Differential expression of the T-cell inhibitor TIGIT in glioblastoma and MS

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Abstract

Objective
To identify coinhibitory immune pathways important in the brain, we hypothesized that comparison of T cells in lesions from patients with MS with tumor-infiltrating T cells (TILs) from patients with glioblastoma multiforme may reveal novel targets for immunotherapy.

Methods
We collected fresh surgical resections and matched blood from patients with glioblastoma, blood and unmatched postmortem CNS tissue from patients with MS, and blood from healthy donors. The expression of TIGIT, CD226, and their shared ligand CD155 as well as PD-1 and PDL1 was assessed by both immunohistochemistry and flow cytometry.

Results
We found that TIGIT was highly expressed on glioblastoma-infiltrating T cells, but was near-absent from MS lesions. Conversely, lymphocytic expression of PD-1/PD-L1 was comparable between the 2 diseases. Moreover, TIGIT was significantly upregulated in circulating lymphocytes of patients with glioblastoma compared with healthy controls, suggesting recirculation of TILs. Expression of CD226 was also increased in glioblastoma, but this costimulatory receptor was expressed alongside TIGIT in the majority of tumor-infiltrating T cells, suggesting functional counteraction.

Conclusions
The opposite patterns of TIGIT expression in the CNS between MS and glioblastoma reflects the divergent features of the immune response in these 2 CNS diseases. These data raise the possibility that anti-TIGIT therapy may be beneficial for patients with glioblastoma.

*These authors contributed equally to this work.
Immune checkpoint receptors are a family of coinhibitory receptors that modulate T-cell activation. The interactions between coinhibitory receptors on tumor-infiltrating T cells and their ligands expressed by tumor cells is believed to contribute to the failure of the immune system to reject tumors. Therapeutic blockade of this interaction has yielded dramatic results in the therapy of multiple cancer types. To date, this has led to FDA approval of 6 immune checkpoint inhibitors that target cytotoxic T-lymphocyte–associated protein 4 (CTLA-4, ipilimumab) and programmed cell death protein 1 and its ligand (PD-1, pembrolizumab and nivolumab; PD-L1, atezolizumab, avelumab, and durvalumab).

To identify coinhibitory pathways that may be important in the CNS, we hypothesized that comparison of T cells in lesions from patients with the autoimmune disease MS with tumor-infiltrating T cells (TILs) of tumor from patients with glioblastoma multiforme (GBM) may reveal novel targets for immunotherapy in patients with CNS tumors. This approach was suggested by the role in coinhibitory and costimulatory pathways in T-cell regulation in preventing activation of autoreactive T cells and by the high incidence of autoimmune manifestations associated with therapeutic blockade of checkpoint inhibitors. We focused on the T-cell immunoreceptor with Ig and ITIM domains (TIGIT) axis, a recently discovered coinhibitory receptor expressed by activated T cells and natural killer cells, and on PD-1/PDL-1. TIGIT shapes T-cell function directly by repressing proinflammatory Th1 and Th17, but not Th2 responses, and indirectly by enhancing dendritic cell production of IL-10. TIGIT also prevents costimulatory signaling through CD226 by competing for the same ligand, CD155, and by disrupting CD226 homodimerization. Moreover, TIGIT is a marker of highly suppressive regulatory T cells (Tregs) and directly promotes Treg function in environments of Th1 inflammation. TIGIT has been investigated as a novel candidate target of cancer immunotherapy. Indeed, increased TIGIT has been demonstrated on tumor-infiltrating lymphocytes in a number of cancers including non–small-cell lung cancer and melanoma. Moreover, TIGIT blockade in animal models and in CD4 and CD8 T cells isolated from human tumors showed reinvigoration of antitumor immune responses. However, TIGIT blockade also has the potential for inducing autoimmune disease, as expression of the competing costimulatory receptor, CD226, is increased on peripheral T cells of patients with rheumatoid arthritis and lupus. In addition, a coding variant in the CD226 gene is associated with multiple autoimmune diseases, including MS and rheumatoid arthritis. Finally, TIGIT-deficient mice displayed increased susceptibility to developing experimental autoimmune encephalomyelitis (EAE), an animal model of MS, whereas treatment with a CD226-blocking monoclonal antibody delayed onset and reduced severity of EAE.

