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Limited Impact of the Inhibitory Receptor TIGIT on NK and T Cell Responses during Toxoplasma gondii Infection

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ABSTRACT

Resistance to the parasite Toxoplasma gondii is mediated by NK and T cell production of IFN-γ, but the failure to contract this response can lead to severe T cell–dependent immunopathology. Although the cytokines IL-10 and IL-27 prevent immune hyperactivity during toxoplasmosis, inhibitory receptors, expressed by NK and T cells, are also implicated in this process. The inhibitory receptor TIGIT is expressed on NK and T cells and competes with the costimulatory receptor CD226 for binding of the ligand CD155. During toxoplasmosis, the activation of NK and T cells is associated with increased expression of CD226 and TIGIT, whereas DCs express increased levels of CD155. To determine if the loss of TIGIT impacts NK and T cell activities, wild-type and TIGIT knockout mice were infected with T. gondii. During the acute stage of infection, wild-type and TIGIT knockout mice had comparable parasite burdens and similar NK and T cell responses. Likewise, during the chronic phase of this infection, the loss of TIGIT did not affect the magnitude or phenotype of the T cell response nor the ability to control pathogen load. These data suggest that during toxoplasmosis, despite upregulation of relevant ligands, TIGIT signaling does not limit NK and T cell activities. Thus, TIGIT-independent mechanisms dominate the restraint of the immune response during toxoplasmosis. ImmunoHorizons, 2021, 5: 384–394.

INTRODUCTION

Toxoplasma gondii is an intracellular, apicomplexan parasite that infects humans, persists for the life of the infected host, and is typically well controlled in the majority of infections of immunocompetent individuals. However, patients with primary and acquired immunodeficiencies that impact T cell–mediated immunity are susceptible to the development of toxoplasmic encephalitis (TE) (1). During the acute stage of infection with T. gondii, the parasite disseminates from the site of infection to peripheral tissues, which include the lung and liver. NK and innate lymphoid cell (ILC1) production of IFN-γ provide a mechanism for initial resistance to the parasite (2–5), but CD4+ and CD8+ T cells are required for long-term resistance (6). During the chronic phase of infection, the parasite persists within the CNS (7), and in murine models the presence of CD4+ and CD8+ T cells within the CNS is required to limit parasite reactivation (6, 8, 9).

Although the generation of a T cell response and the production of IFN-γ are critical for the control of many intracellular infections, failure to limit this response can lead to severe immunopathology (10). During toxoplasmosis this is apparent...
in several settings (11), such as the development of a CD4+ T cell–mediated ileitis (12) or in the CNS during the chronic phase of infection (13). Additionally, mice that are deficient in IL-10 (14–16) or that lack IL-27 signaling (17) also develop pathological CD4+ T cell responses when challenged with T. gondii. Inhibitory receptors, such as PD-1, CTLA4, and Lag-3, are surface molecules expressed by T cells that function in a cell intrinsic fashion to limit T cell responses during a variety of viral, fungal, and bacterial infections, and mitigate the development of immune pathology (18–22). Furthermore, in mice infected with T. gondii the cytokine IL-27 promotes the expression of the inhibitory receptors TIGIT, Lag-3, and Tim-3 (23). This finding suggests that the suppressive activities of IL-27 may be related to its regulation of inhibitory receptors, but there are open questions about the function of different inhibitory receptors during toxoplasmosis. For example, during toxoplasmosis PD-1 is expressed on CD8+ T cells during toxoplasmosis (23, 27) and suggests that TIGIT may have a role in limiting parasite replication (26). Although there are reports that T cell expression of other inhibitory receptors is increased during toxoplasmosis (23), it is unclear whether these have a significant role in limiting pathologic responses. One example is the inhibitory receptor T cell immunoreceptor with Ig and ITIM domains (TIGIT), which is expressed at high levels on parasite-specific T cells during the acute and chronic phases of toxoplasmosis (23, 27) and suggests that TIGIT may have a role in limiting the immune response during this infection.

