Differential inhibition of T-cell receptor and STAT5 signaling pathways determines the immunomodulatory effects of dasatinib in chronic phase chronic myeloid leukemia

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Abstract

Dasatinib is a multi-kinase inhibitor with activity against the SRC kinase LCK, which plays a critical role in T-cell receptor signaling. Dasatinib, initially developed as an immunosuppressive agent, is by contrast, also noted to result in enhanced tumor immunity in a subset of patients. We studied the impact of dasatinib in chronic myeloid leukemia patients and compared it with patients taking other tyrosine kinase inhibitors (TKI) and healthy controls. We found that patients on dasatinib showed inhibition of both T-cell receptor (TCR) and STAT5 signaling pathways, and reduced expression of T-effector pro-inflammatory cytokines. In addition, dasatinib induced selective depletion of regulatory T cells (Tregs) and effector Tregs, particularly in patients with clonal expansion of effector CD8+ T cells, who demonstrated greater and preferential inhibition of Treg TCR intracellular signaling. In addition, we show that dasatinib selectively reduces Treg STAT5 phosphorylation via reduction of IL-2, in relation with the marked reduction of plasma IL-2 levels in patients taking dasatinib. Finally, patients on other TKI had significantly increased TCR signaling in TIM3+ cells compared to patients taking dasatinib, suggesting that chronic SRC kinase inhibition by dasatinib may play a role in preventing TIM-3-mediated T-cell exhaustion and preserve anti-tumor immunity. These data provide further insight into the selective immunomodulatory effects of dasatinib and its potential use for pharmacologic control of immunotherapies..

Introduction

The second-generation tyrosine kinase inhibitor (TKI) dasatinib (Bristol Myers Squibb) is an effective treatment used in the management of patients with chronic myeloid leukemia (CML).[1-3] It is utilized both as a first-line agent and in patients resistant to other TKI, with an estimated 325-times greater affinity for BCR-ABL1 than imatinib.[4] Dasatinib is a multi-kinase inhibitor with activity against the SRC family kinase LCK, which is inhibited at nanomolar concentrations.[5] LCK plays a critical role in signaling from the T-cell receptor (TCR) and expression is largely restricted to T cells making it a promising target for suppression of T-cell activity.[6,7] Immediate downstream targets from LCK are the key signaling molecules ZAP70

and LAT, which have both been implicated in regulatory T-cell (Treg) development also. Several studies have previously reported on the inhibitory effect of dasatinib on the function of T effectors, with reduction in signaling, proliferation and expression of pro-inflammatory cytokine expression.[8-10] T-cell immunoglobulin domain and mucin domain 3 (TIM-3) is an immune-checkpoint molecule that has been proposed to promote T-cell exhaustion through initial enhancement of TCR signaling pathways.[11]

Tregs are a suppressive subset of CD4+ T cells, identifiable by high expression of the interleukin 2α (IL-2α) receptor CD25 and the transcription factor FOXP3.[12, 13] In vitro analyses of Tregs from healthy controls have shown that increasing concentrations of dasatinib result in reduced expression of FOXP3 and reduced Treg function.[14] Tregs

have been shown to be increased at diagnosis in CML patients, with subsequent reduction following effective TKI therapy.[15,16] They have also previously been shown to be increased in patients with advanced-phase disease and high-risk prognostic scores.[17,18] STAT5 is a critical signaling molecule that propagates cytokine responses and is the downstream target of IL-2. STAT5 signaling plays a critical role in Treg differentiation and the JAK2-STAT5 pathway plays a pivotal role in survival and proliferation of CML leukemic stem cells.

A subset of patients treated with dasatinib are observed to develop clonal large granular lymphocytosis (LGL) which is associated with immune-mediated toxicity and improved CML-related outcomes.[19-21] It has previously been shown that patients who develop LGL have a reduced frequency of Tregs.[22] We hypothesized that these patients have a reduction in both number and function of Tregs, resulting in increased toxicity but also allowing for enhancement of the anti-leukemic immune response. We hereby report findings from an *ex vivo* functional assessment of the effect of dasatinib on immune cell subsets including Tregs in CML patients.

Methods

Patients and controls

Twenty-five Patients with a World Health Organization-defined diagnosis of chronic phase CML (dasatinib n=18, nilotinib n=5, imatinib n=2) and seven healthy controls (HC) (median age 46 years, range, 31-78; 57% male) were recruited in accordance with the Regional Research and Ethics Review Board. Most patients had achieved major molecular response (MMR/MR3) or deeper at the time of inclusion and patient characteristics are summarized in the *Online Supplementary Table S1*. Patients administered TKI therapy at a uniform time on the morning of blood sampling. Peripheral blood mononucelar cells (PBMC) were isolated from venous blood samples using standard Ficoll density centrifugation and were cryopreserved in 30% dimethyl sulfoxide (DMSO) and fetal calf serum (FCS).

Intracellular phospho-specific flow cytometric assay

Phosphoflow cytometry was performed in Tregs, T effectors and natural killer (NK) cells to assess the effect of dasatinib on signaling downstream from the TCR and activating NK-cell receptors as well as cytokine signaling pathways. Briefly, cells were stained with a viability dye prior to activation with H_2O_2 (50 mM), due to its activity as a potent phosphatase inhibitor. Cells were fixed and permeabilized (BD Phosphoflow Buffer) prior to staining with antibodies directed against surface and intracellular markers (*Online Supplementary Appendix*). Cells were analyzed for phosphorylation of the signaling proteins ZAP70, LAT and STAT5. Flow cytometry analysis was performed on BD Fortessa and analysis was performed using FlowJo v10.3.

Intracellular cytokine flow cytometric assay

Intracellular flow cytometry was also performed to assess the impact of dasatinib on T-effector cytokine production including tumor necrosis factor α (TNFα), interferon γ (IFNγ), IL-2, IL-4 and IL-10, after stimulation with OKT3. Cells were thawed, then rested prior to adding OKT3 (BioLegend) and subsequently Brefeldin-A (BFA). Unstimulated cells were used as a negative control and PMA and ionomycin (Millipore) were used as a positive control. Cells were stained with a viability dye, then stained with antibodies directed against surface markers, and fixed and permeabilized (BD, CytoFix/Cytoperm) prior to staining with antibodies directed against intracellular cytokines.

In vitro dasatinib culture

In vitro analysis was performed on PBMC from HC (n=1 per experiment) using the above-mentioned techniques. Fresh cells were cultured alongside increasing concentrations of dasatinib (Cambridge Bioscience) in RPMI and 10% FCS for 3 hours. For phosphoflow assays, cells were stimulated with either H_2O_2 or IL-2 (Merck) for 15 minutes prior to fixation, permeabilization and staining with surface and intracellular antibodies.

Plasma cytokine analysis

Baseline cytokine levels were evaluated using the Luminex Flexmap3D platform with analysis performed on thawed plasma samples using the Thermofisher 9-Plex Human ProcartaPlex Panel as per manufacturer's instructions. Briefly, plasma samples were incubated with magnetic capture beads with specific spectral properties for 18 hours, prior to streptavidin-RPE being added followed by a biotinylated detection antibody. Data was acquired on the Luminex Flexmap3D.

Plasma dasatinib levels

Plasma or serum samples are prepared by methanol/acetonitrile protein precipitation followed by phospholipid depletion using phospholipid-removal plates (Phree, Phenomenex). Extracts were then analyzed directly by mass spectrometry using the Agilent 6460 mass spectrometer and Infinity II MultiSampler.

