CA, USA), CD45RO (ECD; Beckman Coulter), CD69 (APC; Beckman Coulter), CD127 (PE; Beckman Coulter), PD-1 (biotinylated with a secondary stain with streptavidin-Pacific Blue; R&D Systems, Abingdon, UK), CD16 (PE; BD) and CD56 (APC; Miltenyi Biotec).

In study II, fluorochrome-labeled tetrameric peptide/HLA-A*0201 (pHLA-A*0201) complexes were produced and used as previously described. ExtrAvidin-R-PE (Sigma-Aldrich), streptavidin-APC (Prozyme, Hayward, CA, USA) or streptavidin-QDot 705 (Molecular Probes) were used for the tetramerization. In addition, a Live/Dead fixable dye (Molecular Probes) was used to exclude dead cells from the analysis. Cells were stained according to the manufacturer’s recommendations. A sample of 1-5x10⁵ cells was acquired and recorded using FACSArray II (BD Biosciences, San Diego, CA, USA) and analyzed by using FlowJo software (version 9.3.2, Tree Star, Inc., Ashland, OR, USA).

In study III, immunophenotyping of Granzyme B (GrB) positive lymphocytes was done by first staining the surface markers with antibodies against CD45 (APC-H7), CD3 (APC), CD4 (PerCP), CD8 (PE-Cy7), TCR-γδ (PE) or CD45 (APC-H7), CD56 (PE), CD16 (PE-Texas Red; Invitrogen). Second, the cells were fixed and permeabilized with a Cytofix/Cytoperm™-kit according to the manufacturer’s instructions (BD Bioscience), following which intracellular GrB was stained (Alexa Flour 700). All antibodies were purchased from BD Biosciences unless otherwise mentioned. 5x10⁵ cells were acquired and recorded with FACSArray (BD Biosciences) and analysis was done with FlowJo software.

In study IV, the immunophenotyping was done with a 6-color cytometry panel including antibodies against the following antigens: CD3 (PerCP, APC, or PE-Cy7), CD4 (APC), CD8 (APC-H7), CD16 and CD56 (PE), CD45 (PerCP), CD57 (FITC), CD45RA (FITC), CD45RO (PE), TCR-αβ (FITC), TCR-γδ (PE), CCR2 (Alexa 647), CCR3 (Alexa 647), and Vγ9 (FITC) with isotype controls. Regulatory T cells (Tregs) were analyzed with the following antibody combination; CD3 (PerCP), CD4 (FITC), CD25 (PE), and FOXP3 (APC; clone PCH101, eBiosciences, San Diego, CA, USA). All antibodies were acquired from BD Biosciences unless otherwise stated. Cells were stained according to the manufacturer’s recommendations and 1-5x10⁵ cells were analyzed using FACSArray (BD Biosciences). Data analyses were performed with FACS Diva software (version 6.0, BD Biosciences). In addition, the expression of PR1 in T cells was studied in 10 HLA-A*0201 positive patients. Approximately 5x10⁶ frozen PB MNCs were stained with the following antibodies: CD3, CD4, CD8, and PR1 iTAgTM MHC
class I human Tetramer (Beckman Coulter). \(5 \times 10^5\) CD8\(^+\) T cells were acquired and recorded with FACS\(\text{Aria II}\) and the analysis was done with FACSDiva software.

### 9.7 Cell culture

In study II, all functional assays were done in RPMI 1640-medium supplemented with 10% fetal calf serum (FCS), 2 mM of L-glutamine, and 100 u/ml of penicillin and 100 \(\mu\)g/ml of streptomycin. The cell culture media used in study III was RPMI-1640 supplemented with 10% FCS, 1% penicillin, 1% streptomycin, and 1% L-glutamin (all Lonza, Basel, Switzerland). All incubations were done at +37°C with 5% CO\(_2\) in an incubator (Thermo Scientific).

### 9.8 In vitro assays

In study II, cryopreserved or fresh PB MNCs were used for \textit{in vitro} functional analysis of lymphocytes. PB MNCs were taken from the liquid nitrogen and thawed the previous day and then rested over night in RPMI. In contrast, only freshly isolated PB MNCs were used in the \textit{in vitro} assays in study III. The samples were taken before, and 1 or 2 hours after the patient had taken his daily dose of dasatinib, imatinib or nilotinib (Fig. 8).

![Figure 8. Dasatinib, imatinib, and nilotinib concentration in plasma. Plasma was collected from CML patients before and at 1h, 2h, 4h after TKI (imatinib, nilotinib, or dasatinib) intake. TKI plasma concentrations were measured by liquid chromatography-mass spectrometry. Blood samples for functional analysis were collected before the respective TKI intakes commenced (0h) and after TKI intake commenced depending on the plasma peak for the different TKIs; dasatinib 1h, imatinib and nilotinib 2h after drug intake (marked with circles).](image)

#### 9.8.1 T cell stimulation

In studies II and III, the same protocol for T cell activation was used. The previous day, wells on a 48-well plate (Nunclon) were plated with an OKT3-antibody (5 \(\mu\)g/ml) overnight at +4°C. The PB MNCs were counted and resuspended to correct their concentrations in RPMI the following day. The precoated OKT3 plates were
washed once with PBS and 1x10^6 PB MNCs (in 500μl) was added to the plates. In addition, costimulatory anti-CD28 and anti-CD49d antibodies (both 1μg/ml) were added to the wells with OKT3. GolgiStop was added to all wells. Each experiment always included a negative control (without stimulation) and stimulated wells with or without dasatinib. After 6 hours, the cells were harvested and surface markers for the different T cells subpopulations (CD4 and CD8) were stained as described above. After cell surface staining, the cells were fixed and permeabilized with a Cytofix/Cytoperm™-kit according to the manufacturer’s instructions (BD Bioscience). Intracellular TNF-α and IFN-γ were stained, and both were conjugated to FITC. In addition in study III, a GrB antibody was added. All antibodies and reagents were purchased from BD Biosciences unless otherwise mentioned. Samples containing 5x10^5 CD45 positive lymphocytes were acquired and recorded in project II using FACSCalibur (BD Pharmingen) and analyzed by running with CellQuest Pro software (version 5.2.1, BD Biosciences). In study III, the events were acquired and recorded with FACSARia and the analysis was done using FlowJo software.

9.8.2 Apoptosis assay
In study II, 1x10^6 PB MNCs were stimulated with OKT3-antibody as described above. The cells were incubated for four days; thereafter the cells were harvested and stained with Annexin V (PE; BD Pharmingen) and 7-amino-actinomycin D (7AAD; Sigma-Aldrich). Cells were analyzed using FACSCalibur, and apoptotic cells were defined as 7AAD-/Annexin V^+, and late apoptotic/necrotic cells as 7AAD^+/Annexin V^+.

9.8.3 Proliferation assay
In study II, 1x10^6 PB MNCs were suspended in PBS and labeled with vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) at a final concentration of 0.25 μM as described previously. After the stained cells had rested overnight, the cells were stimulated with OKT3 as described above for four days in the presence or absence of dasatinib.

9.8.4 CMV specific CD8^+ T cell assay
In study II, the function of CMV-specific CD8^+ T cells were studied by a CMV pp65_{495-503} peptide NLVPMVATV, which is restricted to HLA-A*0201. Therefore, the following assays were performed only on CMV seropositive patients and healthy controls who had the HLA-A*0201 allele. Proliferation assays were done by stimulating cells with a CMV pp65_{495-503} peptide (2μM) and co-stimulatory CD28 and CD49d antibodies in the presence or absence of dasatinib. After six days, the cells were stained with the surface marker for CMV-specific cells, CMV pp65_{495-503}/HLA-A*0201-APC tetramer and a CD8-specific antibody (PerCP; BD Bioscience).
Degranulation and cytokine production of CMV-specific CD8+ T cells, in the presence or absence of dasatinib, were studied with five hours of assay. The PB MNCs were pretreated with dasatinib for one hour and thereafter stimulated with a CMV pp65_{495-503} peptide in the presence of antibodies to detect degranulation (CD107a and CD107b, both FITC; BD Pharmingen), brefeldin and GolgiStop. After five hours had elapsed, the cells were stained with the appropriate surface marker for CMV-specific cells, CMV pp65_{495-503}/HLA-A*0201-APC tetramer and a CD8 specific antibody. After surface staining, the cells were fixed and permeabilized, and stained for intracellular TNF-α and IFN-γ (both PE; BD Pharmingen) as described above. 5x10^5 lymphocytes were acquired and recorded with FACSCalibur and analyzed with CellQuest Pro software.

9.8.5 Killing assays
In study II, the effect of dasatinib on NK cell-mediated killing was measured by a biophotonic assay. The target cell line K562 was transfected at 300 V with a firefly luciferase (fLuc):zeocin (zeo) mammalian expression vector consisting of an engineered fusion between the fLuc and zeo resistance genes under the control of the CMV IE promoter (Invitrogen) clones inserted into the pcDNA3.1 (+) plasmid backbone. The transfected K562 fLuc+ cells were cultivated in RPMI containing zeoocin. After the PB MNCs recovered, the percentages of NK cells (CD3-, CD16+ and/or CD56+) were determined by flow cytometry. The assay was then conducted in triplicate with effector:target ratios of 0.6:1, 2.5:1, and 10:1 in the presence or absence of dasatinib for four hours. Target cell lysis was detected and monitored as a decrease in detected luminescence, measured as relative light units (RLU) with an Orion II Microplate luminometer (Berthold Detection Systems, Pforzheim, Germany). To measure NK cell anergy, PB MNCs were pretreated with 2000 IU/ml interleukin-2 (IL-2; Proleukin, Chiron, Emeryville, CA, USA) for 18 hours in appointed reactions.

In study III, a flow cytometry based cytotoxicity assay was used. The previous day, the target cell line K562 was stained with Violet Proliferation stain (Invitrogen) according to the manufacturer’s instructions and rested overnight. The following day, PB MNCs were isolated and counted. Three different kinds of cytotoxicity assays were performed. First, the whole PB MNC population was calculated as the effectors (effector:target ratio 0:1, 5:1, 10:1, and 20:1), then unpurified NK cells were calculated as effectors (0:1, 5:1, 10:1), and finally purified NK cells were used as effector cells (0:1, 5:1, 10:1). Both targets and effectors were resuspended in appropriate concentrations in RPMI and co-plated in different ratios onto round bottom 96-well plates and incubated for six hours. Afterwards, the cells were harvested and stained with LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen), according to the manufacturer’s instructions. CountBright-beads (Invitrogen) were added to each tube, and 5000 beads were acquired and recorded with FACSARia. The gate was set at violet K562, and the percentage of live K562 cells was calculated by using the FlowJo software (Fig. 9).
Figure 9. Flow cytometric analysis of NK cell cytotoxicity against K562 in study III. K562 cell line was stained the previous day with a violet stain. The next day, PB MNCs and the stained K562 cells were co-cultured for six hours. (A) K562 cell line only; (B) K562 cell line was co-cultured with a dasatinib-treated patient’s PB MNCs at effector:target ratio 20:1. K562 cells were separated from PB MNCs by gating violet positive cells (middle panel). From the violet K562 cells the percentage of live cells (Calcein AM positive) was measured (right panel).

9.8.6 NK cell stimulation
In study II, PB MNCs were pretreated with 10 nM or 50 nM dasatinib for one hour, and thereafter stimulated with K562 cells in the presence of CD107a and CD107b antibodies, brefeldin and GolgiStop for six hours. The cells where then harvested and stained for CD3 (PerCP; Biolegend), and CD16 and CD56 (both APC; Miltenyi). After surface staining the cells were fixed, permeabilized, and stained for intracellular TNF-α and IFN-γ as described above. 5x10^5 lymphocytes were acquired and recorded with FACSCalibur and analyzed by CellQuest Pro software.

In parallel with the killing assay in study III, degranulation assays were also performed. Freshly isolated PB MNCs (pre and post drug intake) were co-incubated with the K562 cell line (ratio 10:1) for six hours in the presence of CD107a and CD107b antibodies. The cells were then harvested and stained for surface markers against CD45 (APC-H7), CD3 (APC), CD56 (PE), and CD16 (PE-Texas Red; Invitrogen). All antibodies were purchased from BD Biosciences, unless otherwise mentioned. 5x10^5 CD45 positive lymphocytes were acquired and recorded with FACSARia and the analysis was done with FlowJo software.
9.8.7 NK cell expansion
In study II, polyclonal NK cells were generated as described previously. Briefly, after plastic adherence from healthy donors non-adherent PB lymphocytes were cocultured with irradiated EBV-transformed B cell line RPMI 8866 at a ratio of 4:1 for 8-11 days in RPMI. Experiments were conducted when the purity of the expanded NK cells exceeded 75%, as confirmed by flow cytometry by defining NK cells as CD3−, CD16+ and/or CD56+.

