

## Profound Inhibition of Antigen-Specific T-Cell Effector Functions by Dasatinib

Ralf Weichsel,<sup>1</sup> Carolin Dix,<sup>1</sup> Linda Wooldridge,<sup>2</sup> Matthew Clement,<sup>2</sup> Angharad Fenton-May,<sup>2</sup> Andrew K. Sewell,<sup>2</sup> Josef Zezula,<sup>3</sup> Elisabeth Greiner,<sup>3</sup> Emma Gostick,<sup>4</sup> David A. Price,<sup>4</sup> Hermann Einsele,<sup>1</sup> and Ruth Seggewiss<sup>1</sup>

**Abstract Purpose:** The dual BCR-ABL/SRC kinase inhibitor dasatinib entered the clinic for the treatment of chronic myeloid leukemia and Ph<sup>+</sup> acute lymphoblastic leukemia. Because SRC kinases are known to play an important role in physiologic T-cell activation, we analyzed the immunobiological effects of dasatinib on T-cell function. The effect of dasatinib on multiple T-cell effector functions was examined at clinically relevant doses (1-100 nmol/L); the promiscuous tyrosine kinase inhibitor staurosporine was used as a comparator.

**Experimental Design:** Purified human CD3<sup>+</sup> cells and virus-specific CD8<sup>+</sup> T cells from healthy blood donors were studied directly *ex vivo*; antigen-specific effects were confirmed in defined T-cell clones. Functional outcomes included cytokine production (interleukin-2, IFN $\gamma$ , and tumor necrosis factor  $\alpha$ ), degranulation (CD107a/b mobilization), activation (CD69 up-regulation), proliferation (carboxyfluorescein diacetate succinimidyl ester dilution), apoptosis/necrosis induction, and signal transduction.

**Results:** Both dasatinib and staurosporine inhibited T-cell activation, proliferation, cytokine production, and degranulation in a dose-dependent manner. Mechanistically, this was mediated by the blockade of early signal transduction events and was not due to loss of T-cell viability. Overall, CD4<sup>+</sup> T cells seemed to be more sensitive to these effects than CD8<sup>+</sup> T cells, and naïve T cells more sensitive than memory T-cell subsets. The inhibitory effects of dasatinib were so profound that all T-cell effector functions were shut down at therapeutically relevant concentrations.

**Conclusion:** These findings indicate that caution is warranted with use of this drug in the clinical setting and provide a rationale to explore the potential of dasatinib as an immunosuppressant in the fields of transplantation and T-cell – driven autoimmune diseases.

Tyrosine kinase (TK) inhibitors such as imatinib (Glivec, STI571, Novartis) have entered the clinic as specific cancer treatments. Imatinib is now first-line therapy for chronic myeloid leukemia (1, 2) and is increasingly used in conjunction with allogeneic hematopoietic stem cell transplantation (3–5). Recently, *in vitro* data have highlighted the inhibitory

effects of imatinib on leukemia-specific T cells (6). However, its effect on graft-versus-host disease, graft-versus-leukemia, or other immune-mediated processes affecting transplant outcome are not completely understood. We and others have found that imatinib exerts T-cell – suppressive effects *in vitro*, possibly via LCK inhibition (7–9). LCK is a major player in proximal T-cell signaling; CD3/T-cell receptor (TCR) – mediated signal transduction leads to activation of the SRC family TKs LCK and FYN, which are associated with the coreceptors CD4 and CD8 (10). Activated LCK and/or FYN, in turn, phosphorylates the immunoreceptor tyrosine – based activation motifs located within the CD3 and  $\zeta$  chains of the TCR/CD3 complex itself. The phosphorylated immunoreceptor tyrosine – based activation motifs serve as a docking site for ZAP70, which is also a substrate for LCK. ZAP70, in turn, phosphorylates the T cell-specific adapters LAT and SLP-76, which generate the secondary messenger effectors of T-cell activation and proliferation. Patients can become resistant to imatinib, and thus there is a demand for alternative kinase inhibitors. Several TK inhibitors, which are often less specific than imatinib, are currently undergoing preclinical and clinical development or have recently been approved. However, whereas these agents show much promise, there are concerns that concurrent immunosuppressive effects mediated through inhibition of TKs involved in the antigen receptor signaling pathways of T cells as

**Authors' Affiliations:** <sup>1</sup>Immune Recovery Section, Med. Klinik und Poliklinik II, University of Würzburg, Würzburg, Germany; <sup>2</sup>Department of Medical Biochemistry and Immunology, Cardiff University, Cardiff, United Kingdom; <sup>3</sup>Laboratory of Medicinal Chemistry, National Institutes of Diabetes, Digestive and Kidney Diseases, NIH, Bethesda, Maryland; and <sup>4</sup>Weatherall Institute of Molecular Medicine, University of Oxford, John Radcliffe Hospital, Oxford, United Kingdom  
Received 9/20/07; revised 11/28/07; accepted 12/13/07.

**Grant support:** This work was supported by Deutsche Forschungsgemeinschaft grant no. SE 1669/1-1. D.A. Price is a Medical Research Council (UK) Senior Clinical Fellow.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

**Requests for reprints:** Ruth Seggewiss, Med. Klinik und Poliklinik II, University of Würzburg, C11, Room E02, Josef-Schneider-Strasse 2, 97080 Würzburg, Germany. Phone: 49-931-201-36402; Fax: 49-931-201-36409; E-mail: Seggewiss\_R@medizin.uni-wuerzburg.de.

©2008 American Association for Cancer Research.  
doi:10.1158/1078-0432.CCR-07-4393

described above might diminish their benefit/risk ratio. Pancytopenia, herpes virus infections, and sepsis have been observed in chronic myeloid leukemia patients treated with the TK inhibitor dasatinib (Sprycel, BMS-354825, Bristol-Myers Squibb); it seems likely that these clinical sequelae are due to inhibitory effects on TKs that play an important role in T-cell development and function (11–13). We therefore conducted a detailed investigation of the immunobiological effects of dasatinib on T cells.

Dasatinib has been shown to be safe and more effective in chronic myeloid leukemia patients than imatinib and was approved in Europe and the United States in 2006. Because the ability of dasatinib to inhibit LCK is ~1,000-fold higher compared with imatinib [dasatinib  $IC_{50}$ , 0.5 nmol/L (14); imatinib  $IC_{50}$ , 0.6–0.8  $\mu$ mol/L (9)], and it also inhibits FYN (15), the immunosuppressive effects of this SRC kinase inhibitor on T cells are likely to be much more profound than those of imatinib. We determined the effect of dasatinib in comparison with the effects of the promiscuous TK inhibitor staurosporine, which has been the base for several SRC kinase inhibitors in clinical development, on the functional consequences of T-cell activation such as proliferation, cytokine secretion, degranulation, and up-regulation of activation markers such as CD69. Furthermore, we examined the effects of dasatinib on different T-cell subsets and clinically relevant antigen-specific T-cell responses to persistent viruses such as cytomegalovirus (CMV) and Epstein Barr Virus (EBV). Immunosuppression can lead to recrudescence of such viruses and consequent disease manifestations. In addition, we examined the effect of dasatinib on  $\gamma\delta$  T-cell activation because previous studies have suggested a role for these cells in the control of CMV *in vivo* (16, 17). Overall, our data indicate a profound and global inhibitory effect of dasatinib on adaptive T-cell responses and suggest a potential therapeutic role for this drug as a novel immunosuppressant in transplantation and T-cell-driven autoimmune diseases.

