Cutting Edge: TIGIT Has T Cell-Intrinsic Inhibitory Functions

Nicole Joller,* Jason P. Hafler,† Boel Brynedal,‡,§ Nasim Kassam,*, Silvia Spoerl,* Steven D. Levin,*,¹ Arlene H. Sharpe,‖ and Vijay K. Kuchroo*‡

Costimulatory molecules regulate the functional outcome of T cell activation, and disturbance of the balance between activating and inhibitory signals results in increased susceptibility to infection or the induction of autoimmunity. Similar to the well-characterized CD28/CTLA-4 costimulatory pathway, a newly emerging pathway consisting of CD226 and T cell Ig and ITIM domain (TIGIT) has been associated with susceptibility to multiple autoimmune diseases. In this study, we examined the role of the putative coinhibitory molecule TIGIT and show that loss of TIGIT in mice results in hyperproliferative T cell responses and increased susceptibility to autoimmunity. TIGIT is thought to indirectly inhibit T cell responses by the induction of tolerogenic dendritic cells. By generating an agonistic anti-TIGIT Ab, we demonstrate that TIGIT can inhibit T cell responses directly independent of APCs. Microarray analysis of T cells stimulated with agonistic anti-TIGIT Ab revealed that TIGIT can act directly on T cells by attenuating TCR-driven activation signals. The Journal of Immunology, 2011, 186: 000–000.

Recent genome-wide association scans have linked a costimulatory molecule, CD226, to multiple autoimmune diseases in humans (1). CD226, together with T cell Ig and ITIM domain (TIGIT), forms an emerging pathway that has striking similarities to the well-known costimulatory CD28–CTLA-4 pathway. As in the B7-CD28–CTLA-4 pathway, CD226 and TIGIT bind the same set of ligands (CD155 and CD112), and CD226 is a positive regulator of T cell responses, whereas TIGIT inhibits them (2). A recent study suggested that TIGIT does not have any direct effects on T cells, but instead acts on dendritic cells (DCs) through the ligand CD155. TIGIT interaction with CD155 induced tolerogenic DCs that impaired T cell proliferation and inhibited IFN-γ production from responding T cells (3). However, although TIGIT contains two ITIMs in its cytoplasmic tail, it has not been elucidated whether TIGIT can directly inhibit T cell responses. This is partly because of the lack of relevant reagents that allow direct evaluation of its role in T cells.

In this study, we used TIGIT−/− mice and generated anti-TIGIT mAbs to analyze the function of TIGIT in T cells. We showed that loss of TIGIT results in hyperproliferative T cell responses and increased susceptibility to autoimmunity. Furthermore, by generating an agonistic anti-TIGIT Ab, we demonstrate that TIGIT has T cell-intrinsic effects and that signals through TIGIT directly inhibit T cell activation.

Materials and Methods

Animals

C57BL/6-Tg(Tcrα2D2,Tcrβ2D2) (2D2) mice have been previously described (4). TIGIT−/− mice were obtained from ZymoGenetics (Seattle, WA) and were generated by Ozgene (Bentley, Australia) using embryonic stem cells derived from C57BL/6 mice. C57BL/6 (B6) mice were purchased from the The Jackson Laboratory (littermate controls were used for at least one repeat of all of the experiments described, and results obtained did not differ from those using controls from The Jackson Laboratory). B6.SJL-Ptpε−/−BoAlTac (CD45.2 B6) mice were from Taconic (Hudson, NY), and Armenian hamsters were from Harlan Laboratories (Indianapolis, IN). Animals were kept in a conventional, pathogen-free facility at the Harvard Institutes of Medicine (Boston, MA), and all experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at Harvard Medical School.