Here, we examined expression of TIGIT, CD226, their shared ligand CD155, and of PD-1 and its ligand PD-L1 in 2 prototypic neoplastic and autoimmune CNS diseases, glioblastoma and MS. Our data show that TIGIT+ T cells were highly prevalent in glioblastoma infiltrates but not in MS lesions, whereas the frequency of PD-1+ and PD-L1+ lymphocytes was comparable in the 2 conditions. Our findings highlight specific differences in immune checkpoint expression between glioblastoma and MS and provide a strong rationale for developing immunotherapy against TIGIT for glioblastoma.

Methods
Tissue and blood samples
Immunohistochemistry was performed on formalin-fixed tissue from 6 patients with MS (obtained through autopsy) and 6 patients with glioblastoma (resection/biopsy). Flow cytometry was performed on blood from 5 healthy volunteers and on freshly resected glioblastoma tissue and matched blood from 7 patients (1 tumor did not yield enough T cells for analysis, table).

Standard protocol approvals, registrations, and patient consents
All samples were collected according to institutional review board–approved protocols conformed to the principles of the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report; all patients and healthy volunteers gave informed consent.

Immunohistochemistry
Formalin-fixed paraffin-embedded sections were prepared for immunohistochemistry as described elsewhere. Serial sections were stained with primary antibodies against CD68 (Cell Signaling #76437, 1:500), MBP (Millipore Sigma MAB386, 1:500), CD3 (Dako A 40452, 1:200), TIGIT (Santa Cruz sc-103349, 1:800), CD226 (Novus Biologicals NB1-85001, 1:50), CD155 (Bioss Inc. bs-2525R, 1:750), and of PD-1 and its ligand PD-L1 in 2 prototypic neoplastic and autoimmune CNS diseases, glioblastoma and MS. Our data show that TIGIT+ T cells were highly prevalent in glioblastoma infiltrates but not in MS lesions, whereas the frequency of PD-1+ and PD-L1+ lymphocytes was comparable in the 2 conditions. Our findings highlight specific differences in immune checkpoint expression between glioblastoma and MS and provide a strong rationale for developing immunotherapy against TIGIT for glioblastoma.

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Table  Clinical data of patients with MS and GBM

1. Immunohistochemistry

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<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>PMI</th>
<th>Disease course/duration</th>
<th>Lesion types</th>
<th>Lesions examined</th>
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<tr>
<td>MS 1</td>
<td>68</td>
<td>Male</td>
<td>12 h</td>
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<td>MS 2</td>
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<td>3 h</td>
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<td>Chronic active</td>
<td>5</td>
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<tr>
<td>MS 3</td>
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<td>Male</td>
<td>8 h</td>
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<td>MS 4</td>
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<td>RRMS; 6 y</td>
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<tr>
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<td>RRMS; 6 y</td>
<td>Acute</td>
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<table>
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<tr>
<th>Case</th>
<th>Age</th>
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<th>PMI</th>
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<th>MGMT promoter methylation</th>
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<tr>
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<td>Female</td>
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<td>Unmethylated</td>
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<tr>
<td>GBM 2</td>
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<td>Male</td>
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<td>Partially methylated</td>
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<tr>
<td>GBM 3</td>
<td>40</td>
<td>Male</td>
<td>NA\textsuperscript{b}</td>
<td>Right parietal</td>
<td>Unmethylated</td>
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<tr>
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<td>Female</td>
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<td>Left posteriortemporal</td>
<td>ND</td>
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<tr>
<td>GBM 5</td>
<td>64</td>
<td>Male</td>
<td>NA\textsuperscript{b}</td>
<td>Left parietal</td>
<td>ND</td>
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<tr>
<td>GBM 6</td>
<td>43</td>
<td>Male</td>
<td>NA\textsuperscript{b}</td>
<td>Right temporal</td>
<td>ND</td>
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2. Flow cytometry and in vitro experiments

