Tumor-infiltrating Tim-3+ T cells proliferate avidly except when PD-1 is co-expressed: Evidence for intracellular cross talk

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ABSTRACT
Programmed Death 1 (PD-1) and T cell Ig and mucin domain-3 protein (Tim-3) are immune checkpoint receptors highly expressed on tumor infiltrating T lymphocytes (TIL). PD-1 inhibits T cell activation and type 1 T cell responses, while Tim-3 is proposed to mark more extensively exhausted cells, although the mechanisms underlying Tim-3 function are not clear. Trials of anti-PD-1 therapy have identified a large subset of non-responder patients, likely due to expression of alternative checkpoint molecules like Tim-3. We investigated the phenotypic and functional characteristics of T cells with differential expression of PD-1 (high/low) and Tim-3 (positive/negative), using TIL directly isolated from head and neck squamous cell carcinomas (HNSCC). Unexpectedly, we found that expression of Tim-3 alone does not necessarily mark TIL as dysfunctional/exhausted. In Tim-3+ TIL, PD-1 levels correlate with T cell dysfunction, with a PD-1low/intermed phenotype identifying recently activated and still functional cells, whereas PD-1hiTim-3+ T cells are actually exhausted. Nonetheless, PD-1intermed cells are still potently suppressed by PD-L1. PD-1 expression was associated with reduced phosphorylation of ribosomal protein S6 (pS6), whereas Tim-3 expression was associated with increased pS6. Using a novel mouse model for inducible Tim-3 expression, we confirmed that expression of Tim-3 does not necessarily render T cells refractory to further activation. These results suggest the existence of PD-1 and Tim-3 crosstalk in regulating antitumor T cell responses, with important implications for anti-PD-1 immunotherapy.

Introduction
HNSCC is associated with a highly immunosuppressive tumor microenvironment that may limit the efficacy of cancer therapies. Immunosuppression in the HNSCC microenvironment is mediated by regulatory T cells (Treg), myeloid derived suppressor cells (MDSC) and dysfunctional or exhausted antitumor effector T cells. During chronic viral infection, exhausted T cells undergo an altered transcriptional program with upregulation of several transcription factors, including Eomes, Blimp-1 and BATF, and downregulation of T-bet. Another feature of T cell exhaustion is the increased expression of immune checkpoint receptors (ICR) like PD-1, CTLA-4 and Tim-3. Since these ICRs and their ligands are often overexpressed in tumors, they have been recently targeted for therapeutic approach to reverse immunosuppression in cancer patients. Indeed, blockade of cytotoxic T lymphocyte antigen 4 (CTLA-4) and/or PD-1 with monoclonal antibodies (mAb) ipilimumab or nivolumab/pembrolizumab, respectively, has shown clinical efficacy for some patients with melanoma, renal cell carcinoma, non-small cell lung cancer and, more recently HNSCC.

Despite recent progress, improved success of mAbs targeting PD-1 has been hindered by a relatively poor understanding of mechanisms of resistance to these mAbs, which occurs in the majority of patients, as well as a lack of biomarkers of clinical response. One explanation for variable responses to anti-PD-1 therapy is the heterogeneous expression of alternative checkpoint inhibitory receptors on TIL, such as Tim-3, reflecting greater complexity to the phenotypic and functional attributes of T cell “exhaustion” than was initially appreciated. In preliminary studies, dual blockade of PD-1 and Tim-3 has been shown to enhance antitumor T cell responses, compared to PD-1/PD-L1 blockade alone. In addition, PD-1 expression on TIL has actually been identified as a favorable prognostic biomarker for...
HPV+ head and neck cancer, which has raised questions about using PD-1 as the sole defining marker of “exhausted” T cells in the tumor microenvironment. Therefore, we reasoned that TIL from human cancer patients would provide direct evidence for phenotypic and functional consequences of PD-1 and/or Tim-3 expression, in order to verify activation versus exhaustion of these important TIL subsets. Finally, whether blockade of Tim-3 on single positive T cells would be deleterious or beneficial has not been determined.

We segregated subsets of CD8+ and CD4+ effector TIL from HNSCC patients, based on expression of PD-1 and Tim-3, to compare their phenotypic and functional characteristics. Our findings first confirm that expression of high levels of PD-1 are associated with T cell exhaustion, with increased expression of the exhaustion-related transcription factors Blimp-1, BATF and Eomes, deficient production of Th1 cytokines and decreased clonal expansion. By contrast, T cells with intermediate levels of PD-1 appear activated rather than exhausted. Surprisingly, expression of Tim-3 by T cells is also associated with functional competence, at least at the level of proliferation, although this can still be overridden by high levels or ligation of PD-1. In addition, PD-1 appears to interfere with activation events (such as pS6) downstream of TCR and Tim-3 signaling, representing a potential mechanism of crosstalk between PD-1 and Tim-3. Finally, we show that expression of Tim-3 alone augments the activation of events downstream of TCR signaling. Our findings are consistent with those of others who have suggested that the expression of one or more “checkpoint” receptors (e.g. PD-1 and Tim-3) is not necessarily indicative of functional exhaustion. As such, our findings also have implications for reversing immune escape from anti-PD-1 therapy currently in the clinic, and for the development of new therapies targeting Tim-3 itself.

