Inhibition of SHP-1 Expands the Repertoire of Antitumor T Cells Available to Respond to Immune Checkpoint Blockade

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ABSTRACT

The presence and activity of CD8+ T cells within the tumor microenvironment are essential for the control of tumor growth. Utilizing B16-F10 melanoma tumors that express altered peptide ligands of chicken ovalbumin, OVA257-264, we measured high- and low-affinity OVA-specific responses following adoptive transfer of OT-I CD8+ T cell into mice subsequently challenged with tumors. T-cell receptor (TCR) affinity positively correlated with the frequency of OT-I tumor-infiltrating lymphocytes (TIL). Differences in TCR affinity inversely corresponded to in vivo tumor growth rate. Blockade of the PD-1 and CTLA-4 checkpoints preferentially increased the frequency and antitumor function of TIL responding to high-affinity antigens, while failing to enhance the antitumor activity of low-affinity T cells. To determine whether lowering the TCR activation threshold could enhance the breadth and magnitude of the antitumor T-cell response, we inhibited Src homology region 2 domain–containing phosphatase 1 (SHP-1) in OT-I T cells prior to tumor antigen exposure. SHP-1 knockdown increased the cytokine-producing potential of high- and low-affinity T cells but failed to enhance control of tumor growth. In contrast, when SHP-1 knockdown of OT-I T cells was combined with immunotherapy, we observed a significant and long-lasting suppression of tumor growth mediated by low-affinity T cells. We conclude that lowering the TCR activation threshold by targeting SHP-1 expands the repertoire of T cells available to respond to conventional checkpoint blockade, leading to enhanced control of tumor growth.

Introduction

Multiple immunotherapeutic approaches are now available to treat melanoma and other cancers, including administration of high-dose cytokines (1–3), checkpoint blockade inhibitors (4–10), adoptive transfer of in vitro–expanded tumor-specific T cells, engineering of T cells, expression of genetically modified or chimeric antigen receptors, and use of oncolytic viruses (11–13). Although T-cell–directed immunotherapies have successfully induced durable antitumor responses in a subset of patients and increased overall survival, many patients continue to be resistant to such approaches. Consequently, efforts are underway to understand mechanisms of resistance and design strategies for expanding both the tumor types and patient pool that can respond to immunotherapy.

T cells limit tumor growth (14, 15). The presence or migration of tumor-infiltrating lymphocytes (TIL) corresponds to responsiveness to tumor immunotherapies such as checkpoint blockade, as well as overall patient survival for multiple tumor types (16, 17). However, even in settings with brisk TIL responses, response to tumor immunotherapy may be variable. Factors that suppress the ability of TIL to eradicate tumor cells may include inefficient T-cell activation, dysregulated cytokine signaling, acquisition of exhausted or anergic states, and the impact of the immunosuppressive tumor microenvironment (TME; ref. 18). The reasons for failure to generate TIL may also vary. Whereas active immunosuppression may prevent activation or migration of antitumor T cells, an absence of mutated neoantigens may also limit generation of high-affinity T-cell responses. Mutation burden corresponds to resistance to checkpoint blockade therapies and patient outcome (19–21). The impact of existing checkpoint blockade therapies on activation and function of low-affinity T cells specific for tumor-associated self-antigens or weakly reactive neoantigens is not fully understood. Enhancing efficacy of checkpoint blockade therapies in patients with ineffective TIL, or lacking TIL altogether, will likely require development of strategies for expanding the repertoire of tumor-reactive T cells.