TIGIT functions through its ability to interfere with the CD155-CD226 costimulatory pathway. CD155 (or polio virus receptor) is expressed on dendritic cells (DCs), monocytes, and macrophages, and it interacts with CD226 (or DNAM-1) on NK and T cells to provide costimulatory signals that promote cyto-kine production and lymphocyte proliferation (28–30). TIGIT, expressed on activated NK and T cells, has a higher affinity for CD155 than does CD226, which allows TIGIT to outcompete the activating receptor (31). TIGIT can also directly disrupt CD226 dimerization, further inhibiting its ability to signal (32). Upon the binding of CD155 to TIGIT, its cytoplasmic ITIM and ITT motifs are activated, either of which are sufficient to induce inhibitory signaling in NK cells and allow TIGIT to suppress in cis (33). Conversely, when CD155 on DCs is bound by TIGIT, a tolerogenic signal is delivered to DCs. This provides a cell-extrinsic mechanism whereby TIGIT can globally suppress the immune response by functioning in trans (34). Thus, TIGIT appears to have at least four distinct activities that contribute to its function as an inhibitory receptor. The biological significance of these suppressive functions is highlighted by studies in mouse models of autoimmunity, cancer, and chronic viral infection. For example, in the CD4+ T cell–dependent experimental autoimmune encephalomyelitis model, TIGIT KO mice exhibit enhanced disease severity (35). Furthermore, TIGIT KO mice challenged with B16 melanoma have enhanced NK cell effector function and enhanced tumor resistance (36), and in a model of colorectal cancer the combined blockade of PD-L1 and TIGIT resulted in enhanced CD8+ T cell–mediated tumor clearance (32). Similarly, following infection with lymphocytic choriomeningitis virus (LCMV)–clone 13, the absence of TIGIT on T cells led to enhanced responses to infection (32), and its agonistic engagement in both LCMV and influenza infections was recently shown to decrease immune mediated tissue damage (37). However, the role of TIGIT in other infection models is not well defined.

Based on this literature linking TIGIT to the inhibition of NK and T cell responses, studies were performed to characterize its expression and function during toxoplasmosis. Analysis of infected mice revealed that DC expression of CD155 and NK and T cell CD226 and TIGIT levels correlated with activation status. Despite TIGIT expression correlating with activation of NK and T cells, when TIGIT KO mice were infected they showed comparable NK and T cell responses to those of wild-type (WT) mice as well as similar levels of parasite control at both acute and chronic stages of infection. Additionally, the loss of TIGIT did not result in overt signs of increased immunopathology. These data sets indicate a limited role for TIGIT in the restraint of NK and T cell responses during toxoplasmosis and that TIGIT-independent mechanisms dominate the restraint of the immune response to T. gondii infection.

**MATERIALS AND METHODS**

**Mice and infections**

Female and male C57BL/6 mice were purchased (~8–12 wk old) from Taconic Biosciences. TIGIT KO mice were generated by Merck, Kenilworth, NJ (38), and housed at Taconic farms before transfer to our facilities. Female TIGIT KO mice (~8–12 wk) were used in these experiments, with age and sex matched WT C57BL/6 mice indicated above. Mice were housed in specific pathogen-free facilities in the Department of Pathobiology at the University of Pennsylvania in accordance with institutional guidelines. Cysts of the ME49 strain of T. gondii were collected from chronically infected CBA mouse brain tissue. Host mice were then infected i.p. with 20 cysts.

