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Distinct Effects of Saracatinib on Memory CD8+ T Cell Differentiation

Shinji Takai, Helen Sabzevari,1 Benedetto Farsaci, Jeffrey Schlom, and John W. Greiner

Immunologic memory involving CD8+ T cells is a hallmark of an adaptive Ag-specific immune response and constitutes a critical component of protective immunity. Designing approaches that enhance long-term T cell memory would, for the most part, fortify vaccines and enhance host protection against infectious diseases and, perhaps, cancer immunotherapy. A better understanding of the cellular programs involved in the Ag-specific T cell response has led to new approaches that target the magnitude and quality of the memory T cell response. In this article, we show that T cells from TCR transgenic mice for the nucleoprotein of influenza virus NP68 exhibit the distinct phases—priming, expansion, contraction, and memory—of an Ag-specific T cell response when exposed in vitro to the cognate peptide. Saracatinib, a specific inhibitor of Src family kinases, administered at low doses during the expansion or contraction phases, increased CD62Lhigh/CD44high central memory CD8+ T cells and IFN-γ production but suppressed immunity when added during the priming phase. These effects by saracatinib were not accompanied by the expected decline of Src family kinases but were accompanied by Akt-mammalian target of rapamycin suppression and/or mediated via another pathway. Increased central memory cells by saracatinib were recapitulated in mice using a poxvirus-based influenza vaccine, thus underscoring the importance of dose and timing of the inhibitor in the context of memory T cell differentiation. Finally, vaccine plus saracatinib treatment showed better protection against tumor challenge. The immune-potentiating effects on CD8+ T cells by a low dose of saracatinib might afford better protection from pathogens or cancer when combined with vaccine. The Journal of Immunology, 2012, 188: 000–000.

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Abbreviations used in this article: AMPK, AMP-activated protein kinase; m, murine; mTOR, mammalian target of rapamycin; SFK, Src family kinase; Tg, transgenic.

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following T cell stimulation with cognate peptide. Inhibition of the Akt–mTOR or perhaps other molecular pathways, absent any change in the Src pathway, accompanied those immune-potentiating effects. The findings argue for the differential cellular effects of saracatinib: inhibition of Src expression in tumor cells while stimulating CD8+ T cell differentiation through a Src-independent pathway or pathways. Additional study may provide a potential use for combination therapy of saracatinib and vaccine to improve vaccination against infections and cancer.

Materials and Methods

Mice

Female C57BL/6 mice (8–12 wk old) were obtained from the National Cancer Institute, Frederick Cancer Research Facility (Frederick, MD). F5 mice that are transgenic (Tg) for nucleoprotein of influenza virus A/NT/60/68 (366ASNENMDAM374; NP68)-specific, H-2Db–restricted TCR (20, 21) were obtained from Taconic Farms (Hudson, NY). Mice expressing the transgene for human CEA (CEA.Tg) were generously provided by Dr. John Shively (City of Hope, Duarte, CA). The mice were originally generated by microinjecting a 32.6-kb AatII restriction fragment containing the entire human CEA genomic region into a pronucleus of C57BL/6 zygotes (22). Homozygosity for CEA expression was tested and verified using PCR analysis of DNA isolated from the tails of progeny mice (23). All mice were housed and maintained in microisolator cages under specific pathogen-free conditions and in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. All experimental studies were carried out under the approval of the Intramural Animal Care and Use Committee.

Cell lines

Murine colon carcinoma MC38 cells (H-2b) expressing human CEA (designated MC32a) were generated by retroviral transduction with CEA (24). MC32a cells were cultured in MEM medium supplemented (designated MC32a) were generated by retroviral transduction with CEA (24). MC32a cells were cultured in MEM medium supplemented with 1 mmol/L sodium pyruvate, 1× nonessential amino acids, 2 mmol/L t-glutamine, 10 mmol/L HEPES, 300 μg/ml G418 sulfate, and 10% heat-inactivated FBS. Unless otherwise indicated, all media and their components were purchased from Mediatech (Manassas, VA).

Inhibitors

For in vitro study, saracatinib (AZD0530) (kindly provided by AstraZeneca, Wilmington, DE) or dasatinib (LC Laboratories, Woburn, MA) was dissolved in DMSO and diluted in culture media to a respective final concentration. The maximum concentration of DMSO was 0.1%.

For in vivo study, saracatinib was formulated as a 1 mg/ml solution and dasatinib was formulated as a 0.25 mg/ml solution in water with 1% Tween 80. These solutions were administered orally using a plastic feeding tube.

Poxvirus constructs

Recombinant vaccinia and recombinant fowlpox viruses containing murine B7-1, ICAM-1, and LFA-3 genes in combination with human CEA (CEA/TRICOM) have been described previously (25). Recombinant vaccinia and fowlpox viruses containing murine B7-1, ICAM-1, and LFA-3 genes in combination with nucleoprotein of influenza virus A/PR/8/34 (366ASNENMETM374; NP34) (26) were expressed and propagated in 293 cell lines. The recombinant fowlpox virus containing the gene for murine GM-CSF has also been described earlier (27).