T-cell receptor clonality analysis

TCR clonality analysis was performed using the Biomed2 PCR protocol with primers analyzing both *TCR-B* and *TCR-G* genes for evidence of clonality on the CEQ-8000 Genetic Analysis System sequencer (Beckman Coulter, Brea), as described previously.[23]
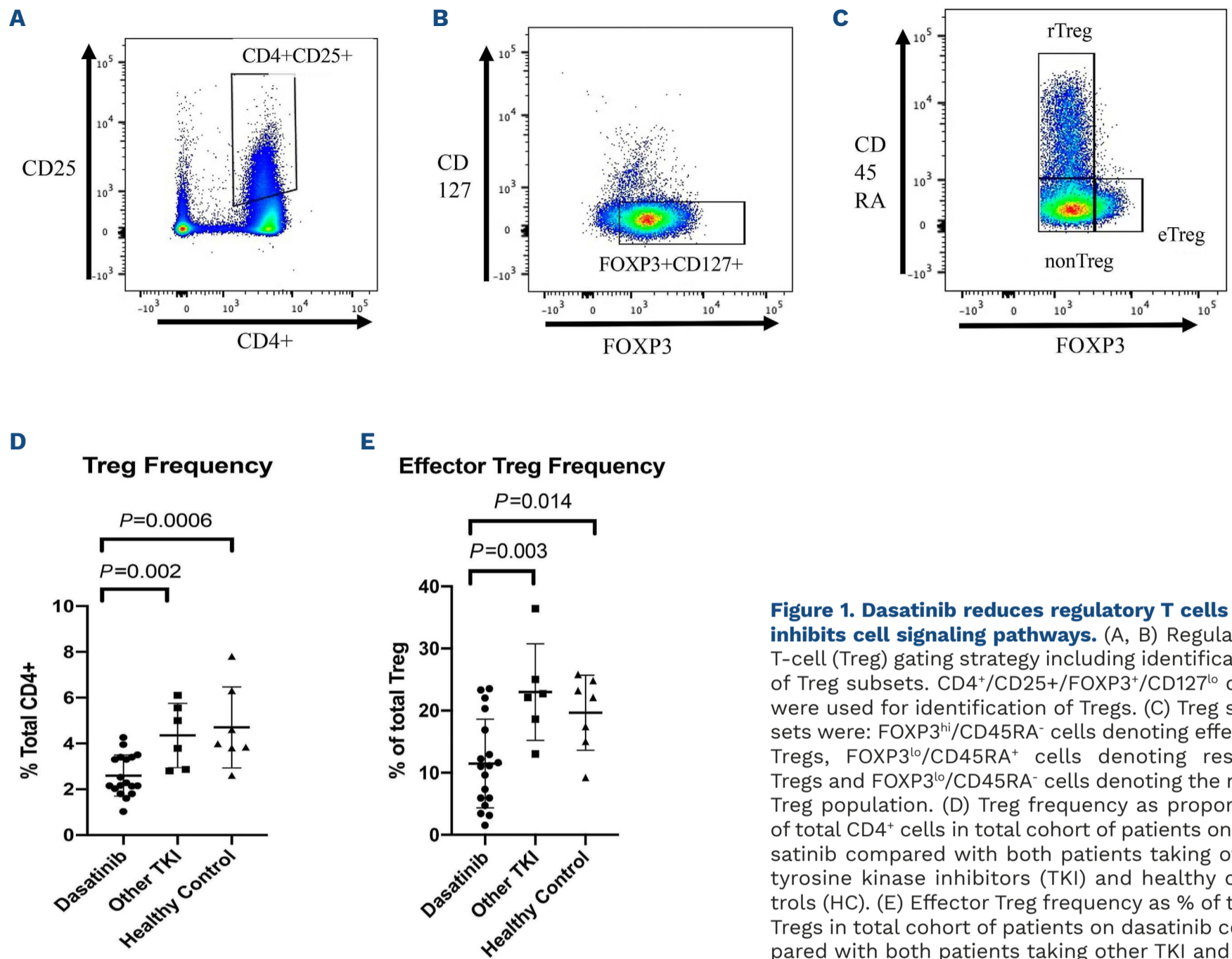
Figure 1. Dasatinib reduces regulatory T cells and inhibits cell signaling pathways. (A, B) Regulatory T-cell (Treg) gating strategy including identification of Treg subsets. CD4+/CD25+/FOXP3+/CD127lo cells were used for identification of Tregs. (C) Treg subsets were: FOXP3hi/CD45RA- cells denoting effector Tregs, FOXP3lo/CD45RA+ cells denoting resting Tregs and FOXP3lo/CD45RA- cells denoting the non-Treg population. (D) Treg frequency as proportion of total CD4+ cells in total cohort of patients on dasatinib compared with both patients taking other tyrosine kinase inhibitors (TKI) and healthy controls (HC). (E) Effector Treg frequency as % of total Tregs in total cohort of patients on dasatinib compared with both patients taking other TKI and HC.

Statistical analysis

Data was reported as mean values and *P* values were from independent sample *t*-tests, with values less than 0.05 considered statistically significant. Distribution of data was assessed using Levene's test for equality of variance. All reported *P* values are two-sided. Analyses were performed using SPSS version 24 (IBM, Armonk, NY) and Prism v8.
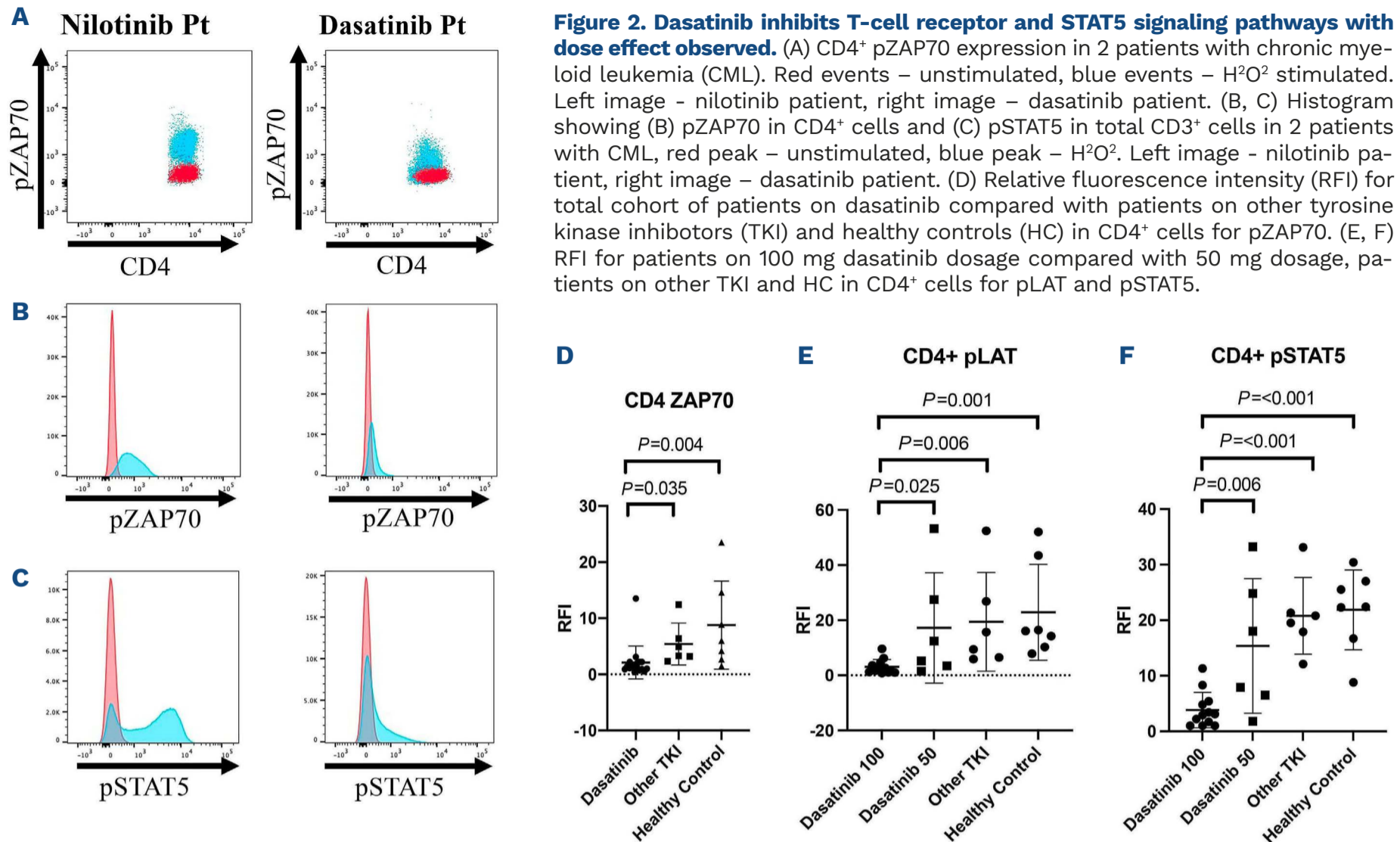
Results

Patients on dasatinib have reduced frequency of Tregs and effector Tregs

Patients on dasatinib had a lower proportion of Tregs compared with the patients taking other TKI and HC, with a mean proportion of Treg from total CD4+ cells of 2.6% in dasatinib-treated patients, 4.4% in CML patients on other TKI without significant SRC kinase inhibitory effects and 4.7% in HC (*P*=0.002, *P*=0.0006; Figure 1A-D). Dasatinib-treated patients also had lower ratio of effector Tregs/total Tregs than the other two groups at 11.5% ver-

sus 23% in patients on other TKI and 19.6% in HC (*P*=0.003 and *P*=0.014 respectively; Figure 1E).