**Cell staining and flow cytometry**

Splenocytes were obtained by grinding spleens through a 70-μm filter and washing with RPMI supplemented with 5% FBS. RBCs were then lysed by incubating samples with ACK lysis buffer (Thermo Fisher Scientific) for 5 min before washing again with RPMI + 5% FBS. Cells were then washed with FACS buffer (1× PBS, 0.2% bovine serum Ag, 1mM EDTA) before incubating with Fc block (99.5% FACS buffer, 0.5% normal rat serum, 1 μg/ml 2.4G2 IgG Ab) prior to staining. Cells were stained with the viability dye Ghost Dye Violet 510 (12-0870; Tonbo Biosciences), and the following Abs were used for subsequent staining: IFN-γ FITC (XMG1.2; Invitrogen), CD11c
FITC (N418; eBioscience), CD11a PerCP-Cy5.5 (M17/4; BioLegend), Eomes eFluor 450 (DanIImg; Invitrogen), F4/80 eFluor 450 (BM8; Invitrogen), CD19 Brilliant Violet 605 (6D5; Bio Legend), CD4 Brilliant Violet 650 (RM4-5; BioLegend), CD11b Brilliant Violet 650 (m1/70; BioLegend), XCR1 Brilliant Violet 650 (ZET; BioLegend), CD19 Brilliant Violet 650 (6D5; Bio Legend), CD11b Brilliant Violet 650 (m1/70; BioLegend), CD155 Brilliant Violet 711 (TX56; BioLegend), CD3 Brilliant Violet 785 (17A2; BioLegend), TIGIT eFluor 660 (GIGD7; Invitrogen), CD4 AF700 (RM4-5; BioLegend), MHC class II (MHC-II) AF700 (M5/14.15.2; BioLegend), NK1.1 allophycocyanin-Cy7 (PK136; BD Biosciences), CD45 allophycocyanin-eFluor780 (30-F11; Invitrogen), CD45 allophycocyanin-eFluor780 (RM4-5; Invitrogen), NK1.1 PE (PK136; BioLegend), CD8 PE-CF594 (53-; BioLegend), CD226 (10E5; BioLegend). MHC class I (MHC-I) tetramers were conjugated with the Tgd-057 peptide (SVLAFRRL) or MHC-II tetramers loaded with the AS15 peptide (AVEHIHRPVG-TAPPS) and conjugated to the PE fluorophore were provided by the National Institutes of Health Tetramer Facility. Intracellular staining was performed using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. Samples were run on a LSR Fortessa (BD Biosciences) and analyzed using the FlowJo Software analysis program (TreeStar).

**Parasite quantification**

Brain and liver tissues were collected, and genomic DNA was isolated using DNeasy Blood and Tissue kits (Qiagen) according to manufacturer’s instruction. Parasite burden was then measured by real-time PCR targeting the 35-fold repetitive *T. gondii* gene *B1* (forward primer, 5'-TCCCCTCTGTGCACGAAAAT-3'; reverse primer, 5'-AGCTTTCTGTGCAACTATCGATTG-3') using the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and run on the ViiA 7 real-time PCR system (Applied Biosystems) at previously published conditions (16). To quantify the cyst number within brain tissue, brain sections (fixed and stained as below) in 7-μm slices were scanned and analyzed using an Aperio ImageScope (Leica). For cytoplasm counts, single cells from the peritoneal cavity (1 × 10⁶ cells per slide) were spun at 500 rpm for 5 min onto glass slides. Cells were stained using the PROTOCOL Hema 3 Manual Staining System (Thermo Fisher Scientific), mounted with CytoSeal (Edmund Sciences), and infected cells manually counted under a 10× objective.

**Histology and immunohistochemistry**

Brain and liver tissues were fixed in 10% buffered formalin before being paraffin embedded and sliced into 7-μm sections. These sections were stained with H&E then scanned and analyzed using an Aperio ImageScope (Leica). Immunohistochemistry for *T. gondii* was performed as previously described (16).

**ELISA for cytokines**

For analysis of serum cytokines, mice were bled at the indicated time points, and then centrifuged to isolate plasma from the cellular components. For soluble *T. gondii* Ag (STAg) restimulations, 4 × 10⁵ splenocytes were plated and restimulated for 72 h with 10 μg/ml of STAg. Supernatants were then collected and analyzed for cytokines. IFN-γ and IL-12 were measured by incubating with capture Abs (AN18 and C17.8, respectively) followed by incubation with a biotinylated detection Ab (R4-6A2 and C15.6, respectively) and then streptavidin conjugated HRP.

**Statistics**

Statistical analyses were performed using the PRISM software package (GraphPad software). Unpaired Welch *t* tests were used to determine significant differences between samples, unless otherwise indicated, with *p* < 0.05 deemed as significant.

