LETTER TO THE EDITOR

BCR–ABL-specific cytotoxic T cells in the bone marrow of patients with Ph+ acute lymphoblastic leukemia during second-generation tyrosine-kinase inhibitor therapy

The emergence of leukemia-specific cytotoxic T lymphocytes and their putative protective role against disease progression have recently been described in patients undergoing imatinib mesylate (IM) therapy, affected by either chronic myeloid leukemia or Philadelphia chromosome-positive acute lymphoblastic leukemia (Ph+ALL).[1,2] In particular, we have reported the immunological monitoring of 10 Ph+ALL patients during long-term IM maintenance treatment, demonstrating the inverse correlation between the frequencies of cytotoxic p190BCR–ABL-specific T cells in the bone marrow (BM) and minimal residual disease (MRD) values.[2] Whether similar autologous p190BCR–ABL-specific immune responses may be detectable and associated with the disease course under second-generation tyrosine-kinase inhibitors (TKIs), remains to be assessed.

To address this issue, we have prospectively analyzed five Ph+ALL patients treated with either dasatinib or nilotinib (Figure 1a). Patient 1 was a 60-year-old woman, who switched from a 3-years IM maintenance (800 mg/die) to dasatinib therapy (140 mg/die since June 2007; reduction to 100 mg/die since December 2007 and to 70 mg/die since October 2008), due to molecular relapse with fast-growing MRD values. The patient rapidly achieved a second complete molecular remission of the disease, but then (February 2009), she was switched to nilotinib (800 mg/die) because of recurrent episodes of severe fluid retention with pleural effusions. At present, she is well and still in complete molecular remission. Patient 2 was a 66-year-old woman, presenting a marked increment of the MRD on month 53 (January 2007) of a 4-years IM maintenance (800 mg/die). The patient was switched to dasatinib (140 mg/die; reduction to 100 mg/die since March 2007 and to 70 mg/die since November 2007) and achieved the control of the disease. Three years later (August 2010), she presented with severe cardiac toxicity and died of heart failure, still in complete hematologic remission. Patient 3 was a 66-year-old man, who was started on IM (800 mg/die) and steroids. The patient obtained complete molecular remission of leukemia and was subsequently maintained with IM alone (800 mg/die). Eventually, after 5 months (March 2010), the patient showed hematologic relapse and underwent therapy with dasatinib alone (140 mg/die; reduction to 100 mg/die since April 2010), obtaining a second complete hematologic remission of leukemia. The patient was lost in follow-up still in complete hematologic remission. Patients 4 and 5, a 57- and 56-year-old man, respectively, were treated upfront with dasatinib (140 mg/die; reduction to 100 mg/die since September 2007 and October 2007, respectively), obtaining complete hematologic remission. Both patients underwent allogeneic stem cell transplantation, because of either hematologic relapse or the progressive increase of MRD values, after 7 months (November 2007) and 8 months (January 2008) of treatment, respectively.

All patients underwent monthly collection of BM and peripheral blood samples (total of 59 samples) for either MRD or immunological monitoring (median follow-up time 11 months, range 8–43 months). The latter has been performed through an interferon-γ (IFNγ) enzyme-linked immunospot assay, using, as antigenic stimulation, different pools of short and long peptides (9–20 mers), deriving from the complete spanning of p190BCR–ABL protein fusion region, as previously described.[2] IFNγ-producing p190BCR–ABL-specific T cells were observed in five out of five (100%) Ph+ALL patients under second-generation TKIs. The median number of p190BCR–ABL-specific T cells resulted 54.7 ± 17.4 spot forming cells/10^6cells (range 35–78) upon nilotinib and 27.5 ± 28.4 spot forming cells/10^6cells (range 20–115) upon dasatinib, respectively. The comparison, through the median test, between the frequencies of anti-leukemic T cells detected upon dasatinib and nilotinib and those observed upon IM therapy (median spot forming cells/10^6 cells 70.3 ± 79.7; range 20–350) in the 10 Ph+ALL patients previously reported,[2] demonstrated statistical significant differences between IM and dasatinib ($P = 0.01$) and between nilotinib and dasatinib ($P = 0.003$), but not between IM and nilotinib ($P = 0.85$) (Figure 1b). Moreover, p190BCR–ABL-specific T cells were more frequently detectable in BM than peripheral blood samples (62.2 vs 20%, $P < 0.01$), as early as 45 days from the beginning of the treatment, and were associated with MRD values lower than 10^{-3}, in a statistically significant manner ($P < 0.001$). In addition, the dynamics of p190BCR–ABL-specific BM T cells appeared to be inversely correlated with the kinetics of MRD by showing the following: (a) increased or sustained number of specific T cells with either absent or decreasing MRD values (patients 1–3); (b) decreasing number or absence of specific T cells with growing MRD values (patients 2–5) or with hematologic relapse (patient 4) (Figure 1a).

