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Functional Anti-TIGIT Antibodies Regulate Development of Autoimmunity and Antitumor Immunity

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Coinhibitory receptors, such as CTLA-4 and PD-1, play a critical role in maintaining immune homeostasis by dampening T cell responses. Recently, they have gained attention as therapeutic targets in chronic disease settings where their dysregulated expression contributes to suppressed immune responses. The novel coinhibitory receptor TIGIT (T cell Ig and ITIM domain) has been shown to play an important role in modulating immune responses in the context of autoimmunity and cancer. However, the molecular mechanisms by which TIGIT modulates immune responses are still insufficiently understood. We have generated a panel of monoclonal anti-mouse TIGIT Abs that show functional properties in mice in vivo and can serve as important tools to study the underlying mechanisms of TIGIT function. We have identified agonistic as well as blocking anti-TIGIT Ab clones that are capable of modulating T cell responses in vivo. Administration of either agonist or blocking anti-TIGIT Abs modulated autoimmune disease severity whereas administration of blocking anti-TIGIT Abs synergized with anti–PD-1 Abs to affect partial or even complete tumor regression. The Abs presented in this study can thus serve as important tools for detailed analysis of TIGIT function in different disease settings and the knowledge gained will provide valuable insight for the development of novel therapeutic approaches targeting TIGIT. ***The Journal of Immunology*****, 2018, 200: 3000–3007.**

Coinhibitory receptors play an essential role in maintaining immune homeostasis. Loss of coinhibitory receptor expression can lead to autoimmunity whereas persistent expression can suppress effective immunity in the setting of chronic viral infection or cancer. Blocking Abs to coinhibitory receptors PD-1 and CTLA-4 are being harnessed clinically to improve antitumor T cell responses. In addition to these well-studied coinhibitory molecules, we have been studying a number of novel coinhibitory molecules that regulate autoimmunity and antitumor immunity. TIGIT (T cell Ig and ITIM domain; also called Vsig9, Vstm3, or WUCAM), one of these novel coinhibitory receptors, is expressed on NK cells, activated T cells, memory T cells, and a subset of regulatory T cells (Tregs), and was shown to inhibit immune responses by affecting T cells and APCs (1–3). We (1, 2, 4) and others (5, 6) have recently shown that TIGIT also plays an important role in modulating immune responses in the context of autoimmunity and cancer. TIGIT shares its two ligands, CD155 (PVR) and CD112 (PVRL2, nectin-2), with the costimulatory molecule CD226 and together they comprise a pathway that strongly resembles the well-known B7/CD28/CTLA-4 pathway (3, 6, 7). Similar to CTLA-4, TIGIT can inhibit immune responses by delivering inhibitory signals to effector T and NK cells, by inducing tolerogenic dendritic cells, and by enhancing the suppressive capacity of Tregs (1–3).

The TIGIT/CD226 pathway has been linked to multiple autoimmune diseases in humans including type 1 diabetes, multiple sclerosis (MS), and rheumatoid arthritis (8, 9). Indeed, data from experimental models have confirmed the protective role of TIGIT in autoimmune settings. TIGIT$^{-/-}$ mice show increased susceptibility to experimental autoimmune encephalomyelitis (EAE),

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Abbreviations used in this article: EAE, experimental autoimmune encephalomyelitis; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; TIGIT, T cell Ig and ITIM domain; TIL, tumor-infiltrating lymphocyte; Treg, regulatory T cell.

a mouse model of the human disease MS (1, 6). Furthermore, TIGIT blockade leads to exacerbation of collagen-induced arthritis and graft-versus-host disease (6). In contrast, TIGIT has been shown to suppress antitumor immunity because loss of TIGIT enhances antitumor immunity and results in improved tumor control (4, 5). In this regard, a number of recent publications indicate that TIGIT synergizes with the PD-1/PD-L1 pathway in promoting $CD8^+$ T cell dysfunction, thereby directly impairing protective antitumor T cell responses (5, 10). In addition, TIGIT can further suppress antitumor immunity indirectly through promotion of Treg function in tumor-infiltrating lymphocytes (TILs) (4).

Therapeutic targeting of the CTLA-4 and PD-1 pathways is able to improve $CD8^+$ T cell function and has shown remarkable efficacy in treating several human cancers. However, CTLA-4 and PD-1 Ab blockade results in stable responses in only ~10–30% of patients, and a number of human cancers are refractory to treatment with these two Abs. Due to its expression on TILs and its ability to synergize with the PD-1/PD-L1 pathway, TIGIT has recently received much attention as a potential therapeutic target for promoting antitumor immunity and inducing tumor regression (4, 5, 10, 11). However, the molecular mechanisms by which TIGIT modulates immune responses are still insufficiently understood and tools to modulate the TIGIT pathway in vivo have been lacking.

We have generated a panel of functional anti-TIGIT Abs that can serve as a tool to study the underlying mechanisms of TIGIT function in detail. We have identified and characterized different clones of Ab-producing hybridomas (agonistic and blocking) that are able to modulate T cell responses in vivo. Importantly, functional modulation of immune responses by these anti-TIGIT Abs translates into differences in disease severity and progression in models of autoimmunity and cancer. The monoclonal anti-TIGIT Abs we have generated thus represent a valuable set of tools that allows us to deepen our understanding of the underlying mechanisms by which TIGIT modulates immune responses in vivo in the context of disease.

Materials and Methods

Animals

$TIGIT^{-/-}$ and TIGIT transgenic mice were obtained from ZymoGenetics (Seattle, WA). C57BL/6 (B6) and BALB/c mice were purchased from the Jackson Laboratory, Janvier, or were bred at the Cox-7 gnotobiotic animal facility operated by the Edwin L. Steele Laboratory at Massachusetts General Hospital. Armenian hamsters were purchased from Harlan (Indianapolis, IN). Animals were kept in a conventional, pathogen-free facility at the Harvard Institutes of Medicine (Boston, MA), Massachusetts General Hospital, or at the Laboratory Animal Services Center (Zurich, Switzerland) and all experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at Harvard Medical School, Massachusetts General Hospital, or institutional policies at the Laboratory Animal Services Center and have been reviewed by the cantonal veterinary office.