## Materials and Methods

**Reagents.** Dasatinib was synthesized according to the published procedure (14). Imatinib was extracted from a commercially available 400-mg imatinib mesylate tablet as described (9). The identity and purity of dasatinib and imatinib were established as described (9). Staurosporine was obtained from Biomol. All TK inhibitors were dissolved in DMSO at stock concentrations of 100  $\mu$ mol/L (staurosporine), 10  $\mu$ mol/L (dasatinib), and 10 mmol/L (imatinib). The biological activities of the extracted imatinib, synthesized dasatinib, and commercially available staurosporine were tested in a cell death titration assay on BA/F3 bcr-abl<sup>+</sup> cells as described (18).

**Cell culture and activation.** Peripheral blood mononuclear cells were collected from healthy volunteer donors at the Frankfurt Red Cross Transfusion Medicine department in Germany after obtaining written informed consent. Jurkat T cells and primary human T cells from healthy blood donors were cultured in RPMI 1640 (PAN) containing 10% FCS, 2 mmol/L L-glutamine, 50 units/mL penicillin, and 50  $\mu$ g/mL streptomycin (R10). Primary T cells were purified by Ficoll-Hypaque (PAN) density gradient centrifugation, followed by negative selection with magnetic beads coated with an antibody mix and an LS magnetic-activated cell sorting column according to the manufacturer's instructions (Pan T Cell Kit Untouched, Miltenyi Biotec). CD3<sup>+</sup> T-cell purity, determined by flow cytometry, ranged from 80% to 97.5% (median, 90.5%); viability was always >90% as determined by trypan blue staining (Trypan Blue Solution 0.2%, Sigma). After overnight rest, the

cells were incubated with or without TK inhibitors for 1 h and then stimulated with 5  $\mu$ g/mL OKT3 (Orthoclone, Janssen-Cilag). Unstimulated T cells as a negative control and DMSO diluted 1:10 in R10 as a solvent control were included in all assays.

**Antibodies and basic flow cytometry.** All antibodies for flow cytometry were obtained from BD PharMingen. Stained samples were collected on a four-color FACSCalibur flow cytometer (BD Immunocytometry Systems). List mode files were analyzed using CellQuest software (BD PharMingen). In all cases, at least 30,000 events were collected for analysis. Gates were set on live cells and on total CD3<sup>+</sup>, cytotoxic (CD3<sup>+</sup>CD8<sup>+</sup>), and helper (CD3<sup>+</sup>CD4<sup>+</sup>) T cells, as well as naïve (CD4<sup>+</sup> or CD8<sup>+</sup>/CD45RO<sup>-</sup>/CD27<sup>+</sup>) and memory (CD4<sup>+</sup> or CD8<sup>+</sup>/CD45RO<sup>+</sup>/CD27<sup>+</sup>; CD4<sup>+</sup> or CD8<sup>+</sup>/CD45RO<sup>+</sup>/CD27<sup>-</sup>) T-cell subsets, to evaluate proliferative responses to OKT3, and on CD8<sup>+</sup> T cells to evaluate responses to CMV or EBV peptides. Activation of T cells was evaluated by analysis of CD69 expression on gated cells after 24-h stimulation with 5  $\mu$ g/mL OKT3 in the presence or absence of TK inhibitors. The following antibodies were used: CD3-FITC/allophycocyanin (UCHT1), CD4-phycoerythrin (RPA-T4), CD4-peridinin chlorophyll protein (PerCP; SK3), CD8-FITC (RPA-T8), CD8-PerCP (SK1), CD27-phycoerythrin (M-T271), CD45RO-allophycocyanin (UCHL1), CD69-phycoerythrin (FN50), CD69-PerCP (L78), CD107a-FITC (H4A3), CD107b-FITC (H4B4), Annexin V-phycoerythrin, IFN $\gamma$ -phycoerythrin (B27), and tumor necrosis factor  $\alpha$  (TNF $\alpha$ )-phycoerythrin (Mab11); 7-amino-actinomycin D (7-AAD) was used to discriminate live from dead cells.

**Carboxyfluorescein diacetate succinimidyl ester proliferation assay.** Purified human T cells were suspended in PBS ( $1 \times 10^6$ /mL) and labeled with the vital dye carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) at a final concentration of 0.25  $\mu$ mol/L as described (9). After overnight rest, CFSE-labeled cells were preincubated with TK inhibitors as indicated for 1 h, and then cultured for 4 d in the presence of 5  $\mu$ g/mL OKT3 and antibodies against the costimulatory molecules CD28 and CD49d (1  $\mu$ g/mL each; BD PharMingen). In washout experiments, T cells were incubated for 24 h with dasatinib or staurosporine, then harvested and washed twice before assay.

**Apoptosis assay.** T cells ( $1 \times 10^6$ /mL) were stimulated with 5  $\mu$ g/mL OKT3 and antibodies against the costimulatory molecules CD28 and CD49d (1  $\mu$ g/mL each), with or without 50 nmol/L dasatinib or staurosporine, for 4 d after overnight rest in R10, and then harvested and stained with Annexin V-phycoerythrin and 7-AAD. Apoptotic cells were defined by flow cytometry as 7-AAD<sup>+</sup>/Annexin V<sup>+</sup>; necrotic cells were defined as 7-AAD<sup>+</sup>/Annexin V<sup>-</sup>.

**Cytokine secretion, degranulation, and proliferation of epitope-specific CD8<sup>+</sup> T cells stimulated with HLA A\*0201-restricted CMV and EBV peptides.** T cells from HLA A2<sup>+</sup> CMV and/or EBV IgG seropositive healthy donors were magnetic-activated cell sorted and CFSE labeled as described above. Purified cells were stimulated with 2  $\mu$ mol/L of the HLA A\*0201-restricted peptides (purity >70%; IPT Peptide Technologies GmbH) CMV pp65<sub>495-503</sub> (NLVPMVATV) or EBV BMLF1<sub>259-267</sub> (GLCTLVAML) in the presence of antibodies against the costimulatory molecules CD28 and CD49d (1  $\mu$ g/mL each). Proliferation of CMV- or EBV-specific CD8<sup>+</sup> cells was determined after 6 d of culture by staining with anti-CD8-PerCP and allophycocyanin-labeled HLA A\*0201 tetrameric complexes (tetramers) refolded with CMV pp65<sub>495-503</sub> or EBV BMLF1<sub>259-267</sub> peptides (19); tetramers were produced as previously described (20). Expression of the degranulation markers CD107a/b, together with intracellular production of IFN $\gamma$  and TNF $\alpha$ , was evaluated in 5-h assays using Golgi Stop and Cytofix/Cytoperm (BD PharMingen), with and without dasatinib or staurosporine, at the indicated concentrations as previously described (21). The percentages of antigen-specific CD8<sup>+</sup> cells secreting cytokine and expressing CD107a/b were determined by flow cytometry using a FACSCalibur instrument with CellQuest software.

**Interleukin-2 ELISA.** Purified T cells ( $2 \times 10^5$  per well) were plated in 96-well plates with or without TK inhibitors at the indicated

concentrations for 18 h and stimulated with 5  $\mu\text{g}/\text{mL}$  OKT3 in the presence of antibodies against the costimulatory molecules CD28 and CD49d (1  $\mu\text{g}/\text{mL}$  each). Interleukin-2 (IL-2) was measured with a commercially available ELISA kit according to the manufacturer's instructions (OptEIA Human IL-2; BD Biosciences).

