PD-1/SHP-2 Inhibits Tc1/Th1 Phenotypic Responses and the Activation of T Cells in the Tumor Microenvironment

Jing Li1, Hyun-Bae Jie2, Yu Lei2, Neil Gildener-Leapman2, Sumita Trivedi2, Tony Green3, Lawrence P. Kane4, and Robert L. Ferris2,4,5

Abstract

Immune rejection of tumors is mediated by IFNγ production and T-cell cytolytic activity. These processes are impeded by PD-1, a coinhibitory molecule expressed on T cells that is elevated in tumor-infiltrating lymphocytes (TIL). PD-1 elevation may reflect T-cell exhaustion marked by decreased proliferation, production of type I cytokines, and poor cytolytic activity. Although anti–PD-1 antibodies enhance IFNγ secretion after stimulation of the T-cell receptor (TCR), the mechanistic link between PD-1 and its effects on T-cell help (Tc1/Th1 skewing) remains unclear. In prospectively collected cancer tissues, we found that TIL exhibited dampened Tc1/Th1 skewing and activation compared with peripheral blood lymphocytes (PBL). When PD-1 bound its ligand PD-L1, we observed a marked suppression of critical TCR target genes and Th1 cytokines. Conversely, PD-1 blockade reversed these suppressive effects of PD-1-PD-L1 ligation. We also found that the TCR-regulated phosphatase SHP-2 was expressed higher in TIL than in PBL, tightly correlating with PD-1 expression and negative regulation of TCR target genes. Overall, these results defined a PD-1/SHP-2/STAT1/T-bet signaling axis mediating the suppressive effects of PD-1 on Th1 immunity at tumor sites. Our findings argue that PD-1 or SHP-2 blockade will be sufficient to restore robust Th1 immunity and T-cell activation and thereby reverse immunosuppression in the tumor microenvironment.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

H.-B. Jie and Y. Lei contributed equally to this article.

Corresponding Author: Robert L. Ferris, University of Pittsburgh Cancer Immunology Program, Hillman Cancer Center Research Pavilion, 5117 Centre Avenue, Room 2,26, Pittsburgh, PA 15237-1863. Phone: 412-623-0327; Fax: 412-623-4840; E-mail: ferrisrl@upmc.edu

doi: 10.1158/0008-5472.CAN-14-1215

©2014 American Association for Cancer Research.
Patients and Methods

Patients and specimens

Peripheral blood samples and fresh tumor specimens were obtained from 41 patients with HNSCC. All patients were seen in the Department of Otolaryngology at the University of Pittsburgh Medical Center, and all subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh (IRB# 99-06; Pittsburgh, PA). The clinicopathological features of the patients with HNSCC in this study are shown in Table 1. The patient cohort included 7 females and 34 males with a mean age of 58 years (range: 26–74 years).

Collection of peripheral blood mononuclear cells and tumor-infiltrating lymphocytes

Venous blood from patients with HNSCC was drawn into heparinized tubes and centrifuged on Ficoll–Hypaque gradients (GE Healthcare Life Sciences). Peripheral blood mononuclear cells (PBMC) were recovered, washed in RPMI-1640 medium (Sigma), and either used immediately for experiments or resuspended in freezing media containing 10% DMSO, transferred to Mr. Frosty containers (Thermo Scientific), and stored at −80°C until flow-cytometric analysis. For TIL isolation, fresh tumors from patients with HNSCC were minced into small pieces manually or using a gentleMACS Dissociator (Miltenyi Biotec), then transferred to 70-μm cell strainers (BD) and mechanically separated using the plunger of a 5-ml syringe. The cells passing through the cell strainer were collected and subjected to Ficoll–Hypaque gradient centrifugation. After centrifugation, mononuclear cells were recovered and stored at −80°C until flow-cytometric analysis or immediately used for experiments.

