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Abrogation of Src Homology Region 2 Domain-Containing Phosphatase 1 in Tumor-Specific T Cells Improves Efficacy of Adoptive Immunotherapy by Enhancing the Effector Function and Accumulation of Short-Lived Effector T Cells In Vivo

Ingunn M. Stromnes,* Carla Fowler,* Chanel C. Casamina,* Christina M. Georgopolos,* Megan S. McAfee, ‡ Thomas M. Schmitt, ‡ Xiaoxia Tan,* Tae-Don Kim,§ Inpyo Choi,* Joseph N. Blattman,‡ and Philip D. Greenberg*,†,¶

T cell expression of inhibitory proteins can be a critical component for the regulation of immunopathology owing to self-reactivity or potentially exuberant responses to pathogens, but it may also limit T cell responses to some malignancies, particularly if the tumor Ag being targeted is a self-Ag. We found that the abrogation of Src homology region 2 domain-containing phosphatase-1 (SHP-1) in tumor-reactive CD8+ T cells improves the therapeutic outcome of adoptive immunotherapy in a mouse model of disseminated leukemia, with benefit observed in therapy employing transfer of CD8+ T cells alone or in the context of also providing supplemental IL-2. SHP-1−/− and SHP-1+/+ effector T cells were expanded in vitro for immunotherapy. Following transfer in vivo, the SHP-1−/− effector T cells exhibited enhanced short-term accumulation, followed by greater contraction, and they ultimately formed similar numbers of long-lived, functional memory cells. The increased therapeutic effectiveness of SHP-1−/− effector cells was also observed in recipients that expressing the tumor Ag as a self-antigen in the liver, without evidence of inducing autoimmune toxicity. SHP-1−/− effector CD8+ T cells expressed higher levels of eomesodermin, which correlated with enhanced lysis of tumor cells. Furthermore, reduction of SHP-1 expression in tumor-reactive effector T cells by retroviral transduction with vectors that express SHP-1–specific small interfering RNA, a translatable strategy, also exhibited enhanced antitumor activity in vivo. These studies suggest that abrogating SHP-1 in effector T cells may improve the efficacy of tumor elimination by T cell therapy without affecting the ability of the effector cells to persist and provide a long-term response. The Journal of Immunology, 2012, 189: 1812–1825.

Adoptive immunotherapy by transfer of tumor-reactive T cells has proven successful for treatment of some established malignancies (1, 2), including leukemia (3). Several obstacles can limit the efficacy of this approach, such as the ability of donor T cells to adequately recognize the tumor and to persist and function (1). Poor recognition often reflects the low avidity of the donor T cells for the targeted tumor cells. Defects in Ag processing and presentation and/or a lack of costimulatory molecule expression by the tumor can contribute to a lack of T cell recognition and/or activation. Additionally, if the tumor Ag is not a foreign protein but rather a self-Ag, self/tumor-reactive T cells that develop and express a high-affinity TCR are often deleted or rendered tolerant. To overcome this obstacle of inefficient recognition, genetic modification of donor T cells to express high-affinity receptors may provide T cells that efficiently recognize the tumor (4, 5). Another common obstacle is poor persistence of transferred T cells and resulting failure to sustain an antitumor response, reflecting in part a consequence of the requirement of first having to expand the donor T cells to large numbers in vitro for therapy, which generally produces a population of effector T cells that depend on exogenous cytokines for proliferation and survival. This has been addressed by several strategies, including administration of exogenous IL-2 (although this often has toxicities in patients) (6), induction of lymphopenia to take advantage of the homeostatic proliferative drive (7, 8), and, more recently, exploration into the use of other γ-chain cytokines (9–11). An approach that our laboratory is pursuing that can potentially concurrently address these obstacles is to abrogate expression of negative regulators of lymphocyte function prior to T cell transfer, as this may improve responses to Ag stimulation, reduce the threshold...

*Department of Immunology, University of Washington, Seattle, WA 98195; ‡Arizona State University Biodesign Institute, Arizona State University, Tempe, AZ 85287; ¶Program in Immunology, Fred Hutchinson Cancer Research Center, Seattle, WA 98109; ‡Cell Therapy Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Republic of Korea; and †Department of Medicine (Oncology), University of Washington, Seattle, WA 98195

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Address correspondence and reprint requests to Dr. Philip D. Greenberg, Department of Medicine, University of Washington, Office H-461 Health Sciences Center, Box 356425, 1959 NE Pacific Street, Seattle, WA 98195-6425. E-mail address: pgreen@u.washington.edu

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; B6, C57BL/6; Cy, cyclophosphamide; Eomes, eomesodermin; FBL, Friend murine leukemia virus; KLRG1, killer cell lectin-like receptor subfamily G member 1; L. monocytogenes-gag, Listeria monocytogenes engineered to express the gag epitope; MFI, mean fluorescence intensity; SHP-1, Src homology region 2 domain-containing phosphatase-1; shRNA, short hairpin RNA; siRNA, small interfering RNA; SLEC, short-lived effector cell; 3min effector, effector cell stimulated for three cycles; TCRgag, TCR transgenic T cells specific for an epitope derived from the Gag protein of the Friend murine leukemia virus; Tg, transgenic.

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for T cell activation, and enhance effector T cell function (12–14).

The Src-homology domain-containing protein tyrosine phosphatase-1 (SHP-1) is a negative regulator of signaling expressed in all hematopoietic cells (15). In T cells, SHP-1 recruitment to membrane lipid rafts is inversely correlated with the strength of the antigenic signal, thereby enforcing the discrimination between weak or antigenic ligands and agonistic ligands (16–20). SHP-1-dependent dephosphorylation of signaling proteins after Ag encounter, including Lck (21, 22), Zap70 (23, 24), Vav (25), PI3K (26), and TCRz (27), has been shown to limit naive T cell responsiveness. Naïve T cells from mice with a loss-of-function mutation in SHP-1 exhibit increased proliferation to Ag and cytolytic activity in vivo after activation compared with wild-type T cells (28). In tumor settings, SHP-1 is detected at high levels in tumor-infiltrating lymphocytes that lack lytic activity, and the abrogation of SHP-1 expression in tumor-infiltrating lymphocytes was found to restore lytic function in vitro (29). Recently, our laboratory demonstrated that SHP-1 negatively regulates the accumulation of short-lived, Ag-specific effector cells derived from either naive or memory virus-specific CD8 precursors in response to acute virus infection without affecting memory T cell formation (30). These studies suggest that ablating SHP-1 in tumor-reactive effector cells has the potential to improve antitumor activity following T cell therapy by several possible mechanisms.

Many previous studies have assessed the role of SHP-1 in T cells isolated from the moth-eaten mouse strain, which has a null mutation in SHP-1 protein in all cells, but there are difficulties studying T cells from such mice as T cells develop abnormally in the context of severe autoimmune inflammatory disease (31–34). To overcome this limitation, we have used a conditional knockout of SHP-1 in which mature CD8 T cells lack SHP-1 protein to assess the impact of abrogation of SHP-1 expression in tumor-reactive effector T cells during immunotherapy of disseminated leukemia. We have previously generated a TCR transgenic (Tg) mouse strain (TCRgag) with CD8 T cells specific for the immunodominant epitope derived from the FMuLV-transformed erythroleukemia, Friend murine leukemia virus (FBL) (35), as well as Tg mice that express the gag tumor Ag as a self-Ag in the liver (Alb-Gag) (36). Because human adoptive immunotherapy protocols rely on the expansion of effector T cells in vitro prior to transfer, we generated TCRgag mice that had SHP-1 conditionally knocked out specifically in mature T cells to elucidate whether the complete or partial abrogation of SHP-1 regulates the antitumor activity of effector T cells. Our results demonstrate that SHP-1 abrogation in vitro-expanded effector CD8 T cells improves the efficacy of T cells in adoptive immunotherapy of FBL leukemia and is associated with enhanced function and accumulation of short-lived effector cells in a preclinical setting. This study has implications for the development of effective therapies to treat established tumors, as this strategy, which improves the generation and expansion of short-lived tumor-reactive effector cells in vivo without affecting long-term memory formation or inducing toxicity, increases the in vivo antitumor activity of the infused T cells during the time when the peak response is needed.

Materials and Methods

**Mice**

SHP-1+/+ mice (37), a gift from L. Pao and B. Neel (Beth Israel Deaconess Medical Center, Boston, MA) and K. Rajewsky (Harvard Medical School, Immune Disease Institute, Boston, MA), were crossed with Lck-Cre mice (distal Lck-Cre) (38, 39), a gift from P. Fink (University of Washington with permission from N. Killeen), and TCRgag Tg mice (36). Alb-Gag Tg mice have been previously described (36).

SHP-1Flox/Flox mice (37), a gift from L. Pao and B. Neel (Beth Israel Deaconess Medical Center, Boston, MA), were crossed with Lck-Cre mice (distal Lck-Cre) (38, 39), a gift from P. Fink (University of Washington with permission from N. Killeen), and TCRgag Tg mice (36). Alb-Gag Tg mice have been previously described (36).

C57BL/6 (B6) mice were purchased from The Jackson Laboratory. Studies were executed according to our approved animal protocol and to the policies of the Institute for Animal Care and Use Committee in the Department of Comparative Medicine, University of Washington, and mice were maintained under specific pathogen-free conditions.