The role of T-cell receptor (TCR) affinity during an in vivo antitumor response is complex. High-affinity CD8+ T cells may become tolerized once in the TME (22–24). Indeed, continual or prolonged periods of antigen stimulation via the TCR can induce functional exhaustion (25, 26). However, T-cell function may be rescued and enhanced through antibody blockade of T-cell activation checkpoints, most prominently, CTLA-4 and PD-1 (immune checkpoint blockade, ICB; ref. 27). Although T cells in the tumor setting may respond to neoantigens, T cells also respond robustly across a range of affinities to tumor-associated self-antigens. For example, CD8+ T cells specific for the human melanoma antigen, gp100, exhibited a range of antigen affinities with similar antitumor activity (28). In addition, two different TCR transgenic T-cell lines specific for the tissue-restricted TRP-1 antigen which exhibited disparate affinities displayed no significant differences in their ability to control tumor growth (29). Furthermore, CD8+ T cell specific for the human telomerase reverse transcriptase (hTERT) reacting to a range of hTERT altered peptide ligands (APL) demonstrated no optimal affinity at which maximum sensitivity and polyfunctionality occur. Thus, low-affinity T cells may demonstrate antitumor activity. These studies suggest existence of a TCR affinity...
threshold for T-cell activation and also that functional differentiation of activated T cells is not dependent on TCR affinity; a concept we have validated in infectious disease model systems (30, 31).

Efforts are underway to identify additional checkpoints on T-cell activation. However, immunotherapy based on such checkpoints might fail to target previously activated T cells, expand the repertoire of tumor-responsive T cells, or enhance recruitment or increase activation of low-affinity T cells. We hypothesized that targeting the T-cell activation threshold could expand the repertoire of T cells available to respond to ICB.

To address the role of TCR affinity in antitumor recruitment and function, as well as the impact of existing immunotherapies on low-affinity T cells, we utilized a model system in which mouse B16 tumor cells were engineered to express wild-type (WT) chicken ovalbumin (OVA), or OVA with point mutations in the immunodominant epitope recognized by the OT-I TCR. Because these point mutations impair TCR binding, but not MHC binding, the resulting APLs can be used to measure OT-I T-cell responses across a wide range of TCR affinities (38, 39). Using this model system, we found that TCR affinity regulated the antitumor activity of adoptively transferred OT-I T cells. Furthermore, treatment with ICB targeting PD-1 and CTLA-4 selectively enhanced the antitumor activity of high-affinity, but not low-affinity, OT-I. In order to test the impact of strategies aimed at improving the recruitment and activation of low-affinity OT-I, we targeted SHP-1. Although SHP-1 inhibition alone failed to increase the ability of OT-I to control tumor growth expressing either high- or low-affinity OVA variants, it enhanced recruitment and cytokine production by low-affinity T cells. Combined ICB and SHP-1 inhibition enhanced the ability of low-affinity T cells to control tumor growth. Overall, we conclude that targeting SHP-1 expands the repertoire of T cells available to respond to ICB and induces antitumor activity by low-affinity T cells.

**Materials and Methods**

**Mice**

C57BL/6 (6 to 8 weeks old) mice were purchased from Jackson Laboratories. OT-I transgenic mice (on Thy.1+ background) were maintained in our colony at the University of Utah. All mouse experiments were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the University of Utah.

**Tumor cell lines**

Mouse B16-F10 cells were purchased from the American Type Culture Collection in 2014 and grown in DMEM with 10% FBS, penicillin, and streptomycin at 37°C with 5% CO2, and then frozen in liquid nitrogen. For all experiments, cells were recovered from frozen aliquots and cultured for 1 to 2 weeks prior to inoculation of mice. The B16-F10 parental cell line has not been reauthenticated or subjected to mycoplasma testing in the past year. B16 cells expressing OVA or its variants were all derived from the same parental B16-F10 line. All MiR1 retroviral vectors that expressed OVA250-290 or the indicated single amino acid variants (39), and a GFP or mCherry fluorescent reporter under the control of an IRES were transfected into 293T cells, a virus-producing cell line (Polyplus jetPRIME, Polypus Transfection). B16-OVA(APL) cell lines were created by harvesting viral supernatant from retrovirally transfected 293T cells and placing on top of B16-F10 cells in a 6-well plate in the presence of polybrene (1 µg/ml). Transduction of cells with viral supernatant was promoted by spinning at 2,500 rpm and 32°C for 90 minutes. Twenty-four hours after transduction, B16-OVA(APL) cell lines were purified via FACS gated on GFP or mCherry fluorescence.