Dasatinib inhibits T-cell receptor downstream signaling pathway compared to other tyrosine kinase inhibitors

In order to assess if TKI inhibit TCR downstream signaling, we examined their impact on phosphorylation of ZAP70 and LAT on gated T cells. We demonstrated a reduction in levels of pZAP70 in patients on dasatinib as assessed by relative fluorescence intensity (RFI, RFI=mean fluorescence intensity [MFI] of stimulated sample/MFI of unstimulated sample), compared with HC and patients taking non-SRC kinase inhibitor TKI in CD4+ cells, CD8+ cells and Tregs (Figure 2A, B). The mean RFI of pZAP70 was 2.1 in patients on dasatinib *versus* 8.8 in HC in CD4+ cells, 3.1 *versus* 11.4 in CD8+ cells and 2.1 *versus* 6.3 in Tregs (*P*=0.004, *P*=0.005, *P*=0.041) (Figure 2D). This was also lower in dasatinib patients compared with patients on other TKI, who had mean RFI of 5.4 in CD4+, 6.1 in CD8+ and 4.4 in Treg (*P*=0.035, *P*=0.093, *P*=0.176) (Figure 2D). Analysis of patients on 100 mg dosage of dasatinib

Figure 2. Dasatinib inhibits T-cell receptor and STAT5 signaling pathways with dose effect observed. (A) CD4^{+} pZAP70 expression in 2 patients with chronic myeloid leukemia (CML). Red events – unstimulated, blue events – H_2O_2 stimulated. Left image - nilotinib patient, right image – dasatinib patient. (B, C) Histogram showing (B) pZAP70 in CD4^{+} cells and (C) pSTAT5 in total CD3^{+} cells in 2 patients with CML, red peak – unstimulated, blue peak – H_2O_2. Left image - nilotinib patient, right image – dasatinib patient. (D) Relative fluorescence intensity (RFI) for total cohort of patients on dasatinib compared with patients on other tyrosine kinase inhibotors (TKI) and healthy controls (HC) in CD4^{+} cells for pZAP70. (E, F) RFI for patients on 100 mg dasatinib dosage compared with 50 mg dosage, patients on other TKI and HC in CD4^{+} cells for pLAT and pSTAT5.

showed greater inhibition of RFI compared with HC and patients on other TKI in CD4^{+} at 1.2, CD8^{+} 2.3 and Treg 1.2 (CD4^{+} HC *P*=0.004; other TKI *P*=0.001; CD8^{+} *P*=0.007, *P*=0.011; Treg *P*=0.01, *P*=0.005).

Similarly, the dasatinib group had lower mean RFI in all T-cell subsets evaluated for pLAT (Figure 1Gii). In CD4^{+} cells mean RFI was 7.8 *versus* 22.9 in healthy controls and 19.5 in other TKI, (*P*=0.026, *P*=0.097), in CD8^{+} 6.9 *versus* 15 *versus* 19.5 (*P*=0.012, *P*=0.14) and Tregs mean RFI was 5.3 *versus* 14.6 in HC and 14.7 in other TKI (*P*=0.05, *P*=0.037). There was no difference in levels of pLAT in Tregs between patients on other TKI and HC. Comparison of patients on 100 mg dosage of dasatinib and other TKI showed greater difference in RFI, in the 100 mg group mean RFI was 3.1 in CD4^{+} cells, four in CD8^{+} cells, and 2.7 in Tregs (CD4^{+} HC *P*=0.001; other TKI *P*=0.006; CD8^{+} *P*=<0.001, *P*=0.013; Treg *P*=0.005, *P*=0.01) (Figure 2E).

Dasatinib inhibits STAT5 signaling pathway compared to other tyrosine kinase inhibitors

Patients on dasatinib showed significantly reduced pSTAT5 compared to HC and patients on other TKI. In CD4^{+} cells mean RFI for pSTAT5 was reduced at 7.7 in the dasatinib group *versus* 21.9 in HC and 20.8 in patients on other TKI (*P*=0.001 and *P*=0.004, respectively). Similarly, in CD8^{+} cells pSTAT5 RFI was 10.1 *versus* 31.1 and 28.1, respectively (*P*<0.001 and *P*=0.002) and in Tregs 7.3 *versus*

21.9 and 19.1, respectively (*P*=0.005 and *P*=0.011) (Figure 2C). In patients on 100 mg dasatinib there was greater difference in pSTAT5 RFI when compared with HC and other TKI groups with mean RFI of 3.8 in CD4^{+}, 6.1 in CD8^{+} and 3.9 in Tregs (CD4^{+} HC *P*<0.001; other TKI *P*<0.001; HC *P*<0.001, other TKI *P*<0.001; Treg *P*<0.001; HC *P*<0.001) (Figure 2F). There was no difference in levels of pSTAT5 in Tregs between patients on other TKI and HC.

Dasatinib inhibits signaling pathways within natural killer cells

Signaling pathways within CD56^{+} NK cells were also inhibited in patients taking dasatinib when compared with HC. Patients on dasatinib had mean pZAP70 RFI within CD56^{+} cells of 4.6 compared with 17.8 in HC. Phosphorylated LAT and pSTAT5 were also reduced at 6.1 *versus* 33.5 and 15.2 *versus* 46.2 respectively (*P*=0.014, *P*=0.032 and *P*=0.008) (*Online Supplementary Figure S1*). Moreover, patients taking 100 mg dosage of dasatinib had a mean RFI for pZAP70 of 8.1 *versus* 3 in other TKI (*P*=0.008) and for pSTAT5 of 46.5 *versus* 8.2 (*P*<0.0001; *data not shown*).

Dasatinib reduces expression of pro-inflammatory cytokines in CD4^{+} and CD8^{+} cells, including interleukin 2