Antibodies and flow cytometry

The following anti-human antibodies were used for staining: CD3-Alexa Fluor 700, FOXP3-PerCP/Cy5.5, phospho-STAT1 (pY701)-PE, phospho-STAT4 (pY693)-Alexa Fluor 488, and T-bet-BV711 purchased from BD Biosciences, CD3-PE-Cy7, PD-1-PerCP/Cy5.5, and CD25-PE-Cy7 purchased from Biolegend, CD8-PE-TR and CD4-PE-TR purchased from Life Technologies, PD-1-APC (Clone: MIH-4, eBioscience), phospho-S6 (Ser235/236)-Alexa Fluor 488 (Cell Signaling Technology), SH-PTP2 (C-18; Santa Cruz Biotechnology), and APC-conjugated F(ab’2) fragment goat anti-rabbit IgG (Jackson ImmunoResearch). Intracellular staining of FOXP3, SHP-2, p-STAT1, T-bet, p-STAT4, and p-S6 was performed as follows: PBMCs or TILs were stained with surface marker antibodies, fixed with fixation/permeabilization buffer (eBioscience), washed, and stained for intracellular antigens in 1× permeabilization buffer. Cells were analyzed on an LSRFortessa (BD) or CyAn (Dako) flow cytometer, and data analyzed using FlowJo (Tree Star) or Summit V4.3 software (Dako), respectively. The acquisition and analysis gates were restricted to the lymphocyte gate based on characteristic properties of the cells in the forward and side scatter. Dead cells were excluded on the basis of viability dye staining (Zombie Aqua Fixable Viability Dye, Biolegend).

Immunohistochemistry

Formalin-fixed paraffin-embedded tissue sections were deparaffinized and dehydrated in xylene and graded ethanol solutions. Antigen retrieval was conducted in Tris-EDTA buffer. Immunoperoxidase stains were performed according to a standard protocol on the Ventana Benchmark Ultra platform. PD-L1 antibody was provided by Dr. Gordon Freeman at the Dana Farber Cancer Institute (Boston, MA). PD-1 was stained using a mAb NAT105 at 1:500 titration. IFNγ was purchased from Abcam and incubated at 1:500 titration. Staining was interpreted by an oral and maxillofacial pathologist. Both the intensity and percentage of area of staining were evaluated. Representative pictures of matching areas were taken at ×400.

Restimulation of TIL using anti-CD3/-CD28/hlgG1 or anti-CD3/-CD28/PD-L1 beads

LEAF purified anti-human CD3 (clone UCHT1, Biolegend), LEAF purified anti-human CD28 (clone CD28.2, Biolegend) plus PD-L1-hlgG1Fc fusion protein (R&D Systems) or control human IgG1 (Southern Biotech) was covalently coupled to Dynabeads M-450 Epoxy beads according to the manufacturer’s protocol (Life Technologies). We kept constant the total amount of protein at 5 μg per 10⁷ beads as previously described (20). Generally, 10⁷ beads were coated with 1 μg of anti-CD3 (20% of total protein), 1 μg of anti-CD28, and 60% of either PD-L1-hlgG1Fc fusion protein or control human IgG1. Covalent coupling of the proteins to the beads was performed in 0.1 mol/L sodium phosphate buffer for 24 hours at room temperature with gentle tilting and rotation.

TILs were freshly isolated from tumor specimens and subjected to restimulation experiments. TILs were cultured with beads at a fixed cell:bead ratio of 1:10. Briefly, 0.5 × 10⁶ TILs were plated in a 96-well U-bottom tissue culture plate with beads in 200 μL RPMI-1640 complete media in the presence of 100 μg/mL anti-CD3 (BMS-936558) or hlgG4 isotype control provided by Bristol–Myers–Squibb, or 50 μmol/L fumaric acid (6) as indicated. After 48-hour incubation at 37°C with 5% CO₂, supernatants were collected and cells were stained and subjected to flow-cytometric analysis.

Western blot analysis

Western blot analysis was performed with phospho-SPH-2 (Tyr580) antibody (Cell Signaling Technology), SH-PTP2 Antibody (C-18; Santa Cruz Biotechnology), and monoclonal anti-β-actin antibody (Sigma).
Luminex assay

TIL culture supernatant levels of IFN-γ, TNF-α, IL2, IL4, IL5, and IL10 were tested using a human magnetic cytokine/chemokine panel 6-plex kit (Millipore) and analyzed by the UPCI Luminex Core Facility.

