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Involvement of TIGIT in Natural Killer Cell Exhaustion and Immune Escape in Patients and Mouse Model With Liver *Echinococcus multilocularis* Infection

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BACKGROUND AND AIMS: Alveolar echinococcosis (AE) is a lethal helminthic liver disease caused by persistent infection with *Echinococcus multilocularis*. Although more attention has been paid to the immunotolerance of T cells caused by *E. multilocularis* infection, the role of natural killer (NK) cell, a critical player in liver immunity, is seldom studied.

APPROACH AND RESULTS: Here, we observed that NK cells from the blood and closed liver tissue (CLT) of AE patients expressed a higher level of inhibitory receptor TIGIT and were functionally exhausted with a lower expression of granzyme B, perforin, interferon-gamma (IFN-γ), and TNF-α. Addition of anti-TIGIT (T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain) monoclonal antibody into AE patients' peripheral blood mononuclear cell culture significantly enhanced the synthesis of IFN-γ and TNF-α by NK cells, indicating the reversion of exhausted NK cells by TIGIT blockade. In the mouse model of *E. multilocularis* infection, liver and splenic TIGIT$^+$ NK cells progressively increased dependent of infection dosage and timing and were less activated and less degranulated with lower cytokine secretion. Furthermore, TIGIT deficiency or blockade *in vivo* inhibited liver metacestode growth, reduced liver injury, and increased the level of IFN-γ produced by liver NK cells. Interestingly, NK cells from mice with persistent chronic infection expressed a higher level of TIGIT compared to self-healing mice. To look further into the mechanisms, more regulatory CD56^{bright} and murine CD49a$^+$ NK cells with higher TIGIT expression existed in livers of AE patients and mice infected with *E. multilocularis*, respectively. They coexpressed higher surface programmed death ligand 1 and secreted more IL-10, two strong inducers to mediate the functional exhaustion of NK cells.

CONCLUSIONS: Our results indicate that inhibitory receptor TIGIT is involved in NK cell exhaustion and immune escape from *E. multilocularis* infection. (HEPATOLOGY 2021;74:3376-3393).

Alveolar echinococcosis (AE) is a lethal zoonotic helminthic liver disease caused by persistent infection with cestode *Echinococcus multilocularis*.[1] Humans are accidental intermediate hosts; the larval cells mimic a slowly growing liver cancer and progressively invade neighboring tissues

Abbreviations: AE, alveolar echinococcosis; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CLT, close liver tissue; cNK, conventional NK; DLT, distant liver tissue; Em-VF, E. multilocularis vesicular fluid; Fgl2, fibrinogen-like protein 2; HCs, healthy controls; HD, high dose; IFN-γ, interferon-gamma; LAG3, lymphocyte-activation gene 3; lrNK, liver-resident NK; mAb, monoclonal antibody; NK, natural killer; NKG2A, natural-killer group 2 member A; NKG2D, natural-killer group 2 member D; NKT, natural killer T; PBMC, peripheral blood mononuclear cell; PD-1, programmed cell death 1; PD-L1, programmed death ligand 1; TIGIT, T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain; TIM-3, T-cell immunoglobulin and mucin domain 3; trNK, tissue-resident NK; WT, wild type.

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and organs.[2] Chronic inflammation and granuloma located around the parasitic vesicles, along with extensive fibrosis, are the pathological characteristics of AE.[2] Much evidence has demonstrated that the inflamed liver microenvironment plays vital roles in hepatic echinococcosis[2,3]; therefore, how the parasite evades host immunity becomes an interesting question in several aspects of the pathogenesis of *E. multilocularis* infection. Previous research has reported T-cell tolerance as a key factor in the pathogenesis of *E. multilocularis* infection[4,5]; however, the role of innate immune cells in this process is still open for study.