Peptides

H-2Db–restricted influenza virus A/NT/60/68 peptide (366ASNENMDAM374; NP68), influenza virus A/PR/8/34 (366ASNENMETM374; NP34), and H-2Dd–restricted peptide (27)=(GIONSVSA) (379; CEA peptide) were synthesized by CPC Scientific (Sunnyvale, CA).

In vitro assay

Primary splenocytes were dispersed into single-cell suspensions, the RBCs were removed by lysis, and the remaining cells were seeded into six-well plates at 6 × 10^5 cells/ml in complete RPMI (RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin) media. Splenocytes from C57BL/6 mice were stimulated with 10 μg/ml soluble anti-CD3e, and splenocytes from F5 mice were stimulated with 10–4 μg/ml NP68 peptide, then used in the appropriate experiments. For kinase assay and Western blot analysis, cells were collected at the indicated time points and the CD8+ T cells were selected using magnetic cell sorting (CD8+ T Cell Isolation Kit II; Miltenyi Biotec, Auburn, CA).

Ex vivo assays

Primary splenocytes from either vaccinated or naive C57BL/6 mice were dispersed into single-cell suspensions, followed by removal of RBCs, and 5 × 10^6 cells/ml were cultured in 1.6 ml complete RPMI containing 1 μg/ml cognate peptide with or without 10 ng/ml murine IL-2 (mIL-2) in a 24-well plate. Cells were used for subsequent intracellular cytokine staining or dextramer staining assays. For intracellular staining, GolgiPlug was added to the culture media 2 h after peptide stimulation and incubated overnight, at which time the cells were harvested and stained with IFN-γ. Cell supernatants were analyzed for IFN-γ and IL-2, using ELISA-based cytokine detection assays. For IL-2 measurement in cell supernatants, the ex vivo assay using primary splenocytes was carried out without the addition of exogenous mIL-2.

Flow cytometry analysis

Either primary or cultured splenocytes were stained with Abs to cell-surface markers. Abs against CD8a, CD4, CD19, CD44, and CD62L were purchased from BD Biosciences (Mountain View, CA). Annexin V staining was performed using an Annexin V staining kit (BD Biosciences). Abs against IL-7R (CD127) was purchased from eBioscience (San Diego, CA). Mouse regulatory T cell staining was performed using a Foxp3 staining kit from eBioscience. Cells were also stained with appropriate isotype-matched controls. To identify influenza A NP34-specific cells, splenocytes were stained with NP34 dextramer (ASNENMETM) (Immudex, Copenhagen, Denmark) or lymphoctic choriomeningitis virus dextramer (366FQPQNGQFI404) (Immudex). Intracellular cytokine staining was performed using BD GolgiPlug, BD Cytofix/Cytoperm, and anti-mouse IFN-γ Ab (BD Biosciences). Stained cells were acquired on a FACScan or LSR II flow cytometer (BD Biosciences). Dead cells were excluded from the analysis based on scatter profile.

CFSE assay

Cells (10^7/ml) were labeled with 1 μM CFSE, incubated for 10 min at 37˚C and washed twice with PBS, and then seeded into six-well plates at 5 × 10^5 cells/ml in complete RPMI with or without 10^{-4} mg/ml NP68 peptide.

Cytokine assays

Mouse IFN-γ and IL-2 ELISAs were performed using the Quantikine ELISA Kit (R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol.

Kinase assays

Kinase assay was performed using the Universal Tyrosine Kinase Assay Kit (TAKARA BIO, Tokyo, Japan) according to the manufacturer’s protocol. Briefly, cultured cells were collected and washed twice with PBS, and then live cells were collected by density gradient centrifugation (Histopaque 1119). Cells (2 × 10^6) were lysed in extraction buffer provided in the kit. The samples were immunoprecipitated with Src MAb (#2110; Cell Signaling Technology, Danvers, MA), Lck MAb (ab80583; Abcam, Cambridge, MA), or Fyn MAb (ab1881; Abcam) with protein G-agarose, and then ATP was added to the samples, followed by measurement of kinase activity using a plate reader.

Western blot analysis

Cultured cells were washed twice with PBS and then lysed, homogenized, and sonicated in a lysis buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 50 mM DTT, and 10% glycerol. SDS-PAGE was performed according to Laemmli in 10% polyacrylamide gel. Western blot analysis was performed using β-tubulin Ab (Sigma-Aldrich, St. Louis, MO), phospho-specific (Thr172) Src MAb (#2101; Cell Signaling Technology), Src MAb (#2110; Cell Signaling Technology), phospho-specific (Thr308) AKT MAb (#20231; Cell Signaling Technology). AKT MAb (#2927; Cell Signaling Technology), phospho-specific (Thr68) Ampkα Ab (#2531; Cell Signaling Technology), AMPK-α Ab (#2532; Cell Signaling Technology), phospho-specific (Thr286) Akt Ab (#9275; Cell Signaling Technology), and Akt Ab (#9272; Cell Signaling Technology), with phospho- and nonphospho-labeled antibodies. Peroxidase activity on the polyvinylidene fluoride membrane was visualized by x-ray film by means of the ECL Western Blotting Detection System.