Results

Definition of HNSCC TIL by expression of PD-1 and Tim-3

Levels of PD-1 and Tim-3 are upregulated on TIL in tumor-bearing mice and on antigen-specific CD8+ T cells in melanoma patients, and PD-1/Tim-3 co-expression has been associated with T cell exhaustion in chronic viral infection. In order to establish the expression pattern of PD-1 and Tim-3 on TIL from a cohort of stage III–IV HNSCC patients, and to facilitate analysis of phenotypic and functional characteristics of the TIL subsets, we assessed PD-1 and Tim-3 expression on these TIL by flow cytometry. Consistent with previous reports, we observed high expression of PD-1 or Tim-3 individually on CD8+ TIL, as well as co-expression of PD-1 on a subset of Tim-3+ CD8+ TIL. Interestingly, PD-1+Tim-3+ CD8+ TIL had the highest expression level of PD-1 (Fig. 1). Among CD4+ TIL, Tim-3 was mainly expressed on Treg (CD25hiFoxp3+CD4+) but not on Foxp3–CD4+ effector TIL, whereas a distinct population of PD-1+Tim-3– cells was sometimes seen in these Foxp3+ CD4+ effector T cells. By contrast, most of the PD-1 single positive Treg had an intermediate level of PD-1 expression (Fig. 1).

Having defined the various subsets of TIL based on PD-1 and Tim-3 expression, we further characterized these cells (gating strategies described in Materials and Methods) using classical phenotypic markers of naïve (Tn), central memory (TcM) versus effector memory (Tem) T cell populations. Most of the CD8+ and Foxp3–CD4+ effector T lymphocytes at tumor sites

Figure 1. Definition of TIL subsets, based on PD-1 and Tim-3 expression. Expression of PD-1, Tim-3, CCR7 and CD45RA on CD8+ and CD4+ peripheral blood T lymphocytes (PBL) and tumor infiltrating T lymphocytes (TIL) from HNSCC patients (n = 7) was analyzed by flow cytometry. Representative figures showing expression patterns of PD-1 and Tim-3 on CD8+ Treg (CD25hiFoxp3+CD4+) but not on Foxp3–CD4+ effector TIL, whereas a distinct population of PD-1+Tim-3– cells was sometimes seen in these Foxp3+ CD4+ effector T cells. By contrast, most of the PD-1 single positive Treg had an intermediate level of PD-1 expression (Fig. 1).

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were $T_{CM}$ (CCR7$^+$CD45RA$^-$) or $T_{EM}$ (CCR7$^+$CD45RA$^+$), compared to those in the peripheral circulation (Fig. S1A). PD-1$^{hi}$Tim-3$^-$ and PD-1$^{hi}$Tim-3$^-$ CD8$^+$ and Foxp3$^+$CD4$^+$ TIL comprised a significantly higher proportion of effector memory cells than other subsets (Fig. S1B–C), suggesting that most tumor antigen-experienced T cells in the tumor microenvironment are PD-1$^{hi}$Tim-3$^-$ or PD-1$^{hi}$Tim-3$^-$ in CD8$^+$ and lower levels of T-bet, a marker of robust and functional TCR signaling, and to inhibit activation of pS6 in T cells.27 We hypothesized that high PD-1 expression and the concomitant high expression of SH2 domain-containing tyrosine phosphatase-2 (SHP-2)27 could interfere with the putative enhancement of TCR signaling by Tim-3, leading to suppression of T cell activation, as a mechanism of crosstalk between PD-1 and Tim-3. To test this hypothesis, we stimulated sorted TIL subsets from HNSCC patients with anti-CD3/CD28 beads for 48 h and performed intracellular flow cytometry to quantify pS6, as a readout of TCR and Tim-3 signaling. As expected, PD-1$^{hi}$Tim-3$^-$ and PD-1$^{hi}$Tim-3$^-$ effector TIL subsets showed dampened pS6 induction upon anti-CD3/CD28 stimulation (Fig. 4A–B), consistent with their impaired proliferation (Fig. 3). Moreover, among Tim-3$^-$ cells, PD-1$^{hi}$Tim-3$^-$ TIL expressed lower pS6 than PD-1$^{hi}$Tim-3$^-$ cells (Fig. 4A–B). Among Tim-3$^-$ cells, the levels of TCR-stimulated pS6 followed a trend of PD-1$^{hi}$Tim-3$^-$ < PD-1$^{int}$Tim-3$^-$ < PD-1$^{lo}$Tim-3$^-$ (Fig. 4A–B). To further

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**Table 1.** Proportion of PD-1$^{hi}$Tim-3$^-$ and PD-1$^{int}$Tim-3$^-$ cells in CD8$^+$ and CD4$^+$ effector TIL.

| Subset | CD8$^+$ | CD4$^+$CD25$^{hi}$ |
|--------|---------|------------------|
| PD-1$^{hi}$Tim-3$^-$ | 7.67 ± 3.75% | 6.68 ± 3.08% |
| (3.23%–14.8%) | (3.43%–11.6%) |
| PD-1$^{int}$Tim-3$^-$ | 24.39 ± 7.11% | 30.90 ± 9.44% |
| (12.2%–37.6%) | (16.67%–52.63%) |

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**PD-1$^{hi}$Tim-3$^-$ and PD-1$^{int}$Tim-3$^-$ CD8$^+$ TIL display molecular signatures associated with T cell exhaustion**

