Published in final edited form as:

Leuk Lymphoma. 2011 April ; 52(4): 668–679. doi:10.3109/10428194.2010.550074.

A Molecular and Functional Analysis of Large Granular Lymphocyte Expansions in Chronic Myelogenous Leukemia Patients Treated With Tyrosine Kinase Inhibitors

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Abstract

Tyrosine kinase inhibitor (TKI) therapy has become the standard treatment for chronic myelogenous leukemia (CML). Off-target kinase inhibition has been implicated in the appearance of unique adverse effects, such as colitis and pleural effusions. In addition, some patients present oligoclonal expansions of large granular lymphocytes (LGLs). We sought to further investigate this phenomenon in 64 patients treated with 5 different TKIs. Clonal expansions of cytotoxic T lymphocytes (CTLs) were identified in all TKI-treated patient groups, but only in dasatinib-treated patients were these expansions characterized as LGLs. Survival factors known to be important in LGL leukemia (IL-15 transpresentation, plasma PDGF BB levels, NF- κ B, and T-bet activation) were found to be associated with TKI-induced LGL expansions. Interestingly, patients with LGL expansions had increased cytotoxicity against non-transformed endothelial cells, which may play a role in observed autoimmune-like side effects. Our results indicate that CML patients treated with TKIs can develop T cell expansions, which can in certain cases be related with some adverse effects.

Keywords

Chronic myeloid leukemia; tyrosine kinase inhibitors; large granular lymphocytes

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DECLARATION OF INTEREST

Javier Pinilla-Ibarz and Satu Mustjoki have received honoraria from Novartis and Bristol Myer Squibb.

INTRODUCTION

Tyrosine kinase inhibitors (TKIs) have dramatically changed the prognosis of patients with chronic myelogenous leukemia (CML)[1]. Imatinib, the lead compound, has a unique mechanism of action, interfering with the ATP binding to ABL and BCR-ABL, making this agent the first truly targeted therapy for CML[2]. Currently, TKI therapy is considered appropriate for life-long management of disease. Nevertheless, this treatment is not curative, and some patients become intolerant or develop resistance to imatinib after acquiring mutations in the BCR-ABL kinase domain, eventually resulting in treatment failure[3].

The curative potential of graft-versus-leukemia derived from successful hematopoietic stem cell transplantation has shed light on the prospective benefit of directed immunotherapy for CML. Several immunotherapeutic approaches are currently under investigation outside the realm of stem cell transplantation [4].

Interestingly, there is a growing but conflicting body of evidence supporting an as yet uncharacterized role for TKIs in their interaction with the cells of the immune system. Although some *in vitro* studies have shown deleterious effects of TKIs on antigen-presenting cells and T cells function [5-8], imatinib has been shown to elicit an increase in IFN- γ -producing T cells in human patients, and, in our own animal model studies, imatinib enhanced the ability of antigen-presenting cells to overcome tumor-induced tolerance [9,10]. For CML patients post-allogeneic transplant, imatinib in conjunction with donor lymphocyte infusion has also been shown to be synergistic, implicating a beneficial role on the immune arm in the eradication of the disease [11-13]. Furthermore, vaccination of patients with CML under imatinib therapy has generated a specific immune response toward BCR-ABL-specific peptides [14]. Moreover, recent reports demonstrated an *in vivo* clonal expansion of lymphocytes with large granular lymphocyte (LGL) morphologic features in patients being treated with dasatinib [15-18]. Intriguingly, these lymphocyte proliferations are seen in some cases with fevers, colitis, and pleural effusions, suggesting an aberrant immune response mediated by these cytotoxic cells [15,19-22].

To further understand the immunological effects of TKIs, we investigated the lymphocyte populations of CML patients receiving FDA-approved TKI therapy (imatinib, nilotinib, dasatinib). Our results unveiled an increase in memory T lymphocytes characterized by expansion of CTL clones that were dominant within the respective T cell receptor (TCR) beta-chain variable (V β) family. Further analysis of T cell phenotype revealed in some cases LGL expansions, which correlated to specific TKI therapy and may be perpetuated by increased IL-15 transpresentation, serum PDGF BB levels, NF- κ B, and T-bet activity. LGLs from CML patients treated with TKIs were able to induce a significant cytotoxicity against an NK-resistant endothelial cell line. Further analysis of patient history revealed, in some cases, certain side effects correlated to dasatinib therapy and likely LGL proliferation. These observations were supported by our findings that indicated lower levels of circulating T-regulatory cells.