Tumor challenge of vaccinated mice

CEA.Tg mice were vaccinated with MVA-CEA-TRICOM plus rF-GM-CSF on day 0, and boosted with rF-CEA-TRICOM plus rF-GM-CSF on day 15.
Groups of vaccinated mice also received either saracatinib or vehicle at the indicated time intervals. Age-matched untreated CEA.Tg mice were used as controls. All mice were inoculated s.c. in the shaved flank with 3 × 10⁶ MC32a cells, and tumor volumes were measured twice per week.

Statistical analysis

Statistical significance was calculated using GraphPad Prism statistical software (Version 5.0c) (GraphPad Software, La Jolla, CA). Where not specified, results of tests of significance are reported as **p** < 0.05, compared with control (0 μM).

**Results**

**In vitro effects of saracatinib on nonactivated and activated T cells**

Western blot analysis confirmed that saracatinib suppressed SFK phosphorylation in tumor cells. Suppression of SFK phosphorylation in both PancO2 and MC38 tumor cells was dose dependent, ranging between 0.3 and 10 μM (Supplemental Fig. 1).

Next, saracatinib effects on nonactivated and activated T cells in vitro were evaluated by measuring apoptosis and cell number. Saracatinib treatment of non-activated CD4⁺ or CD8⁺ T cells significantly increased apoptosis, as measured by annexin V staining, with a commensurate decrease in cell number beginning at 1.0 μM (Table I, upper rows). In contrast, when the T cells were activated with the addition of anti-CD3 no detrimental effects were noted with the addition of 1.0 μM saracatinib. Increased apoptosis and reduction in the number of activated CD4⁺ and CD8⁺ T cells were observed only after increasing the concentration of saracatinib to 3 or 10 μM (Table I, lower rows). Those results suggest that activated T cells are more resistant than nonactivated T cells to the saracatinib-mediated cytotoxicity and the effects of this Src inhibitor on the generation of Ag-specific CD8⁺ T cells should be examined at doses not to exceed 1.0 μM.

**Table I. Effects of saracatinib on the apoptosis and cell number of nonactivated and activated T cells**

<table>
<thead>
<tr>
<th>Saracatinib Dose (μM)</th>
<th>Nonactivated CD4⁺</th>
<th>Nonactivated CD8⁺</th>
<th>Activated CD4⁺</th>
<th>Activated CD8⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Annexin V (%)</td>
<td>Cell number</td>
<td>Annexin V (%)</td>
<td>Cell number</td>
</tr>
<tr>
<td>0</td>
<td>60.1</td>
<td>1.9 × 10⁷</td>
<td>58.6</td>
<td>3.1 × 10⁷</td>
</tr>
<tr>
<td>0.1</td>
<td>59.0</td>
<td>1.8 × 10⁷</td>
<td>60.3</td>
<td>3.3 × 10⁷</td>
</tr>
<tr>
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<td>56.3</td>
<td>1.8 × 10⁷</td>
<td>57.1</td>
<td>3.2 × 10⁷</td>
</tr>
<tr>
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<tr>
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<td>73.6</td>
<td>0.9 × 10⁷</td>
<td>69.7</td>
<td>1.8 × 10⁷</td>
</tr>
<tr>
<td>10</td>
<td>73.8</td>
<td>0.8 × 10⁷</td>
<td>72.3</td>
<td>1.1 × 10⁷</td>
</tr>
</tbody>
</table>

For nonactivated T cells, primary splenocytes (6 × 10⁶ cells/ml) from C57BL/6 mice were incubated with various doses of saracatinib for 48 h continuously. For activated T cells, primary splenocytes (6 × 10⁶ cells/ml) from C57BL/6 mice were stimulated with 10 μg/ml of anti-CD3 for 24 h. After that, cells were incubated with various doses of saracatinib for 48 h continuously and then analyzed for apoptosis by Annexin V staining. Cell numbers were determined by trypan blue exclusion test.

*Significant difference is indicated, **p** < 0.05, compared with control (0 μM).
In vitro effects of saracatinib on Ag-specific CD8 T cells during the priming phase

To evaluate saracatinib effects on Ag-specific CD8+ T cells, splenocytes from TCR-Tg mice (F5 mice) were isolated and stimulated in vitro with cognate peptide. Because the generation of memory CD8+ T cells can be divided into four distinct phases (priming, expansion, contraction, and memory), saracatinib’s effects on cell number and IFN-γ production were evaluated during each phase, beginning with the priming phase. The priming phase was defined as the initial 24 h after peptide stimulation, a time during which T cells were activated but did not proliferate (Fig. 1A). Indeed, virtually all of the Ag-specific CD8+ T cells expressed the activation marker CD44, 24 h after cognate peptide stimulation, indicating activation (Fig. 1B).