Exhaustion of T cells in chronic viral infection is associated with a transcriptional program distinct from that of functional effector or memory T cells,22 including upregulation of Blimp-1 and BATF.7 However, the expression of these exhaustion-associated transcription factors in freshly isolated TIL from human cancer have not yet been well characterized. Therefore, we sought to define the T cell exhaustion signatures of TIL expressing PD-1 and or Tim-3 isolated from HNSCC patients. Interestingly, PD-1$^{hi}$Tim-3$^-$ and PD-1$^{int}$Tim-3$^-$ CD8$^+$ TIL had higher levels of transcripts for Blimp-1 (encoded by PRDM1) and BATF, transcription factors previously shown to impair T cell proliferation and cytokine secretion and thereby foster T cell exhaustion.23,24 Moreover, higher expression of Eomes, which is associated with exhausted terminal progeny T cells, and lower levels of T-bet, a marker of robust and functional progenitors,25 were observed primarily in PD-1$^{hi}$Tim-3$^-$ and PD-1$^{int}$Tim-3$^-$ CD8$^+$ TIL (Fig. 2A). However, PD-1$^{lo}$Tim-3$^-$ CD8$^+$ TIL expressed higher BATF and lower TBX21, but similar levels of PRDM1 and EOMES transcripts, compared with PD-1$^{hi}$Tim-3$^-$ and PD-1$^{int}$Tim-3$^-$ CD8$^+$ TIL. Thus, PD-1$^{hi}$Tim-3$^-$ and PD-1$^{int}$Tim-3$^-$ CD8$^+$ TIL possessed molecular signatures associated with exhausted T cells found in chronic viral infection, and are most likely to be dysfunctional.

**High levels of PD-1 are associated with reduced S6 phosphorylation, while Tim-3 expression itself drives increased S6 phosphorylation**

Tim-3 has been shown to augment TCR downstream signaling, including increased downstream phosphorylation of ribosomal protein S6 (pS6),26 which promotes proliferation of T cells. In contrast, PD-1:PD-L1 ligation has been shown to suppress TCR signaling, and to inhibit activation of pS6 in T cells.27 We hypothesized that high PD-1 expression and the concomitant high expression of SH2 domain-containing tyrosine phosphatase-2 (SHP-2)27 could interfere with the putative enhancement of TCR signaling by Tim-3, leading to suppression of T cell activation, as a mechanism of crosstalk between PD-1 and Tim-3. To test this hypothesis, we stimulated sorted TIL subsets from HNSCC patients with anti-CD3/CD28 beads for 48 h and performed intracellular flow cytometry to quantify pS6, as a readout of TCR and Tim-3 signaling. As expected, PD-1$^{hi}$Tim-3$^-$ and PD-1$^{int}$Tim-3$^-$ effector TIL subsets showed dampened pS6 induction upon anti-CD3/CD28 stimulation (Fig. 4A–B), consistent with their impaired proliferation (Fig. 3). Moreover, among Tim-3$^-$ cells, PD-1$^{hi}$Tim-3$^-$ TIL expressed lower pS6 than PD-1$^{hi}$Tim-3$^-$ cells (Fig. 4A–B). Among Tim-3$^-$ cells, the levels of TCR-stimulated pS6 followed a trend of PD-1$^{hi}$Tim-3$^-$ < PD-1$^{int}$Tim-3$^-$ < PD-1$^{lo}$Tim-3$^-$ (Fig. 4A–B). To further
Figure 2. PD-1<sup>hi</sup>Tim-3<sup>−</sup> and PD-1<sup>int</sup>Tim-3<sup>−</sup> CD8<sup>+</sup> TIL expressed higher amount of Blimp-1, BATF, Eomes and lower amount of T-bet transcripts. (A) PD-1<sup>hi</sup>Tim-3<sup>−</sup>, PD-1<sup>int</sup>Tim-3<sup>−</sup>, PD-1 Tim-3<sup>−</sup> and PD-1 Tim-3<sup>−</sup> CD8<sup>+</sup> T cells were sorted from TIL. Each TIL subset from four different HNSCC patients was subjected to RNA purification and then analyzed by real time quantitative PCR. Summarized graphs of relative expression of PRDM1 (Blimp-1), BATF, EOMES and TBX21 (T-bet) transcripts in each subset are shown. The quantity of each cDNA sample was normalized by GUSB. All analyses were performed in triplicate. The graphs present the mean ± SD from replicates. Statistical significance was determined by RM One-way ANOVA analysis followed by multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001. (B) PD-1<sup>hi</sup>Tim-3<sup>−</sup> and PD-1 Tim-3<sup>−</sup> effector TIL display defective Th1 cytokine production. Summary data of IFNγ production by each sorted TIL subset (n = 3), measured by IFNγ ELISPOT after anti-CD3/CD28 stimulation. Experiments were performed in duplicate. Statistical significance was determined by RM One-way ANOVA analysis followed by multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001.
explore a potential positive role for Tim-3, we also examined the state of S6 phosphorylation under basal (no additional stimulation) conditions. Thus, as shown in Fig. 4C–D, expression of Tim-3 alone on TIL was associated with a higher basal level of pS6, a phenotype also observed in T cells from HIV-infected individuals28 and consistent with our previous studies in T cell lines with ectopic expression of Tim-326.