**Cell lines, Abs, and peptides**

The Friend virus-induced erythroleukemia of B6 origin, FBL, expresses the FMuLV-encoded gag epitope (peptide CCGCTCCTACTGTTGGTGAGCCGC-3, CCGGGATCGAGGACTGATGCTA-CTGATACCTGCTGG-3, Eomes, 5'-GCTATCCAAACACGGA-3').

**Intracellular staining for SHP-1**

TCRgag SHP-1+/+, SHP-1+/−, and SHP-1−/− cells were surface stained for CD8 and Thy1.1 for 30 min at 4°C. Cells were washed twice in FACS buffer (PBS containing 1% FBS) and fixed using BD Phosflow Perm Buffer I (BD Biosciences) at 37°C for 10 min according to the manufacturer’s protocol. Cells were washed once with FACS buffer and permeabilized using BD Phosflow Perm Buffer II and incubated for 30 min on ice. Cells were washed and incubated with rabbit anti-mouse SHP-1 (C1486; Cell Signaling Technologies) diluted 1:50 in FACS buffer at 4°C. Cells were washed twice with FACS buffer and incubated with anti-rabbit 547 (F(ab′)2 fragment; Molecular Probes). SHP-1 staining was immediately analyzed on a flow cytometer by gating on CD8+Thy1.1+ cells.

**Expansion of effector TCRgag cells in vitro**

TCRgag SHP-1+/+, SHP-1+/−, and SHP-1−/− in vitro-derived effector cells were generated as previously described (14). Briefly, TCRgag SHP-1+/+, SHP-1−/−, and SHP-1−/− Tg cells (1 × 105) were cultured with irradiated, syngeneic splenocytes (5 × 105), irradiated FBL leukemia (3×105), and IL-2 (20 U/ml) in 10 ml complete medium (RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml penicillin/streptomycin, 10% FCS, and 30 μM 2-ME). T cells were similarly restimulated every 7–10 d. Five days following the third in vitro cycle of Ag stimulation, effector T cells were used in various assays or transferred for adoptive immunotherapy.

**T cell proliferation and cytokine production in vitro**

CFSE was purchased from Molecular Probes. Tg T cells were incubated with 1 μM CFSE for 20 min at 37°C, and excess CFSE was washed from T cells before three times using complete media. CFSE-labeled Tg T cells (1 × 105) were incubated in round-bottom, 96-well plates with 5 × 104 congenic (Thyl.2) splenocytes pulsed with titrating concentrations of gag peptide. After 4 d, CFSE dilution of Thyl.1 CD8+ Tg cells was assessed by flow cytometry. Intracellular cytokine staining was performed by incubating Tg T cells with congenic (Thyl.2), peptide-pulsed splenocytes in the presence of brefeldin A (BD Biosciences). After 5 h, cells were stained with Abs to CD8, Thyl.1, and intracellular cytokines according to BD Biosciences protocols.

**CFSE-based cytotoxicity assay**

EL-4 and FBL tumors were incubated 20 min at 37°C with 0.1 μg or 10 μM CFSE. Excess dye was removed by washing tumor cells in serum-containing media. A 1:1 mixture of EL-4 and FBL tumor cells was incubated with titrating numbers of SHP-1−/−, SHP-1+/−, or SHP-1+/+ in vitro-expanded effector T cells for 5 h in 96-well, round-bottom plates at 37°C. 5% CO2. FBL lysis was determined by FACS analyses of the number of CFSE− (FBL) remaining in the well.

**Real-time PCR and gene expression analysis**

RNA was isolated from 5 × 106 naïve or effector T cells at various time points after activation with Ag with or without IL-2 (20 U/ml) using the RNeasy Plus Mini kit (Qiagen). Reverse transcription to generate cDNA from RNA samples was conducted using the SuperScript first-strand synthesis system and oligo(dT) primers (Invitrogen). The following primer sequences (Invitrogen) were used (forward, reverse): β-actin, 5′-AATCTG-CAGAGGACTCCTATGTTGGTGACGC-3′, 5′-CCGGATCGAGGACTGATGCTACTGATACCTGGCTGG-3′; Eomes, 5′-GCTATCCAAACACGGA-3′, 5′-
TCTGTTGGGTGTAGAGG-3′; Rune-3, 5′-TCCAAGTGTACCTGTTGGATGAG-3′; granzyme A, 5′-TTTCCATTTGGAATGG-3′; granzyme B, 5′-TGGCCATATCTGGCTTCT-3′; Cyt B, 5′-TTGAGGATTGATTACCTC-3′; granzyme C, 5′-TCCCTGACCTCCATTCT-3′, 5′-TGTTAGCAGAATTGTTCTC-3′; T-bet, 5′-GTCTCATCCTCTCTC-3′; 5′-CTCTTGTGTGTTGAGCTT-3′; perforin, 5′-GAGTTGACCCCTGACAGA-3′; 5′-GTTTGTATTCAACGCAGA-3′. Real-time PCR reactions were in a total volume of 25 μl containing 1× Power SYBR Green PCR Master Mix (Applied Biosystems), 1.25 μl primer mix, and 2 μl cDNA. All reactions were performed in duplicate and each plate contained the endogenous control (β-actin). The ABI Prism 7000 sequence detection system was used to amplify target genes with the following PCR reactions were in a total volume of 25 μl containing 1× Power SYBR Green PCR Master Mix (Applied Biosystems), 1.25 μl primer mix, and 2 μl cDNA. All reactions were performed in duplicate and each plate contained the endogenous control (β-actin). The ABI Prism 7000 sequence detection system was used to amplify target genes with the following conditions: denaturing at 95˚C for 10 min; 40 cycles at 95˚C for 15 s and 60˚C for 1 min. Gene expression was calculated by the ΔΔCt method and was normalized to the endogenous control (β-actin) and gene expression in naive or wild-type effector T cells as indicated.

Adoptive immunotherapy of disseminated FBL leukemia

B6 or AlbE Gmace were injected with 5 × 10⁵ live FBL leukemia i.p. as described (14, 36). After 5 d, after the leukemia had disseminated, mice received cyclophosphamide (Cy; 180 mg/kg), and after 6 h to permit clearance of the Cy, TCRgag SHP-1−/−, SHP-1+/+, and SHP-1−/− effector cells. Mice were injected with 2 × 10⁶ FBL tumor cells in three cycles in vitro prior to transfer into tumor-bearing mice. Cohorts of recipient mice also received exogenous IL-2 injections (1 × 10⁶ U/ml/mouse/day i.p.). Mice were monitored for tumor burden (ascites, splenomegaly, and nodal masses) and euthanized when the detectable tumor mass reached proportions that potentially led to mortality within 24 to 48 h.

Immunization with Listeria monocytogenes engineered to express the gag epitope after transfer of memory TCRgag cells

At 120 d after tumor therapy, splenocytes were harvested from cured mice and sorted for Thy1.1 CD8+ donor cells. Equivalent numbers of each type of donor cell (1 × 10⁷) were transferred into B6 recipients. Two days later, mice were immunized with an attenuated (ΔΔCt) recombinant L. monocytogenes engineered to express the gag epitope (L. monocytogenes-gag; 3 × 10⁷ CFU). After 6 d, splenocytes were harvested, counted, and stained with Abs to CD8 and Thy1.1 and various other surface markers and analyzed by flow cytometry.

Administration of the anti–IL-7 mAb (M25)

B6 or IL-15−/− mice were injected with irradiated FBL tumor (2 × 10⁷ cells) and/or Cy (180 mg/kg) 6 h prior to administration of effector T cells and irradiated tumor. Some recipients also received exogenous IL-2 injections (1 × 10³ U/ml/mouse/day i.p.). Effector T cells that had undergone three cycles of in vitro expansion with Ag and IL-2 (20 U/ml) were transferred (1 × 10⁵) into either B6 mice, IL-15−/− mice, or B6 and IL-15−/− mice that had been injected with 1 mg IL-7-depleting M25 Ab (1 mg/mouse/mouse/day). The M25 Ab was provided by Amgen) beginning 1 d prior to and then every other day following effector T cell transfer. After 7 d, the percentage and number of donor cells in the spleen were calculated by counting live splenocytes and FACS analysis of CD8+ Thy1.1+ cells.

Short hairpin RNA silencing of SHP-1 in tumor-reactive effector T cells

SHP-1 was targeted with the short hairpin RNA (shRNA) 5′-AACGCAGTCTACGGTATAG-3′ (NM_085095.3), a sequence that is homologous between mice and humans and has been successfully targeted in humans (40, 41) and mouse (29) cells. We generated a MG91 retroviral vector that expressed GFP and the SHP-1-specific shRNA under the control of the U6 promoter. A control vector that had a scramble shRNA sequence was used to control for nonspecific effects of T cell transduction. Viral supernatant was harvested from transfected packaging cells on days 2 and 3 after transfection. Wild-type TCRgag effector cells were transfected by incubating effector T cells with viral supernatant and spinning cells for 90 min at 32°C, at 2500 rpm. Transduced cells were purified by sorting for GFP+CD8+ cells and maintained in vitro by restimulating with Ag and IL-2 (20 U/ml) every 7 d.