Saracatinib (1.0 μM) was added to the CD8+ T cells at different times (i.e., 0, 6, 12, or 24 h; Fig. 1C) after cognate peptide stimulation. The addition of saracatinib during the initial 6 h after peptide stimulation decreased both the total number of CD8+ T cells (Fig. 1D) and IFN-γ production (Fig. 1E). In contrast, delaying the addition of saracatinib (1.0 μM) to 12–24 h after peptide stimulation abrogated any deleterious effects it had on either cell number or IFN-γ production. Of interest, the addition of saracatinib 24 h after peptide stimulation increased the amount of IFN-γ produced by the CD8+ F5 cells (Fig. 1D, 1E), suggesting that the introduction of this Src inhibitor near the end of the priming phase of T cells not only averts its immunosuppressive or toxic actions but also leads to higher production levels of a potent Th1 cytokine.

**FIGURE 2.** In vitro saracatinib effects on Ag-specific CD8+ T cells isolated from F5 mice during the expansion phase. (A) Experimental design to evaluate the in vitro effects of saracatinib on Ag-specific CD8+ T cells during the expansion and contraction phases. Primary splenocytes (6 × 10^5 cells/ml) from F5 mice were stimulated with 10^-24 μg/ml of NP68 peptide and then 24 h later treated with vehicle or the indicated doses of saracatinib. Supernatants were collected at 72 h after peptide stimulation, and IFN-γ was measured by ELISA. (B) CFSE-labeled splenocytes were used for proliferation assays by gating CD8a-positive cells (solid line, unstimulated cells; filled area, stimulated cells). (C) Saracatinib doses of 0.1–1.0 μM enhanced IFN-γ production, as measured by ELISA, by Ag-specific CD8+ T cells. Results are the mean and SEM of triplicates from three independent experiments. (D) Saracatinib enhances IFN-γ production at different NP68 peptide doses. Primary splenocytes (6 × 10^5 cells/ml) from F5 mice were stimulated with indicated amounts of NP68 peptide. Saracatinib (0.3 μM) was added to culture media 24 h after peptide stimulation; IFN-γ production was measured by ELISA at 72 h after peptide stimulation. Results are the mean and SEM of triplicate samples from three independent experiments. *p < 0.05 versus control. (E) Saracatinib treatment enhanced the differentiation of CD62L^{lo}CD44^{hi} central memory CD8+ T cells. Cells were stained with anti-CD62L, CD44, and CD8a. Inset denotes percentage of cells in the upper right quadrant. Data are representative of four to six independent experiments. (F) Number of CD62L^{lo}CD44^{hi} effector memory versus CD62L^{lo}CD44^{hi} central memory CD8+ T cells recovered following stimulation with cognate peptide. Total cell numbers in culture were determined by trypan blue exclusion, and the total number of CD8+, CD62L^{lo}CD44^{hi}, CD62L^{hi}CD44^{hi} cells were determined on the basis of flow cytometry data. Data are the mean and SEM of triplicate samples of at least three independent experiments. *p < 0.05 versus no saracatinib.
In vitro effects of saracatinib on Ag-specific CD8+ T cells during the expansion phase

Next, the in vitro effects of saracatinib during the expansion phase on the proliferation, function, and memory differentiation (effector memory versus central memory) of Ag-specific CD8+ T cells were examined (Fig. 2A). During the 72 h after stimulation with cognate peptide, Ag-specific CD8+ T cells had gone through approximately five cell divisions (Fig. 2B), and saracatinib addition during the expansion phase (24 h post peptide stimulation) had no discernible effect on cell proliferation (Fig. 2B). In three separate experiments, the addition of saracatinib during that time interval resulted in a dose-dependent increase in IFN-γ production up to 1.0 μM (Fig. 2C). A further increase in saracatinib to 3 μM significantly suppressed IFN-γ production. That potentiation of IFN-γ production by saracatinib (0.3 μM) was present at peptide doses ranging from 10−7 to 10−5 μg/ml (Fig. 2D). Consensus with the enhanced IFN-γ production was an increase in the percentage (Fig. 2E) as well as the absolute number (Fig. 2F) of CD62Lhigh/CD44high central memory CD8+ T cells at 72 h after stimulation. Those observations suggested that in vitro addition of saracatinib during the expansion phase shifts the differentiation of CD8+ T cells to a central memory phenotype (28–30).

In vitro effects of saracatinib on Ag-specific CD8+ T cells during the contraction and memory phases

After 5 d of cognate peptide stimulation, CD8+ T cells had differentiated into either CD62Llow/CD44high central memory or CD62Lhigh/CD44high effector memory cells (76.7% and 23.0%, respectively). At this point, the cognate peptide was removed and replaced with IL-2 to mimic T cells in their memory phase (Fig. 3A, 3B), and saracatinib was added to the culture. In the presence of IL-2, the percentage of T cells expressing a central memory (CD62Lhigh/CD44high) fell from 76.7% to 38.5%, indicating a shift toward effector memory (CD62Llow/CD44high) cells (Fig. 3B). The addition of saracatinib during this 72-h interval, however, maintained a higher percentage of central memory cells (54.6% versus 38.5%) without affecting the total number of memory CD8+ T cells (Fig. 3B), suggesting that saracatinib administration during the contraction phase is beneficial for the maintenance of central memory CD8+ T cells.