Our studies thus far suggest that, contrary to many current models, expression of Tim-3 by T cells among TIL is not necessarily indicative of a dysfunctional state, since Tim-3 itself seems to result in a “split” exhaustion phenotype, with enhanced TCR signaling and proliferation, while cytokine production is still suppressed. In order to obtain a better mechanistic understanding of the intrinsic role of Tim-3 on T cells, we generated a novel knock-in mouse model, in which we can induce Tim-3 expression in a Cre-dependent fashion (Fig. 5A). We purified T cells from the spleens and LN of these mice and cultured them overnight with purified Tat-Cre fusion protein. As shown in Fig. 5B, this resulted in robust expression of Tim-3 on the knock-in (but not WT) T cells, without upregulating PD-1. When these cells were subsequently stimulated with anti-CD3/CD28 antibodies, we observed that the induction of pS6 was significantly greater on T cells expressing Tim-3 (Fig. 5C). This finding is consistent with results shown above, in which we observed that TIL expressing Tim-3 (but not PD-1) were still capable of proliferating upon re-stimulation. In order to better define possible cross-talk between PD-1 and Tim-3, we transfected a T cell line (D10. G41)26,29 with cDNAs for human PD-1 and/or Tim-3 (Fig. 5D and Fig. S3), along with an NFAT/AP1-luciferase reporter, as a read-out for TCR signaling. Consistent with data presented in Fig. 5B, and above, expression of Tim-3 alone enhanced reporter activation. However, D10.G41 T cells co-transfected with PD-1 and Tim-3 displayed less upregulation of TCR signaling than cells transfected with Tim-3 alone (Fig. 5E). Taken together, our data indicate that while Tim-3 has an intrinsic ability to augment T cell activation, high expression of PD-1 interferes with both TCR signaling and the apparently positive effects of Tim-3. Nonetheless, PD-1 activity is known to be regulated at least in part by binding to its ligands. We next sought to examine this aspect of PD-1 function in the TIL samples.
Figure 4. High levels of PD-1 are associated with reduced S6 phosphorylation, while Tim-3 expression drives increased S6 phosphorylation. T cells were purified from isolated TIL from HNSCC patients (n = 3). Then, PD-1<sup>hi</sup>Tim-3<sup>−</sup>, PD-1<sup>int</sup>Tim-3<sup>−</sup>, PD-1<sup>−</sup>Tim-3<sup>C</sup>, PD-1<sup>C</sup>Tim-3<sup>C</sup> and PD-1<sup>−</sup>Tim-3<sup>−</sup>CD8<sup>+</sup> and CD4<sup>+</sup>CD25<sup>lo/-</sup>T cells were sorted from purified T cells. After sorting, cells were rested overnight and stimulated with anti-CD3/CD28 microbeads (bead: cell = 5:1) for 48 h. Cells were harvested and pS6 levels were analyzed by intracellular flow cytometry. (A) Representative figures showing expression of pS6 in each subset after 48 h stimulation. (B) Summary data showing frequency of pS6<sup>(S235/236)</sup> cells in each TIL subset after TCR stimulation. (C) Representative figures showing expression of pS6<sup>(S235/236)</sup> cells in each subset without stimulation. (D) Summary data showing frequency of pS6<sup>(S235/236)</sup> cells in each TIL subset without stimulation. Statistical significance was determined by RM one-way ANOVA analysis followed by multiple comparisons. *p < 0.05, **p < 0.01.
**TIL with intermediate levels of PD-1 can still be suppressed by PD-L1**

The *in vivo* function of exhaustion markers like PD-1 is critically regulated by interaction with specific ligands. PD-L1, one of the ligands for PD-1, is often expressed on tumor cells, which is thought to facilitate immune escape of tumors. We therefore investigated how the various populations of PD-1 and/or Tim-3-expressing effector TIL respond to TCR stimulation in the presence of PD-L1.Sorted PD-1**hi**Tim-3**¡** and PD-1**C**Tim-3**C** TIL are likely to be effector memory cells, which is consistent with the prior chronic TCR-stimulation induced expression of these two receptors on T cells in the microenvironment. Gene expression analysis of sorted CD8**C** TIL subsets showed that Blimp-1, BATF and Eomes, transcription when stimulated with anti-CD3/-CD28/PD-L1 beads (Fig. 6). These results suggest that PD-1**inf**Tim-3**inf** TIL are the cells most likely to be suppressed by PD-L1 expressed on tumor cells in the tumor microenvironment and to be reinvigorated by anti-PD-1 targeted immunotherapy.

**Discussion**

In the present study, we set out to provide a more precise and thorough phenotypic and functional profile of TIL from HNSCC patients with heterogeneous expression of PD-1 and Tim-3. Our data demonstrate that PD-1**hi**Tim-3**¡** and PD-1**¡**Tim-3**®** TIL are likely to be effector memory cells, which is consistent with the prior chronic TCR-stimulation induced expression of these two receptors on T cells in the microenvironment. Gene expression analysis of sorted CD8**C** TIL subsets showed that Blimp-1, BATF and Eomes, transcription...
factors previously implicated in CD8\textsuperscript{+} T cell exhaustion, were upregulated, while T-bet, which is important for sustained T cell responses, was reduced in PD-1\textsuperscript{hi}Tim-3\textsuperscript{−} and PD-1\textsuperscript{C}Tim-3\textsuperscript{C} effector TIL. In vitro stimulation of the sorted TIL subsets also revealed that PD-1\textsuperscript{hi}Tim-3\textsuperscript{−} and PD-1\textsuperscript{C}Tim-3\textsuperscript{C} effector TIL are the most dysfunctional in terms of Th1 cytokine production and proliferation after TCR stimulation, whereas PD-1\textsuperscript{int}Tim-3\textsuperscript{−} TIL remained competent to produce Th1 cytokines and proliferate after stimulation. Nonetheless, PD-1\textsuperscript{int}Tim-3\textsuperscript{−} effector TIL were the most susceptible to suppression by PD-L1 treatment, indicating that PD-1\textsuperscript{int}Tim-3\textsuperscript{−} TIL is the major subset to be targeted for anti-PD-1 therapy. Furthermore, the mTOR/pS6 pathway, which is activated by TCR/CD28 signaling and is further enhanced by Tim-3\textsuperscript{26}, was suppressed by high expression levels of PD-1. These findings suggest that low expression of PD-1 in the absence of Tim-3 correlates with the activation status of T cells, while high levels of PD-1, or co-expression of PD-1 and Tim-3\textsuperscript{26}, are markers of T cell dysfunction in the tumor microenvironment.