Furthermore, we have functionally characterized the p190BCR–ABL-specific T cells through a cytokine secretion assay for IFNγ, tumor necrosis factor-α or interleukin-2, as previously described.[2] IFNγ-producing specific immune responses were confirmed in all patients (median percentage of positive cells: 1.48, range 0.1–2.8), mediated mainly by CD8+ T cells (median 0.86%) and, to a lower extent, by CD4+ T lymphocytes (median 0.62%). The former showed a predominantly effector memory (EM) phenotype (median EM/central memory frequencies 0.52%/0.34%), whereas the latter were either EM (0.33%) or central memory (0.29%) (Figure 1c). p190BCR–ABL-specific tumor necrosis factor-α-secreting T cells were also detected at similar frequencies (median % positive cells: 1.51, range 0.1–2.7), due to not only CD8+ lymphocytes, either EM (0.56%) or central memory (0.41%) subsets, but also to CD4+ T cells, predominantly of EM phenotype (median EM/central memory frequencies 0.43%/0.11%) (Figure 1c). Moreover, interleukin-2-producing specific T-cell responses were detected at lower frequencies (median % positive cells: 0.38, range 0.1–0.7) and almost equally distributed between CD8+ and CD4+ T lymphocytes, mostly of EM phenotype (Figure 1c).