Comparative studies of saracatinib and dasatinib

Dasatinib is a well-studied, FDA-approved SFK inhibitor and is known to target Lck and Fyn, two SFK family members involved in the earliest steps of TCR activation (15). It was of interest, therefore, to compare the effects of dasatinib with those of saracatinib on the generation of central memory T cells. Initial molecular studies revealed disparate effects of dasatinib and saracatinib on their relative abilities to influence kinase pathways. Those studies confirmed the ability of dasatinib, but not saracatinib, to suppress Src, Lck, and Fyn in CD8+ T cells after 2 h of treatment (Fig. 4A). Similar results were found in kinase activity assays at 24 h after either saracatinib or dasatinib treatment (data not shown). When 0.03 or 0.1 μM dasatinib was added to F5 CD8+ T cells during their expansion phase (see Fig. 2A for treatment sequence), a significant reduction in the amount of IFN-γ produced in response to cognate peptide stimulation resulted (Fig. 4B). The addition of dasatinib also failed to alter F5 central memory cells (Fig. 4C) and, in fact, reduced the number of central memory (CD62Lhigh/CD44high) and effector memory (CD62Llow/CD44high) cells (Fig. 4D). These findings clearly showed dramatic differences between saracatinib and dasatinib and further indicate that the immune-potentiating effects of saracatinib may not involve SFK inhibition.

Possible molecular mechanisms of increased central memory cell differentiation by saracatinib

Those observations led us to check the effects of saracatinib on Akt, AMPK, and mTOR, which are involved in central memory cell differentiation. Western blot analyses revealed that saracatinib suppressed phosphorylation of Akt (Thr308) and p70S6K at 12 and 24 h, whereas AMPK-α phosphorylation remained unchanged (Fig. 5A). These results suggest that the enhancing effect of central memory CD8+ T cells by saracatinib is mediated, at least in part, through inhibition of the Akt–mTOR pathway (Fig. 5B).

In vivo effects of Src inhibitors on vaccine-induced host immunity

Initial studies were carried out to establish the dose and scheduling of the Src inhibitors prior to examining their immune-potentiating effects in vivo. A previous pharmacokinetic study (14) reported that 10 mg/kg of saracatinib administered by oral gavage twice daily for 5 d consecutively resulted in maximum and minimum blood concentrations (Cmax and Cmin) of 1.09 μM and 0.45 μM, respectively, which approximated the 1.0 μM dose shown to immunosuppress (19). In vitro experiments indicated that the Src inhibitors should be administered after the priming phase and during the expansion and contraction phases, coincident at a time when T cells express CD44. To establish that time interval in vivo, F5 TCR-Tg mice were immunized with cognate peptide, and the peripheral blood was analyzed for the emergence of activated CD8+ T cells on days 0, 3, and 6 postimmunization. On day 3, a significant reduction in the amount of IFN-γ production by saracatinib (0.3 μM) was present at peptide doses ranging from 10−7 to 10−5 μg/ml (Fig. 2D). Consensus with the enhanced IFN-γ production was an increase in the percentage (Fig. 2E) as well as the absolute number (Fig. 2F) of CD62Lhigh/CD44high central memory CD8+ T cells at 72 h after stimulation. Those observations suggested that in vitro addition of saracatinib during the expansion phase shifts the differentiation of CD8+ T cells to a central memory phenotype (28–30).

In vitro saracatinib effects on Ag-specific CD8+ T cells isolated from F5 mice during the memory phase. (A) Experimental design to evaluate saracatinib effects on the differentiation of Ag-specific CD8+ T cells when added in vitro during the memory phase. Primary splenocytes (6 × 10^6 cells/ml) from F5 mice were stimulated with 10−7 μg/ml of NP68 peptide for 5 d, isolated over Histopaque 1119, and incubated in the presence of 10 ng/ml of IL-2 and either 1.0 μM saracatinib or vehicle for 72 h. (B) Flow cytometric analysis of saracatinib effects on the differentiation of CD62Lhigh/CD44high central memory CD8+ T cells during the memory phase. Cells were stained with anti-CD62L, CD44, and CD8a. Insert denotes percentage of cells in each quadrant, and the data are representative of three independent experiments.

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Saracatinib and dasatinib were administered at 10 and 2.5 mg/kg, respectively, by gavage, two times per day, and beginning 3 d postvaccination, using rV-NP34-TRICOM in C57BL/6 mice (Fig. 6A).