Generation of anti-TIGIT Abs

Armenian hamsters and $TIGIT^{-/-}$ mice were immunized with recombinant mouse TIGIT tetramers (ZymoGenetics) by a combination of s.c. and footpad injections as described previously (1). Briefly, animals were immunized with 50 μg of TIGIT tetramers in CFA on day 0, followed by booster injections of 25 μg protein on days 3, 10, and 18 and 25 μg protein in IFA on days 7 and 14. Draining lymph nodes were collected on day 21 and cells were fused with Sp2/0-Ag14 cells using polyethylene glycol 1450 and selected in hypoxanthine/aminopterin/thymidine medium. The supernatants were screened for specific binding by anti-TIGIT ELISA and by flow cytometry using P815 cells transfected with mouse TIGIT and primary mouse splenocytes activated with plate-bound anti-CD3 (2 μg/ml) for 48 h. All hybridoma wells that showed TIGIT-specific binding were expanded and subcloned four times by limiting dilution and single colonies were sorted by flow cytometry. Binding specificity of the Ab-producing hybridomas was confirmed by staining of activated primary TIGIT-expressing wild-type T cells and $TIGIT^{-/-}$ T cells as controls. Anti-TIGIT clone 4D4 is an Armenian hamster IgG Ab, and anti-TIGIT clones 1G9 and 1B4 are mouse IgG1 Abs.

Blocking of CD155 binding to TIGIT was determined on P815 cells transfected with mouse TIGIT (ZymoGenetics). P815 TIGIT cells were incubated with anti-TIGIT or isotype control Abs (Armenian hamster IgG for clone 4D4 or mouse IgG1 for 1G9, 1B4, and 2F6) for 30 min, followed by incubation with recombinant mouse CD155 for 30 min, staining with anti-CD155, and analysis by flow cytometry. Inhibition of CD155 binding was calculated as 100 − (% of $CD155^+$ in sample / % of $CD155^+$ in "no Abs" sample) × 100.

In vitro stimulations

$CD8^+$, $CD4^+Foxp3^-$, and $CD4^+Foxp3^+$ cells were sorted from *Foxp3*-GFP reporter mice (12) and 4×10^4 cells per well ($CD8^+$and $CD4^+$) or 2×10^4 cells per well ($CD4^+Foxp3^+$) were seeded into U-bottom 96-well plates and treated with 25 μg/ml anti-TIGIT (clones 4D4, 1G9, and 1B4) or isotype control (mouse IgG1, Armenian hamster IgG) Abs together with 4×10^4 mouse T activator CD3/CD28 Dynabeads (Life Technologies) per well. For the culture of $CD4^+Foxp3^+$ cells, 3 ng/ml TGF-β (RayBiotech) and 20 U/ml IL-2 (BioLegend) were added to the culture medium. After 48 h, [^{3}H]thymidine (PerkinElmer) was added for the last 18–22 h before [^{3}H]thymidine incorporation was analyzed to assess proliferation. Where indicated, cells were harvested after 48 h, RNA was extracted with the ReliaPrep RNA Tissue Miniprep System (Promega), cDNA was prepared using the GoScript Reverse Transcriptase kit (Promega), and expression was analyzed by real-time PCR (RT-PCR) using a CFX384 Cycler (Bio-Rad Laboratories); *Il10* was analyzed by TaqMan (*Il10*: Mm01288386_m1 and *Actb*: Mm00446968_m1; Applied Biosystems), and *Ifng* was analyzed with SYBR Green assay. Primer sets used were: *Ifng* 5′-GCATTCATGAGTATTGCCAAG-3′ and 5′-GGTGGACCACTCGGATGA-3′, *PolII* 5′-CTGGTCCTTCGAATCCGCATC-3′ and 5′-GCTCGATACCCTGCAGGGTCA-3′. *Ifng* expression was normalized to *PolII* and *Il10* expression to *β-actin*. Relative expression was calculated as $2^{-dCT} \times 10^4$.

Immunizations

Mice were immunized s.c. into the flanks with 200 μl of an emulsion containing 100 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35–55}$ peptide (MEVGWYRSPFSRVVHLYRNGK) and 250 μg of *Mycobacterium tuberculosis* extract H37 Ra (Difco) in adjuvant oil (CFA) on day 0 and treated i.p. with 100 μg of anti-TIGIT (clone 4D4, 1G9, or 1B4) or isotype control (Armenian hamster IgG or mouse IgG1) on days 0, 2, and 4. For Ag-specific proliferation assays, spleens and lymph nodes were collected on day 10 and 2.5×10^6 cells/ml were restimulated in the presence of a range of concentrations of $MOG_{35–55}$ peptide (0.01–50 μg/ml). After 48 h, culture supernatants were collected for the determination of secreted cytokines and cells were pulsed with [^{3}H]thymidine for an additional 18 h before [^{3}H]thymidine incorporation was analyzed. Cytokine concentrations in culture supernatants were determined by cytometric bead array (BD Biosciences) according to the manufacturer's instructions.

Induction of EAE

EAE was induced by s.c. immunization of mice into the flanks with 200 μl of an emulsion containing either 100 μg of $MOG_{35–55}$ peptide in CFA (optimal conditions) or 10–15 μg of $MOG_{35–55}$ peptide in CFA (suboptimal conditions). In addition, the mice received 100 ng of pertussis toxin (List Biological Laboratories) i.v. on days 0 and 2 and were treated i.p. with 100 μg of anti-TIGIT (clone 4D4, 1G9, or 1B4) or isotype control (Armenian hamster IgG or mouse IgG1) on days 0, 2, 4, 10, and 17. Clinical signs of EAE were assessed according to the following scores: 0, no signs of disease; 1, loss of tone in the tail; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; 5, moribund. Mice were sacrificed for flow cytometric and histopathological analysis at day 14 (4D4, 1G9, and respective isotype controls) or day 17 (1B4 and isotype control) after disease induction.

For histopathology, brains and spinal cords were fixed in 10% neutral buffered formalin and processed routinely for paraffin embedment. Slides were stained with Luxol fast blue H&E stains. Inflammatory foci (>10 mononuclear cells) were counted in leptomeninges and parenchyma in a blinded fashion in that the pathologist was unaware of the clinical status and treatment that the mice had received. For flow cytometric analysis, brain and spinal cord of perfused mice were cut into pieces, digested for 30 min at 37°C with collagenase D (2.5 mg/ml; Roche) and DNase I (1 mg/ml; Sigma-Aldrich), passed through a 70-μm filter, and mononuclear cells were isolated over a 37%/70% Percoll gradient (GE Healthcare) as described previously (13).

Flow cytometry

For intracellular cytokine staining, single cell suspensions from the indicated organs were restimulated using PMA (50 ng/ml; Sigma-Aldrich), ionomycin (1 μM; Sigma-Aldrich), and monensin (GolgiStop; BD Biosciences) for 4 h at 37°C, before staining and fixation/permeabilization was performed using the BD Fixation/Permeabilization Solution kit (BD Biosciences). In the glioblastoma model, single cell suspensions of the cervical lymph nodes were stimulated with Leukocyte Activation Cocktail (BD Biosciences) for 4 h at 37°C and then stained for intracellular cytokines. Fluorescently labeled Abs were from BioLegend, or eBioscience (anti-Foxp3). Samples were acquired on a FACSCalibur, LSR II, or Fortessa flow cytometer (BD Biosciences) or sorted on BD FACS Aria III 5L (BD Biosciences) and analyzed using FlowJo software (FlowJo).