Statistical analysis

Data in graphs represent the means ± SEM. Statistical analyses of the data were performed using a one-way ANOVA followed by Tukey post hoc testing to reduce the type I errors, with p values of < 0.05 considered significant for the ANOVA/Tukey testing and p values of >0.05 considered not significant. For comparisons of only two groups, a Student t test was used to determine significance. *p < 0.05, **p < 0.005, ***p < 0.0005. Analysis of survival curves was performed using a log-rank Mantel–Cox test.

Results

Cell-intrinsic SHP-1 abrogation lowers the threshold for proliferation of naïve T cells specific to a tumor Ag

To study the T cell-intrinsic effects of abrogating SHP-1 in tumor-specific T cells, mice that express a floxed knock-in SHP-1 gene (37, 42) and Cre recombinase under control of the distal Lck promoter (39) were crossed to TCRgag mice (35). We have previously shown that this approach permits the study of T cell-intrinsic effects of SHP-1 deletion, with nearly all CD8 T cells becoming SHP-1 deficient (>93%) and developing in the absence of the abnormal host environmental milieu and lethal autoimmune disease that occurs when SHP-1 is deleted in all hematopoietic cells (30). The mean fluorescence intensity (MFI) of SHP-1−/− cells in CD8+ tetramer+ cells demonstrated complete absence of SHP-1 protein in TCRgag SHP-1−/− cells (both alleles floxed) and a 50% reduction in SHP-1−/− cells in SHP-1−/− cells (one allele floxed) compared with SHP-1+/− cells, confirming gene disruption and loss of SHP-1 protein expression in TCRgag cells (Fig. 1A). Expression levels of CD44, CD62L, and CD127 of CD8 T cells did not result in persistent or intermittent T cell activation.

We previously demonstrated that cell-intrinsic expression of SHP-1 regulates naïve T cell activation toward viral Ag (30), and we performed initial studies to verify that SHP-1 deficiency also improves tumor Ag recognition in TCRgag cells. CFSE-labeled naïve TCRgag SHP-1−/−, SHP-1+/+ and, SHP-1−/− T cells (congenic for Thy1.1) were incubated with synecytic Thy1.2+ splenocytes pulsed with titrating concentrations of gag peptide. After 4 d, CFSE dilution of Thy1.1 CD8+ cells was quantified by flow cytometric analysis (FACS). TCRgag SHP-1−/− cells proliferated in response to a log10 lower concentration of Ag compared with TCRgag SHP-1+/− and SHP-1+/- cells (Fig. 1C), indicating that complete abrogation of SHP-1 in naïve T cells lowers the threshold for inducing T cell proliferation in response to tumor Ag recognition. Despite the enhanced proliferation of SHP-1−/− cells, the absence of SHP-1 did not enhance T cell apoptosis, as indicated by annexin V and 7-aminoactinomycin D (7-AAD) staining of T cells 8 d after activation (Fig. 1D). The enhanced proliferation and survival in the absence of SHP-1 resulted in ~2-fold increase in the number of SHP-1−/− cells relative to SHP-1+/+ cells (mean T cell number 2.1 × 10⁷, SHP-1−/− = 2.9 × 10⁷, SHP-1−/− = 4.3 × 10⁷).

Functional impact of SHP-1 abrogation of in vitro-expanded effector cytotoxic T cells

Most studies evaluating SHP-1 regulation of T cells have assessed naïve T cell responses. Naïve T cells have superior proliferative potential compared with effector T cells, which lose the ability to produce IL-2 and often die or fail to respond after subsequent Ag encounter (43, 44). However, to obtain a sufficient number of T cells with the intended Ag specificity, clinical adoptive immunotherapy protocols require T cells to be expanded in vitro prior to transfer into patients (12, 45). Genetic modification of donor T cells to express an Ag receptor of a defined specificity can reduce the number of in vitro stimulations of T cells needed prior to
Splenocytes from naive TCRgag SHP-1+/+, SHP-1+/- mice were stained with Abs to CD8 and Thy1.1 and stained intracellularly for SHP-1. The MFI of SHP-1 expression was determined by gating on CD8+Thy1.1+ cells. Data are pooled from three independent experiments. (C) Purified CD8 T cells from splenocytes isolated from naive TCRgag SHP-1+/+, SHP-1+/-, and SHP-1+/- mice were labeled with CFSE and incubated with Thy1.2+ syngeneic splenocytes pulsed with titrating concentrations of gag peptide. After 4 d, CFSE dilution of Tg cells was determined by FACS analysis of CD8+Thy1.1+ cells. Data are pooled from two independent experiments. (D) The percentage of T cells undergoing apoptosis at 8 d after activation with Ag was determined by analyzing the percentage of CD8+Thy1.1+ cells that stained positive for annexin V and 7-AAD by FACS. Statistical analysis between the groups was conducted using a one-way ANOVA followed by a Tukey post hoc correction. *p < 0.05, **p < 0.005.

FIGURE 1. Cell-intrinsic SHP-1 deficiency does not modify the phenotype of mature TCRgag cells, but it does lower the threshold for activation. (A) Splenocytes from naive TCRgag SHP-1+/+, SHP-1+/-, and SHP-1+/- mice were stained with Abs to CD8 and Thy1.1 and stained intracellularly for SHP-1. The MFI of SHP-1 expression was determined by gating on CD8+Thy1.1+ cells. Data are pooled from three independent experiments. (B) Splenocytes isolated from naive TCRgag SHP-1+/+, SHP-1+/-, and SHP-1+/- mice were stained for CD8 and Thy1.1 and the indicated Abs. Representative histograms of the intensity of Ab staining are gated on CD8+Thy1.1+ cells. (C) Purified CD8 T cells from splenocytes isolated from naive TCRgag SHP-1+/+, SHP-1+/-, and SHP-1+/- mice were labeled with CFSE and incubated with Thy1.2+ syngeneic splenocytes pulsed with titrating concentrations of gag peptide. After 4 d, CFSE dilution of Tg cells was determined by FACS analysis of CD8+Thy1.1+ cells. Data are pooled from two independent experiments. (D) The percentage of T cells undergoing apoptosis at 8 d after activation with Ag was determined by analyzing the percentage of CD8+Thy1.1+ cells that stained positive for annexin V and 7-AAD by FACS. Statistical analysis between the groups was conducted using a one-way ANOVA followed by a Tukey post hoc correction. *p < 0.05, **p < 0.005.

transfer, but activation and expansion of T cells leading to the differentiation of donor T cells into effector cells is still required (3, 46). Therefore, to model immunotherapy protocols, TCRgag SHP-1+/+, SHP-1+/-, and SHP-1+/- cells were expanded in vitro with Ag (irradiated FBL tumor), syngeneic feeder cells, and exogenous IL-2 (20 U/ml) for three cycles of stimulation (3stim effectors). Five days after the third stimulation, SHP-1 protein was readily detectable in wild-type TCRgag effector cells, decreased by ∼50% in SHP-1+/- cells (as indicated by MFI analysis), and absent in SHP-1+/- cells (Fig. 2A). The effector cells expressed similar levels of Vα3 and were CD44hi, CD62Llo, and KLRG1hi, indicative of an effector phenotype (Fig. 2B). The TCRgag SHP-1+/+, SHP-1+/-, and SHP-1+/- effector cells expressed equivalent and high levels of CD25 (IL-2Rx), IL-15Rx, and downregulated CD127 (IL-7Rx) compared with naive CD8 T cells (Fig. 2B). The similar cytokine receptor expression in the effector cell groups suggests that all have the ability to initiate signaling through these critical proliferative and survival pathways. To determine the effect of SHP-1 on Ag-dependent expansion of effector cells, we seeded an equal number of effector cells at the start of the third in vitro simulation and quantified the number of live Tg cells after 7 d. In the absence of exogenous IL-2, there was a 2-fold increase in the number of live Tg SHP-1+/- effectors compared with SHP-1+/- effectors, and the partial decrease in SHP-1 in cells from heterozygous mice resulted in a modest increase in cell number (Fig. 2C). Supplementation of exogenous IL-2 significantly enhanced the accumulation of effector T cells, overcoming the impact of SHP-1 deficiency and making the total cell numbers equivalent and independent of SHP-1 levels (Fig. 2C). Seven days after effector cell expansion with Ag, the percentage of effector T cells undergoing apoptosis (annexin V+annexin V+) correlated with the expression of SHP-1 in T cells (Fig. 2D), indicating that the abrogation of SHP-1 improves effector cell survival after Ag encounter. Increased survival of SHP-1+/- and SHP-1+/- effectors correlated with a modest increase in expression of the prosurvival molecule Bcl-xL (Fig. 2F), with no difference in expression of the prosurvival molecule Bcl-2 (data not shown). To determine whether the abrogation of SHP-1 impacts susceptibility of effector T cells to FasL-induced apoptosis, naive and three time in vitro-expanded SHP-1+/+, SHP-1+/-, and SHP-1+/- effector T cells were incubated with anti-Fas Ab and a secondary Ab to cross-link Fas as described (47). After 12 h, cells were stained for CD8 and apoptosis markers annexin V and 7-AAD and analyzed by flow cytometry. Fas cross-linking on naive T cells did not substantially result in apoptosis (∼4%), but it did induce apoptosis of most (77%) SHP-1+/- effector T cells, and this was substantially reduced in the effector T cells that lacked SHP-1 (28%) (Supplemental Fig. 1). As there were no detectable differences in Fas expression between SHP-1+/- and SHP-1+/- cells, these data suggest that one mechanism of increased survival of SHP-1+/- effector T cells may be resistance to Fas signaling.