T cell exhaustion was first defined, and has been most extensively studied, in the mouse model of chronic lymphocytic choriomeningitis virus (LCMV) infection.\textsuperscript{32,33} As defined in that system, exhaustion is a special state of T cell dysfunction with gradual loss of effector function, starting with IL-2, cytolytic activity and proliferative potential at early stages and IFN\textgreek{g} production at later stages.\textsuperscript{22} Exhaustion of CD8\textsuperscript{+} and CD4\textsuperscript{+} T cells during chronic viral infection is associated with—and possibly driven by—co-expression of multiple inhibitory receptors (including PD-1, LAG3, 2B4 and CD160), in addition to those that we have studied here. This dysfunctional state is also associated with a pattern of transcription factor expression distinct from those of functional effector or memory T cells.\textsuperscript{7,8,23,24}

Functional exhaustion of CD8\textsuperscript{+} T cells is also observed in cancers and is associated with co-expression of PD-1 and Tim-3.\textsuperscript{15,16} However, exhaustion of CD4\textsuperscript{+} effector T cells in cancer is still not well defined. In the cohort of HNSCC patients, we have studied sorted PD-1\textsuperscript{hi}Tim-3\textsuperscript{−} and PD-1\textsuperscript{int}Tim-3\textsuperscript{−} CD8\textsuperscript{+} TIL manifested exhausted phenotypes with defective Th1 cytokine production and proliferation (Figs. 2B and 3), concomitant with increased exhaustion-associated molecular signatures such as high Blimp-1, BATF, Eomes and low T-bet (Fig. 2A). Among CD4\textsuperscript{+} effector TIL, PD-1\textsuperscript{hi}Tim-3\textsuperscript{−} and PD-1\textsuperscript{int}Tim-3\textsuperscript{−} cells also showed a similar exhausted phenotype, but we were not able to analyze the changes in their transcriptional programs by real time qPCR because of the scarcity of these cells in the tumor microenvironment. The exhausted PD-1\textsuperscript{hi}Tim-3\textsuperscript{−}
subpopulation of tumor-infiltrating T cells has been overlooked in past studies, since it has not been appreciated that the PD-1"Tim-3" T cell subset is actually a mixed population with heterogeneous PD-1 expression. Indeed, cells with high versus low levels of PD-1 appear phenotypically and functionally distinct. Thus, according to a previous report, a two subpopulations of exhausted CD8 T cells with high or intermediate expression of PD-1 were identified during chronic LCMV infection in mice. Although both subsets were functionally exhausted compared to memory CD8 T cells in acute infection, the PD-1"0" subset had higher proliferative potential and was preferentially reinvigorated by PD-L1 blockade than were the PD-1"+" cells, indicating that the exhaustion status of T cells can be tuned by the relative expression level of PD-1. In our study, the PD-1""Tim-3" CD8 T and CD4 effector TIL from HNSCC patients were clearly activated, manifesting high TCR-stimulated production of Th1 cytokines and proliferation. Their function was dramatically impaired in the presence of PD-L1 (Fig. 6), suggesting a potential therapeutic benefit of using PD-1 blockade to reinvigorate the PD-1""Tim-3" subset, which represents a large proportion of TIL in our studies (24 ± 7% in CD8 and 31 ± 9% in CD4 effector TIL). We propose the responsiveness of this particular subset as a biomarker for patients treated with anti-PD-1 based immunotherapy. PD-1""Tim-3" cells may represent a negative prognostic biomarker of anti-PD-1 therapy, unless the function of these cells can be restored with Tim-3 blockade.

PD-1 drives T cell exhaustion by recruiting the tyrosine phosphatase SHP-2 to inhibit TCR-proximal phosphorylation events and downstream pSTAT1/T-bet driven Tc1/Th1 differentiation. Nonetheless, how Tim-3 mediates suppression of T cell activation or effector function is poorly understood, even though Tim-3 has been recognized as an additional marker of exhaustion on PD-1" T cells in cancers and chronic viral infection. Recently, Tim-3 was proposed to help drive T cell exhaustion by augmenting TCR/CD28 signaling, since excessive and constitutive TCR signaling, especially mTORC1, has been suggested to be critical for the development of T cell exhaustion. Another non-exclusive possibility is that the expression of Tim-3 observed in settings of high levels of PD-1 (including in HNSCC TIL) may represent a pro-survival mechanism in such cells. Thus, high levels of PD-1 would be expected to more severely depress signaling pathways that regulate both activation and survival of effector T cells. Possible signaling crosstalk between the phosphatase(s) downstream of PD-1 and the kinases activated by Tim-3 needs to be further investigated.