Statistical analysis

Averages were calculated as means. For nonparametric distribution of samples, P values were calculated by Wilcoxon–Mann–Whitney tests using GraphPad Prism (GraphPad). P values of <0.05 were considered to be significant. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

TILs have dampened Tc1/Th1 phenotypic responses and activation status compared with PBL

To determine the status of Tc1/Th1 activation of T cells in the tumor microenvironment, we analyzed expression of p-STAT1, T-bet, and p-STAT4 in baseline (Fig. 1A and B, P-STAT1: P = 0.005; T-bet: P = 0.005, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet expression at baseline (Fig. 1A and B, p-STAT1: P = 0.005; T-bet: P = 0.005, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 1C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL, which also correlates with deficient expression of perforin in CD8+ TIL (unpublished data). CD4+ TIL possessed similar p-STAT1 and slightly higher T-bet but dramatically lower p-STAT4 at baseline (Fig. 2A and B, T-bet: P = 0.002 and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 2C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL. Foxp3+CD4+ T cells also manifested a similar abortive Th1 differentiation program in TIL. Next, we investigated the activation status of TIL marked by expression of phosphorylated ribosomal protein S6 (p-S6), a downstream target of the PI3K pathway (21). As expected, expression of p-S6 was significantly lower in CD8+ TIL at baseline (Figs. 1A and B and 2A and B, CD8: P = 0.0001 and CD4: P = 0.005) and poststimulation (Figs. 1C and D and Fig. 2C and D, CD8, CD4: P = 0.03), suggesting a dampened activation status of tumor-infiltrating T cells, significantly lower p-STAT1, T-bet, and p-STAT4 expression at baseline (Fig. 1A and B, p-STAT1: P = 0.005; T-bet: P = 0.0003, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 1C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL, which also correlates with deficient expression of perforin in CD8+ TIL (unpublished data). CD4+ TIL possessed similar p-STAT1 and slightly higher T-bet but dramatically lower p-STAT4 at baseline (Fig. 1A and B, p-STAT1: P = 0.005; T-bet: P = 0.005, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 1C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL, which also correlates with deficient expression of perforin in CD8+ TIL (unpublished data). CD4+ TIL possessed similar p-STAT1 and slightly higher T-bet but dramatically lower p-STAT4 at baseline (Fig. 1A and B, p-STAT1: P = 0.005; T-bet: P = 0.005, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 1C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL, which also correlates with deficient expression of perforin in CD8+ TIL (unpublished data). CD4+ TIL possessed similar p-STAT1 and slightly higher T-bet but dramatically lower p-STAT4 at baseline (Fig. 1A and B, p-STAT1: P = 0.005; T-bet: P = 0.005, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 1C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL, which also correlates with deficient expression of perforin in CD8+ TIL (unpublished data). CD4+ TIL possessed similar p-STAT1 and slightly higher T-bet but dramatically lower p-STAT4 at baseline (Fig. 1A and B, p-STAT1: P = 0.005; T-bet: P = 0.005, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 1C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL, which also correlates with deficient expression of perforin in CD8+ TIL (unpublished data). CD4+ TIL possessed similar p-STAT1 and slightly higher T-bet but dramatically lower p-STAT4 at baseline (Fig. 1A and B, p-STAT1: P = 0.005; T-bet: P = 0.005, and p-STAT4: P = 0.02), lower p-STAT1 and T-bet after TCR stimulation (Fig. 1C and D, p-STAT1 and T-bet: P = 0.03) compared with PBL, which also correlates with deficient expression of perforin in CD8+ TIL (unpublished data).
despite the presence of multiple tumor antigenic stimulus in the tumor microenvironment from which they were freshly isolated.

PD-1 suppresses TCR-stimulated upregulation of p-STAT1, T-bet, and p-S6

Despite the fact that PD-L1, the major ligand for PD-1, is variably and heterogeneously expressed on HNSCC tumor cells (4 and Supplementary Fig. S1), we observed colocalization of PD-1\(^+\) TIL with PD-L1\(^+\) tumor cells in vivo in the tumor microenvironment (Fig. 3), indicating that the PD-1 inhibitory signaling is relevant and functional in the tumor-infiltrating T cells. Having demonstrated impaired Tc1/Th1 responses and activation of TIL, we investigated whether PD-1 signaling could directly regulate p-STAT1 and T-bet, which are important regulators of the Th1 phenotype, in the tumor microenvironment. To explore this possibility, we generated anti-CD3/-CD28/PD-L1 or anti-CD3/-CD28/hIgG1 (control Ab)-coated beads. Total TILs isolated from patients with HNSCC were stimulated with these beads in the presence or absence of anti–PD-1 blockade (BMS-936558). Interestingly, TIL that highly expressed PD-1 showed lower p-STAT1 and T-bet, when stimulated with anti-CD3/-CD28/PD-L1 beads, than when stimulated with anti-CD3/-CD28/hIgG1 beads. This result indicates that PD-1 ligation with polyvalent PD-L1 suppresses upregulation of p-STAT1 and T-bet due to TCR stimulation. In addition, anti–PD-1 blockade can restore p-STAT1 and T-bet expression in TIL stimulated with anti-CD3/-CD28/PD-L1 beads (Fig. 4A–C, \(P = 0.02\) and Supplementary Fig. S2A and S2B), which suggests that inhibition of PD-1 signaling using a clinically
effective blocking mAb could reverse the suppressive effects of PD-1 on Th1 phenotypic responses. However, anti–PD-1 blockade did not increase p-STAT1 or T-bet expression in TIL stimulated with anti-CD3/-CD28/hIgG1 (isotype control mAb) beads.