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<th>Case</th>
<th>Age</th>
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<th>PMI</th>
<th>Tumor location</th>
<th>IDH1 status/MGMT methylation</th>
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<td>GBM 9</td>
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<tr>
<td>GBM 10</td>
<td>40</td>
<td>Female</td>
<td>NA\textsuperscript{b}</td>
<td>Left cerebellar</td>
<td>Negative/methylated</td>
</tr>
<tr>
<td>GBM 11</td>
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<td>Negative/methylated</td>
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<td>Right parietal</td>
<td>Negative/unmethylated</td>
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<tr>
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<td>Female</td>
<td>NA\textsuperscript{b}</td>
<td>Right occipital</td>
<td>Negative/methylated</td>
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<tr>
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<td>Male</td>
<td>NA\textsuperscript{b}</td>
<td>Left frontal</td>
<td>Negative/unmethylated</td>
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</table>

<table>
<thead>
<tr>
<th>Case</th>
<th>Age</th>
<th>Sex</th>
<th>PMI</th>
<th>Tumor location</th>
<th>IDH1 status/MGMT methylation</th>
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<tr>
<td>GBM 16</td>
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<td>Female</td>
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<td>Right parietal</td>
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<tr>
<td>GBM 17</td>
<td>76</td>
<td>Female</td>
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<td>Right temporal-parietal</td>
<td>Negative/methylated</td>
</tr>
<tr>
<td>GBM 18</td>
<td>78</td>
<td>Male</td>
<td>NA\textsuperscript{b}</td>
<td>Left temporal</td>
<td>Negative/methylated</td>
</tr>
<tr>
<td>GBM 19</td>
<td>70</td>
<td>Male</td>
<td>NA\textsuperscript{b}</td>
<td>Right temporal</td>
<td>Negative/unmethylated</td>
</tr>
<tr>
<td>GBM 20</td>
<td>77</td>
<td>Female</td>
<td>NA\textsuperscript{b}</td>
<td>Left occipital</td>
<td>Negative/partially methylated</td>
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</tbody>
</table>

Abbreviation: GBM = glioblastoma multiforme. All GBM cases were negative for IDH mutations. \textsuperscript{a} Discovered at autopsy. \textsuperscript{b} Biopsy.
Cellular quantification
We identified multiple areas of lymphocytic infiltration both in the perivascular space and the parenchyma in MS lesion and GBM tissue sections that were immunolabeled with antibody against CD3. In GBM tissue, this included infiltrates in vital tumor tissue and in areas surrounding tissue necrosis. In MS tissue, the vast majority of infiltrates were present in perivascular spaces. In these regions of interest (ROIs), CD3+ cells were quantified manually. The same ROIs were selected in adjacent sections immunolabeled with antibodies against TIGIT, CD226, CD155, PD-1, or PDL-1. Immunolabeled cells with a lymphocytic morphology were manually quantified. We distinguished cells present in the perivascular space, i.e., within the borders of the glia limitans, and in the parenchyma. All cells were counted independently by 3 investigators (D.D., V.P.K., and G.P.), and the counts were averaged. The number of TIGIT–, CD226–, CD155–, PD-1–, or PDL-1–positive cells was correlated with the number of CD3+ lymphocytes found in each ROI.

