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Imatinib Mesylate Inhibits Antigen-Specific Memory CD8 T Cell Responses In Vivo¹

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Imatinib mesylate (IM) is effective at inducing complete cytogenetic remission in patients with chronic myelogenous leukemia. Because its influence on CD8 T cell responsiveness in vivo is unknown, we investigated the effects of IM by analyzing the response of OT-1 CD8 T cells to Listeria monocytogenes (LM) that express the cognate epitope OVA257–264 (LM-OVA). In vitro, IM had no effect on Ag-specific expansion, cell division, cell cycle progression, or IFN-γ expression in naive or memory OT-1 T cells. However, IM induced apoptosis of naive and memory OT-1 T cells at doses of >5 μM. At 15 μM IM, OT-1 T cells did not survive in vitro cultures. The primary response of OT-1 T cells in vivo to LM-OVA infection was unaltered. In contrast, continuous IM treatment resulted in a diminished memory OT-1 response. The expression of IL-7Rα, a receptor required for memory cell survival, was lower (on OT-1 cells) in animals receiving IM. These results indicate that IM treatment affects the ability of the CD8 memory pool to respond to Ag and has the potential to increase susceptibility to infection. The Journal of Immunology, 2007, 178: 2028–2037.

Imatinib mesylate (IM) (Gleevec) is a 2-phenylaminopyrimidine derivative that functions as a specific inhibitor of Abl protein tyrosine kinases (v-Abl, Bcr-Abl, and c-Abl) (1). It is now known that it is also active against the platelet-derived growth factor receptor, c-Kit, the (CD117) macrophage CSF receptor, the Abl-related gene, and their fusion proteins (2–6). IM is therefore commonly used in the treatment of chronic myelogenous leukemia (CML; Bcr-Abl oncogene) and has had promising results in the treatment of gastrointestinal stromal tumors (c-kit mutation), myeloproliferative disorders (platelet-derived growth factor receptor β mutation), eosinophilic disorders, and mast cell disease (1–3, 7–9). IM binds the kinase pocket of these targets and blocks access to ATP, thereby preventing the phosphorylation of downstream substrates.

IM affects many branches of the immune system. In addition to the inhibitory effects of IM on CD34⁺ peripheral blood progenitor cells, monocyte-derived dendritic cells, and monocytes, IM has been shown to alter T cell responses (4, 10–14). When stimulated through the TCR and CD28 or by mitogens or Ags, IM reduced the expansion of CD4 and CD8 T cells (15–17). IM at low doses does not affect the viability of T cells but does reduce the expression of CD25 and CD69 early activation markers (15–17). IM also causes a reduction of TCR-induced IL-2 production, and in CD4 T cells from CML patients undergoing IM treatment a reduction in IL-2 and Th1 cytokines was observed (18, 19). IM, upon TCR stimulation, interferes with the TCR/Abl tyrosine kinase signaling pathway resulting in lack of phosphorylation on ZAP70 and reduced signaling through ERK on Jurkat and murine primary Abl1⁺/⁻ Abl2⁺/⁻ T cells (18). In addition, IM reduces the production of NF-κB in conjunction with a reduction in phosphorylation of LCK and ERK1/2 in stimulated T cells (16). Moreover, IM affects c-Kit signaling leading to deficits in pro-T and -B cell development (20).

If IM affects the immune response, it could impede the ability of host T cells to control recurrent or residual tumors. In the case of CML, Bcr-Abl fusion region peptides avidly bind to specific human HLA alleles such as HLA-A3 (21, 22). Consistent with this, circulating HLA-A3 Bcr-Abl tetramer-positive cells and an anti-CML cytotoxic response have been observed (21, 23). The importance of immune surveillance mechanisms for the control of Bcr-Abl clones is further emphasized by clinical observations in CML, where the depletion of T cells in allogeneic stem cell transplantation results in an increased probability of relapse (24, 25). Likewise, relapse after allogeneic stem cell transplantation can be cured with donor lymphocyte infusions (26–29). Ag-specific leukemia-reactive T cells can be found in CML patients after successful treatment with IFN-α or allogeneic stem cell transplantation (30–32). Treatment of patients with IM could influence the graft-vs-leukemia effect of lymphocytes (including CD8 T cells) in allogeneic transplantation and also lead to increased susceptibility to infections with viruses and other pathogens (33–37). In addition, the treatment of patients with higher doses of IM could result in a more profound suppression of CD8 T cells (38, 39).
affects CD8 T cell responses to an in vivo infection with *Listeria monocytogenes* (LM). Our results indicate that IM diminishes the recall response of specific CD8 T cells to pathogen exposure and could therefore affect the ability of CD8 T cells to function optimally against relapsed tumors or other infections.