Tumor experiments

A total of 1×10^6 MC38 cells were implanted s.c. into the right flank of C57BL/6 mice. When tumor size reached ~25 mm^2, mice were randomized and treated as follows: anti–PD-1 (RMP1-14) and its isotype control (rat IgG) were given as a single administration of 100 μg i.p. Two hundred micrograms of anti-TIGIT (1B4) or isotype control (mouse IgG1) were given i.p. every third day for a total of four shots. Tumor size was measured in two dimensions by caliper and is expressed as the product of two perpendicular diameters.

A total of 1×10^5 Gl261-GFP-Gluc cells were stereotactically implanted in the brains of 6–8-wk-old male or female C57BL/6 mice. Tumor size was assessed by measuring circulating Gaussia luciferase (Gluc) activity in the blood (14). Treatment was initiated in size-matched tumors after achieving sizes of ~1 mm^3, and tumor burden was measured over time by serial blood Gluc measurements. Animals were treated with IgG ($n = 14$), anti-TIGIT (1B4, $n = 12$), anti–PD-1 (RMP1-14, $n = 12$), or anti-TIGIT + anti–PD-1 ($n = 12$ mice) every 3 d. The anti-TIGIT Ab was administered four times at 200 μg per mouse; anti–PD-1 was given as one dose of 500 μg per mouse followed by five injections of 250 μg per mouse. Animals were sacrificed when clinical symptoms developed or mice lost more than 20% of their initial body weight.

In rechallenge experiment, mice treated with anti-TIGIT + anti–PD-1 that initially rejected tumors or naive mice as controls were implanted with 1×10^5 Gl261-GFP-Gluc cells in the contralateral hemisphere. One hundred days post-rechallenge mice were sacrificed and splenocytes and cervical lymph node cells were analyzed by flow cytometry. Moribund tumor-bearing mice that had been naive served as controls for comparison.

Results

Generation and properties of anti-TIGIT Abs

To be able to modulate TIGIT function in multiple experimental models in vivo, we set out to generate a set of monoclonal anti-TIGIT Abs that would have agonistic or antagonistic (blocking) properties. We had previously reported the generation of an agonistic anti-TIGIT Ab in Armenian hamster that inhibits T cell proliferation in vitro (clone 4D4, Armenian hamster IgG) (1). However, because of the species difference, this clone is not suitable for prolonged in vivo treatment of mice. We thus generated a second panel of monoclonal mouse anti-TIGIT Abs by immunizing $TIGIT^{-/-}$ mice with recombinant mouse TIGIT tetramers. Positive clones were selected by specific binding in ELISA (Fig. 1A) as well as by specific staining of activated primary T cells. We then tested the ability of positive clones to block ligand binding in a competition assay with recombinant CD155, the high affinity ligand of TIGIT. Two clones (1G9 (1) and 1B4, both mouse IgG1) were able to fully block CD155 binding, as did the previously reported clone 4D4 (1) (Fig. 1B). The fact that the 1G9 and 1B4 Ab clones both block CD155 binding indicated that they recognize similar epitopes on TIGIT. Indeed, Ab competition experiments support that these Abs interfere with each other's binding and thus bind to the same or overlapping epitopes on TIGIT (Fig. 1C). In addition, their ability to compete with CD155 for TIGIT binding suggests that they might show functional properties in vivo.

We next addressed whether the 1G9 and 1B4 Ab clones could deplete $TIGIT^+$ T cells in vivo under steady-state conditions. For this we took advantage of TIGIT transgenic mice that constitutively express high levels of TIGIT on peripheral $CD4^+$ and $CD8^+$ T cells (6). We administered 1G9, 1B4, or mouse IgG1 isotype control Abs to TIGIT transgenic mice and assessed the number of $CD4^+$ and $CD8^+$ T cells 48 h later. We found that administration of 1G9 and 1B4 had no effect on the total number of $CD4^+$ or $CD8^+$ T cells (Fig. 1D). This is in line with the inability of mouse IgG1 to promote Ab-dependent cell-mediated cytotoxicity. Thus, the anti-TIGIT Ab clones 1G9 and 1B4 block CD155 binding but do not deplete $TIGIT^+$ cells in vivo under steady-state conditions.

Anti-TIGIT Abs modulate T cell responses in vivo

Next we addressed whether the hamster anti-mouse TIGIT Ab clone 4D4 and the two mouse anti-mouse clones 1G9 and 1B4 might have functional properties in vitro and in vivo. TIGIT has T cell intrinsic inhibitory properties in that it inhibits T cell proliferation and production of proinflammatory cytokines, such as IFN-γ, while enhancing IL-10 production (1, 2). So to test the functional properties of our anti-TIGIT Abs in vitro, we sorted $CD4^+Foxp3^-$ effector T cells and $CD4^+Foxp3^+$ Tregs, stimulated them with anti-CD3/anti-CD28 beads in vitro, and tested how the anti-TIGIT Abs affected cell proliferation and cytokine production. As described previously (1), we could observe decreased proliferation in the presence of clone 4D4, suggesting that it acts as an agonistic anti-TIGIT Ab. However, clones 1G9 and 1B4 did not affect T cell proliferation in vitro (Fig. 2A). Anti-TIGIT clone 4D4 also enhanced *Il10* transcription in $CD4^+Foxp3^+$ Tregs but had no effect on *Ifng* transcription in effector T cells (Fig. 2B, 2C). Although anti-TIGIT clones 1G9 and 1B4 did not affect T cell proliferation in vitro, the presence of clone 1B4 resulted in increased *Ifng* transcription in $CD4^+Foxp3^-$ effector T cells and decreased *Il10* levels in $CD4^+Foxp3^+$ Tregs (Fig. 2B, 2C), indicating that it might act as a TIGIT blocking Ab. Anti-TIGIT clone 1G9 had no effect on *Ifng* expression but slightly enhanced *Il10* transcription in $CD4^+Foxp3^+$ Tregs, although this increase did not reach significance (Fig. 2B, 2C).