MATERIALS AND METHODS

Patients

Plasma and peripheral blood mononuclear cells (PBMCs) were obtained from 59 patients with CML (28 males and 31 females, mean age 50 years, median 51 years, range 17-76 years). At the time of analysis, 30 patients were receiving imatinib therapy, 15 were treated with dasatinib, 14 with nilotinib therapy; all patients included in the study received TKI therapy for at least 6 months before blood draw. Additional samples were obtained from 4 newly diagnosed patients. All subjects gave written Institutional Review Board-approved informed consent for their blood products to be used for immunological research. Blood was collected at the Moffitt Cancer Center (Tampa, FL), plasma was stored in aliquots at -80°C until used, and PBMCs were stored at -140°C until used.

PDGF-BB Enzyme-linked Immunosorbent Assay

Enzyme-linked immunosorbent assay (ELISA) was performed using plasma obtained from the resulting undiluted top fraction of whole blood isolated centrifugally on Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden); 96-well EIA/RIA flat-bottom plates were coated overnight with recombinant human PDGF R β /Fc Chimera capture antibody (385-PR/CF, R&D Systems, Minneapolis, MN) at 10 $\mu\text{g}/\text{mL}$ with 100 μL per well. Plates were washed with PBS and blocked. Undiluted and serially diluted plasma was used in conjunction with a standard of recombinant human PDGF BB (220-BB-010, R&D Systems) serially diluted from 2000 to 16 pg/mL . A biotinylated, PDGF BB polyclonal detection antibody (BAF220, R&D Systems) was used in conjunction with streptavidin-horseradish peroxidase and SureBlue TMB microwell peroxidase substrate (1-component) (52-00-01 KPL, Kirkegaard & Perry Laboratories, Gaithersburg, MD).

T-cell Receptor Beta-Chain Variable Region (V- β)

Total cellular DNA was extracted, isolated, and subjected to PCR amplification with a series of primers specific for the TCR genes in multiplex amplification. DNA fragments were fluorescently labeled, separated in polyacrylamide, and identified by capillary electrophoresis on the ABI 3130. Clonal peaks are generally identified as a 3- to 5-fold prominence over the background polyclonal pattern.

Flow Cytometry Immunophenotyping

Flow cytometric analysis of PBMCs was performed using fluorochrome-labeled monoclonal antibodies (anti-CD3, -CD4, -CD8, -CD25, -CD45RA, -CD57, -CD62L, -CD127, and IL-15; Becton Dickinson, San Jose, CA, and eBiosciences, San Diego, CA) and the vitality dye 4',6-diamidino-2-phenylindole (Sigma). Data were acquired on an LSRII cytometer (Beckman Coulter, Fullerton, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

CRL2598 Cytotoxicity Assay

We isolated CD8 $^{+}$ cells from CML patients with LGL-like expansion with or without TCR clonality regardless of the TKI therapy using a CD8 $^{+}$ positive selection kit (130-045-201,

Miltenyi, Bergisch Gladbach, Germany). Cells (at an effector-to-target ratio of 50:1; 5×10^5 CD8⁺ cells/well to 1×10^4 CRL2598 cells/well) were incubated at 37°C for 5 hours in phenol red-free RPMI (11835, GIBCO Invitrogen, Carlsbad, CA). Cytotoxicity was measured by using a CytoTox 96 non-radioactive cytotoxicity assay (G1781, Promega, Madison, WI) and following established protocols. The assay was performed as established by Chen et al. [23].

Target CRL2598 cells, a kind gift from Dr. Sheng Wei, is a pulmonary artery endothelial cell line is particularly sensitive to lysis from CD8⁺ LGL cells yet highly resistant to lysis by allogenic NK cells from healthy donors[23].

DNA Binding ELISA for Activated NF- κ B Family or T-bet

Nuclear fractions from CD8⁺-selected (130-045-201 Miltenyi) CML patients and from donor control PBMCs were obtained using a nuclear extraction kit and following the manufacturer's protocol (40010, Active Motif, Carlsbad, CA). Protein concentrations of the nuclear fractions were determined using a standard Bradford reagent protocol. Each patient sample was performed in duplicate, using 1 or 0.5 μ g of nuclear extract for each test. We used the manufacturer's protocol to determine concentration of activated transcription factor (for T-bet: 51396 TransAM T-bet Active Motif; for NF- κ B family: 43296 TransAM NF- κ B family active motif).