Both innate and adaptive immunity are pivotal to the parasitic infection.[6] As an active member of innate immunity, natural killer (NK) cells represent ~25%-50% of human liver lymphocytes and 10%-15% of mouse liver lymphocytes. Accumulating findings have demonstrated their special importance in liver immunity and even proposed "liver as an innate immune organ" since this discrimination.[7] NK cells are a heterogeneous population, consisting of two phenotypically and functionally distant subsets. CD49a and CD49b (DX5) are commonly used to distinguishing these two NK subsets in mouse liver, in which $CD49a^{-}CD49b^{+}$ NK cells are recognized as conventional NK (cNK) cells and $CD49a^{+}CD49b^{-}$ NK cells as liver-resident NK (lrNK) cells[8]; the latter has been recently reported by us as a negative regulator in antiviral immunity to keep liver homeostasis.[9] In the human liver, the proportion of $CD56^{bright}CD16^{-}$ NK cells among NK cells is higher and they display some similarities with mouse lrNK cells with a high production of cytokines (i.e., interferon-gamma [IFN-γ]), whereas $CD56^{dim}CD16^{+}$ NK cells, similar to mouse cNK cells, serve primarily as cytotoxic effectors.[10] A few studies have described the existence of NK cells in AE patients and infected mice[11-14]; however, a detailed and systemic study on NK cells in relation to *E. multilocularis* infection remains a goal for research.

Activation and function of NK cells are controlled by a dynamic balance between arrays of inhibitory (recently called checkpoint) and activating receptors, differentially expressed on cell surfaces.[15] The expression pattern of immune checkpoint receptors and the

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exact regulation of NK cells in the *E. multilocularis*–infected liver microenvironment provide basic knowledge to understand the process of *E. multilocularis* immune escape, indicating the importance of such research. Chronic *E. multilocularis* infection is often associated with functional impairment of NK cells by regulating the expression of their inhibitory and activating receptors. For example, impairment of NK cell activity in the periparasitic area of the liver is partly attributable to the reduced expression of activating receptor natural-killer group 2 member D (NKG2D) and enhanced expression of inhibitory receptor natural-killer group 2 member A (NKG2A).[13,16] *In vitro* exposure to *E. multilocularis* vesicular fluid (*Em*-VF) induces the expression of the immune checkpoint receptors (i.e., programmed death-1 [PD-1]; lymphocyte-activation gene-3 [LAG-3]; and T-cell immunoglobulin and mucin-domain containing-3 [TIM-3]) and down-regulates the functions of NK cells.[12] Altogether, these data suggest that NK cells and their surface checkpoint receptors might play critical roles in the tolerated pathogen-host interaction in human echinococcosis.

T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain (TIGIT) is a recently discovered inhibitory receptor expressed mainly on T cells in its early discovery and is involved in the setting of chronic viral infections and tumors.[17] Our previous research has reported that TIGIT is a critical checkpoint to induce functional exhaustion of NK cells as well as T cells, and its blockade significantly reduces tumor growth in preclinical mouse models.[18] Additionally, evidence from our recent finding has indicated that TIGIT works as a checkpoint to mediate T-cell exhaustion in the process of *E. multilocularis* immune escape.[5] However, whether TIGIT regulates the functions of NK cells in *E. multilocularis* infection remains to be explored.

Here, we observed that NK cells from blood and liver tissues close to the parasitic lesions (close liver tissue; CLT) of AE patients expressed a high level of TIGIT, and these NK cells were functionally exhausted with impaired expression of granzyme B, perforin, IFN-γ, and TNF-α. Anti-TIGIT monoclonal antibody (mAb) successfully reversed the functional exhaustion of NK cells in AE patients' peripheral blood mononuclear cell (PBMC) culture. Furthermore, in the mouse model of *E. multilocularis* infection, the proportion of liver or splenic $TIGIT^+$ NK cells progressively increased depending on infection dosage and time. These $TIGIT^+$ NK cells were significantly less activated, less degranulated, and showed lower cytokine secretion. Importantly, TIGIT deficiency or blockade with mAb inhibited liver metacestode growth by reversing NK cell exhaustion. In addition, we found more regulatory $TIGIT^+$ NK cells within human $CD56^{bright}$ and murine $CD49a^+$ NK cells, with higher IL-10 secretion and highly coexpressed surface programmed death ligand 1 (PD-L1), two strong inducers to mediate the functional exhaustion of immune cells. Our results indicate that NK cell exhaustion induced by TIGIT is involved in the immune escape of *E. multilocularis* infection.

