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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

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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-protein. 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 expressed 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.
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 antagonistic 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 TCRζ (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 motheaten 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 gag 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 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±Cre+ 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 (35, 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 CCGCGGATCCTATGGGTTGACG3′). The fluorochrome-conjugated Abs CDSa, Thy1.1, CD44, CD45, CD69, CD62L, and CD127, killer cell lectin-like receptor subfamily G member 1 (KLRG1), CD5, comedosinomin (Eomes), granzyme B, B-het, perforin, IL-2, and IFN-γ were purchased from eBioscience. The Ab to IL-15Rα was purchased from R&D Systems. The Immune Monitoring Core at the Fred Hutchinson Cancer Research Center (Seattle, WA) synthesized the gag-H2-Dd tetramer. Neutralizing Ab to mouse IL-7 (M25) was a gift from Amgen (Seattle, WA). Human recombinant IL-2 (National Institute of Allergy and Infectious Diseases, Division of Acquired Immunodeficiency Syndrome) was used to stimulate cultures.

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 Fix Buffer I (BD Biosciences) at 37°C for 10 min according to the manufacturer’s protocol. Cells were washed once with FACS buffer and permeablized using BD Phosflow Perm Buffer I 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 (1×106) were cultured with irradiated, syngeneic splenocytes (5×106), irradiated FBL leukemia (3×106), and IL-2 (20 U/ml) in 10 ml complete medium (RPMI 1640 supplemented with 2 μM 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 prior to use after complete mixing. CFSE-labeled Tg Tg cells (1×105) were cultured in round-bottom, 96-well plates with 5×105 congenic (Thy1.2+) splenocytes pulsed with titrating concentrations of gag peptide. After 4 d, CFSE dilution of Thy1.1+ CD8+ Tg cells was assessed by flow cytometry. Intracellular cytokine staining was performed by incubating Tg T cells with congenic (Thy1.2+), peptide-pulsed splenocytes in the presence of brefeldin A (BD Biosciences). After 5 h, cells were stained with Abs to CD8, Thy1.1, 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 μM 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+ (FL1) remaining in the well.

Real-time PCR and gene expression analysis

RNA was isolated from 5×10⁶ naive or effector T cells at various time points after Ag 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). Real-time PCR and gene expression analysis was performed using the SuperScript one-step protocol in the ABI Prism 7700 (Applied Biosystems). Gene expression was normalized to the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as fold change over a control condition.
TCTGTGGGTTTGAGGAGG-3'; Runx-3, 5'-TCAAAGGTACACTGTTGG-ATGGA-3'; 5'-AGCTCTGAGGACCTTGGAT-3'; granzyme A, 5'-TTT-TCATCCTGTAAATGGACTA-3'; 5'-GGATCTCCACATCTC-3'; granzyme B, 5'-TGGACCTCATGCTTGTAC-3'; 5'-TGGAGGATT-CATTTTACCAT-3'; granzyme C, 5'-TCTCCCTGACCTCTTGCTT-3'; 5'-GTAGTGGACGAATTGTGCT-3'; T-bet, 5'-GTCCCTATCCCTGTC-CTC-3'; 5'-CTCTGTTGGTTGGTGAAGTT-3'; perforin, 5'-GATGTTGACACCATGGGCAAGA-3'; 5'-GGTTTTATTAACGGCCAAAA-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 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 Alb.Gag mice were injected with 5 × 10⁶ 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, TCRαβ SHP-1+/−, SHP-1+/−, and SHP-1−/− effector cells that had undergone three cycles in vitro was transferred into tumor-bearing mice. Cohorts of recipient mice also received exogenous IL-2 injections (1 × 10⁶ U/mouse/day i.p.). Mice were monitored for tumor burden (ascites formation, splenomegaly, and nodal masses) and euthanized when the detectable tumor mass reached proportions that predictably led to mortality within 24 to 48 h.