T cell activation and proliferation

Cells were cultured in DMEM with 10% FCS, 50 μM 2-ME, 1 mM sodium pyruvate, nonessential amino acids, l-glutamine, penicillin, and streptomycin. For in vitro T cell activation, CD4+ and CD8+ T cells were isolated using anti-CD4 or anti-CD8 beads (Miltenyi Biotec) and stimulated with plate-bound anti-CD3 (145-2C11, 2 μg/ml) and anti-CD28 (PV-1, 2 μg/ml) or soluble anti-CD3 (0.025 μg/ml) with irradiated splenocytes as APCs. Where

*Center for Neurologic Diseases, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115; †Department of Medical Genetics, Juvenile Diabetes Research Foundation/Welcoming Trust Diabetes and Inflammation Laboratory, Cambridge Institute for Medical Research, Addenbrooke’s Hospital, University of Cambridge, Cambridge, United Kingdom; ‡Neurology Department, Yale Medical School, New Haven, CT 06510; †Medical and Population Genetics, Broad Institute of MIT and Harvard, Cambridge, MA 02142; ‡Department of Immunology, ZymoGenetics, Seattle, WA 98102; and §Department of Pathology, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA 02115

¹Current address: Novo Nordisk Inflammation Research Center, Seattle, WA.

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Address correspondence and reprint requests to Dr. Vijay K. Kuchroo, Center for Neurologic Diseases, Brigham and Women’s Hospital, 27 Avenue Louis Pasteur, HIM 785, Boston, MA 02115-5817. E-mail address: vkuchroo@rics.bwh.harvard.edu

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Abbreviations used in this article: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; GSEA, gene set enrichment analysis; IPA, ingenuity pathway analysis; KO, knockout; LN, lymph node; MOG, myelin oligodendrocyte glycoprotein; RT-PCR, real-time PCR; TIGIT, T cell Ig and ITIM domain.
indicated, cells were labeled with 2 μM CFSE. For costimulation with agonistic anti-TIGIT, CD4+MHC class II+ cells were sorted by flow cytometry and stimulated with plate-bound anti-CD3 (0.5 μg/ml), anti-CD28 (0.5 μg/ml), and anti-TIGIT (clone 4D4, 50 μg/ml) or isotype control (BioLegend). To determine proliferation, cells were pulsed with 1 μCi [3H]thymidine (PerkinElmer) after 48 h and incubated for an additional 18 h before incorporation was analyzed using a beta counter (1450 MicroBeta TriLux; PerkinElmer).

**Immunizations**

Where indicated, 10^5 TCR transgenic CD4+ T cells were transferred i.v. 1 d prior to immunization. Mice were immunized s.c. with 100 μg myelin oligodendrocyte glycoprotein (MOG)35-55 peptide (MEGVYRRPSFVR-VHLYRNGK) emulsified in CFA. Organs were collected 7 or 8 d after, cells were restimulated with MOG35-55 peptide, and proliferation was determined by [3H]thymidine incorporation. Frequencies of MOG-specific cells were determined after 5 d of restimulation with 30 μg/ml MOG35-55 peptide using MHC class II tetramers (I-A^d) loaded with MOG35-55, or CLIP peptide (PVSKMRMATPLIMQCA, control) (20 μg/ml, 1.5 h at room temperature; National Institutes of Health Tetramer Core Facility, Atlanta, GA). Cytokine concentrations in culture supernatants were determined by ELISA (IL-17) or cytokine bead array (BD Biosciences, other cytokines).

**Experimental autoimmune encephalomyelitis**

Experimental autoimmune encephalomyelitis (EAE) was induced by s.c. immunization of mice with 10–15 μg of MOG35-55 peptide emulsified in CFA followed by 100 ng pertussis toxin (List Biological Laboratories) i.v. on day 0 and day 2, and classical clinical signs of EAE were scored as described previously (4). Atypical signs were scored as 0.5 for each of the following: dyskinesia, ataxia, and clasp phenotype.