Materials and Methods

HUMAN DONORS

Liver specimens were obtained from 18 AE patients undergoing liver resection. Patients with immunesuppression–associated conditions, as described in Chauchet et al., were excluded.[4] AE diagnosis was confirmed on liver biopsies in all patients. One specimen was taken close to the parasitic lesion, including the metacestode (CLT, ~0.5 cm from the lesion), and/or one was taken in the macroscopically normal liver distant from the lesion (distant liver tissue [DLT], ≥2 cm distant from the lesion), as described in our previous publication.[5] Blood samples were obtained from 10 AE patients paired with tissue samplesand from 10 healthy age- and sex-matched controls (healthy controls; HCs). The number of liver and blood samples, types of measurements (given in detail in the Supporting Information), and purpose of comparisons are precisely given in Table 1. The study protocol was approved by the ethics committee of the First Affiliated Hospital of Xinjiang Medical University (no.: S20130418-3; Urumqi, China), and all patients provided informed written consent in accordance with the Declaration of Helsinki (1975) of the World Medical Association. All the data of the human subjects are summarized in Supporting Table S1.

TABLE 1. Liver and Blood Samples From AE Patients and HCs Used for Immunological Studies

Experiment						
Liver			AE Patients			
			CLT No. of Samples	DLT No. of Samples	Paired CLT and DLT No. of Sample Pairs	Comparison Between Groups
	FCM	$CD3^-$ $CD56^+$ NK cells	18	10	10	CLT vs. DLT
		NK cells, TIGIT	10	10	10	CLT vs. DLT
		NK cells, granzyme B, perforin, IFN-γ, TNF-α	10	10	10	CLT vs. DLT
			CLT No. of Samples	Comparison Between Groups		
		NK cells, CD226	10	$TIGIT^+$ NK cells vs. $TIGIT^-$ NK cells		
		NK cells, TIGIT, PD-1, 2B4	10	$TIGIT^+PD\text{-}1^-$ NK cells vs. $TIGIT^+PD\text{-}1^+$ NK cells vs. $TIGIT^-PD\text{-}1^+$ NK cells or $TIGIT^+2B4^-$ NK cells vs. $TIGIT^+2B4^+$ NK cells vs. $TIGIT^-2B4^+$ NK cells	
		NK cells, granzyme B, perforin, IFN-γ, TNF-α	10	$TIGIT^+$ NK cells vs. $TIGIT^-$ NK cells		
		NK cells, CD56, CD16	10	$CD56^{bright}CD16^-$ NK cells vs. $CD56^{dim}CD16^+$ NK cells		
		NK cells, TIGIT	10	$CD56^{bright}CD16^-$ NK cells vs. $CD56^{dim}CD16^+$ NK cells		
		NK cells, granzyme B, perforin, IFN-γ, TNF-α, IL-10, PD-L1	10	$CD56^{bright}CD16^-$ NK cells vs. $CD56^{dim}CD16^+$ NK cells		
		$CD56^{bright}CD16^-$ NK cells, granzyme B, perforin, IFN-γ, TNF-α, IL-10, PD-L1	10	$TIGIT^+$ $CD56^{bright}CD16^-$ NK cells vs. $TIGIT^-$ $CD56^{bright}CD16^-$ NK cells		
Blood			AE Patients and HCs			
			AE Patients No. of Samples	HCs No. of Samples	Comparison Between Groups	
	FCM	NK cells, TIGIT	10	10	AE vs. HC	
			AE Patients No. of Samples	Comparison Between Groups		
	FCM	NK cells, TIGIT, PD-1, 2B4	10	$TIGIT^+PD\text{-}1^-$ NK cells vs. $TIGIT^+PD\text{-}1^+$ NK cells vs. $TIGIT^-PD\text{-}1^+$ NK cells or $TIGIT^+2B4^-$ NK cells vs. $TIGIT^+2B4^+$ NK cells vs. $TIGIT^-2B4^+$ NK cells	
		NK cells, granzyme B, perforin, IFN-γ, TNF-α	10	$TIGIT^+$ NK cells vs. $TIGIT^-$ NK cells		
	Blockade	NK cells, IFN-γ, TNF-α	10	IgG vs. anti-TIGIT mAb		

Abbreviation: AE, alveolar echinococcosis; HCs, healthy control; CLT, close liver tissue; DLT, distant liver tissue; FCM, flow cytometry; vs, versus.

Key reagents or resources used in this study are listed in Supporting Table S2. Other materials and methods are included in the Supporting Materials and Methods.

STATISTICAL ANALYSIS

Student's *t* test was used to compare between two groups, and one-way analysis of variance (ANOVA) was used to compare between three or more groups. All data are presented as the mean ± standard deviation (SD). Values of $P < 0.05$ were considered statistically significant.

