Dasatinib Changes Immune Cell Profiles Concomitant with Reduced Tumor Growth in Several Murine Solid Tumor Models

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Abstract

Dasatinib, a broad-range tyrosine kinase inhibitor, induces rapid mobilization of lymphocytes and clonal expansion of cytotoxic cells in leukemia patients. Here, we investigated whether dasatinib could induce beneficial immunomodulatory effects in solid tumor models. The effects on tumor growth and on the immune system were studied in four different syngeneic mouse models (B16.OVA melanoma, 1936 sarcoma, MC38 colon, and 4T1 breast carcinoma). Both peripheral blood (PB) and tumor samples were immunophenotyped during treatment. Although in vitro dasatinib displayed no direct cytotoxicity to B16 melanoma cells, a significant decrease in tumor growth was observed in dasatinib-treated mice compared with vehicle-treated group. Further, dasatinib-treated melanoma-bearing mice had an increased proportion of CD8+ T cells in PB, together with a higher amount of tumor-infiltrating CD8+ T cells. Dasatinib-mediated antitumor efficacy was abolished when CD4+ and CD8+ T cells were depleted with antibodies. Results were confirmed in sarcoma, colon, and breast cancer models, and in all cases mice treated daily with dasatinib had a significant decrease in tumor growth. Detailed immunophenotyping of tumor tissues with CyTOF indicated that dasatinib had reduced the number of intratumoral regulatory T cells in all tumor types. To conclude, dasatinib is able to slow down the tumor growth of various solid tumor models, which is associated with the favorable blood/tumor T-cell immunomodulation. The assessment of synergistic combinatorial therapies with other immunomodulatory drugs or targeted small-molecule oncokinase inhibitors is warranted in future clinical trials. Cancer Immunol Res; 5(2): 157–69. ©2017 AACR.

Introduction

Cancer is recognized and controlled by the immune system via a multifaceted process called immunoediting (1), and the escape of tumor cells from immune surveillance is now considered as one of the hallmarks of cancer (2). Thus, stimulating the immune system to overcome the immunosuppression caused by tumor cells is an attractive treatment strategy (3). Current immunotherapy approaches comprise cancer vaccines, immunomodulatory agents, adoptive T-cell therapy, oncolytic viruses, and immune checkpoint blockade. Among these, immune checkpoint inhibitors against programmed cell death 1 (PD-1) or cytotoxic T-lymphocyte antigen–4 (CTLA-4) have already been successful in the treatment of melanoma, renal cell carcinoma, and non–small cell lung carcinoma (4, 5).

Dasatinib is a broadly specific BCR/ABL and SRC-family tyrosine kinase inhibitor (TKI) used in the treatment of chronic myeloid leukemia (CML) and BCR-ABL+ acute lymphoblastic leukemia (ALL; ref. 6). SRC kinases are key moderators of T-cell activation and proliferation (7). Several in vitro results have suggested that dasatinib has immunosuppressive effects due to SRC kinase inhibition (8–11). However, the short half-life of dasatinib (3–4 hours) alters the situation in vivo. A short exposure to dasatinib can actually activate NK cells (12, 13). Similarly, in clinical use, immunostimulatory effects have been observed during the long-term use of dasatinib (14–17). For example, in a subset of leukemia patients, dasatinib treatment causes a clonal expansion of large granular lymphocytes (LGL) in blood, including expansion of CD8+ T cells and NK cells (17, 18) and a decrease of regulatory T cells (Treg; refs. 17, 19). The expansion of LGLs has also been associated with improved treatment responses (17). In addition, dasatinib causes a rapid mobilization of cytotoxic lymphocytes that closely follows the drug plasma concentration (16). This phenomenon is accompanied by an enhanced NK cell cytotoxicity and transmigration through the endothelial cell layer. Similar effects have not been observed with other TKIs, imatinib, or nilotinib (16).

Even though dasatinib causes several changes in the immune cell profile in leukemia patients, it is currently unknown whether...
these effects could contribute to cancer control in solid tumor models, nor whether mobilized T cells are able to migrate into the tumor. To study these aspects, we first used a solid subcutaneous B16.OVA melanoma tumor model and monitored the tumor growth and lymphocyte responses during the treatment. Our results illustrate that dasatinib could slow down the growth of aggressive B16.OVA melanoma tumors, which was accompanied by increased amounts of tumor-infiltrating CD8+ T cells and reduced CD4/CD8 T-cell ratio in the peripheral blood, indicating that dasatinib could induce the migration of CD8+ T cells into the tumor tissue. The immunomodulatory effects were confirmed using three other models (1956 sarcoma, MC38 colon, and 4T1 breast carcinoma), and detailed immunophenotyping with CyTOF indicated that dasatinib also decreased the amount of regulatory T cells within the tumor. Finally, the depletion of T cells in the B16.OVA model abrogated the antitumor effects of dasatinib, emphasizing the potent immunomodulatory properties of this TKI.