10. Statistical calculations
All statistical analysis in studies II and III were performed with GraphPad Prism software (version 5.0c, GraphPad, La Jolla, CA, USA). Statistical significance was determined by using the non-parametric Kruskal-Wallis test, the paired two-tailed Student’s t-test or using a one-way analysis of variance (ANOVA). In study III, the comparisons of pre and post drug intake, and follow-up samples were evaluated by the nonparametric-paired Wilcoxon t test. Multiple groups were compared with one-way ANOVA test. All statistical analysis was done with the GraphPad Prism software (version 5.0c). The statistics of immunophenotyping results in study IV were done using SPSS (version 16.0). The statistical analyses of the other experiments used GraphPad Prism software by using the nonparametric Mann Whitney test and t test. In all studies the statistically significant results were defined as p< 0.05.

11. Ethical permissions
Approvals for patient sample collection and laboratory analyses for all four studies mentioned above were obtained from Helsinki University Central Hospital Ethics committee (Dnro HUS 364/E5/07). In addition, approval from the University Hospital Olomouc ethics committee for study IV was obtained. Signed informed consents were obtained from all patients and healthy volunteer controls.
Results

12. Assessment of clonal lymphocytes in CML patients (I, IV)

It has been previously shown, that treatment of Ph + positive leukemia patients with the second-generation TKI dasatinib causes a clonal expansion of cytotoxic T and NK cells.\textsuperscript{115-117,136} The expansion occurs in a distinct group of patients and eventually leads to an absolute lymphocytosis in the blood followed by an excellent therapy response.\textsuperscript{115,137} However, the evolution and prevalence of the clonal lymphocytes was unknown and required further investigation.

12.1 Clonal BCR-ABL1 negative lymphocytes are present at diagnosis (I)

A total of 18 DNA samples were collected from diagnostic phase CML patients later treated with either imatinib or dasatinib to study if dasatinib therapy induces the clonality of lymphocytes. The analysis was performed by detecting clonal rearrangements in TCR $\delta$- and $\gamma$-genes by PCR.

At diagnosis, 15/18 of CML CP patients (83%) had a clonal, BCR-ABL1 negative lymphocyte population, which was uncommon in healthy individuals of the control group (1/12; 8%). Most patients displayed either monoclonal or oligoclonal cytotoxic lymphocytes at diagnosis. The sensitivity of the assay was 1-5% of clonal cells, which indicates that a significant proportion of diagnostic phase lymphocytes is clonal. Of note, if the proportion of the clone is at the time of sampling less than 1%, it could not be detected with conventional PCR. However, if patient-specific ASO-primers were used, the sensitivity increased significantly (up to 0.001%) when using quantitative RQ-PCR.

To determine whether the clonal lymphocytes belonged to the malignant Ph + leukemic clone, FISH analysis with a BCR-ABL1 fusion gene probe was performed on two diagnostic phase samples. From these two CML patients, lymphocytes were first sorted into CD4$^+$, CD8$^+$, NK, and B cell fractions. The fractions were then studied separately by FISH analysis. In the first patient, 1-2% of CD4$^+$, CD8$^+$, and NK cells were positive for the Ph$^+$ chromosome, whereas up to 9% of B cells were positive. In the second patient, all analyzed lymphocyte subpopulations were negative. These observations were in accordance with previously published results by Takahashi et al. who showed that T and NK cells in untreated CML patients are often negative for the Ph chromosome.\textsuperscript{155}

12.2 Clones detected at diagnosis persist during TKI therapy (I)

Of the 18 studied diagnostic phase samples, 15 patients had at least one clonal lymphocyte population at diagnosis. A follow-up sample was taken typically one year after the start of the therapy (either imatinib or dasatinib) from all 18 patients.
Clonal rearrangements were examined in a similar fashion as the diagnostic phase samples.

After the patients began TKI therapy, the clone found in the follow-up sample was identical to the diagnostic phase clone in nine of 15 patients (60%). The clones disappeared (could no longer be detected by PCR) in two patients treated with imatinib and in one patient treated with dasatinib. In three patients a clone was detected also one year after the start of the therapy, but the clone was not identical to the one detected at diagnosis.

DNA samples were available from five CML patients who had first been treated with imatinib and then switched to dasatinib due to intolerance or resistance. The clones observed during dasatinib therapy were also detected by quantitative PCR at low levels in the samples when the patients were treated with imatinib. It was noted that during imatinib therapy the proportion of the clones remained low and the absolute lymphocyte counts stayed below the normal upper limit. Furthermore, a diagnostic phase sample was available from two of these patients, and by using the patient specific ASO-primers the clones could already be detected at diagnosis. Figure 10 presents one CML patient from whom samples were available from time-points prior to the start of any therapy (at diagnosis) and later when she was on imatinib and later still on dasatinib therapy. The relative proportion of one clone was high and the other clone low at diagnosis. Further, the proportion of both clones was low at diagnosis and during imatinib therapy. However, approximately two months after the start of dasatinib therapy the proportion of the clones increased 4-fold, simultaneously with the increased absolute lymphocyte count.

Figure 10. Evolution of two lymphocyte clones in a dasatinib-treated CML patient before and during TKI therapy. Two ASO-primers, which recognize the clonally rearranged TCR \( \gamma \) and \( \delta \)-gene, were designed for this patient. A diagnostic phase sample was taken (day -739), another sample was taken during imatinib therapy (day -187), and more samples were taken after day 0 from the period during which the patient was using dasatinib. Further analysis revealed that the clones resided in \( \alpha \delta^+CD4^+ \) T cells and \( \gamma\delta^+CD4^+ \) T cells.
12.3 Clonal lymphocytes during different treatments (I, IV)
As clonal lymphocytes are common in CML patients at diagnosis, the prevalence of clonal lymphocytes was assessed in CML patients treated with three different therapies: imatinib, dasatinib, or IFN-α monotherapy. The analysis was performed as described above, by detecting clonal rearrangements in the TCR δ- and γ-genes by PCR.

The majority of the clonal rearrangements detected with the primers for the TCR δ-genes resided in the Vδ2 gene in all patient groups. Compared to the time of diagnosis, the dasatinib-treated group (n=20) had approximately the same number of clones detected by Vδ2-primers (Table 4). In contrast, the clone disappeared or decreased in proportion below the detection limit (1%) of the assay in patients who had a clonal rearrangement of the Vδ2 gene at diagnosis and who were started on imatinib (n=3). Further, no clonal TCR-δ rearrangements were observed in any of the studied imatinib-treated patients (n=14) (Table 4). However, patients who were or had been treated with IFN-α monotherapy (n=19) had an increased prevalence of lymphocytes with complete clonally rearranged Vδ2-genes (primer pair Vδ2-Jδ1); 53% compared to 6% at diagnosis (Table 4).

Table 4. Percentages of positive clonal rearrangements detected in TCR δ-genes.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Vδ1-Jδ1</th>
<th>Vδ2-Jδ1</th>
<th>Vδ2-Jδ3</th>
<th>Vδ3-Jδ1</th>
<th>Dδ2-Jδ1</th>
<th>Dδ2-Jδ3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>22</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>18</td>
<td>-</td>
<td>17</td>
<td>6</td>
<td>-</td>
<td>6</td>
<td>-</td>
</tr>
<tr>
<td>IFN-α</td>
<td>19</td>
<td>11</td>
<td>37</td>
<td>53</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Dasatinib</td>
<td>20</td>
<td>5</td>
<td>35</td>
<td>10</td>
<td>5</td>
<td>5</td>
<td>-</td>
</tr>
<tr>
<td>Imatinib</td>
<td>14</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Clonally rearranged TCR γ-genes in patients treated with imatinib and dasatinib did not show any patterns, and the findings were similar to the clones detected in the diagnostic samples. In contrast, patients who were or had been on IFN-α monotherapy had an increased prevalence of clones detected with the primer pair Vγ2-Jγ1.2 (74% compared to 28% at diagnosis) (Table 5). However, no homology was observed between the clonally rearranged TCR δ- or γ-genes of the patients.

Table 5. Percentages of positive clonal rearrangements detected in TCR γ-genes.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Vγ2-Jγ1.1/2.1</th>
<th>Vγ1-Jγ1.1</th>
<th>Vγ2-Jγ1.1/2.1</th>
<th>Vγ2-Jγ1.1/2.3</th>
<th>Vγ3-Jγ1.1/2.3</th>
<th>Vγ4-Jγ1.1/2.3</th>
<th>Vγ4-Jγ1.2/2.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy controls</td>
<td>22</td>
<td>-</td>
<td>5</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>18</td>
<td>11</td>
<td>17</td>
<td>39</td>
<td>28</td>
<td>11</td>
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<td>6</td>
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<td>11</td>
<td>17</td>
<td>26</td>
<td>74</td>
<td>16</td>
<td>-</td>
<td>21</td>
</tr>
<tr>
<td>Dasatinib</td>
<td>20</td>
<td>20</td>
<td>15</td>
<td>20</td>
<td>15</td>
<td>15</td>
<td>5</td>
<td>25</td>
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<tr>
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<td>14</td>
<td>14</td>
<td>7</td>
<td>29</td>
<td>7</td>
<td>-</td>
<td>7</td>
</tr>
</tbody>
</table>

No clones were detected with primer pairs Vγ1-Jγ1.3/2.3 and Vγ3-Jγ1.2 (not shown).
Comparison of IFN-ON (patients who were on IFN-α monotherapy) and IFN-OFF (patients who had successfully discontinued IFN-α monotherapy) patient subgroups did not reveal any significant differences in the pattern of clonal lymphocytes.

In contrast, upon dividing dasatinib-treated CML patients into two groups, the LGL\textsuperscript{pos} patients and patients whose lymphocyte counts stayed below the normal upper limit (LGL\textsuperscript{neg}), differences were observed. First of all, clonal lymphocytes were detected in all LGL\textsuperscript{pos} patients (10/10). Notably clones were also detected in all patients with NK cell expansion during the dasatinib therapy (n=6). The frequency of clonal TCR γ- and δ-gene rearrangements in LGL\textsuperscript{neg} patients on dasatinib therapy (70%) was similar to the percentage of clones found in imatinib-treated patients (64%). Furthermore, most patients who had LGL expansions (90%) also had TCR δ-rearrangements, which were uncommon in those patients who had no LGL expansion (10%) (Table 6). No homology was observed between clonally rearranged TCR-sequences of IFN-α patients in any of the studied groups.

<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>LGL\textsuperscript{pos}</td>
<td>10</td>
<td>10</td>
<td>60</td>
<td>20</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>LGL\textsuperscript{neg}</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**12.4 Correlation between absolute lymphocyte count and the relative proportion of lymphocyte clone (I)**

During dasatinib therapy, a proportion of patients developed a lymphocyte expansion in their blood. However, the lymphocyte counts fluctuated markedly during treatment. Therefore, the aim was to quantify the amount of the lymphocyte clones at different time-points by using patient-specific ASO-primers to see whether the proportion of the clones also correlated with the absolute lymphocyte counts. Primers were designed to four LGL\textsuperscript{pos} and two LGL\textsuperscript{neg} patients, and RQ-PCR was performed on several follow-up samples. The median follow-up was 21 months (range 8-41 months).

The estimation of the proportion of clones was done by using one sample as a reference with the value of 1. The results of follow-up samples were then compared to the reference value. This method allowed a quantitative comparison of the clones for different samples. The analysis revealed that, in most cases, LGL\textsuperscript{pos} patients had from one to two predominant clones, which were responsible for the clonal expansion, reflected as lymphocytosis in the peripheral blood (an example of one patient is given in Fig. 11). In addition, several minor clones were often detected. In contrast, the proportion of clones in LGL\textsuperscript{neg} patients did not vary significantly between the different follow-up samples (Fig. 12).
Figure 11. Evolution of two lymphocyte clones in a dasatinib-treated CML patient during imatinib and dasatinib therapies. Two ASO-primers, which recognize the clonally rearranged TCR γ- and δ-genes, were designed for this patient. Samples taken on days -103 and 0 present the imatinib therapy period, and the samples after day 0 were taken during dasatinib therapy. Further analysis revealed that the clones resided in NK cells and αβ+CD8+ T cells.

Figure 12. Quantitative follow-up of a T cell clone in a dasatinib-treated CML patient who did not develop absolute lymphocytosis during treatment. An ASO-primer, which recognizes the clonally rearranged TCR γ-gene, was designed for this patient. Four samples were taken at different dates during the dasatinib therapy and were used to estimate the proportion of the lymphocyte clone.

