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, Neil Gildener-Leapman, Sumita Trivedi, Tony Green, Lawrence P. Kane, and Robert L. Ferris

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 1 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.

Introduction

Although recent advances in surgery, chemotherapy, and radiotherapy have been developed, the overall 5-year survival rate for head and neck squamous cell carcinoma (HNSCC) remains at about 50%. The tumor microenvironment in patients with HNSCC is highly immunosuppressive, suggesting a potential to use immunotherapies to improve survival of patients with HNSCC (1). One of the most important immune resistance mechanisms involves coinhibitory pathways mediated by immune checkpoint receptors (ICR), such as CTLA-4, PD-1, BTLA, and LAG-3. These ICRs and their ligands are commonly overexpressed in the tumor microenvironment (2–6), suggesting a promising approach to activate antitumor immune response of T cells by blockade of ICRs (7). CTLA-4 antagonistic mAb (ipilimumab) has significant activity in patients with metastatic melanoma (8) and was approved by FDA in 2010 for melanoma. Anti–PD-1 mAbs have also demonstrated clinical efficacy in early-stage clinical trials for various tumor types and may provide durable antitumor responses (9). However, despite the clinical benefit of ICR antagonist antibodies, the mechanism of improved immune response is still poorly understood.

Optimal T-cell–based antitumor immunity requires both Tc1-biased CD8+ T cells acting as cytolytic effector cells and CD4+ Th1 cells, to enhance the potency and duration of antitumor response. In response to IFNγ and IL12, STAT1 and STAT4 bind to the Tbx21 (encoding T-bet) enhancer and induce a T-bet–dependent Tc1 response (including IFNγ production and cytolytic development) in CD8+ T cells (10). Development of Th1 cells requires a multistep mechanism in which the transcription factors STAT1, T-bet, and STAT4 are sequentially activated (11, 12). Sustained tumor regression that results from antitumor therapies such as cancer vaccines is dependent on a strong type I immune response. In contrast, CD4+ Th2 cells induce M2-biased tumor-associated macrophages and suppress CD8+ antitumor response, driving a more tumor-permissive microenvironment (1). Therefore, skewing to a type I–dominant tumor microenvironment is indispensable to enhance efficacy of antitumor immunotherapy.

Although blockade of the PD-1 pathway (PD-1/PD-L1) enhances production of IFNγ (a hallmark Th1 cytokine) and cytolytic activity of tumor-infiltrating T cells in both tumor-bearing mice and patients with cancer (13, 14), the link between PD-1 and type 1 immunity remains vague. After clustering with the T-cell receptor (TCR) for antigen during inflammatory conditions, PD-1...
can recruit the phosphatase SHP-2 (15, 16) and relieve SHP-2 from its autoinhibited state (17). In contrast, blockade of PD-1 signaling inhibits phosphorylation of SHP-2 (18). We therefore hypothesized that PD-1 suppresses beneficial type 1–dominant immune responses in the tumor microenvironment through the PD-1/SHP-2/p-STAT1/T-bet axis. Stimulation of the TCR and CD28 would lead to activation of the PI3K/Akt/mTOR/p-S6 pathway, which is important for sustaining T-cell survival and expansion (19). Thus, PD-1 might interfere with TCR/CD28 signaling to mediate the suppression of T-cell survival and proliferation in patients with cancer. In addition, PD-1 can turn off TCR/CD28 signals by inhibiting TCR-proximal kinases in T cells. In this study, we used unique, paired, freshly isolated tumor-infiltrating lymphocytes (TIL) and peripheral blood lymphocytes (PBL) specimens to investigate the in vivo phenotypic and functional impact of PD-1 expression on TCR signaling and Th skewing directly in the tumor microenvironment of patients with cancer, since these TILs have appeared to be more suppressed than in the peripheral circulation (2).

**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 Meso Scale Discovery (W Germantown, MD). Both the intensity and percentage of area of staining were excluded on the basis of viability dye staining (Zombie Aqua Fixable Viability Dye, Biolegend). Patients and specimens

**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 dilution. IFNα/β 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 dilution. IFNα/β antibody was purchased from Abcam and incubated at 1:500 dilution. Staining was interpreted by an oral and maxillofacial pathologist. The sensitivity 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-hlgG1 Fc 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 10^7 beads as previously described (20). Generally, 10^7 beads were coated with 1 μg of anti-CD3 (20% of total protein), 1 μg of anti-CD28, and 60% of either PD-L1-hlgG1 Fc 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 × 10^6 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–PD-1 (BMS-936558) or hlgG4 isotype control provided by Bristol–Myers Squibb, or 50 μM/ml Fura-2 AM (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 IFNγ, TNFα, 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.

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 T cells from paired PBLs and TILs from patients with HNSCC. Interestingly, CD8+ TIL had 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. 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+ and CD4+ TIL than in PBL 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 2C and D, CD8, CD4: P = 0.03), suggesting a dampened activation status of tumor-infiltrating T cells.

Table 1. Clinicopathological features of the patients with head and neck cancer in this study

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Abbreviations: L, larynx; OC, oral cavity; OP, oropharynx.
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 DAB chromogen, and PD-L1 was distinguishing areas in double immunoperoxidase stainings (right) were performed, and representative matching areas in five (n = 5) tumors are shown. PD-1-positive lymphocytes are labeled with a red chromogen, and PD-L1-positive HNSCC cells are labeled with a brown chromogen. Images were taken at ×200.

Figure 3.
PD-1 TIL colocalize with PD-L1 HNSCC cells in the tumor microenvironment. Hematoxylin and eosin (H&E; left) and PD-1/PD-L1 double immunoperoxidase stainings (right) were performed, and representative matching areas in five (n = 5) tumors are shown. PD-1-positive lymphocytes are labeled with a red chromogen, and PD-L1-positive HNSCC cells are labeled with a brown chromogen. Images were taken at ×200.

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 Th1/Tc1 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 fusaruside 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 fusaruside, 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 fusaruside (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 fusaruside with TCR stimulation. Consistent with decreases in p-STAT1 and T-bet, activation of SHP-2 by fusaruside 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 Th1/Th2 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 I immunity and T-cell activation in the tumor microenvironment. First, we show that TILs that express significantly more PD-1 manifest dampened Th1/Tc1 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 fusaruside can bypass PD-1 signaling to induce suppression of Th1/Th2 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 I 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...
Figure 4.
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.)
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 IFN\(\gamma\)-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 TGF\(\beta\), 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 IFN\(\gamma\), IL2, TNF\(\alpha\), 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 (IFN\(\gamma\), IL2, and TNF\(\alpha\)) 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 in the supernatants of TIL cultured under indicated conditions. The graphs present the mean \(\pm\) 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.).
References

6. Woo SR, Turnis ME, Goldberg MV, Bankoti J, Selby M, Nirschl CJ, et al. 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.

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No potential conflicts of interest were disclosed.

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

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