Patients on dasatinib had lower absolute increase in TNFα and IFNγ expression in CD4^{+} cells after activation with OKT3, when compared with patients on other TKI and HC.
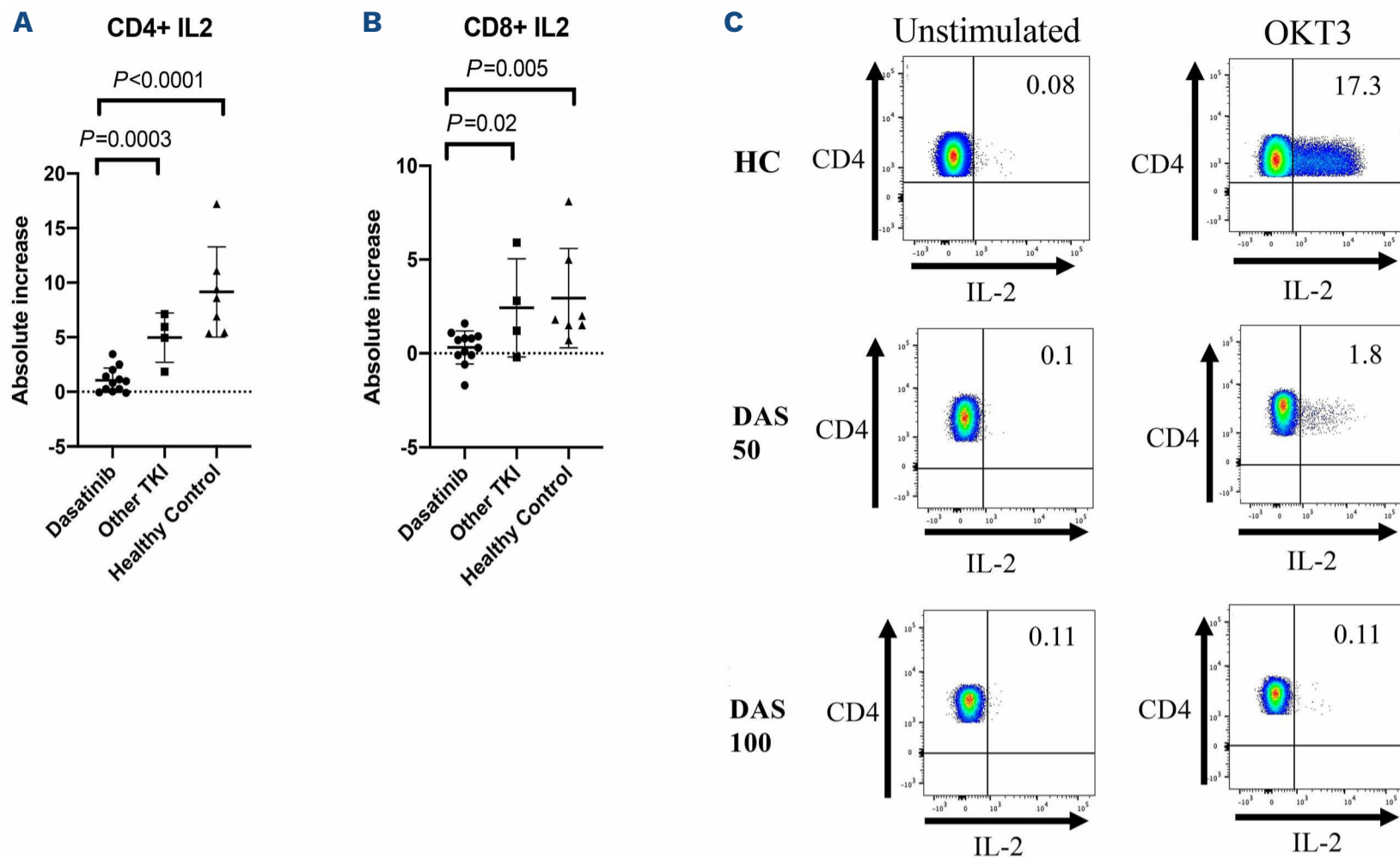
Figure 3. Dasatinib inhibits pro-inflammatory cytokines including interleukin 2. (A) Absolute increase in interleukin 2 (IL-2) expression in CD4^{+} cells (IL-2 expression in unstimulated sample subtracted from IL-2 expression in OKT3 stimulated sample). (B) Absolute increase in IL-2 expression in CD8^{+} cells. (C) IL-2 expression in CD4^{+} cells in 2 patients and a healthy control (HC). Left column – unstimulated, right column – OKT3. Top – HC, middle – intermediate dose (50 mg) dasatinib (DAS), bottom – full dose dasatinib (100 mg). TKI: tyrosine kinase inhibitor.

Mean absolute increase in the total dasatinib group was 9.5 for TNFα and 5.1 for IFNγ, compared with 25.7 and 16.3 for other TKI, and 26.8 and 16.9 for HC (*P*=0.027, *P*=0.028, *P*=0.001, *P*=0.007; *Online Supplementary Figure S2*).
The mean absolute increase in IL-2 expression was also lower in the dasatinib group in both CD4^{+} and CD8^{+} cells when compared with that of other TKI and HC. Mean absolute increase in CD4^{+} cells in dasatinib patients was 1.1, compared to 5 in other TKI and 9.1 in HC (*P*=0.0003, *P*<0.0001) (Figure 3A, C). Mean absolute increase in CD8^{+} cells in dasatinib patients was 0.3, compared to 2.4 in other TKI and 2.9 in HC (*P*=0.02, *P*=0.005) (Figure 3B).

Patients on reduced dasatinib dosage have preserved T-cell function

Six patients were managed with reduced dose dasatinib at a dose of 50 mg daily. These patients had significantly less inhibition of T-cell signaling within immune cell subsets when compared with those taking 100 mg daily. For pLAT RFI was higher at the 50 mg dosage in CD4^{+} cells at 17.2 *versus* 3.1, CD8^{+} T cells at 12.9 *versus* 4 and in Tregs at 10.4 *versus* 2.7 (*P*=0.025, *P*=0.083, *P*=0.049) (Figure 2E). Comparison of pSTAT5 between patients on different dosage also showed those on 50 mg dose to have significantly higher RFI than in those on the 100 mg dose within CD4^{+} cells at 15.4 *versus*

3.8 respectively, CD8^{+} cells at 18.1 *versus* 6.1 and within Tregs at 14 *versus* 3.9 (*P*=0.006, *P*=0.03, *P*=0.028) (Figure 2F).
Similarly, patients at the 50 mg dose had significantly higher proportional increase in CD8^{+} cell TNF expression at 6 *versus* 3 (*P*=0.044). Proportional increase in IL-2 expression was also significantly higher at the 50 mg dose in both CD4^{+} cells, at 24.3 *versus* 2.5, and CD8^{+} cells at 4.8 *versus* 1.5 (*P*=0.035, *P*=0.006). No significant differences were observed in the frequency of Tregs between patients on different dasatinib dosage.
Dasatinib plasma levels were measured in ten patients (100 mg dosage n=6, 50 mg dosage n=4). Patients on 50 mg dosage had levels of <5 µg/mL whilst patients on 100 mg had a mean level of 14.7 µg/L (range, 5-26 µg/L). We observed a moderate inverse correlation between dasatinib level and RFI for pZAP70, pLAT and pSTAT5 across immune cell subsets (*Online Supplementary Appendix; Online Supplementary Figure S3*).

Effect of dasatinib on T-cell signaling is reversible

One patient was analyzed at three time points due to the development of pleural effusion and brief drug discontinuation. This patient demonstrated strong inhibition of cell signaling at both time points when taking dasatinib for each phosphoprotein evaluated. However, when ana-
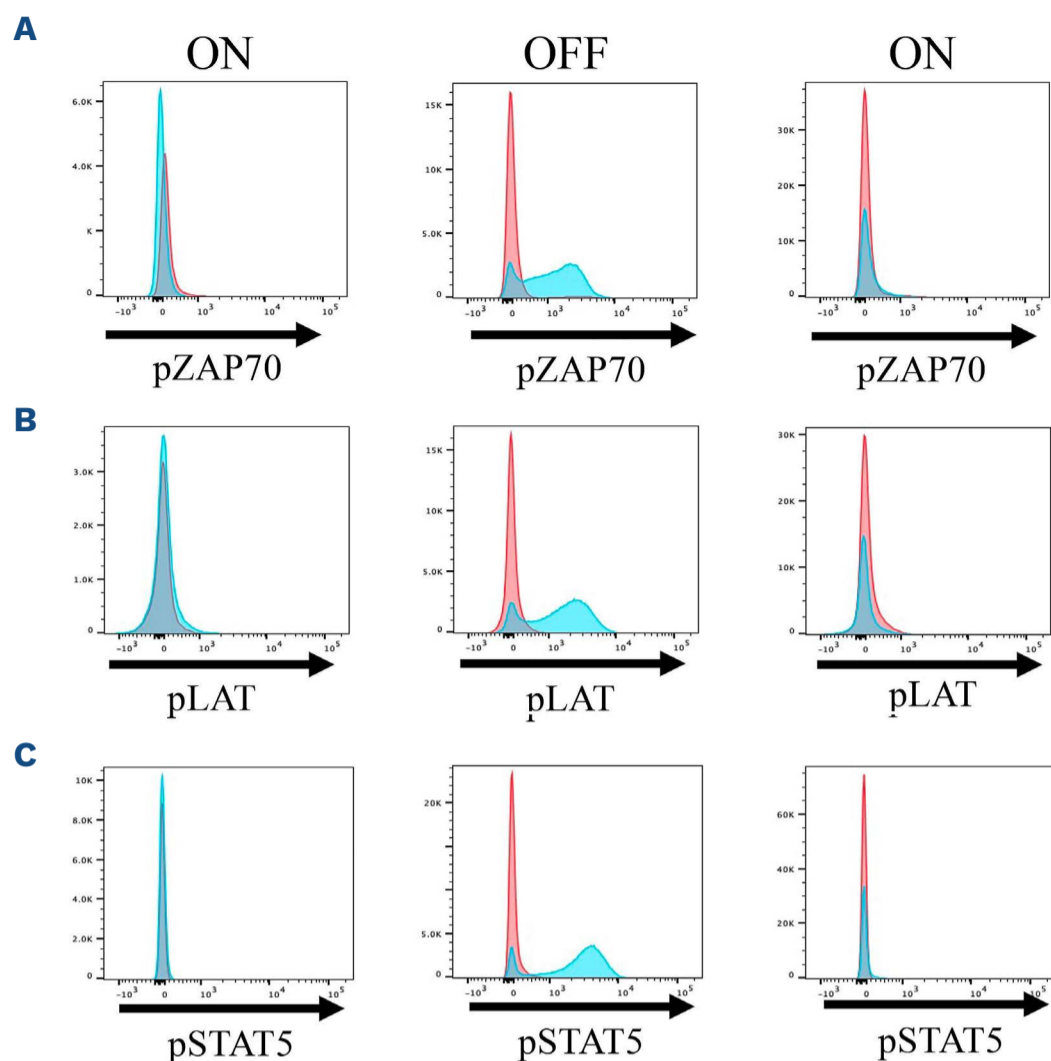
Figure 4. Patients on reduced dosage of dasatinib have preserved cell signaling. (A-C) Total CD3^{+} T cells show inhibition of pZAP70 expression when taking dasatinib (left and right plots), however, during treatment 'holiday' restoration of (D) ZAP70, (E) pLAT and (F) pSTAT5 phosphorylation is evident (middle plots).