12.5 Clones in different lymphocyte subpopulations (I, IV)
At the following stage the lymphocyte subpopulation, in which the clonal lymphocytes reside, was investigated. Lymphocytes were first sorted into T cell subsets (CD4+ and CD8+ T cells, or αβ+ and γδ+ T cells), NK, or B cells. DNA was then extracted from the pure subpopulations and analyzed by RQ-PCR with the
patient specific ASO-primers, or by conventional PCR using the same primers that were used in the detection of the clones.

In the dasatinib-treated CML patient group, sorting and further analysis was performed for the eight LGL<sup>pos</sup> and two LGL<sup>neg</sup> patients. The results showed that the clonal lymphocytes in dasatinib-treated patients resided in all studied T cell subsets; CD4<sup>+</sup> and CD8<sup>+</sup> T cells, in both αβ<sup>+</sup> and γδ<sup>+</sup> T cells. Notably, NK cells could also be detected by clonal, incomplete TCR δ-gene rearrangements (primer pair Vδ2-Dδ3, Table 3). More precisely, the TCR δ clones were confined to γδ<sup>+</sup> T or NK cell compartments and the TCR γ clones to CD4<sup>+</sup>/CD8<sup>+</sup> αβ<sup>+</sup> fractions. No clones were detected in the B cells (CD45<sup>+</sup>CD3<sup>neg</sup>CD16/56<sup>neg</sup>), which confirmed the specificity of the PCR assay.

The same experiment was performed on eight CML patients who were treated with IFN-α monotherapy. Earlier analysis showed that the majority of IFN-α-treated patients had clonal lymphocytes detected with the same Vγ2-Jγ1.2 primers, which are known to detect clonal Vγ9Vδ2 T cells, further suggesting that the clones are in the Vγ9 subset of γδ<sup>+</sup> T cells. This was confirmed by sorting and further analyzing the subpopulation with PCR. Furthermore, the proportion of Vγ9<sup>+</sup> cells of the total γδ<sup>+</sup> T cell population was measured. The majority of γδ<sup>+</sup> T cells were Vγ9<sup>+</sup> in healthy controls (78%) and IFN-OFF patients (92%), whereas IFN-ON patients had a decreased proportion of these cells (57%). The control group that consisted of patients with myeloproliferative neoplasm (MPN) treated with IFN-α also had a decreased proportion of these cells (62%) (Fig. 13; p=0.03). As the two main γδ<sup>+</sup> T cell subsets are Vγ9Vδ2 and Vδ1<sup>+</sup>, it is possible that IFN-α therapy change the Vδ1<sup>+</sup> to be the dominant γδ<sup>+</sup> T cell population. This hypothesis is supported by the fact that a clonal Vδ1<sup>+</sup> cell population was detected in 11% of IFN-α-treated patients (Table 4), meaning that their percentage of the whole lymphocyte population is at least 1%.

![Figure 13. Proportion of Vγ9 cells from the total γδ<sup>+</sup> T cell population in healthy controls and CML patients who were on IFN-α monotherapy (IFN-ON) or had been able to discontinue the therapy (IFN-OFF). An additional control group consisted of three patients with myeloproliferative neoplasms treated with IFN-α.](image-url)
13. Immunophenotyping in CML patients on different treatments (II, IV)

13.1 Phenotype of lymphocytes in dasatinib-treated patients (II)

To study the detailed phenotype of the expanded T and NK cells in dasatinib-treated patients a detailed immunophenotyping analysis was performed. A majority of the patients included in study II experienced LGL lymphocytosis during treatment (LGLpos, n=16). The LGLpos patients were further divided into three subgroups based on the phenotype of the expanded cells; T cell (n=7), NK cell lymphocytosis (n=3), and patients who had both lymphocyte subtypes expanded (n=6). Patients with normal lymphocyte counts during treatment (LGLneg, n=3) were used as a control group.

Patients with T cell expansion had a higher frequency of CD8+ T cells, and hence a lower frequency of CD4+ T cells in comparison to patients with NK cell expansion or LGLneg patients. In addition, the majority of the T cells in these patients, as well as patients with both T and NK cell expansion, were CD45RO/CD27neg. Many of these cells co-expressed CD57, which was higher in CD8+ than in CD4+ T cells. The frequency of PD-1+CD57negCD8+ T cells was highest in LGLneg patients, and consequently the frequency of PD-1+CD57+CD8+ T cells was higher in the LGLpos patients (Table 7).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>T LGL n=5</th>
<th>NK LGL n=5</th>
<th>T + NK LGL n=1</th>
<th>No LGL n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8+ (% of CD3+ T cells)</td>
<td>76.0 (31.6-80.3)</td>
<td>36.8 (33.8-42.6)</td>
<td>35.3</td>
<td>30.9 (27.3-48.9)</td>
</tr>
<tr>
<td>CD4+ (% of CD3+ T cells)</td>
<td>17.1 (14.0-57.8)</td>
<td>42.2 (29.8-61.1)</td>
<td>37.4</td>
<td>54.2 (35.1-67.7)</td>
</tr>
<tr>
<td>CD57+ (% of CD8+ T cells)</td>
<td>78.2 (51.9-83.8)</td>
<td>47.3 (26.5-70.3)</td>
<td>76.1</td>
<td>12.2 (8.0-16.6)</td>
</tr>
<tr>
<td>PD-1+CD57+ (% of CD8+ T cells)</td>
<td>3.4 (0.9-7.6)</td>
<td>9.4 (1.4-15.6)</td>
<td>14.9</td>
<td>18.9 (5.2-22.1)</td>
</tr>
<tr>
<td>PD-1+CD57+ (% of CD3+ T cells)</td>
<td>5.2 (1.0-19.3)</td>
<td>7.1 (0.5-17.2)</td>
<td>23.9</td>
<td>3.7 (0.7-4.0)</td>
</tr>
<tr>
<td>CD45RO/CD27+ (% of CD8+ T cells)</td>
<td>80.5 (63.8-90.5)</td>
<td>54.8 (27.2-80.6)</td>
<td>90.5</td>
<td>17.6 (15.6-29.0)</td>
</tr>
<tr>
<td>CD57+ (% of CD8+CD45RO/CD27)</td>
<td>86.2 (55.2-87.6)</td>
<td>78.9 (73.9-81.2)</td>
<td>79.4</td>
<td>30.0 (20.4-34.2)</td>
</tr>
<tr>
<td>CD57+ (% of CD4+ T cells)</td>
<td>39.4 (21.6-80.7)</td>
<td>4.5 (1.3-72.6)</td>
<td>67.6</td>
<td>2.3 (1.0-5.6)</td>
</tr>
<tr>
<td>CD45RO/CD27+ (% of CD4+ T cells)</td>
<td>79.1 (35.1-87.6)</td>
<td>14.6 (4.2-90.8)</td>
<td>90.1</td>
<td>16.5 (5.8-26.7)</td>
</tr>
<tr>
<td>CD57+ (% of CD4+CD45RO/CD27)</td>
<td>65.6 (44.9-88.0)</td>
<td>23.5 (6.6-78.8)</td>
<td>73.8</td>
<td>11.1 (3.1-14.2)</td>
</tr>
</tbody>
</table>

Medians with ranges in parentheses are shown.

In addition, a total of three LGLpos patients had CD8+ T cell populations, which were specific for the PR1 leukemia-associated peptide derived from proteinase 3 (1.1, 0.6, and 0.05% of the total CD8+ population). These three patients had an advanced disease (ALL or CML BC) and had relapsed after allogeneic bone marrow transplantation. Two of these patients have subsequently been in remission for over two years with dasatinib monotherapy, which suggests an unexpectedly good response to treatment. In the other studied patients, no PR1-specific T cells were observed.

The median value of CD57 expressing NK cells was the lowest in patients with T cell expansions (31.6% vs >77.3% in patients with expanded NK cells or LGLneg
patients). Further, the majority of all NK cells (>70%) were CD45RO<sup>neg</sup>CD27<sup>neg</sup> in all studied patients. The majority of NK cells expressed a CD45RO<sup>neg</sup>CD27<sup>neg</sup>CD57<sup>+</sup> phenotype, especially in patients with expanded NK cells (NK cells only or both T and NK cell expansion) (summarized in Table 8).

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>T LGL</th>
<th>NK LGL</th>
<th>T + NK LGL</th>
<th>No LGL</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD16&lt;sup&gt;+&lt;/sup&gt; (% of live CD45&lt;sup&gt;+&lt;/sup&gt; cells)</td>
<td>27.7 (3.5-84.2)</td>
<td>91.8 (64.3-97)</td>
<td>63.4 (49.4-72.7)</td>
<td>9.6 (7.0-12.2)</td>
</tr>
<tr>
<td>CD56&lt;sup&gt;+&lt;/sup&gt; (% of live CD45&lt;sup&gt;+&lt;/sup&gt; cells)</td>
<td>28.7 (6.2-65.0)</td>
<td>28.5 (19.8-93.7)</td>
<td>37.1 (29.7-59.6)</td>
<td>12.5 (8.3-16.7)</td>
</tr>
<tr>
<td>CD16&lt;sup&gt;+&lt;/sup&gt; (% of CD56&lt;sup&gt;+&lt;/sup&gt; NK cells)</td>
<td>59.7 (36.2-95.1)</td>
<td>96.7 (77.1-98.2)</td>
<td>73.8 (65.5-88.0)</td>
<td>43.9 (27.9-59.8)</td>
</tr>
<tr>
<td>CD56&lt;sup&gt;+&lt;/sup&gt; (% of CD16&lt;sup&gt;+&lt;/sup&gt; NK cells)</td>
<td>68.9 (25.8-75.5)</td>
<td>30.3 (19.5-77.8)</td>
<td>42.9 (29.0-70.7)</td>
<td>55.9 (32.9-78.8)</td>
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<td>CD57&lt;sup&gt;+&lt;/sup&gt; (% of CD56&lt;sup&gt;+&lt;/sup&gt;CD16&lt;sup&gt;+&lt;/sup&gt; NK cells)</td>
<td>55.2 (31.4-91.8)</td>
<td>81.9 (72.2-89.7)</td>
<td>77.2 (30.9-85.2)</td>
<td>86.4 (83.0-89.7)</td>
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<tr>
<td>CD57&lt;sup&gt;+&lt;/sup&gt; (% of CD56&lt;sup&gt;+&lt;/sup&gt;CD45RO&lt;sup&gt;-&lt;/sup&gt;CD27&lt;sup&gt;-&lt;/sup&gt;)</td>
<td>40.6 (26.2-90.9)</td>
<td>81.6 (75.4-88.8)</td>
<td>73.0 (76.1-81.3)</td>
<td>75.9 (70.1-81.6)</td>
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<tr>
<td>CD57&lt;sup&gt;+&lt;/sup&gt; (% of CD56&lt;sup&gt;+&lt;/sup&gt;CD16&lt;sup&gt;+&lt;/sup&gt; NK cells)</td>
<td>55.2 (31.4-91.8)</td>
<td>81.9 (72.2-89.7)</td>
<td>77.2 (30.9-85.2)</td>
<td>86.4 (83.0-89.7)</td>
</tr>
<tr>
<td>CD45RO&lt;sup&gt;-&lt;/sup&gt;CD27&lt;sup&gt;-&lt;/sup&gt; (% of CD56&lt;sup&gt;+&lt;/sup&gt;CD16&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>30.7 (11.0-40.6)</td>
<td>45.6 (35.6-46.5)</td>
<td>19.6 (17.1-33.9)</td>
<td>18.2 (13.1-23.2)</td>
</tr>
<tr>
<td>CD45RO&lt;sup&gt;-&lt;/sup&gt;CD27&lt;sup&gt;-&lt;/sup&gt; (% of CD56&lt;sup&gt;+&lt;/sup&gt;CD16&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>66.2 (50.3-80.9)</td>
<td>54.2 (53.4-64.3)</td>
<td>79.5 (64.6-81.5)</td>
<td>80.6 (74.6-88.5)</td>
</tr>
</tbody>
</table>

**Table 8. Phenotypic features (median proportions) of NK cells in patients with T, NK or T + NK LGL expansion**

Medians with ranges in parentheses are shown.

13.2 Immunophenotype of IFN-α-treated CML patients (IV)

A comprehensive flow cytometric analysis was performed on 19 patients treated with IFN-α monotherapy to study the immunomodulatory effects of IFN-α therapy in CML patients. Nine of the patients had been able to stop treatment without disease relapse (IFN-OFF), whereas the remaining 10 patients were still on therapy (IFN-ON).