Next, we investigated whether PD-1 signaling interferes with signals downstream of TCR activation. Of interest, p-S6, which can be upregulated by TCR signaling, was decreased by the ligation of PD-1 using PD-L1–coated beads. Consequently, blockade of PD-1 by anti–PD-1 Ab (BMS-936558) restored upregulation of p-S6 (Fig. 4A and D, $P = 0.02$ and Supplementary Fig. S2C). These findings suggest that PD-1 signaling interferes with activation of downstream T-cell activation molecules (such as p-S6) induced by TCR stimulation, promoting dysfunction of T cells in the tumor microenvironment.

PD-1 suppresses secretion of Th1 cytokines but not Th2 cytokines by TIL upon TCR stimulation

Because we observed that PD-1 could suppress p-STAT1 and T-bet, the transcription factors regulating production of Th1 cytokines by CD8$^+$ and CD4$^+$ T cells, we next investigated whether production of Th1 cytokines upon TCR stimulation is influenced by PD-1 ligation or anti–PD-1 blockade. Supernatants of TIL cultured with anti-CD3/-CD28/hlgG1 or anti-CD3/-CD28/PD-L1 beads for 48 hours, with or without anti–PD-1 blockade (BMS-936558), were analyzed by Luminex for Th1/Th2 cytokines secretion. As expected, secretion of the Th1 cytokines IFNγ ($P = 0.008$), TNFα (data not shown), and IL2 ($P = 0.02$, $P = 0.04$) was lower in TIL with anti-CD3/-CD28/PD-L1 stimulation, compared with those stimulated with anti-CD3/-CD28/hlgG1, whereas PD-1 blockade Ab could reverse the inhibitory effects of PD-1/PD-L1.
positive lymphocytes are labeled with a red chromogen, and PD-1-positive HNSCC cells are labeled with a brown chromogen. Images were double immunoperoxidase stainings (right) were performed, and microenvironment. Hematoxylin and eosin (H&E; left) and PD-1/PD-L1 analysis of PD-1 and IFNγ by PD-1 ligation or PD-1 blockade (Fig. 4E). To validate our ligation. Production of the Th2 cytokine IL10 was not altered by PD-1 ligation or PD-1 blockade (Fig. 4E). To validate our ex vivo findings in vivo, we conducted immunohistochemistry analysis of PD-1 and IFNγ in serial sections of the original tumor tissues. When expression of PD-1 on TIL was high, the amount of IFNγ in the tumor microenvironment was low (Tumor 1), and vice versa (Tumor 2, Fig. 4F). Taken together, these findings suggest that PD-1 signaling negatively regulates Tc1/Th1 responses by suppressing activation of p-STAT1 and T-bet and secretion of Th1 cytokines.

**SHP-2 is overexpressed in TIL and strongly correlates with PD-1 expression**

After ligand binding, PD-1 clusters with the TCR and can recruit SHP-2 to its immunoreceptor tyrosine-based switch motif (ITSM), where SHP-2 is phosphorylated (22). SHP-2 has been suggested to be a mediator of PD-1 inhibitory signals (23, 24). Because PD-1 is highly expressed in TIL (22, 24), we examined whether expression of SHP-2 itself correlates with PD-1 expression on TIL from patients with HNSCC. Expression of SHP-2 was tested in paired PBL and TIL from patients with HNSCC by flow cytometry. Although SHP-2 is ubiquitously expressed in T cells, the MFI of SHP-2 was significantly higher in TIL, compared with PBL (Fig. 5A, CD8, CD4: P = 0.002). In addition, the MFI of SHP-2 was even higher in PD-1+ TIL than in PD-1- TIL (Fig. 5B, CD8, CD4: P = 0.002). We also observed that the levels of SHP-2 expression in tumor-infiltrating T cells from patients with HNSCC positively correlated with PD-1 expression (Fig. 5C, R² = 0.8643, P = 0.02). Together, these observations strongly suggest that expression of SHP-2 correlates with PD-1 expression, particularly at the tumor sites.