RESULTS

TKI Therapy Increases the Percentage of Effector and Terminal Memory Lymphocytes Within Peripheral Blood of CML Patients

We have shown here that TKIs can shift the peripheral T-lymphocyte pool toward memory and away from naïve compartments. To elucidate the TKI role in immunoregulation, we analyzed the T-lymphocyte subpopulations *in vivo*. In brief, we analyzed the naïve (CD62L⁺ CD45RA⁻), central memory (CD62L⁺ CD45RA⁺), effector memory (CD62L⁻ CD45RA⁺), and terminal memory (CD62L⁻ CD45RA⁻) T-lymphocyte compartments via flow cytometry. The percentage of each lymphocyte pool was calculated for both CD4⁺ and CD8⁺ T-cells from patients under TKI therapy and from a set of age-matched healthy donor control patients (Figure 1). Results confirmed that TKIs affect the CD4⁺ memory subset with a reduction in the central memory or naïve and a shift toward effector and terminal memory. A similar trend was observed in CD8⁺ T-cells; however, imatinib was the only TKI that significantly increased the percentage of CD8⁺ T-cells within the terminal memory subset.

T Cell Clonality and Specific Lymphocyte Expansions Can Be Found in the Peripheral Blood of Patients Receiving TKIs

Recent evidence has shed light on the potential for clonal expansion of LGLs in specific patients treated with dasatinib who experienced specific side effects [15-18]. After validating the memory shift in the circulating lymphocyte pool of randomly selected patients, we wanted to further investigate the potential expansion of T cell clones that were dominant within the respective TCR V β family. Using PCR-based clonality testing, we

identified multiple TKI therapy patients with significant clonal expansions of a single V β receptor (Table I). Our results showed that 44% (7 of 16) of dasatinib, 46% (12 of 26) of imatinib, and 33% (4 of 12) of nilotinib treated patients had dominant clonal expansions of the T-lymphocyte repertoire. Of the two untreated CML control patients tested, neither exhibited clonal expansions.

To better correlate the lymphocyte expansions to treatment with TKIs, we sequentially examined two dasatinib-treated patients (patients 13 and 6, Table I and Figure 2). Shortly after initiation of TKI therapy, both of these patients displayed episodic lymphocyte expansions, characterized primarily by NK or CD8⁺ T lymphocytes. Interestingly, these lymphocyte expansions correlated with clinically noted side effects, including pleural effusions, colitis, and fevers.

Given the aforementioned evidence regarding expansion of LGLs in dasatinib-treated patients, we measured the circulating LGLs CD3⁺CD57⁺ (upper limit of normal range = 500 cells/ μ l) and CD3⁺CD8⁺CD57⁺ (upper limit of normal range = 300 cells/ μ l) in peripheral circulation as well as LGL morphology, which was confirmed using Giemsa-stained peripheral blood smear (data not shown). Our data showed that 31% (5 of 16) of dasatinib-treated patients had LGL expansions, whereas the likelihood of such expansions under other TKI treatments was relatively low: 3% (1 of 29) of imatinib-treated and 14% (2 of 14) of nilotinib-treated patients. The odds ratio of identifying LGL expansions in dasatinib-treated patients was 10.5 (95% confidence interval 1.8 to 62.0), compared with imatinib-treated patients.

TKI Therapy Recapitulates Many of the Survival Factors Considered Necessary for LGL Survival and Expansion

Recent efforts in bioinformatics and literature compilation have verified IL-15 transpresentation, PDGF BB signaling, and NF- κ B and T-bet activation as critical factors in the survival and expansion of leukemic LGLs [24]. Although the LGL expansions that we have identified in our current and prior studies do not persist after discontinuation of therapy and therefore cannot be considered LGL leukemia, we wanted to know whether similar survival factors were transiently induced by certain TKIs[15].

We first investigated the possibility that deregulation of IL-15 transpresentation may be responsible for the increased T cell memory population and clonal lymphocyte expansions observed in CML patients receiving TKIs. As shown in Figure 3A, the percentage of CD3⁺CD57⁺ cells with transpresentation of IL-15 in patients treated with dasatinib and imatinib ($p < 0.05$ and $p < 0.001$, respectively) was markedly increased, with a lesser extent in those treated with nilotinib, compared to that shown in age-matched healthy donor controls. It should be noted that although the levels of CD3⁺CD57⁺ cells found in imatinib-treated patients are lower the overall proportion with transpresentation of IL-15 is markedly higher. The CD3⁺CD57⁺ cells were only part of our comprehensive analysis, and no other lymphocyte lineage transpresented IL-15 on their surface at elevated levels (data not shown).