To evaluate whether SHP-1 influenced the lytic activity of effector cells, we used a CFSE-based cytotoxicity assay that allows visualization of tumor cell lysis mediated by CTLs. TCRgag SHP-1+/+, SHP-1+/-, or SHP-1+/- 3stim effectors were incubated with a mixture of live FBL tumor (CFSEhi) and control tumor (EL4, CFSElo). After 5 h, CTL-mediated specific lysis of FBL tumor was assessed by comparing the percentage of FBL (CFSEhi) to the partial decrease in SHP-1 in cells from heterozygous mice resulted in a modest increase in cell number (Fig. 2C). Supplementation of exogenous IL-2 significantly enhanced the accumulation of effector T cells, overcoming the impact of SHP-1 deficiency and making the total cell numbers equivalent and independent of SHP-1 levels (Fig. 2C). Seven days after effector cell expansion with Ag, the percentage of effector T cells undergoing apoptosis (annexin V+) correlated with the expression of SHP-1 in T cells (Fig. 2D), indicating that the abrogation of SHP-1 improves effector cell survival after Ag encounter. Increased survival of SHP-1+/- and SHP-1+/- effectors correlated with a modest increase in expression of the prosurvival molecule Bcl-xL (Fig. 2F), with no difference in expression of the prosurvival molecule Bcl-2 (data not shown). To determine whether the abrogation of SHP-1 impacts susceptibility of effector T cells to FasL-induced apoptosis, naive and three time in vitro-expanded SHP-1+/+, SHP-1+/-, and SHP-1+/- effector T cells were incubated with anti-Fas Ab and a secondary Ab to cross-link Fas as described (47). After 12 h, cells were stained for CD8 and apoptosis markers annexin V and 7-AAD and analyzed by flow cytometry. Fas cross-linking on naive T cells did not substantially result in apoptosis (∼4%), but it did induce apoptosis of most (77%) SHP-1+/- effector T cells, and this was substantially reduced in the effector T cells that lacked SHP-1 (28%) (Supplemental Fig. 1). As there were no detectable differences in Fas expression between SHP-1+/- and SHP-1+/- cells, these data suggest that one mechanism of increased survival of SHP-1+/- effector T cells may be resistance to Fas signaling.

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FIGURE 2. SHP-1 abrogation improves cell survival and cytolytic activity of in vitro-expanded effector T cells. Splenocytes from naive Thy1.1+ TCRgag SHP-1+/+, SHP-1+/−, and SHP-1−/− mice were stimulated in vitro with Ag and recombinant human IL-2 (20 U/ml) for three cycles of expansion (3stim effectors). On day 5 after the third stimulation, T cells were stained for CD8, Thy1.1, and intracellular SHP-1 (A) or the indicated Ags (B) and analyzed by FACS. Representative histograms are gated on CD8+Thy1.1+ cells. (C) On day 7 after the third in vitro stimulation with Ag with or without IL-2 (20 U/ml), the number of live Tg T cells in expanded cultures was derived from total cell counts and FACS analysis of CD8+Thy1.1+ cells. In the absence of exogenous IL-2, the number of SHP-1−/− effector cells was significantly increased compared with SHP-1+/+ cells (p = 0.01). The dashed line indicates the number of Tg effectors (1 × 10⁶) at the start of the third stimulation. Data are combined from four independent experiments. (D) Apoptosis of 3stim effectors was determined 7 d after the third stimulation of Ag and IL-2 by analyzing the percentage of annexin V+7-AAD cells of the total Tg CD8+Thy1.1+ cells by FACS. (E) Seven days after stimulation with Ag and IL-2, 3stim effector cells were stained for intracellular Bcl-xL and the MFI of Bcl-xL staining was determined by FACS analysis of the CD8+Thy1.1+ cell population. (F) 3stim effectors were incubated at a 1:1 ratio with a mixture of FBL targets labeled with 10 μM CFSE and control EL4 tumor cells labeled with 0.1 μM CFSE. After 5 h, the percentage of FBL cells was compared with control tumor by FACS analysis. A standard CFSE-based cytotoxicity assay as described in (F) was performed using titrating numbers of 3stim effectors. The percentage lysis was determined by dividing the number of FBL cells incubated with T cells by the number of live FBL cells incubated without T cells. (H) CD8+ effectors were stained for the intracellular expression of the indicated proteins and analyzed by FACS. Representative histograms (n = 4) are shown. (I) The percentage of CD8+ Tg effectors that express Eomes (left panel) and the MFI of Eomes in CD8+ Tg effectors (right panel) was pooled from four experiments. (J) Relative expression of Eomes and perforin mRNA was determined by real-time PCR. Data are normalized to the housekeeping gene β-actin and to the gene levels in wild-type effectors. Data are pooled from four independent experiments. Statistical analysis between the groups was conducted using a one-way ANOVA followed by a Tukey post hoc correction. *p < 0.05, **p < 0.005, ***p < 0.0005.
SHP-1 (48, 49), T-bet (50), and Runx-3 (49). A higher percentage of in the absence of SHP-1, we compared expression of effector (Fig. 2G). To investigate the mechanism of enhanced CTL activity and function, Eomes (48, 49), T-bet (50), and Runx-3 (49). A higher percentage of SHP-1+/− effector cells expressed the transcription factor Eomes (Fig. 2H), and the MFI of Eomes was also significantly higher in SHP-1−/− effector cells, indicating a higher level of Eomes expression on a per cell basis (Fig. 2I). T-bet and granzyme B protein were expressed at similar levels in SHP-1+/+ and SHP-1−/− effector cells, but a modest significant (p = 0.0165, n = 4) increase in the expression of perforin protein was detected in the SHP-1−/− cells as compared with wild-type effectors (Fig. 2H). Consistent with the protein analysis, real-time PCR showed that SHP-1+/− effectors expressed 3-fold more Eomes mRNA and 2-fold more perforin mRNA as compared with SHP-1+/+ effectors (Fig. 2J). Both Runx-3 and T-bet mRNA were increased in wild-type and knockout effector T cells as compared with naive T cells, but expression levels of these genes did not differ between SHP-1+/− and SHP-1−/− effector cells (data not shown). Thus, the enhanced CTL activity of SHP-1+/− effector cells may partially reflect higher expression levels of Eomes, a transcription factor that has been shown to promote the lytic program in CD8 T cells (48, 51).

*Abrogating SHP-1 expression increases therapeutic efficacy in adoptive immunotherapy of disseminated leukemia in mice*

We used our preclinical mouse model of therapy for disseminated FBL leukemia (52, 53) to determine whether abrogating SHP-1 in tumor-specific T cells improves therapeutic activity of T cells during adoptive immunotherapy of cancer. B6 mice were injected with a lethal dose of FBL leukemia, which expresses the gag epitope (35), and 5 d later, after the leukemia is disseminated, Cy (180 mg/kg) was administered, which reduces tumor burden and renders mice lymphopenic. After 6 h, Thy1.1+ TCRgag SHP-1+/+ or SHP-1−/− 3gum effectors (1 × 10⁶ cells) were transferred into cohorts of tumor-bearing mice. Administration of exogenous IL-2 (10⁵ U/mouse daily for 10 d) following T cell transfer is required to promote in vivo persistence, expansion, and therapeutic activity of wild-type effector T cells in the disseminated FBL leukemia model (54). However, transferred TCRgag SHP-1−/− effectors, in distinction to the other cell types, prolonged survival and cured 50% of treated mice in the absence of providing exogenous IL-2 at this cell dose (Fig. 3A). Providing exogenous IL-2 enhanced the therapeutic activity of all of the transferred cells, but mice treated with TCRgag SHP-1−/− effectors still exhibited significantly enhanced survival compared with TCRgag SHP-1+/+ and SHP-1−/− cells (Fig. 3A, right panel). We stained blood mononuclear cells with Abs to CD8 and Thy1.1 to determine the percentage of donor T cells in the blood in therapy recipients at 7 d after transfer. Both in the presence and absence of exogenous IL-2, there was an increase in the percentage of TCRgag SHP-1−/− cells in the blood compared with wild-type TCRgag cells (Fig. 3B, p < 0.001), which correlated with a 2-fold decrease in the percentage of SHP-1+/− effector cells that were annexin V+ compared with SHP-1+/+ cells (Fig. 3C). Cells with a partial deficiency in SHP-1 (SHP-1+/−) were indistinguishable from wild-type cells, indicating that a >50% reduction of SHP-1 in donor T cells is required for the enhanced therapeutic effect.

We tracked the percentage of donor T cells by staining PBMCs with Abs to CD8 and Thy1.1 to investigate whether SHP-1 influenced the contraction and/or persistence of effector T cells. In recipients that did not receive IL-2, the percentage of SHP-1−/− and SHP-1−/− cells was similar at day 30 and undetectable by FACS by day 60 (Fig. 3D). In mice that received IL-2 injections, there was a significantly higher percentage of TCRgag SHP-1−/− effector cells compared with wild-type cells 30 d after T cell transfer (p < 0.01), consistent with the enhanced expansion resulting from both abrogation of SHP-1 expression and administration of IL-2 during adoptive immunotherapy (Fig. 3D, right panel). However, even in the presence of exogenous IL-2, donor cells could not be readily detected at 60 d after transfer of 1 × 10⁶ effectors. Thus, abrogating SHP-1 expression in effector T cells appeared to increase the efficacy of adoptive immunotherapy by enhancing the short-term accumulation of effector cells.