Materials and Methods

Cell lines
Dasatinib was purchased from LC Laboratories (Cat. D-3307) and dissolved in 80 mmol/L citrate buffer pH 3.0 in a concentration of 15 mg/mL. The ovalbumin-expressing murine melanoma cell line B16.OVA was provided by Richard Ville (Mayo Clinic, Rochester, MN; September 30, 2010), and the human CML cell line K562 was purchased from Sigma-Aldrich (Cat. 89121407, year 2011). 4T1 breast and MC38 colon carcinoma cells were purchased from ATCC (Wesel, Germany, both in year 2013). The 1956 sarcoma line was a kind gift from Dr. Robert Schreiber (20) in year 2015 (Washington University School of Medicine. St. Louis, MO, USA). All cell lines were cryopreserved until use and cultured in RPMI 1640 medium (Lonza, Cat. 12167F) containing 10% FBS, 2 mmol/L l-glutamine (EuroClone, ECB3000D), and penicillin/streptomycin (EuroClone, ECB3001D). Prior to start of the experiments, B16.OVA cells were cultured for 5 days and passed once, K562 cells for 2 months and passed twice a week, 1956 and MC38 cells for 9 days and passed twice, and 4T1 cells for 17 days and passed 4 times during this period. B16.OVA cells were either preincubated with a concentration series (0, 1, 10, 100, 1,000, and 10,000 nmol/L) for 1 hour at 37°C or alternatively dasatinib was added to the top layer of agar. B16.OVA cells/well (1,000) were embedded in 0.3% agar, and culture media (100 μL) were added on top. Embaded cells were cultured for 12 days, and colonies were counted.

In vitro tumor models
To study the antitumor effects of dasatinib treatment in vivo B16.OVA cells (250,000 cells/mouse) in 100 μL of RPMI 1640 medium were injected s.c. into dorsal flank of each C57BL/6J female mice (The Jackson Laboratory and Envigo). Mice were 14 to 16 weeks old when the experiment started. After shaving the hair from the tumor site, tumor sizes were measured manually by a digital caliper and calculated according to the formula A = width x length. Specific growth rate (SGR) was calculated based on the first and the last day of the treatment according to the formula SGR = ln(Vf/V1) / (t1 - t0) (21). The mice (n = 6/group) were treated daily intragastric (i.g.) either with dasatinib (30 mg/kg) or vehicle only (citrate buffer). All antibodies for depletion were purchased from Bio X Cell Inc. Depletion of the CD4+ , CD8+ , and NK cell populations was performed by injecting antibodies to mouse CD4 (clone GK1.5, Cat. BE0003-1), CD8a (clone 2.43, Cat. BE0061), and NK1.1 (clone PK136, Cat. BE0036), respectively. Rat IgG2b (clone LT2-2, Cat. BE0090) was injected as control. Depleting antibodies (200 μg) were injected i.p. 5 days before and the day treatment started. Depletion was confirmed by analyzing the peripheral blood of the mice by flow cytometry.

To validate the antitumor effects of dasatinib more broadly, we utilized several additional tumor models including the MC38 murine colon carcinoma, the 3-methylcholanthrene (MCA) induced sarcoma 1956, both grown in C57BL/6J mice (Charles River Laboratory), and the 4T1 breast carcinoma model grown in Balb-c mice (Envigo). Tumors were initiated from cells maintained in cell cultures in log growth phase in RPMI medium supplemented with 25 mmol/L HEPES and 10% FBS at 37°C in a humidified atmosphere (5% CO2). The required number of animals needed to detect a meaningful response was calculated at the start of the experiment. Each mouse was given a subcutaneous inoculation of 1 x 10^6.
tumor cells. With 4T1 breast carcinoma cells, we also performed an orthotopic model by injecting the cancer cells directly to the mouse mammary fat pad to better mimic the natural environment. For treatment randomization, the tumors were allowed to grow to 50–100 mg (tumors outside the range were excluded), and mice were evenly distributed to various treatments and control groups. Treatment of each mouse was based on individual body weight. Treated animals were checked daily for treatment-related toxicity/mortality. Tumor sizes were measured manually by a digital caliper and calculated with the same formula as described above for the B16.OVA melanoma model.