The median proportions of CD3<sup>+</sup> T cells of the total lymphocyte population were decreased in both patient groups (IFN-ON 67%, IFN-OFF 56% vs. 73% in healthy volunteers; p=0.0002). In addition, the absolute T cell counts were decreased in the patients (IFN-ON 0.89x10<sup>9</sup>/L, IFN-OFF 0.82x10<sup>9</sup>/L vs. healthy volunteers 1.3x10<sup>9</sup>/L, respectively; p=0.005). Distribution of CD4 and CD8 cells within the T cell population was skewed in the patient groups. The CD4/CD8 ratio was lower in IFN-ON (1.3) and IFN-OFF (0.8) patients, compared to healthy volunteers (2.2; p=0.04) (Fig. 14). Moreover, the absolute CD4<sup>+</sup> T cell numbers were decreased in IFN-OFF (0.33x10<sup>9</sup>/L) when compared to normal reference values (0.46-1.41x10<sup>9</sup>/L).

Further, the memory and activation status of the T cells were studied. Both patient groups had significantly higher proportions of antigen encountered memory CD45RO<sup>+</sup> T cells (IFN-ON 58% and 74% in IFN-OFF vs. 44% in healthy subjects; p=0.006). In contrast, no differences in CD57 expression were observed, which is another activation marker for T cells. However, IFN-OFF patients had a higher proportion of proliferating CD3<sup>+</sup> T cells (9.8%) as detected by Ki-67 antigen expression compared to the IFN-ON (2.6%) and the healthy volunteer (1.9%) groups.

The median proportion of NK cells of all lymphocytes in IFN-OFF patients was significantly increased compared to IFN-ON patients and healthy volunteers...
(26% vs. 12% and 11%, respectively; p=0.0005) (Fig. 14). No differences in the proportion or absolute B cell counts were observed.

![Figure 14. The proportions of lymphocyte subpopulations in healthy controls and CML patients treated with IFN-α monotherapy. The figure shows the median value sectors for each lymphocyte subgroup.](image)

In addition, follow-up samples were analyzed from seven patients (four IFN-ON and three IFN-OFF). The samples were taken 15 to 31 months after the first batch of analyzed samples. The noted differences (proportion of NK cells, CD8+ T cells, and CD45RO+ cells) in the immunoprofiles did not markedly differ between these samples. The other control group used in this study, namely MPN patients treated with IFN-α, had similar numbers of NK and CD8+ T cells as healthy subjects.

An analysis of PR1 expressing T cells was performed only in HLA-A2 positive patients as the antibody, PR1 MHC class I human tetramer, was restricted to the HLA-A2 allele. The HLA-A2 analysis revealed seven HLA-A*0201 positive patients and all of these had PR1 positive CD8+ T cells (proportion varied between 0.3% and 3.9% of total CD8+ T cell population).

**13.3 Regulatory T cells in CML patients (I,IV)**

Previous results showed that CML patients treated with dasatinib, who experienced LGL lymphocytosis during treatment, had decreased amounts of Tregs. It was suggested that this could be one of the mechanisms that may cause the clonal expansion of lymphocytes in the blood. The dasatinib-associated decreased amount of Tregs was confirmed in study I (Fig. 15).
Figure 15. Proportions of regulatory T cells of all CD4+ T cells in healthy controls, imatinib (IM), and dasatinib (DA) treated patients with (LGLpos) or without (LGLneg) lymphocytosis during treatment.

Notably, the patients who had been treated with IFN-α monotherapy and stayed in remission without any treatment (IFN-OFF) had an increased median proportion of Tregs in their CD4+ T cells (6.1%) when compared to those of IFN-ON (5.2%) and healthy subjects (3.8%) (p=0.01). However, no difference was observed in absolute Treg numbers.

14. The role of CMV in dasatinib induced lymphocytosis (I, II)

The hypothesis of a causative role of CMV in LGL lymphocytosis originates from a publication reporting dasatinib-associated LGL lymphocytosis, whereby half of the patients experienced CMV reactivation during dasatinib therapy. In addition, CMV-specific T cells were detected in the patients with LGL lymphocytosis. Therefore, the association of CMV to LGL lymphocytosis was studied in more detail in studies I and II.

In study I, the clonal lymphocytes were characterized in more detail. Notably, 21 out of the 23 dasatinib-treated patients who had a confirmed clonal lymphocyte population were positive for CMV immunoglobulin G (IgG). In contrast, only two out of six patients without clonal TCR rearrangement were positive for CMV IgG. Furthermore, all patients who had lymphocytosis during dasatinib treatment (n=10) were CMV positive, whereas only four out of eight patients with normal lymphocyte counts during therapy were positive. However, none of the CMV negative patients (n=4) had clonally rearranged TCR γ- or δ-genes.

In study II, the function and phenotype of CMV-specific T cells found in dasatinib-treated patients were assessed. A high proportion of LGLpos patients (40%) experienced symptomatic CMV reactivation during dasatinib therapy, while...
there was no evidence for reactivation of other viruses. In addition, all patients with LGL expansion had increased frequencies of CMV-specific CD8\(^+\) T cells (Fig. 16). These cells also exhibited a predominant CD45RO\(^+\)CD27\(^{neg}\) phenotype and co-expressed CD57 (Table 9). It is worth noting that this experiment could not be performed on LGL\(^{neg}\) patients since the majority of these patients were HLA-A*02\(^{neg}\) or CMV IgG seronegative.

![Figure 16. Proportions of CMV-specific (pp65\(^+\)) obtained from all CD8\(^+\) T cells in dasatinib-treated patients. (A) A patient without absolute LGL lymphocytosis. (B) a patient with NK expansion (approximately 65\% of lymphocytes were NK cells) during treatment, (C) a patient with both T and NK expansion, and (D) a patient with a dominant CD3\(^+\) T cell expansion (approximately 90\% of lymphocytes were T cells) and CMV reactivation during treatment.](image)

### Table 9. Phenotypic features of CMV-specific T cell populations.

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>T LGL</th>
<th>NK LGL</th>
<th>T + NK LGL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=5</td>
<td>n=5</td>
<td>n=1</td>
</tr>
<tr>
<td>CD8(^+)NLV(^+) % of CD3(^+) T-cells</td>
<td>7.9 [5.7-11.2]</td>
<td>2.6 [0.7-4.2]</td>
<td>15.6</td>
</tr>
<tr>
<td>CD45RO(^+)CD27(^+) % of NLV(^+) T-cells</td>
<td>84.8 [79.4-88.9]</td>
<td>53.3 [39.9-76.2]</td>
<td>93.2</td>
</tr>
<tr>
<td>CD57% of NLV(^+) T-cells</td>
<td>39.8 [26.6-88.1]</td>
<td>29.7 [24.8-46.8]</td>
<td>70.6</td>
</tr>
<tr>
<td>CD57% of NLV(^+) CD45RO(^+)CD27(^+)</td>
<td>41.6 [27.7-89.6]</td>
<td>46.3 [30.6-54.2]</td>
<td>72.4</td>
</tr>
</tbody>
</table>

Median percentages with ranges in parentheses are shown. This analysis could not be performed in LGL\(^{neg}\) patients as they were HLA-A*02 negative.

Since 5/16 LGL\(^{neg}\) patients experienced symptomatic CMV reactivation during dasatinib therapy, the proliferation of CD8\(^+\) T cells in response to the CMV pp495-503 peptide antigen (NLVPMVATV) was studied. A 50 nM dasatinib dosage effectively inhibited the proliferation of these CMV-specific T cells, whereas 10 nM
did not. In addition, no differences were observed when LGL patients (n=4) were compared to healthy controls (n=2) or an LGL$^{\text{neg}}$ patient (Fig. 17). The LGL$^{\text{neg}}$ control group was limited since the majority of these patients were either HLA-A*02$^{\text{neg}}$ or CMV IgG seronegative.

Figure 17. The inhibition of proliferation in CMV-specific CD8$^+$ T cell populations by dasatinib. PB MNCs taken from healthy controls and dasatinib-treated patients with a dominant T or NK cell expansion in the blood during therapy, PB MNCs were first stained with CFSE and then stimulated with a CMV pp65 peptide for six days in the presence or absence of dasatinib.

**14.1 Two distinct CD8 populations in dasatinib-treated patients**

Distinct CD8$^{\text{high}}$ and CD8$^{\text{low}}$ subpopulations were observed within the NLVPMVATV-specific CD8$^+$ T cell population in dasatinib-treated patients with LGL expansion (n=8). In a LGL$^{\text{neg}}$ patient and healthy controls (n=3) only a NLVPMVATV-specific CD8$^{\text{high}}$ population was observed, whereas the CD8$^{\text{low}}$ subpopulation was only seen in the LGL$^{\text{pos}}$ patients.

The NLVPMVATV-specific CD8$^+$ T cell populations were studied separately in functional assays. When stimulated with a cognate peptide, both NLVPMVATV-specific CD8$^+$ T cells degranulated and produced cytokines. These functions were inhibited by dasatinib in a dose-dependent manner. In addition, both degranulation and cytokine production were significantly more pronounced in the CD8$^{\text{high}}$ subpopulation in LGL$^{\text{pos}}$ patients (Fig. 18). Dasatinib *in vitro* also effectively abrogated downregulation of both TCR and CD8 in the CD8$^{\text{high}}$ subpopulation (data not shown).
Figure 18. Degranulation and cytokine production in CMV-specific CD8⁺ T cells in response to stimulation with a CMV-peptide. PB MNCs were stimulated with a CMV-peptide (2 μg/ml) for five hours. (A) IFN-γ and TNF-α production and (B) CD107a/b expression were measured in CMV-specific CD8⁺ T cells, CMV-specific CD8⁺⁺ T cells and CD8⁺⁺ T cells.

Clonotyping of CMV-specific CD8⁺ T cells showed that both subsets are similar (Table 10). This indicates that these cells were derived from the same precursors and that the observed CD8 downregulation probably reflected a state of recent activation.

Table 10. Clonotyping of CMV-specific CD8⁺ T cells.

<table>
<thead>
<tr>
<th>CD8⁺⁺</th>
<th>TRBV</th>
<th>CDR3</th>
<th>TRBJ</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD8⁺⁺⁺⁺</td>
<td>12-3⁺/12-4⁺</td>
<td>CASSSANYGYT</td>
<td>1-2</td>
<td>45.2</td>
</tr>
<tr>
<td></td>
<td>6-5</td>
<td>CASTLGTGREL</td>
<td></td>
<td>28.8</td>
</tr>
<tr>
<td>CD8⁺⁺⁺</td>
<td>TRBV</td>
<td>CDR3</td>
<td>TRBJ</td>
<td>Frequency (%)</td>
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<td>-------</td>
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<td>------</td>
<td>------</td>
<td>---------------</td>
</tr>
<tr>
<td></td>
<td>12-3⁺/12-4⁺</td>
<td>CASSSANYGYT</td>
<td>1-2</td>
<td>63.2</td>
</tr>
<tr>
<td></td>
<td>6-5</td>
<td>CASTLGTGREL</td>
<td>2-2</td>
<td>32.9</td>
</tr>
</tbody>
</table>

The clonotypic composition for CD8⁺⁺⁺ and CD8⁺⁺⁺ T cells of a dasatinib-treated patient who experienced CMV reactivation during therapy. TRBV and TRBJ usage, CDR3 amino acid sequence and the frequency are shown for each distinct clonotype. ¹TRBV12-3 and TRBV12-4 could not be distinguished.

15. Functional properties of lymphocytes in CML patients (II, III)

15.1 The function of T cells in dasatinib-treated patients (II)
In study II, a panel of different functional in vitro assays was performed on PB MNCs from dasatinib-treated patients. The majority of these patients experienced LGL lymphocytosis during treatment (LGL⁺⁺). LGL⁺⁺ patients were further divided into three subgroups based on the phenotype of the expanded cells: T cell lymphocytosis, NK cell lymphocytosis, and patients who had both lymphocyte
subtypes expanded. Patients who had normal lymphocyte counts during treatment were used as a control group together with healthy volunteers.

The CD3$^+$ T cells that were obtained from patients with LGL expansion (n=8) showed a significantly higher rate of apoptosis compared to LGL$^{neg}$ patients (n=6) and healthy volunteers (n=4) after culturing for four days without stimulation (p<0.05 for both experiments). In addition, T cells from LGL$^{pos}$ patients were more sensitive to activation-induced cell death after stimulation compared to the control group (p<0.05). A trend towards increased apoptosis was also seen when LGL$^{pos}$ patients were compared to LGL$^{neg}$ patients. However, no differences were noted when comparing apoptotic/necrotic cells (7AAD$^+$/annexin V$^+$).

Next, the ex vivo modulation of T cell activation, proliferation, degranulation and cytokine production by dasatinib was studied. When T cells were stimulated in the presence of dasatinib, a significant dose-dependent inhibition was observed in T cell activation, proliferation, degranulation, and cytokine production in both CD4$^+$ and CD8$^+$ T cells. However no differences in T cell function were observed between the studied groups (LGL$^{pos}$, LGL$^{neg}$ patients, and healthy controls).