**Analysis of TCR signal transduction in Jurkat T cells.** Jurkat T cells ( $1 \times 10^7/\text{mL}$ ) were incubated for 1 h with dasatinib at the indicated concentrations and stimulated for 5 min with 5  $\mu\text{g}/\text{mL}$  OKT3. Cells were lysed in 1-mL lysis buffer containing 1% Triton X-100. Protein concentrations were adjusted by detergent-compatible protein assay kit according to the manufacturer's instructions (Bio-Rad). For each sample, 20- $\mu\text{g}$  protein was loaded on a 10% Bis-Tris Gel (Invitrogen) and transferred to nitrocellulose. Western blots were incubated with the indicated antibodies in PBS containing 3% to 4% milk/Tween 0.1%. Antibodies were as follows: mouse monoclonal IgG2b antibody clones PY20 and PY99 (Santa Cruz Biotechnology) specific for phosphotyrosine mixed 1:1, a polyclonal antibody specific for LCK (Cell Signaling), and a monoclonal antibody specific for  $\beta$ -actin (clone AC-74, Sigma). Blots were developed by enhanced chemiluminescence.

**Cell culture of antigen-specific CD8<sup>+</sup> and  $\gamma\delta$  T-cell lines.** The CD8<sup>+</sup> T-cell clones EBV-C, ILA-1, and Mel-13 were isolated from peripheral blood mononuclear cells of healthy donors as described (22). EBV-C is specific for the HLA A\*0201-restricted, EBV-derived *BMLFI*-encoded epitope GLCTLVAML; ILA-1 recognizes the HLA A\*0201-restricted human telomerase reverse transcriptase epitope ILAKFLHWL; and Mel-13 is specific for the HLA A\*0201-restricted MelanA epitope ELAGIGILT. The  $\gamma\delta$  line AJH is >99%  $\text{V}\gamma 9/\text{V}\delta 2^+$  and was generated as described (23). Antigen-specific CD8<sup>+</sup> T-cell clones and the  $\gamma\delta$  T-cell line were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% FCS (Life Technologies), 2 mmol/L L-glutamine (Life Technologies), 100 units/mL penicillin (Life Technologies), 100  $\mu\text{g}/\text{mL}$  streptomycin (Life Technologies), 5% Cellkines (Helvetica Healthcare), 200 units/mL IL-2 (Chiron), and 25 ng/mL IL-15 (Peprotech). Clones and lines were periodically restimulated using 5  $\mu\text{g}/\text{mL}$  phytohemagglutinin with irradiated allogeneic peripheral blood mononuclear cells from at least three individuals as feeder cells.

**Cytometric bead arrays.** EBV-C CD8<sup>+</sup> T cells ( $2 \times 10^4$ ) or AJH  $\gamma\delta$  T cells ( $1 \times 10^4$ ) were treated with either dasatinib or staurosporine for 1 h at the concentrations indicated. EBV-C CD8<sup>+</sup> T cells were then stimulated with  $2.5 \times 10^4$  C1R A2 B cells that had been prepulsed with the indicated concentrations of specific peptide (GLCTLVAML) for 1 h and washed twice with R10 as above. The AJH  $\gamma\delta$  line was stimulated by the addition of 1  $\mu\text{g}/\text{mL}$  OKT3. Cells were incubated in a 96-well U-bottomed plate for 4 h, then pelleted by centrifugation. The supernatant was harvested and assayed with the human TH1/TH2 cytokine kit (BD Biosciences) according to the manufacturer's instructions. Analysis was done with a FACSCalibur flow cytometer.

**Cell-surface TCR expression assays.** EBV-C, ILA-1, or Mel-13 CD8<sup>+</sup> T cells ( $1 \times 10^5$ ) were resuspended in 100  $\mu\text{L}$  of R10 as above. Cells were then treated with either dasatinib or staurosporine at 0, 2, 10, or 100 nmol/L for 3 h. Following incubation with TK inhibitors, cells were stained with anti- $\alpha\beta\text{TCR-FITC}$  (Serotec), anti-CD8-allophycocyanin (BD PharMingen), and 7-AAD for 30 min on ice, washed twice in PBS, and resuspended in 200- $\mu\text{L}$  PBS before analysis.

**Statistical analyses.** Results were expressed as mean  $\pm$  SD. Statistical significance was determined by the two-tailed Student *t* test or one-way ANOVA and considered statistically significant at  $P < 0.05$ . The mean  $\text{IC}_{50}$  was calculated with GraphPad Prism software (sigmoidal dose response, variable slope).

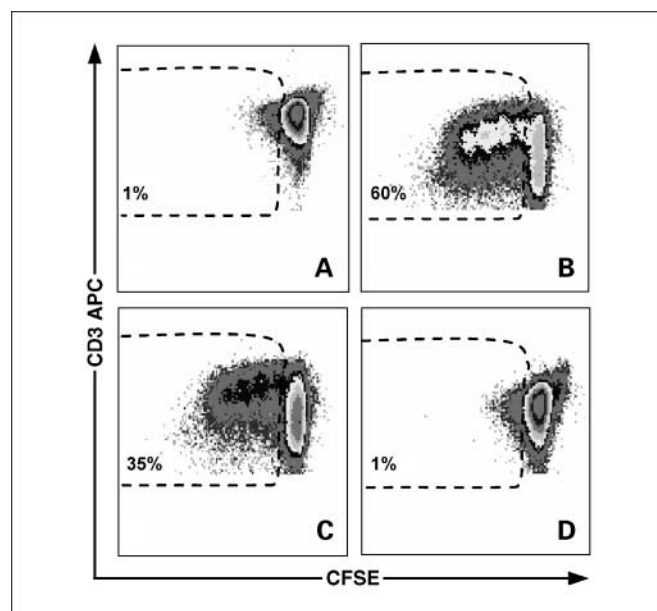
## Results

All assays described herein were done at clinically relevant doses of dasatinib between 1 and 100 nmol/L according to the published serum levels achieved in patients taking 70 mg

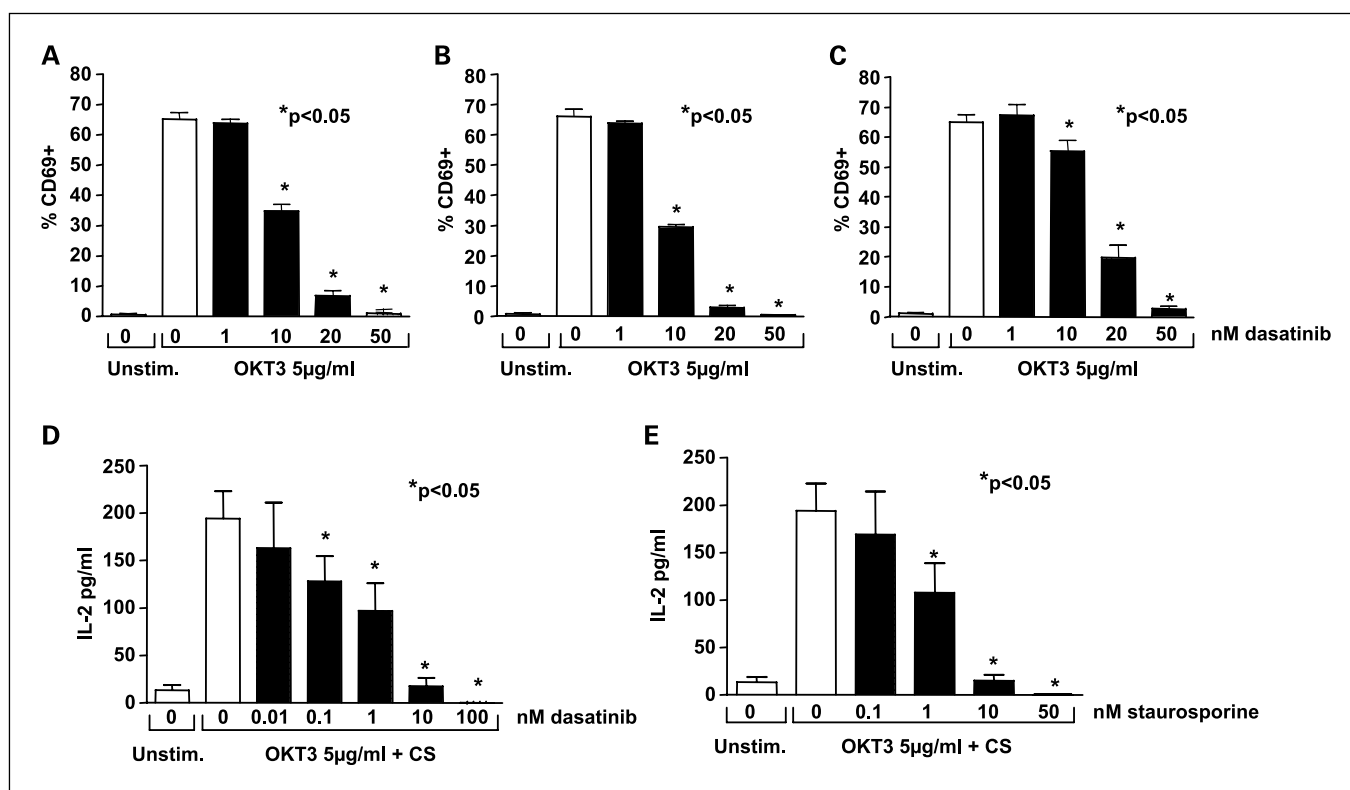
dasatinib twice daily (24). Positive, negative, and solvent controls (DMSO) were included in all assays. The effects of the dual SRC/BCR-ABL inhibitor dasatinib were compared with the promiscuous natural TK inhibitor staurosporine.