The animal experiments were approved by the Animal Experiment Board of the State Provincial Office of Southern Finland. Animal studies conducted in the United States were approved by the appropriate ethics committee. All studies have been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Blood sampling and leukocyte differential counts
Peripheral blood (PB) was collected into heparin-precoated tubes by making an incision in the tail of the mice before tumor transplantation, before treatment, and on treatment days 4, 8, and 11. After treating the blood samples with ammonium–chloride–potassium (ACK) lysing buffer (Life Technologies, Cat. A10492-01), white blood cells (WBC) were counted manually under microscope by hemocytometer. Differential counts were performed manually from the blood smears. Fold-change values were used to analyze the cell counts before dasatinib intake and 1 hour after due to large variation in baseline cell counts between individual animals.

Flow cytometry of PB samples
PB samples were stained with fluorochrome-conjugated mAbs to CD45.1-APC-Cy7 (Cat. 557659, BD Biosciences), CD3-Pe-Cy7 (Cat. 560591, BD Biosciences), CD4-PerCP (Cat. 560591, BD Biosciences), CD8-FITC (Cat. 121611, BioLegend) and NK1.1-PE (Cat. 557391, BD Biosciences). To assess the functional properties of lymphocytes, we used conjugated antibodies specific for immune checkpoint inhibitors CTLA-4 (BV421, Cat. 106311, BioLegend) and PD-1 (PerCP-Cy5.5, Cat. 135207, BioLegend), and a degranulation marker CD107a (PE, Cat. 121611, BioLegend).

Tumor samples
Tumors were dissociated with gentle pipetting in PBS, and immunophenotyping of tumors was done by flow cytometry using antibodies to CD45–APC-Cy7, CD3–Pe-Cy7, CD8–FITC, CD107a–PerCP-Cy5.5, CTLA-4–BV421, and CD107a–PE. Tumors were surgically removed with a scalpel and then divided in half. One part of the tumor was fixed in 4% paraformaldehyde (PFA) for immunohistochemistry while the other half was transferred into 1.5-mL tubes containing RPMI 1640 medium and then homogenized manually using micro tube pellet pestle (Fisher Scientific, Cat. 12-141-367). After centrifugation at 300 × g, the supernatant was discarded, and cells were resuspended in RPMI medium equal to the tumor weight. Tumor homogenate was diluted 1:1 in PBS and stained. Countbright beads (Invitrogen, Cat. C36950) were added and 2,000 beads were counted with FACsAria II (BD Biosciences).

Immunohistochemistry
After fixing in 4% PFA, the tumor tissues were dehydrated and embedded into paraffin blocks and cut at 4 μm thickness. Anti-CD8 (orb10325, Biorbyt) was used for the immunostainings. Brieﬂy, sections were washed on the glass slides, deparaffinized, and rehydrated. Antigen unmasking was done by Lab Vision PreTreatment module in Tris–HCL buffer pH 8.5 followed by peroxide blocking, primary antibody incubation, and HRP detection steps, which were all done by Lab Vision autostainer (Thermo Scientiﬁc). The sections were counterstained with Mayer-Hematoxylin. The counting of the tumor infiltrating CD8+ T cells was done at 50× magniﬁcation starting from capsule area and counting 3 ﬁelds toward to the core of the tissue. The same process was repeated 3 times. All counts were summed, and median was calculated.

CyTOF mass cytometry for tumor samples
Fresh mouse tumors were collected in RPMI medium, and then enzymatically dissociated using a mouse tumor dissociation kit and the gentleMACS dissociator (Miltenyi Biotech) according to the manufacturer’s protocol. The cells were stained with a panel of metal isotope conjugated anti-mouse antibodies (DVS Sciences; Supplementary Table S1), all of which have been validated by the manufacturer. Normalized, background-subtracted FCS files were imported to Cytobank (www.cytobank.org, Cytobank, Inc.) for single cell and population gating. EQ Calibration beads (Ce140)-, DNA intercalator (Ir191/193)+, Single (Event Length 20–50), Viable (Rh103), CD45+ population were manually gated as input for SPADE tree analysis. An unsupervised agglomerative hierarchical clustering step consolidated CD45+ cells into clusters of phenotypically similar cells based on a set of “core” markers (indicated with an asterisk in Supplementary Table S1). The percentage of total or the median intensity of a particular parameter within each node (or cluster) for each experimental condition was used for data visualization and statistical analysis. The CyTOF assay was initially validated with patient blood samples to conﬁrm that the data are comparable with the traditional ﬂow cytometry.