In these experiments, a dichotomy was observed in the CD8$^+$ T cells in LGL$^{pos}$ patients. The population could be divided into CD8$^{high}$ and CD8$^{low}$ subsets according to the intensity of CD8 expression (Fig. 19). Furthermore, the CD8$^{high}$ cells exhibited a greater degranulation and cytokine production in response to OKT3 stimulation when compared to CD8$^{low}$ or CD4$^+$ T cells (Fig. 20). Again, the distinct CD8 subpopulations were not observed in LGL$^{neg}$ or in healthy controls, with the exception of one LGL$^{neg}$ patient.

![Figure 19. The distribution of the two distinct CD3$^+$CD8$^+$ T cell populations in a dasatinib-treated CML patient who had LGL lymphocytosis during treatment.](image-url)
15.2 Cytotoxicity of NK cells in dasatinib-treated patients (II)

Cytotoxicity assays in study II showed that the expanded NK cells from LGL\textsuperscript{pos} patients had a significantly impaired cytotoxic activity against K562 cells (Fig. 21A). Of note, patients with a prominent CD8 expansion did not have enough NK cells for these experiments and therefore, their samples were not included in this assay. Pretreatment with IL-2 restored the cytotoxicity of NK cells (Fig. 21B). Further, dasatinib in vitro inhibited NK cell mediated cytotoxicity, including experiments with IL-2 pretreatment. Similar inhibition was found in all the studied groups (data not shown).

In contrast, when PB MNCs were stimulated with K562 cells in the presence of degranulation markers (CD107a/b) without IL-2 pretreatment, no differences were detected between LGL\textsuperscript{pos} (n=5) with NK cell expansion compared to LGL\textsuperscript{neg} (n=3)
and healthy controls (n=3). *In vitro*, dasatinib had already significantly reduced CD107a/b degranulation at the concentration of 10 nM in LGL$^{\text{pos}}$ and those of healthy controls, whereas no significant reduction was observed for cells of LGL$^{\text{neg}}$ patients. Further, degranulation and cytokine production were totally inhibited by dasatinib at 50 nM in all three groups.

15.3 The function of large granular lymphocytes in dasatinib-treated patients (III)

In order to analyze the expanded LGLs in more detail, it was first examined whether LGLs could be identified by flow cytometry with antibodies that detect intracellular Granzyme B (GrB), a component found in cytotoxic granules. The numbers of LGLs were calculated from MGG-stained PB smears from TKI-treated patients (n=17). GrB was stained in PB MNCs and used to measure in parallel the frequency of GrB$^+$CD45$^+$ lymphocytes by flow cytometry. The numbers of MGG-stained LGLs correlated well with GrB$^+$ cell counts ($r=0.90; p<0.0001$), which showed that the results from both methods are equivalent (Fig. 22). This marker enabled the distinction between LGL and non-LGLs, and the study the function of these separately. In addition, to studying the effects of TKI in vivo exposure, samples in this substudy (III) were collected before and after the patients took their daily dose of dasatinib, imatinib, or nilotinib (Fig. 8).

![Figure 22. Correlation of the number of granzyme B (GrB) positive lymphocytes measured by flow cytometry and LGLs obtained from MGG smears. GrB$^+$ lymphocytes were measured by flow cytometry pre dasatinib (n=8), imatinib (n=3), and nilotinib (n=6) intake. In parallel, LGLs obtained from MGG smears were counted. Correlation was $r=0.90$ ($p<0.0001$).](image-url)
15.3.1 The function of T-LGLs in TKI treated patients (III)
As LGL lymphocytosis in dasatinib-treated patients had previously been correlated with superior therapy responses, the function of these cells was studied by using a GrB-specific antibody as a marker for LGLs. In addition, the long-term effects of TKIs in healthy immune effectors were studied by taking samples at a time-point when no TKI was present in the plasma. Samples were collected from patients who had been on therapy for a time frame ranging between six and 72 months for dasatinib, for 12 and 30 months for imatinib, or six and 42 months for nilotinib. Th1-type cytokine production (TNF-α and IFN-γ) in T cells was measured by flow cytometry and cells were gated into GrB⁺ (T-LGL) and GrB⁻ (T-nonLGL) T cell populations.

In unstimulated samples collected on the morning before the patient took the drug (12 or 24h after last capsule), neither T-LGL nor T-nonLGLs produced significant amounts of the studied cytokines (Fig. 23A, upper panels). In contrast, 13.7% of the T-LGLs in the stimulated pre-dasatinib samples produced TNF-α and IFN-γ (median value of 8 patients, range 4.1-40.8%; Fig. 23A, lower panels). Only 2.3% of non-T-LGLs were positive for these cytokines (median value of 9 patients, range 0.8-4.4%), which indicated that the T-LGLs were the main cytokine producers in the T cells. When studied separately, both CD4-LGL and CD8-LGL types produced the cytokines (Fig. 23B). In contrast, T-LGLs in healthy volunteers (n=5; median 5.0%), imatinib-treated patients (n=4; 2.4%) or nilotinib-treated patients (n=6; 5.5%) were significantly less active in producing Th-1 type cytokines (p=0.015 for pre TKI samples).
Figure 23. Cytokine production in granzyme B positive and negative T cells after stimulation with an OKT3 antibody. (A) PB MNCs were stimulated with OKT3 and co-stimulatory molecules (α-CD28 and α-CD49d) for six hours in the presence of GolgiStop. After, TNF-α and IFN-γ production in T cells were measured by flow cytometry. The figure presents results of representative cases: (A) a healthy volunteer, a dasatinib patient, an imatinib patient, and a nilotinib patient. Each plot shows 20,000 events. (B) Cytokine production in the CD4-LGLs and CD8-LGLs of the patient treated with dasatinib.

Next the TNF-α and IFN-γ production was studied separately. Samples from five patients (frozen PB MNCs from one imatinib, two nilotinib, and two dasatinib-treated patients) were analyzed using the antibodies for the cytokines in different reaction tubes. In dasatinib and nilotinib-treated patients T-LGL cells (both CD4- and CD8-LGLs) were responsible for producing IFN-γ (Fig. 24A and B), and non-T-LGLs produced considerably less of this cytokine. In the imatinib-treated patient, only a very few T-cells produced IFN-γ upon stimulation. In all TKI groups, T-LGLs and non-T-LGLs produced TNF-α. The increased cytokine production observed in dasatinib-treated patients is mainly due to highly active CD4-LGLs and CD8-LGLs, which produce large amounts of IFN-γ and TNF-α upon stimulation.
To investigate whether dasatinib *in vivo* affects T cell cytokine production, or whether larger proportions of T cells in patients on dasatinib had differentiated into Th-1 type effector cells, samples were collected and analyzed before and after drug intake. The difference in cytokine producing cells observed in the pre-TKI samples was significant (p=0.015), however; this amount decreased after drug intake (p=0.66) (median for dasatinib patients 8.8%, imatinib 3.9%, nilotinib 5.3%). An exposure to dasatinib *in vivo* after one hour decreased cytokine production in both CD4-LGL (p=0.0044) and CD8-LGL (p=0.0039), which indicated that dasatinib inhibits cytokine-production in T cells as reported previously.\textsuperscript{99,101,106}

When the absolute numbers of cytokine producing T cells were measured before TKI intake, dasatinib patients had more circulating cytokine-producing T cells (median 60563 cells per ml), than healthy volunteers (14084 cells per ml), imatinib (17574 cells per ml), or nilotinib (20383 cells per ml) treated patients (p=0.09). Importantly, the difference remained similar after the drug intake (dasatinib patients 66084 cells per ml, imatinib 22326 cells per ml, and nilotinib 15205 cells per ml, p=0.08), though it was previously shown that dasatinib intake slightly decreases the cytokine production.
15.4 The role of mobilization in NK cell function (III)
Since it has previously been shown that TKIs inhibit NK cell cytotoxicity in vitro, \(^{107-109}\) the function of NK cells pre and post TKI intake (one to two hours of in vivo exposure) was studied. Freshly isolated PB MNCs, or purified NK cells were co-cultured with the target cell line K562. After six hours, the percentage of dead K562 cells and degranulation of NK cells (CD107ab assay) was measured.

When the PB MNC fraction was used as the effector population (effector:target ratio 20:1), the median percentage of dead K562 cells was 18% in patient samples taken before, and 32% taken after dasatinib intake (p=0.004; Fig. 25A). No differences in NK cell cytotoxicity were observed after imatinib (11% before vs. 8% after; Fig. 25B) or nilotinib (10% before vs. 10% after; Fig. 25C) intake. The results were confirmed by using purified NK cells (effector:target ratio 10:1): the median percentage of dead K562 cells was 12% in pre-dasatinib samples, and 29% in post-dasatinib samples (p=0.06), whereas no differences were noticed with imatinib (30% before vs. 28% after) or nilotinib (14% before vs. 15% after) patients (Fig. 25A-C). The median percentage of dead K562 cells after incubation with purified NK cells obtained from healthy volunteers was 20%.

![Graphs A, B, and C showing cytotoxicity of NK cells pre and post TKI intake.](image)

**Figure 25. Cytotoxicity of NK cells pre and post TKI intake.** Freshly isolated effectors were incubated with the target cell line K562 for six hours. As effectors were counted PB MNC in ratios 5:1, 10:1, and 20:1, and purified NK cells in ratios 5:1 and 10:1. The cytotoxicity assay of samples taken from (A) dasatinib (PB MNC n=10, pure NK n=6), (B) imatinib (PB MNC n=3, pure NK n=3), (C) nilotinib (PB MNC n=5, pure NK n=3) treated patients pre- and post-drug intake and healthy controls (PB MNC n=6, pure NK n=4).

In order to study if the higher cytotoxicity capacity measured in samples taken one hour after dasatinib intake was caused by an increased number of K562-reactive NK cells, degranulation assays were performed in parallel. The results demonstrated that the proportions of degranulated NK cells after K562 stimulation between healthy controls and patients on dasatinib therapy (both pre and post TKI)
were equal (p=0.98). However, when the absolute number of CD107ab\(^+\) NK cells was calculated, there was a trend towards a higher absolute number of CD107ab\(^+\) NK cells for dasatinib-treated patients after TKI intake (median cell count for dasatinib-treated patients 61594, imatinib 19249, nilotinib 5598; p=0.055), which indicates that the increased number of NK-LGLs measured after dasatinib intake possess a higher cytotoxic capacity that results in a higher killing percentage.

15.5 Correlation of NK cytotoxicity to TKI responses (III)
While analyzing the cytotoxicity results in dasatinib-treated patients, a wide variation between individual patients was noticed. Therefore, to study whether cytotoxicity correlated with therapy responses, first-line and imatinib intolerant second-line dasatinib-treated patients were divided into two groups based on how fast they reached CMR. The PB MNCs obtained from patients who had achieved CMR within 12 months of dasatinib treatment (n=4) had significantly higher cytotoxic capability compared to those who had not (n=6): 46% vs. 29% of dead K562 cells in post-dasatinib samples (p=0.02, Fig. 26A). Notably, no differences in the percentage of NK cells between these two groups were seen (p=1.00 for both pre or post dasatinib intake samples). Also, the difference in cytotoxic capacity remained when purified NK cells were used as effectors (Fig. 26B).

![Graphs A and B](imageURL)

*Figure 26. Cytotoxicity of NK cells in dasatinib-treated patients with optimal and suboptimal responses. Freshly isolated effectors were incubated with the target cell line K562 for six hours. As effectors were counted PB MNC (A) in ratios 5:1, 10:1, and 20:1, patients with optimal (n=4) and suboptimal response (n=6). As effector (B) purified NK cells were used in ratios 1:5 and 10:1, patients with optimal (n=2) and suboptimal response (n=4).*
16. Dasatinib intake is associated with rapid increase of circulating LGLs (III)

Plasma peak concentration of dasatinib occurred approximately one hour after drug intake and decreased rapidly thereafter (see Fig. 8). Thus, the influence of sampling time on the proportion of GrB+ lymphocytes (LGLs) was studied in patients who received dasatinib, imatinib, or nilotinib by comparing the counts before (pre-count) and after TKI intake (post-count). LGL pre-counts and post-counts did not differ in patients who were either treated with imatinib (n=4; p=1.00) or nilotinib (n=7; p=0.42). However, total LGL counts increased in the dasatinib-treated patients (n=10; p=0.012) as early as one hour after dasatinib intake i.e. simultaneously with the plasma peak concentration of dasatinib. LGLs were further divided into CD4-LGL and CD8-LGL subgroups to reveal whether the increase in total LGL-count was associated with the T-LGL-count. No differences were seen in pre and post-dasatinib samples (CD4-LGL p=0.20; CD8-LGL p=0.56). These results suggest that the expansion of LGLs is mainly due to the increased number of NK cells. This was confirmed by analyzing the number of CD3negCD56+CD16+ cells in samples prior to and after TKI intake. Dasatinib intake increased the amount of NK cells (n=10; p=0.04), whereas no differences were seen after imatinib (n=4; p=0.63) or nilotinib (n=7; p=0.84) intake.