**Dasatinib and staurosporine inhibit T-cell proliferation in a dose-dependent manner.** To investigate the effects of dasatinib and staurosporine on induced T-cell proliferation, we applied the murine monoclonal antibody OKT3, which cross-links the CD3 component of the CD3/TCR complex. To visualize cell proliferation, we labeled the cells with the vital dye CFSE. This dye is retained in the cytoplasm and is diluted out with each cell division, thereby allowing visualization of successive cell divisions by flow cytometry. A dose-dependent inhibition of T-cell proliferation was detected with almost complete inhibition (96%) occurring at a concentration of 20 nmol/L dasatinib ( $\text{IC}_{50}$ , 11 nmol/L; Fig. 1). T cells that were incubated with dasatinib for 24 hours and then removed from dasatinib proliferated as well as untreated T cells over a period of 4 days ( $P = 0.156$ ;  $n = 5$ ); this argues for a reversible blockade of T-cell proliferation. In contrast, staurosporine led to a dose-dependent, but irreversible, inhibition of proliferation at a concentration of 10 nmol/L (mean of 90% inhibition;  $P = 0.027$ ;  $n = 4$ ).

**Dasatinib and staurosporine inhibit T-cell activation and IL-2 release in a dose-dependent manner.** We further investigated the functional effects of dasatinib and staurosporine on T-cell activation and cytokine release. A statistically significant inhibition of OKT3-induced up-regulation of the early T-cell activation marker CD69 was observed at concentrations as low as 10 nmol/L, and almost complete inhibition (99%)



**Fig. 1.** Dasatinib and staurosporine inhibit anti-CD3–induced T-cell proliferation in a dose-dependent manner. Purified CD3<sup>+</sup> T cells were labeled with 0.25  $\mu\text{mol}/\text{L}$  CFSE, preincubated with 10 to 50 nmol/L dasatinib or staurosporine (data for the latter not shown), and then stimulated with 5  $\mu\text{g}/\text{mL}$  OKT3 and antibodies against the costimulatory molecules CD28 and CD49d (1  $\mu\text{g}/\text{mL}$  each) for 4 d. Representative of four experiments (A–D). Gates were set on live lymphocytes. A, unstimulated T cells. B, T cells stimulated with 5  $\mu\text{g}/\text{mL}$  OKT3 and  $\alpha\text{CD}28/\alpha\text{CD}49\text{d}$  at 1  $\mu\text{g}/\text{mL}$  each. C, same as in B, but preincubated with 10 nmol/L dasatinib. D, same as in B, but preincubated with 50 nmol/L dasatinib.



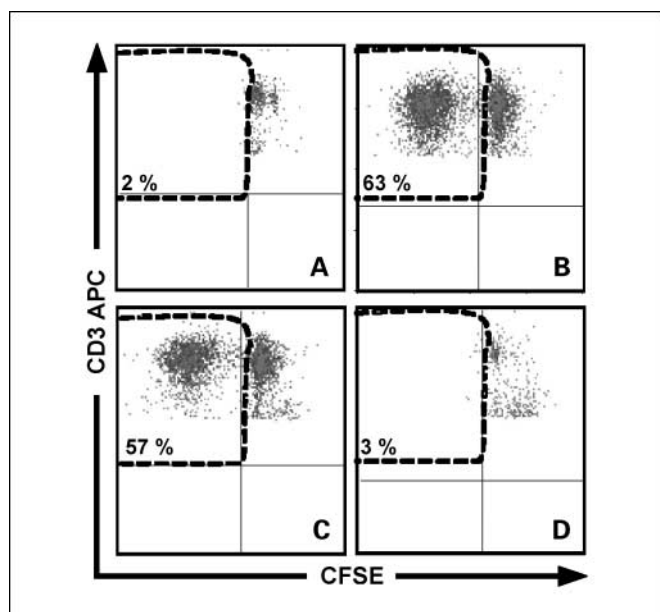
**Fig. 2.** Dasatinib and staurosporine inhibit T-cell activation and IL-2 release in a dose-dependent manner. T cells untreated (*open columns*) and preincubated with dasatinib (*closed columns*) at 1, 10, 20, or 50 nmol/L for 1 h, as indicated, were stimulated for 24 h with 5 μg/mL OKT3. Columns, mean CD69 expression in gated live lymphocytes from four independent experiments for total CD3<sup>+</sup> T cells (A), helper CD4<sup>+</sup> T cells (B), and cytotoxic CD8<sup>+</sup> T cells (C); bars, SD. Unstimulated T cells (*open columns*) are shown in each case for comparison. For analysis of effects on IL-2 production, untreated (*open columns*) and CD3<sup>+</sup> T cells pretreated (*closed columns*) with dasatinib (D) or staurosporine (E) at the indicated concentrations for 1 h were incubated for 18 h either unstimulated (*open columns*) or stimulated with 5 μg/mL OKT3 in the presence of costimulatory molecules (CS) αCD28/αCD49d at 1 μg/mL each. Columns, mean from six independent ELISA experiments, each done with purified CD3<sup>+</sup> T cells from different donors and assayed in duplicates; bars, SD.

occurred after 24 hours at a concentration of 50 nmol/L (IC<sub>50</sub>, 11 nmol/L; Fig. 2); corresponding effects for staurosporine occurred at IC<sub>50</sub> of 4 to 5 nmol/L (data not shown). In line with the inhibition of CD69 up-regulation, we also observed a profound reduction of IL-2 secretion measured by ELISA in T cells treated with dasatinib and staurosporine ( $n = 6$ ; IC<sub>50</sub>, 2 nmol/L for both; Fig. 2).