Statistical analysis
Values in the ﬁgures are shown as mean ± SEM, mean ± SD, or median, as indicated. The Mann–Whitney test and the Student unpaired t test were used when comparing two groups and one- or two-way ANOVA were used for multiple comparisons. Statistical calculations were performed using Prism program 6.0 (GraphPad Software Inc.).

Results
Dasatinib induced rapid mobilization of lymphocytes in mice
To validate whether dasatinib induces rapid lymphocyte mobilization similar as in humans, we administered dasatinib in a series of concentrations into naïve mice (16). Dasatinib caused a rapid increase in the WBC count in a dose-dependent fashion (Fig. 1A). Although when compared with the vehicle, 5 and 10 mg/kg concentrations caused a modest increase, 50 and 150 mg/kg concentrations gave mean fold changes of WBC counts of 3.95 (P = 0.03) and 5.31 (P = 0.04), respectively. However, in all groups, there was a clear variation in the extent of mobilization between individual mice, and some mice had more marked
Figure 1.
WBC counts before and after 1 hour of dasatinib administration. A, Dasatinib dissolved in citrate buffer was given i.g. at concentrations 0, 5, 10, 50, and 150 mg/kg. Peripheral blood samples were collected from tail veins before and 1 hour after dasatinib administration. WBC counts were analyzed manually under microscope by hemocytometer, and fold changes were counted based on baseline values. Horizontal lines show median values, and error bars SD. One-way ANOVA and unpaired parametric t-test were used for statistical comparisons. B–E, Mice were treated with 30 mg/kg dasatinib or vehicle only (citrate buffer), and WBC counts were analyzed on day 8 after the start of therapy. Blood samples were taken both pre-dasatinib and after 1 hour of dasatinib administration. P values were analyzed with an unpaired parametric t-test.
leukocytosis induced by dasatinib treatment (Fig. 1A). Similarly, we tested the immunomodulatory effects of dasatinib (30 mg/kg) in B16.OVA tumor-bearing mice and observed a 5.36-fold increase in the WBC counts 1 hour after dasatinib administration (control 2.0, \( P = 0.02 \); Fig. 1B). However, among the six mice tested, two had only modest leukocytosis, and no effect was observed in one mouse. Differential WBC count analysis showed a significant fold increase of lymphocytes (mean dasatinib 5.17-vs. control 2.16-fold increase, \( P = 0.02 \); Fig. 1C), whereas in monocytes and neutrophils only a trend toward increased blood mobilization was observed (Fig. 1D and E).

**In vitro cytotoxic effect of dasatinib on B16.OVA cells**

To assess the direct cytotoxic effect of dasatinib on B16.OVA cells, we performed an *in vitro* cell viability assay after incubating the cells with dasatinib in a concentration series (0, 1, 10, 100, 1,000, and 10,000 nmol/L) for 48 hours. A moderate unspecific cytotoxicity of B16.OVA cells was observed only with the two highest concentrations (1,000 and 10,000 nmol/L), whereas in K562 cells (a CML blast crisis cell line) almost complete killing was observed already with the 100 nmol/L concentration (Fig. 2A). The cell viability of B16.OVA cells was 90% with the 100 nmol/L of dasatinib concentration, suggesting that there is no direct dasatinib-sensitive oncokines target in this cell line.

To study the cytotoxic effects of dasatinib on B16.OVA cells in a three-dimensional setting, we performed a colony-formation assay. Either preincubation with dasatinib or constant exposure to dasatinib did not have an effect on the colony formation of B16. OVA cells (Fig. 2B).

**Dasatinib reduces B16.OVA melanoma tumor growth in vivo**

To assess the antitumor effect of dasatinib, we first treated B16. OVA tumor-bearing mice with dasatinib (30 mg/kg/day i.g.) or with vehicle. The subcutaneously injected B16.OVA cells appeared to grow very rapidly and the mice needed to be sacrificed already at day 11. Mice treated with dasatinib displayed greater tumor control than after treatment with vehicle, with smaller tumor volumes and reduced specific tumor growth rate (0.06 vs. 0.18, \( P = 0.03 \)). The experiment was repeated two times showing similar results. When results from the two experiments were combined, a significant reduction of the tumor volumes was observed in the dasatinib-treated group (Fig. 2C).