**RESULTS**

**Impact of *T. gondii* infection on innate expression of TIGIT, CD226, and CD155**

To begin to define the role of TIGIT during toxoplasmosis, WT C57BL/6 mice were infected with the ME49 strain of *T. gondii* and expression of TIGIT, the activating receptor CD226, and their shared ligand, CD155, was assessed by flow cytometry in naive mice (day 0) and at days 5, 8, and 13 postinfection. These time points correspond to times of peak NK (days 5–8) and T cell (days 8–13) responses. In naive mice, DCs (CD3⁻, TCRβ⁻, CD19⁻, B220⁺, F4/80⁺, CD11c⁺, MHC-II⁺), subsetted into XCR1⁺ and XCR1⁻ cells, showed a low basal proportion of cells expressing CD155 (Fig. 1A). Postinfection, there was no impact on the proportion of XCR1⁺ DCs expressing CD155 until 13 dpi, when there was a reduction in the proportion of cells expressing CD155 and a marked decrease in the number of CD155⁺ cells. The expression of CD155, as measured by mean fluorescence intensity (MFI), on these cells also did not show a change in expression level from baseline until day 13, when it also decreased. XCR1⁻ DCs showed an increase in the proportion of cells expressing CD155, beginning at 8 dpi, and this was mirrored by both an increase in the numbers of XCR1⁻ CD155⁺ cells and the expression of CD155 on these cells (Fig. 1A).

In uninfected mice, ~46% of NK cells (F4/80⁻, CD9⁻, B220⁺, CD3⁻, NK1.1⁻) express CD226 (Fig. 1B). The proportion and overall numbers of CD226⁺ NK cells peaked at 8 dpi before returning to basal levels by day 13, and the expression of CD226 on these cells remained consistent throughout infection until decreasing at 13 dpi. In naive mice, TIGIT is expressed on ~0.5% of NK cells (Fig. 1B), but by 8 dpi there was an increase in the proportion and numbers of this population before a return to basal levels at day 13 (Fig. 1B). Despite this, TIGIT expression on these cells remained the same throughout infection. Because TIGIT counter-regulates CD226 activities, it...
FIGURE 1. CD155, CD226, and TIGIT are expressed on innate immune cells during acute toxoplasmosis.

Bulk splenocytes from mice infected with \textit{T. gondii} were analyzed for expression of CD155, CD226, and TIGIT on DC (A) and NK (B) cells throughout the course of acute infection (0, 5, 8, and 13 dpi). (A) DCs (CD3−, TCRβ−, CD19−, B220−, NK1.1−, F4/80−, CD11c+, MHC-II+) were subsetted into XCR1+ and XCR1− cells and expression of CD155 measured at the indicated time points. Representative flow plots from naive mice and mice 8 dpi are shown (left). Percentages, numbers, and MFI of CD155+ cells are quantified throughout infection (right). Percentages are measured as a percentage of either XCR1+ or XCR1− DC populations. MFI is calculated as the geometric MFI (gMFI) and is measured on CD155+ cells. (B) NK cell (F4/80−, CD19−, B220−, CD3−, NK1.1+) expression of CD226 (top) and TIGIT (bottom) was analyzed at the indicated time points. Representative flow plots from naive mice and mice 8 dpi are shown (left). Percentages, numbers, and MFI of CD226+ and TIGIT+ cells are quantified throughout infection (right). Percentages are measured as a percentage of the NK cell population and MFI was calculated as above for CD155 expression. (C) Representative plots of naive and infected mice 8 dpi analyzing coexpression of CD226 and TIGIT on NK cells are shown (left) and quantified throughout infection (right).
FIGURE 2. CD226 and TIGIT are expressed on CD4+ and CD8+ T cells during acute toxoplasmosis.

Bulk splenocytes from mice infected with T. gondii were analyzed for expression of CD226 and TIGIT on CD4+ (A) and CD8+ (B) T cells throughout the course of acute infection (0, 5, 8, and 13 dpi). (A) CD4+ T cell (F4/80−, CD19−, B220−, CD3+, CD4+) expression of CD226 (top) and TIGIT (bottom) was measured at the indicated time points. Representative flow plots of T cells from naive mice with CD11alo, tetramer− (Continued)
seemed likely that TIGIT would be coexpressed with CD226 on NK cells. However, in uninfected mice, there was limited coexpression of these molecules and during infection the population of TIGIT\(^+\) NK cells that emerged was primarily CD226\(^-\) (Fig. 1C). Interestingly, CD226 can also be observed on DCs, with the number of CD226\(^+\) DCs peaking at 8 dpi, suggesting CD226 also tracks with DC activation during infection (Supplemental Fig. 1A).