In two out of three patients tested (patients 1, 3, 5), p190BCR–ABL-specific cytotoxic CD8+ and CD4+ T-cell subsets were expandable *ex-vivo* in short-term BM cultures, and were demonstrated to be able to specifically lyse p190BCR–ABL-positive
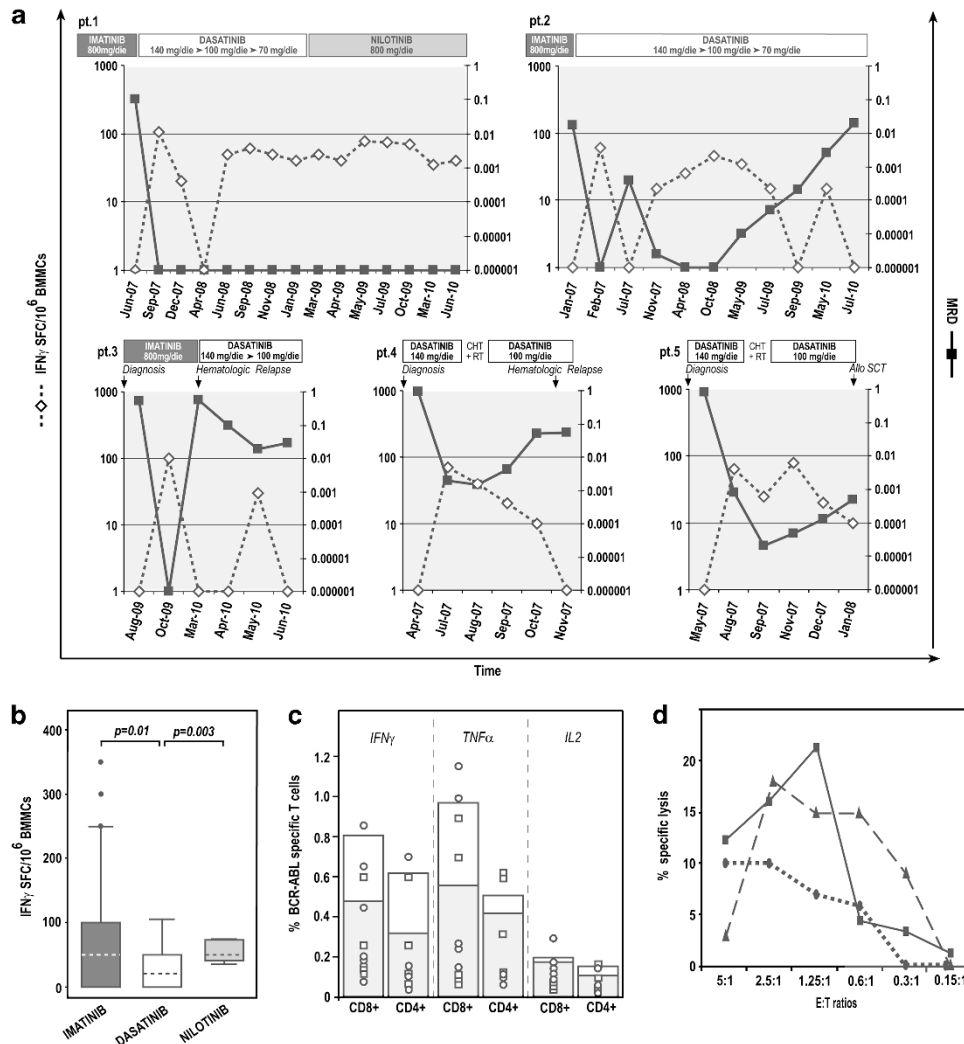
Figure 1 (a) Immunological monitoring of ^{p190}BCR–ABL-specific memory T-cell responses in the BM of five Ph $+$ ALL patients on TKIs therapy, and correlation with the disease course. Longitudinal data tracking MRD kinetics (right, y axis; continuous line with black squares) and IFNγ-producing ^{p190}BCR–ABL-specific T-cell responses as detected by the enzyme-linked immunospot assay (left, y axis, dotted line with white squares) are summarized in a single time-course graph (months, x axis) for each patient (patient (pt.) 1–5). Imatinib = dark gray bar; dasatinib = white bar; nilotinib = light gray bar. AlloSCT = allogeneic stem cell transplantation; BMMCs = bone marrow mononuclear cells; CHT = consolidation chemotherapy; IFNγ = interferon γ; RT = central nervous system radiotherapeutic prophylaxis; SFC = spot forming cells. (b) Median frequencies of ^{p190}BCR–ABL-specific T cells upon treatment with the different TKIs. Dashed lines represent the median values. The upper and lower hinges of the boxes represent the 75th percentile and the 25th percentile values, respectively. Dots represent outside values. Statistically significant differences are indicated. (c, d) Analysis of cytokine profile and cytolytic activity of ^{p190}BCR–ABL-specific BM T cells from the Ph $+$ ALL patients. (c) The frequencies of ^{p190}BCR–ABL specific BM T cells producing IFNγ, tumor necrosis factor (TNF)-α or interleukin-2, either as effector memory (CCR7$-$) (EM, gray) or central memory (CCR7 $+$) (CM, white), from all the five patients, are shown in columns as median percentage of positive cells, and expressed as percentages of CD8$^+$ or CD4$^+$ T cells. Single values of specific cytokine-producing T cells are represented on each column, either as EM ($\bigcirc$) or CM ($\square$). (d) Cytotoxicity profile of cultured BMMCs obtained from pt.1 under treatment with different TKIs. The figure reports percentage of specific lysis against autologous PHA blasts pulsed with ^{p190}BCR–ABL peptides of BMMC cultured for 13 days in the presence of ^{p190}BCR–ABL-derived peptides and obtained during imatinib (continuous black line with black squares), dasatinib (segmented black line with black triangles) and nilotinib treatment (dotted black line with black dots). The mean percentage of lysis of duplicate wells for six different effector to target (E:T) ratios is shown.