Flow cytometry
Peripheral blood mononuclear cells were isolated from whole blood by Ficoll-Paque gradient centrifugation. Freshly resected tumor specimens were manually disrupted followed by digestion with collagenase IV (2.5 mg/mL) and DNase I (0.2 mg/mL) (Worthington Biochemical Corporation) for 1 hour. Tumor homogenates were separated on discontinuous 70%–30% Percoll (Sigma-Aldrich) gradients. Flow cytometric analysis was performed with antibodies targeting CD4 (BD Biosciences clone RPA-T4, V450 conjugated), CD8 (BD Biosciences clone RPA-T8, V500 conjugated), TIGIT (eBioscience clone MBSA43, RPA-T4, V450 conjugated), CD226 (eBioscience clone RX25, FITC conjugated), and PD-1 (BD Biosciences clone EH12.1, Alexa Fluor® 647 conjugated). Cell viability was assessed using Live/Dead Cell Viability Assays (Life Technologies). Samples were run on a BD LSRRFortessa or BD FACSAria II, as previously described. FlowJo software (Tree Star Inc.) was used for analysis after gating on live cells, with doublet exclusion followed by gating on CD4 and CD8 T cells.

In vitro proliferation assay with TIGIT blockade
Peripheral blood from patients with glioblastoma was obtained on the day of surgery, and mononuclear cells were isolated from whole blood by Ficoll-Paque gradient centrifugation and cryopreserved in GemCell human AB serum (GemBio) with 10% DMSO in liquid nitrogen. After thawing, cells were stained with LIVE/DEAD™ Fixable Dead Cell Stain kit (Invitrogen), aTIGIT (clone MBSA43 from eBioscience) and aCD226 (clone DX11 from eBioscience). Stained Tregs were washed and acquired on a BD Fortessa™ flow cytometer.

Statistics and data availability
Statistical analysis was performed in GraphPad Prism. When comparing 2 conditions within the same individual, we used paired 2-tailed t tests. For comparisons across 2 groups of unrelated individuals, we used unpaired 2-tailed t tests. p Values <0.05 were considered significant. All anonymized data will be shared by request with any qualified investigator.

Results
We examined the expression of TIGIT, CD226, CD155, PD-1, and PD-L1 in tumor-infiltrating T cells in glioblastoma and demyelinating lesions from patients with MS by immunohistochemistry. The percentage of TIGIT+ T cells was substantially higher in GBM infiltrates compared with MS lesions (figure 1, A and C). Similarly, the percentage of CD226+ lymphocytes was higher in glioblastoma than in MS, although cellular expression was overall low and had a punctate appearance. CD155, the ligand for TIGIT and CD226, was present at a low level in infiltrating lymphocytes in glioblastoma and MS, but was highly expressed by GBM tumor cells, perivascular monocytes and, to a lower degree, by cortical neurons (figure 1B). Finally, the percentage of infiltrating lymphocytes that expressed PD-1 and PD-L1 was similar in GBM and MS (figure 2, A and B). In addition, we compared the percentage of TIGIT+ and CD226-expressing lymphocytes in perivascular cuffs and in the lesion parenchyma. In GBM, we found that the percentage of TIGIT+ T cells was significantly higher in tumor tissue than in perivascular infiltrates. Furthermore, we observed a gradient in the opposite direction for CD226+ lymphocytes, with higher frequencies in perivascular cuffs compared with tumor parenchyma. In MS lesions, the prevalence of TIGIT+ and CD226+ lymphocytes did not differ significantly between perivascular and parenchymal infiltrates (figure 2C).

We used freshly resected (unfixed) tumor tissue from newly diagnosed patients with glioblastoma to examine expression of TIGIT and CD226 by tumor-infiltrating T cells with flow cytometry. We observed that the majority of CD8 but not CD4 T cells were TIGIT positive, whereas CD226 was expressed at higher rates by CD4 T cells compared with CD8 (figure 3, A and B). Moreover, because TIGIT and CD226 supplemented with 2 nM 1-glutamine, 5 mM HEPES, 100 U/μL penicillin/streptomycin (Biowhittaker), 0.5 mM sodium pyruvate, 0.05 mM nonessential amino acids (Life Technologies; complete RPMI), and 5% GemCell human AB serum (GemBio) with soluble agonistic αCD28 antibody (clone 28.2, BD Biosciences) at 1 μg/mL, and blocking αTIGIT antibody (clone 3F9, a kind gift of Pr. Vijay K. Kuchroo) at 20 μg/mL or isotype control (mouse IgG1 kappa, clone P3.6.2.8.1 from eBioscience) at 20 μg/mL. After 5 days, cells were stained with LIVE/DEAD™ Fixable Dead Cell Stain kit (Invitrogen), αTIGIT (clone MBSA43 from eBioscience) and aCD226 (clone DX11 from eBioscience). Stained Tregs were washed and acquired on a BD Fortessa™ flow cytometer.
have been reported to interact in cis, i.e., on the same cell,\textsuperscript{15} we assessed coexpression of the 2 molecules by flow cytometry and found that the majority of tumor-infiltrating CD8 T cells expressing CD226 were also positive for TIGIT (figure 3C).