**Expression of TIGIT and CD226 by T cells during toxoplasmosis**

CD4\(^+\) and CD8\(^+\) T cells can also express CD226 and TIGIT, and these are influenced by T cell activation (23, 29). Therefore, the expression of these molecules on pathogen-specific T cells, identified using MHC-I– and MHC-II–specific tetramers during the early phase of infection (days 8–13) was examined. In infected mice, naive T cells are CD11a\(^lo\) whereas Ag-experienced T cells are CD11a\(^hi\), and these levels of CD11a aid in identifying populations of pathogen-specific T cells (39–41). Thus, effector T cells can be identified as CD11a\(^hi\) tetramer\(^+\) cells, and naive cells as CD11a\(^lo\) tetramer\(^-\). In naive mice, ~50% of naive CD4\(^+\) T cells expressed CD226 (Fig. 2A). In infected mice, these naive CD4\(^+\) T cells had reduced proportions of CD226\(^+\) cells whereas effector T cells at 8 and 13 dpi showed the highest proportions of CD226\(^+\) cells (Fig. 2A). The percentage of TIGIT\(^+\), naive CD4\(^+\) T cells was low in both uninfected and infected mice (Fig. 2A). However, by 8 dpi, the percentage and number of effector TIGIT\(^+\) CD4\(^+\) T cells had increased in the population of activated, effector cells and contracted by day 13 (Fig. 2A).

In contrast to CD4\(^+\) T cells, murine CD8\(^+\) T cells are reported to constitutively express CD226 (29), regardless of CD11a levels or tetramer binding; this was confirmed in naive and infected mice (Fig. 2B). Naive CD8\(^+\) T cells in uninfected or infected mice had a low proportion of TIGIT\(^+\) cells, but effector CD8\(^+\) T cells had high percentages and numbers of TIGIT\(^+\) cells at 8 dpi that declined by 13 dpi (Fig. 2B). Analysis of the coexpression of CD226 and TIGIT on CD4\(^+\) and CD8\(^+\) T cells revealed two notable patterns of expression. For effector CD8\(^+\) T cells, those that expressed CD226 were predominantly either TIGIT\(^+\) or TIGIT\(^-\). In contrast, parasite-specific CD4\(^+\) T cells showed relatively equal proportions of cells that coexpressed the receptors or expressed TIGIT or CD226 individually. Thus, three main populations can be observed in these cells: a CD226\(^+\)TIGIT\(^+\), a CD226\(^+\)TIGIT\(^-\), and a CD226\(^-\)TIGIT\(^+\) population (Fig. 2C). These populations are most apparent at 8 dpi, whereas by 13 dpi CD226 singly expressing cells dominate. During acute toxoplasmosis, then, CD226 and TIGIT show expression on parasite-specific CD4\(^+\) and CD8\(^+\) T cells, whereas CD155 expression can be seen on DCs.