Due to the paucity of TIL that could be isolated from most HNSCC tumor specimens, replicates in our study required larger tumors, which could skew the results and our interpretation of earlier stage disease. However, the differences of these TIL subsets were consistent among the patients studied, in spite of some variation. In our ex vivo analyses, we first stimulated sorted TIL subsets for cytokine expression and proliferation in the absence of ligands of PD-1 and Tim-3 expressed on antigen-presenting cells or tumor cells. We introduced PD-1 ligand by coating PD-L1 together with anti-CD3 and anti-CD28 on beads to see which subset is most susceptible to suppression by PD-1 signaling. However, since there is still not a unique, specific, ligand defined for Tim-3, we were not able to test how PD-1"+"Tim-3" TIL respond to simultaneous engagement by ligand.

Anti-PD-1 immunotherapy is clinically effective in some patients with solid tumors in early clinical trials. However, this promising cancer immunotherapy still lacks complete response (objective response rate: ~30%) despite the high frequency of PD-1" tumor-reactive T cells in the treated tumors. The limited efficacy of PD-1 blockade to reinvigorate exhausted T cells in the tumor microenvironment could be partly explained by the presence of Tim-3 on a subset of the PD-1" cells. Dual blockade of PD-1 and Tim-3 might be needed to generate improved clinical responses. Moreover, since PD-1""Tim-3" TIL were the most susceptible to suppression by PD-L1 (Fig. 6), clinical responses of anti-PD-1 immunotherapy should not only be evaluated in the context of PD-L1 expression in the tumor microenvironment, but also the frequency of PD-1""Tim-3" TIL, in order to improve the efficacy of anti-PD-1 therapies, and to predict those most likely to benefit from the strategies.

Materials and methods

Patients and specimens

Peripheral blood samples and fresh tumor specimens were obtained from 26 HNSCC patients. All patients were treated in the Department of Otolaryngology at the University of Pittsburgh Medical Center, and all subjects signed written informed consent approved by the Institutional Review Board of the University of Pittsburgh (UPCI# 99-069). The clinical-pathological features of the HNSCC patients in this study are shown in Table 2. The patient cohort included six females and 20 males with a mean age of 62.5 y (range: 39–89 y).

Peripheral blood mononuclear cells (PBMC) and tumor infiltrating lymphocytes (TIL)

Venous blood from HNSCC patients was drawn into heparinized tubes and centrifuged on Ficoll-Hypeaque gradients (GE Healthcare Life Sciences 17-1440-03). PBMC were recovered, washed in RPMI-1640 medium (Sigma R8758), and either used immediately for experiments or resuspended in freezing media containing 10% DMSO, transferred to Mr. Frosty containers (Thermo Scientific, 5100-0001), and stored at -80°C until flow cytometry analysis. For TIL isolation, fresh tumors from HNSCC patients were minced into small pieces manually or using a gentleMACS Dissociator (Miltenyi Biotec 130-093-235), 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-Hypeaque gradient centrifugation. After centrifugation, mononuclear cells were recovered and stored at -80°C until flow cytometry analysis or immediately used for experiments.

Antibodies and flow cytometry

The following anti-human antibodies were used for staining of PBMC or TIL: CD3-Alexa Fluor 700 (BD 557917), CD8 PE (BD 555367), CD4-PerCP/Cy5.5 (BD 560650), FOXP3-PerCP/Cy5.5
Table 2. Clinicopathological features of the HNSCC patients in this study.

| HN#    | Gender | Age (at diagnosis) | Tumor site | T-stage, path | N-stage, path | M-stage, path |
|--------|--------|--------------------|------------|---------------|---------------|---------------|
| HN13-7001 | M     | 68                 | Oral cavity | T1            | N0            | M0            |
| HN14-7661 | M     | 41                 | Oropharynx  | TX            | N2B           | M0            |
| HN14-7668 | M     | 39                 | Hypopharynx | TX            | N2A           | MX            |
| HN14-7675 | M     | 50                 | Oropharynx  | TX            | N2A           | MX            |
| HN14-7682 | F     | 82                 | Oropharynx  | T2            | N2B           | MX            |
| HN14-7684 | F     | 81                 | Oropharynx  | T2            | N2B           | MX            |
| HN14-7710 | F     | 65                 | Oral cavity | T3            | N0            | MX            |
| HN14-7721 | M     | 71                 | Oral Cavity | T2            | N1            | MX            |
| HN14-7749 | M     | 50                 | Larynx      | T3            | N0            | MX            |
| HN14-7750 | M     | 77                 | Oral cavity | T4A           | N0            | MX            |
| HN14-7752 | M     | 62                 | Oral cavity | TX            | N2C           | MX            |
| HN14-7778 | M     | 55                 | Oral cavity | T2            | N1            | MX            |
| HN14-7787 | M     | 75                 | Hypopharynx | T3            | N2B           | MX            |
| HN14-7767 | F     | 89                 | Oral cavity | T2            | N2B           | MX            |
| HN14-7941 | M     | 47                 | Larynx      | T4A           | N0            | MX            |
| HN14-7950 | M     | 67                 | Larynx      | T4A           | N2B           | M0            |
| HN14-7960 | M     | 69                 | Parotid      | T4A           | N2B           | MX            |
| HN14-7971 | M     | 78                 | Larynx      | TX (cT4A)     | NX (cN1)      | MX (cM1)      |
| HN15-7990 | M     | 66                 | Oral cavity | T1            | N0            | M0            |
| HN15-8010 | M     | 53                 | Oropharynx  | TX (cT2)      | NX (cN2b)     | MX (cM0)      |
| HN15-8038 | M     | 49                 | Oral cavity | T4            | N2C           | M0            |
| HN15-8045 | M     | 58                 | Oral cavity | T3            | N0            | M0            |
| HN15-8069 | F     | 58                 | Larynx      | T4B           | N2            | MX            |
| HN15-8121 | F     | 63                 | Larynx      | TX (cT3)     | NX (cN0)      | MX (cM0)      |
| HN15-8144 | M     | 50                 | Larynx      | T1            | N1            | MX            |
| HN15-8157 | M     | 62                 | Oropharynx  | T3            | N1            | M0            |