We also tested plasma levels of PDGF BB, a known LGL survival signal, to investigate its possible role in these T cell expansions. We found that patients who received imatinib and dasatinib had particularly high PDGF BB levels (2000 to 3000 pg/ml) (Figure 3B). High levels were not seen in nilotinib-treated patients, untreated CML patients, or age-matched healthy donor controls, indicating that these factors may be specific to the kinase inhibition profile of imatinib and dasatinib. Increased plasma levels of PDGF BB could be attributed to inhibition of kinases involved in PDGFR α/β phosphorylation. However, in light of the IC₅₀s of PDGFR α/β phosphorylation (71nM imatinib, 74nM nilotinib and 28nM dasatinib) and the fact that plasma PDGF BB levels do not correlate, we find this mechanism unlikely [25,26].

Finally, we study the transcription factor activation in CD8 T lymphocytes. Using an ELISA-based transcription factor activation analysis, we assayed basal activation of NF- κ B and T-bet in purified CD8 T cells from CML patients under TKI therapy (Table II). Our results revealed that two of three tested patients with LGL expansions and TCR clonality had a basal NF- κ B and T-bet activation. Unfortunately, because of the large amount of purified CD8 cells and nuclear extract, the number of patients that we were able to investigate was limited. However, serum IL-2 has been shown to inversely correlate to T-bet activation in the context of LGL leukemia; thus we decided to examine plasma levels of IL-2 as a potential surrogate for T-bet activation [24,27,28]. Our analysis revealed that IL-2 levels were generally lower in patients treated with TKIs than in untreated CML patients; this trend was significant in patients treated with dasatinib and imatinib (Figure 3C). In addition, IL-15 is a direct transcriptional target of NF- κ B (p65/p60); thus our data on IL-15 transpresentation indirectly confirm our results on NF- κ B activation.

Taken together, these data implicate that a deregulation of IL-15 transpresentation in the NK-T-cell lineage, an increased PDGF BB signaling, and a NF- κ B and T-bet activation in the CD8 T cell lineage may coordinate to result in the observed increase in clonal LGL expansion in certain patients (Figure 5). Our data also indicate that dasatinib is more likely to coordinately align these multiple molecular factors in an LGL favorable manner, a result that supports our clinical observations.

TKIs Significantly Reduce T-Regulatory Cell Levels in Peripheral Blood of CML Patients

Our analysis of CML patient lymphocytes, thus far, has elucidated a possible cellular mechanism underlying the proliferation of clonal LGLs. However, our evidence provides little mechanism for the evasion of immune tolerance, a proposed immunomodulatory activity of TKIs, and a purported cause of immune-mediated side effects induced by dasatinib[29]. It has been well demonstrated that T-regulatory cells potently suppress anti-self and anti-leukemic immune responses[30]. Thus, in an effort to elucidate a cellular mechanism, we evaluated the percentage of T-regulatory (CD25^{hi}CD4⁺CD127^{lo}) cells in the peripheral blood of CML patients receiving dasatinib, imatinib, or nilotinib and compared these percentages to those of healthy age-matched blood donor controls. Our analysis revealed significantly reduced levels of T-regulatory cells in nilotinib- and dasatinib-treated patients (Figure 4A). Although we observed a decreased percentage of T-regulatory cells in the peripheral circulation of imatinib-treated patients, our data did not

demonstrate statistical significance. While it would have been ideal to compare T-regulatory populations to age-matched untreated CML patients, our analysis was limited to the use of healthy donors due to the rarity of such samples.

These results imply that the expansion of the T cell clones may be enhanced by the decreased percentage of T-regulatory cells that normally stem the expansion of autoreactive T effector cells.

TKI Therapy Is Associated with Proliferation of Cytotoxic CD8 T Cells

Our data indicate that clonal expansions of T cells often dominate the TCR V β repertoire in patients treated with various TKIs. Moreover, our analyses of clinical data combined with our investigation of cellular signaling networks indicate that dasatinib can induce transient LGL proliferation in 31% of patients. Although this proliferation accompanies decreased levels of T-regulatory cells, it is still unclear if the proliferation of autoreactive T cell clones is responsible for the observed immune mediated side effects. LGL leukemia can be associated with pulmonary artery hypertension (PAH) and these T cell have acquired the ability to directly lyse endothelial cells [23]. PAH is also been described in CML patients under dasatinib who had pleural effusions [17,21]. To further investigate this potential, we assayed CD8 T cell cytotoxicity against CRL2598 cells, a human pulmonary artery endothelial cell line in one nilotinib- and four dasatinib-treated patients (Figure 4B). It has been previously demonstrated that the CRL2598 cell line is resistant to allogenic NK cell cytotoxicity, yet susceptible to lysis by autoreactive LGL cells[23]. Our analysis revealed significant increases in endothelial cytotoxicity in dasatinib- and nilotinib-treated patients (30-60%) compared to results shown in healthy donor control or untreated CML patients (5-20%). CML patients under TKI therapy that had high cytotoxicity also maintained clonal LGL expansions.