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

At 120 d after tumour therapy, splenocytes were harvested from cured mice and sorted for Thy1.1+/CD8+ donor cells. Equivalent numbers of each type of donor cell (1 × 10⁶) were transferred into B6 recipients. Two days later, mice were immunized with an attenuated (ΔlcrA) 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 × 10⁶ 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 × 10⁶ U/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 each B6 mouse, 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 i.p.). IL-2 (104 U/ml/mouse/day i.p.) was given 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'-AAGCGAGCTGACATTGAA-3' (NM_080549.3), a sequence that is homologous between mice and humans and has been successfully targeted in humans (40, 41) and mouse (29). We generated an MigR1 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 TCRαβ effector cells were transduced by incubating effector T cells with virus supernatant and spinning cells for 90 min. 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 risk of 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 naive 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 TCRαβ mice (35). We have previously shown that this approach permits the study of T cell-intrinsic effects of SHP-1 deletion, with nearly all C57 B6 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 (MI) of SHP-1 staining of CD8+ tetramer+ cells demonstrated complete absence of SHP-1 protein in TCRαβ SHP-1−/− cells (both alleles floxed) and a 50% reduction in SHP-1 protein levels in SHP-1+/− cells (one allele floxed) compared with SHP-1+/− cells, confirming gene disruption and loss of SHP-1 protein expression in TCRαβ cells (Fig. 1A). Expression levels of CD44, CD62L, and CD127 of CD8 T cells from TCRαβ SHP-1−/− mice were not distinguishable from wild-type TCRαβ cells, and they were consistent with the cells retaining a naive phenotype despite the loss of the SHP-1 protein (Fig. 1B). The activation markers CD25 and CD69 were not increased on SHP-1−/− T cells, further indicating that ablating SHP-1 in mature CD8 T cells does not result in persistent or intermittent T cell activation.

We previously demonstrated that cell-intrinsic expression of SHP-1 regulates naive T cell activation toward viral Ag (30), and we performed initial studies to verify that SHP-1 deficiency also improves tumor Ag recognition in TCRαβ cells. CFSE-labeled naive TCRαβ SHP-1+/+, SHP-1−/−, and SHP-1−/− T cells (congenic for Thy1.1) were incubated with syngeneic 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). TCRαβ SHP-1−/− cells proliferated in response to a log10 lower concentration of Ag compared with TCRαβ SHP-1+/+ and SHP-1−/− cells (Fig. 1C), indicating that complete abrogation of SHP-1 in naive 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 7 d after activation, SHP-1−/− = 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 naive T cell responses. Naive 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...
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, and SHP-1+/−/− effector cells expressed equivalent and high levels of CD25 (IL-2Rα), IL-15Rα, and downregulated CD127 (IL-7Rα) 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+) 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 pro-survival 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) and control tumor (EL4, CFSElo) remaining in the well by percentage AnnexinV/7AAD. 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 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 × 10^6) 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 effectors 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. (G) A standard CFSE-based cytotoxicity assay as described in (F) was performed using titrating numbers of 3stim effectors. (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 expressed Eomes (left panel) and the MFI of Eomes in CD8+ 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.
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 TCRγγ 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 × 10^6 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 × 10^5 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 3sum effectors (5 × 10^7) 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, KLRG1lo), 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 TCRγγ 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 × 10^6 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-
Enhanced short-term accumulation of TCR gag SHP-1−/− effector cells following Ag encounter. It does regulate the ability of those memory cells to generate acute converted to long-lived memory cells following immunotherapy, but does not impact the ability of in vitro-expanded effector T cells to enhanced in the absence of SHP-1 (Fig. 4H). Thus, SHP-1 ablation of the expanded effector response after immunization was enhanced in the absence of SHP-1.