**Tumor experiments**

C57BL/6 mice received 1 to 2 × 10⁶ OT-I CD8⁺ T cells (unsorted total splenocytes) i.v., followed by 1 × 10⁶ B16-OVA(APL) cells injected into the hind flank 1 day later. B16-OVA(APL) cells were cultured in such a way that they were 60% to 80% confluent in a 25 cm² tissue culture flask at the day of injection. Tumor volume was calculated with the formula \( V = \frac{a \times b^2}{2} \), where \( a \) and \( b \) are tumor length and width (mm), respectively (40). On various days after tumor implantation, mice were euthanized, and analysis of T cells in tumors and draining lymphoid organs was performed. For tumor killing assay experiments, the initial tumor cell implantation consisted of a 1:1 mixture of B16-F10 cells (EV) and a B16-OVA(APL) cell line, each expressing a different fluorescent marker (GFP or mCherry).

**Checkpoint blockade**

Mice were treated at 7 and 10 days after tumor implantation. Each mouse in the treatment group received an i.p. injection of anti–PD-1 (250 µg/dose, clone RMP1-14), anti–PD-L1 (250 µg/dose, clone 10F.9G2), and anti–CTLA-4 (100 µg/dose, clone 9H10; BioXCell) as well as anti–PD-L1 (250 µg/dose, clone 10F.9G2) and anti–CTLA-4 (400 µg/dose, clone 9H10; BioXCell) as described previously (41).
Antibodies
The following fluorophore-conjugated antibodies were used for flow cytometry: anti-CD8 (53-6.7), anti-Thy1.1 (OX-7), anti-Vα2 (B20.1), anti-CD45 (30-F11), anti-CXCR3 (CXCR3-173), anti-IFNγ (XMG1.2), anti-IL2 (JES6-5H4), anti-TNFα (MP6-XT22), anti-CD28 (4B10; Biolegend); anti-CD27 (LG.7F9), anti-LAG3 (C9B7W), anti-granzyme B (Ngzb), anti-Eomes (Dan11mag; Thermo Fisher); anti-pCD3ζ (pY142) (K25-407.69), anti-CXCR5 (CXCR5-1). SHP-1 retroviral knockdown
Retroviral vectors (pMig-R1) were used to express shRNA KD constructs specific for SHP-1 (SHP-1 KD) as previously described (42). Vectors that expressed this shRNA construct utilized a human miRNA (mir-30) flanking sequence allowing for optimal expression and processing of siRNA (43). KD was confirmed in an EL-4 thymoma cell line. KD in primary OT-I CD8+ T cells was accomplished by transducing OT-1 bone marrow with SHP-1 KD or EV retrovirus and transplanting into irradiated Rag−/− mice. After reconstitution (8–10 weeks later), OT-I CD8+ T cells (GFP+ and GFP-) were then isolated from the spleen and transferred into B6 recipient mice that received B16-OVA(APL) cells 1 day later (31).

Statistical analysis
Graphical representation of data along with statistical analysis was performed using Prism (Graphpad v8.2.1) software. Experimental replicates are denoted in figure legends. Statistical analyses were performed using an unpaired two-tailed t test. Significant P values

Figure 1.
Antitumor responses are dominated by high-affinity CD8+ T cells. A, Line graph indicates the growth of B16-OVA(N4) or B16-OVA(V4) tumors in the presence or absence of OT-I CD8+ T cells. The bar graph depicts the final tumor diameter at day 15 after implantation. B, The line graph indicates the growth kinetics of B16-OVA(APL) tumors in B6 mice that received OT-I CD8+ T cells. The bar graph shows the final tumor diameters for all six different OVA(APL)s. C, Representative flow plots show the gating scheme used to determine the frequency of OT-I CD8+ T cells (CD45+ Thy1.1+ Vα2+) within the tumor. D, Bar graph indicates the frequency of OT-I cells within the CD45+ cell population in B16-OVA(APL) tumors 14 days after implantation. E, Bar graphs show the expression of PD-1, CXCR3, and Granzyme B (GzB) in either frequency or mean fluorescence intensity (MFI) via flow cytometry of OT-I CD8+ T cells within the tumor. F, Bar graphs indicate the frequency of single- or multi-cytokine-producing OT-I T cells after ex vivo restimulation with corresponding OVA(APL) peptide. Error bars, SD. Values that are not detectable above background are labeled “ND.” Statistical significance was determined by an unpaired t test: *, P < 0.05 and **, P < 0.01 (n = 5–8 mice per group, representative of two independent experiments).
are marked with ".", "P < 0.05; ".", "P < 0.01; ".", "P < 0.001; and ".", "P < 0.0001.