**Materials and Methods**

**Imatinib mesylate**

One hundred-milligram tablets of IM (Gleevec; Novartis) were pulverized and dissolved in 10% DMSO at a 10 mM stock and used at the indicated concentrations. Drug calculations were based on the pure drug. For delivery to mice, IM was dissolved in water. Because the $t_{1/2}$ of IM in mice is ~8 h, IM was injected i.p. into each mouse twice daily at a dose of 75 mg/kg (40). Control animals received vehicle without the drug. IM injections started on day 0 and continued daily through the primary and secondary responses.

**Mice**

C57BL/6J (B6), C57BL/6.PL-Thy1a/Cy (B6.Thy1.1) and OT-1 TCR transgenic mice (B6 background) were bred and maintained at the University of Texas Southwestern Medical Center (Dallas, TX) animal facility under the approval of the Institutional Animal Care and Use Committee.

**Bacteria**

Log phase cultures of LM expressing the full-length OVA protein (LM-OVA), provided by Dr. H. Shen (University of Pennsylvania School of Medicine, Philadelphia, PA) were washed twice and diluted in PBS to the desired concentration (41).
IM measurement
Mice injected with IM were bled at 2 h. Blood was collected in heparinized tubes and plasma was prepared by centrifugation. IM levels in plasma were quantitated following a liquid chromatography/mass spectrometry assay (42).

Generation of LM-specific primary and secondary OT-1 cells
Lymph node (LN; 1 × 10^6) cells from OT-1 TCR transgenic mice were injected i.v. into B6.Thy1.1 recipient mice, rested for 1–3 days, and infected i.v. with LM-OVA at a 0.1 LD50 dose to generate a primary response. LM-OVA immune animals that were infected 28 days previously were challenged with one LD50 of LM-OVA to generate a secondary response.

Cell culture and in vitro stimulation
For in vitro experiments, mouse splenocytes were cultured in 96-well plates at 0.5–1 × 10^6 cells per 200 μl of medium. The cultures were grown in complete RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% FCS (Atlanta Biologicals) and 10U/ml IL-2 (provided by Dr. M. Bennett, University of Texas Southwestern Medical Center, Dallas, TX) in the presence of 10 nM SIINFEKL peptide (Peptide Synthesis Facility, University of Texas Southwestern Medical Center). Control cultures that did not receive SIINFEKL were supplemented with 10 ng/ml IL-7 (BD Biosciences) to promote cell viability. The cells were allowed to grow for 3–4 days at which time IFN-γ production, T cell expansion, and viability were assessed.

Abs and cell staining
For flow cytometry the following Abs or dyes from BD Biosciences were used: anti-CD8α (clone 53-6.7), anti-CD90.2 (clone 53-2.1 and 30-H12), anti-IFN-γ (XMG1.2), Annexin V (AnnV), anti-BrdU mAb (clone B44), and 7-aminoactinomycin D (7-AAD). Anti-CD127 (IL-7Rα; clone 7R34) was obtained from eBioscience. Intracellular cytokine staining (ICS) was performed as described previously (43). For staining with anti-BrdU FITC, the cells were fixed as in the ICS protocol, treated with DNase 1 (Sigma-Aldrich) at 500U/ml, and incubated with anti-BrdU FITC (44, 45). Data were acquired using a FACSCaliber flow cytometer and were analyzed using CellQuest (BD Biosciences) or Flow Jo (Tree Star) software.

CFSE labeling
For in vitro assays, CFSE (Molecular Probes) labeling of splenocytes was performed at a final concentration of 1 μM for OT-1 T cells. For the in vivo CFSE transfer assays, the OT-1 lymph node cells were labeled and transferred as described with a subsequent LM-OVA challenge (46).

Propidium iodide staining
Cultured naive OT-1 cells were fixed in 70% ethanol and treated with propidium iodide/Triton X-100 staining solution containing RNase A. Data were acquired using a FACScan flow cytometer and were analyzed with Flow Jo (Tree Star) software.

BrdU labeling
For splenocyte labeling with BrdU, naive mice on day 0 and memory mice on the day of recall (day 28) were given BrdU (0.8 mg/ml) in the drinking water continuously for 6 days. BrdU staining was performed as described above.

Data analysis
To combine the results of several independent experiments, the responses in the IM-treated (+IM) group for a given experiment were expressed as a percentage of the average response in the untreated (–IM) control group for that experiment. Statistical significance for the outcomes (percentage of OT-1 cells, IFN-γ expression of OT-1 cells, and IL-7Rα expression on OT-1 cells) were determined by Student’s t test, two-tailed distribution, and two-sample unequal variance. Because in some experiments multiple (three) outcomes were being compared on the same samples, the resulting p value for each was adjusted using the Bonferroni correction by multiplying the resulting p value by three.