**Impact of the loss of TIGIT on acute NK and T cells responses to T. gondii**

To determine if the loss of TIGIT would enhance NK and T cell responses to infection, WT and TIGIT KO mice were infected i.p. with *T. gondii* and systemic cytokine production, NK and T cell responses, and parasite burden were compared. Serum levels of IL-12 and IFN-γ in uninfected mice are low to negligible, and in these experiments the infection-induced levels of these cytokines were comparable in WT and TIGIT KO mice at 7 dpi (Fig. 3A). In addition, flow cytometric analysis of NK cells isolated from WT and TIGIT KO mice 8 dpi showed that NK cell numbers were comparable at multiple sites of infection (Fig. 3B) and restimulation of these cells with PMA and ionomycin resulted in similar numbers of IFN-γ-producing cells (Fig. 3C) as well as similar amounts of IFN-γ (as determined by MFI, data not shown). To assess the impact of TIGIT-deficiency on T cell responses, we quantified the number of parasite-specific effector T cells, identified as above, via flow cytometric analysis of tissues isolated from infected WT and KO mice. Consistent with previous studies, infection resulted in a marked increase in T cell expression of CD11a, and the use of the tetramers identified a subset of parasite-specific CD4\(^+\) and CD8\(^+\) T cells. The numbers of CD11a\(^hi\), tetramer\(^+\) CD4\(^+\), and CD8\(^+\) T cells from the spleen (Fig. 3D) as well as from the liver and lung (data not shown) were similar in the WT and TIGIT KO mice. Furthermore, when splenocytes from infected mice were restimulated with *T. gondii* peptides similar numbers of IFN-γ-producing CD4\(^+\) and CD8\(^+\) T cells were observed (Fig. 3E). CD4\(^+\) and CD8\(^+\) IFN-γ\(^+\) T cells also showed similar MFI values for IFN-γ between WT and KO splenocytes (data not shown), indicating similar IFN-γ production between these conditions as well. This could be further seen in the stimulation of splenocytes from infected WT and TIGIT KO mice with STAg, which resulted in the secretion of comparable levels of IFN-γ (Fig. 3F). To assess if parasite control was impacted by the absence of TIGIT, cytopsins of peritoneal exudate cells at 8 dpi were used to calculate the number of infected mice 8 dpi with either CD11a\(^lo\), tetramer\(^-\) and CD11a\(^hi\), tetramer\(^-\) expression are shown (left). Numbers of CD11a\(^lo\), tetramer\(^-\) CD226\(^-\) and TIGIT\(^-\) cells are quantified throughout infection, and percentages were determined from the populations of effector CD4\(^+\) T cells (right). (B) CD8\(^+\) T cell (F4/80\(^-\), CD19\(^-\), B220\(^-\), CD3\(^+\), CD8\(^+\)) expression of CD226 (top) and TIGIT (bottom) was measured as above. Representative flow plots are shown as above (left). Numbers of CD11a\(^lo\), tetramer\(^-\) CD226\(^-\) and TIGIT\(^-\) cells are quantified throughout infection, as above, and percentages measured in the effector CD8\(^+\) population (right). (C) Coexpression of CD226 and TIGIT was analyzed on CD4\(^+\) (top) and CD8\(^+\) (bottom) effector T cells. Representative plots are shown of the expression on CD11a\(^lo\), tetramer\(^-\) cells from naive mice (left plots) compared with CD11a\(^hi\), tetramer\(^+\) cells from infected mice 8 dpi (right plots). The CD11a\(^hi\), tetramer\(^+\) populations were then quantified throughout infection (right graphs).
FIGURE 3. Loss of TIGIT during the acute phase of *T. gondii* infection does not impact NK or T cell responses. WT and TIGIT KO mice were infected with *T. gondii* and analyzed during the acute phase of infection. (A) Serum levels of IL-12 and IFN-γ were measured at 7 dpi in both WT and TIGIT KO mice. (B) NK cell (CD19<sup>-</sup>, CD3<sup>-</sup>, NK1.1<sup>+</sup>, Eomes<sup>+</sup>) numbers were quantified at 8 dpi (Continued)
infected cells and quantitative PCR was used to estimate parasite burden in the liver (Fig. 3G). Across multiple experiments there was no statistical difference in parasite levels at these sites. Together, these data show that during the acute phase of T. gondii infection the loss of TIGIT does not impact the induction of NK or T cell responses required for parasite control.

**Role of TIGIT in the CNS during toxoplasmosis**

To determine if the TIGIT/CD226 axis was operational during the chronic phase of this infection, which predominantly affects the CNS, CD155, TIGIT, and CD226 expression was measured on brain mononuclear cells isolated from chronically infected mice (Fig. 4A, 4B). Examination of the microglia, monocytes/macrophages, and DCs revealed that infiltrating monocytes and macrophages had the highest expression of CD155 (Fig. 4A). Furthermore, expression of CD226 and TIGIT was detected on these intracerebral T cells (Fig. 4B), suggesting that the CD155-CD226-TIGIT axis would be operational during TE. However, analysis of the T cell responses in the brains of chronically infected WT and TIGIT KO mice revealed comparable numbers of effector CD4+ and CD8+ T cells (Fig. 4C). Furthermore, stimulation of mononuclear cells isolated from the CNS of WT and KO mice with T. gondii–specific peptides revealed similar numbers of CD4+ and CD8+ IFN-γ-producing T cells (Fig. 4D) as well as similar MFI of IFN-γ (data not shown). Histological analysis of brain sections taken from WT and TIGIT KO mice showed similar levels of immune cell infiltration (Fig. 4E) and similar numbers of parasite cysts (Fig. 4F). Thus, the absence of TIGIT did not have any overt effect on the T cell response to T. gondii and parasite control within the CNS.