As functional properties of Abs might differ in vitro and in vivo, we next tested how our anti-TIGIT clones affected T cell function in vivo. We immunized mice with $MOG_{35–55}$ peptide in CFA and treated them with the anti-TIGIT Abs or the appropriate isotype control on days 0, 2, and 4. After 10 d, cells from spleens and draining lymph nodes were isolated and restimulated with $MOG_{35–55}$ peptide in vitro. Treatment with clone 4D4 strongly reduced dose-dependent proliferation upon restimulation with antigenic peptide (Fig. 3A). In line with this finding, $MOG_{35–55}$-restimulated splenocytes and lymph node cells from 4D4-treated mice produced lower levels of the proinflammatory cytokines IFN-γ and IL-17 (Fig. 3B). Similarly, mice treated with clone 1G9 also displayed reduced Ag-specific proliferation upon restimulation, although to a lesser degree than mice treated with clone 4D4 (Fig. 3C). Nevertheless, a similar reduction in the secretion of IFN-γ and IL-17 could be observed in 1G9-treated mice (Fig. 3D). In contrast, treatment with clone 1B4 resulted in enhanced Ag-specific proliferation concomitant with increased secretion of IFN-γ and IL-17 (Fig. 3E, 3F). Importantly, for all three clones, Ab treatment had no effect on cell composition, as frequencies and total numbers of lymphocytes in the anti-TIGIT– or isotype control–treated animals were comparable (Supplemental Fig. 1). Taken together, these results indicate that all three anti-TIGIT clones show functional properties in vivo and are capable of modulating T cell responses by affecting their magnitude and the cytokine profile. Although clones 4D4 and 1G9 appear to have agonistic anti-TIGIT activity, clone 1B4 acts as a TIGIT blocking Ab in vivo.

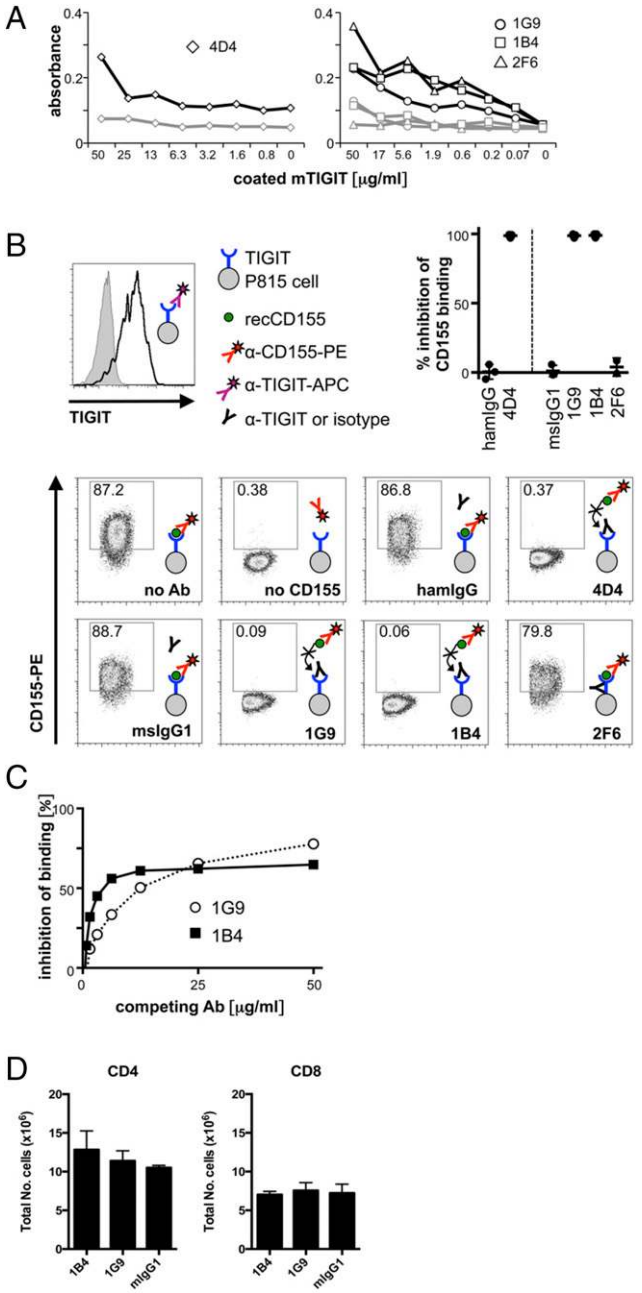


FIGURE 1. Anti-TIGIT Abs compete with CD155. TIGIT-specific Abs were generated in Armenian hamster (clone 4D4) or TIGIT$^{-/-}$ mice (clones 1G9, 1B4, and 2F6). (**A**) Anti-TIGIT Abs were titrated in an ELISA against recombinant mouse TIGIT (black) or a control (gray) protein. (**B**) P815 cells transfected with mouse TIGIT that uniformly express TIGIT (top left) were incubated with anti-TIGIT or isotype control Abs (Armenian hamster IgG for clone 4D4 or mouse IgG1 for 1G9, 1B4, and 2F6). Samples were then incubated with recombinant mouse CD155, then stained with anti-CD155 and analyzed by flow cytometry. A sample not incubated with CD155 was used as a positive control for blocking of CD155 binding. Representative FACS plots and summary data of inhibition of CD155 binding of three to five independent experiments are shown. (**C**) TIGIT-expressing splenocytes from TIGIT transgenic mice were incubated with purified 1G9 or 1B4 at the concentrations indicated for 15 min, then washed, and stained with 1G9-PE. The inhibition of binding of 1G9-PE is shown. (**D**) Two hundred micrograms of purified 1G9, 1B4, or mouse IgG1 were administered i.p. to TIGIT transgenic mice. Forty-eight hours later spleens were harvested and the presence of CD4$^+$ and CD8$^+$ T cells analyzed by flow cytometry. The average of two independent experiments is shown (n = 2 per group).