*Effector T cells persist long term and convert to memory T cells independent of SHP-1 during adoptive immunotherapy of cancer*

The ability of donor T cells to persist after transfer into tumor-bearing hosts is a major determinant for sustained antitumor activity (55), and strategies that enhance T cell persistence during immunotherapy are avidly being pursued (56). The transfer of a 1 × 10⁶ effector T cells revealed that SHP-1 ablation improves therapeutic efficacy of adoptive immunotherapy of leukemia by enhancing the short-term accumulation of effector T cells (Fig. 3), but it did not directly address whether SHP-1 abrogation impacts long-term T cell persistence since both wild-type and SHP-1−/− cells were largely undetectable at 60 d after transfer. As the ability to detect persisting T cells reflects in part the number of effector cells initially transferred, we infused 50-fold more 3gum effectors (5 × 10⁶) into tumor-bearing mice treated with Cy prior to transfer, and IL-2 was injected after the cell infusion. With this therapeutic regimen, all recipients survived disseminated tumor. At 120 d after T cell transfer, the CD8+Thy1.1+ donor T cells in the blood were readily detectable in recipients of either SHP-1+/+, SHP-1−/−, or SHP-1−/− cells (Fig. 4A), and there were no significant differences in the percentage (Fig. 4B) or number (data not shown) of T cells. We compared the nature of persisting cells by staining donor cells for markers indicative of effector, effector memory, or central memory phenotypes at 60 and 120 d after transfer. Despite initial transfer of a uniform population of phenotypic effector T cells (CD62Llo, CD127lo, KLRG1hi), at 60 d after transfer donor cells expressed intermediate levels of CD62L and CD127 and most were KLRG1lo (Fig. 4C). By 120 d, donor T cells were CD62Llo, KLRG1lo, and CD127lo, a phenotype more consistent with central memory cells. To determine whether the persisting T cells had the capacity to proliferate to Ag presented in an immunogenic context, we immunized mice that had been cured of tumor with an attenuated recombinant L. monocytogenes-gag. The percentage of TCRgag SHP-1+/+, SHP-1−/−, and SHP-1−/− donor cells (CD8+Thy1.1+) increased in response to L. monocytogenes-gag immunization, indicating that the memory cells generated following tumor elimination from transferred effectors have the capacity to proliferate upon Ag encounter (Fig. 4D). To determine whether SHP-1 regulated the generation of effector cells from these persisting memory cells, we transferred 1 × 10⁶ purified Thy1.1+CD8+ SHP-1+/+, SHP-1−/−, and SHP-1−/− cells (obtained at 120 d after therapy) into Thy1.2+ hosts. Two days later, these recipients were immunized with L. monocytogenes-gag. At the peak of the response, a significantly higher percentage (Fig. 4E) and number (Fig. 4F) of SHP-1+/+ and SHP-1−/− cells compared with wild-type T cells was detected in the boosted re-
FIGURE 3. Abrogation of SHP-1 in TCRgag in vitro-expanded effector cells improves therapeutic outcome of adoptive immunotherapy of disseminated FBL leukemia. (A) B6 mice (Thy1.2) were injected with 5 × 10^6 FBL leukemia. After 5 d when the leukemia had disseminated, mice were injected with Cy (180 mg/kg), followed by 1 × 10^7 Thy1.1+ TCRgag SHP-1+/+, SHP-1−/−, or SHP-1−/− 3sim effectors. The right panel depicts T cell recipients that also received exogenous IL-2 (10^5 U/mouse/day i.p. for 10 d beginning on the day of T cell transfer). (B) At 7 d after T cell transfer, mononuclear blood cells were isolated from therapy recipients and analyzed for the percentage of donor cells (CD8Thy1.1+) by FACS. Numbers in the FACS plots indicate the percentage of CD8Thy1.1+ cells of the total percentage of CD8+ cells. Data are representative of 12–15 mice per group. (C) At 7 d after T cell transfer, CD8Thy1.1+ cells isolated from the blood of Thy1.2 recipient mice (without exogenous IL-2) were analyzed for apoptosis by annexin V staining. Histograms are representative of three to five mice per group and gated on CD8Thy1.1+ cells. (D) The percentage of CD8Thy1.1+ cells in the blood of therapy recipients was determined by FACS at the indicated time points (n = 8–12 mice/group). **p < 0.005.

response, indicating that even a 50% reduction in SHP-1 has a biological effect on the response of memory T cells. The vast majority of memory T cells responding to L. monocytogenes-gag immunization acutely converted to an effector phenotype (CD62Llo, KLRG1hi, CD127lo; Fig. 4G) with the only phenotypic difference detected among the groups being slightly higher levels of CD5 expression on SHP-1−/− cells compared with SHP-1+/+ cells (Fig. 4G). A higher percentage of SHP-1−/− cells secreted IFN-γ and TNF-α compared with wild-type cells, indicating that the quality of the expanded effector response after immunization was enhanced in the absence of SHP-1 (Fig. 4H). Thus, SHP-1 ablation does not impact the ability of in vitro-expanded T effector cells to convert to long-lived memory cells following immunotherapy, but it does regulate the ability of those memory cells to generate accumulation of effector cells following Ag encounter.

Enhanced short-term accumulation of TCRgag SHP-1−/− cells is not associated with autoimmunity in mice that express the gag tumor Ag as a self-antigen in the liver

We have previously described Tg mice that express the FBL gag tumor Ag as a self-Ag in the liver (Alb:Gag mice) (36) and have used the Alb:Gag mice as hosts for T cell therapy to better model human cancer in which the tumor Ags are often also self-Ags. Transfer of naive TCRgag cells into Alb:Gag mice results in the rapid deletion and/or tolerization of the T cells (10, 35, 57), but expression of the gag Ag in the liver does not preclude therapeutic activity when high numbers (1 × 10^6) of in vitro-expanded TCRgag effector cells are transferred into Alb:Gag hosts with disseminated leukemia in the presence of exogenous IL-2 (36). Therefore, we transferred 5 × 10^6 in vitro-expanded TCRgag SHP-1+/+ and SHP-1−/− effectors into B6 or Alb:Gag mice with disseminated leukemia treated with Cy (as described in Fig. 4) and administered exogenous IL-2 after transfer to evaluate whether abrogation of SHP-1 in tumor-reactive T cells that can potentially also recognize normal tissues influenced therapeutic efficacy in this setting and/or induced autoimmunotoxicity. Seven days after the infusion of effector T cells, we stained blood mononuclear cells from control B6 or Alb:Gag recipients with Abs to CD8 and Thy1.1. A significantly higher percentage of TCRgag SHP-1−/− cells compared with wild-type effectors were found in both B6 and Alb:Gag recipients (Fig. 5A, 5B). Independent of SHP-1, there was a modest, but not statistically significant, reduction in the percentage of effector T cells in Alb:Gag hosts (Fig. 5B), which correlated with less therapeutic activity (Fig. 5C). TCRgag SHP-1−/− cells had enhanced therapeutic activity compared with TCRgag SHP-1+/+ cells in Alb:Gag mice treated with exogenous IL-2 (Fig. 5C). Additionally, ablating SHP-1 reduced the difference in antitumor activity of effector CTL in hosts that express the tumor Ag as a self-Ag as compared with wild-type hosts in which the gag tumor Ag is not expressed in self-tissue (Fig. 5C, 5D). Therefore, recognition of the self-Ag is likely promoting deletion of a portion of the transferred T cells, the benefit of the abrogation of SHP-1 continues to be sustained in the presence of self-Ag.

The abrogation of SHP-1 in persisting transferred effector CTL could potentially result in autoimmune toxicity to the liver cells that express the gag epitope. However, the Alb:Gag recipients of TCRgag SHP-1−/− cells that were cured of tumor remained healthy without overt clinical systemic signs of autoimmune disease. At the time of T cell transfer, serum levels of the liver enzymes aspartate aminotransferase and alanine aminotransferase, indicators of liver injury, were found to be ∼2-fold elevated in tumor-bearing mice due to the fact that leukemia is disseminated in multiple tissues, including the liver (data not shown). Two days after transfer, liver enzymes were increased 3- to 4-fold in recipients of either SHP-
1+/+ or SHP-1−/− cells, and by 15 d after transfer, the levels of these enzymes had resolved to ∼1.5-fold as compared with normal Alb:Gag mice. Histological analysis of liver sections on day 12 after T cell transfer also revealed no evidence of lymphocytic cellular infiltration or injury to hepatocytes engineered to express the gag Ag (Supplemental Fig. 2). Thus, abrogation of SHP-1 in CTLs still increases the therapeutic efficacy of adoptive immunotherapy in hosts in which the tumor Ag is expressed as a self-Ag in the liver and does not necessarily increase the likelihood for autoimmune toxicity.