Taken together, our data suggest a model for LGL expansion in which TKI therapy under a variety of unknown circumstances creates an ideal microenvironment for potentially autoreactive clonal T lymphocytes to expand unabated. This signaling is predicated upon IL-15 transpresentation, increased serum PDGF BB, NF- κ B, and T-bet activation, and the lack of T-regulatory cell supervision (Figure 5).

DISCUSSION

In this study, we report the presence and clonal expansion of T cells in CML patients who underwent TKI therapy. In certain dasatinib-treated patients, this can manifest itself as an LGL expansion and could be responsible for some of side effects seen with this drug. In our studies the LGL clonal expansions accompany the increased transpresentation of IL-15 by T cells, plasma levels of PDGF BB, NF- κ B and T-bet signaling; all of these molecular signals have been positively associated with the expansion and survival of LGL leukemic cells in prior independent studies[24]. Moreover, we observed a decreased T-regulatory cell presence and an increased CD8 cytotoxic potential *in vitro*, supporting the possible relation with immune-mediated side effects. We hypothesize that autoreactive LGL expansions occur when PDGF BB, NF- κ B, T-Bet, and IL-15 signaling simultaneously persists in the absence of T regulator cells, a condition which was only observed in dasatinib treated

patients. The observed shift toward a memory phenotype indicates cognate antigen education/reencounter and subsequent proliferation of naïve or central memory cytotoxic and helper T-cells. Presumably, these lymphocytes then join the effector and terminal memory pools, resulting in a shift of overall peripheral blood phenotype. Although we had comprehensive longitudinal data on two dasatinib-treated patients, we were unable to obtain such data on other patients. We plan to closely monitor current and future CML patients so that such experiments can be conducted.

Similar cytotoxic T cell clonal expansions are frequently seen as an immune response against viruses and in hematological conditions such as acute promyelocytic leukemia, multiple myeloma, or myelodysplastic syndrome [31-35]. In multiple myeloma, these expansions were associated with superior prognosis. A recent report in CML also found an association between clonal lymphocytosis and durable responses in patients with advanced and poor prognostic Ph+ leukemia [15]. Based on this observation, it is possible that anergic leukemic-specific T cell clones can expanded under certain TKIs and other unknown conditions. These T cell clones could theoretically cross-react against normal tissues and produce some side effects. We could not find in our patients any association between the TCR clonality, LGL expansion, and response to therapy, but most of our cases were in chronic phase. Interestingly, the presence of clonal TCR rearrangements has been recently described in more than 80% of CML patients at diagnosis as well as in patients with lymphocytosis under dasatinib therapy, although this is the first time other TKI therapies have been shown to have this effect. [15,16].

The relatively recent development of targeted small molecule therapy has led to promising results. Despite their remarkable clinical success, these inhibitors tend to possess wide-spectrum kinase inhibition profiles leading to pleiotropic effects. This is especially true for dasatinib because of its Src and Tec kinase inhibitory capacity and their known important roles in the immune responses. Unintended kinase inhibition leads to a variety of unknown cellular side-effects, only a minority of which can be clinically appreciated, such as hypophosphatemia [36]. Recently published clinical data found that a history of maculopapular rash while receiving imatinib was statistically associated with an increased risk of developing immune-mediated side-effects while taking dasatinib, indicating that TKIs may activate an underlying genetic proclivity toward formation of immune-mediated side effects [22].

In vitro data generated within the past decade indicate potential inhibitory effect of TKIs on immune system activation and proliferation [5-8,37,38]. Interestingly however, the immunosuppressive effects of TKIs are quite minor from a clinical point of view and opportunistic infections are relatively rare. It has been hypothesized that the blatant discrepancy between data indicating immune cell enhancement and data indicating immune cell suppression may be resolved by a better understanding of the off-target inhibitory effects of various TKIs. It is possible that an alternative, non-TCR-dependent pathway, such as the IL-2R-mediated pathway, may circumvent the reported inhibitory effects of TKIs in certain subpopulation of T/NK cells. Our data provide new evidence supporting an alternative activation signal in lymphocyte activation, especially since IL-15 signals are transmitted to lymphocytes via IL-2R. In the presence of lowered proportions of T-

regulatory cells, this may increase the likelihood of developing immune-mediated side effects.