**Results**

Tumor antigen affinity affects CD8\(^{+}\) T-cell tumor trafficking and controls tumor growth rate

In order to establish a system for tracking high- and low-affinity T-cell responses to antigens expressed by tumor cells, we employed the previously characterized APLs of the immunodominant H-2K\(^b\)-restricted epitope of chicken ovalbumin, OVA\(_{257-264}\). APLs of this epitope are recognized by OT-I TCR transgenic T cells across an approximately 700-fold range of TCR affinities (38, 39). Because single amino acid substitutions affect TCR contact but not MHC binding for each peptide, high- and low-affinity OT-I responses to each peptide are directly comparable. Previous work utilizing these APLs found that OT-I T cells respond to very low-affinity APLs in an acute bacterial infection model, although the magnitude and kinetics of the response was altered (39). The OVA\(_{257-264}\) APLs we assessed were in order of decreasing affinity for the OT-I transgenic TCR: N4 (WT), A2 (~50% lower affinity as compared with N4), Y3 (~75% lower affinity), Q4 (~88% lower affinity), T4 (~98.75% lower affinity), and V4 (~99.8% lower affinity). OVA constructs expressing each of the OVA APLs were individually retrovirally transfected into B16-F10 melanoma cells. Due to the presence of a GFP reporter, antigen expression was normalized by FACS sorting based on fluorescence intensity.

To determine the response of high- and low-affinity T cells to B16 tumor growth, B6 mice received \(1 \times 10^5\) naive OT-I CD8\(^{+}\) T cells 1 day before being subcutaneously inoculated with \(1 \times 10^6\) B16 cells expressing WT OVA or an OVA APL in the hind flank. Tumors expressing WT OVA were effectively controlled by OT-I T cells beginning at day 7. Control of tumor growth required the presence of OT-I T cells, as mice that did not receive OT-I T cells failed to slow the rate of tumor growth regardless of tumor affinity (Fig. 1A).

Although mice receiving OT-I cells controlled the growth of N4 (WT) tumors, B6 mice that were not given OT-I cells demonstrated no observable difference in basal tumorigenicity of N4 (WT) versus V4 (low-affinity) tumors (Fig. 1A), suggesting that endogenous CD8\(^{+}\) T-cell responses to OVA or its antigens were not sufficient to delay tumor growth. OT-I affinity for OVA APLs inversely corresponded to...
the rate of tumor growth starting at day 7, as well as tumor size at day 15 (Fig. 1B). These results show that OT-I affinity for tumor antigen corresponds to the ability of CD8$^+$ T cells to control tumor growth in vivo.

The recruitment of TIL OT-I T cells also correlated with TCR affinity (Fig. 1C). Although the high-affinity ligands N4 and A2 readily induced OT-I TIL, low-affinity ligands Y3 and Q4 recruited reduced frequencies of OT-I TIL (Fig. 1C and D). Very low-affinity ligands (T4, V4) failed to induce OT-I infiltration into the tumor (Fig. 1C and D). The number of OT-I T cells recruited by the low-affinity antigen, Q4, was low, and the phenotypes displayed by OT-I T cells specific for this APL are uncertain. Determination for positive staining was ascertained using a negative control B16 that did not express OVA or any of its variants. TCR affinity for tumor antigen correlated with surface expression of CXCR3, a chemokine receptor expressed by activated TILs in melanoma (44). Higher affinity interactions resulted in increased expression. Despite differences in infiltration, OT-I T cells did not demonstrate TCR affinity–dependent differences in expression of PD-1 and granzyme B (Fig. 1E) or differences in frequency of OT-I TIL–expressing IFN$\gamma$, TNF$\alpha$, or IL2 (Fig. 1F).