In vivo effects of the Src inhibitors combined with vaccine

The addition of either Src inhibitor, saracatinib or dasatinib, with vaccine did not change either splenic cell number or individual immune cell populations when compared with vaccine alone (Supplemental Table I). Neither Src inhibitor had any adverse effects on the generation of Ag-specific CD8\(^+\) T cells in terms of frequency and absolute number, as determined by dextramer staining (Fig. 6B, 6C). A significant increase in the number of NP34 dextramer\(^+\)/CD62L\(^{low}\)/CD44\(^{high}\) CD8\(^+\) T cells (central memory cells) was seen only in splenocytes analyzed from mice given the vaccine combined with saracatinib, which was consistent with in vitro findings (Fig. 6D). The central memory T cell phenotype was confirmed by the presence of IL-7R expression on \(\sim 80\%\) of CD62L\(^{high}\)/CD44\(^{high}\) CD8\(^+\) T cells (data not shown). When the splenocytes from each treatment group were restimulated ex vivo with cognate peptide, a trend to a higher percentage of intracellular IFN-\(\gamma\)/CD8\(^+\) T cells from the vaccine plus saracatinib treatment group was noted (Fig. 7A). In continuing the ex vivo expansion of dextramer-positive CD8\(^+\) T cells for 4 d, a difference persisted, but was not significant, in both the percentage and the absolute numbers of dextramer-positive CD8\(^+\) T cells from the vaccine plus saracatinib treatment group (Fig. 7B, 7C). Yet, when IFN-\(\gamma\) production levels were measured in the mice receiving saracatinib plus vaccine, those cultures produced significantly higher levels than did ex vivo peptide-stimulated splenocytes from either the vaccine alone or the vaccine plus dasatinib treatment groups (Fig. 7D).

In vivo recall response of saracatinib-treated mice

To evaluate the polyfunctionality of memory CD8\(^+\) T cells generated by vaccine plus saracatinib, we chose the “CEA-self-Ag system,” which is in ongoing development as an immunotherapeutic agent (32). Mice expressing CEA as a transgene were found to mount CEA-specific host immunity following vaccination with diversified prime-boost poxvirus-based vaccines (MV A-CEA-TRICOM and rF-CEA-TRICOM) alone or combined with saracatinib (Fig. 8A). Prior to tumor challenge, splenocytes from naive (untreated) mice and mice administered the vaccine alone or combined with saracatinib were stimulated ex vivo with a CEA peptide for 4 d. Splenocytes from either group of vaccinated mice produced higher IFN-\(\gamma\) and IL-2 levels than did naive mice, underscoring the ability to immunize the CEA.Tg mice against a self-Ag (Fig. 8B, 8C). Higher IFN-\(\gamma\) levels were also produced by splenocytes from the mice administered the CEA-based vaccines combined with saracatinib, which agreed with previous results using the NP34-based vaccine (Fig. 7D). In addition, although comparison of IL-2 levels between vaccine plus vehicle
and vaccine plus saracatinib did not reach statistical significance ($p = 0.058$), we noted a clear incremental increase of IL-2 production by splenocytes from mice treated with vaccine combined with saracatinib. Those findings also highlight the polyfunctionality of the generated memory CD8+ T cells from that treatment group (Fig. 8C). The remaining mice in the three treatment groups received a challenge of CEA-expressing tumors on day 31 (Fig. 8A). Mice that were previously vaccinated against CEA and administered saracatinib had reduced tumor growth following challenge (Fig. 8D). Average tumor volume at the termination of the study was significantly lower in the vaccine and saracatinib group than in the naive control mice. For comparison, there was no significant difference between vaccine plus vehicle and naive control mice (Fig. 8E). These results suggest the polyfunctionality of memory CD8+ T cells generated by vaccine plus saracatinib, as evidenced by their ability to produce higher IFN-$\gamma$ levels in response to cognate peptide as well as mediate significant regression of CEA-expressing tumors.

**FIGURE 5.** Molecular mechanisms of increased central memory cell differentiation by saracatinib. (A) Effects of saracatinib on the Akt–mTOR pathway in CD8+ T cells during the expansion phase. Splenocytes from F5 mice were stimulated with 10^{-4} mg/ml of NP68 peptide for 24 h, followed by the addition of vehicle or saracatinib. Cells were incubated with vehicle or saracatinib for indicated time periods, and the CD8+ T cells were negatively isolated by magnetic bead selection used for subsequent Western blot analysis for phospho–AMPK-$\alpha$, total AMPK-$\alpha$, phospho-Akt (Thr308), total Akt (Thr308), phospho-p70S6K, and total p70S6K. Data are representative of two independent experiments. (B) Illustrative hypothesis of the possible molecular mechanisms of mTOR inhibition by saracatinib in CD8+ T cells. The Akt–mTOR pathway is activated in response to IL-2, whereas mTOR activation is suppressed in response to a decrease in ATP. Saracatinib inhibits an unknown molecule or molecules that consequently inhibit the Akt–mTOR pathway at late time points, and this inhibition induces central memory cell differentiation of CD8+ T cells.