lyzed 1 week following treatment discontinuation a significant increase in phosphorylation was noted for all signaling proteins evaluated and across immune cell subsets, with strong inhibition again observed upon analysis 1 week after treatment re-initiation (Figure 4A-C).

Dasatinib enhances clonal expansion of CD8^{+} T cells
TCR gene rearrangement analysis was performed in 16 patients taking dasatinib. We identified seven patients as having clonal T-cell populations (Figure 5A), with nine having a polyclonal pattern. Of the patients with confirmed TCR clonality there was a reversal of the normal CD4:CD8 ratio in six of the seven in keeping with expansion of a clonal CD8^{+} T-cell population. Of note, two of the patients with confirmed clonality were taking a reduced dosage of dasatinib at 50 mg orally once daily (OD).

Patients with clonal CD8^{+} lymphocytosis have greater inhibition of Treg intracellular signaling
Patient with clonal T-cell populations had a lower Treg proportion of total CD3^{+} cells when compared with other patients on dasatinib with a mean value of 0.9 *versus* 1.7 (*P*=0.018). Moreover, these patients also had a lower RFI for pSTAT5 within isolated Tregs when compared with other patients on dasatinib with polyclonal T cells (mean RFI 1.7 *vs.* 11.8; *P*=0.04) (Figure 5B, C). Patients with clonal T-cell populations also had greater inhibition of TCR signaling within Tregs compared with other patients on da-

satinib (mean RFI pZAP70 0.9 *vs.* 3.2; *P*=0.24, mean RFI pLAT 1.8 *vs.* 8.5; *P*=0.1).

Preferential inhibition of Tregs in patients on dasatinib
Patients on 100 mg dasatinib had significantly reduced pZAP70 RFI in Tregs compared to effector cells, with mean of 1.2 in Tregs compared with 2.3 in CD8^{+} cells and 3.0 in NK cells (*P*=0.059, *P*=0.019) (Figure 6A, B). This effect was particularly noted in patients with clonal CD8^{+} lymphocytosis (Figure 6A). In addition, patients on the 50 mg dosage of dasatinib had greater difference in pSTAT5 RFI between Tregs and CD8^{+} T cells and NK cells, again suggesting a relative sparing of effector immune-cell inhibition. The mean difference in pSTAT5 RFI between Tregs and CD8^{+} T cells was 0.7 for those on 100 mg dasatinib compared with 5.4 in those on 50 mg dasatinib (*P*=0.005) (Figure 6B). Similarly, the mean difference in pSTAT5 RFI between Tregs and NK cells was 3 in those on 100 mg dose compared to 14.6 in those on 50 mg (*P*=0.08) (Figure 6D).

Dasatinib reduces phosphorylation of STAT5 via reduction of interleukin 2
We next investigated the mechanism through which dasatinib exerts an inhibitory effect on Treg STAT5 signaling, performing experiments on Tregs isolated from HC treated with dasatinib. We compared H_2O_2 and IL-2 as alternate methods of cell stimulation for STAT5 signaling. Using a uniform concentration of IL-2 (2U) to stimulate cells there was no in-
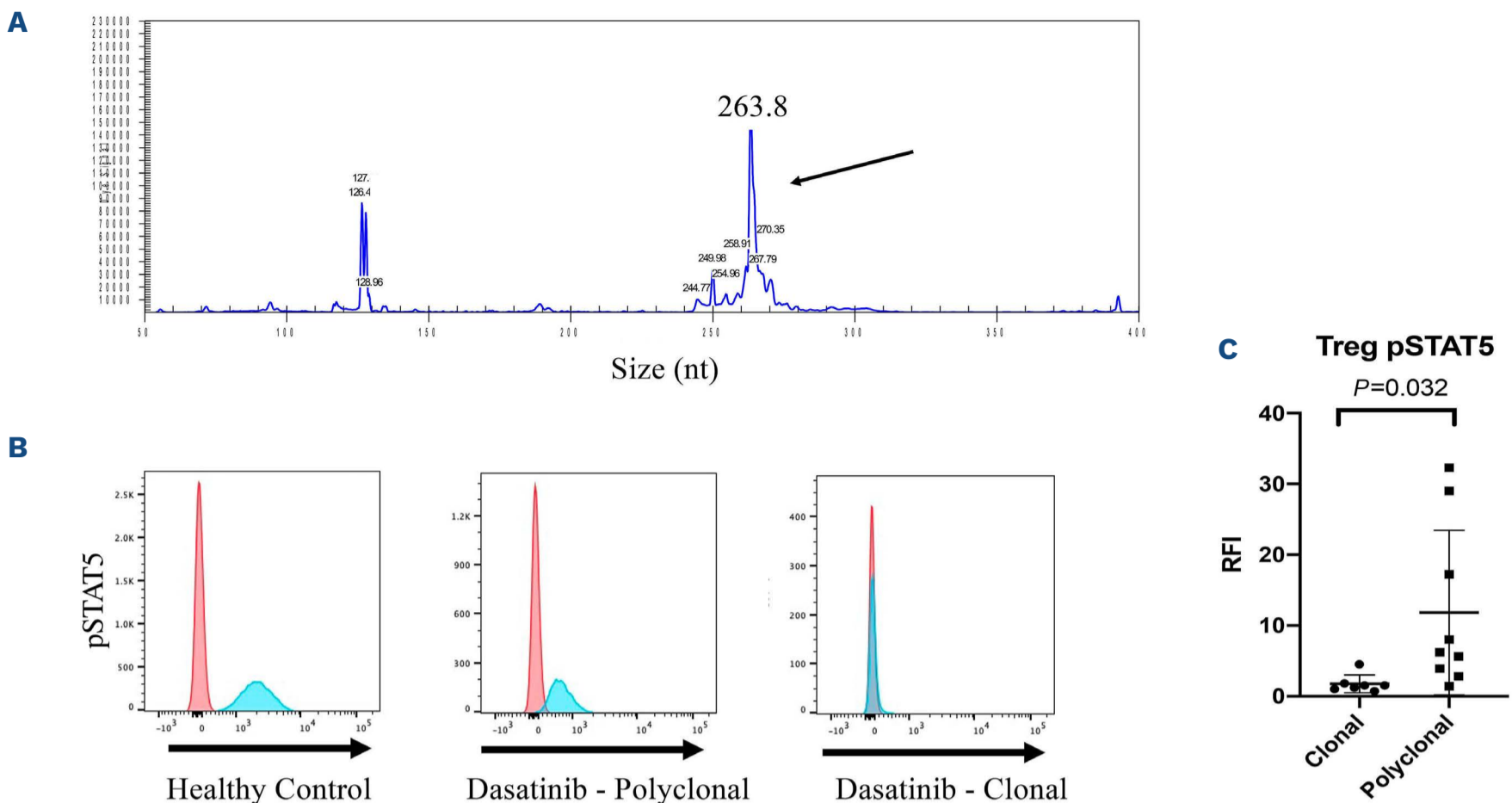
A

B

Healthy Control   Dasatinib - Polyclonal   Dasatinib - Clonal

C

Figure 5. Patients on dasatinib with clonal lymphocytosis have increased inhibition of regulatory T-cell signaling. (A) Representative T-cell receptor (TCR) clonality analyses showing peaks in *TCR B* gene consistent with clonal T-cell population, in patient treated with dasatinib. (B) Histogram showing regulatory T cells (Treg) pSTAT5 mean fluorescence intensity/relative fluorescence intensity (MFI/RFI). Left – healthy control, middle – dasatinib patient with polyclonal T cells, right – dasatinib patient with clonal CD8^{+} lymphocytosis. (C) Comparison of RFI for Treg pSTAT5 between patients on dasatinib with clonal CD8^{+} T-cell populations and those with polyclonal T cells.

hibitory effect on pSTAT5 from dasatinib, even at supra-therapeutic concentrations (Figure 7B, C). In contrast, there was clear and dose-dependent inhibition of pSTAT5 by dasatinib when cells were activated with H_2O_2 (Figure 7A, C) suggesting that inhibition is dependent on reduction in IL-2. Supporting evidence for the central role of IL-2 inhibition came from additional experiments investigating effects of dasatinib on T-effector pro-inflammatory cytokine expression. Cells from HC were cultured with increasing concentrations of dasatinib, and then stimulated with OKT3. Levels of TNF, IFN and IL-2 were reduced in dose-dependent manner in cells treated with both 100 nM and 500 nM concentrations of dasatinib, to levels approaching or less than the unstimulated control (Figure 7D-F).