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**Figure 2.** *In vitro* and *in vivo* effects of dasatinib. **A**, B16.OVA cells were incubated for 48 hours with an increasing concentration of dasatinib, and the viability of the cells was measured with an MTS assay. The dasatinib-sensitive K562 cell line was used as a control. **B**, B16.OVA cells were embedded in agar together with dasatinib. Colonies were counted on day 12. **C**, Two separate tumor experiments were performed in identical manner regarding the dasatinib dose (30 mg/kg/day) with the same administration route (i.g.). At both times, 6 mice were in each treatment group (total \( n = 12 \)/group). The tumor volumes were manually measured by digital caliper and calculated with the formula \( \text{tumor size (volume) = width}^2 \times \text{length}/2 \). Error bars, SEM. One-way ANOVA was used for statistical comparisons except in **C**, where two-way ANOVA was applied.
PB immune cell composition changed with dasatinib treatment

Disregarding outliers, early lymphocytosis in peripheral blood correlated inversely with specific tumor growth rate after dasatinib administration ($R^2 = 0.878$; Supplementary Fig. S1), suggesting that the antitumor effects of dasatinib are influenced by its capacity to mobilize immune cells. Over the course of the entire 11-day treatment period, however, the proportion of blood lymphocytes decreased in dasatinib-treated mice compared with the control group. At the same time, the number of WBCs in the dasatinib group tended to decrease, whereas the proportion of monocytes and granulocytes increased.

To assess changes in the lymphocyte profile, we monitored their phenotype in peripheral blood before the tumor challenge and before the start of the treatment, as well as during the therapy (days 4, 8, and 11). On day 8, dasatinib-treated mice had increased the proportion of CD8$^+$ T cells in circulation compared with vehicle-injected mice (median 16.6% vs. 13.2%, $P = 0.001$, Fig. 3A), and supporting this, the CD4/CD8 ratio was significantly decreased (1.39 vs. 1.52, $P = 0.04$, Fig. 3B). During tumor growth, the mean expression of the immune checkpoint molecule CTLA-4 on CD8$^+$ T cells increased from 1.2% to 9% in the control group, whereas the dasatinib group showed a more modest increase (1.2%–5.7%, Fig. 3C).

The proportion of NK cells from total lymphocytes increased marginally during the dasatinib treatment (5.1% on day 8 vs. 4% at the baseline, $P = 0.04$, Fig. 3D). On day 11, the proportion of degranulating (CD107$^+$) NK cells seemed to be larger in dasatinib-treated mice than in the control group, but due to variation no statistical significance was reached (51.5% vs. 38.3% of NK cells, $P = 0.08$, Fig. 3E).

Increased infiltration of CD8$^+$ T cells into tumors

To study lymphocyte infiltration, tumor content was analyzed both with the immunohistochemistry and flow cytometry (Fig. 4). The number of tumor infiltrating CD8$^+$ T cells within and in the proximity of the capsular region was counted from the anti-CD8–immunostained tissue slides (Fig. 4A and B; Supplementary Fig. S2). An increased number of tumor infiltrated CD8$^+$ T cells was observed in the dasatinib-treated group when compared with the control group (median 17 vs. 4 cells/counted microscope fields, $P = 0.03$, Fig. 4C).

However, flow analysis of the homogenized tumor tissue showed that 80% of the tumor-infiltrating CD8$^+$ T cells expressed PD-1 antigen compared with <5% of peripheral blood CD8$^+$ T cells, suggesting that these T cells are activated and may be prone to anergy or exhaustion caused by the immunosuppressive tumor (Fig. 4D and E). The control group had less tumor-infiltrating CD8$^+$ T cells expressing PD-1 compared with the dasatinib-treated mice (dasatinib median 83.7%, control 71.3%, $P = 0.06$).

T and NK cell depletion abolished dasatinib-associated effects on tumor growth

To functionally assess the impact of immune cells on dasatinib-induced tumor growth, we depleted CD4$^+$ and CD8$^+$ T cells and NK cells from B16.OVA tumor-bearing animals. Before the start of dasatinib treatment, T-cell and NK cell counts were analyzed with flow cytometry, and total depletion was observed (Supplementary Fig. S3). In T cell– and NK cell–depleted animals, the overall tumor growth in the non–dasatinib-treated group was slightly faster than in the animals without lymphocyte depletion, but no statistically significant difference was observed (Fig. 4F). In contrast, both IgG + dasatinib and dasatinib alone treated mice had significantly smaller tumors than dasatinib + T/NK antibody–treated mice (Fig. 4F). Thus, both the depletion of T cells and the combination of T and NK cells prevented the effects of dasatinib to slow down the tumor growth.