**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 foot pad immunization and booster injections. Draining lymph nodes were fused mouse TIGIT tetramers (ZymoGenetics) by a combination of s.c. and foot pad immunization and booster injections. Draining lymph nodes were fused

**Flow cytometry**

Cells were stained in PBS, 0.1% sodium azide, and 0.5% BSA (20 min at 4°C). Abs were from BioLegend, eBioscience (anti-Foxp3), BD Biosciences (7-aminomucopycinin) D), or they were generated as part of this study (anti-TIGIT, clone 1G9). Samples were acquired on a FACSCalibur or LSR II flow cytometer (BD Biosciences) and analyzed using the Flowjo software (Tree Star).

**Quantitative real-time PCR**

RNA was extracted with RNaseasy Mini kits (Qiagen, Valencia, CA) and was analyzed by real-time PCR (RT-PCR) according to the manufacturer’s instructions (Applied Biosystems, Carlsbad, CA). Primers–probe mixtures were: CD226 (Mm01301769m1), β-actin (Mm00469668-m1), TCRα (Mm0131039_g1), CD3ε (Mm01179194_m1), phospholipase C-γ1 (Mm01247293_m1), IL-2Rγ (Mm01178285_m1), CD25 (Mm01340213_m1), and Bcl-xL (Mm00437-783_m1). For TIGIT, primers and probe were: forward primer, 5′-CTGATACAAGGCTGTCCCTTCCTC-3′; reverse primer, 5′-TGGGTCACTTCAGCTGTGTC-3′; probe, 5′-AGGAGCCAGCAGCCAGGA-3′ (FAM, TAMRA).

**Microarray**

Cells were harvested after 24 h stimulation, and RNA was isolated using RNaseasy kits (Qiagen). GeneChip hybridization, staining, and scanning of the arrays were performed by the Partners HealthCare Center for Personalized Genetic Medicine (Cambridge, MA), according to the manufacturer’s instructions (Affymetrix). Summarization of probe set intensity, background correction, and normalization was done using the Bioconductor implementation of the GCRMA algorithm (5). Expression signals were compared using linear regression (6). In the single probe set analysis we used an α level of 0.05 and regarded fold change ≥1.4 or ≤0.71 as significant. Ingenuity pathway analysis (Ingenuity Systems) was used to identify groups of genes or pathways that show enrichment in significant molecules (fold change ≥1.2, p value of ≤0.1) and gene set enrichment analysis (GSEA) (7) to identify significant coordinate expression (using the Kyoto Encyclopedia of Genes and Genomes database, http://www.genome.jp/kegg). We created an additional two pathways (see T cell activation and T cell survival in Supplemental Table II). The microarray data are deposited at http://www.ebi.ac.uk/arrayexpress with the accession number E-MEXP-2847.

**Results and Discussion**

**TIGIT is expressed upon initial T cell activation**

TIGIT is upregulated on human T cells upon activation and expressed on human memory and regulatory T cells (3). However, owing to lack of reagents, the expression of TIGIT on mouse T cells has not been analyzed. To monitor TIGIT surface expression, we generated a panel of TIGIT-specific mAbs in TIGIT−/− mice and screened them for TIGIT specificity. Clone 1G9 showed the best binding properties when screened by ELISA (Supplemental Fig. 1A). It also specifically stained TIGIT-transfected P815 cells and activated primary mouse T cells (Supplemental Fig. 1B, 1C) as assessed by flow cytometry. We therefore used the 1G9 anti-TIGIT Ab to analyze the kinetics of TIGIT expression and compared its expression to its costimulatory receptor CD226 in mouse T cells.

TIGIT expression was induced upon stimulation, and mRNA levels steadily increased during the first 3 d of activation in both CD4+ and CD8+ T cells (Fig. 1A). Interestingly, surface expression of TIGIT peaked at 24 h and then decreased over time, even though mRNA levels kept increasing, suggesting that TIGIT expression was tightly regulated posttranscriptionally (Fig. 1B). This could be due to degradation and possibly also to receptor internalization, which would functionally decrease surface TIGIT levels and limit inhibitory signals at the initiation of the T cell response. Despite a decrease in CD226 mRNA after 24 h, CD226 cell surface protein expression was transiently upregulated on CD4+ T cells upon activation. Although CD8+ T cells constitutively express CD226 (8), T cell activation induced only minimal changes in CD226 mRNA and cell surface protein expression on CD8+ T cells (Fig. 1).