Dasatinib reduces plasma levels of pro-inflammatory cytokines including interleukin 2

Plasma cytokine levels were evaluated in CML patients, including 11 taking dasatinib (100 mg dosage n=8, 50mg dosage n=3) and four taking nilotinib, and compared with six HC. In support of the above findings, most patients on dasatinib had undetectable levels of IL-2 (<0.571 pg/mL), significantly lower than both HC and patients taking other TKI (mean level dasatinib 0.9, HC 3.8, other TKI 7.5; *P*=0.0075, *P*=0.01). Moreover, levels of other pro-inflammatory cytokines including TNFα, IFNγ, IL-6 and IL-4 were lower in pa-

tients on dasatinib compared with HC and patients taking other TKI (mean levels TNFα – dasatinib 0.86, HC 20.4, other TKI 21.5, *P*=0.006/0.005; IFNγ - 7.3, 24, 18.5, *P*=0.2/0.3; IL-6 - 2.6, 16.1, 13.6, *P*=0.0004/0.004; IL-4 – 6, 36.3, 36.3, *P*=0.01/0.05) (*Online Supplementary Figure S4B, C*). No differences in plasma cytokine levels were observed between patients taking dasatinib 50 mg and 100 mg dosage.

Increased T-cell receptor signaling in TIM3^{+} T cells in chronic myeloid leukemia patients treated with other tyrosine kinase inhibitors is limited in those taking dasatinib

In keeping with previous studies that have shown a role for excessive TCR signaling in TIM-3-mediated T-cell exhaustion, we observed an increase in pZAP70 in TIM3^{+} cells in four patients analyzed on non-SRC kinase inhibitor therapy (3 nilotinib, 1 imatinib). The MFI was 2,313 in CD8^{+}TIM3^{+} and 1,899 in CD4^{+}TIM3^{+} cells, compared with 1,628 and 1,424 in CD4^{+}TIM3^{-} and CD8^{+}TIM3^{-} cells (*P*=not significant [ns]) (Figure 8Ai, ii). In contrast, this effect was not observed in four patients taking dasatinib at 100 mg dosage. The mean difference in MFI between CD4^{+}TIM3^{+} cells was 475 in the non-SRC kinase inhibitor TKI group, compared with 66 in patients on full dosage dasatinib (*P*=0.0001) (Figure 8B). Similarly, in CD8^{+} cells the mean difference in MFI between TIM3^{+} and TIM3^{-} cells was 95 in patients on 100 mg dasatinib compared
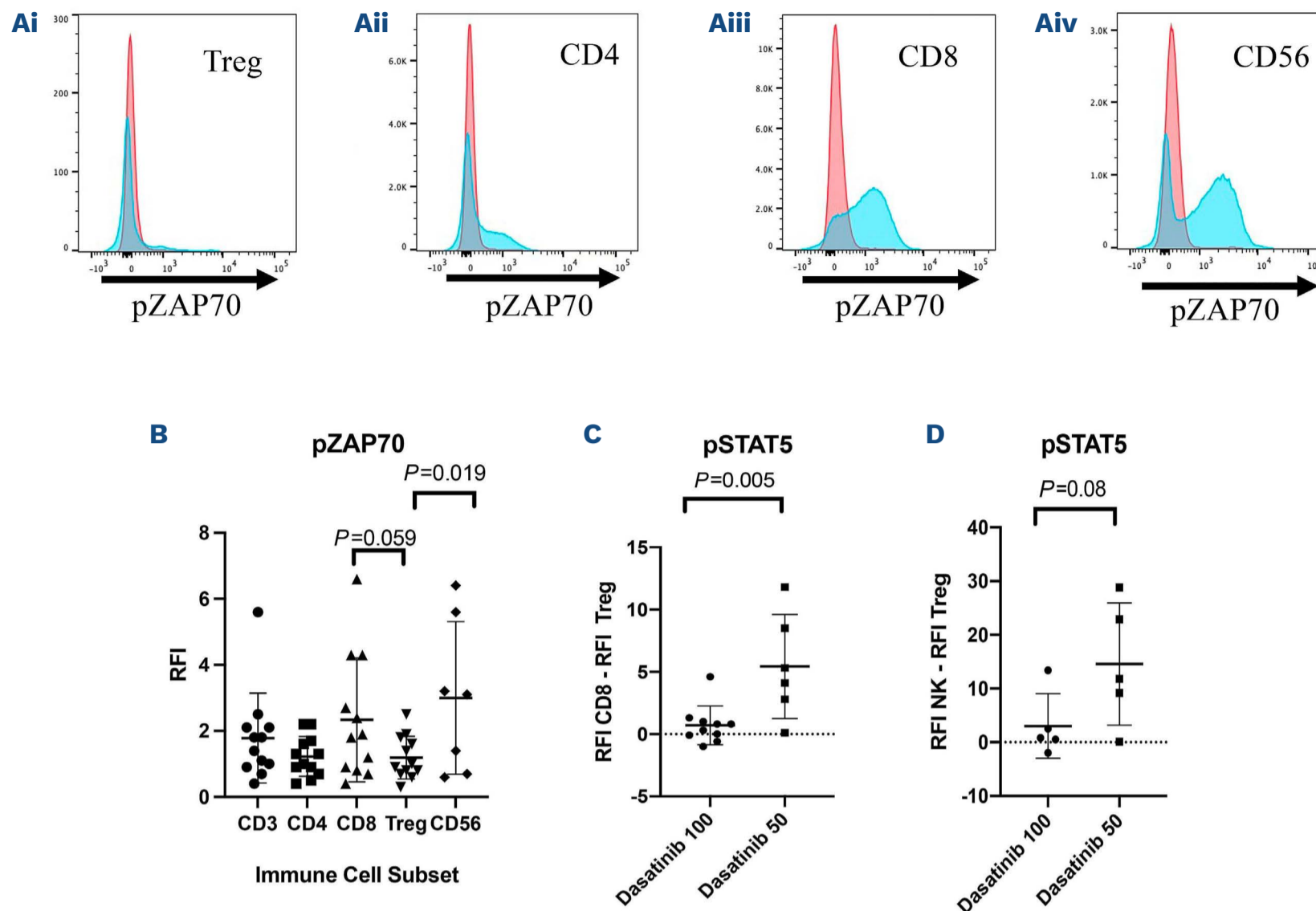
Figure 6. Preferential inhibition of regulatory T cells when compared with effector immune cell subsets. (A) Patient on dasatinib with clonal CD8+ lymphocytosis and showing preferential inhibition of pZAP70 in regulatory T cells (Tregs), (Ai) pZAP70 expression in Tregs, (Aii) pZAP70 in CD4+ cells, (Aiii) pZAP70 in CD8+ cells and (Aiv) pZAP70 in natural killer (NK) cells. (B) Comparison of mean florescence intensity (MFI) in immune cell subsets in patients on 100 mg dosage of dasatinib showing increased inhibition in Tregs compared with effector cell subsets. (C) Comparison of relative fluorescence intensity (RFI) pSTAT5 in CD8+ T cells – RFI pSTAT5 in Tregs, between patients on 50 mg and 100 mg doses of dasatinib. (D) Comparison of RFI pSTAT5 in CD56+ NK cells – RFI pSTAT5 in Tregs, between patients on 50 mg and 100 mg doses of dasatinib.

with 685 in those on other TKI (*P*=0.0016) (Figure 8C). Analysis was also performed in two patients on reduced dosage of dasatinib at 50 mg daily who had a mean difference in MFI in TIM3+ and non-TIM-3 expressing cells of 475 and 462 in CD4+ and CD8+ cells respectively (*P*=0.12/*P*=0.16).