Results

HIGH EXPRESSION OF TIGIT AND FUNCTIONAL EXHAUSTION OF NK CELLS IN AE PATIENTS

To investigate the expression of TIGIT on different cell subsets in HCs and AE-infected patients, we analyzed the expression of TIGIT on $CD3^+$ T cells, NK cells, and natural killer T (NKT) cells in peripheral blood of HC and AE patients, respectively. We found that TIGIT was highly expressed on $CD3^+$ T cells, NK cells, and NKT cells of AE patients compared to

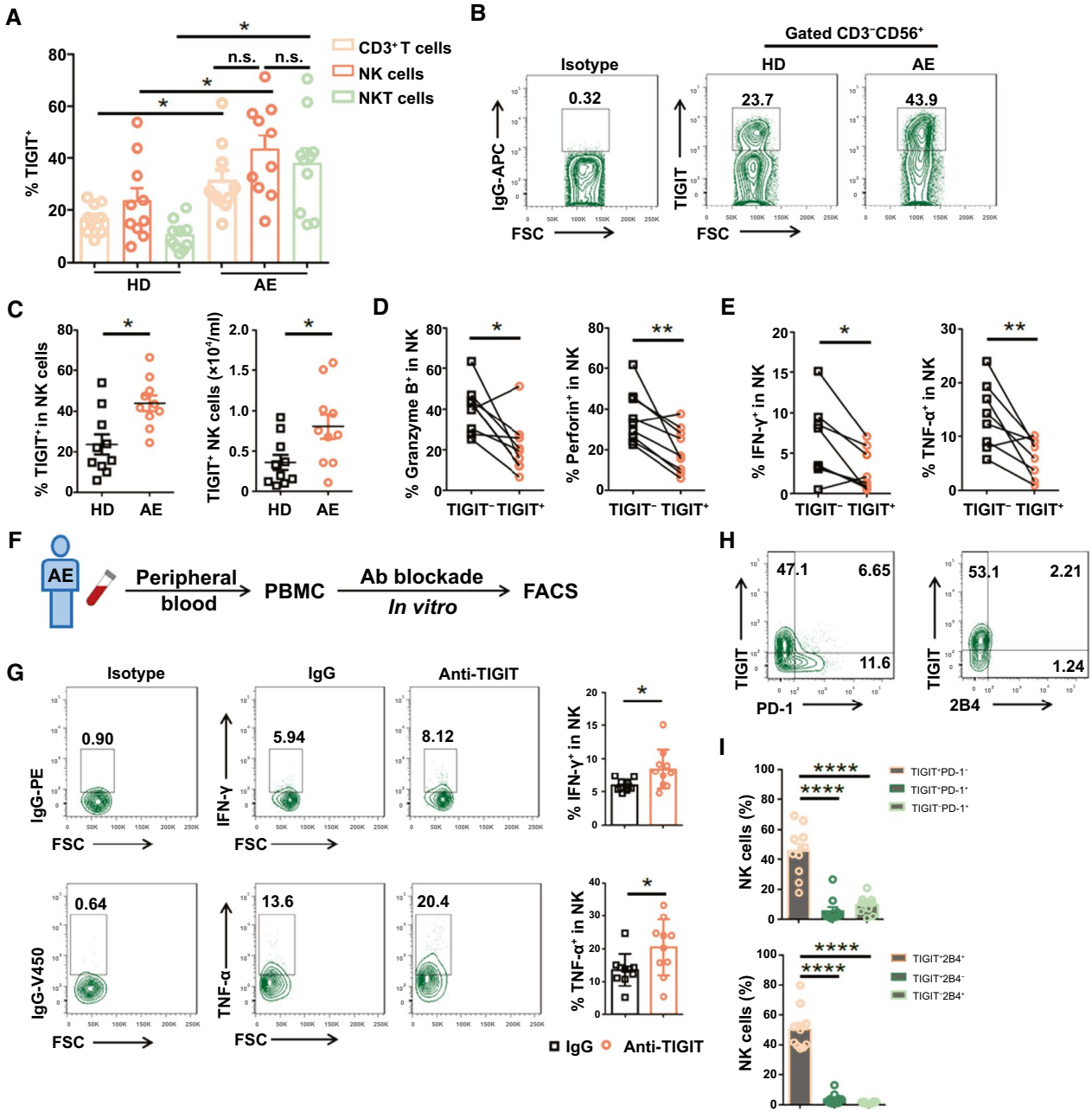

FIG. 1. TIGIT expression and dysfunction of blood NK cells in AE patients. (A) Percentage of $CD3^+$ T cells, NK cells, or NKT cells expressing TIGIT in PBMCs of HCs and AE patients (n = 10). (B) Representative flow cytometry plot of NK cells expressing TIGIT in PBMCs of HCs and AE patients (AE). (C) Percentage and absolute number of NK cells expressing TIGIT in PBMCs of HCs (n = 10) and AE patients (n = 10). (D) Expression of granzyme B and perforin in $TIGIT^+$ or $TIGIT^-$ NK cells from PBMCs of AE patients (n = 10). (E) Expression of IFN-γ and TNF-α in $TIGIT^+$ or $TIGIT^-$ NK cells from PBMCs of AE patients (n = 10). (F) Working model for (G). (G) Representative flow cytometry plot (left) and percentage (right, n = 10) of IFN-γ and TNF-α secretion by NK cells in the presence of control IgG or anti-TIGIT mAb. (H) Representative flow cytometry plot of NK cells coexpressing TIGIT with PD-1 or 2B4 in PBMCs of AE patients. (I) Percentage of NK cells coexpressing TIGIT with PD-1 or 2B4 in PBMCs of AE patients (n = 10). Data were analyzed using paired Student *t* tests or the one-way ANOVA test. All data are presented as mean ± SD. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s., $P > 0.05$. Abbreviations: Ab, antibody; APC, allophycocyanin; FACS, fluorescence-activated cell sorting; FSC, forward scatter.

those of HC (Fig. 1A); furthermore, TIGIT showed the highest expression on NK cells among the three cell subsets, indicating the importance of $TIGIT^+$ NK cells in the progression of AE infection. We then reconfirmed TIGIT expression on $CD3^-CD56^+$ NK cells in HCs and AE patients and observed a higher percentage and absolute number of $TIGIT^+$ NK cells in peripheral blood of AE patients (Fig. 1B,C). Furthermore, $TIGIT^+$ NK cells in peripheral blood of AE patients showed impaired degranulation (as indicated by expression of granzyme B and perforin; Fig. 1D) and cytokine secretion (as indicated by secretion of IFN-γ and TNF-α; Fig. 1E) compared to $TIGIT^-$ NK cells. To determine whether *in vitro* blocking of TIGIT on NK cells could