**SHP-2 activation by fusaraside suppresses p-STAT1/T-bet and production of Th1 cytokines**

Because SHP-2 can function as a negative regulator of p-STAT1 in tumor cells (15) and lymphocytes (16), we next investigated whether activation of SHP-2 regulates the inhibitory effects of PD-1 signaling on p-STAT1 and T-bet. To investigate this possibility, we used fusaraside, a small-molecule compound that specifically induces phosphorylation of SHP-2 (Supplementary Fig. S3; refs. 16, 25), to activate SHP-2 directly in TIL, bypassing ligand engagement of PD-1. As shown in Fig. 6A, upregulation of p-STAT1 and T-bet by TCR/CD28 stimulation was inhibited by fusaraside (50 μmol/L for 48 hours), even when the PD-1 signaling pathway was blocked (P = 0.03). These results indicate that activation of SHP-2 suppresses p-STAT1 and T-bet in a fashion similar to PD-1 signaling. We also tested Th1/Th2 cytokines in the supernatants of TIL cultured in the presence or absence of fusaraside with TCR stimulation. Consistent with decreases in p-STAT1 and T-bet, activation of SHP-2 by fusaraside suppresses production of the Th1 cytokines IFNγ (P = 0.02, P = 0.008), TNFα (data not shown) and IL2, but not the Th2 cytokine IL10 (Fig. 6B). Therefore, PD-1 appears to suppress Tc1/Th1 phenotypic responses that are controlled by p-STAT1/T-bet in the tumor microenvironment by recruiting and activating SHP-2 to skew away from a Th1-biased antitumor response.

**Discussion**

In this study, we provide a mechanistic explanation for how PD-1 suppresses type 1 immunity and T-cell activation in the tumor microenvironment. First, we show that TILs that express significantly more PD-1 manifest dampened Tc1/Th1 phenotypic responses and activation status compared with T cells in PBL. Second, ligation of PD-1 to PD-L1–coated beads suppresses p-STAT1, T-bet, p-S6 and production of Th1 cytokines due to TCR stimulation, while an antagonist PD-1 mAb (BMS-936558) can reverse the negative effects of PD-1 signaling. Third, we demonstrate that SHP-2, the downstream mediator of PD-1, is increased in TIL and is tightly correlated with PD-1 expression. Furthermore, activation of SHP-2 by fusaraside can bypass PD-1 signaling to induce suppression of Tc1/Th1 phenotypic responses marked by expression of p-STAT1 and T-bet and secretion of Th1 cytokines. Taken together, our study describes a novel function for PD-1 in suppressing type 1 immunity, through inhibition of p-STAT1/T-bet, via SHP-2 activation, and in antagonizing TCR/CD28 signaling to decrease p-S6 expression.

Immune escape of tumors results from loss of tumor antigen expression (due to loss of expression of strong rejection antigens...
PD-1 ligation with bead-coated PD-L1 suppresses p-STAT1, T-bet, p-S6, and production of Th1 cytokines upon TCR stimulation, while anti–PD-1 blockade could reverse the suppressive effects of PD-1. Total TILs were stimulated with anti-CD3/-CD28/hIgG1 or anti-CD3/-CD28/PD-L1–coated beads (bead: cell = 10:1) for 48 hours in the presence of 100 µg/mL hIgG4 or anti–PD-1 (BMS-936558), then p-STAT1, T-bet, and p-S6 were analyzed by flow cytometry. Supernatants from each condition were collected and stored at −80°C. Th1 (IFNγ and IL2) and Th2 (IL10) cytokines in the supernatants were determined by Luminex. A, representative data showing p-STAT1 (Y701), T-bet, and p-S6 (S235/236) levels in CD8⁺ TIL under the described conditions. (Continued on the following page.)