**TIGIT−/− mice show augmented T cell responses upon immunization**

To begin to determine the role of TIGIT in vivo, TIGIT−/− mice were immunized s.c. with MOG35-55, and the T cell...
response was analyzed 8 d later. When compared with B6 mice, T cells from TIGIT–/– mice displayed increased dose-dependent proliferation upon restimulation with antigenic peptide. T cell hyperproliferation was observed in draining lymph nodes (LNs) and spleens of TIGIT–/– mice as well as in nondraining LNs, whereas no response was observed in the nondraining LNs of wild-type mice (Fig. 2A). Consistent with these results, tracking of Ag-specific CD4+ T cells with MOG35–55/I-Ab tetramers confirmed that TIGIT–/– mice had higher frequencies of MOG-specific T cells in these organs (Fig. 2B). Similarly, MOG35–55−restimulated splenocytes and LN cells from TIGIT–/– mice produced higher levels of proinflammatory cytokines, including IL-6, IFN-γ, and IL-17 (Fig. 2C). Interestingly, TIGIT–/––derived cells produced reduced basal levels of IL-10, which is in line with previous reports indicating that TIGIT induces IL-10 production in DCs (3). However, in addition to reduced basal levels, IL-10 was also not induced by Ag-specific stimulation in TIGIT–/– cultures, suggesting that production of IL-10 by T cells is also impaired. In summary, these data demonstrate that TIGIT acts as a negative regulator of T cell responses in mice.

TIGIT–/– mice are more susceptible to EAE

Because CD226 has been genetically linked to susceptibility to autoimmunity (1), and TIGIT, which shares the same ligands, seems to act as an inhibitory molecule, we next tested whether the absence of TIGIT affects the development of autoimmunity. We immunized TIGIT–/– and B6 mice for induction of EAE with suboptimal doses of MOG35–55 (10–15 μg), such that B6 mice could not develop severe EAE but TIGIT–/– mice might display full-blown disease. Indeed, in contrast to B6 mice, the vast majority of TIGIT–/– mice developed severe EAE (Fig. 3A), supporting the notion of TIGIT as a coinhibitory molecule and suggesting an important role for the CD226–TIGIT pathway in regulating autoimmune responses.

To assess the role of TIGIT in the control of spontaneous autoimmunity, we crossed the TIGIT–/– mice to MOG35–55−specific TCR transgenic mice (2D2 mice) (4). 2D2 mice do not develop spontaneous EAE but mostly develop spontaneous optic neuritis. If TIGIT is an inhibitory molecule, we reasoned that in the absence of TIGIT, 2D2 mice might develop EAE spontaneously. Indeed, 2D2 × TIGIT–/– mice showed atypical signs of neurologic dysfunction as early as 28 d after birth (Fig. 3B), and with advancing age all 2D2 × TIGIT–/– mice displayed dyskinesia, ataxia, and a claspng phenotype (9). Some of the 2D2 mice also showed these atypical symptoms. However, onset was strongly delayed and, in contrast to 2D2 × TIGIT–/– mice, 2D2 mice never progressed to classical paralytic disease. These results emphasize the role of TIGIT as a negative regulator of T cell responses and indicate that TIGIT plays a role in limiting autoimmune responses, and loss or dysfunction of this coreceptor may likely contribute to susceptibility to autoimmunity.