reactivate their functional responsiveness, PBMCs were isolated from AE patients and restimulated with recombinant human interleukin 2 in the presence of anti-TIGIT mAb (Fig. 1F). Significant elevation of IFN-γ and TNF-α levels were observed in NK cells upon addition of anti-TIGIT mAb, indicating that TIGIT blockade could restore cytokine secretion of exhausted NK cells in AE patients (Fig. 1G). We also assessed the expression of PD-1 and 2B4 on $TIGIT^+$ NK cells, the two critical molecules in T-cell exhaustion, and the results indicated that $TIGIT^+$ NK cells were mostly PD-1 negative and 2B4 negative (Fig. 1H,I), indicating that TIGIT plays a more critical role under the scenario of NK cells during AE infection.

Next, we analyzed the expression of TIGIT on different cell subsets in liver tissues close to the parasitic lesion (CLT, ~0.5 cm from the lesion) of AE patients. Consistent with our findings in peripheral blood, TIGIT was highly expressed on NK cells in the CLT of AE patients compared to either $CD3^+$ T cells or NKT cells (Fig. 2A). Furthermore, by comparing the CLT and paired liver tissues distant from the parasitic lesion (DLT, ≥2 cm distant from the lesion), we found that the percentage and absolute number of NK cells were both higher in CLT compared to those in DLT (Fig. 2B), suggesting that more NK cells were infiltrated into liver lesions. However, NK cells in the CLT showed impaired degranulation (as indicated by the expression of granzyme B and perforin; Fig. 2C) and cytokine secretion (as indicated by the secretion of IFN-γ and TNF-a; Fig. 2D) compared to NK cells in the DLT. We then analyzed the expression of TIGIT on $CD3^-CD56^+$ NK cells in DLT and CLT, and observed a higher percentage and absolute number of $TIGIT^+$ NK cells in CLT of AE patients (Fig. 2E,F). TIGIT expression was colocalized with $CD56^+$ NK cells in CLT using immunofluorescence staining (Fig. 2G). Furthermore, $TIGIT^+$ NK cells in the CLT of AE patients showed significantly lower CD226 expression (Fig. 2H), were less degranulated (Fig. 2I), and produced less cytokines (Fig. 2J) compared to $TIGIT^-$ NK cells, suggesting that $TIGIT^+$ NK cells in the CLT are functionally impaired. We also assessed the expression of PD-1 and 2B4 on $TIGIT^+$ NK cells; the results indicated that $TIGIT^+$ NK cells were mostly PD-1 negative and 2B4 positive (Fig. 2K,L), suggesting that coexpression of TIGIT with 2B4 exists more commonly. Collectively, these results suggested that TIGIT played an important role in the regulation of NK cells during AE infection.