In addition, changes in the absolute LGL counts after TKI intake (Fig. 27) was measured. Before TKI intake, absolute LGL counts in all groups were equal (median for healthy controls 0.3x10^6 LGLs per ml, pre imatinib 0.5x10^6 LGLs per ml, pre nilotinib 0.6x10^6 LGLs per ml, and pre dasatinib 0.5x10^6 LGLs per ml) (p=0.50). However, post TKI the difference became significant (p=0.013). No significant changes were seen after imatinib or nilotinib intake (p=0.06 and p=0.16, respectively), whereas dasatinib intake significantly more than doubled (p=0.002) the number of circulating LGLs (from 0.5x10^6 LGLs per ml to 1.2x10^6 LGLs per ml).
Figure 27. Only dasatinib intake increases the absolute number of circulating CD4-LGLs, CD8-LGLs, and NK-LGLs. The absolute counts of LGLs in CML patients before (pre) and after (post) treatment daily dosage of (A) dasatinib, (B) imatinib, and (C) nilotinib. Absolute counts before and after TKI intake were compared between all of the different LGL subgroups CD4-LGLs, CD8-LGLs, and NK-LGLs. Nilotinib intake significantly decreased CD4-LGLs, whereas no significant changes were observed for other cell types.

17. Evolution of LGL subgroups during TKI (III)

In previous publications, LGLs were regarded as one population and it was not possible to distinguish the subgroups CD4-LGL, CD8-LGL, and NK-LGL from each other. It became possible to study the frequencies and phenotype of different LGL subgroups in samples taken at diagnosis and during TKI (dasatinib, imatinib, and nilotinib) therapy using GrB-positivity as a marker for LGLs.

At diagnosis, CML patients had significantly increased relative amounts of CD8-LGLs from all CD8^+ T cells (median 38%, p=0.028) compared to healthy controls (11.0%) (Fig. 28A). Moreover, the proportion of CD4-LGLs of all CD4^+ T cells was increased (median 3.6%) but did not significantly differ from that of healthy controls (median 0.8%, p=0.08) (Fig. 28B). No differences were seen in the proportion of NK-LGLs between healthy subjects (median 11.6%) and CML patients at diagnosis (median 11.4%, p=0.85) (Fig. 28C).

Follow-up samples from 16 newly diagnosed CML patients were collected during the first, third, sixth and 12th months after the start of the therapy. The relative frequencies of both CD4-LGLs (Fig. 28D) and CD8-LGLs increased (at diagnosis 36.5%, six months on therapy 70.9%; p=0.016) in patients treated with dasatinib as first-line therapy. In contrast, similar increases were not observed in patients on either imatinib (30.1% vs. 30.0%) or nilotinib (31.3% vs. 41.2%) as
first-line treatments. Furthermore, the frequency of NK-LGLs in the total lymphocyte population was increased one month after the start of dasatinib therapy (p=0.031). Imatinib-treated patients also had a significantly increased number of NK-LGLs one month after the start of therapy (p=0.031), whereas no differences were observed in nilotinib-treated patients (p=0.63).

**Figure 28.** The proportion of GrB positive T and NK cells in CML patients at diagnosis and during TKI therapy. GrB+ T and NK cells were measured by flow cytometry using a GrB-specific antibody or measured as NK cells (CD3negCD16+CD56+). (A-C) The percentage of GrB+CD4+ and GrB+CD8+ T cells and NK cells at diagnosis (n=18) compared to healthy controls (n=5). (D) Percentage of GrB+CD4+ T cells six months after the start of dasatinib (DA), imatinib (IM), and nilotinib (NI) therapy.

The absolute numbers of the various LGL subgroups (CD4-LGLs, CD8-LGLs, and NK-LGLs) were also calculated. After six months of therapy, imatinib and nilotinib-treated patients had similar numbers of LGLs in their blood as the healthy controls (Fig. 29A). In contrast, dasatinib-treated patients were clearly divided into two subgroups based on the absolute number of circulating LGLs (Fig. 29A). Three patients (LGLhigh group) had over 1.4x10^6 LGLs per ml (mean value for the whole group), whereas in other patients (LGLlow group), the absolute LGL counts were below 0.8x10^6 LGLs per ml. In the LGLhigh group both NK-LGL and CD4-LGL cells tended to be higher than in the LGLlow group (Fig. 29B and D). The absolute CD8-LGL counts did not differ significantly between the groups (Fig. 29C). Notably, the LGLhigh patients already had a greater number of LGLs at the diagnosis compared to the LGLlow patient subgroup (Fig. 29E).
Figure 29. Dasatinib-treated patients can be divided into two subgroups based on their absolute LGL counts six months after the start of therapy. (A) Absolute LGL counts per ml of PB in patients treated with dasatinib (DA), imatinib (IM), nilotinib (NI) six months after the start of therapy and healthy controls. Dasatinib-treated patients were divided into two groups (LGL high and LGL low) based on their absolute number of circulating LGLs per ml at the six months time point. Absolute NK-LGL (B), CD8-LGL (C), and CD4-LGL (D) counts were compared between the two groups of dasatinib-treated patients after 6 months on therapy. (E) Absolute LGL counts in LGL high and LGL low groups at diagnosis.

18. Immunomodulatory effects of dasatinib and IFN-α (II, IV)

To study the immunomodulatory effects of IFN-α, imatinib, and dasatinib therapies further, a total of 25 different plasma cytokines and chemokines were measured with a multiplex bead-based cytokine assay (Luminex®).

Ph+ leukemia patients treated with the second-generation TKI dasatinib, had several cytokines elevated when compared to healthy subjects: IL-6, IL-7, IP-10, MIG, and MCP-1 (Table 11). Notably, the highest plasma levels of these soluble factors were detected in patients who experienced symptomatic CMV reactivation during dasatinib therapy. No significant differences were observed between these two groups in any of the other 22 soluble factors (Table 12).
Table 11. Median plasma cytokine concentrations in healthy controls and dasatinib-treated patients.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Healthy n=10</th>
<th>Dasatinib n=23</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>12.3 (10.0-20.0)</td>
<td>19.5 (9.5-3577)</td>
<td>0.002*</td>
</tr>
<tr>
<td>IL-7</td>
<td>16.0 (13.0-20.0)</td>
<td>19.5 (11.0-58.3)</td>
<td>0.036*</td>
</tr>
<tr>
<td>IP-10</td>
<td>161.2 (100.8-383.8)</td>
<td>723.8 (144.8-4105)</td>
<td>0.002*</td>
</tr>
<tr>
<td>MIG</td>
<td>7.8 (6.0-23.5)</td>
<td>14.0 (5.0-621.0)</td>
<td>0.033*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>106.5 (50.8-361.0)</td>
<td>174.0 (63.0-1006)</td>
<td>0.048*</td>
</tr>
</tbody>
</table>

Medians and ranges are given for both groups. Cytokine concentrations are given in pg/ml. *median values are significantly different, nonparametric Mann Whitney test. Only cytokines with a significant p-value are shown.

Table 12. Median plasma cytokine concentrations in dasatinib-treated patients with (LGL<sup>hi</sup>) or without (LGL<sup>lo</sup>) LGL lymphocytosis during treatment.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>LGL&lt;sup&gt;hi&lt;/sup&gt; n=15</th>
<th>LGL&lt;sup&gt;lo&lt;/sup&gt; n=8</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-2R</td>
<td>55.5 (15.0-369.5)</td>
<td>21.5 (8.8-38.8)</td>
<td>0.018*</td>
</tr>
<tr>
<td>IL-5</td>
<td>2.0 (1.0-17.8)</td>
<td>3.0 (2.0-5.3)</td>
<td>0.08</td>
</tr>
<tr>
<td>IL-12</td>
<td>127.2 (28.0-277.0)</td>
<td>86.8 (46.5-118.0)</td>
<td>0.10</td>
</tr>
<tr>
<td>IP-10</td>
<td>1308 (144.8-4105)</td>
<td>271.7 (148.5-1287)</td>
<td>0.042*</td>
</tr>
<tr>
<td>MIG</td>
<td>79.8 (6.0-621.0)</td>
<td>9.8 (5.0-29.5)</td>
<td>0.031*</td>
</tr>
</tbody>
</table>

Medians and ranges are given for both groups. Cytokine concentrations are given in pg/ml. *median values are significantly different, nonparametric Mann Whitney test. Only cytokines with a p-value less than 0.1 are shown.

CML patients on IFN-α monotherapy had a significantly different cytokine profile compared to healthy volunteers. IFN-ON patients had several cytokines elevated in their plasma: IP-10, IL-6, IL-12, and MCP-1. It has been previously shown that IFN-α therapy induces the expression of several of these cytokines,<sup>159</sup> thus the cytokine profiles were compared between healthy volunteers and IFN-OFF patients (Table 13; Fig. 30). The greatest differences were seen in eotaxin (p<0.0001) and MCP-1 (p=0.0003) levels, which were highly elevated in patients who had been in remission for years without treatment (Table 13). Further, IL-6 (p=0.024), IL-12 (p=0.010), IP-10 (p=0.035), and IFN-γ (p=0.037) were elevated in the IFN-OFF group compared to healthy controls. Furthermore, both αβ<sup>+</sup> and γδ<sup>+</sup> T cells in IFN-α-treated patients express the corresponding receptors (CCR2 and CCR3) for the cytokines (eotaxin and MCP-1, respectively) over varying ranges (0.1%-8.0% of αβ<sup>+</sup> and γδ<sup>+</sup> T cells).
Table 13. Median plasma cytokine concentrations in healthy controls and CML patients treated with IFN-α monotherapy (IFN-ON patients are still on therapy, IFN-OFF have successfully discontinued therapy).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Healthy n=10</th>
<th>IFN-ON n=10</th>
<th>IFN-OFF n=9</th>
<th>p (healthy vs IFN-OFF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>12.3 (10.0-20.0)</td>
<td>25.5 (8.0-90.0)</td>
<td>15.5 (12.0-50.0)</td>
<td>0.024*</td>
</tr>
<tr>
<td>IL-12</td>
<td>70.5 (56.3-146.0)</td>
<td>124.0 (49.5-393.5)</td>
<td>103.8 (68.0-204.8)</td>
<td>0.010*</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>3.9 (2.0-11.0)</td>
<td>4.3 (2.5-20.8)</td>
<td>2.8 (2.0-6.0)</td>
<td>0.07</td>
</tr>
<tr>
<td>IP-10</td>
<td>161.2 (100.8-383.8)</td>
<td>1676 (446.8-3018)</td>
<td>299.5 (58.0-641.0)</td>
<td>0.035*</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>427.2 (127.5-749.3)</td>
<td>705.0 (346.3-2589)</td>
<td>1173 (699.3-2162)</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>MCP-1</td>
<td>106.5 (50.8-361.0)</td>
<td>631.5 (305.8-1765)</td>
<td>458.5 (153.5-767.5)</td>
<td>0.0003*</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.0 (4.0-13.0)</td>
<td>5.5 (4.0-10.0)</td>
<td>4.0 (3.5-6.0)</td>
<td>0.037*</td>
</tr>
</tbody>
</table>

Medians and ranges are given for each studied group. Cytokine concentrations are given in pg/ml. *median values are significantly different, nonparametric Mann Whitney test. Cytokine concentrations are given in pg/ml. *median values are significantly different, nonparametric Mann Whitney test. Only cytokines with a p-value less than 0.1 are shown.

Figure 30. Median plasma cytokine concentrations of healthy controls, CML patients at diagnosis and CML patients who had been able to discontinue IFN-α treatment. The figure presents the cytokines that were significantly different between the groups.

18.1 Cytokine profiles in CML patients on different treatments
Plasma cytokines and chemokines in healthy controls and dasatinib and IFN-α-treated patients were investigated in studies II and IV. In addition, unpublished observations from imatinib and imatinib + IFN-α-treated CML patients were
compared. Further, diagnostic samples were analyzed in a study by Jalkanen et al. 160

In order to study how the immune system can be restored in CML patients by different therapies, the plasma levels of 25 different cytokines were studied. The samples investigated were from healthy volunteers (n=10), CML patients before the start of treatment (n=7) and Ph+ leukemia patients treated with either dasatinib (n=26), imatinib (n=7), IFN-α (n=10), or IFN-α+imatinib (n=5). Notably, ten cytokines were significantly decreased in untreated CML patients when compared to healthy volunteers (Table 14, Fig. 31). In contrast, imatinib mostly restored the plasma cytokines to the levels found in healthy volunteers, while dasatinib and IFN-α monotherapy significantly modified the cytokine profile (Table 14, Fig. 31). Significant changes in the cytokine profile were seen between the untreated CML patients and dasatinib or IFN-α-treated patients (Table 14, Fig. 31).