**FIGURE 6.** In vivo experiments of Src inhibitors with vaccine. (A) Experimental protocol for mice model of rV/rF-NP34-TRICOM plus the Src inhibitors. C57BL/6 mice were vaccinated with rV-NP34-TRICOM plus rF-GM-CSF on day 0, and then boosted with rF-NP34-TRICOM plus rF-GM-CSF on day 15. Vaccinated mice were orally administered either vehicle (1% Tween 80 solution), 10 mg/kg of saracatinib, or 2.5 mg/kg of dasatinib (twice per day) at the indicated time intervals. Mice were euthanized on day 31, and splenocytes were used for phenotypic analysis. Splenocytes were counted by trypan blue staining. (B) CD8a-NP34 dextramer staining of splenocytes from each group. Results show representative FACS plots for each treatment group. Inset denotes percentage of dextramer-positive cells among the CD8+ T cells. (C and D) Absolute numbers of CD8a+/NP34 dextramer-positive cells (C) and CD8a+/NP34 tetramer'/CD44high/CD62Lhigh cells (D). Each dot represents results from a single mouse; the horizontal line denotes the mean for each group. Similar results were observed in a repeat of this experiment.
SARACATINIB INCREASES CENTRAL MEMORY CD8^+ T CELLS

Discussion

The ability to modulate intrinsic signal transduction pathways that boost immune memory through CD8^+ T cell differentiation offers a powerful new approach to bolster vaccine potency. The actions of metformin, rapamycin (3, 4), and, now, saracatinib to induce better T cell memory seem to depend not only on dose but also, perhaps more importantly, on timing. In all three instances, the changes in cellular metabolism that enhance T cell memory required intervention that was scheduled during the late expansion/contraction phases of the immune response.

Splenocytes from H-2Db–restricted NP68–specific CD8^+ TCR Tg mice (F5 mice) offer a possible in vitro model that could provide important insights into the pharmacologic modulation of intrinsic T cell metabolic pathways and their role or roles in the development of immune memory. Upon stimulation with cognate peptide, the CD8^+ F5 T cells acquire both phenotypic changes and immune effector functions that approximate those described during the generation of an in vivo Ag-specific T cell response—priming, expansion, contraction, and memory. In initial studies, saracatinib treatment of either nonactivated T cells (Table I) or F5 T cells in the priming phase resulted in considerable cytotoxicity and loss of immune effector function (i.e., IFN-γ production; see Fig. 1). However, when addition of a low dose (1.0 μM or lower) of saracatinib was delayed until the F5 cells entered their expansion phase, defined by proliferation and CD44 acquisition, cytotoxicity was averted (Fig. 2). CD44 is activated at the earliest phases of T cell clonal expansion and distinguishes effector and memory T cells from their nonactivated counterparts (33). Recognition of CD44-positive T cells by their ligand on the surface of dendritic cells can promote T cell clustering (34). Although ligand of CD44 does not elicit T cell proliferation, it can affect the T cell response through activation of Lck and Fyn and has been associated with both resistance and susceptibility of activated T cells to apoptosis (35–37). Thus CD44 expression participates in the control of T cell expansion, and the addition of low-dose saracatinib during that time interval results in immune potentiation, as evidenced by the elevated IFN-γ production and increased number of central memory cells. Those results underscore the importance of understanding the timing at which T cells become fully activated, which seems to be tightly linked to the immune-potentiating effects of several pharmacologic agents.

To investigate those intrinsic metabolic pathways through which saracatinib enhanced immunologic memory was of interest. Initial studies clearly showed Src inhibition in murine tumor cells following saracatinib treatment (Supplemental Fig. 1), which agreed with previous reports of tumor cell inhibition by saracatinib using Src-dependent or -independent pathways (13, 38, 39). However, when nonactivated T cells were treated with saracatinib at doses in excess of 1.0 μM, significant cytotoxicity resulted. In those cells, SFK was not activated, as determined by Western blot and kinase activity assays (data not shown), suggesting signaling through an Src-independent mechanism, perhaps inhibition of survival or antiapoptotic pathways. That complexity was emphasized in subsequent comparative studies of saracatinib and dasatinib on F5 T cell biology. Consistent with its well-known immunosuppressive actions, dasatinib treatment of cognate peptide-stimulated F5 T cells significantly reduced IFN-γ production yet had no effect on memory cell differentiation, which was in direct contrast to the enhanced IFN-γ production and memory cell differentiation following low-dose saracatinib. Furthermore, dasatinib inhibited SFK in T cells, whereas saracatinib did not, suggesting that SFK inhibition was associated with immunosuppression, not T cell differentiation. The IC_{50} for SFK for dasatinib is ~5– to 10-fold lower than that for saracatinib (i.e., 0.5 versus 2.7 nM; Refs 14 and 15), indicating that the different doses used for the two compounds were comparable. In other studies, a variety of tumor cell types were reported to have sensitivities to saracatinib-induced inhibition, and those differences did not correlate with Src activation levels (38–41). Moreover, some cell lines are resistant to Src inhibition by saracatinib or dasatinib, even though Src is constitutively phosphorylated (37, 42, 43). The results, unexpectedly, suggest that molecules other than SFK are modulated by low-dose saracatinib and are responsible for the immune potentiation. Thus, other factors may influence the efficacy of the pharmacologic effects of saracatinib on T cells that are resistant to SFK inhibition by low-dose saracatinib while remaining sensitive to dasatinib. Another possibility is that activated T cells have switched into specific metabolic pathways to provide the energy necessary to sustain the high rates of cell proliferation and the acquisition of effector functions (44). Indeed, by upregulating the antiapoptotic protein Bcl-2, memory T cells (45, 46) would resist the cytotoxic effects of such agents as saracatinib while simultaneously initiating cellular metabolic pathways to acquire the defined cellular functions. Nonetheless, Akt and mTOR phosphorylation was inhibited in the activated T cells, indicating those signal transduction pathways are saracatinib sensitive (Fig. 5). Because
inhibition of the Akt–mTOR pathway occurred at 12 and 24 h after saracatinib administration, these actions may be indirect through an unknown molecule or molecules that reside upstream of the Akt–mTOR pathway. Yet in other reports (3, 4, 11) of pharmacologic manipulation of the mTOR and other pathways, central memory T cells, but not IFN-γ production, were enhanced by Ag-specific T cells. Those observations suggest that a yet undefined molecular pathway or pathways controlling IFN-γ production may be involved in saracatinib actions. Efforts to identify this unknown molecule might open a new window to understand the molecular mechanisms of controlling memory cell differentiation.