The enhanced accumulation of TCR gag SHP-1−/− effectors during therapy is Ag-dependent and enhanced in mice rendered lymphopenic

The relative differences in the accumulation of SHP-1−/− compared with SHP-1+/+ effectors in vivo were much more pronounced than what we had initially observed in our in vitro studies. This disparity suggested that environmental factors in vivo might be contributing to the increased short-term accumulation of SHP-1−/− effector cells. Lymphodepletion enhances the antitumor effects of T cells during T cell-based immunotherapy at least in part by increasing serum levels of homeostatic cytokines IL-7 and IL-15 in both mouse tumor models and in patients with cancer (4, 7). Because our adoptive immunotherapy protocol transferred T cells into mice rendered lymphopenic by Cy, we hypothesized that abrogation of SHP-1 might also be enhancing signaling from the available cytokines in vivo such as IL-7 and/or IL-15 (58). We transferred 1 × 10^6 SHP-1+/+ or SHP-1−/− in vitro-expanded effectors into either naive B6 mice (negative control), B6 mice rendered lymphopenic from Cy, B6 mice that received irradiated FBL tumor (Ag), or B6 mice that received irradiated FBL Ag in the context of Cy-induced lymphopenia.

FIGURE 4. SHP-1 abrogation does not impact the formation of memory after immunotherapy, but it does enhance the expansion of effector cells from memory cells in response to Ag. B6 mice were injected with 5 × 10^6 FBL and, after 5 d, received Cy (180 mg/kg) and 6 h later were injected with 5 × 10^6 TCRgag SHP-1+/+, SHP-1+/−, and SHP-1−/− 3dme effectors and IL-2 (10^4 U/mouse/day i.p. for 10 d). (A) At 120 d after T cell transfer, spleens were harvested from recipient mice and stained for CD8Thy1.1 donor cells and analyzed by FACS. The number in the FACS plots indicates the percentage of donor T cells of total mononuclear splenocytes. (B) The percentage of CD8Thy1.1 donor cells in the spleens of recipient mice was determined by FACS (100–120 d after T cell transfer). Each dot represents an individual mouse. (C) The phenotype of persisting cells at 60 and 120 d after transfer was determined by staining splenic cells with Abs to CD8, Thy1.1, and the indicated markers. Histograms are gated on CD8Thy1.1 cells. (D) Cured mice (120 d after T cell therapy) were immunized with L. monocytogenes-gag (3 × 10^7 CFU) and 6 d later, splenocytes were analyzed for the percentage (left panel) and number (right panel) of CD8Thy1.1 T cells. (E) Persisting TCRgag SHP-1+/+, SHP-1+/−, and SHP-1−/− cells isolated from cured mice were purified using Thy1.1 magnetic beads, and aliquots of 1 × 10^6 CD8Thy1.1 cells were transferred into naive B6 recipients. Two days later, recipients were immunized with L. monocytogenes-gag. After 6 d, spleens were harvested and analyzed by FACS for the percentage (E) (representative plots) and number (F) of CD8Thy1.1 cells. (G) Splenocytes from L. monocytogenes-gag–immunized mice in (E) were stained for CD8, Thy1.1, and the indicated Ags to compare the phenotype of expanded effector cells. Histograms are representative mice and are gated on CD8Thy1.1 cells. (H) Splenocytes isolated at 6 d after immunization with L. monocytogenes-gag were restimulated with Ag and the protein transport inhibitor brefeldin A in vitro. After 5 h, cells were stained for CD8, Thy1.1, and for intracellular expression of IFN-γ and TNF-α. Plots are gated on CD8Thy1.1 cells. Representative plots are shown from n = 5. Statistical analysis between the groups was conducted using a one-way ANOVA followed by a Tukey post hoc correction. *p < 0.05, **p < 0.005.
SHP-1+*/ and SHP-1−/− effector cells were not detected 6 d after transfer into naive B6 mice, but they were both detected at similarly low frequencies in the spleen 7 d after transfer into mice rendered lymphopenic by Cy (Fig. 6A). These results suggest that the lymphopenia-dependent accumulation of effector cells in the absence of an antigenic signal, at least in this time frame studied, is not regulated by SHP-1. In contrast, a higher percentage and number of SHP-1−/− effector cells were detected in response to Ag alone compared with SHP-1+/+ T cells, resulting in approximately a 3-fold difference in the number of T cells (Fig. 6A). Ag in a lymphopenic setting amplified the expansion of both SHP-1+/+ and SHP-1−/− T cells, similar to what is observed in a therapeutic setting, and also resulted in a 3-fold increase in the number of SHP-1−/− compared with SHP-1+/+ T cells. Even in the context of Ag and lymphopenia, exogenous IL-2 administration still enhanced proliferation of SHP-1+/+ and SHP-1−/− effector cells (Fig. 6A). To determine whether SHP-1−/− effector cells depended on the homeostatic cytokines IL-7 and IL-15 for the enhanced Ag-dependent expansion in a lymphopenic setting, we transfected effector T cells into Cy-treated, FBL-bearing IL-15−/− mice that received injections of a mouse anti-human mAb specific to human IL-7 that cross-reacts and neutralizes murine IL-7 (M25) (59). Because IL-7 is critical for naive T cell homeostasis (38), we first verified that the anti–IL-7 treatment neutralized the biologically active IL-7 in these experiments by transferring naive, CFSE-labeled Thy1.1+ TCRgag cells into wild-type mice treated with either anti–IL-7 or an isotype control. The administration of anti–IL-7 inhibited the low level of homeostatic proliferation of naive TCRgag cells, as measured by the absence of CFSE dilution by donor T cells, indicating the dose of anti–IL-7 administered was sufficient to neutralize IL-7 in vivo (Fig. 6B). Therefore, we transferred wild-type and SHP-1−/− effector cells into FBL-bearing, Cy-treated IL-15−/− mice that also received anti–IL-7. After 7 d, we determined the percentage of donor cells (CD8+Thy1.1+) in the blood by FACS. The absence of IL-7 and IL-15 reduced the percentage and number of both SHP-1+/+ and SHP-1−/− effector T cells present at day 7 (Fig. 6C, data not shown), resulting in a 3- to 4-fold decrease in the acute expansion of both wild-type and SHP-1−/− cells (Fig. 6D). Thus, similar to wild-type cells, SHP-1−/− T cells also depended on IL-7 and IL-15 homeostatic cytokine signals in vivo for their proliferation and survival, suggesting that abrogation of SHP-1 amplifies in vivo responses to Ag but does not bypass the requirement for cytokine signals for antitumor activity.

**Targeting SHP-1 expression in tumor-reactive T cells by retroviral transduction of SHP-1–specific shRNAs**

Translating these findings from the mouse model to clinical studies will require the ability to reduce SHP-1 expression in effector T cells specific to tumor Ags. One approach to reduce SHP-1 protein expression in tumor-reactive T cells for therapy would be to transduce T cells in vitro with retroviral vectors engineered to express shRNAs targeting the specific protein prior to T cell infusion. To investigate the feasibility, efficiency, and biologic impact of reducing SHP-1 levels by shRNA-mediated gene silencing in T cells, we targeted SHP-1 in TCRgag cells by transducing in vitro-activated TCRgag effector cells with a retroviral construct that expresses a SHP-1 shRNA specific for a conserved sequence in SHP-1. This sequence, when used as a small interfering RNA (siRNA), has previously been shown to reduce specific SHP-1 expression in both human and mouse cells (29, 40, 41). Transfection of EL-4 thymoma cells with this specific siRNA sequence effectively knocked down SHP-1 by ~80% (data not shown). Therefore, we constructed a retroviral vector to constitutively express SHP-1 shRNA and GFP for in vitro sorting and in vivo tracking of transduced effector T cells. Wild-type TCRgag cells were stimulated in vitro with peptide-pulsed splenocytes and IL-2 (20 U/ml). After 2 d, during active cell division, the proliferating wild-type TCRgag cells were retrovirally transduced and the T cells analyzed for GFP expression (Fig. 7A). By gating on the CD8+GFP+ cells, we found that cells transduced with the retrovirus that expressed the SHP-1–specific siRNA had reduced levels of SHP-1 (66% average reduction) as compared with cells transduced with a control vector containing a scramble siRNA sequence (Fig. 7B, right panel). On a per cell basis, some GFP+ T cells had greatly reduced SHP-1 expression whereas others exhibited less reduction of SHP-1.
(Fig. 7B), presumably reflecting influences of integration sites and indicating that this approach does not generate a uniform reduction of SHP-1 protein levels. TCRgag cells transduced with SHP-1 shRNA retrovirus displayed an enhanced ability to lyse FBL tumor in vitro using a standard CFSE-based cytotoxicity assay (Fig. 7C). To compare the ability of transduced TCRgag cells to acutely expand in response to FBL in vivo, we transferred $6 \times 10^4$ SHP-1 shRNA transduced effector cells that had been expanded in vitro for an additional two cycles of stimulation into recipients that were treated with Cy 5 h prior to T cell transfer and