Effector immune cells are activated during acute viral infection in patients treated with dasatinib

A patient taking 100 mg dasatinib OD was analyzed during acute viral infection with varicella zoster, prior to initiation of antiviral therapy. Baseline plasma IL-6 levels and IFNγ, were elevated at 76.081 pg/mL/14.6 pg/mL (average in HC 17.63 pg/mL/2.69 pg/mL) and C reactive protein levels were raised at 160 mg/L consistent with acute infection. There was significant expansion of CD56dim NK cells, accounting for >50% of the total live lymphocyte population (*Online Supplementary Figure S5A*). In addition, there was a notable reduction in the effector Treg (FOXP3hi/CD45-) subset of Tregs, with expansion of resting Tregs (FOXP3hi/ CD45+) (*Online Supplementary Figure S5B*). Of note, there was also pre-

served signaling within effector cells following stimulation with H$_2$O$_2$, however the signaling from the TCR and of pSTAT5 within Tregs remained largely inhibited (*Online Supplementary Figure S5C, D*). Repeat analysis performed 1 week after the resolution of infection showed a reduction in NK cells at 30% of total lymphocytes, although remaining Tregs increased (normal range in healthy controls, 5-20%).

Discussion

The positive effect of the development of lymphocytosis on CML response in patients treated with dasatinib was demonstrated from a review of 1,402 patients, in whom a third developed lymphocytosis.[24] Patients with lymphocytosis were more likely to meet major response milestones including MMR/MR3 and deep molecular response (DMR/MR4). Iriyama and colleagues analyzed lymphocyte dynamics in patients treated with dasatinib and found that cytotoxic lymphocyte or NK-cell counts at 1 month were
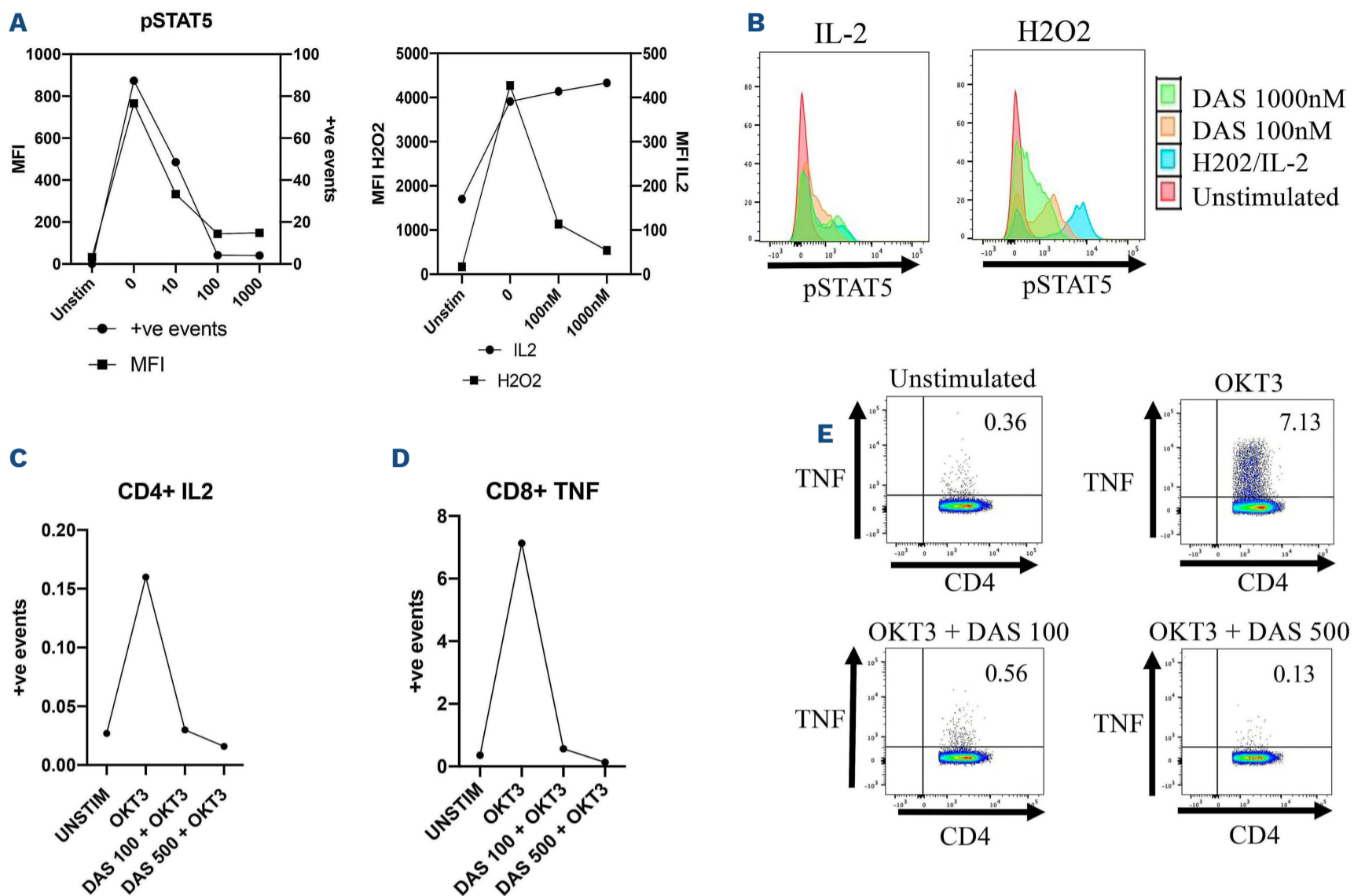
Figure 7. Dasatinib inhibits STAT5 signaling within regulatory T cells via inhibition of interleukin 2. (A) Dose-dependent inhibition of pSTAT5 with increasing concentrations of dasatinib (DAS) in gated regulatory T cells (Tregs) from healthy control (HC, n=1), following stimulation with H_2O_2. Circles on graph represent positive events and squares represent mean florescence intensity (MFI). (B) Direct comparison between H_2O_2 and interleukin 2 (IL-2) stimulation techniques, showing dose-dependent inhibition of pSTAT5 by dasatinib in gated Tregs from HC (n=1), in H_2O_2-stimulated samples, with lack of inhibition in IL-2-stimulated samples, even at supra-therapeutic concentrations. (C) Histograms showing pSTAT5 in gated Tregs from HC (n=1) following treatment with increasing doses of dasatinib, left histogram shows IL-2-stimulated cells and right shows H_2O_2-stimulated cells. (D) IL-2 expression in $CD4^+$ T cells from HC (n=1) treated with increasing concentrations of dasatinib, following OKT3 activation. (E) TNFα expression in $CD8^+$ T cells from HC (n=1) treated with increasing concentrations of dasatinib, following OKT3 activation. (F) TNFα expression in $CD8^+$ T cells from HC (n=1). Top left – unstimulated, top right – OKT3, bottom left – dasatinib 100 nM + OKT3, bottom right – dasatinib 500 nM + OKT3. +ve: positive; UNSTIM: unstimulated.

significantly higher in those patients who went on to achieve DMR.[25] Further analysis from the same study showed that patients with Tregs below a determined threshold at 12 months, had significantly improved rates of DMR.[16] It was also shown that Treg inhibition was inversely correlated with the NK-cell differentiation, with an increased proportion of mature $CD57^+$ NK cells observed as Treg number reduced, suggesting that Tregs may impede the immune response against CML cells by restricting NK cell differentiation.

In our study treatment with dasatinib resulted in a reduction in the proportion of Tregs in CML patients when compared with HC and patients taking other TKI. We also demonstrated, for the first time, that dasatinib reduces the effector Treg cell subset, which are the subset with most suppressive activity and highest expression of key suppressor molecules such as CTLA-4.[13] A recent study

suggests that imatinib causes depletion of effector Tregs, through inhibition of LCK, due to the relatively low expression of LCK in effector Tregs in comparison with other T-cell subsets, rendering them selectively susceptible to signal-deprived apoptosis.[26] From our analysis dasatinib causes greater depletion of effector Tregs, as would be expected in view of its significantly greater inhibitory effect on LCK.