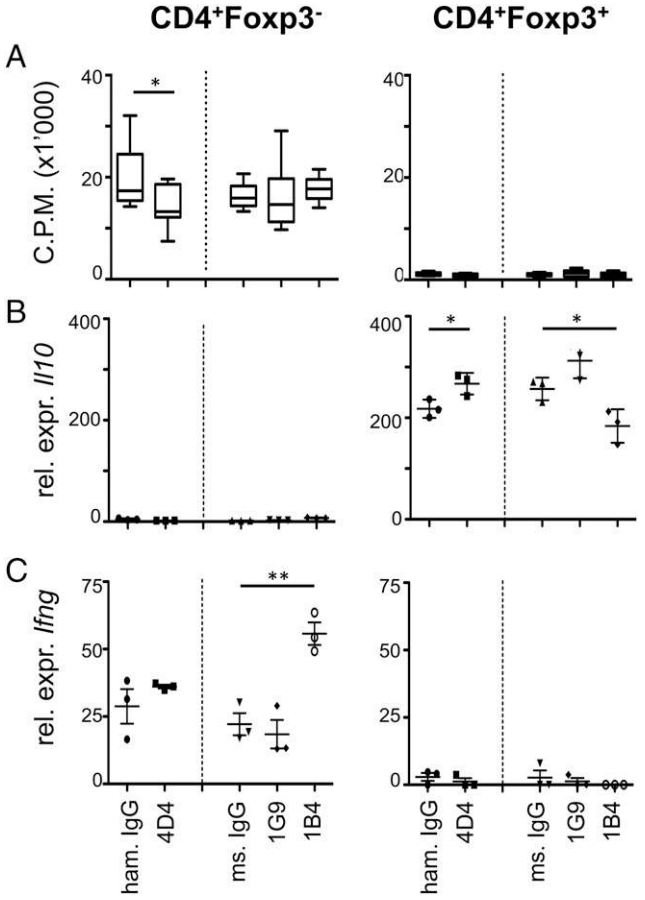


FIGURE 2. Anti-TIGIT Abs show limited functional properties in vitro. CD8$^+$, CD4$^+$, and CD4$^+$Foxp3$^+$ cells were sorted from *Foxp3*-GFP reporter mice; 4×10^4 cells per well (CD8$^+$ and CD4$^+$) or 2×10^4 cells per well (CD4$^+$Foxp3$^+$) were seeded into 96-well plates and stimulated with 4×10^4 CD3/CD28 Dynabeads in the presence of 25 μg/ml anti-TIGIT (clones 4D4, 1G9, and 1B4) or isotype control (Armenian hamster IgG, mouse IgG1) Abs. (**A**) Proliferation was measured after 48 h based on [^{3}H]thymidine incorporation. Pooled data of two to three independent experiments is shown, mean ± SEM (CD8$^+$and CD4$^+$ n = 9; CD4$^+$Foxp3$^+$ n = 6). (**B** and **C**) After 48 h, cells were harvested and expression of (B) *Il10* and (C) *Ifng* mRNA was determined by quantitative RT-PCR (representative plot, n = 3, mean ± SD). $^*p < 0.05$, $^{**}p < 0.01$ (Student t test).

Functional anti-TIGIT Abs modulate disease severity in EAE

As all three anti-TIGIT Ab clones were able to modulate T cell responses in vivo, they were next tested for their ability to affect the development of T cell driven autoimmunity. Mice were immunized with $MOG_{35–55}$ peptide in CFA to induce EAE and treated with an anti-TIGIT Ab (or the appropriate isotype control). In line with the observed reduction in Ag-specific T cell responses, treatment with the agonistic clones 4D4 and 1G9 was able to reduce EAE severity (Fig. 4A, 4B). Interestingly, clone 1G9 had a similar if not stronger beneficial effect in this setting despite its slightly reduced potency in modulating the recall T cell responses (Fig. 3). This is most likely due to decreasing activity of the hamster Ab 4D4 in this prolonged experimental setting, highlighting the importance of species-specific tools for studies in long-term chronic disease models. We reasoned that treatment with the blocking Ab clone 1B4 would result in a similar phenotype as observed in TIGIT$^{-/-}$ mice, where, in contrast to wild-type mice, suboptimal doses of $MOG_{35–55}$ are sufficient to induce full-blown EAE (1). Indeed, 1B4-treated mice developed