**FIGURE 6.** The enhanced expansion of SHP-1$^{-/-}$ effector T cells in vivo depends on Ag and is enhanced in a lymphopenic setting. (A) To temporarily induce lymphopenia, cohorts of B6 mice were administered Cy (180 mg/kg) with or without irradiated FBL ($1 \times 10^7$) and exogenous IL-2 (10^4 U/mouse/day) as indicated. Treated mice received $1 \times 10^5$ TCRgag 3stim SHP-1+/+ or SHP-1$^{-/-}$ effectors. After 6 d, splenocytes were harvested and the percentage (left panel) and number (right panel) of donor CD8$^+$Thy1.1$^+$ cells were calculated. (B) CFSE-labeled, naive Thy1.1$^+$ TCRgag cells (5 $\times 10^5$) were transferred into wild-type B6 mice treated with the IL-7–depleting mAb M25 (1 mg/mouse every other day i.p.) or an isotype control. After 10 d, splenocytes were harvested, stained for CD8 and Thy1.1, and CFSE dilution of the CD8$^+$Thy1.1$^+$ cells was assessed by FACS. (C) Wild-type and IL-15$^{-/-}$ mice received Cy (180 mg/kg), irradiated FBL (1 $\times 10^7$ cells per mouse) with or without anti-IL-7–depleting Ab (M25), and were administered $1 \times 10^5$ 3stim effectors. The percentage of CD8$^+$Thy1.1$^+$ cells in the spleen 6 d after T cell transfer was determined by FACS. Representative plots are shown (n = 5–8 mice/group). (D) The relative impact of abrogating homeostatic cytokine signals IL-7 and IL-15 in the expansion of 3stim SHP-1+/+ or SHP-1$^{-/-}$ effectors was determined by calculating the fold decrease in the number of donor T cells recovered from recipients that lacked IL-7 and IL-15 signals as compared with the number of effector cells in control mice (the number of donor T cells from isotype treated B6 mice/the number of donor T cells from M25 treated IL-15$^{-/-}$ treated mice). *p < 0.05, **p < 0.005.

**FIGURE 7.** Retroviral transduction of TCRgag cells with SHP-1–specific shRNA reduces SHP-1 expression and improves effector T cell function in response to leukemia. (A) Naive TCRgag cells isolated from wild-type Tg mice were retrovirally transduced with a retroviral vector engineered to express SHP-1–specific shRNA or a control scramble shRNA sequence and GFP under control of the U6 promoter. After 5 d, cells were stained for CD8 and analyzed for expression of GFP. (B) SHP-1 levels were determined by FACS analysis of the gated CD8$^+$GFP$^+$ cells that had been stained intracellularly with a SHP-1–specific mAb. (C) Retrovirally transduced TCRgag CD8$^+$GFP$^+$ 3stim effectors ($6 \times 10^5$) were transferred into recipient mice that received Cy (180 mg/kg) and irradiated FBL (1 $\times 10^7$ cells) prior to transfer. After 7 d, the percentage of CD8$^+$Thy1.1$^+$ donor cells of the total mononuclear blood cells was determined by FACS. Each circle represents an individual mouse. Statistical analysis was performed using a Student t test to compare cell expansion. (D) CFSE-based cytotoxicity assays were performed by incubating retrovirally transduced TCRgag 3stim effectors at a 1:1 ratio with a mixture of FBL (CFSE$^+$) and control tumor EL4 (CFSE$^-$). After 5 h, the ratio of FBL tumor to control tumor was compared by FACS analysis. These data are representative of two independent experiments. **p < 0.005.
irradiated FBL at the time of T cell transfer. There was a 3-fold increase in the percentage of TCRgag cells expressing the targeted shRNA in the blood compared with TCRgag cells transduced with a control retrovirus that expressed a nonspecific scramble siRNA sequence (Fig. 7D). Thus, the retroviral transduction of effector CTL with SHP-1–specific shRNA can improve effector T cell responses to tumors and represents a novel strategy that may improve antitumor activity of effector T cells in humans with established malignancies.

**Discussion**

This study describes the potential value of disrupting the regulatory signaling pathways mediated by SHP-1 as a strategy to improve the efficacy of adoptive T cell therapy of cancer. We used a model of cell-intrinsic SHP-1 deficiency in mature T cells to overcome major limitations of studying T cells derived from mice globally deficient for SHP-1. We found that the abrogation of SHP-1 in tumor-specific, effector T cells significantly improved the efficacy of the T cells in therapy of disseminated leukemia, which in part reflected an increase after transfer in the in vivo expansion and accumulation of short-lived effector T cells. This rapid expansion after transfer of short-lived effector cells derived from the transferred SHP-1–deficient population did not impact the ability of donor T cells to establish a functional long-lived memory T cell population after successful tumor elimination.

A key obstacle to the success of adoptive immunotherapy of cancer is the low level of Ag presentation by tumors. Malignant cells often have defects in Ag processing and presentation (60), including the downregulation or complete loss of MHC molecules (61), resulting in the lack of T cell recognition. We investigated the impact of abrogating SHP-1 in tumor-reactive T cells, in part because of the described role for SHP-1 in serving as a rheostat for regulating Ag signaling in T cells. SHP-1 is rapidly recruited to lipid rafts after TCR binding to low-avidity peptide/MHC complexes, resulting in the rapid dephosphorylation of Lck and the prevention of potential detrimental activation of T cells directed toward low-avidity interactions such as self-Ags (17, 18). After TCR binding to higher avidity ligands, SHP-1 is also recruited to the TCR complex, potentially turning off prolonged responses to Ag (15). Our observation that naive T cells with cell-intrinsic SHP-1 deficiency have enhanced functional avidity, as measured by proliferation in response to a log lower concentration of peptide-loaded targets, suggests that one approach to engineering T cells to respond better to reduced Ag levels would be to ablate SHP-1 expression. These data are consistent with a previous study that demonstrated SHP-1 regulates cell cycle entry after Ag encounter in part by diminishing T cell/APC conjugate formation in vitro (28).

Strategies to enhance T cell avidity and function may overcome obstacles associated with T cell tolerance and dysfunction during adoptive immunotherapy. However, because most tumor Ags are also expressed to some degree in normal tissues, one unintended consequence of increasing T cell function could be immune-mediated toxicity directed at self-tissues (62). The death of a patient shortly after receiving T cells engineered to express a high-affinity chimeric Ag receptor specific to ERBB2 (HER-2/neu), a tumor Ag overexpressed in malignant cells but also expressed at low levels in the lung, was likely the result of T cell recognition of ERBB2 expressed by normal lung cells, resulting in a cascading cytokine storm and multiorgan failure (63). The affinity and signaling capabilities of chimeric Ag receptors, which are a fusion protein composed of an extracellular Fv fragment and often multiple intracellular signaling domains (CD28, 4-IBB, and CD3ζ), are dramatically different from TCRs. The ablation of SHP-1 in the infused effector CD8 T cells that express TCRs specific to the gag self/tumor Ag enhanced tumor immunity without causing autoimmunity when the self-Ag was expressed in the liver. Similarly, we previously observed that abrogation of another negative regulator of TCR signaling, Cbl-b, in tumor-reactive effector T cells also enhanced therapeutic efficacy of adoptive immunotherapy of FBL leukemia without increasing the likelihood for toxicity in recipient mice that expressed the gag tumor Ag as a self-Ag in the liver (14). The lack of autoimmunity cannot be explained by insufficient Ag expression in hepatocytes to be recognized, because naive TCRgag cells transferred into Alb:Gag mice have been shown to directly recognize hepatocytes, although the outcome of this event is tolerance induction or deletion (57, 64). Because hepatocytes are inherently resistant to cytotoxic T cell-mediated killing (65, 66), and the liver may be a more naturally immunosuppressive environment than other organs (67), ablation of SHP-1 might increase autoimmunity if the self/tumor Ag was expressed in tissues other than the liver. However, because autoimmune injury in the liver has been observed in other settings, the liver is not completely resistant to autoimmune damage (68–71). Although additional studies will be required to extrapolate the impact of abrogating SHP-1 during T cell-based therapy in settings in which the self/tumor Ag is expressed on other tissues, the present data demonstrate that toxicity is not an inherent consequence of increasing effector T cell function during adoptive immunotherapy, even when the tumor Ag is a self-protein.

Various laboratories are exploring the impact of inclusion of CD4 T cells in therapeutic efficacy and safety during T cell-based therapy of cancer (72). SHP-1 may regulate various functions in CD4 T cells, and abrogation of SHP-1 has been reported to bias naive CD4 T cells toward a Th2 (73) or Th17 phenotype (74). Abrogating SHP-1 or other negative regulators in CD4 helper T cells would certainly have the potential to further increase the therapeutic activity of CD8 T cells, but it may also potentially increase the likelihood for autoimmune injury. A greater understanding of how abrogation of SHP-1 in CD4 T cells specific to tumor Ags influences the therapeutic efficacy and toxicity could potentially lead to novel strategies that target malignancies.