TIGIT has T cell-intrinsic effect

We next tested whether the augmented T cell responses displayed by TIGIT–/– mice were exclusively mediated through APCs or whether TIGIT can also directly affect T cells. To discriminate the effects mediated by APCs from those that are T cell intrinsic, we isolated CD4+ T cells from B6 and TIGIT–/– mice, labeled them with CFSE, and stimulated them with either B6 or knockout (KO) APCs together with anti-CD3. Analysis of B6 T cell proliferation confirmed that TIGIT could mediate effects indirectly through APCs, as KO APCs were better at promoting proliferation than were their wild-type counterparts (Fig. 4A, Supplemental Fig. 2A). However, when wild-type APCs were used to stimulate B6 or TIGIT–/– T cells, the TIGIT–/– T cells also showed increased proliferation. The strongest proliferation was observed when combining KO APCs with KO T cells, suggesting that TIGIT has synergistic roles on T cells and APCs.

To dissect T cell-intrinsic and indirect effects of TIGIT in vivo, we transferred 2D2 or 2D2 × TIGIT–/– CD4+ T cells into CD45.1 B6 hosts. These mice were immunized with MOG35–55 and the T cell response was analyzed 7 d later. Despite comparable T cell expansion in vivo, TIGIT–/–
in the presence of irradiated B6 or TIGIT−/− APCs. Proliferation was analyzed after 60 h using flow cytometry, and proliferation indices were determined using the FlowJo software (A) (n = 6). B and C, CD45.1 B6 recipients received CD4+ 2D2 or 2D2 × TIGIT−/− cells i.v. 1 d before s.c. immunization with MOG35–55 peptide. On day 7 spleens and LNs were harvested, cells were restimulated with MOG35–55 peptide (0–100 ng/ml, 10× dilution steps), and proliferation was measured after 48 h ([3H]thymidine incorporation) (B) (n = 4). C, Cytokines were measured in the supernatants derived from the same cultures as in B at 48 h using ELISA (IL-17) and cytokometric bead array (others; n = 4). D, CD4+ T cells were sorted from B6 and TIGIT−/− mice and stimulated with plate-bound anti-CD3 and anti-CD28 plus 4D4 or isotype control Ab. Proliferation was assessed by [3H]thymidine incorporation (mean ± SD). s, spleen; d, draining LN; nd, nondraining LN.

T cells showed increased proliferation upon restimulation with antigenic peptide and produced higher levels of proinflammatory cytokines (Fig. 4B, 4C, Supplemental Fig. 2B). Importantly, 2D2 × TIGIT−/− recipients also showed reduced IL-10 levels, confirming a T cell-intrinsic defect in IL-10 production in TIGIT−/− T cells.

To exclude any effects that might be due to conditioning of the APCs, we analyzed the T cell-intrinsic effects of TIGIT in an APC-free system. To completely eliminate any cells other than T cells from our experiments, we tested our panel of TIGIT-specific Abs for functional agonistic activity in vitro. Generating agonistic Abs directed against Ig superfamily members, such as CD28, has proven to be a challenging endeavor, and when tested for functional effects, none of our Abs showed agonistic activity. We therefore generated a second panel of TIGIT-specific Abs in Armenian hamsters and screened them for specificity (Supplemental Fig. 2C–E) and functional activity. Out of these Abs, only one clone (4D4) affected T cell proliferation in vitro. Clone 4D4 proved to be agonistic, as the addition of plate-bound 4D4 to anti-CD3/anti-CD28–stimulated T cells inhibited their proliferation (Fig. 4D). Importantly, when TIGIT−/− T cells were stimulated with agonistic anti-TIGIT, no functional inhibition in T cell proliferation was observed, confirming the specificity of the Abs. Because no APCs were present in these in vitro experiments, these data clearly demonstrate that TIGIT can act directly on T cells.

**TIGIT engagement modulates T cell activation**

Coinhibitory receptors on T cells are fundamental constituents of the adaptive immune system necessary to limit T cell responses to prevent chronic T cell activation, immunopathology, and autoimmunity, as demonstrated by the autoimmune-mediated lethality of the CTLA-4−/− mice as well as the therapeutic approaches using CTLA-4-Ig (10–12). Although all co-inhibitory molecules have the ability to dampen T cell activation, they differ in potency, kinetics of expression, and the cellular pathways they alter. To understand how each co-inhibitory molecule alters T cell responses, it is critically important to elucidate which signaling pathways are modulated by each of them.