To design in vivo protocols to test the effects of Src inhibitors on a primary immune response, it was important to determine when T cells expressed CD44 postvaccination as an indicator of their entering the expansion phase. We reported, using F5 mice, that >95% of Ag-specific T cells expressed CD44 on day 3 postvaccination (Supplemental Fig. 2), which is consistent with a previous report that Ag presentation by dendritic cells takes place within 2–3 d postinfection (47, 48). The subsequent in vivo studies again highlighted the differences between the two Src inhibitors. Saracatinib administration 3 d after primary and booster vaccinations resulted in immune potentiation, as measured by an increase in IFN-γ production, arguing that treatment might be tied to the dose or bioavailability of dasatinib and/or the treatment schedule. Dasatinib showed strong immunosuppression from 10 nM levels of IC50 in vitro, yet it requires a dose of 25 mg/kg to induce measurable immunosuppressive effects in vivo (19). Another plausible explanation is that IL-2 signaling can blunt the immunosuppressive effects of dasatinib (50); in the current study, dasatinib was administered during the expansion phase, a time when Ag-specific CD8+ T cells begin proliferation via IL-2 signaling.

The CEA–self-Ag system has been used extensively to investigate the ability of recombinant poxviruses expressing CEA to overcome host tolerance to a self-Ag and to induce CEA-specific antitumor immunity (33). For the most part, the relative strength of the CEA-specific host immune response in CEA.Tg mice has been blunted when directly compared with that generated in wild-type B6 mice, using the same recombinant poxviruses expressing CEA vaccine. Those observations were recapitulated in the current study. Addition of saracatinib to the foreign Ag flu-based (NP34-TRICOM) vaccine resulted in a strong, statistically significant increase of IFN-γ production by the NP34-specific memory T cells (Fig. 7D). In contrast, treatment of CEA.Tg mice with a combination of the MVA/rF-CEA-TRICOM vaccine and saracatinib produced an incremental increase of CEA peptide-specific IFN-γ production (Fig. 8B). Yet that incremental increase in IFN-γ was enough to establish statistical significance when compared with control mice as well as significant protection of CEA.Tg mice following challenge with CEA-expressing tumors (Fig. 8E). These and previous in vivo results demonstrate that the addition of saracatinib to a vaccine protocol at a time of T cell expansion/contraction can result in polyfunctional T cells capable of producing higher IFN-γ levels in response to cognate peptide as well as a more potent recall response to tumor challenge. The findings also suggest that the addition of saracatinib to vaccines for infectious diseases (e.g., malaria, HIV) in which the target Ag

FIGURE 8. Recall response to tumor Ag rechallenge. (A) Experimental protocol for CEA.Tg model of MVA/rF-CEA-TRICOM plus saracatinib. CEA.Tg mice (n = 8 mice/group) were vaccinated with MVA-CEA-TRICOM plus rF-GM-CSF on day 0, then boosted with rF-CEA-TRICOM plus rF-GM-CSF on day 15. Vaccinated mice were orally administered either vehicle (1% Tween 80 solution) or 10 mg/kg of saracatinib (twice per day) for the indicated time periods. Mice were challenged with 3 × 10^6 of MC32a tumor cells on day 31. (B and C) Mice from each group (n = 3) were euthanized prior to tumor challenge (day 31), and splenocytes were stimulated with 1 μg/ml of CEA peptide for 4 d, at which time the supernatants were collected and IFN-γ (B) and IL-2 (C) levels were measured by ELISA. Each dot represents results from a single mouse, and the horizontal line denotes the mean. (D) Average tumor volume (mm^3 ± SEM) of the MC32a-challenged mice. (E) Each dot represents individual tumor volume for each treatment group on day 24 after tumor challenge.
is foreign might result in the more pronounced increase in Ag-specific central memory T cells.

This study presents several intriguing avenues for future investigation. First, studies should address the mechanisms by which low-dose saracatinib inhibits Src phosphorylation in murine tumors, but not in T cells. Second, saracatinib can be added to the list of seemingly different compounds that share similar abilities to increase the functional qualities of memory T cells. Finally, it is within the context of the present findings to suggest a bimodal action for saracatinib that includes suppression of tumor growth via Src inhibition and enhanced memory T cell function through some yet to be determined signaling pathway.

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