(BD 561493), Tim-3-BV421 (Biolegend 345007), CD25-PE-Cy7 (Biolegend 302612), CD4+PE-TR (Life Technologies MHC0D417), PD-1-APC (eBioscience 17-9969-42) and CD45RA-PE-Cy7 (eBioscience 25-0458-71), CCR7-FITC (R&D Systems FAB197F) and phospho-S6 (Ser235/236)-Alexa Fluor 488 (Cell Signaling Technology 4803). Antibodies to mouse proteins were as follows: Tim-3-PE (R&D, 215008); PD1-APC (Biolegend 109111); CD3-biotin (BD 553060); CD28-biotin (BD 553296). Streptavidin was from Calbiochem. In D10 transfection experiments, human Tim-3 and PD-1 were stained with APC-conjugated clone 344823 (R&D FAB2365A) and BV421-conjugated clone EH12.1 (BD 562516), respectively. Intracellular staining for FOXP3 and pS6 was performed as follows: PBMC or TIL were stained with surface marker antibodies, fixed with fixation/permeabilization buffer (eBioscience 00-5521), washed, and stained for intracellular antigens in 1X permeabilization buffer. Cells were analyzed on an LSRFortessa (BD Biosciences) flow cytometer, and data analyzed using Flowjo. 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 based on viability dye staining (Zombie Aqua Fixable Viability Dye, Biolegend 423101).

**Gating strategies for TIL subsets**

PD-1 or Tim-3 positivity was determined by isotype control. PD-1+ Tim-3- CD8+ or Foxp3+ CD4+ TIL with similar fluorescence intensity of PD-1 with CD8+ or Foxp3+ CD4+ T cells from matched PBMC were recognized as PD-1hiTim-3-, while those with higher expression of PD-1 than peripheral blood T cells were PD-1hiTim-3+. To set gates consistently across different patients, we used the same amount of antibodies for staining and the same voltage on the cytometer.

**Anti-CD3/-CD28/hIgG1 and anti-CD3/-CD28/PD-L1 beads**

LEAF purified anti-human CD3 (clone UCHT1, Biolegend, 300414), LEAF purified anti-human CD28 (clone CD28.2, Biolegend 302914) plus PD-L1-hIgG1 Fc fusion protein (R&D Systems, Q9NZQ7) or control human IgG1 (Southern Biotech, Birmingham, AL) were covalently coupled to Dynabeads M-450 Epoxy beads according to the manufacturer’s protocol (Life Technologies 14011). The total amount of protein was kept constant at 5 μg per 10⁷ beads as previously described. 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-hIgG1 Fc fusion protein or control human IgG1. Covalent coupling of the proteins to the beads was performed in 0.1 M sodium phosphate buffer for 24 h at room temperature with gentle tilting and rotation.

**Sorting of TIL subsets**

TIL were isolated from tumor specimens as described above and T cells were purified using EasySep™ Human T Cell Enrichment Kit (Stemcell technologies 19051). Then, the T cells were stained with CD4+PE-Cy5.5, CD8-PE, CD25-PE-Cy7, PD-1-APC (eBioscience 17-9969-42) and Tim-3-BV421 (Biolegend 345007) antibodies. PD-1hiTim-3-, PD-1loTim-3-, PD-1-Tim-3+, PD-1-Tim-3+, PD-1-Tim-3+ and PD-1-Tim-3+ CD8+ and CD4+CD25−/− cells were sorted using Beckman Coulter MoFlo Astrios. Post-sort purity of the sorted populations was ~94% (Fig. S4).

**Re-stimulation of TIL using anti-CD3/CD28 beads, anti-CD3/-CD28/hIgG1 or anti-CD3/-CD28/PD-L1 beads**

T lymphocyte subsets were sorted based on PD-1 and Tim-3 expression from freshly isolated TIL from HNSCC.
patients, rested in 30% Human AB serum overnight and then subjected to re-stimulation experiments. Sorted TIL were cultured with Dynabeads® Human T-Activator CD3/CD28 beads at a fixed cell: bead ratio of 1:5 or anti-CD3/-CD28/hlgG1 and anti-CD3/-CD28/PD-L1 at a cell: bead ratio of 1:30. The cultures were incubated at 37°C with 5% CO2 for indicated time periods. Magnetic beads were removed before analysis.

**Quantitative Real-Time RT-PCR**

Sorted TIL subsets were either subjected to RNA extraction or stimulated with anti-CD3/CD28 beads for 6 h as described above and harvested before RNA extraction. Total RNA was extracted using Trizol reagent (Invitrogen 15596018) and purified using RNA cleanup (Qiagen), followed by Purelink on-column DNase digestion (Invitrogen 12185-010) according to manufacturer’s instructions. The concentration and purity of RNA was determined by measuring absorbance at 260 and 280 nm. RNA was used for first strand cDNA synthesis using random hexamers and MultiScribe reverse transcriptase (Applied Biosystems) according to manufacturer’s instructions. PCR probes for PRDM1 (Hs00153357_m1), BATF (Hs00232390_m1), EOMES (Hs00172872_m1), TBX21 (Hs00203436_m1), IFNG (Hs00989291_m1), TNF (Hs99999043_m1) and GUSB (Hs99999908_m1) were purchased from Applied Biosystem for TaqMan® Gene Expression Assay. Real-time PCR cycling was performed using StepOne™ Real-Time PCR Systems (Applied Biosystems). GUSB was amplified as an internal control. All of the experiments were performed in triplicate. Relative expression of the target genes to endogenous control gene (GUSB) was calculated using the ∆CT method: relative expression = 2^−∆CT, where ∆CT = C_T (target gene) − C_T (GUSB).