To confirm that control of tumor growth was antigen specific, we transduced B16-F10 cells with retroviruses generated using an empty expression vector with a mCherry reporter (B16-EV). We then coinoculated those cells in a 1:1 ratio with B16-F10 expressing an empty vector (GFP$^+$) or an OVA APL (N4, A2, Y3, Q4) (GFP$^+$) into the hind flank of B6 mice. OT-I T cells selectively controlled the growth of B16-EV/N4 coimplanted tumors beginning after day 6, as compared with B16-EV/EV tumors alone (Supplementary Fig. S2A). By day 7, OT-I T cells only controlled growth of tumors expressing high-affinity antigen (N4). Tumors expressing lower-affinity antigens (A2, Y3, Q4) were not controlled by the presence of OT-I (Supplementary Fig. S2B and S2C). By day 14, however, OT-I T cells were able to delay the growth of B16 cells expressing lower-affinity antigens, and EV B16 cells made up 90% to 95% of all tumor cells (Supplementary Fig. S2B). These experiments show targeted tumor cell elimination by OT-I T cells that is TCR affinity dependent and occurs in a temporal manner. Our findings confirm that TCR affinity for tumor antigen guides development of TIL responses during B16 tumor growth.

**ICB preferentially rescues high-affinity T cells**

We next sought to test whether ICB enhances the antitumor activity of high-affinity T cells only or additionally broadens the antitumor T-cell response by enhancing the recruitment and effector response of low-affinity T cells. A previous study employed a model of adoptive cell therapy combined with peptide vaccination to conclude that PD-1 blockade could rescue low-affinity T-cell responses (45). However, an in-depth analysis of the impact of ICB on low- and high-affinity T cells responding de novo without additional manipulation has not been performed. We adoptively transferred OT-I CD8$^+$ T cells into B6 mice that were subcutaneously inoculated with B16 cells expressing WT OVA (N4) or an OVA APL (A2, Y3, Q4, T4, V4) 1 day later. Although previous treatment approaches have initiated ICB at day 3 after
inoculation, we wished to allow sufficient time for establishment of the effector T-cell response prior to treatment. Therefore, we delayed our ICB regimen until 7 and 10 days after tumor cell transplantation using an antibody cocktail consisting of anti–PD-1, anti–PD-L1, and anti–CTLA-4. Following treatment, only OT-Is responding to WT OVA significantly limited tumor growth, as compared with untreated controls (Fig. 2A). ICB induced a significant increase in the frequency of OT-I TIL in response to tumors expressing WT OVA (N4), but not to tumors expressing lower-affinity APLs (A2, Y3, Q4; Fig. 2B). Furthermore, ICB treatment of mice inoculated with tumors expressing very low-affinity antigens (T4, V4) failed to induce recruitment of OT-I TIL (Fig. 2B).

Despite their inability to efficiently control tumor growth, a number of functional and phenotypic changes to low-affinity T cells were observed following ICB. These included increased expression of CXCR5 by both high- and low-affinity OT-I (Fig. 3A and B), a marker associated with an ICB response. In addition, low-affinity T cells responded to ICB by increasing expression of Granzyme B, although high-affinity T cells decreased expression of CD27 (Fig. 3A and B), both associated with increased effector differentiation. However, OT-I T cells did not alter their expression of PD-1, and cytokine production following restimulation was unchanged by ICB (Fig. 3B and C). Furthermore, OT-I presence and activity within the draining lymph nodes was not significantly altered upon ICB (Supplementary Fig. S3). From these results, we concluded that although ICB has some effects on low-affinity T cells, it only enhanced control of tumor growth in the presence of high-affinity T cells. ICB failed to expand the repertoire of T cells responding to the tumor, as very low-affinity T cells that initially failed to become activated (T4, V4) were nonresponsive to ICB.