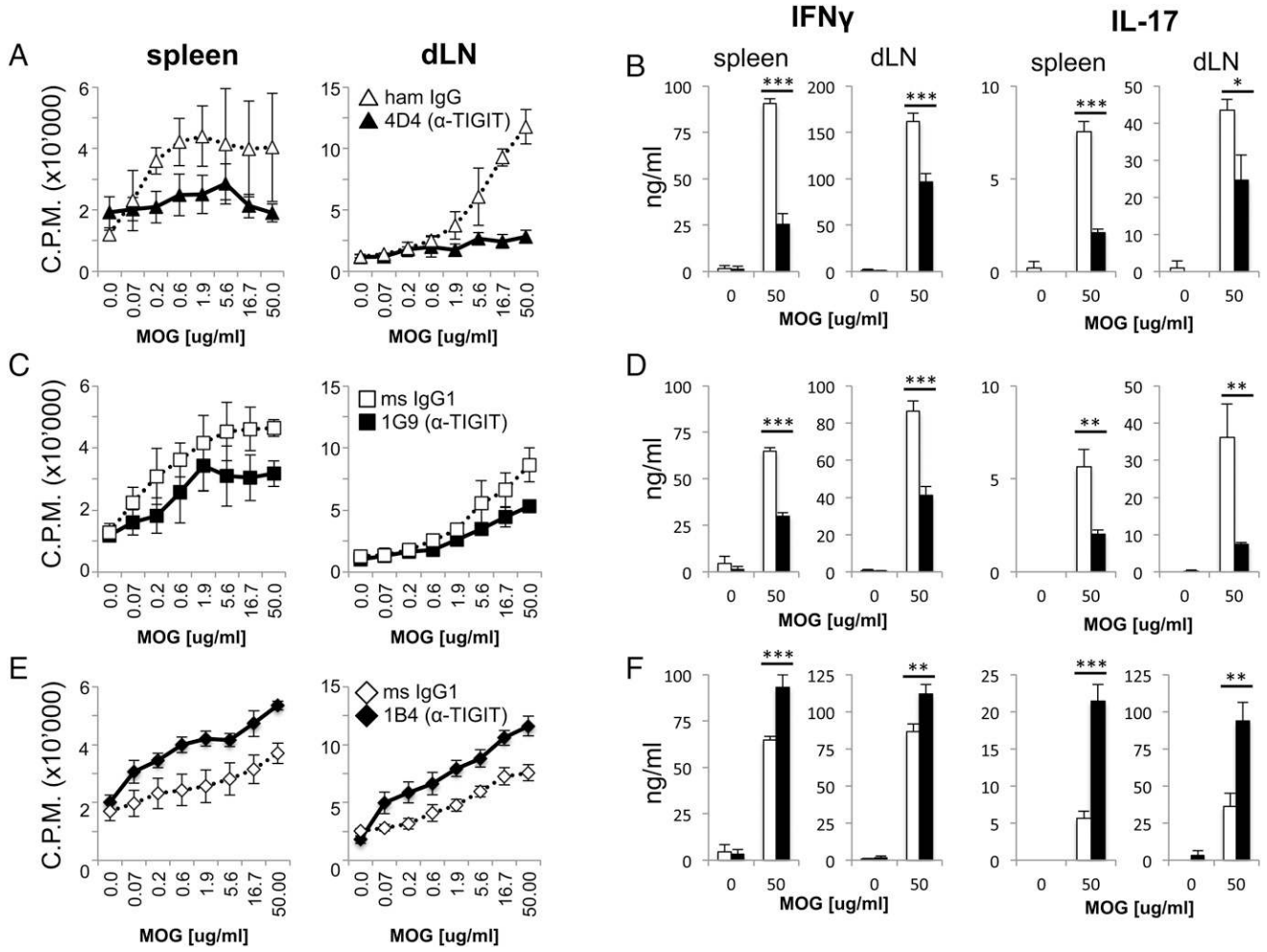
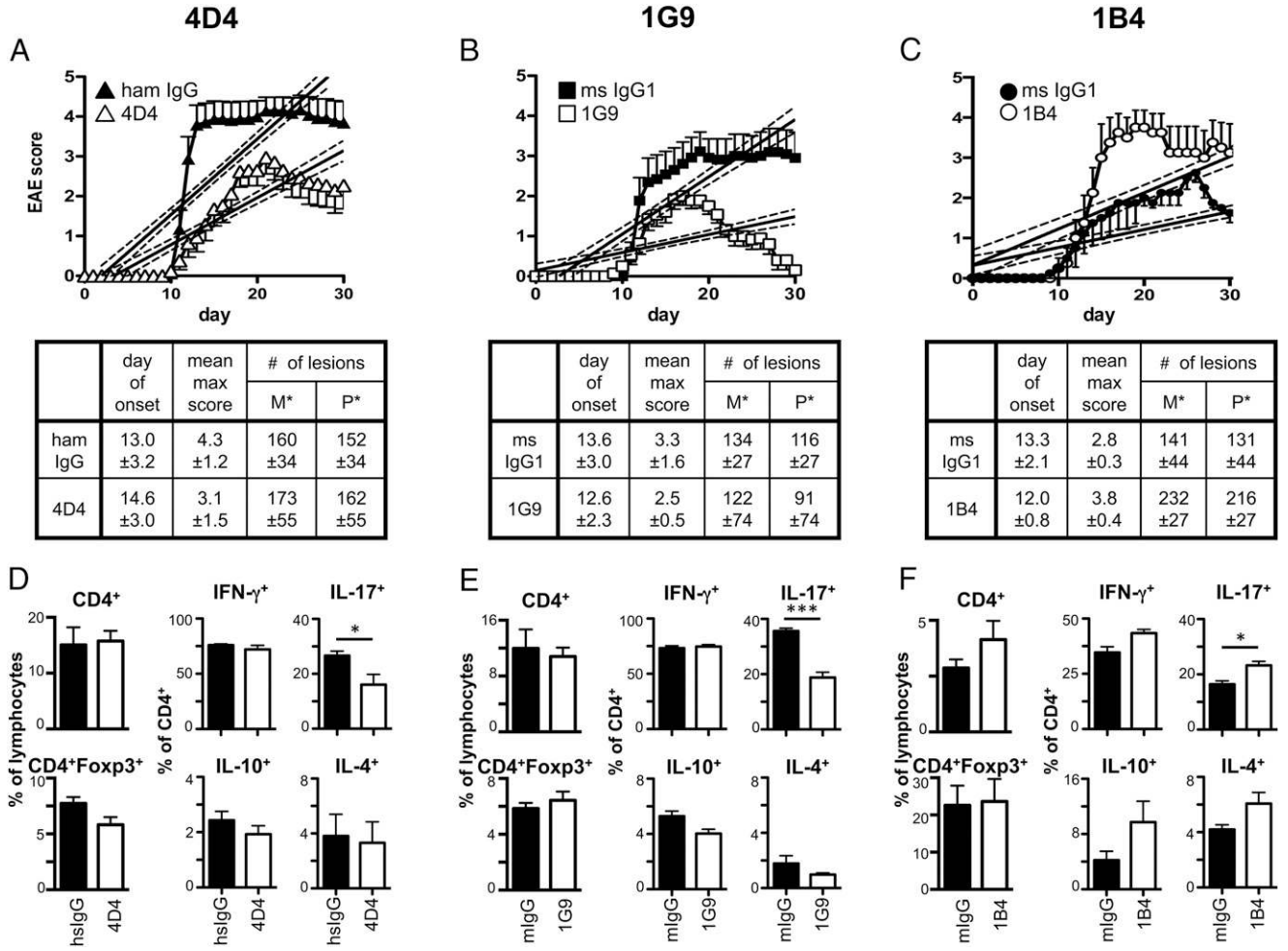


FIGURE 3. Anti-TIGIT Abs modulate T cell responses in vivo. Wild-type B6 mice were immunized s.c. with 100 μg $MOG_{35–55}$ peptide in CFA and received 100 μg anti-TIGIT (or isotype control: Armenian hamster IgG for clone 4D4 or mouse IgG1 for 1G9 and 1B4) Abs i.p. on days 0, 2, and 4. On day 10 spleens and lymph nodes were harvested and cells were restimulated with $MOG_{35–55}$ peptide (dLN: draining lymph node). (**A**, **C**, and **E**) After 48 h, [^{3}H]thymidine was added for the last 18–22 h before [^{3}H]thymidine incorporation was analyzed to assess proliferation (mean ± SD of triplicate wells from three to four animals per group, three to six independent experiments). (**B**, **D**, and **F**) IFN-γ and IL-17 were measured in the supernatants derived from the same cultures at 48 h using cytometric bead array. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ (Student t test).

exacerbated disease in this experimental setting, confirming that clone 1B4 acts as a blocking anti-TIGIT Ab in vivo (Fig. 4C). Interestingly, treatment with both agonistic anti-TIGIT Abs (clones 4D4 and 1G9) did not result in significant differences in the number of lesions or CNS-infiltrating cells but significantly reduced the frequencies of IL-17$^+$ Th17 cells (Fig. 4) infiltrating the CNS. In contrast, blocking of TIGIT with clone 1B4 significantly increased the number of lesions in both the meninges ($p = 0.029$) and the parenchyma ($p = 0.045$) and also led to increased frequencies of Th17 cells in the CNS (Fig. 4C, 4F). Collectively, these results show that the functional modulation of T cell responses with respect to magnitude and cytokine profile through the agonistic and blocking anti-TIGIT clones 4D4, 1G9, and 1B4 also translates into differences in disease severity during EAE.