We describe the first report of the differential inhibitory effect of dasatinib on signaling pathways within immune cell subsets including Tregs and NK cells in an *ex vivo* analysis. We show that TCR signaling is inhibited most strongly in Tregs when compared with effector cell subsets in patients on dasatinib, again likely explained by low Treg expression of LCK. This may have potential implications given the well-demonstrated impact of NK cell subsets around TKI discontinuation outcomes.[27] Our data also supports evaluating Treg
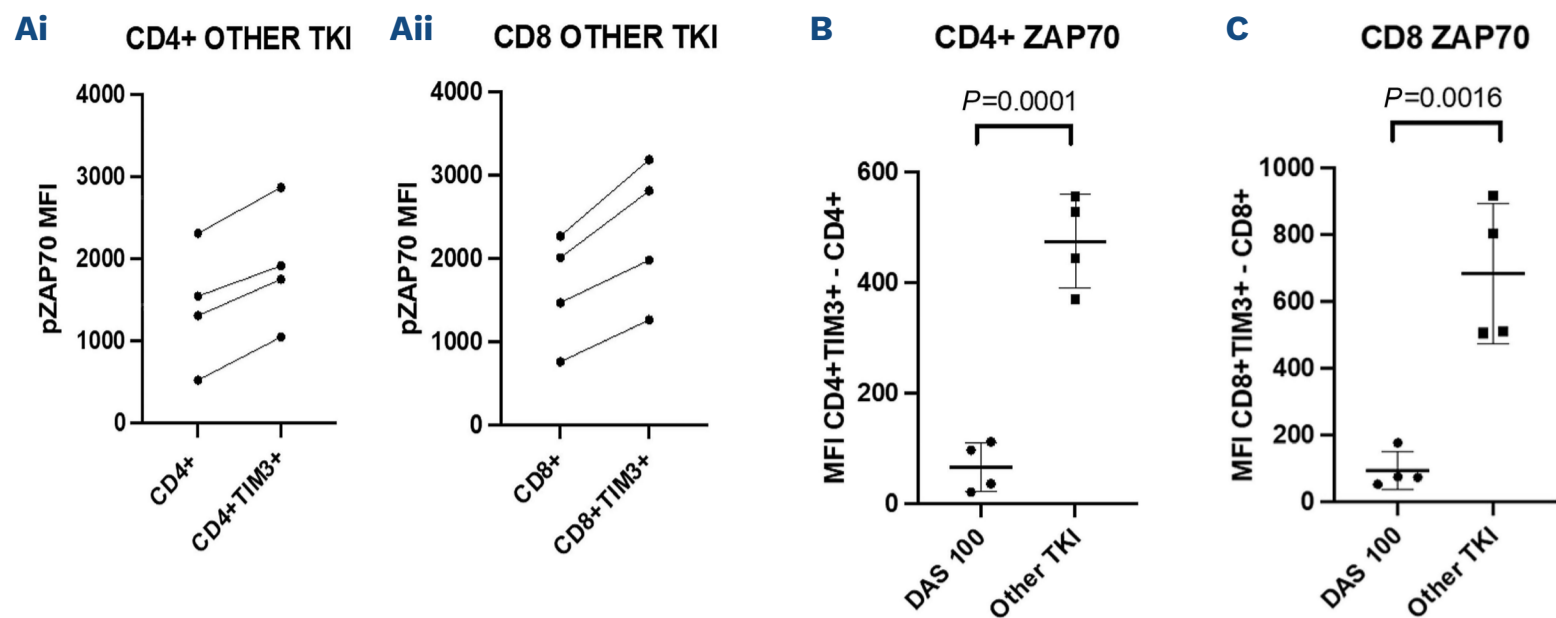
Figure 8. Effect of dasatinib on TIM3⁺ T-cell receptor signaling and reversibility of dasatinib effect. (A) Increased mean florescence intensity (MFI) for pZAP70 in (Ai) CD4⁺ and (Aii) CD8⁺ cells expressing TIM3 compared with non-TIM-3⁺ cells. (B, C) Comparison of difference in MFI between TIM3⁺ and TIM3⁻ (B) CD4⁺ and (C) CD8⁺ cells show significant increase in patients taking other tyrosine kinase inhibitors (TKI) compared to that observed in dasatinib (DAS)-treated patients.

function as an additional potential biomarker for TFR outcome in patients treated with dasatinib as suggested in a study on dasatinib discontinuation, which found reduced Tregs to correlate with improved TFR outcome.[28]

We found that patients with clonal CD8⁺ T cells have a lower proportion of Tregs when compared with patients on dasatinib without clonality, as well as reporting a novel finding of increased Treg STAT5 inhibition in patients with clonal T-cell populations.[22] Dasatinib-treated patients with clonal lymphocytosis regularly develop immune-mediated adverse effects, with accumulation of clonal CD8⁺ T cells within affected organs.[29,30] Reduction in number and function of Tregs may explain the enhanced tumor immunity effects seen in this group.

The roles of IL-2 and its downstream target STAT5 in the function of Tregs are well recognized. Tregs have abundant expression of the IL-2α receptor on the cell surface and binding results in STAT5 engaging with the promoter region of the FOXP3 gene, controlling Treg differentiation through expression of FOXP3. We have shown that dasatinib causes a significant reduction in pro-inflammatory cytokine expression within CD4⁺ cells, with greatest effect seen against IL-2, providing a mechanistic insight into the inhibition of Treg function by dasatinib through reduction of STAT5 phosphorylation. Our *in vitro* analysis of Tregs suggests pSTAT5 inhibition by dasatinib is primarily dependent on reduction in IL-2 levels. This was supported by the finding of undetectable IL-2 levels in patients receiving dasatinib, as well as confirmation of *in vitro* inhibition of IL-2 production in effector T cells following OKT3 activation, at nanomolar concentrations of dasatinib.

We also describe the effect dasatinib exerts on restricting the increased TCR signaling that is observed in TIM-3⁺ T cells in CML patients taking other TKI. TIM-3 contains no recognized inhibitory motifs and as such is thought to result in T-cell exhaustion through sustained and uncontrolled increased TCR signaling under acute conditions, which is mediated by SRC kinases.[31] In view of these observations, we suggest that chronic SRC kinase inhibition may play a role in preventing TIM-3-mediated T-cell exhaustion and enhance the immunostimulatory effects observed in certain patients on dasatinib.

Interestingly we demonstrate, for the first time in an *ex vivo* analysis, that patients on reduced dasatinib dosage have preserved T-cell function, suggestive of a dose-dependent effect of dasatinib. This may have important clinical implications when determining dasatinib dose reduction in certain cases. Moreover, we report *ex vivo* reversibility of the dasatinib effect on T-cell signaling in one patient. Importantly, we found expansion of NK cells and restoration of TCR downstream signaling in effector immune cells during acute viral infection in another patient taking dasatinib, suggesting the presence of mechanisms to overcome inhibitory effects in acute infection.

Our study is limited by relatively small sample size, and as such there was no identifiable association between presence of clonality and outcome in dasatinib-treated patients, although this has been demonstrated in other large clinical studies. In addition, patients in the dasatinib group had lower rates of DMR compared to those taking other TKI, however as most patients on dasatinib had achieved MMR or greater, we feel this is unlikely to cause significant differences in immune cell populations between groups. We also recognize that dasatinib has diffuse inhibitory effects across the kinome and that it has not been possible to evaluate all aspects in this *ex vivo* analysis.

In summary, dasatinib exerts selective depletion of effector Tregs and functional impairment of Tregs through in-

hibition of TCR and STAT5 signaling leading to increase in effector CD8^{+} T cells. We show that Treg STAT5 inhibition is primarily mediated via reduction of IL-2 signaling and that SRC kinase inhibition may also prevent TIM-3-mediated CAR T-cell exhaustion. Our data are also of relevance to the potential use of dasatinib for pharmacologic control of CAR-T cell function as well as management of cytokine release syndrome and may help with future use of dasatinib alongside other immunotherapeutic approaches.[32-34] The use of small molecule inhibitors that selectively target Tregs is an attractive option that might be beneficial across oncology practice and warrants further investigation.

Disclosures
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Data-sharing statement
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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