**Dasatinib and staurosporine do not increase apoptosis in T cells at clinically relevant concentrations.** To investigate whether the induction of apoptosis contributes to the dose-dependent inhibition of T-cell activation and proliferation induced by dasatinib and staurosporine, we measured apoptosis and necrosis by Annexin V and 7-AAD staining after 4 days of stimulation with OKT3 and antibodies against the costimulatory molecules CD28 and CD49d. Neither dasatinib nor staurosporine increased activation-induced cell death in T cells ( $n = 5$ ). Instead, the addition of dasatinib and staurosporine led to a statistically significant increase in necrosis compared with stimulated T cells alone ( $n = 5$ ; Supplementary figure). Overall, the induction of apoptosis and necrosis was more pronounced in CD8<sup>+</sup> compared with CD4<sup>+</sup> T cells. In time course experiments conducted over 4 days ( $n = 2$ ), we observed a peak in apoptosis/necrosis induction at days 3 to 4 in samples pretreated with TK inhibitors and stimulated with OKT3 and antibodies against the costimulatory molecules CD28 and CD49d; the corresponding peak in stimulated samples occurred at day 2.

**Differential sensitivity of helper and cytotoxic T cells to the inhibitory effects of dasatinib and staurosporine.** Helper T cells (CD4<sup>+</sup>) and cytotoxic T cells (CD8<sup>+</sup>) differ substantially in terms of their development from antigen-inexperienced naïve T cells to effector and long-lived central memory T cells. For example, the kinetics and efficiency of CD8<sup>+</sup> T-cell proliferation differ from those of CD4<sup>+</sup> T cells. Overall, naïve CD8<sup>+</sup> T cells develop more readily into effector T cells after short-term primary stimulation than naïve CD4<sup>+</sup> T cells (25). In our assays, CD4<sup>+</sup> T cells were marginally more sensitive than CD8<sup>+</sup> T cells to the inhibitory effects of staurosporine on activation and proliferation (data not shown). The same held true for dasatinib; the IC<sub>50</sub> for inhibition of activation was 10 nmol/L for CD4<sup>+</sup> cells and 15 nmol/L for CD8<sup>+</sup> cells (Fig. 2), whereas the IC<sub>50</sub> for inhibition of proliferation was 10 nmol/L for CD4<sup>+</sup> cells and 13 nmol/L for CD8<sup>+</sup> cells (data not shown).

**Naïve T cells are more sensitive than memory T cells to the inhibitory effects of dasatinib.** Murine cytotoxic memory T cells are more sensitive than naïve CD8<sup>+</sup> T cells to the inhibitory effects of imatinib (26, 27). In contrast, we observed that naïve T cells were more sensitive to the inhibitory effects of dasatinib (activation: IC<sub>50</sub>, 16 nmol/L for naïve CD8<sup>+</sup> cells, 12 nmol/L for naïve CD4<sup>+</sup> cells; proliferation: IC<sub>50</sub>, 15 nmol/L for naïve CD8<sup>+</sup> cells, 9 nmol/L for naïve CD4<sup>+</sup> cells) than memory subsets (activation: IC<sub>50</sub>,



**Fig. 3.** Inhibition of virus-specific CD8<sup>+</sup> T-cell proliferation in response to cognate antigen. T cells from healthy HLA A2<sup>+</sup> human blood donors with tetramer-defined CD8<sup>+</sup> T-cell responses to CMV pp65<sub>495-503</sub> were purified, labeled with CFSE, and cultured for 6 d in the presence of 2 μmol/L CMV peptide and αCD49d/αCD28 antibodies at 1 μg/mL each. Representative of four experiments. *A*, unstimulated CD8<sup>+</sup> T cells. *B*, CD8<sup>+</sup> T cells stimulated with cognate CMV peptide and αCD49d/αCD28 as above. *C*, same as in *B*, but preincubated with 10 nmol/L dasatinib for 1 h. *D*, same as in *B*, but preincubated with 100 nmol/L dasatinib for 1 h. Similar data were obtained with HLA A\*0201-restricted EBV-specific T cells (*n* = 3) in response to cognate peptide (data not shown). Staurosporine exhibited comparable inhibition of CMV-specific and EBV-specific CD8<sup>+</sup> T cells at concentrations of 10 and 50 nmol/L (data not shown).

20 nmol/L for CD45RO<sup>+</sup>CD27<sup>+</sup> CD8<sup>+</sup> cells and 26 nmol/L for CD45RO<sup>+</sup>CD27<sup>-</sup> CD8<sup>+</sup> cells, 13 nmol/L for CD45RO<sup>+</sup>CD27<sup>+</sup> CD4<sup>+</sup> cells and 15 nmol/L for CD45RO<sup>+</sup>CD27<sup>-</sup> CD4<sup>+</sup> cells; proliferation: IC<sub>50</sub>, 21 nmol/L for CD45RO<sup>+</sup>CD27<sup>+</sup> CD8<sup>+</sup> cells and 22 nmol/L for CD45RO<sup>+</sup>CD27<sup>-</sup> CD8<sup>+</sup> cells, 13 nmol/L

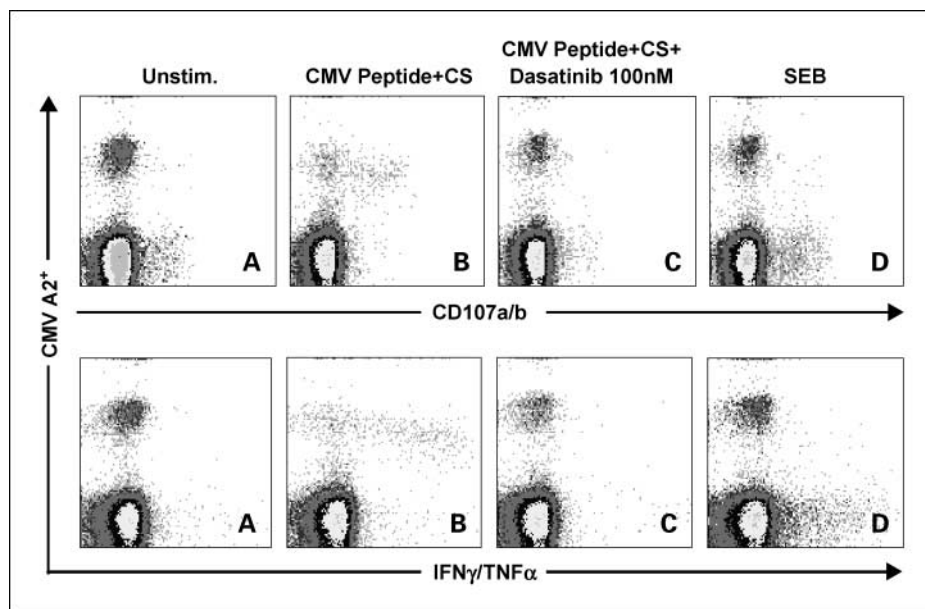
for CD45RO<sup>+</sup>CD27<sup>+</sup> CD4<sup>+</sup> cells and 16 nmol/L for CD45RO<sup>+</sup>CD27<sup>-</sup> CD4<sup>+</sup> cells). Similar results were obtained for staurosporine (data not shown).

**Virus-specific CD8<sup>+</sup> T-cell responses are suppressed by dasatinib and staurosporine in a dose-dependent manner.** Because CD8<sup>+</sup> T-cell-mediated immunity is essential for the long-term control of persistent DNA viruses, we evaluated the effect of dasatinib and staurosporine on antigen-specific T-cell responses to CMV and EBV. Proliferation of both CMV-specific (*n* = 4) and EBV-specific (*n* = 3) CD8<sup>+</sup> T cells was suppressed in a dose-dependent manner by both TK inhibitors, with complete inhibition observed at 100 nmol/L dasatinib and 50 nmol/L staurosporine (Fig. 3). We also observed a dose-dependent inhibition of IFNγ and TNFα secretion as well as an inhibition of CD107a/b mobilization (CMV *n* = 3, EBV *n* = 2); surface expression of CD107a/b follows activation-induced degranulation and is thus a necessary precursor to perforin/granzyme-mediated cytotoxicity (Fig. 4). These results were confirmed with an EBV-specific CD8<sup>+</sup> T-cell clone (Fig. 5A).