To model human adoptive immunotherapy protocols, we expanded naive CD8 T cells with Ag and IL-2 for several cycles of stimulation prior to transfer, producing a uniform population of cells that phenotypically resembled short-lived effector cells (CD44hi, CD62Llo, CD122hi, KLRG1hi) independent of SHP-1 expression. Although KLRG-1 has been suggested to be a marker of terminally differentiated short-lived effector cells (SLECs) in mouse models of virus infection (50), we found that KLRG1hi effector T cells can eradicate disseminated tumor. Although most transferred effectors undergo programmed cell death, at least a minor fraction eventually transition to cells with phenotypic and functional characteristics of central memory cells, again independent of SHP-1 expression. Such plasticity of in vitro-expanded CD8 effector cells from apparent differentiated effector cells to memory cells has also been reported in humans and animal models (75, 76). The finding that SHP-1 ablation, which increases the sensitivity of TCR signaling to Ag, also increases the abundance of SLECs without impacting memory precursor cells is not entirely consistent with a proposed model suggesting that less TCR activation favors memory precursor cell survival and formation, whereas a stronger TCR signal favors effector differentiation (77). Potentially SHP-1 deficiency may impact subpopulations of the responding cells differently, such as driving an enhanced proliferative response by the population that differentiates to SLECs. The amount of IFN-γ production induced shortly after T cell activation (20–24 h) has been shown to depend on the TCR signal strength (i.e., the amount of MHC/peptide expressed on the cell surface), and triggering by targets with high
Ag densities led to the development of polyfunctional effector cells in the IFN-γβ clonal progeny (78). SHP-1−/− effector cells were found to produce ~5-fold greater amount of IFN-γ 24 h after TCR stimulation as compared with SHP-1+/+ cells (data not shown), suggesting that abrogation of SHP-1 may result in cells with enhanced effector functions.

We observed a modest but reproducible increase in expression of the transcription factor Eomes in SHP-1−/− effectors shortly after activation, as compared with wild-type cells, which may also contribute to the increased cytolytic activity of SHP-1−/− effectors, as Eomes has been described to be involved in the cytolytic program of both CD4 and CD8 T cells (48, 49). The increase in Eomes in SHP-1−/− cells was evident in T cells that were stimulated with Ag in the presence and absence of exogenous IL-2, suggesting increased Eomes is not a result of greater proliferation but rather the specific lack of SHP-1 after Ag encounter (data not shown). It is surprising that we did not observe any difference in T-bet levels in SHP-1−/− effectors at any time point tested (days 1, 3, 5, and 7 after primary activation, and day 5 after the third in vitro stimulation with Ag; Fig. 2 and data not shown), yet we still observed enhanced IFN-γ production in the absence of SHP-1 at early time points (day 1 after activation). The enhanced cytolytic capacity of SHP-1−/− effector T cells was apparent at high tissue densities and correlated with a modest but significant increase in perforin expression, but it did not correlate with an increase in granzyme A, B, or C expression or Fas and FasL expression. These results suggest that although the increase in perforin expression may contribute to the enhanced lytic capacity of SHP-1−/− effectors, it also remains a likely possibility that the capacity to deliver cytotoxic molecules after TCR signaling is also improved in the absence of SHP-1. SHP-1 interfaces with multiple pathways, including key TCR signaling intermediates Lck (21, 22), Zap70 (23, 24), Vav (25), PI3K (26), and TCRζ (27). Defining precisely how the abrogation of SHP-1 enhances cytolytic activity will require further investigation.

The increase in the generation and expansion of SHP-1−/− effector cells derived initially from the transferred tumor-specific in vitro-generated effector cells and subsequently from the in vivo persisting memory cells are consistent with a recent study from our laboratory showing that SHP-1 limits the accumulation of virus-specific SLECs derived from both naive and memory precursors after acute lymphocytic choriomeningitis virus infection in mice (30). The increase in effector cells at peak time points in response to disseminated tumor in the present study could not be attributed to SHP-1−/− mediated regulation of the differentiation of SLECs from naive precursors, indicating that SHP-1 may also act at the effector T cell stage to limit cell expansion and survival. In both the setting of lymphocytic choriomeningitis virus infection and in the present analysis of response to tumor Ag, SHP-1 was found to regulate secondary responses to Ag similar to primary responses (~3-fold increase in the absence of SHP-1). However, in both settings, memory cells partially deficient in SHP-1 (derived from heterozygous SHP-1+/− mice) expanded better in response to Ag as compared with wild-type T cells, and similar as compared with SHP-1−/− T cells. These results indicate that memory cells are likely more sensitive to changes in SHP-1 levels than are naive T cells. Preliminary studies with therapy by transferring central memory T cells deficient or heterozygous for SHP-1 deficiency suggest that both populations are better than wild-type central memory T cells (data not shown). Thus, the fact that abrogation of SHP-1 enhances the quantity and quality of effector cells derived from distinct cell subsets suggests that targeting SHP-1 may prove useful independent of the CD8 cell subset used for therapy.

Because SHP-1 has been implicated in a variety of cytokine and costimulatory pathways in addition to TCR signaling, including carcinoembryonic Ag cell adhesion molecules (40, 42, 79), B and T lymphocyte attenuator (80), programmed death-1 (81) CD5 (82), IL-10 (83, 84), and TGF-β (85), ablating SHP-1 likely influences how effector cells respond to suppressive cytokines and/or inhibitory receptor signaling that can also influence the survival and accumulation of effector T cells during adoptive immunotherapy. TGF-β has been shown to limit the number of SLECs formed in response to infection in vivo (86), and SHP-1 has been implicated in transmitting inhibitory signals by TGF-β binding, suggesting that differential signaling in response to TGF-β may influence the survival of SLECs following transfer. The fact that we did not observe any difference in the survival or accumulation of SHP-1−/− and SHP-1−/− cells shortly after transfer into lymphopenic hosts in the absence of an antigenic signal indicates that TCR signaling is a necessary component of the differential responses, but does not rule out the contribution of additional pathways. Response to lymphopenic cytokines IL-7 and IL-15 was not impaired in the absence of SHP-1, indicating that the absence of SHP-1 does not perturb these vital pathways that impact T cell activity during adoptive immunotherapy and are also important for memory T cell homeostasis. Although we did not observe an increase in proliferation of SHP-1−/− effectors to Ag and IL-2 in vitro, SHP-1−/− effectors responded better than do wild-type cells in the presence of Ag and exogenous IL-2 in vivo, suggesting that the in vitro conditions do not adequately reflect the extent of the biological impact of SHP-1 ablation. It may be that settings with a high tumor burden, in which effector cells are challenged to function and are being induced to undergo apoptosis or become dysfunctional, provide a more discriminating setting for evaluating the role of SHP-1. Indeed, upregulation of SHP-1 has been observed in dysfunctional T cells isolated from solid tumors in mice, and abrogation of SHP-1 in tumor-infiltrating lymphocytes has been shown to restore T cell lytic function in vitro (29). Preliminary studies in our laboratory using a solid tumor model have further suggested that abrogating SHP-1 may be useful for overcoming some of the obstacles associated with adoptive immunotherapy of solid tumors, such as effector T cell accumulation.

Precisely how much SHP-1 protein levels will need to be reduced to have a beneficial impact on effector T cell activity is not yet defined. Previous studies have transiently reduced SHP-1 protein levels by transient transfection of T cells (29, 40, 41), but we are currently not aware of any studies that have permanently reduced SHP-1 levels in effector T cells. Our observation that retroviral transduction of tumor-reactive T cells with viral vectors engineered to express a SHP-1–specific shRNA can stably knockdown a sufficient amount of SHP-1 protein in wild type T cells to have a biological impact. Because we obtained a broad range of protein levels in cells in which SHP-1 was reduced after retroviral transduction by expression of an shRNA, we postulate that the T cells that had the most levels of SHP-1 protein reduced are the cells responsible for the increased functional activity. Although this study suggests that abrogating SHP-1 for clinical trials should be pursued, translating this strategy would require additional investigation, including the optimization of the retroviral vector and determining whether there are more effective SHP-1 sites to target to achieve more uniform downregulation of SHP-1 protein in all transduced T cells. At present, our laboratory, as well as others, has focused on the genetic modification of T cells to express tumor-reactive Ag receptors (4, 5) to overcome some of the obstacles associated with T cell–based therapy of cancer, such as the expression of only low-affinity TCRs in naturally derived self/tumor-reactive T cells. The abrogation of SHP-1 in effector T cells is a therapeutic strategy that
References


Supplementary Figure 1. Abrogation of SHP-1 protects effector CD8 T cells from Fas-dependent apoptosis. Naïve (left panel) or 3×\textsubscript{stim} SHP-1\textsuperscript{+/-}, SHP-1\textsuperscript{+/-} and SHP-1\textsuperscript{-/-} TCR\textsubscript{gag} effectors (right panel) were cultured with anti-CD95 (clone Jo2, BD Biosciences)+ anti-hamster IgG (BD Biosciences) to cross-link and induce Fas signaling in T cells. After 12h, flow cytometry was used to analyze the percentage of Annexin-V+ 7AAD+ cells of CD8+ T lymphocytes. Representative plots from two independent experiments.
Supplementary Figure 2. Abrogation of SHP-1 in donor tumor-reactive effector CTL does not cause immunopathology in the liver in which the gag tumor antigen is also expressed as a self-antigen in the liver. B6 and Alb:Gag mice with disseminated FBL leukemia were treated with Cy (180 mg/kg), and 6 h later, injected with $5 \times 10^6$ TCR<sub>gag</sub> SHP-1<sup>+/+</sup> or TCR<sub>gag</sub> SHP-1<sup>−/−</sup> 3<sub>stim</sub> effectors. Recipient mice also received daily IL-2 injections ($10^4$ U/ml i.p for 10 days). Twelve days after T cell transfer, livers were isolated, fixed in 10% neutral buffered formalin, embedded in paraffin and stained using Hematoxylin and Eosin for detection of mononuclear inflammatory cells and loss of hepatocytes. Arrowheads depict hepatocytes.