Figure 4.
or loss of MHC class I molecules; refs. 26, 27) and establishment of the immunosuppressive tumor microenvironment. Immunosuppression of effector T cells in the tumor microenvironment is mediated by increased expression of coinhibitory receptors (such as PD-1 and CTLA-4) that inhibit activation of T cells, or by immunosuppressive cytokines (such as TGF-β and IL10) derived from both tumor cells and infiltrating Treg and MDSC (28–31). These inhibitory mechanisms are consistent with our observation of a dampened Th1/Tc1 phenotypic response in TIL (Figs. 1 and 2).

In the ongoing clinical trials of anti–PD-1/PD-L1 therapies, there is discussion of whether clinical responses to PD-1/PD-L1 blockade correlate with PD-L1 expression on tumor cells or immune cells. Recently, a report on the clinical trial of anti–PD-1 mAb (BMS-936558) therapy in patients with cancer showed that pretreatment expression of PD-L1 on tumors was associated with enhanced clinical responses (9). In our restimulation system, anti–PD-1 blockade did not increase expression of p-STAT1 and T-bet or production of Th1 cytokines in TIL stimulated with anti-CD3/CD28 alone. This might be because

Figure 5.
SHP-2 is overexpressed in TIL and correlates with PD-1⁺ expression. Expression level of SHP-2 in TIL and paired PBL from patients with HNSCC (n = 10) was assessed by flow cytometry. A, representative figure (top) and summary data (bottom) showing MFI of SHP-2 in CD8⁺ and CD4⁺ TIL compared with PBL. B, representative figure (top) and summary data (bottom) showing MFI of SHP-2 in PD-1⁻ and PD-1⁺ CD8⁺ and CD4⁺ TIL. Statistical significance was determined by the Wilcoxon (nonparametric paired) test. **, P < 0.01. C, expression levels of PD-1 and SHP-2 (shown by MFI) in tumor-infiltrating T cells from 5 patients with HNSCC.

(Continued.) Summary data of frequency of p-STAT1 (Y701)⁺ (B), T-bet⁺ (C), and p-S6 (S235/236)⁺ (D) in CD8⁺ and CD4⁺ TIL with indicated conditions are shown (n = 7). E, summary data of amount of IFNγ, IL2, and IL10 in the supernatants of TIL cultured under indicated conditions are shown. The graphs present the mean ± SEM from 8 patients with HNSCC. F, immunohistochemistry analysis of PD-1 and IFNγ in serial sections of representative HNSCC tumors. Statistical significance was determined by the Wilcoxon (nonparametric paired) test. **, P < 0.05; ***, P < 0.01. P > 0.05 was considered to be not significant (n.s.).
when we isolated TIL, PD-L1+ tumor cells interacting with PD-1+ TIL in the tumor microenvironment (Fig. 3) were depleted, so that PD-1 ligands were much less abundant than in the tumor sites. Therefore, we might only observe beneficial effects of anti–PD-1 blockade on type I antitumor immunity when PD-L1 is reintroduced into the culture to mimic the real tumor microenvironment. Our findings also suggest an important role for PD-L1 expression on tumor cells in triggering PD-1 inhibitory signaling in the interacting T cells (Fig. 4).

Type I–biased innate effector cells (such as IL12-producing DC and IFNγ-producing NK/NKT cells) are crucial for inducing Th1 CD4+ cells and Tc1 CD8+ cells with optimal cytotoxicity and effector functions for tumor cell lysis. Adoptive transfer of Th1 cells (32) and antigen-specific Tc1 cells (33) elicits strong antitumor activity. In contrast, type II–biased effector cells, which produce IL4, IL10, and TGFβ, negatively regulate type I antitumor immunity and make the tumor microenvironment more tumor permissive. Thus, Th1/Tc1-biased antitumor immunity is highly desirable for rejection of tumors by the host immune system. Alteration of the Th1/Th2 balance should therefore be considered as a strategy for cancer immunotherapy, too.

PD-1 blockade has been shown to augment Th1 and Th17 responses (as evidenced by increased production of IFNγ, IL2, TNFα, IL6, and IL17) and to suppress production of the Th2 cytokines IL5 and IL13 in reactivated T cells from peripheral blood of patients with prostate and advanced melanoma cancer (34). However, in our system, PD-1 signaling inhibits production of Th1 cytokines (IFNγ, IL2, and TNFα) by TIL, without altering production of the Th2 cytokine IL10 (Fig. 4E). In contrast, IL4 and IL5, the other two Th2 cytokines, were below the limit of detection.