MCA-induced 1956 sarcoma, MC38 colon carcinoma, and 4T1 breast carcinoma models

To confirm the results in other solid tumor types, we used MCA-induced sarcoma, MC38 colon carcinoma, and 4T1 breast carcinoma models. First, we assessed the direct cytotoxicity of dasatinib on these cells in vitro (Fig. 5A–C). Dasatinib had no effect on the growth of MC38 and 4T1 cells at the physiologic 125 nmol/L concentration (Figs. 5A and B), but a moderate cytotoxic effect was observed at higher concentrations (300 nmol/L, $P < 0.05$ for 4T1 cells). The 1956 sarcoma cells were slightly more sensitive to dasatinib at lower concentrations, with cell counts declining more moderately even at 31 nmol/L concentration (Fig. 5C). However, statistical difference in pair-wise correlations was observed only at 500 nmol/L concentration when compared with vehicle-treated cells (Fig. 5C).

To study the effects in vivo, we injected MCA-induced 1956 sarcoma, MC38 colon, and 4T1 breast carcinoma cells s.c. in C57BL/6j mice and treated them either with dasatinib (30 mg/kg/day i.g.) or with vehicle. In addition, we also performed an orthotopic tumor model with 4T1 breast carcinoma cells by injecting them directly into the mouse mammary fat pads. The tumor growth was slower in these models than in the more aggressive B16 melanoma model, and mice were followed up for 3 weeks. Similarly, dasatinib treatment caused significant inhibition of the tumor growth in all four models including the orthotopic 4T1 breast cancer model (Fig. 5D–G, $P < 0.001$).

Reduced number of regulatory T cells in tumors from dasatinib-treated mice

Tumor tissues from four different models (1956, MC38, 4T1, and B16.OVA) were further analyzed with CyTOF to examine the changes induced by dasatinib treatment. The proportion of CD8$^+$ T cells did not significantly change when measured as a percentage of total CD45$^+$ WBCs. However, the proportion of CD4$^+$ T cells was reduced in all four models ($P < 0.05$ in three of four models; Fig. 6A–D; Supplementary Figs. S4–S7), which was a result of selective depletion of FoxP3$^+$ Tregs (Fig. 6E; Supplementary Table S2). Additionally, in 1956 sarcoma and MC38 colon carcinoma models, the number of monocytes within the tumor tissue increased in the dasatinib group (Fig. 6A and B; Supplementary Figs. S5E and S6E).

Discussion

In addition to direct onco kinase inhibition in tumor cells, the effects of TKI treatment may also be contributed by alterations to the immune system (22–24). In the syngeneic melanoma mouse model used here, we demonstrated that the broad-spectrum TKI dasatinib decreased tumor growth, although it had no direct effect on the tumor cells. This effect could be abolished by depleting the functional immune cells, thus suggesting an important role for the immune system. Our findings were validated when we observed similar reduction of tumor growth in three other slowly growing syngeneic mouse models (1956 sarcoma, MC38 colon, and 4T1
breast carcinoma models). With a moderate toxicity profile in patients, dasatinib could be a useful immunomodulatory add-on drug in combination regimens of nonhematologic tumors, regardless of direct antitumor efficacy. Further clinical studies in cancer patients are warranted.

Dasatinib, besides inhibiting BCR/ABL oncokinase and SRC family kinases, targets a wide range of other tyrosine kinases, such as c-KIT, EphA2, and TEC kinases (25). Inhibition of these kinases affects cell growth and migration and modulates immune responses. For example, inhibition of c-KIT impairs tumor growth

**Figure 3.**
Immunophenotype of lymphocytes during dasatinib treatment. Mice bearing B16.OVA melanoma tumors were treated either with vehicle or dasatinib (50 mg/kg). Blood samples were collected from tail vein, and lymphocyte subclasses and immunophenotype were analyzed with flow cytometry at the baseline and on day 8. A, Proportion of CD8⁺ T cells of lymphocytes; B, CD4/CD8 ratio throughout the treatment (median ± SEM); C, CTLA-4 expression on CD8⁺ T cells; D, the proportion of NK cells from lymphocytes; E, the expression of degranulation marker CD107 on NK cells on day 11. Horizontal lines indicate the median values; P values were analyzed by the Mann-Whitney U test. Two-way ANOVA was used in B.
in systemic mastocytosis (26, 27). Similarly, inhibition of EphA2 and SRC family kinases prevents growth, migration, and invasion of breast and prostate cancer. Several phase I and II trials have evaluated the efficacy of dasatinib in solid tumors, and some clinical efficacy has been observed (28). This has been thought to occur primarily through inhibition of SRC kinases, whose activation mediates invasive growth in many tumors.