We then compared TIGIT/CD226 expression in tumor-infiltrating and blood-derived lymphocytes of patients with glioblastoma and healthy controls. The frequency of TIGIT\textsuperscript{+} CD8 but not CD4 T cells was increased in tumor tissue compared with peripheral blood of patients with glioblastoma, whereas CD226\textsuperscript{+} CD8 lymphocytes were significantly reduced in the tumor compared with blood, where they coexpressed similar levels of TIGIT (figure 3D). Moreover, comparing the frequency of circulating TIGIT\textsuperscript{+} and CD226\textsuperscript{+} T cells between patients with glioblastoma and healthy controls highlighted a peripheral signature of the disease. Overall, patients with glioblastoma showed strong enrichment of TIGIT but not PD-1 expressing CD4 and CD8 T cells compared with healthy controls (figure 4). Although the frequency of CD226\textsuperscript{+} CD4 and CD8 T cells was not significantly different between patients and controls (figure 4C), CD226\textsuperscript{+} T cells from patients with glioblastoma coexpressed higher levels...
of TIGIT compared with healthy controls (figure 4, C and D).

Finally, we asked whether the enlarged TIGIT+ compartment in the circulation of patients with glioblastoma could be functionally modulated by TIGIT blockade. For this, we sorted CD4 T effectors (after removal of the CD25highCD127low Treg population) and CD8 T cells from the peripheral blood of 6 additional patients with glioblastoma and measured proliferation in response to αCD3/αCD28 stimulation in the presence of an αTIGIT blocking antibody or isotype control. When gating on TIGIT+ cells after 4 days of stimulation, we observed that TIGIT blockade conferred a significant increase in proliferation, which was more pronounced in CD4 than CD8 T cells and in cultures with low proliferation at baseline. Moreover, blocking TIGIT also induced heightened expression of CD226 on TIGIT+ cells (figure 5, A and B).

**Discussion**

Immune checkpoint receptors are a family of co-inhibitory receptors that modulate T-cell activation. The interactions between co-inhibitory receptors on tumor-infiltrating T cells and their ligands expressed by tumor cells are believed to contribute to the failure of the immune system to reject tumors.1,2 Although therapeutic blockade of this interaction has yielded dramatic results in the therapy of multiple cancer types, therapeutic trials with the immune checkpoint inhibitors anti-PD-1 and CTLA-4 in patients with glioblastoma have not been successful.25 This suggests that PD-1 signaling might be redundant in the tumor microenvironment of glioblastoma, where additional co-inhibitory pathways may be operative. To identify co-inhibitory pathways that are important in the brain, we hypothesized that comparison of T cells in lesions from patients with MS with TILs from patients with...
glioblastoma may reveal novel targets for immunotherapy of brain tumors.

Previous studies have reported frequencies of TIGIT+ tumor-infiltrating lymphocytes in glioblastoma (25%–60%), similar to the ones observed by us, although these studies have not addressed whether TIGIT expression on T cells occurs in any CNS environment or is a feature specific to the glioblastoma infiltrate. Here, we report that TIGIT-expressing lymphocytes were substantially higher in glioblastoma infiltrates than in normal white matter and MS lesions. Given the abundant expression of the TIGIT ligand, CD155, on glioblastoma cells, this suggests that TIGIT signaling critically limits antitumor responses in GBM. In contrast, the relative absence of TIGIT/CD155 in normal white matter and MS lesions indicates that TIGIT signaling does not occur constitutively in the CNS. Moreover, PD-1/PD-L1+ lymphocytes were present in both conditions, indicating that PD-1 signaling is not a distinguishing feature between inflammatory responses in GBM and MS.