**DISCUSSION**

As CD155 is upregulated during both the acute and chronic phases of infection, and as CD226 and TIGIT are also expressed at these times by activated NK cells and parasite-specific T cells, it would appear that these pathways are operational during infection. Furthermore, these data show a heterogeneity of CD226 and TIGIT coexpression, indicating that TIGIT and CD226 are not always coexpressed on NK and T cells during toxoplasmosis. TIGIT and CD226 can define educational states of NK cells (42, 43), and so variable expression of these on NK cells might not be surprising, but coexpression on T cells could be expected based on a model of TIGIT’s primary role being regulation of costimulation through CD226. The existence of CD226 TIGIT+ cells, then, was unexpected. Although CD226 expressing cells most likely identify effector populations (29, 44, 45), the CD226 TIGIT+ cells may represent a terminal differentiation state or a cell type that can provide tolerogenic signals to DCs independent of any impact on CD226 (34).

Based on the previous studies in models of autoimmunity, cancer, and viral infection, combined with the dynamic expression of TIGIT during toxoplasmosis, it seemed likely that the loss of TIGIT would result in enhanced NK and T cell responses that, in turn, would lead to improved parasite control or even immune pathology. However, the experiments performed in this study provide no evidence that the loss of TIGIT significantly altered the immune response to T. gondii. Of relevance to this, previous reports using the chronic infection model established by the clone 13 strain of LCMV found that the deletion of TIGIT from T cells resulted in enhanced T cell responses and improved viral control (32). However, when these authors used the Armstrong strain of LCMV, the loss of TIGIT did not result in an improved ability to control this acute infection. These latter results parallel our own experience and argue that the immune context, and/or pathogen virulence, may determine whether the inhibitory effects of TIGIT on NK and T cell responses are biologically relevant.

Although the studies presented in this study have focused on the potential role of TIGIT in resistance to T. gondii, it is still not clear whether the costimulatory properties of CD226 contribute to the induction of NK and T cell responses to T. gondii. Other costimulatory receptors, such as CD28, are not required for resistance to T. gondii (46), and, furthermore, under certain immunological conditions, such as transplantation, CD226 is dispensable for the allogenic T cell response (47). In particular, the latter finding indicates that despite expression of the activating ligands for CD226 in the effector site, CD226 was still dispensable for effector T cell function. Thus, one possible reason the loss of TIGIT does not impact the immune response to toxoplasmosis is because the costimulatory receptor it regulates, CD226, is not required for resistance to T. gondii. Similarly, other inhibitory receptors, such as

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**In the spleen, liver, and lung of WT and TIGIT KO mice.** (C) Bulk splenocytes from WT and TIGIT KO mice were restimulated 8 dpi with PMA + ionomycin and IFN-γ responses of splenic NK cells (CD19–, CD3–, NK1.1+) were measured by intracellular staining and flow cytometric analysis. (D) Spleenocytes from WT and TIGIT KO mice at 8 dpi were stained with MHC-I and MHC-II tetramers to identify parasite-specific CD8+ and CD4+ T cells, respectively. Representative flow plots are shown of CD4+ T cells (top), and the numbers of tetramer specific CD4+ and CD8+ T cells are quantified (bottom). (E) Bulk splenocytes from WT and TIGIT KO mice were restimulated 8 dpi with T. gondii–specific peptides and IFN-γ responses measured by intracellular staining and flow cytometric analysis. Representative flow plots of CD4+ T cells are shown (top) with IFN-γ CD4+ and CD8+ T cell numbers quantified (bottom). (F) Bulk splenocytes from WT and TIGIT KO mice were restimulated with STAg for 72 h. Their supernatants were collected and measured for IFN-γ by ELISA. (G) Cytospins were performed on peritoneal exudate cells isolated from the peritoneal cavity of WT and TIGIT KO mice 8 dpi. These cells were then stained for parasite, analyzed by light microscopy, and the number of infected cells counted (left). DNA was extracted from the livers of WT and TIGIT KO mice 8 dpi and analyzed for parasite DNA by quantitative PCR. Amount of parasite was normalized to the total amount of input DNA (right).
FIGURE 4. Loss of TIGIT during the chronic phase of T. gondii infection does not impact T cell responses or parasite control.