**Dasatinib and staurosporine inhibit γδ T-cell activation.** Interestingly, dasatinib was also shown to inhibit γδ T-cell activation. The secretion of both TH1 (IFNγ, TNFα, and IL-2) and TH2 (IL-4) cytokines was inhibited to the same extent (Fig. 5B).

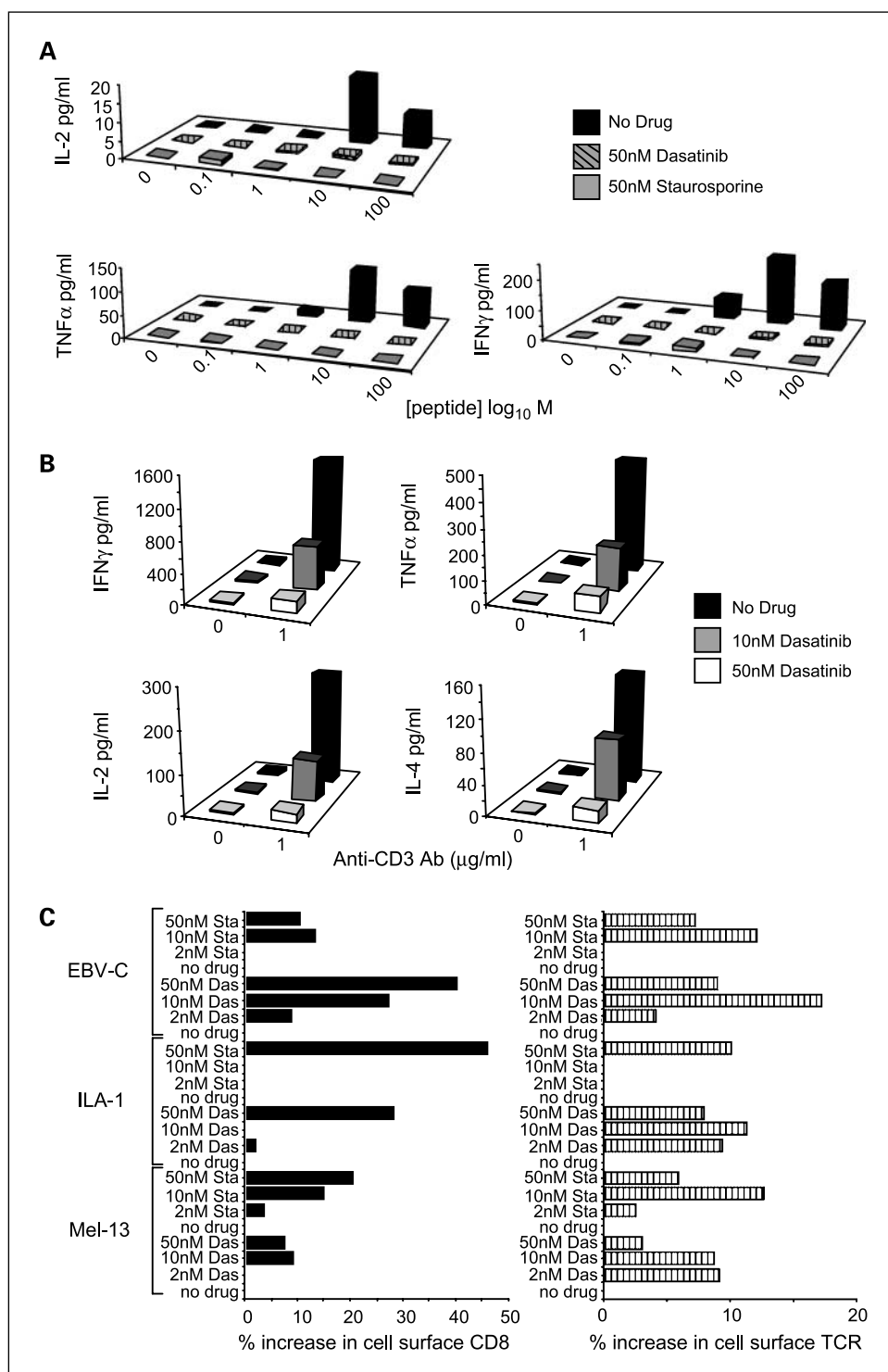
**Dasatinib and staurosporine inhibit TCR and CD8 down-regulation from the surface of cytotoxic T cells.** Both dasatinib and staurosporine exhibited dose-dependent effects on the levels of TCR and CD8 expressed on the surface of T-cell clones. Indeed, expression levels of TCR and CD8 at the cell surface of the antigen-specific T-cell clone EBV-C increased by ~15% and 40%, respectively, after 3 hours of exposure to 50 nmol/L staurosporine or dasatinib (Fig. 5C). These observations suggest that dasatinib and staurosporine block TCR down-regulation from the cell surface (28), one of the initial events that occur after TCR engagement.

**Dasatinib inhibits proximal transduction components of the CD3/TCR complex and decreases LCK phosphorylation.** To



**Fig. 4.** Modulation of CD107a/b mobilization and IFNγ/TNFα secretion by dasatinib and staurosporine. Frequencies of tetramer-positive CD8<sup>+</sup> T cells specific for CMV pp65<sub>495-503</sub> that expressed CD107a/b (top) and produced IFNγ/TNFα (bottom) in response to cognate antigen were evaluated in 5-h assays (4 h in the presence of Golgi Stop). *A*, unstimulated. *B*, stimulated with CMV pp65<sub>495-503</sub> peptide at 2 μmol/L and costimulatory molecules (CS) αCD49d/αCD28 antibodies at 1 μg/mL each. *C*, same as in *B*, but preincubated for 1 h with 100 nmol/L dasatinib. *D*, positive control (SEB; 1 μg/mL). Representative of five experiments done with different donors (CMV, *n* = 3; EBV, *n* = 2). Similar data were obtained with staurosporine (not shown).

**Fig. 5.** Effects of dasatinib and staurosporine on antigen-specific CD8<sup>+</sup> T-cell clones and  $\gamma\delta$  T cells. **A**, EBV-C CD8<sup>+</sup> T cells ( $2 \times 10^4$ ) were pretreated with either 50 nmol/L dasatinib or 50 nmol/L staurosporine, or left untreated, for 1 h in a total volume of 100  $\mu$ L. C1R B cells that express HLA A\*0201 were pulsed with GLCVTLVAML peptide at concentrations of 0 to 100  $\mu$ M for 1 h, then washed twice. Pulsed target cells ( $2.5 \times 10^4$ ) were then added to the previously treated EBV-C CD8<sup>+</sup> T cells in a final volume of 200  $\mu$ L and incubated for 4 h at 37°C. After pelleting the cells by centrifugation, supernatant was collected and assayed for IL-2, TNF $\alpha$ , and IFN $\gamma$  levels using a TH1/TH2 cytokine kit (BD). **B**, AJH  $\gamma\delta$  T cells ( $1 \times 10^4$ ) were treated with either 10 or 50 nmol/L dasatinib, or left untreated, for 1 h at 37°C. After addition of 1  $\mu$ g/mL OKT3, cells were incubated for a further 4 h at 37°C. Supernatant was then harvested and analyzed by cytometric bead array. **C**, cells ( $1 \times 10^5$ ) of the CD8<sup>+</sup> T-cell clones EBV-C, MEL-13, or ILA-1 were either left untreated or treated with either dasatinib or staurosporine at concentrations of 2, 10, or 50 nmol/L for 3 h at 37°C and analyzed for TCR $\alpha\beta$  and CD8 expression as described in Materials and Methods.