leukemic blasts or target cells pulsed with ^{p190}BCR–ABL-derived peptides, using standard ^{51}chromium-release cytotoxicity assays (specific lysis: 2500 and 5263 LU$_{10}$/10^5, respectively), performed as previously described.[2] Of note, in patient 1, similar levels of leukemia-specific lysis were shown by different ^{p190}BCR–ABL-specific cytotoxic T cells, derived from BM samples obtained during initial IM maintenance, later on, during dasatinib treatment, and, eventually, after the switch to nilotinib (Figure 1d).

In this work, by applying different antigen-specific immunological assays (namely, IFNγ-enzyme-linked immunospot,

cytokine secretion assay and ^{51}chromium-release cytotoxicity assays), we have demonstrated that ^{p190}BCR–ABL specific T-cell responses may occur in Ph $+$ALL patients also during second-generation TKIs therapy. Similarly to those previously observed under IM maintenance therapy in the same clinical setting,[2] such leukemia-specific T cells may emerge early in the course of the treatment and fluctuate or persist for several months, mainly reside in BM, may be maintained or recovered by switching from a TKI to another, are significantly associated with lower MRD values and able to either produce cytotoxic cytokines or mediate leukemia-specific lysis. Although the median frequencies

of the [p190]BCR–ABL-specific T cells seem higher upon IM and nilotinib than upon dasatinib, the number of patients studied is too small to draw any firm conclusion. Moreover, data from the literature about the effect of dasatinib on the immune system are still controversial. Several in vitro studies have reported that dasatinib, more than IM and nilotinib, may exert a suppressive activity on the development and function of adaptive immunity, due to the additional inhibition of the Src-family kinases.[3,4] Nonetheless, recent clinical observations have demonstrated that dasatinib treatment may induce chronic mono/oligoclonal large granular lymphocytes proliferations, of either natural killer or natural killer/T phenotype in the patients' peripheral blood, which are strongly associated with the improvement of patients' outcome.[5–7] Of note, none of our Ph[+]ALL patients under dasatinib developed clinically significant large granular lymphocytes proliferation in the peripheral blood, but an increase of the natural killer/T and natural killer subsets was observed in their BM (data not shown), consistently with the BM immune profile of chronic myeloid leukemia patients treated with TKIs, recently reported.[8]

The immunomodulatory effects of TKI treatment on generation, function and maintenance of anti-leukemic immunity are still largely unclear. Regarding to this issue, our data seem to suggest that long-term therapy with either first or second generation TKIs, despite prevalent inhibitory effects on T-cell proliferation and functions in vitro, may not exert the expected suppressive activity on patients' anti-leukemic immune cells. Furthermore, the continuous killing of Ph[+] blasts by TKI therapy could be functional to provide an optimal amount of tumor antigens, this having, in turn, a pivotal role for maintaining and shaping the protective specific T-cell repertoire. In our series of Ph[+]ALL patients, treated with either first[2] or second generation TKIs, the detection of [p190]BCR–ABL-specific T cells was significantly associated with lower levels of MRD. However, these specific cytotoxic T cells may likely constitute one subset of the total anti-leukemic immunity occurring in Ph[+]ALL patients, as other leukemia-associated antigens have been reported to be immunogenic targets in Ph[+]ALL in different therapeutic settings.[9,10]

In conclusion, in vivo data, both from this report and the literature, seem to suggest that, although preferentially prompting the innate immunity, also dasatinib, as well as the other TKIs, may allow the emergence of leukemia-specific T cells with direct anti-leukemic activity and, possibly, contributing to the control of the disease. Additional studies on larger cohorts of patients are warranted to confirm our findings, to define all the factors affecting the occurrence and persistence of [p190]BCR–ABL-specific immune responses, and to investigate whether such specific T cells or other specific immune responses to different leukemia-associated antigens may be harnessed either as further prognostic factors or as a novel source for autologous T-cell therapy in Ph[+]ALL patients, during TKIs maintenance treatment.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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