HIGH TIGIT EXPRESSION CORRELATES TO FUNCTIONAL EXHAUSTION OF NK CELLS IN THE MOUSE MODEL OF *E. multilocularis* INFECTION

To investigate the role of TIGIT on NK cells during infection, we constructed a mouse model of *E. multilocularis* infection. We found that the percentage of liver $TIGIT^+$ NK cells progressively increased, depending on the infection dosage (Fig. 3A), and the highest percentage of liver $TIGIT^+$ NK cells was found in high-dose (HD)-infected mice after 24 weeks (Fig. 3B); therefore, we decided to infect mice with HD *E. multilocularis* for 24 weeks for the following experiments. We first analyzed the activation of liver NK cells by the expression of CD69 and CD44, and found that liver $TIGIT^+$ NK cells were significantly less activated compared to $TIGIT^-$ NK cells in mice after 24 weeks of HD infection (Fig. 3C); in addition, they also expressed lower CD226 (Fig. 3C). Furthermore, consistent with our findings in humans, liver $TIGIT^+$ NK cells from HD-infected mice showed significantly impaired degranulation (as indicated by the expression of granzyme B and CD107a; Fig. 3D) and cytokine secretion (as indicated by the secretion of IFN-γ and TNF-α; Fig. 3E). Also, we found that liver $TIGIT^+$ NK cells were mostly PD-1 negative, 2B4 negative, and TIM3 negative (Fig. 3F) in this mouse model.

Consistent with our findings in liver NK cells, the percentage of splenic $TIGIT^+$ NK cells increased after *E. multilocularis* infection (Supporting Fig. S1A,B), and splenic $TIGIT^+$ NK cells were less activated compared to $TIGIT^-$ NK cells in mice after 24 weeks of infection