We used a whole genome microarray approach to identify the pathways affected by TIGIT engagement and compared the gene expression in B6 CD4+ T cells stimulated with agonistic anti-TIGIT 4D4 Ab to that of isotype and TIGIT−/− controls (Supplemental Table I). We found most differences observed to be small, likely representing alterations of pathways induced by T cell activation rather than separate pathways that are induced through TIGIT (Supplemental Fig. 3A). Ingenuity pathway analysis (IPA) and GSEA (7, 13) were used to identify pathways that are differently regulated if TIGIT is engaged, and in line with the functional effects we observed upon TIGIT activation, we found several pathways that are associated with T cell activation and cell cycle progression to be enriched in the controls, indicating that TIGIT downregulates these pathways. An overlay of the microarray data with the T cell activation pathway showed that a number of molecules involved in TCR and CD28 signaling are significantly downregulated upon TIGIT engagement and that many other key molecules involved, although not significantly decreased, show the same trend (Supplemental Fig. 4A).

Furthermore, we generated a gene set comprising key molecules for the TCR and CD28 signaling pathways as well as cell cycle progression and could verify that this gene set is enriched in the control group using both GSEA and IPA (Supplemental Fig. 3B). Additionally, we have verified the TIGIT-mediated downregulation of three key molecules of this pathway by RT-PCR (Supplemental Fig. 4C). TIGIT seems to block productive T cell activation by directly acting on TCR expression itself, as engagement of TIGIT induced a downregulation of the TCR α-chain as well as molecules that comprise the TCR complex. Therefore, in contrast to other coinhibitory receptors (e.g., programmed death-1) that interfere with processes that are further downstream in the TCR-induced signaling cascade (14), TIGIT acts upstream.

Although TIGIT engagement downregulated TCR activation pathways, it did not inhibit cellular processes in general. We found expression of cytokine receptors that are associated with T cell maintenance (i.e., IL-2R, IL-7R, and IL-15R) as well as antiapoptotic molecules, such as Bcl-xL, to be upregulated by TIGIT (Supplemental Fig. 4B). As for the T cell activation, we used RT-PCR to validate the differential expression of key genes of this pathway (Supplemental Fig. 4D) and generated a core gene set for T cell maintenance and survival, which was found to be enriched in the TIGIT-stimulated sample using GSEA and IPA (Supplemental Fig. 3C). In summary, TIGIT also inhibits T cell responses directly by targeting molecules that are upstream in the T cell activation process while promoting T cell maintenance and survival; thus, although T cells are not activated and expanded, the cells do not undergo anergy and are not deleted from the repertoire.

Our data support the idea that TIGIT is an inhibitory molecule, and loss of TIGIT in vivo increases T cell proliferation, proinflammatory cytokine production, and accelerates development of autoimmunity. In addition to inhibiting...
T cell responses via APCs, TIGIT can act directly on T cells, as an agonistic anti-TIGIT Ab was able to inhibit T cell proliferation in the absence of any other cell type. Overall, our results support the notion that TIGIT is an inhibitory molecule that, in addition to affecting APC function, suppresses T cell responses directly in a T cell-intrinsic manner.

Although TIGIT deficiency per se does not result in the induction of spontaneous autoimmunity or tissue inflammation, loss of TIGIT in a susceptible background can result in autoimmune disease as was observed in 2D2 × TIGIT−/− mice, which spontaneously developed EAE. TIGIT might therefore regulate the threshold of T cell activation and may be involved in the maintenance of peripheral tolerance. Finally, the genetic linkage of the CD226–TIGIT pathway to autoimmune disease as was observed in 2D2 mice and recombinant proteins. We thank Vance Morgan and the Partners HealthCare Center for Personalized Genetic Medicine for performing the microarrays, Deneen Kozoriz for cell sorting, Sharon Kunder and the Partners HealthCare Center for Personalized Genetic Medicine for discussions.

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Disclosures
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