Expanding the TCR Repertoire during ICB by SHP-1 Inhibition

Figure 4. Inhibition of SHP-1 in antitumor CD8+ T cells expands available repertoire for low-affinity tumor antigen. We generated OT-I bone marrow chimeras expressing an SHP-1-specific shRNA, along with a GFP reporter. One day before tumor cell implantation, OT-I T cells were adoptively transferred into naive B6 recipient mice. GFP+ (SHP-1 KD) and GFP+ (nontransduced, SHP-1 WT) OT-I T cells in the tumor were analyzed. A, Line graphs indicate the growth kinetics of the different B16-OVA(APL) tumors in the presence of SHP-1 KD (dashed) or WT (solid) OT-I CD8+ T cells. B, Representative flow plots show the frequency of GFP+ (SHP-1 KD) compared with GFP+ (SHP-1 WT) OT-I T cells within the OT-I CD8+ T-cell population of a single tumor at time of harvest, including the frequency of the OT-I population upon initiation of the experiment (d0). Bar graph indicates the ratio of SHP-1 KD to WT OT-I cells found in the tumor at day 14 after implantation. C, Bar graph shows the percentage of OT-Is within the CD45+ cell population SHP-1 KD OT-I T cells (dashed) compared with WT counterparts (filled) within the same tumor. Frequencies are normalized to input of KD:WT OT-Is at time of adoptive transfer. Error bars, SD. Values that are not detectable above background are labeled “ND.” Statistical significance was determined by comparing the area under the tumor growth curve (A) or group mean (B and C) using an unpaired t test (n = 5–8 mice per group, representative of two independent experiments). *, P < 0.05.
SHP-1 controls antitumor response of low-affinity T cells

Due to their ability to influence T-cell activation, differentiation, and function, protein tyrosine phosphatases that regulate intracellular signaling within T cells are an attractive target for improving antitumor activity. SHP-1 phosphatase modulates TCR-mediated activation threshold and signal strength. We have previously reported that SHP-1 regulates TCR-dependent effector and memory T-cell differentiation (31). Therefore, we hypothesized that modulation of SHP-1 would enhance the ability of low-affinity T cells to join the antitumor response. In support of this hypothesis, SHP-1 inhibition promotes antitumor immunity in some experimental settings (46). However, the mechanisms underlying protection are unclear, and the role of SHP-1 has largely been defined in high-affinity T cells. Complete abrogation of SHP-1 activity through genetic mutation impairs T-cell selection in the thymus (47–49). Therefore, we pursued a targeted approach to partially inhibit SHP-1 activity in OT-I T cells. We generated bone marrow chimeras by transducing OT-I bone marrow with SHP-1 shRNA retroviral vectors (SHP-1 KD), or an empty vector (EV) control, and transplanting into irradiated Rag−/− recipients. We have previously used this method to achieve approximately 70% knockdown (KD) of SHP-1 expression (31). Eight to 10 weeks later, GFP+ (transduced) and GFP− (nontransduced) OT-I T cells were isolated from the spleen and adoptively transferred into recipient B6 mice that received B16-OVA(APL) cells 1 day later. We compared OT-I T cells with decreased SHP-1 (GFP+) with those with normal amounts (GFP−) in the same mouse, as well as OT-I cells expressing an EV in separate control mice.

SHP-1 KD OT-I T cells did not limit tumor growth compared with WT counterparts, regardless of the presence of a high- or low-affinity epitope (Fig. 4A). We additionally compared recruitment of SHP-1 KD and WT OT-I with the tumor in the same mouse by measuring their relative ratio as compared with the initial ratio at the time of OT-I transfer. Although the ratio of KD to WT OT-I remained unchanged for high-affinity OT-I responses, the low-affinity response to Q4 favored SHP-1 KD OT-I (Fig. 4B and C). However, SHP-1 KD failed to induce a response to low-affinity antigens (T4, V4), as these tumors failed to recruit either WT or SHP-1 KD OT-I (Fig. 4C). SHP-1 KD resulted in a significant increase in frequency of OT-I that made IFNγ, TNFα, and IL-2 following activation by both high-affinity (N4) and low-affinity (A2, Y3, Q4) tumors (Fig. 5A and B). These differences were measured in draining lymph nodes as well but not observed in the EV control tumors (Supplementary Fig. S4).
Although no differences in CXCR5 or Granzyme B expression were observed, there were significant increases in the markers CD27 and PD-1 (Fig. 5C), which have been linked to increased TCR-stimulated proliferation of T cells in humans (50–52). There was also a significant difference in TCR signal duration observed during in vitro stimulations, as knockdown of SHP-1 caused an increase in CD3ζ phosphorylation over a sustained period of time across a range of TCR affinities (Supplementary Fig. S4). Overall, these findings demonstrate that SHP-1 KD functionally enhances low-affinity T cells, but with limited therapeutic benefit.