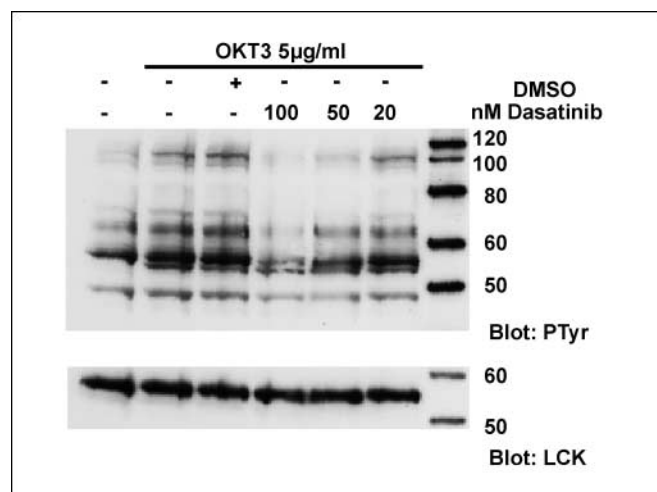


dissect the mechanism by which dasatinib and staurosporine inhibit all T-cell effector functions, we stimulated Jurkat T cells in the presence or absence of dasatinib or staurosporine with OKT3. Decreased phosphorylation of proteins in the 50 to 120 kDa range was observed; reprobing of the Western blots with a specific LCK antibody showed the dephosphorylation of LCK by dasatinib (Fig. 6). We did not observe dephosphorylation with staurosporine at 50 and 100 nmol/L; only at

1  $\mu$ mol/L staurosporine was general dephosphorylation observed (data not shown).

## Discussion

In this study, we examined the effects of the dual BCR-ABL/SRC kinase inhibitor dasatinib on multiple T-cell effector functions. Using the promiscuous TK inhibitor staurosporine



**Fig. 6.** Dasatinib inhibits signal transduction through the CD3/TCR complex. Jurkat T cells were stimulated with OKT3 for 5 min in the presence or absence of dasatinib. Whole-cell lysates were analyzed by Western blotting for tyrosine phosphorylation (PTyr). To quantify LCK expression and to control for protein loading, the antiphosphotyrosine antibody was stripped off and the membrane was reprobed with an LCK-specific antibody.

as a comparator, we found that dasatinib exerted global inhibitory effects on T-cell effector functions including proliferation, activation, cytokine production, and degranulation. Furthermore, we observed that different T-cell subsets exhibited different thresholds for inhibition, with CD4<sup>+</sup> T cells being marginally more sensitive than CD8<sup>+</sup> T cells and naïve T cells being more sensitive than memory T-cell subsets. This latter observation might reflect differential expression levels of LCK, a SRC kinase with an important role in proximal signal transduction. Indeed, it is known that antigen sensitivity correlates with LCK expression (29), and differential requirements for LCK during primary and memory CD8<sup>+</sup> T-cell responses have been observed in mice (30). Nevertheless, all T-cell subsets were inhibited by dasatinib within the therapeutic range achieved with current standard dosing schedules.

In contrast to the immunomodulatory effects of imatinib, which inhibits secretion of the proinflammatory cytokines IFN $\gamma$  and TNF $\alpha$  but does not affect cytolytic effector functions to the same extent (26, 31), dasatinib and staurosporine exhibited suppressive effects on both the production of cytokines and degranulation. These latter effects might affect CD8<sup>+</sup> T-cell-mediated control of intracellular infections and tumors because degranulation is a necessary precursor to the release of cytolytic granules located in the cytoplasm (32). Notably, we also observed that dasatinib halted production of TH1 and TH2 cytokines by  $\gamma\delta$  T cells. In some situations, this might be advantageous given the role of these cells in certain adverse immune-mediated syndromes (33) and the potential role for these cells in autoimmune disease (34, 35). On the other hand, significant expansions of  $\gamma\delta$  T cells have been documented in response to multiple intracellular pathogens including EBV

infection, and recent studies have suggested a role for  $\gamma\delta$  T cells in the control of human and murine CMV viruses *in vivo* (16, 17). Therefore, inhibition of  $\gamma\delta$  T-cell activation could increase host susceptibility to viral infection.

The *in vitro* assays used here may not completely reproduce *in vivo* conditions, but our data still suggest that dasatinib and staurosporine attenuate T-cell function at the level of effector cytokine secretion and degranulation at therapeutically relevant concentrations (24). These findings suggest that dasatinib could be considered as a potential therapeutic strategy in T-cell-mediated autoimmune disorders or as an immunosuppressant in the transplantation setting. Inevitably, the observation that both cytokine production and degranulation are impaired is a concern from the clinical standpoint given the potential increased susceptibility to infections. Indeed, in clinical trials conducted to evaluate the safety and efficacy of dasatinib before approval for the treatment of imatinib-refractory chronic myeloid leukemia and Ph<sup>+</sup> acute lymphoblastic leukemia, a small percentage of patients developed infections, especially pneumonia, which might reflect not only myelosuppression but also inhibition of T-cell effector functions (11–13). However, as our washout experiments show, the effects of dasatinib on T cells are transient; thus, dose reduction or treatment interruption could rapidly reverse any dasatinib-induced immune insufficiency, which is in line with what is observed in current clinical practice. Furthermore, we observed no obvious increase in activation-induced cell death with dasatinib. Apoptosis is one mechanism through which immune responses are controlled, and LCK is required for activation-induced cell death to occur in T cells (36). In line with the literature, we observed a decreased rate of activation-induced cell death in T cells treated with dasatinib and activated with OKT3 and costimulatory molecules. However, in contrast to the effects of imatinib on apoptosis (6, 9), the apoptosis rate of OKT3-stimulated T cells in the presence of dasatinib was higher than in the untreated T cells, thereby arguing for a potential interaction of dasatinib with other apoptosis pathways.

Mechanistically, the inhibitory effects of dasatinib documented above seem to be mediated primarily by inhibition of LCK. We observed profound dephosphorylation of LCK in Jurkat T cells (Fig. 6); furthermore, the striking inhibition of TCR and CD8 down-regulation on the surface of antigen-specific T-cell clones attests to the potency with which very proximal events are inhibited by dasatinib (Fig. 5C).

Based on our findings, close monitoring of patients undergoing treatment with TK inhibitors seems to be warranted with respect to reactivation of persistent viral infections and newly acquired opportunistic infections. However, these findings also indicate a potential role for dasatinib as an immunosuppressant in the fields of transplantation and autoimmunity. Of note, a recent case report showed the efficacy of dasatinib in the treatment of a patient with thymoma (37). Thus, whereas caution is advised with respect to the clinical use of dasatinib, the data presented here also suggest new therapeutic uses for this novel drug.

## References

1. Druker BJ, Talpaz M, Resta DJ, et al. Efficacy and safety of a specific inhibitor of the BCR-ABL tyrosine kinase in chronic myeloid leukemia. *N Engl J Med* 2001;344:1031–7.
2. Savage DG, Antman KH. Imatinib mesylate—a new oral targeted therapy. *N Engl J Med* 2002;346:683–93.
3. Bornhauser M, Kroger N, Schwerdtfeger R, et al. Allogeneic haematopoietic cell transplantation for chronic myelogenous leukaemia in the era of imatinib: a retrospective multicenter study. *Eur J Haematol* 2006;76:9–17.