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Healthy controls</th>
<th>Diagnosis</th>
<th>Dasatinib</th>
<th>IFN-α</th>
<th>Imatinib</th>
<th>Imatinib + IFN-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1Ra</td>
<td>20.7</td>
<td>11.0**</td>
<td>20.5</td>
<td>30.3</td>
<td>16.5</td>
<td>24.5</td>
</tr>
<tr>
<td>IL-12</td>
<td>70.5</td>
<td>49.0*</td>
<td>104.8</td>
<td>124.0*</td>
<td>73.5</td>
<td>102.8</td>
</tr>
<tr>
<td>IL-15</td>
<td>12.8</td>
<td>9.0*</td>
<td>14.3</td>
<td>19.0</td>
<td>13.0</td>
<td>14.5</td>
</tr>
<tr>
<td>TNF-α</td>
<td>12.0</td>
<td>8.5**</td>
<td>11.3</td>
<td>14.4</td>
<td>10.0</td>
<td>12.5</td>
</tr>
<tr>
<td>IFN-α</td>
<td>5.8</td>
<td>4.5*</td>
<td>5.5</td>
<td>9.8</td>
<td>4.9</td>
<td>10.5**</td>
</tr>
<tr>
<td>IP-10</td>
<td>161.2</td>
<td>119.5**</td>
<td>723.8**</td>
<td>2225****</td>
<td>275.0</td>
<td>548.5**</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>427.2</td>
<td>74.5***</td>
<td>423.3</td>
<td>705.0*</td>
<td>359.0</td>
<td>291.8</td>
</tr>
<tr>
<td>RANTES</td>
<td>5558</td>
<td>4242**</td>
<td>4932</td>
<td>548</td>
<td>5564</td>
<td>5647</td>
</tr>
<tr>
<td>MCP-1</td>
<td>106.5</td>
<td>47.5*</td>
<td>174.0</td>
<td>631.5****</td>
<td>119.5</td>
<td>179.0</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5.0</td>
<td>4.0*</td>
<td>5.0</td>
<td>5.5</td>
<td>4.0</td>
<td>6.0</td>
</tr>
</tbody>
</table>

All patient groups on therapy were compared to healthy subjects. Only cytokines with a significant p-value (<0.05) are shown. Analysis was done with the nonparametric Mann Whitney test and the results are shown as median pg/ml. Results are flagged with a single asterisk when the p-value was less than 0.05, with two asterisks when p<0.01, and three asterisks p<0.001, and four asterisks p<0.0001.
Figure 31. Plasma cytokines in healthy controls, CML patients at diagnosis, and during different treatments. The figure presents the cytokines that were significantly different between healthy controls, CML diagnosis, and CML patients on dasatinib, IFN-α, imatinib, and imatinib+IFN-α treatment.
19. Discussion

After decades of investigation, increasing evidence support the immunosurveillance hypothesis in cancer as reviewed by Lesterhuis et al. (2011). This process includes recognition and elimination of transformed cells by the immune system, and is part of a more general process called cancer immunoediting, which protects the body from cancer growths. One common aim in cancer research is to find factors within the immunoediting process, which could be utilized in treating cancer.

In the case of CML, the malignant leukemic cells have finally escaped the immune system and subsequently caused the disease. At this point, tumor-reactive cytotoxic cells have been deleted or suppressed, or they are anergic and therefore unable to function properly and failed to respond to their specific antigens. Yet, CML is known to be one of the most immunogenic tumors and leukemia antigen-specific (proteinase-3; PR1, and Wilms tumor 1 protein; WT1) cells have been found in CML patients, although they are shown to be anergic. However, in some cases, CML can be successfully treated with immunomodulatory therapies, such as IFN-α and donor lymphocyte infusions. It is therefore thought that the immune system in CML patients is not permanently compromised. Further evidence of the usefulness of inducing an anti-CML immune response comes from clinical trials with BCR-ABL1 peptide vaccines.

Treatment of CML has improved significantly during the last 10 years after the discovery of the first tyrosine kinase inhibitor (TKI) imatinib. Further improvement has been achieved with the second-generation TKIs dasatinib and nilotinib. Of the three TKIs that are currently used to treat Ph+ leukemia, dasatinib has the widest inhibition profile, which results in side effects not observed in imatinib or nilotinib-treated patients. Increasing evidence suggests that dasatinib has pronounced immunomodulatory off-target effects. However, the results are rather controversial. In vitro studies have indicated that the effects on immune cells are merely suppressive or inhibitory, although no increased infection rates have been reported in large clinical trials. In contrast to the in vitro findings, several groups have reported immunostimulatory effects in vivo as a proportion of patients have developed clonal expansion of large granular lymphocytes (LGLs) during treatment. Importantly, there is growing proof that indicates that lymphocytosis is associated with excellent therapy responses. Therefore, the purpose of this PhD project was to investigate the observed anti-leukemic effect of dasatinib in more detail.

In the first study, the prevalence of clonal lymphocytes was investigated in untreated, recently diagnosed CML patients. Remarkably, most patients already had clonal Ph-negative lymphocyte populations at diagnosis. Moreover, the sensitivity of the used PCR assay was 1-5%, which suggests that a significant proportion of lymphocytes were clonal at diagnosis. The results also showed that the clones were
present at low levels in patients on imatinib therapy, but if the patients switched to dasatinib, the clones rapidly expanded several-fold. A similar clonal expansion was also seen in some first-line dasatinib-treated patients. However, the expansion seemed to appear only in a proportion of these patients. Clonal non-malignant lymphocytes have previously been found in other hematologic malignancies such as B chronic lymphocytic leukemia, Waldenstrom’s macroglobulinemia, multiple myeloma and importantly, have also been associated with improved prognosis.

The clones were further confined to all three groups of cytotoxic lymphocytes; NK cells, CD8+ T cells, and γδ T cells. These cells are key effectors involved in controlling hematologic malignancies and are capable of eliminating CML cells. Notably, patients with LGL lymphocytosis had significantly more δ-gene rearrangements, which indicates an increased prevalence of both clonal NK and γδ+ T cell types. Such increased prevalences were not detected in patients without lymphocytosis. Based on these findings, it could be argued that these cells contribute to the excellent, long-lasting responses seen in dasatinib-treated patients with poor-prognosis disease (Ph+ ALL or CML BC) who developed marked LGL expansion during therapy.

These results strengthened the original theory that dasatinib induced LGL lymphocytosis is an expansion of pre-existing T-memory cell clones, which were already present in the patient before the start of treatment. In addition, the results suggested that dasatinib therapy might restore the function of unresponsive, anergic CML-specific cytotoxic cells.

Encouraged by these results, the function and immunophenotype of the expanded T and NK cells were studied in more detail. T cells in dasatinib-treated patients with LGL expansion were found to share several characteristics of typical T cells observed after viral infections. Previous publications support this finding, as LGL expansions have been observed to occur during viral infections and after allogeneic stem cell transplantations. Moreover, expansions that occur after transplantations are mostly seen in the context of viral infections. These observations suggest that chronic antigen-driven T cell stimulation may be a part of or even the cause of LGL expansions.

CMV had already been associated with dasatinib-induced lymphocytosis in our original publication, as over 40% of the patients had CMV reactivation during treatment. This finding was further confirmed in study I, as an association between positive CMV IgG serology and clonal lymphocyte populations was observed. Moreover, in study II high frequencies of CMV-specific CD8+ T cells in patients with LGL expansion was observed. Notably, a high proportion of the studied LGL patients also experienced symptomatic CMV reactivation during dasatinib therapy. It is therefore possible that the immunosuppressive effects of dasatinib triggered the CMV reactivation and consequently led to the clonal expansion of CD8+ T cells and/or NK cells. This hypothesis was supported by the
present finding that dasatinib at low concentrations did not inhibit the proliferation of CMV-specific CD8+ T cells. Further, these results led to the speculation that the observed CMV-specific CD8+ T cells could be cross-reactive and also target leukemic cells in addition to virally infected cells. Therefore, the observed CMV-specific CD8+ T cells could have a role in the beneficial anti-leukemic effects of LGL expansions.

CD8+ T cells in patients treated with dasatinib were observed to share another typical feature seen during viral infections. Both CD8+ and CMV-specific CD8+ T cells could each be divided into two distinct populations (CD8high and CD8low) based on the intensity of the CD8 expression. Similar phenotypic dichotomy has also been described in healthy individuals and has been associated with CMV.177,178 Moreover, a small CD8low population (<7%) can be found in nearly all individuals and is not affected by the CMV status, whereas the CD8high population is significantly increased in CMV seropositive individuals.178,180 The present results show that dasatinib-treated patients with LGL expansion have often an increased CD8low population as well. Circulating CD8low cells are often oligoclonal cytotoxic effector cells and represent a subset of activated CD8 effector cells, which is likely due to a long-term, low-level response driven by chronic antigen exposure.179 In addition, it was shown that the two distinct CD8 populations in dasatinib-treated patient groups were phenotypically and clonotypically similar, and therefore it is possible that the same antigen-specific clonotypes could exist in different states of activation during dasatinib-induced LGL expansion.

The analysis of plasma cytokines showed that IP-10, MIG, IL-6, and IL-2R levels were increased in these patients with high LGL counts during therapy. These cytokines are known to be chemotactic for monocytes, NK cells, and activated T cells. A review by Frederick and Clayman (2001) suggests that these cytokines may also be involved in the enhanced therapy outcomes such as chemotactic effects, which have been shown to be relevant in controlling leukemia.181 Furthermore, a study found that some of these cytokines are produced by antigen-specific CD8+ T cells in response to viral infections and are associated with anti-viral processes.182 Consistent with the publication, it was found in study II that the highest plasma concentrations of IP-10, IL-6, MIG, and IL-2R were in patients with the highest LGL expansions and symptomatic CMV reactivation during treatment. Furthermore, two previous publications demonstrated that increased levels of IFN-γ stimulated gene products, such as IP-10 and MIG, were found in the plasma of patients with LGL expansions unrelated to dasatinib treatment.183,184

The phenotype of the expanded lymphocytes in the dasatinib-associated LGL expansions was shown to be predominantly CD27negCD57+. This phenotype has previously been associated with late differentiated antigen-specific effector memory cells.185 Furthermore, the immunophenotype of dasatinib-treated patients showed less than 20% PD-1+CD8+ T cells, regardless if they had or did not have LGL expansion during therapy. This was an unexpected finding as PD-1 expression in imatinib-treated patients has been shown to be around 60% and less than 10% in healthy subjects.163 This finding indicates that unlike imatinib, dasatinib can
decrease PD-1 expression to be near the levels of healthy controls, which could be of importance as the lack of PD-1 signaling pathway is associated with an improved survival and might restore the function of CML-specific CTLs as shown in a CML mouse model.\textsuperscript{163}

Further proof of cytotoxic immunoactivation in CML patients was obtained in study III, as this study showed that CML patients had increased numbers of cytotoxic CD8- and CD4-LGLs at the time of diagnosis. These results support the hypothesis based on the first study (I) that CML patients already have clonal, putative anergic anti-leukemia cells at the time of diagnosis. The results further suggested that dasatinib therapy might restore the function of unresponsive, exhausted CML-specific cytotoxic cells. It was therefore interesting to observe that dasatinib therapy not only increased the percentage and the absolute numbers of cytotoxic CD8- and NK-LGLs, it also increased the amount of CD4-LGLs: an opposite response to those of imatinib and nilotinib. Furthermore, the unique CD4-LGLs observed in dasatinib-treated patients were highly active and responded to stimulation by secreting considerable amounts of the Th1 type cytokines IFN-\(\gamma\) and TNF-\(\alpha\). In healthy individuals, similar cytotoxic CD4\(^+\) T cells are rare.\textsuperscript{186} However, a few reports have suggested that these cells are involved in anti-tumor activity in other malignancies such as melanoma \textsuperscript{187} and myeloma.\textsuperscript{188} Further, it has been shown that CD4\(^+\) T cells in humans are able to recognize and lyse foreign leukemic target cells.\textsuperscript{189} Moreover, CD4-LGLs may have a role in tumor immunosurveillance as it has been reported that effector CD4\(^+\) T cells are able to impair and kill tumor-induced immunosuppressive Tregs by producing GrB.\textsuperscript{190,191} Remarkably, the results in study III showed that GrB is expressed in a high proportion of CD4\(^+\) T cells of dasatinib-treated patients. This is in accordance with the previous observations that long-term dasatinib therapy decreases the number of Tregs, which is especially prominent in patients with LGL lymphocytosis.\textsuperscript{115,192,193}