Figure 6. SHP-2 activation by fusaromide suppresses p-STAT1/T-bet and production of Th1 cytokines upon TCR stimulation. Total TILs were stimulated with anti-CD3/-CD28/hIgG1 beads (bead: cell = 10:1) or anti-CD3/-CD28/PD-L1 beads plus 100 μg/mL anti–PD-1 blockade (BMS-936558) for 48 hours in the presence of 50 μmol/L fusaromide or DMSO. Then p-STAT1 and T-bet were analyzed by flow cytometry. Supernatants were collected and stored at −80°C. Th1 (IFNγ and IL2) and Th2 (IL10) cytokines in the supernatants were determined by Luminex. A, summary data of frequency of p-STAT1+ and T-bet+ cells in CD8+ and CD4+ TIL at different conditions are shown (n = 6). B, summary data of amount of IFNγ (n = 8), IL2 (n = 4), and IL10 (n = 8) in the supernatants of TIL cultured under indicated conditions. The graphs present the mean ± SEM from different patients with HNSCC. Statistical significance was determined by the Wilcoxon (nonparametric paired) test. *, P < 0.05; **, P < 0.01. P > 0.05 was considered to be not significant (n.s.).
tumor-bearing mice, injection of anti-PD-1 antibody induces higher levels of T-bet in CD8+ TIL (35). What is more, phosphorylated SHP-2 can selectively sequester STAT1 from kinases that mediate phosphorylation and thus suppress the STAT1-dependent Th1 immune responses (16). Taken together, these findings suggest that Th1 immunity can be efficiently modulated by PD-1 or SHP-2.

In conclusion, anti–PD-1 blockade, which is being actively explored as an immunotherapy agent in clinical trials and has shown clinical efficacy in several solid tumors, can improve T-cell–based immunotherapy by restoring a robust type I antitumor immunity and enhancing T-cell activation. Biomarkers of anti–PD-1 activity are needed to monitor the efficacy of this type of immunotherapy, which we suggest should include successful restoration of Th1 phenotypes. In addition, SHP-2 inhibitory strategies might be a powerful tool for cancer immunotherapy. Thus, SHP-2 not only suppresses Tc1/Th1 skewing of tumor-infiltrating T cells, but also inhibits pSTAT1-dependent expression of HLA/APM (elements of the antigen processing machinery), and secretion of T-cell attracting chemokines RANTES and IP10 (15) and the cytokine IL12 (unpublished data) by head and neck cancer cells. Therefore, development of a specific SHP-2 inhibitor would be a promising strategy for cancer immunotherapy in the future.

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

Authors’ Contributions
Conception and design: J Li, H.-B. Jie, R.L. Ferris
Development of methodology: J Li, H.-B. Jie, R.L. Ferris
 Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J Li, H.-B. Jie, N. Gildener-Leapman, S. Trivedi, Y. Lei
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J Li, Y. Lei, L.P. Kane
Writing, review, and/or revision of the manuscript: J Li, S. Trivedi, L.P. Kane, R.L. Ferris
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J Li, T. Green, R.L. Ferris
Study supervision: R.L. Ferris

Acknowledgments
The authors thank Dr. Gordon Freeman (Dana-Farber Cancer Institute) for PD-L1 Ab, Dr. Renxiang Tan (Nanjing University, China) for generating and providing fusaric acid (25), and Dr. Yang Sun, Qiang Xu (Nanjing University, China), and Gang Liu (Tsinghua University, China) for generous assistance.

Grant Support
This work was supported by NIH grants R01 DE019727 and P50CA097190. This project used the UPCI Cancer Biomarkers Facility: Luminex Core Laboratory and Flow Cytometry Facility that are supported in part by award P30 CA047904. J Li was supported by the China Scholarship Council. Y. Lei was supported by T32 CA06397 (R.L. Ferris) and NIH K99 DE024173.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 23, 2014; revised September 17, 2014; accepted October 31, 2014; published OnlineFirst December 5, 2014.

References


PD-1/SHP-2 Inhibits Tc1/Th1 Phenotypic Responses and the Activation of T Cells in the Tumor Microenvironment

Jing Li, Hyun-Bae Jie, Yu Lei, et al.

Cancer Res  Published OnlineFirst December 5, 2014.

Updated version  Access the most recent version of this article at: doi:10.1158/0008-5472.CAN-14-1215

Supplementary Material  Access the most recent supplemental material at: http://cancerres.aacrjournals.org/content/suppl/2014/12/06/0008-5472.CAN-14-1215.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.