WT and TIGIT KO mice were infected with T. gondii and analyzed at 27 dpi. (A) Brain, mononuclear cells were analyzed for expression of CD155. The gating strategy for identifying microglia (CD19−, CD3−, CD45int, CD11b+), monocytes/macrophages (CD19−, CD3−, CD45hi, F4/801, CD11cint), and DCs (CD19−, CD3−, CD45hi, F4/80−, CD11c−) is shown (left). Representative plots of CD155 expression on the above cell types in WT infected mice are shown and quantified (right). (B) Brain infiltrating, CD11ahi (top) or CD44hi (bottom), tetramer^1CD41 and CD81T cells from WT mice were analyzed for expression of CD226 and TIGIT. Representative flow plots are shown. (C) Brain-infiltrating CD41 and CD81 T cells from WT and TIGIT KO mice were stained with parasite Ag-specific class I and II tetramers. Representative flow plots are shown for tetramer stained CD41 T cells (top) and the numbers of tetramer staining CD41 and CD81 T cells are quantified (bottom). (D) Brain mononuclear cells were restimulated with T. gondii-specific peptides and intracellularly stained for IFN-γ before being analyzed by flow cytometry. Representative plots of CD41 T cells are shown (top), and numbers of IFN-γ1 CD41 and CD81 T cells are quantified (bottom). (E) Brains from infected WT and TIGIT KO mice were isolated and sectioned before staining for infiltrating cells and parasite cysts (arrows). Representative images are shown at original magnification x20. (F) Stained sections from (E) were counted for parasite cysts by light microscopy.
assess the impact of CD96 on resistance to phases of infection on NK and T cells (data not shown), and continued suppression in the absence of TIGIT. Our data indicate that CD96 is upregulated during both acute and chronic phases of infection on NK and T cells (data not shown), and this corresponds with a report of increased levels of CD96 transcripts during chronic infection (49). Additional studies to assess the impact of CD96 on resistance to T. gondii would help determine its effects during TE.

Finally, whereas the loss of TIGIT does not appear to impact the outcome of infection with T. gondii, TIGIT does limit NK and T cell responses in certain tumor and chronic viral models (32, 36). However, there are multiple TIGIT-independent mechanisms that limit the immune response during toxoplasmosis, and these may explain this difference. As activated parasite-specific T cells express numerous inhibitory receptors, perhaps these have overlapping functions and the blockade of several receptors may be required to distinguish their relative contributions. This would mirror the role of inhibitory receptors in other infectious settings, such as malaria, in which the blockade of PD-1 or Lag-3 alone had minimal effects on pathogen control while combined blockade resulted in significantly increased resistance (50). Similarly, in a mouse model of colorectal cancer, blockade of TIGIT had no effect on tumor control, but when combined with the blockade of PD-L1 the combination resulted in a dramatic improvement in antitumor responses (32). Thus, although toxoplasmosis may engage multiple inhibitory mechanisms to limit T cell hyperactivity, not all may contribute equally. Of the suppressive mechanisms that are present during T. gondii infection, then, it appears that the suppressive effects of the cytokines IL-10 and IL-27 (14, 17), the eicosanoid mediator LXA4 (51), and glucocorticoids (52) are dominant pathways in protecting from immune pathol-
gogy during toxoplasmosis. This would suggest a hierarchal role for these suppressive mechanisms during infection and that TIGIT-independent mechanisms dominate the restraint of the immune response during experimental toxoplasmosis.

DISCLOSURES

The authors have no financial conflicts of interest.

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A LIMITED ROLE FOR TIGIT IN T. GONDII INFECTION


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