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 × 10⁶ FBL leukemia. After 5 d when the leukemia had disseminated, mice were injected with Cy (180 mg/kg), followed by 1 × 10⁹ Thy1.1+ TCRgag SHP-1−/−, SHP-1−/+, or SHP-1−/− 3₅₉ responding to L. monocytogenes-gag immunization acutely converted to an effector phenotype (CD62L lo, 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 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 effector T 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−/− effector 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) 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-Ag. 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 × 10⁹) 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 × 10⁹ in vitro-expanded TCRgag SHP-1−/− and SHP-1−/− effector cells 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 autoimmune toxicity. 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, p < 0.05). Thus, while 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−/−.
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+/+, SHP-1+/−, and SHP-1−/− 3_dose effector cells into naive 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 TCR_gag SHP-1+/+, SHP-1+/−, and SHP-1−/− 3_dose 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 CD8+Thy1.1+ donor cells and analyzed by FACS. The number in the FACS plots indicates the percentage of donor T cells of total mononuclear splenocytes. The percentage of CD8+Thy1.1+ 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. (B) 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 CD8+Thy1.1+ cells. (C) Cured mice (120 d after T cell therapy) were immunized with L. monocytogenes-gag (3 × 10^7 CFU) and 6 d later, splenocytes were harvested from 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 of CD8+Thy1.1+ T cells. (E) Persisting TCR_gag 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 CD8+Thy1.1+ 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 (left panel) and number (right panel) of CD8+Thy1.1+ T cells. (F) Persisting TCR_gag 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 CD8+Thy1.1+ 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 (left panel) and number (right panel) of CD8+Thy1.1+ T cells. (G) Splenocytes from L. monocytogenes-gag-immunized mice in (F) 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 CD8+Thy1.1+ 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 CD8+Thy1.1+ 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 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 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 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.
FIGURE 6. The enhanced expansion of SHP-1<sup>−/−</sup> 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 × 10<sup>7</sup>) and exogenous IL-2 (10<sup>4</sup> U/mouse/day) as indicated. Treated mice received 1 × 10<sup>5</sup> TCR<sub>gag</sub> 3<sub>stim</sub> SHP-1+/+ or SHP-1<sup>−/−</sup> effector cells. After 6 d, splenocytes were harvested and the percentage (left panel) and number (right panel) of donor CD8<sup>+</sup>Thy1.1<sup>+</sup> cells were calculated. (B) CFSE-labeled, naive Thy1.1<sup>+</sup> TCR<sub>gag</sub> cells (5 × 10<sup>5</sup>) 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<sup>+</sup>Thy1.1<sup>+</sup> cells was assessed by FACS. (C) Wild-type and IL-15<sup>−/−</sup> mice received Cy (180 mg/kg), irradiated FBL (1 × 10<sup>7</sup> cells per mouse) with or without anti-IL-7-depleting Ab (M25), and were administered 1 × 10<sup>5</sup> 3<sub>stim</sub> effectors. The percentage of CD8<sup>+</sup>Thy1.1<sup>+</sup> 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 3<sub>stim</sub> SHP-1+/+ or SHP-1<sup>−/−</sup> 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<sup>−/−</sup> treated mice). *p < 0.05, **p < 0.005.

FIGURE 7. Retroviral transduction of TCR<sub>gag</sub> cells with SHP-1–specific shRNA reduces SHP-1 expression and improves effector T cell function in response to leukemia. (A) Naive TCR<sub>gag</sub> 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<sup>+</sup> and analyzed for expression of GFP. (B) SHP-1 levels were determined by FACS analysis of the gated CD8<sup>+</sup>GFP<sup>+</sup> T cells that had been stained intracellularly with a SHP-1–specific mAb. (C) Retrovirally transduced TCR<sub>gag</sub> CD8<sup>+</sup>GFP<sup>+</sup> 3<sub>stim</sub> effectors (6 × 10<sup>4</sup>) were transferred into recipient mice that received Cy (180 mg/kg) and irradiated FBL (1 × 10<sup>7</sup> cells) prior to transfer. After 7 d, the percentage of CD8<sup>+</sup>Thy1.1<sup>+</sup> 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 TCR<sub>gag</sub> 3<sub>stim</sub> effectors at a 1:1 ratio with a mixture of FBL (CFSE<sup>+</sup>) and control tumor EL4 (CFSE<sup>−</sup>). 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 TCR<sub>ag</sub> cells expressing the targeted shRNA in the blood compared with TCR<sub>ag</sub> 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 immunemediated 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, 4-1BB, 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 TCR<sub>ag</sub> cells transferred into AlbGag 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 (CD44<sup>hi</sup>, CD62L<sup>lo</sup>, CD127<sup>lo</sup>, KLRI<sup>hi</sup>) 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 KLRG1<sup>hi</sup> 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-γ3 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 tumor 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 carcinomembryonic 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 or 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 knock down 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
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can be used alone or in combination with genetic modification of T cells to express Ag receptors, and our data indicate that this approach has the potential to increase therapeutic activity of the T cells in some malignancies.

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Disclosures
The authors have no financial conflicts of interest.

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Supplementary Figure 1. Abrogation of SHP-1 protects effector CD8 T cells from Fas-dependent apoptosis. Naïve (left panel) or 3_sim SHP-1+/+, SHP-1+/−, and SHP-1−/− TCR_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 x 10^6 TCR_{gag} SHP-1^{+/+} or TCR_{gag} SHP-1^{-/-} 3_{gag}^m 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.