Our research indicates that TKIs may have a greater impact on T-regulatory cell numbers, skewing the balance of T-cell suppression toward activation and proliferation. This association might be reinforced by the fact that TEC kinase and Bruton agammaglobulinemia tyrosine kinase, both targets of TKIs, are differentially involved in the signaling of various T-lymphocyte lineages [39,40]. One particular study found that these immunosuppressive effects were most potent on the naïve T-cell subsets, something that could explain the memory phenotype polarization observed in our investigation [6]. Although the exact mechanism by which T-regulatory cells are preferentially suppressed remains unclear, our research has unveiled that other TKIs, besides dasatinib, also have an effect on the T-regulatory cells and may be useful for future accompanying immunotherapeutic strategies [15].

Our investigation identified an increase in IL-15 transpresentation via NK-T cells, elucidating a previously unknown effect of TKIs on the immune system. Conceivably, increased IL-15 transpresentation is responsible for eliciting oligoclonal lymphocyte expansions and immune-mediated phenomena observed in some dasatinib-treated patients. The straightforward concept implies that this result is due to the well-known NK and T-cell stimulatory activity of IL-15 signaling. In recent months, an alternative and potentially complementary hypothesis has arisen thanks to evidence indicating that dasatinib can increase the binding affinity of the TCR complex [41]. The researchers found that this effect peaked in low-affinity TCR T-cell clones, such as those recognizing prominent cancer or self antigens. In addition, it was found that cells treated with TKIs maintained a lower level of activation-induced cell death after tetramer staining. The *in vivo* implications of these TKI-mediated effects have yet to be tested; however, such studies imply that the immunosuppressive nature of TKIs may be limited to specific myeloid and lymphoid subsets.

PAH has been described in LGL leukemia patients and elegant studies conducted by our group related this phenomenon to the T cell ability to directly lyse endothelial cells [23]. PAH is also been described in CML patients under dasatinib who had pleural effusions [21]. Interestingly we have demonstrated an increased endothelial cytotoxicity by LGL cells in dasatinib- and one nilotinib-treated patient. Only one patient has pleural effusion in the past suggesting that other factors could to be involved.

In conclusion, our results identify clonal lymphocyte expansions in CML patients receiving a variety of TKIs. In the case of dasatinib, we find that this often results in LGL expansions, in some cases associated to immune-mediated side effects. In an effort to delineate these effects, we focused on known molecular and cellular survival factors for LGL leukemia, in the hopes that similar signals would provide the stimulus for clonal expansion of T lymphocytes in TKI-treated CML patients. Our results associated IL-15 transpresentation and serum PDGF BB levels with certain TKI treatments. In addition, we identified basal activation of NF- κ B and T-bet in CD8 T lymphocytes from treated CML patients, data corroborated by IL-2 downregulation and increased IL-15 signaling. Moreover, we

identified a cytotoxic effect on endothelial cells in the setting of a decrease in T-regulatory cell populations, which could perpetuate the autoimmune effects that we clinically identified and experimentally verified. All in all, our data correlate TKI therapy with multiple factors considered necessary for clonal lymphocyte expansion data, which should be considered a starting point for future research and high-power association studies.

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Figure 1. CD4+ and CD8+ T-cell memory repertoires shift toward an effector and terminal memory phenotype during treatment with various TKIs

TKI treated CML patient CD4+ or CD8+ memory subsets: naïve (CD45RA+CD62L+), central memory (CD45RA–CD62L+), terminal memory (CD45RA+CD62L–), and effector memory (CD45RA–CD62L–) were quantified. Error bars represent standard error of the mean. Student's t-test was performed to determine significance difference between age-matched healthy donor controls and CML patients. Outcomes represent response at the time of analysis.

A

B

Figure 2. Longitudinal analysis of 2 patients receiving dasatinib (patients 13 and 6) reveals NK and T cell lymphoproliferation and the presentation of adverse side effects
 A time course of lymphocyte populations is shown in conjunction with adverse side effects (indicted with arrows, panel A). Arrows indicate time point of initiation of dasatinib therapy. Expanded detailed lymphocyte count time course is displayed in panel B.

Figure 3. Several survival signals for large granular lymphocytes (LGLs) are present in CML patients undergoing TKI therapy

A, FACS analysis of surface IL-15 on the CD3+CD57+ population of 26 patients (9 imatinib-, 8 nilotinib-, and 9 dasatinib-treated, 2 with no TKI, in addition to 4 age-matched healthy donors). **B**, plasma from 29 patients with CML were subjected to PDGF BB-specific ELISA (4 control, 3 with no TKI, 12 imatinib-treated, 4 nilotinib-treated, and 10 dasatinib-treated). **C**, plasma from 29 patients with CML were subjected to IL-2 cytometric bead array (4 control patients, 3 with no TKI, 12 imatinib-treated, 4 nilotinib-

treated, and 10 dasatinib-treated). All experimental samples were tested in triplicate, and each experiment was performed at least two independent times. P values were determined by Students t-test.