In addition to the differences in kinase inhibition profiles of the studied TKIs, their \textit{in vivo} pharmacokinetics differ significantly.\textsuperscript{194} In contrast to imatinib and nilotinib, the peak concentration (Cmax) of dasatinib occurs as early as one hour after drug intake and its half-life is short. Therefore it could be argued that previous studies, which had dasatinib constantly present in \textit{in vitro} cultures did not mimic the \textit{in vivo} pharmacokinetics. Those same studies showed that dasatinib inhibit T cell function,\textsuperscript{99-101,106} whereas our results showed that dasatinib exposure \textit{in vivo} does not completely inhibit T cells. The functional assays in study III were performed on fresh samples collected both before and after TKI intake in order to imitate the \textit{in vivo} situation as closely as possible. It was further observed that even though the proportion of cytokine producing T-LGLs did decrease, the absolute number of these cells remained the same due to the rapid increase of lymphocytes and especially the LGLs as early as one hour after dasatinib intake as recently described by us.\textsuperscript{195} In contrast to previous publications, the results also indicated that TKIs \textit{in vivo} do not inhibit NK cell function; NK cells that were obtained either pre or post
TKI intake were able to degranulate and kill K562-targets *ex vivo*. Importantly, NK cells obtained only one hour after dasatinib intake possessed significantly improved cytotoxicity. Of note, the observed cytotoxicity varied notably between the studied patients and correlated with therapy response. In accordance with this finding, it was previously reported that patients with LGL expansions had enhanced NK cell function, which could be one mechanism of the observed improved therapy responses. Although these results need to be proven in larger prospective studies, patient-specific determinants may exist and they may play additional roles in the immunomodulatory effects of dasatinib. This is supported by the observation that patients, who had significantly increased LGLs in circulation six months after commencing with dasatinib, already had higher numbers of these cells at diagnosis.

Of note, the cytotoxicity results in study III differed from those found in study II. These discrepancies could be partly explained by the differences in the studied patient cohorts. For instance in study II the patient groups consisted mostly of second-line treated patients with advanced CML or Ph+ ALL, whereas in study III the patient groups comprised mostly of first-line CML CP patients. Furthermore, in study II, the NK cells studied were obtained from patients whose LGL expansions dominantly consisted of NK cells (over 70% of the total lymphocyte population was NK cells). This finding is indicative of an excessive expansion of these cells *in vivo* and as shown, the cells were anergic. In contrast, none of the patients in study III had such a significant NK cell expansion. Further, the contrasting results could also be attributed to the different assays used and, as observed in study III, to the different time-point that the samples were taken after dasatinib intake. Moreover, cryopreserved cells were used in study II, whereas in study III only freshly isolated cells were used, which could further affect the results.

Another drug used to treat CML, which is known to have immunomodulatory effects, is IFN-α. Increasing evidence support the benefits of IFN-α therapy and currently several centers are investigating the possibility of re-introducing IFN-α in the treatment of CML. IFN-α is known to improve responses to imatinib therapy and it is possible that IFN-α could increase the likelihood of discontinuing imatinib treatment successfully. However, the most promising proof of the beneficial immunomodulatory effects of IFN-α comes from a study by Mahon et al. The study reported that most patients, who had successfully stopped treatment, still had minimal residual disease but the disease stayed under control and did not progress. The anti-CML effect of IFN-α has typically been thought to be due to the effects on immune cells such as cytotoxic T cells. This has been shown by an improved function of T and NK cells in CML patients treated with IFN-α monotherapy. In addition, Essers et al. provided an interesting alternative hypothesis, when they showed that IFN-α pre-treatment in CML patients might have activated and, therefore, sensitized CML stem cells to imatinib.

In study IV, the immunomodulatory effects of IFN-α were studied in CML patients who were either on IFN-α monotherapy (ON), or had been treated with
IFN-α monotherapy and then discontinued treatment successfully (OFF). Immunophenotyping revealed features that distinguished this unique group of patients from healthy volunteers. Specifically, the lymphocyte profile in IFN-α-treated patients was shifted to a more cytotoxic phenotype, such as increased amounts of CD8+ T cells. Notably, the specific immunophenotypic changes were also observed in IFN-OFF-patients who had been without any treatment for at least two years. The most prominent change was observed in the NK cells as the OFF-patients had the highest number and proportion of these cells. Furthermore, they had increased amounts of CD3+CD8+CD45RO+ memory T cells, which is in accordance with the report by Usuki et al.199 Furthermore, the expanded CD8+ T cells were mostly CD45RO+, which was also found in dasatinib-treated patient groups.120 As discussed above, CD8+ T cells and NK cells may have a role within tumor surveillance and in eliminating CML cells.162,173,174

Cytokines are important in mediating signaling between immune cells and chemokines are known to direct the migration of immune cells. Elevated plasma levels of IL-6, IL-12, IP-10, eotaxin, MCP-1, and IFN-γ were observed in CML patients treated with IFN-α monotherapy. It is known that IFN-α therapy induce the production of IP-10,159 however the patients who had been without treatment for years also had increased levels of IP-10. In accordance with the observed changes in the immunoprofiles of IFN-α-treated patients, Saudemont and colleagues reported that IP-10 can increase the percentage of NK cells, activate them and improve their cytotoxic capacity.145 Intriguingly, this study also suggested that IP-10 could be part of the elimination process of dormant tumor cells, which could be the case in the IFN-α-treated CML patients included in study IV. Moreover, the same analysis of plasma of patients treated with dasatinib revealed increased IP-10 levels in those who had achieved exceptionally good therapy responses. Furthermore, it was observed that eotaxin and MCP-1 levels were significantly higher in the IFN-α-treated patient group. MCP-1 has been associated with Th1 type immune response, whereas eotaxin has been suggested to have a role within Th2 type responses.200,201 MCP-1 levels were especially high in patients on IFN-α monotherapy, whereas the patients who had successfully discontinued treatment had higher levels of eotaxin in their plasma, which suggests that the discontinuation of IFN-α treatment switches the immune response to the Th2 type. However, both cytokines have been related to better therapy responses in solid tumors.202,203 It is noteworthy that these two cytokines were at the same level in TKI treated patients as in healthy volunteers, which revealed yet another unique immunomodulatory effect of IFN-α therapy.

In accordance with the results in study I, clonal lymphocytes in the majority of IFN-α-treated patients were observed. In contrast to that found in TKI-treated patients, a pattern of clonal TCR rearrangements was observed in IFN-α patients i.e. 79% of IFN-α-treated patients had a clonal rearrangement detected with the same pair of primers. The percentage of these clones in TKI-treated patients was only between 10-20%, which indicates that this finding is characteristic of IFN-α monotherapy. As there was no pattern in the clonal sequences, it is uncertain
whether same antigens induced the clonality. However, further analysis of these clonal lymphocytes revealed that the clones resided in the $\gamma\delta^+$ T cells in all analyzed patients. Even though it is thought that $\gamma\delta^+$ T cells can recognize transformed cells it remains controversial whether $\gamma\delta^+$ T cells have a role in tumor immunology. It is hypothesized that $\gamma\delta^+$ T cells are activated by phosphoantigens. Such antigens can be produced by certain tumors and are recognized by $\gamma\delta^+$ T cells. Increasing numbers of clinical trials have shown that co-injection of synthetic phosphoantigens with $\gamma\delta^+$ T cells to cancer patients improved responses. Thus, it is possible that even though, the observed clonal $\gamma\delta^+$ T cells do not share same TCR receptor sequences, they could be activated by the same phosphoantigens that are induced by malignant cells. In accordance with this hypothesis, an increased number of $\gamma\delta^+$ T cells in acute leukemia patients has been associated with improved survival after bone marrow transplantation. A more recent study showed that ex vivo expanded $\gamma\delta^+$ T cells can kill K562 cells, which further suggests that they could also have the ability to eliminate leukemic cells. The fact that CML patients who can be considered to be cured with IFN-α monotherapy have unusual clonal $\gamma\delta^+$ T cells is an additional evidence that these cells possess an anti-leukemic effect in vivo. In combination with the increased numbers of NK cells and CD8$^+$ T cells, the clonal $\gamma\delta^+$ T cells could possess an anti-leukemic activity, which allows leukemia patients to discontinue therapy without disease progression.
Figure 32. The immunomodulatory effects of IFN-α and dasatinib therapies in CML patients. IFN-α therapy was observed to increase the concentration of several cytokines and chemokines in the plasma. It also induced the clonality of γδ+ T cells and upregulated CD8+ T and NK cells. However, dasatinib seemed to have wider effects on the immune system. First, dasatinib therapy decreased the number of suppressive Tregs, and increased the number of cytotoxic effector cells (γδ+ T cells, CD8-LGL, CD4-LGL and NK-LGLs). Second, dasatinib increased the levels of several cytokines that are known to be chemotactic for Th1 type immune cells. A proportion of dasatinib-treated patients also develop CMV reactivation during the treatment, which may lead to the expansion of CMV-specific CD8+ T cells which could possibly cross-react with leukemia cells. Third, CMV could also indirectly affect immunoactivation through the induction of cytokine production.
20. Conclusions and future directions

This thesis provides further proof to support the immunoediting theory in CML. The results show that CML patients have Ph- lymphocyte clones at diagnosis and an increased proportion of cytotoxic CD4- and CD8-LGLs, which are all signs of a cytotoxic immune activation. These results support the hypothesis of the beneficial immunomodulatory effects of dasatinib, as the presented results show that dasatinib therapy further increases the number of circulating CD4- and CD8-LGLs, and importantly also generates a strong Th1-type immune response in these cells. In accordance with this finding, the expanded T cells or NK cells in dasatinib-treated patients were shown express a predominantly late or terminally differentiated phenotype. In contrast to previous reports, the results presented in this thesis indicate that dasatinib enhances the cytotoxic capability of NK cells. This data supports the dual mode of action of dasatinib: the effective inhibition of BCR-ABL1 in leukemic cells is complemented with enhanced immune responses, which in turn may have a role in the long-term control of Ph+ leukemia. However, the specific epitopes of the clonally expanded lymphocytes and their possible anti-CML function require further studies.

This thesis also provides strong evidence that dasatinib induced LGL expansions are associated with CMV reactivation. The observed CMV-specific CD8+ T cells could, in fact, be cross-reactive and target leukemia-associated antigens. Therefore, CMV-specific CD8+ T cells might have a role in the beneficial anti-leukemic effects of LGL expansions. Another possibility worth studying is that CML stem cells could be infected with CMV and CMV-specific T cells could therefore also target CML stem cells. However, further experiments are needed to explore this hypothesis.

In addition to data on dasatinib, the findings in this thesis present distinct immunomodulatory effects of IFN-α treatment. Patients who had successfully discontinued the IFN-α therapy and who can be considered cured, had characteristic lymphocyte and cytokine profiles. Together with the increased number of NK and CD8+ T cells, the observed clonal γδ+ T cells could possess anti-leukemic activity, which allows these patients to discontinue therapy without disease progression. These results suggest that IFN-α therapy, which is likely supported by an active immune defense against CML, can be considered curative for a small subset of CML patients. To study this theory, functional studies are ongoing.

The treatment of leukemia is evolving at a rapid pace and new therapies are constantly entering the clinics. Yet, one remaining obstacle to curing leukemia is the difficulty to access and eliminate all leukemic stem cells. An interesting approach to solve this problem is to utilize the patients’ own immune system, as it evidently has the specific tools to fight leukemia. To be able to re-initiate the anti-leukemic response, the immunosuppressive environment and a state of anergy caused by the disease itself need to be reversed. The results provided in this dissertation suggest that this may be accomplished in the future with small molecular drugs such as dasatinib.
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References


22. Elefanty AG, Hariharan IK, Cory S. bcr-abl, the hallmark of chronic myeloid leukaemia in man, induces multiple
haemopoietic neoplasms in mice. EMBO J 1990;9:1069-78.


145. Saudemont A, Jouy N, Hetuin D, Quesnel B. NK cells that are activated by CXCL10 can kill dormant tumor cells that resist CTL-mediated lysis and can express B7-H1 that stimulates T cells. Blood 2005;105:2428-35.


196. Nicolini FE, Hayette S, Legros L, et al. Pegylated IFN-alpha2a combined to imatinib mesylate 600mg daily can induce complete cytogenetic and molecular responses in a subset of chronic phase CML patients refractory to IFN alone or to imatinib 600mg daily alone. Leuk Res 2011;35:80-6.
Original publications