Blocking anti-TIGIT Abs have synergistic effects with PD-1 blockade in cancer

The blockade of coinhibitory receptors is highly relevant in cancer where therapeutic effects are being exploited clinically. Anti–PD-1 therapy has shown great success but resistance to this therapy is increasing and some tumors (like colorectal carcinomas) are resistant to anti–PD-1 or anti–CTLA-4 immunotherapy. Accordingly, combination therapies are currently the most promising avenue for achieving durable responses. We therefore tested the efficacy of the anti-TIGIT blocking Ab 1B4 that we have generated in the setting wherein mice fail to respond to anti–PD-1 therapy. We found that anti-TIGIT 1B4 treatment alone led to a small but uniform retardation of established MC38 colon carcinoma growth. As expected, anti–PD-1 treatment alone resulted in a variable response with most mice showing initial tumor regression followed by escape and only one mouse showing complete tumor regression. However, we observed complete tumor regression in all mice when they were additionally treated with anti-TIGIT (Fig. 5A). Analysis of TILs showed no consistent effects on the frequencies of CD4$^+$ and CD8$^+$ T cells across groups. Importantly, only the anti-TIGIT/anti–PD-1 coblockade group showed increased IFN-γ secretion by CD4$^+$ TILs (Fig. 5B) and increased production of IFN-γ, TNF-α, and IL-2 in CD8$^+$ TILs (Fig. 5C). Combined blockade of PD-1 and TIGIT thus markedly enhanced functionality of tumor-infiltrating T cells, resulting in improved tumor control. To test whether these findings could be extended to other tumor models, we tested the efficacy of anti-TIGIT alone or in combination with anti–PD-1 in established GL261 glioblastoma. As observed for MC38 colon carcinomas, treatment with anti-TIGIT alone led to a small, but consistent



	day of onset	mean max score	# of lesions M*	# of lesions P*
ham IgG	13.0 ±3.2	4.3 ±1.2	160 ±34	152 ±34
4D4	14.6 ±3.0	3.1 ±1.5	173 ±55	162 ±55

	day of onset	mean max score	# of lesions M*	# of lesions P*
ms IgG1	13.6 ±3.0	3.3 ±1.6	134 ±27	116 ±27
1G9	12.6 ±2.3	2.5 ±0.5	122 ±74	91 ±74

	day of onset	mean max score	# of lesions M*	# of lesions P*
ms IgG1	13.3 ±2.1	2.8 ±0.3	141 ±44	131 ±44
1B4	12.0 ±0.8	3.8 ±0.4	232 ±27	216 ±27

FIGURE 4. Functional anti-TIGIT Abs modulate disease severity in EAE. Wild-type B6 mice were immunized s.c. with 100 μg (**A**, **B**, **D**, and **E**) or 10–15 μg (**C** and **F**) $MOG_{35–55}$ peptide in CFA, followed by injection of 100 ng pertussis toxin i.v. on day 0 and day 2. Mice also received 100 μg anti-TIGIT (or isotype control: Armenian hamster IgG for clone 4D4 or mouse IgG1 for 1G9 and 1B4) Abs i.p. on days 0, 2, 4, 10, and 17 and were monitored daily for EAE. Mean clinical score ± SEM is shown and linear regression curves of the disease for each group are depicted (the 95% confidence intervals are represented with dashed lines; combined data of two to three independent experiments). The number of lesions in the meninges (M*) and parenchyma (P*) were determined by histopathology when control mice were at the peak of disease: (A and B) day 14; (C) day 17. (D–F) At the peak of disease, CNS-infiltrating cells were isolated, restimulated with PMA/ionomycin for 4 h, and analyzed for the production of IFN-γ, IL-17A, IL-10, and IL-4 by intracellular cytokine staining. $*p < 0.05$, $***p < 0.001$ (Student *t* test).

improvement of tumor control and a modest survival benefit (Fig. 5D). However, combination of anti–PD-1 and anti-TIGIT markedly increased survival, with 17% of mice even showing long-term survival. Importantly, these mice had a durable antitumor response, as they remained tumor free for >100 d when rechallenged with GL261 implanted in the contralateral hemisphere (Fig. 5E). Functional analysis on T cells in the cervical lymph nodes again revealed improved functionality in that TNF-α production in $CD4^+$ T cells was increased (Fig. 5F) and $CD8^+$ T cells showed enhanced proliferation and contained elevated levels of granzyme B (Fig. 5G). These data support the combination of TIGIT and PD-1 blockade in cancer therapy. Collectively, our data demonstrate that the monoclonal anti-TIGIT Abs we have generated represent a valuable set of tools that can be used to modulate TIGIT function in different disease models in vivo.

Discussion

Coinhibitory receptor pathways have gained much attention as potential targets for immune modulation. Although a broad spectrum of pathologies that are caused or aggravated by dysregulated immune responses, such as cancer, autoimmunity, and chronic infection, can potentially be modulated by targeting coinhibitory receptor pathways, the development of such therapies is spearheaded by cancer therapy, where blockade of the coinhibitory receptors CTLA-4 and PD-1 has shown remarkable success (15). Despite these promising developments, low response rates, adverse autoimmune-like toxicity, and resistance highlight the need to identify additional pathways to broaden the therapeutic repertoire of coinhibitory receptors and overcome these hurdles. TIGIT represents such a potential therapeutic target; however, its mechanism of action has not been fully elucidated. In this study we have identified two monoclonal anti-TIGIT Abs that can be used to manipulate TIGIT function in vivo and investigate its function in detail.

Although Abs targeting coinhibitory receptors have successfully been used for the treatment of cancer, our studies show that they could also be exploited to treat chronic autoimmune diseases. One of the identified clones (1G9) acts agonistically in vivo and leads to a reduction in the overall T cell expansion and proinflammatory cytokine production. This also translated into beneficial effects in the EAE model of CNS autoimmunity, which is driven by