Figure 4. TKI therapy significantly reduces regulatory T cell populations potentially leading to elevated endothelial cell cytotoxicity

A, FACS analysis of T-regulatory cells ($CD4+CD25^{\text{high}}CD127^{\text{lo}}$) from CML patients and healthy age-matched blood donor controls (6 control patients, 10 imatinib-treated, 8 nilotinib-treated, and 10 dasatinib-treated). Graph show the percentage of CD4+ T lymphocytes. **B**, Cytotoxicity of lymphocytes in CML patients with LGL phenotype was assayed by lysis of the LGL susceptible CRL2598 human endothelial cell line at a 50:1 effector-to-target ratio [23]. Experiments were repeated at least three independent times with

at least three replicates each for both CML patients and controls. Significance was assessed using Students t-test.

Figure 5. Potential cellular mechanism underlying the functional and molecular characteristics of LGL phenotype expansions in CML patients on TKI therapy

Our studies of CML patients receiving TKI therapy have elucidated modulation of IL-15 transpresentation, PDGF BB plasma levels, and NF-κB and T-bet activation. Each factor has been recently associated with the proliferation and survival of LGLs in leukemic patients. Dasatinib was the only drug consistently capable of eliciting LGL-like proliferations, which accompanied autoimmune-like side effects. Our data indicate that dasatinib was the only TKI tested that has the potential to modulate all factors tested in addition to lowering

regulatory T cell populations. Conceivably, a simultaneous initiation of these factors could result in the clinically observed side effects.

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Table I
Clinical characteristics of CML patients

		Disease Phase	Age, years	Sex	TCR Clonality	CD3+ CD57+ 500 cells/ul	CD3+ CD8+ CD57+ 300 cells/ul	Side Effects	Outcome
DASATINIB	1	CP	64	F	Pos	2669	1039	NC	MMR
	2	CP	65	F	Pos	1557	--	D(PE), N (R)	CMR
		CP	65	F	Pos	1024	--		
	3	BC	56	M	Pos	730	--	D(R)	PD
		BC	56	M	Pos	--	--	D(PE)	PD
		BC	56	M	--	2590	--		PD
	4	CP	51	M	Pos	380	314	NC	CMR
		CP	52	M	Pos	560	--		
	5	CP	48	F	Neg	615	--	I (R)	--
	6	CP	56	F	Pos	365	282	I(PE), D(PE)	CMR
	7	AP	42	M	Pos	164	69	NC	CCR
		AP	42	M	Pos	160	--		
	8	CP	52	M	Pos	219	--	NC	CCR
		CP	52	M	Pos	--	--		
	9	CP	36	M	Neg	135	--	I(R)	CCR
	10	CP	31	F	Neg	96	85	D(PE)	CCR
		CP	32	F	Neg	155	--		
	11	AP	41	M	--	180	140	NC	CCR
	12	CP	28	M	Neg	193	109	NC	PCR
		CP	29	M	Neg	255	--		
	13	CP	43	F	Neg	231	155	D(F,PE)	MMR
		CP	45	F	Neg	228	--		
	14	CP	53	M	--	80	48	NC	CMR
		CP	53	M	Neg	45	--		
		CP	54	M	Neg	35	--		
	15	CP	33	M	Neg	85	--	NC	CMR
	16	CP	49	F	Neg	--	--	NC	MMR
	17	CP	49	M	Neg	--	--	NC	PD
IMATINIB	1	CP	43	M	Pos	664	--	NC	PCR
	2	CP	63	F	Pos	94	43	NC	MMR
	3	CP	59	F	Pos	41	--	I(R)	--
	4	CP	66	F	Pos	--	--	I(R)	CCR
	5	CP	51	M	--	84	64	NC	MMR
		CP	53	M	Pos	139	--		
	6	CP	54	M	Pos	209	--	NC	CMR