Although we observed a greater percentage of CD226 expressing lymphocytes in tumor infiltrates compared with MS lesions, its coexpression with TIGIT is likely to disrupt CD226 homodimerization and thereby renders CD226 nonfunctional. In contrast, the nearly absent expression rate of TIGIT in infiltrating lymphocytes in MS lesions suggests low TIGIT/CD226 coexpression, resulting in undisrupted CD226 function.
The increased percentage of TIGIT+ lymphocytes in tumor parenchyma compared with perivascular infiltrates, the high expression CD155 in glioblastoma cells, and the relative decrease of CD226+ T cells within the tumor tissue all suggest that CD155-induced TIGIT signaling is most pronounced in direct proximity to tumor tissue. Further investigations will elucidate whether the tumor microenvironment locally induces upregulation of TIGIT and downregulation of CD226 or preferentially attracts and retains TIGIT+CD226− lymphocytes.

Finally, TIGIT expression was increased in peripheral T cells in patients with glioblastoma compared with healthy controls, whereas expression of CD226 was decreased. This novel observation could indicate a systemic leakage of TIGIT-inducing factor and/or recirculation of TIGIT+ T cells between the periphery and the tumor bed. Trafficking of T cells between tumor and periphery would present an opportunity to gain insight into induction of TIGIT expression, to monitor the state of tumor-infiltrating lymphocytes, and to block TIGIT activity peripherally, thereby circumventing the obstacle of the blood-brain barrier for therapeutic antibodies. Indeed, circulating TIGIT+ T cells from patients with GBM stimulated in vitro in the presence of a blocking αTIGIT antibody displayed increased proliferation and expression of...
CD226, an effect that was more pronounced in CD4 than CD8 T cells. The elevated expression of TIGIT but only moderate response to TIGIT blockade of circulating CD8 T cells could indicate that these cells represent a terminally differentiated subpopulation with limited self-renewal and reinvigoration properties. Moreover, although cytotoxic CD8 T cells can directly kill tumor cells, mechanisms other than intrinsic coinhibitory receptor signaling can control their cytotoxic functions. Indeed, recent studies in melanoma models have shown that CD4 T cell help is needed for CD8 T cells to acquire cytotoxic functions that mediate tumor rejection\textsuperscript{27} and that the effect of TIGIT blockade in vivo was mediated predominantly by CD4\textsuperscript{+} Tregs but resulted in increased proinflammatory activity of CD8 T cells.\textsuperscript{28}

In summary, our study provides evidence for a critical role for the CD155/TIGIT axis on T cells in immune evasion of glioblastoma. Further work is warranted to elucidate the role of these molecules on the function of other immune cell populations capable of tumor rejection, such as NK cells. Together with existing studies, our results contribute a mechanistic rationale for the development of immunotherapy that targets TIGIT signaling in GBM, either alone or in combination with anti PD-1 or anti-CTLA-4 checkpoint inhibition.

Limitations of our study include that we did not explore expression of CD112, a low-affinity ligand for TIGIT and CD226. Further work exploring the pattern of expression of CD112 might help quantify the overall level of engagement that each receptor receives in the tumor microenvironment. Moreover, our staining experiments have been performed with brightfield single-antibody labeling rather than fluorescent double labeling, which allows only an estimate of the percentage of checkpoint receptor/ligand...
expressing lymphocytes but permits a better assessment of cells within the lesion environment.

Acknowledgment
The authors thank the operating room staff at Yale-New Haven Hospital and Jonathan Cruz for assistance in obtaining patient samples.

Study funding
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Disclosure
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Appendix

### Authors

<table>
<thead>
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<tbody>
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### References


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Liliana E. Lucca, Benjamin A. Lerner, Calvin Park, et al.

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