Previous in vitro studies have suggested that dasatinib causes the impairment of cytotoxic T-cell functions (8, 10). This is controversial with regard to human patient findings that demonstrate lymphocyte expansion and activation during dasatinib treatment (17). Furthermore, like in leukemia patients (16), we saw a rapid, dasatinib-induced increase in lymphocyte counts in mice after dasatinib administration. However, despite this rapid mobilization, the total number of blood lymphocytes decreased at later treatment days, while the proportion of CD8$^+$ T cells, monocytes, granulocytes, and NK cells increased. This could be due to cell-type and dose-dependent effects of dasatinib as a recent ex vivo study by Wu and colleagues demonstrated that CD8$^+$ T cells and NK cells react differently to dasatinib exposure depending on incubation times and concentrations used (15). At lower doses, dasatinib increased the expansion of $\gamma\delta$ T cells, whereas higher doses inhibited them, but increased the number and function of NK cells. In addition, dasatinib reduces the adhesion of granulocytes to blood endothelium, perhaps contributing to the increased accumulation of these cells in blood over the course of the treatment and accounting for the observed increased granulocyte counts (29). The secretion of granulocyte–macrophage colony-stimulating factor (GM-CSF) has also been shown to be increased by dasatinib treatment (30).

Previously, Fraser and colleagues (31) reported that a higher dose of dasatinib (25 mg/kg/day) reduced the number of extrapulmonary B16.OVA metastases compared with a lower dose (5 mg/kg/day). The higher dose used by Fraser and colleagues was
Figure 5.
Confirmation of results with 1956 sarcoma, 4T1, and MC38 tumors. The direct cytotoxicity of dasatinib on MC38 (A), 4T1 (B), and 1956 cells (C) was studied with an increasing concentration of dasatinib, and the viable cells were counted on day 4. One-way ANOVA was used for statistical comparisons. In vivo effects were studied in mice, which had been given a subcutaneous inoculation of 1 × 10E6 tumor cells. For treatment randomization, the tumors were allowed to grow to 50 to 100 mg (tumors outside the range were excluded) and mice were evenly distributed to dasatinib (daily dose of 30 mg/kg) and vehicle groups. Tumor growth curves are shown for 1956 sarcoma tumors (D), MC38 colon carcinoma (E), and 4T1 breast carcinoma (F). G, 4T1 breast carcinoma cells were also directly injected to the mammary fat pad to better mimic the natural environment of tumor cells. Similar growth inhibition induced by dasatinib treatment was also observed in this model. P < 0.001 in all models analyzed by a two-way ANOVA test.
Figure 6.
Analysis of tumor-infiltrating leukocytes by CyTOF. Fresh mouse tumors were collected in RPMI medium, and after the enzymatic dissociation, cells were stained with a panel of metal isotope-conjugated anti-mouse antibodies and analyzed with CyTOF. Data were analyzed with Cytobank software, and an unsupervised agglomerative hierarchical clustering step consolidated CD45+ cells into clusters of phenotypically similar cells. A and B, SPADE tree presentation of CD45+ leucocytes in tumors from vehicle-treated (A) and dasatinib-treated (B) 1956 sarcoma bearing mice. C and D, CD25+FOXP3+ Tregs in vehicle-treated (C) and dasatinib-treated (D) 1956 sarcoma bearing mice. E, Intratumoral Treg frequencies in B16.OVA, 1956 sarcoma, MC38 colon carcinoma, and 4T1 breast carcinoma. P values were analyzed with one-way ANOVA and the Mann-Whitney U test.
comparable with the dose used in our study (30 mg/kg/day). If higher doses of dasatinib are better for NK cell activation, as shown by Wu and colleagues (15), the results could suggest an important role for NK cells, as they are known to exhibit antitumor activity against B16 melanoma cells (32), and they can induce CD8\(^+\) T-cell activation (33). Also, in our experiments, the number and degranulation function of NK cells was enhanced. However, the results from our depletion experiments suggest that, despite their enhanced activity, NK cells mediate antitumor efficacy through the regulation of CD8\(^+\) T cells, rather than via direct cytolysis of tumor cells. Taken together, the maximal antitumor efficacy and CD8\(^+\) T-cell-mediated cell killing may rely on optimal dosing of dasatinib, as also pulse treatment (3-day treatment with 150 mg/kg) has been reported to induce beneficial immunomodulatory effects (34).