Results
Effect of IM on Ag-specific CD8 T cells in vitro
To determine the effect of IM on CD8 T cells, we used OT-1 T cells specific for the OVA-SIINFEKL epitope as an indicator of
CD8 T cell function. Prior studies have shown that IM reduces proliferation but does not cause apoptosis of PBLs stimulated in vitro with PHA, staphylococcal enterotoxin B, and anti-CD3 Ab (15, 17). To investigate the effect of IM on CD8 T cell proliferation, we cultured splenocytes from naive OT-1 mice with their cognate Ag SIINFEKL in the presence or absence of IM for 4 days. To identify viable vs nonviable cultured OT-1 cells, we analyzed the cells for AnnV and 7-AAD staining. 7-AAD$^-$AnnV$^-$ cells were easily distinguished from 7-AAD$^+$AnnV$^+$ cells in a forward scatter vs side scatter plot (Fig. 1A). Accordingly, the effects of IM on cell division and function were analyzed from the live cell population exclusively (Fig. 1A, right panel, gate A).

The division of OT-1 T cells from IM-treated cultures was similar to control cultures at IM concentrations up to 10$^{-9}$ M (Fig. 1B). At 15$^{-9}$ M IM there were no detectable viable cells. Therefore, we gated on the nonviable population (Fig. 1A, right panel).
Intensity of IL-7Rα dose of IM increased. At 15% positive cells (13%). Apoptosis increased substantially as the cells underwent apoptosis as detected by an increase in AnnV staining were measured as described above. The remaining cells stained for both AnnV and 7-AAD (data not shown). However, at 7.5 μM IM, about half of the cells stained with AnnV only (Fig. 1E). However, because the percentage of viable cells decreases with increasing doses of IM (Fig. 1D), total IFN-γ secretion would be expected to decrease.

To determine whether IM affected IFN-γ secretion, we determined the percentage of IFN-γ+ cells by ICS. Viable OT-1 cells exposed to IM expressed the same percentage of intracellular IFN-γ as control cells (Fig. 1E). However, because the percentage of viable cells decreases with increasing doses of IM (Fig. 1D), total IFN-γ secretion would be expected to decrease.

OT-1 memory cells were obtained from B6.Thy1.1 mice primed with LM-OVA >28 days previously. The cells were cultured with IM for 3 days in the presence of SIINFEKL, and proliferation, apoptosis, and IFN-γ staining were measured as described above. The results are similar to those seen with naive OT-1 T cells in that proliferation (Fig. 2A) and IFN-γ secretion (Fig. 2C) of viable memory cells was not affected by IM. Induction of apoptosis was noted at IM concentrations >5 μM (Fig. 2B).

Effect of IM on the growth of LM in vivo
Because the model uses measures the CD8 T cell response to LM-OVA, we investigated whether IM affects the growth of LM-OVA in vivo. After IM administration of 75 mg/kg twice daily, plasma levels measured in six animals at 2 h indicated that the levels ranged from 4.9 to 11 μM (data not shown). Similar doses have been used in mice to control Bcr-Abl tumors (40, 47). A dose of 4.6 μM IM is the effective pharmacological dose for tumor control and is reached at steady state by the administration of 400 mg of the drug to humans (1, 48). Animals were infected with 0.1 LD₅₀ of LM-OVA, and CFU values were determined in the spleen and liver on days 1 and 3 (Fig. 3). At the IM dose used (75 mg/kg), no effect on LM growth was detected in liver. In the spleen, IM had a small effect on LM growth on day 1. However, this was not seen on day 3 (Fig. 3).
When the data were plotted as the mean fluorescence intensity of IL7R expression, a decrease was noted on OT-1 cells in IM-treated animals compared with controls, whereas the expression on host CD8 T cells was equivalent in both IM and control groups (Fig. 5B). These results indicate that IM did not affect the primary response as measured by the number of Ag-specific cells, IFN-α secretion, or expansion in vivo but that it decreased the percentage of OT-1 cells that expressed high levels of IL-7R.

**Effect of IM on the secondary response of OT-1 T cells in vivo**

Because IM decreased IL-7Rα expression on OT-1 cells during the effector phase of the primary response, we wished to determine whether continuous IM administration would affect the secondary response. B6.Thy1.1 recipients received 10⁶ LN OT-1 T cells and were infected with LM-OVA with or without twice daily injections of IM. On day 28, before rechallenge, we observed that the percentage of OT-1 memory cells in PBLs from mice that received IM was 40% less than in controls, although this difference is not statistically significant (Fig. 6A). On the next day, the animals were re-challenged with one LD₅₀ of LM-OVA, and the CD8 T cell response was assessed 6 days later. Mice that received IM had significantly (60%) fewer OT-1 cells in their PBLs (Fig. 6B).