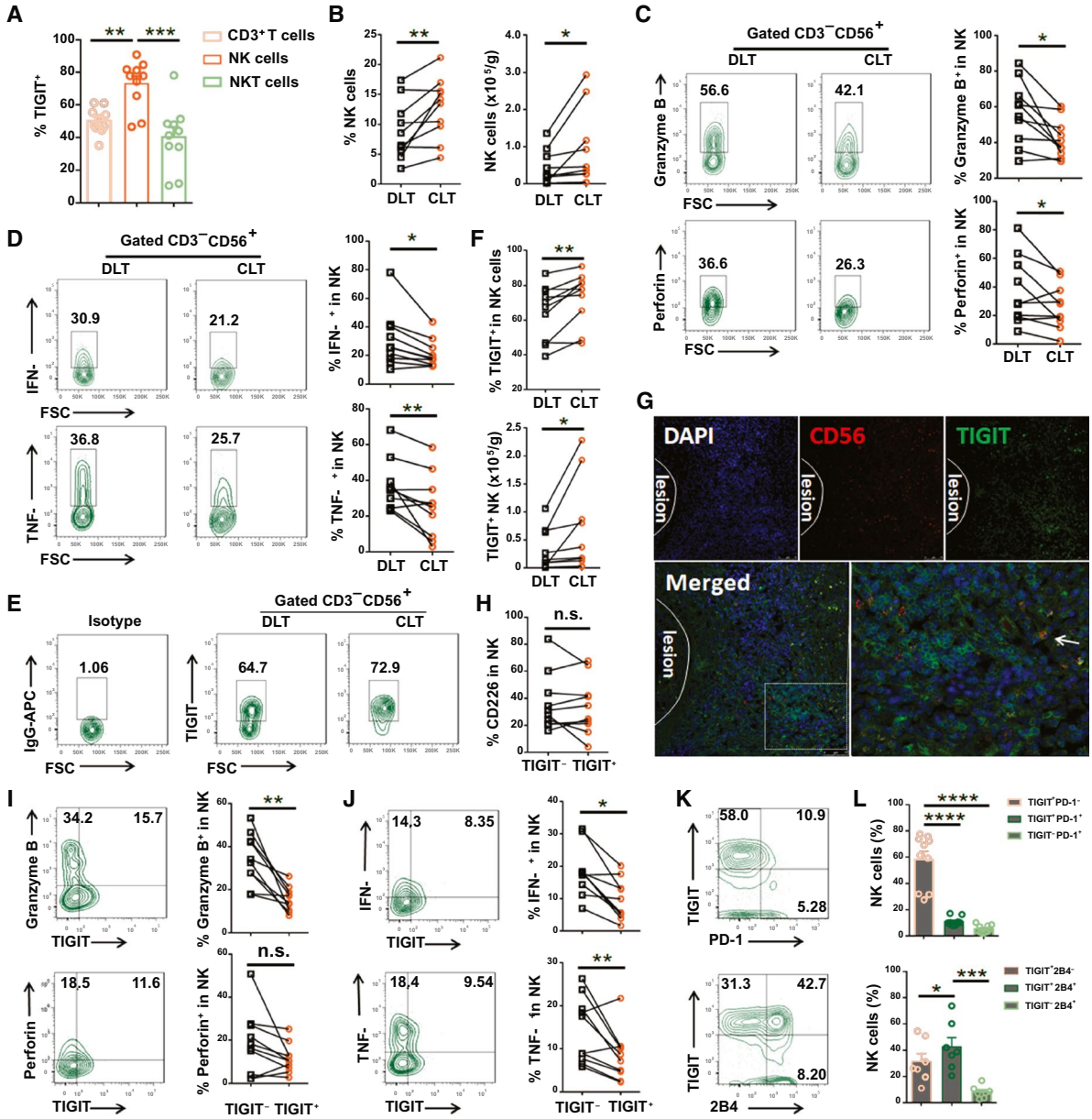

FIG. 2. TIGIT expression and dysfunction of NK cells in liver tissue of AE patients close to (CLT) and distant from (DLT) the parasitic lesions. (A) Percentage of $CD3^+$ T cells, NK cells, or NKT cells expressing TIGIT in CLT of AE patients (n = 10). (B) Percentage and absolute number of infiltrating NK cells isolated from paired DLT and CLT of AE patients (n = 10). (C) Representative flow cytometry plot (left) and percentage (right, n = 10) of granzyme B and perforin production by liver-infiltrating NK cells after phorbol 12-myristrate 13-acetate (PMA)/ionomycin stimulation in paired CLT versus DLT of AE patients. (D) Representative flow cytometry plot (left) and percentage (right, n = 10) of IFN-γ and TNF-α production by liver-infiltrating NK cells after PMA/ionomycin stimulation in paired CLT versus DLT of AE patients. (E) Representative flow cytometry plot of liver NK cells expressing TIGIT in paired CLT versus DLT of AE patients. (F) Percentage and absolute number of liver $TIGIT^+$ NK cells in paired CLT versus DLT of AE patients (n = 10). (G) Representative images from IF costaining of DAPI (blue), CD56 (red), TIGIT (green), and merged image on liver section from CLT of AE patients. (H) Percentage of CD266 expression in liver $TIGIT^+$ or $TIGIT^-$ NK cells in CLT of AE patients (n = 10). (I) Representative flow cytometry plot (left) and percentage (right, n = 10) of granzyme B and perforin production by liver $TIGIT^+$ or $TIGIT^-$ NK cells after PMA/ionomycin stimulation in CLT of AE patients. (J) Representative flow cytometry plot (left) and percentage (right, n = 10) of IFN-γ and TNF-α production by liver $TIGIT^+$ or $TIGIT^-$ NK cells after PMA/ionomycin stimulation in CLT of AE patients. (K) Representative flow cytometry plot of NK cells coexpressing TIGIT with PD-1 or 2B4 in CLT of AE patients (n = 10). (L) Percentage of NK cells coexpressing TIGIT with PD-1 or 2B4 in CLT of AE patients (n = 10). All data are presented as mean ± SD. Data were analyzed using the Student *t* test. $^*P < 0.05$; $^{**}P < 0.01$; n.s., $P > 0.05$. Abbreviations: APC, allophycocyanin; FSC, forward scatter.