In many previous studies, elevated numbers of tumor-infiltrating lymphocytes have been associated with better prognosis, in melanoma (35) and in several other cancers, such as ovarian and colorectal cancers (36–38). Dasatinib-induced lymphocyte mobilization is also related to increased extravasation (16), which provides a potential mechanism for our finding that dasatinib-treated mice had higher numbers of tumor-infiltrating CD8\(^+\) T cells when analyzed by immunohistochemical staining. When tumor tissues were analyzed by CyTOF, the proportion of CD8\(^+\) T cells was counted from the total leukocyte count, and thus it does not give the absolute number of individual cell types within the tumor tissue. Rapid mobilization and extravasation of cytotoxic cells induced by dasatinib treatment could increase the migration and tumor infiltration of CD8\(^+\) cells. As this mobilization has been shown to occur after every dose of dasatinib, it gives a daily boost to immune effector cells (16). This is in accordance with the previous publication by Lowe and colleagues (39) showing that the combination treatment of dasatinib and dendritic cell vaccination is able to induce an infiltration of CD8\(^+\) T cells and dendritic cells into the tumor microenvironment accompanied by tumor size reduction. In addition, combination of dasatinib and antibodies to OX40 has been reported to enhance tumor accumulation of antigen-specific T cells in a mastocytoma mouse model (34). The infiltration of CD8\(^+\) T cells together with the reduction of regulatory T cells, which was noted in all tumor models, could explain the efficacy of dasatinib in solid tumors. This is also supported by our findings showing that the T-cell depletion was sufficient to abolish dasatinib-induced reduction of tumor growth.

Despite the enhanced antitumor immune response and significantly lower growth rate, B16 melanoma tumors eventually grew also in the dasatinib group. This may be due to the aggressiveness of the B16 model, which may activate compensatory survival pathways or display residual signaling (40) or due to the inability of dasatinib to fully break tumor immunosuppression. At day 8, mice still had significantly improved immune response as demonstrated by a reduced CD4/CD8 ratio, increased NK cell proportion, and lower CTLA-4 expression on CD8\(^+\) T cells, indicating the decreased inhibition of T-cell responses (41–43). However, it is also possible that the immune response starts to fail after the tumor reaches a certain size or immunosuppression becomes even more potent. In the more slowly growing tumors (1956 sarcoma, MC38 colon, and 4T1 carcinoma), the inhibition of the tumor growth was more pronounced and long-lasting.

Mice treated with dasatinib and carrying B16.0VA melanoma tumors had significantly increased expression of PD-1 on their tumor-infiltrating CD8\(^+\) T cells. Conventionally, increased PD-1 expression on T cells has been associated with “reactive” T-cell exhaustion (44) as a result of preceding T-cell activation (45, 46). However, a recent study in melanoma suggests that PD-1 expression on tumor infiltrating lymphocytes may have a dual role and is a biomarker of autologous tumor-reactive cells (47). Similarly, we also observed that PD-1 expression on circulating CD8\(^+\) T cells was dramatically lower compared with CD8\(^+\) T cells within the tumor. Taken together, dasatinib may increase the clonal expansion of tumor-reactive CD8\(^+\) T cells in the tumor, and thus combination studies with anti–PD-1 would be of great interest, if the blocking of the PD-1 inside the tumor would be able to rescue exhausted tumor-reactive cells. However, as immune checkpoint inhibitors are known to have many side effects, the safety of the combination treatment needs to be carefully evaluated. In first-line treated CML patients, dasatinib has already been combined with another immunomodulatory drug (interferon-α; ref. 48), and no additional toxicities—including pleural effusions, which are typical dasatinib-related side effects (49, 50)—were observed. Similarly, in a published prostate cancer study, dasatinib was safely combined with chemotheraphy agent docetaxel, and no major additional toxicities occurred (51).

To conclude, broad-spectrum TKI dasatinib induces beneficial immunomodulatory effects in murine solid tumor models, and these effects are not dependent on direct cytotoxicity of dasatinib on tumor cells. Theoretically, the addition of dasatinib to treatment regimens could be advantageous in several solid tumor types to increase the infiltration of tumor-reactive lymphocytes. However, our results suggest that dasatinib monotherapy may not be sufficient to overcome potent tumor-mediated immunosuppression, and the investigation of combination treatment strategies with other immune modulators is warranted.

**Disclosure of Potential Conflicts of Interest**

A. Hemminki is the CEO of TILT Biotherapeutics Ltd, reports receiving a commercial research grant from TILT Biotherapeutics Ltd, has ownership interest (including patents) in TILT Biotherapeutics Ltd and Targovax ASA, and is a consultant/advisory board member for Amgen Inc. S. Mustjoki reports receiving a commercial research grant from Pfizer, Ariad, and Novartis, has received speakers bureau honoraria from BMS, Novartis, and Pfizer, and is a consultant/advisory board member for BMS, Novartis, Pfizer, and Celgene. J. Yan, E. Michaud, R. Smykla, and F.Y.F. Lee are employees of BMS. No potential conflicts of interest were disclosed by the other authors.

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