**IFNγ ELISPOT**

ELISPOT assays were performed as previously described. Briefly, MultiScreen-IP filter plates (Millipore MAIP54510) were coated with anti-human IFNγ mAb 1-D1K (Mabtech 3420-2H) (10 μg/mL in PBS) overnight at 4°C. Plates were washed with PBS and then blocked for 1 h at 37°C with RPMI supplemented with 10% human serum. Sorted cells were added to wells in duplicate (× 10^3) and then stimulated with anti-CD3/CD28 beads (bead: cell = 5:1), anti-CD3/-CD28/hlgG1 or anti-CD3/-CD28/PD-L1 beads (bead: cell = 30:1). Following an 18 h incubation at 37°C, plates were washed with PBS/0.5% Tween 20 (PBS-T), and incubated with biotinylated anti-IFNγ mAb (2 μg/mL in PBS/0.5% BSA) for 2 h at 37°C. Plates were washed with PBS-T and incubated with Streptavidin-HRP (1:500 in PBS/0.5% BSA) for 1 h at 37°C. Plates were washed with PBS-T and TMB substrate solution (Vector Laboratories SK-4400) was added. After distinct spots emerged, color development was stopped by washing extensively in tap water. Plates were dried and spots were counted using CTL ImmunoSpot® Analyzers (CTL) and analyzed by CTL Professional Double Color Software.

**Proliferation assay**

Sorted cells were labeled with CFSE using CellTrace CFSE Cell Proliferation Kit (Life Technologies C34554). Briefly, cells were incubated with 2 uM CellTrace CFSE in 1 mL PBS/0.1% BSA for 10 min at 37°C and then washed twice with complete medium. Then, the labeled cells were cultured anti-CD3/CD28 beads at a fixed cell: bead ratio of 1:5, anti-CD3/-CD28/hlgG1 or anti-CD3/-CD28/PD-L1 beads at a cell: bead ratio of 1:30 for 4 d. Dilution of CFSE was measured by flow cytometry.

**Cre-inducible FSF-Tim-3 mouse model**

A cDNA construct containing the BL/6 allele of murine Tim-3 (gene name Haver2), with an N-terminal Flag tag, was inserted into a targeting vector containing part of the Rosa26 locus (genOway S.A.). The resulting targeting construct contained floxed PGK-neo-PA “STOP” cassette upstream of the Flag-Tim-3 sequence. The STOP cassette was preceded by a hybrid CAG promoter to enhance transcription of the Flag-Tim-3 sequence after removal of the STOP cassette by Cre-mediated recombination. The targeting construct was transfected into BL/6 ES cells and clones with targeted recombination were selected (genOway S.A.). The resulting clones were then injected into BALB/C embryos for the generation of chimeraic animals, which were then bred to BL/6 mice to generate F1 offspring (U.C. Davis Mouse Biology Program). These animals were then maintained on a BL/6 background by breeding to C57BL/6 mice (Jackson).

**Cre-mediated induction of Tim-3 and T cell activation**

Naïve T cells from FSF-Tim-3 mice, or littermate controls, were treated overnight with 1.5 μM Tat-Cre (Excellgen). The next day, cells were stimulated with anti-CD3/CD28 antibodies for 1–4 h. Cells were then fixed and stained with an Alexa647-conjugated antibody to phospho-S6 (pS6(Ser235/236); Cell Signaling 5548). Flow cytometry was performed with a Becton Dickinson LSRFortessa flow cytometer, and data were analyzed with FlowJo (Treestar).

**T cell transfection and luciferase assays**

A fast-growing variant of the D10.G41 murine T cell clone was maintained and transfected as previously described. Cells were transfected with cDNA’s encoding the human allele of Tim-3 and/or PD-1, along with an NFAT/AP1-luciferase reporter construct. The next day, cells were analyzed for protein expression by flow cytometry or stimulated as indicated for 6 h. Luciferase activity was then determined on a 96-well plate luminometer (Promega).

**Statistical analysis**

Averages were calculated as means. p-values were calculated by Wilcoxon–Mann–Whitney tests or RM One-way ANOVA analysis followed by multiple comparisons using GraphPad Prism (GraphPad, La Jolla, CA). p-values < 0.05 were considered to be significant. *p < 0.05, **p < 0.01, ***p < 0.001.
Disclosure of potential conflicts of interest
Dr. Ferris reports research funding and advisory board service for Bristol-Myers Squibb, Merck, Astra-Zeneca/Medimmune and Celgene.

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Author contributions
JL was involved in concept, design and performing of experiments, and wrote the manuscript. GS was involved in performing experiments and revising the manuscript. LA performed experiments and provided feedback on the manuscript. HJ was involved in processing patient samples. NGL and NS helped collect patient samples from the clinic. BL and LPK were involved in experimental design and manuscript revision. RLF supervised the study, was involved in concept and design of experiments, critically involved in writing and editing of the manuscript. All authors read and approved the final manuscript.

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