To determine the functional status of IM-treated memory OT-1 T cells, we cultured the spleen cells from the infected mice on day 6 after recall for 16 h with SIINFEKL and measured IFN-γ by ICS. Even though IM-treated animals had fewer responding cells, no difference in IFN-γ⁺ cells was detected between animals receiving vehicle vs IM (Fig. 6C).

**Effect of IM on expression of IL-7Ra on CD8 T cells**

Because the expression of IL-7Rα was lower on OT-1 effector cells during the primary response, we tested whether IM alters IL-7Rα expression on memory OT-1 cells before and after recall (50). Before recall, the percentage of cells that express IL-7Rαhigh was lower on memory cells in IM-treated mice compared with untreated controls. The percentage of IL-7Rαhigh-expressing cells decreased from 86% (range 78–88%) to 54% (range 46–55%) (Fig. 7A). Furthermore, after recall, IM treatment also resulted in a lower percentage of IL-7Rαhigh memory OT-1 T cells compared with untreated controls. The percentage of IL-7Rαhigh cells in IM-treated mice was 9% (range 8–15%) compared with 34% (range 32–40%) in untreated controls (Fig. 7B). When IL-7Ra expression was plotted as mean fluorescence intensity both before and after recall, IM treatment resulted in lower levels of expression (Fig. 7C). Therefore, not only did IM decrease the number of memory...
cells and their responses to Ag, but it also decreased the percentage of cells that express high levels of IL-7Rα.

**Effect of IM on the expansion of memory OT-1 cells**

Because there were fewer OT-1 cells in IM-treated mice after rechallenge with LM-OVA, we determined whether IM influences the proliferation of OT-1 memory cells. Mice received BrdU continuously during rechallenge with LM-OVA. Although 50% of the cells in IM-treated mice incorporated BrdU as compared with 72% of cells in untreated mice (Fig. 8), this difference was not statistically significant.

**Discussion**

We used CD8 OT-1 T cells to assess the effect of IM on CD8 T cell responses and showed that although IM did not inhibit the primary CD8 OT-1 T cell response to LM-OVA, it inhibited secondary CD8 T cell responses at therapeutically relevant in vivo concentrations (1, 48). This effect correlated with a decreased expression level of IL-7Rα on OT-1 CD8 memory cells both before and after Ag rechallenge.

It is known that IM inhibits proliferation with an arrest in the G1/G0 phase of the cell cycle in Bcr-Abl expressing tumor cells (51–53). Therefore, one might expect a similar outcome in Ag-stimulated CD8 T cells. However, we observed no inhibition of proliferation or cell cycle arrest in viable OT-1 T cells at IM concentrations as high as 10 µM. At concentrations >10 µM there were too few viable cells to analyze in the cultures. In contrast to our report, others have observed an inhibition of proliferation by IM on human PBLs (15–17). This may be due to the toxic effects of IM, because we found that sampling the entire population (live and dead cells) showed an inhibition in proliferation and a G1/G0/G0 arrest that was attributed to the dead cells (data not shown). Although Bcr-Abl in malignant cells allows for proliferation of tumor cells, our data suggest that c-Abl in CD8 T cells is not required for their proliferation through TCR signaling. However, Abl is responsible for downstream phosphorylation of LAT (18). Furthermore, our results show that IM increased apoptosis induced by activation-induced cell death at concentrations >5 µM.

There was a modest effect of IM on bacterial growth during the early stages of infection in that following injection of a low dose of LM-OVA, IM slightly decreased bacterial colonies in the spleen but not the liver. Further, this effect was only detected on day 1 but not on day 3. Because Ag dosage has been shown to not have an effect on CD8 T cell expansion to LM infection, this decrease would not be expected to play a role in the subsequent response (54).
In vivo, the primary response of OT-1 CD8 T cells specific for the cognate OVA epitope was unaffected by IM with respect to percentage and function. This was demonstrated by our findings that the extent of in vivo expansion and IFN-γ production by cells undergoing a primary response was not influenced by IM. However, IL-7Rα expression was lower on OT-1 effector cells in IM-treated mice. When we assayed for memory CD8 T cells before recall there were fewer cells, although that did not represent a significant change in the percentage of CD8 Ag-specific cells. However, the response of memory OT-1 T cells upon rechallenge with LM-OVA was significantly reduced. This result agrees with previous data showing that IM inhibits the delayed type hypersensitivity response, although the cell type(s) involved in the inhibition of this response was not described (16). Furthermore, the decrease in IL-7Rα expression on OT-1 effector cells is consistent with previous data showing this effect on the quality of the memory response (49).