		Disease Phase	Age, years	Sex	TCR Clonality	CD3+ CD57+ 500 cells/ul	CD3+ CD8+ CD57+ 300 cells/ul	Side Effects	Outcome
	7	CP	46	F	Pos	71	--	NC	MMR
		CP	47	F	Pos	--	--		
	8	CP	34	F	Pos	195	--	NC	CMR
		CP	34	F	Pos	339	--		
	9	CP	21	F	Pos	85	--	NC	MMR
	10	AP	49	M	Pos	100	--	NC	CMR
	11	CP	61	F	Pos	493	--	NC	MMR
	12	CP	31	F	Pos	296.5	--	NC	MCR
	13	CP	68	F	Neg	--	--	I(R)	CMR
	14	CP	55	F	--	37	29	NC	CMR
	15	CP	49	F	--	86	40	I(R)	MMR
	16	CP	35	M	--	160	135	NC	MMR
	17	CP	49	F	--	201	134	NC	CMR
	18	CP	65	F	--	100	69	NC	CMR
	19	CP	73	F	Neg	58	--	NC	CMR
	20	CP	61	M	--	--	--	NC	CMR
	21	CP	17	M	Neg	108	--	NC	CCR
	22	CP	42	M	Neg	68	--	NC	HR
	23	CP	75	M	Neg	175	--	D(PE)	MCR
	24	CP	27	F	Neg	55	--	NC	CMR
	25	CP	52	F	Neg	425	--	NC	CMR
	26	CP	64	M	Neg	30	--	NC	CMR
	27	CP	34	F	Neg	230	--	NC	MMR
	28	CP	71	F	Neg	140	--	I(PE)	MMR
	29	CP	71	M	Neg	50	--	NC	MMR
	30	CP	19	M	Neg	29	--	NC	CCR
	31	CP	67	F	Neg	--	--	NC	PCR
	32	CP	63	F	Neg	--	--	NC	CMR
NILOTINIB	1	CP	76	M	Pos	576	--	NC	CMR
	2	CP	69	F	Pos	170	130	I(R)	CMR
		CP	70	F	Neg	314	--		
	3	CP	56	F	Pos	43	18	N(R)	CMR
		CP	57	F	Neg	--	--		
	4	CP	66	M	Pos	420	--	NC	CCR
	5	CP	48	F	Neg	300		I(R)	CCR
	6	CP	46	M	Neg	513	335	NC	MMR
		CP	47	M	Neg	285	--		
	7	CP	44	M	Neg	126	55	NC	MMR

		Disease Phase	Age, years	Sex	TCR Clonality	CD3+ CD57+ 500 cells/ul	CD3+ CD8+ CD57+ 300 cells/ul	Side Effects	Outcome
	8	CP	51	F	--	250	186	NC	MMR
	9	CP	58	M	Neg	68	30	NC	MCR
		CP	60	M	Neg	40	--		
	10	CP	45	F	Neg	80	--	NC	MMR
	11	CP	54	F	--	212	92	NC	PCR
	12	CP	40	M	Neg	350	--	NC	MCR
	13	CP	44	M	Neg	98	--	NC	CCR
	14	CP	59	F	Neg	45	--	NC	CCR
No. Tx. CML Pt.	1	CP	36	F	--	468	202	NC	--
	2	CP	51	M	--	--	--	NC	--
	3	AP	60	M	--	361	162	NC	--
	4	CP	32	F	Neg	807	--	NC	--
	5	CP	65	F	Neg	--	--	NC	--

Peripheral blood mononuclear cells from CML patients were used to identify LGL phenotype and TCR clonality. Patient disease phase, characteristics, therapy, side effects, prior treatments, and clinical outcome are shown. Shaded areas show positive TCR, LGL, and imatinib rashes. R, rash; PE, pleural effusions; F, fever; C, colitis; NC, no relevant complications; N, occurred while on nilotinib; I, occurred while on imatinib; D, occurred while on dasatinib; CCR, complete cytogenetic response; CMR, complete molecular response; CHR, complete hematological response; PCR, partial cytogenetic response; MMR, major molecular response; PD, persistent disease; cells/ul, upper limit of normal number of cells per ul of whole blood.

Table II
Basal Activation of T-bet and NF- κ B

Treatment	Patient No.	T-bet	NF- κ B	TCR	Abnormal Phenotype
CML no treatment	1	–	+	–	None
Dasatinib	1	+	+	+	LGL-like
	2	–	+	+	LGL-like
	12	–	–	–	None
	13	–	+	–	NK-T Pheno
Imatinib	1	–	+	–	None
	4	–	–	+	Unknown
Nilotinib	1	+	+	+	LGL-like
	2	–	–	+	None

Transcription factors T-bet and NF- κ B were checked to determine if they were constitutively active in the CD8+ population of a representative subset of TKI-treated patients. A culmination of two experiments showing that only TCR positive and large granular lymphocyte (LGL)-like phenotype indicated above normal levels of T-bet and NF- κ B.