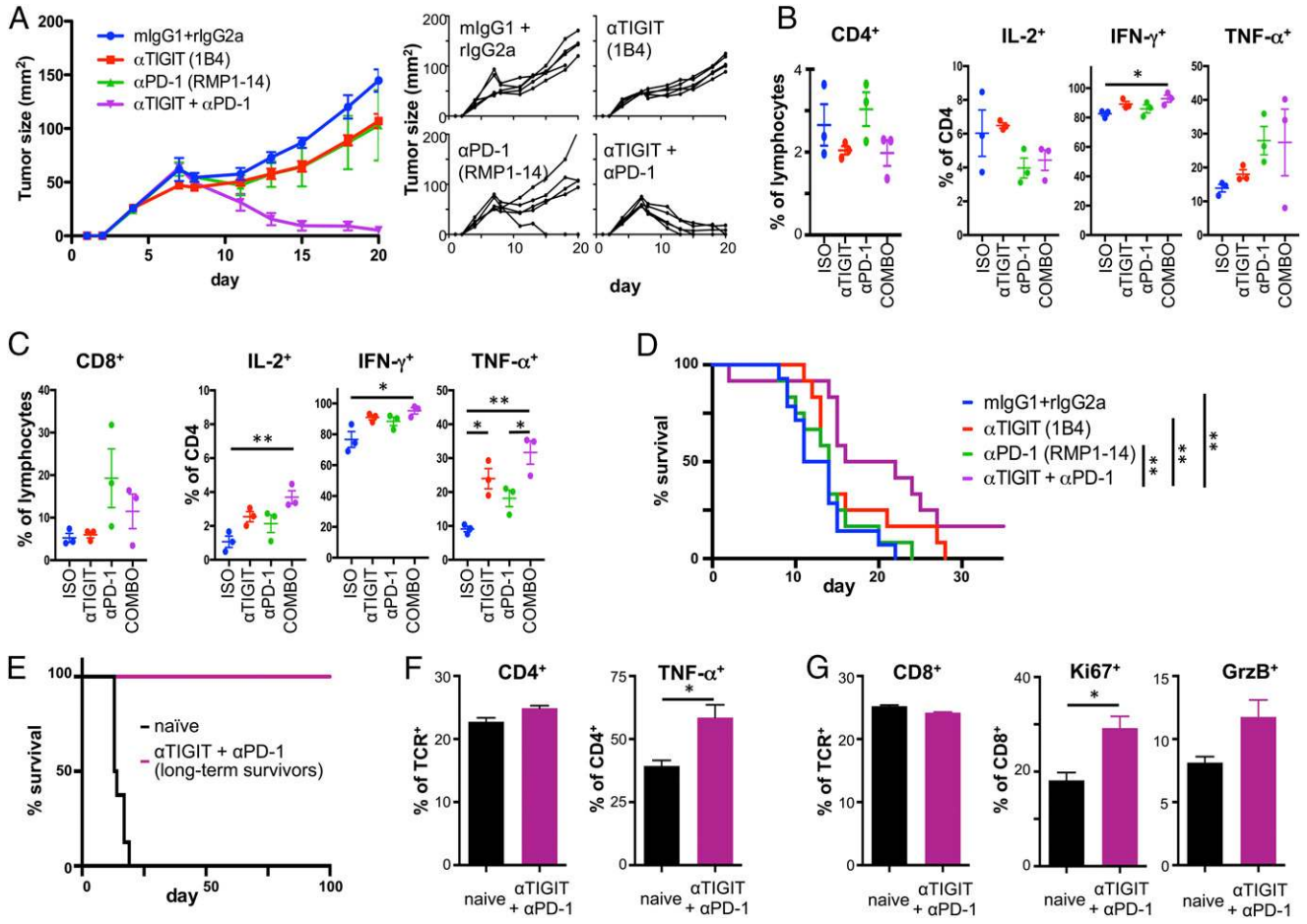


FIGURE 5. Blocking anti-TIGIT Abs have synergistic effects with blockade of other coinhibitory receptors in cancer. (**A**–**D**) Wild-type C57BL/6 mice were implanted with MC38 colon carcinoma. Mice with established tumors were treated with anti–PD-1, anti-TIGIT, anti–PD-1 + anti-TIGIT (COMBO), or isotype controls (ISO) and monitored for tumor growth. (A) Mean (left) and individual (right) tumor growth curves are shown ($n = 5$ per group). Similar results were obtained in an independent experiment. (B and C) TILs were harvested from tumor-bearing wild-type mice ($n = 3$) 9 d after tumor implantation when tumor sizes measured between 33 and 60 mm^2. Frequency (± SEM) of CD4$^+$ (B) and CD8$^+$ (C) TILs was determined by flow cytometry. Cells were stimulated with PMA/ionomycin and frequency (±SEM) of CD4$^+$ (B) and CD8$^+$ (C) TILs producing IL-2, TNF-α, and IFN-γ was determined by intracellular cytokine staining. (D) Wild-type C57BL/6 mice were orthotopically implanted with GL261-GFP-Gluc cells. Mice with established tumors were size matched and treated with anti–PD-1, anti-TIGIT, anti–PD-1 + anti-TIGIT, or isotype controls ($n = 12$ per group) and monitored for survival. (**E**) Long-term survivors ($n = 2$) and naive control mice ($n = 3$) were rechallenged with GL261 tumor cells in the contralateral brain hemisphere and survival was monitored for >100 d. Frequency of CD4$^+$ (**F**) and CD8$^+$ (**G**) in cervical lymph nodes and their expression of TNF-α (F) or Ki67 and granzyme B (GrzB) (G) were determined by flow cytometry. $*p < 0.05$, $**p < 0.01$ (Student t test).

pathogenic IFN-γ– and IL-17–producing T cells. Allelic variants in the TIGIT/CD226 pathway have been associated with susceptibility to autoimmune diseases in humans (8) and we, and others, have previously shown that TIGIT acts as a negative regulator in a number of autoimmune diseases (1, 6). Importantly, TIGIT is expressed at normal levels in MS patients and as such could be targeted therapeutically to dampen autoreactive T cell responses (16). Indeed, in vitro treatment of CD4$^+$ T cells from MS patients with agonistic anti-TIGIT Abs results in decreased T cell proliferation and cytokine production (16). Our results expand this finding and demonstrate that treatment with agonistic anti-TIGIT Abs also dampens autoimmune T cell responses in vivo and the reduction of T cell expansion and proinflammatory cytokines leads to amelioration of autoimmune disease. Although further studies are needed to test the therapeutic potential of targeting TIGIT in human autoimmune diseases, we now have the tools at hand for a detailed analysis of the mechanistic action of agonistic anti-TIGIT treatment that forms the basis for such interventions. In this context, the role that coinhibitory molecules may play in regulating progression in autoimmune diseases in humans has been indicated in a large cohort of patients suffering with multiple autoimmune diseases (17).

Although the blocking anti-TIGIT Ab clone (1B4) had a small effect as a single agent on established colon carcinoma or glioblastoma, combined blockade of TIGIT and PD-1 resulted in complete tumor regression in colon carcinoma and could induce long-term survival and a durable, protective antitumor response in the glioblastoma model. This is in keeping with a recent study where TIGIT blockade synergized with PD-L1 blockade in a model of established colon carcinoma (5). Furthermore, combined treatment significantly improved the function of tumor-infiltrating T cells, which is in line with another study that showed coblockade of TIGIT and PD-1 to improve the effector responses of tumor Ag-specific CD8$^+$ T cells from melanoma patients (10). Together, these observations support synergy between the TIGIT and PD-1 pathways and indicate nonredundant functions for these receptors in controlling the antitumor immune response in mice and humans.

As the current therapeutic targets of coinhibitory receptors expand from CTLA-4 and PD-1 to include novel inhibitory molecules such as TIGIT, it is essential to deepen our understanding of their mechanism of

action and both their cell- and tissue-specific functions. The Abs presented in this study will serve as important tools to the scientific community to address these questions in vivo and the knowledge gained will provide valuable insight for the development of novel therapeutic approaches targeting TIGIT both in autoimmune diseases and cancer.

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Disclosures

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