4. Kantarjian HM, O'Brien S, Cortes JE, et al. Imatinib mesylate therapy for relapse after allogeneic stem cell transplantation for chronic myelogenous leukemia. *Blood* 2002;100:1590–5.
5. Olavarria E, Craddock C, Dazzi F, et al. Imatinib mesylate (STI571) in the treatment of relapse of chronic myeloid leukemia after allogeneic stem cell transplantation. *Blood* 2002;99:3861–2.
6. Chen J, Schmitt A, Chen B, et al. Imatinib impairs CD8<sup>+</sup> T lymphocytes specifically directed against the leukemia-associated antigen RHAMM/CD168 *in vitro*. *Cancer Immunol Immunother* 2007;56:849–61.
7. Dietz AB, Souan L, Knutson GJ, Bulur PA, Litzow MR, Vuk-Pavlovic S. Imatinib mesylate inhibits T-cell proliferation *in vitro* and delayed-type hypersensitivity *in vivo*. *Blood* 2004;104:1094–9.
8. Cwynarski K, Laylor R, Macchiarulo E, et al. Imatinib inhibits the activation and proliferation of normal T lymphocytes *in vitro*. *Leukemia* 2004;18:1332–9.
9. Seggewiss R, Lore K, Greiner E, et al. Imatinib inhibits T-cell receptor-mediated T-cell proliferation and activation in a dose-dependent manner. *Blood* 2005;105:2473–9.
10. Zamoyska R, Basson A, Filby A, Legname G, Lovatt M, Seddon B. The influence of the src-family kinases, Lck and Fyn, on T cell differentiation, survival and activation. *Immunol Rev* 2003;191:107–18.
11. Talpaz M, Shah NP, Kantarjian H, et al. Dasatinib in imatinib-resistant Philadelphia chromosome-positive leukemias. *N Engl J Med* 2006;354:2531–41.
12. Cortes J, Rousselot P, Kim DW, et al. Dasatinib induces complete hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in blast crisis. *Blood* 2007;109:3207–13.
13. Guilhot F, Apperley J, Kim DW, et al. Dasatinib induces significant hematologic and cytogenetic responses in patients with imatinib-resistant or -intolerant chronic myeloid leukemia in accelerated phase. *Blood* 2007;109:4143–50.
14. Lombardo LJ, Lee FY, Chen P, et al. Discovery of *N*-(2-chloro-6-methyl-phenyl)-2-(6-(4-(2-hydroxyethyl)-piperazin-1-yl)-2-methylpyrimidin-4-ylamino)thiazole-5-carboxamide (BMS-354825), a dual Src/Abl kinase inhibitor with potent antitumor activity in preclinical assays. *J Med Chem* 2004;47:6658–61.
15. Das J, Chen P, Norris D, et al. 2-aminothiazole as a novel kinase inhibitor template. Structure-activity relationship studies toward the discovery of *N*-(2-chloro-6-methylphenyl)-2-[(6-(4-(2-hydroxyethyl)-1-piperazinyl)-2-methyl-4-pyrimidinyl)amino]-1,3-thiazole-5-carboxamide (dasatinib, BMS-354825) as a potent pan-Src kinase inhibitor. *J Med Chem* 2006;49:6819–32.
16. Dechanet J, Merville P, Lim A, et al. Implication of  $\gamma\delta$  T cells in the human immune response to cytomegalovirus. *J Clin Invest* 1999;103:1437–49.
17. Ninomiya T, Takimoto H, Matsuzaki G, et al.  $V\gamma 1^+ \gamma\delta$  T cells play protective roles at an early phase of murine cytomegalovirus infection through production of interferon- $\gamma$ . *Immunology* 2000;99:187–94.
18. Magnusson MK, Meade KE, Nakamura R, Barrett J, Dunbar CE. Activity of STI571 in chronic myelomonocytic leukemia with a platelet-derived growth factor  $\beta$  receptor fusion oncogene. *Blood* 2002;100:1088–91.
19. Betts MR, Casazza JP, Patterson BA, et al. Putative immunodominant human immunodeficiency virus-specific CD8(+) T-cell responses cannot be predicted by major histocompatibility complex class I haplotype. *J Virol* 2000;74:9144–51.
20. Hutchinson SL, Wooldridge L, Tafuro S, et al. The CD8 T cell coreceptor exhibits disproportionate biological activity at extremely low binding affinities. *J Biol Chem* 2003;278:24285–93.
21. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8<sup>+</sup> T cells by a flow cytometric assay for degranulation. *J Immunol Methods* 2003;281:65–78.
22. Wooldridge L, Hutchinson SL, Choi EM, et al. Anti-CD8 antibodies can inhibit or enhance peptide-MHC class I (pMHCI) multimer binding: this is paralleled by their effects on CTL activation and occurs in the absence of an interaction between pMHCI and CD8 on the cell surface. *J Immunol* 2003;171:6650–60.
23. Green AE, Lissina A, Hutchinson SL, et al. Recognition of nonpeptide antigens by human  $V\gamma 9V\delta 2$  T cells requires contact with cells of human origin. *Clin Exp Immunol* 2004;136:472–82.
24. Dasatinib (BMS-354825), Oncologic drug advisory committee (ODAC) briefing document, NDA 21-986; Bristol-Myers-Squibb Company; 2006 Jun 2. Available from: <http://www.fda.gov/ohrms/dockets/AC/06/briefing/2006-4220-B1-01BristolMyersSquibb-Background.pdf>.
25. Seder RA, Ahmed R. Similarities and differences in CD4<sup>+</sup> and CD8<sup>+</sup> effector and memory T cell generation. *Nat Immunol* 2003;4:835–42.
26. Mumprecht S, Matter M, Pavelic V, Ochsenbein AF. Imatinib mesylate selectively impairs expansion of memory cytotoxic T cells without affecting the control of primary viral infections. *Blood* 2006;108:3406–13.
27. Sinai P, Berg RE, Haynie JM, Egorin MJ, Ilaria RL, Jr., Forman J. Imatinib mesylate inhibits antigen-specific memory CD8 T cell responses *in vivo*. *J Immunol* 2007;178:2028–37.
28. Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 1995;375:148–51.
29. Slika MK, Whitton JL. Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR. *Nat Immunol* 2001;2:711–7.
30. Tewari K, Walent J, Svaren J, Zamoyska R, Suresh M. Differential requirement for Lck during primary and memory CD8<sup>+</sup> T cell responses. *Proc Natl Acad Sci U S A* 2006;103:16388–93.
31. Leder C, Ortler S, Seggewiss R, Einsele H, Wiendl H. Modulation of T-effector function by imatinib at the level of cytokine secretion. *Exp Hematol* 2007;35:1266–71.
32. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. *Nat Rev Immunol* 2002;2:735–47.
33. Hewitt RE, Lissina A, Green AE, Slay ES, Price DA, Sewell AK. The bisphosphonate acute phase response: rapid and copious production of proinflammatory cytokines by peripheral blood gd T cells in response to aminobisphosphonates is inhibited by statins. *Clin Exp Immunol* 2005;139:101–11.
34. Hayday A, Geng L.  $\gamma\delta$  cells regulate autoimmunity. *Curr Opin Immunol* 1997;9:884–9.
35. Borsellino G, Koul O, Placido R, et al. Evidence for a role of  $\gamma\delta$  T cells in demyelinating diseases as determined by activation states and responses to lipid antigens. *J Neuroimmunol* 2000;107:124–9.
36. Yu XZ, Levin SD, Madrenas J, Anasetti C. Lck is required for activation-induced T cell death after TCR ligation with partial agonists. *J Immunol* 2004;172:1437–43.
37. Chuah C, Lim TH, Lim AS, et al. Dasatinib induces a response in malignant thymoma. *J Clin Oncol* 2006;24:e56–8.