IL-7 is a cytokine involved in the homeostatic proliferation of CD8 T cells in lymphopenic animals (55). It is also needed for the survival of both naïve and memory T cells (55–62). More recent data indicate that IL-7Rα expression is required for effective memory cell generation and that effector CD8 T cells that retain high levels of this receptor give rise to long-lived memory cells (49, 50). Further, adoptive transfer of IL-7Rα-high cells leads to protective immunity (49, 63, 64). The decrease in IL-7Rα expression that we observed on OT-1 memory cells exposed to IM is consistent with their impaired response.

The percentages of OT-1 T cells from both IM-treated and control mice that incorporate BrdU during the recall response were similar. This suggests that an increase in apoptosis is a contributing factor to the lower number of OT-1 cells recovered 6 days later in IM-treated mice. These results are also consistent with our in vitro data showing that IM at concentrations of >5 μM induces apoptosis of Ag-stimulated memory cells. Signaling through the IL-7R has been shown to induce the synthesis of antiapoptotic molecules such as Bcl-2 and to block the proapoptotic proteins Bad and Bax by posttranslational mechanisms (65). This could be one mechanism by which a lower level of IL-7Rα on IM-treated memory cells could lead to increased apoptosis and, therefore, a depressed recall response.

The inhibitory effect on OT-1 T cells observed in vitro is likely through a direct inhibition of the TCR/Abl tyrosine kinase pathway and not other tyrosine kinases such as c-Kit, because c-Kit is expressed on pro-T cells but not on mature T cells (66, 67). However, we cannot rule out the possibility of an indirect effect on other cell types through c-Kit or other kinases on the decrease of the CD8 memory response in vivo.

CD4 cells are not required for the development of efficient primary CD8 T cell responses against infectious agents. However, without CD4 T cells, memory CD8 T cells produce a poorer secondary response than do memory CD8 T cells generated with CD4 help (68–71). The role that CD4 T cells play in the CD8 secondary anti-LM response is not settled. Some reports show that CD4 T cells are crucial only during the initial priming of naïve CD8 T cells to differentiate into functional CD8 memory cells (69–72). However, other studies indicate that CD4 T cell help for the CD8 memory response is essential and plays a role after the primary CD8 T cell response. Adoptive transfer of effector or memory CD8 T cells into CD4 T cell-deficient mice shows that CD4 T cells are required after, not during, the early programming phase (73).

Thus, it is possible that the effect of IM on the generation of memory CD8 T cells is partly mediated through its effect on CD4 Th activity. IM inhibits activation and proliferation of CD4 T lymphocytes in vitro, suppresses CD4 T cell cytokine secretion, and modulates a shift toward a Th2 response (15, 19, 74). In addition, because IM influences Ag-presenting functions of dendritic cells, the effect of IM on APCs may compromise CD4 Th activity as well as have a direct effect on CD8 T cell function (10, 75, 76). The absence of CD4 T cell help compromises the emergence of functionally competent IL-7Rα-high memory cells, which is consistent with our findings of lower IL-7Rα expression on OT-1 CD8 memory T cells in IM-treated animals (63). The effects of IM on CD4 Th cell responses in an in vivo system merit further investigation and could shed light on the depressed memory CD8 T cell response.

There are case reports of increased infections in patients undergoing IM treatment (36, 37). In addition, a reactivation of herpes zoster infection has been observed in patients treated with IM (35). This could be the result of a defective CD8 memory response and would be consistent with the effect we observed with OT-1 T cells. IM has also been shown to have beneficial effects on viral infections. Abl kinase, in conjunction with Src family kinases, stimulates actin-based motility of the vaccinia virus (77, 78). The inhibition of Abl by IM, therefore, reduces vaccinia viral dissemination and promotes survival in mice (78). In addition, the inhibition of platelet-derived growth factor and c-Kit activity by IM may induce regression of AIDS-related Kaposi’s sarcoma (79). Thus, although the regression of viral infection is a positive aspect of IM therapy, the immunosuppressive effects of IM, such as interference with memory CD8 responses, is a negative factor. Recent clinical trials use high dose IM (800 mg) to treat patients that would be expected to produce higher levels of IM in plasma (1, 38, 39, 48). High IM could have a more profound suppression of the CD8 response.

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Disclosures
The authors have no financial conflict of interest.

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