Combining SHP-1 knockdown with ICB enables control of tumor growth by low-affinity T cells

Because SHP-1 KD increased the recruitment and effector function of low-affinity OT-1, we hypothesized that limiting SHP-1 activity would expand the antitumor function of low-affinity T cells following ICB. Although our previous results found that ICB alone only limited tumor growth in the presence of high-affinity OT-1 (N4; Fig. 2), combined ICB and SHP-1 KD resulted in a significant delay in tumor growth in the presence of low-affinity OT-1 (A2, Y3, Q4; Fig. 6A and B). The delay in tumor growth was rapid and durable, resulting in a significant decrease in tumor mass size in the week following treatment (Fig. 6B) and lasting at least 20 days (Fig. 6A). By day 20, the combination of SHP-1 knockdown and checkpoint blockade therapy resulted in no observable tumor for the WT (N4: 6/10) and the lower-affinity variants (A2: 5/10; Y3: 4/10; Q4: 3/10). Tumor eradication was not observed following challenge with lower-affinity tumors in other treatment settings. The reduced tumor growth could not be fully explained by an increase in OT-1 recruitment to the tumor, as combined therapy did not result in an increase in OT-1 frequency in response to the lower-affinity OVA-APLs (Fig. 7A). However, an increase in CXCR3 expression was measured following combined treatment across all affinities (Fig. 7A), suggesting an increase in T-cell activation and antitumor activity of the OT-1 cells in response to increased TCR signaling and PD-1 blockade (53). To explore alternative mechanisms of antitumor activity mediated by low-affinity
T cells, we measured endogenous CD8\(^+\) T-cell responses to WT OVA (N4) or OVA APLs (A2, Y3, Q4). For lower antigen affinities (Y3 and Q4), an increase in the endogenous antitumor response was observed, as measured by endogenous CD8\(^+\) T-cell frequency, CXCR3 expression, and Granzyme B production (Fig. 7B). This phenotype was further supported by the frequency of endogenous CD8\(^+\) T cells (Thy1.1\(^+\)) within the CD45\(^+\) population in the tumor and expression of CXCR3 and Granzyme B (GzB) on endogenous CD8\(^+\) T cells with (red dashed) or without (black dashed) ICB. MFI, mean fluorescence intensity. C, Bar graphs indicate the frequency of IFN\(_{\gamma}\)-producing endogenous CD8\(^+\) T cells after ex vivo restimulation with corresponding OVA(APL) peptide. Error bars, SD, and statistical significance was determined by an unpaired t test (n = 10–15 mice per group, representative of two independent experiments). *, P < 0.05; **, P < 0.01; ***, P < 0.001; and ****, P < 0.0001. Endg, endogenous.

**Discussion**

The results we present in this study demonstrate that inhibition of SHP-1 potentiates the antitumor activity of low-affinity T cells responding to tumor antigen, particularly in combination with blockade of PD-1 and CTLA-4. Our findings clarify the role of TCR affinity in conferring on antitumor CD8\(^+\) T cells the ability to traffic to and control growth of solid tumors. Although TCR affinity enforced a threshold for efficient activation of antimelanoma T cells, as well as their frequency within the tumor, low-affinity T cells demonstrated profound therapeutic potential. Altering the activation threshold of low-affinity T cells via inhibition of SHP-1, combined with ICB, demonstrated that low-affinity T cells can control tumor growth. The mechanism by which SHP-1 knockdown enhances the antitumor function of low-affinity T cells remains a topic of future investigation. Although our results show an increase in TCR signal strength following SHP-1 inhibition, we also observe an increase in IFN\(_{\gamma}\) production that may not be related to the role of SHP-1 in regulation T-cell activation and effector function.
Low-affinity T cells may represent a source of antitumor activity during natural tumor growth, as supported by our finding that antitumor responses induced by ICB are dominated by high-affinity T cells. The induction of high-affinity TCRs for either tumor-associated self-antigens or neoantigens presents the risk of off-target effects that may lead to autoimmune activity. One study that induced tumor antigen expression in off-target organs determined that low-affinity T-cell interactions avoid concomitant autoimmunogenic in an ovarian carcinoma model (55). Other studies observed no differences in the antitumor capability of high-affinity and moderate-affinity antitumor TCRs (28, 29, 56). High-affinity T cells may be more prone to increased checkpoint molecule expression and the development of functional exhaustion (24). In sum, we argue that moderate-to-low-affinity TCRs are present less danger of off-target toxicity.

Cytolytic antitumor activity is mediated by CD8⁺ T cells, and the frequency of CD8⁺ T cells in tumors corresponds to their ability to control tumor growth. Our findings show that ICB effectiveness corresponds to an increase in the frequency of high-affinity T cells in the tumor. In contrast, low-affinity T cells rescued by SHP-1 inhibition may utilize distinct mechanisms for controlling tumor growth. One possibility is that low-affinity TILs reshape the TME to promote antitumor immunity. Two pieces of evidence in support of that are the increased IFNγ production by TIL after SHP-1 knockdown and the increase in the frequency of endogenous CD8⁺ T cells responding to OVA after combined ICB and SHP-1 knockdown. These results may relate to a prior study that found that cytotoxic and cytokine-dependent antitumor responses could be uncoupled in a TCR-affinity-dependent manner (57). Future studies are needed to elucidate the mechanisms by which SHP-1-deficient T cells mediate their antitumor function.

SHP-1-regulated TCR signal strength plays a role in the functional differentiation of T cells (31). For example, there is an optimal TCR affinity for tumor antigen that generates intense and sustained TCR signals within NY-ESO-1-specific CD8⁺ T-cells, with a role for SHP-1 in determining signal strength (35). Although in vitro studies have shown that the phosphatases SHP-1 and SHP-2 have overlapping specificities, other studies have indicated that they preferentially colocalize with the TCR and PD-1, respectively. Therefore, in vivo SHP-1 and SHP-2 appear to act in distinct and separate pathways (36, 37, 58, 59). SHP-1 and PD-1 inhibit T-cell activation independently, with PD-1 (and possibly SHP-2) preferentially inhibiting high-affinity T-cells and SHP-1 limiting activation incrementally as TCR affinity increases (11). Deficiency in SHP-1 confers resistance to Treg suppression in both in vitro and in vivo settings (34). Previous work in a leukemic model supports the rationale for targeting SHP-1 activity in antitumor T cells (46).

Although we demonstrate that ICB preferentially rescues high-affinity antitumor responses, ICB has been previously shown to increase the antitumor activity of both high- and low-affinity TILs in a B16-OVA melanoma model (45). However, that study utilized a model of therapeutic cell transfer, suggesting that ICB may target low-affinity T cells if T-cell activation thresholds are lowered or bypassed. This may correspond to our finding that SHP-1 inhibition, which lowers TCR activation threshold, combines with ICB to enhance the antitumor function of low-affinity T cells. In fact, there are likely a number of activation thresholds that must be overcome in order to efficiently activate and recruit antitumor CD8⁺ T cells, including thresholds for activation, migration to the tumor, and antitumor effector functions. In an adoptive cell therapy model, activation of OT-1 T cells before adoptive transfer into a tumor-bearing mouse was necessary for antitumor activity (60), supporting a connection between proper T-cell activation and effector function within the tumor. Our current study, focused on the role of SHP-1 inhibition in T cells activated by in vivo tumor growth, suggests SHP-1 may prove a viable target in settings of adoptive cell therapy or CAR T-cell therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: J.P. Snook, A.J. Soedel, H.A. Ekiz, M.A. Williams
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References


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