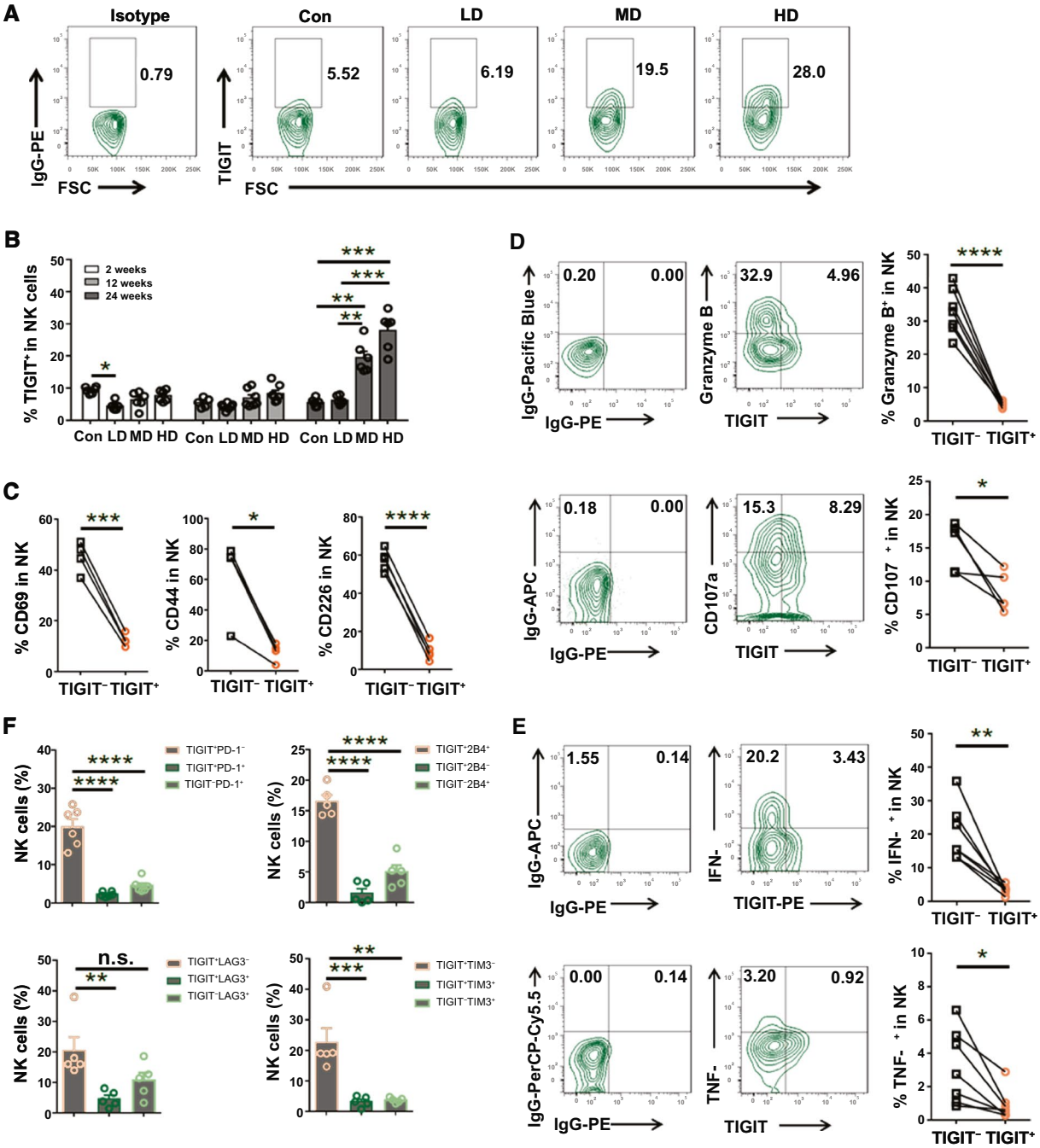

FIG. 3. High TIGIT expression and dysfunction of liver NK cells in mouse model of *E. multilocularis* infection. (A) Representative flow cytometry plot of liver NK cells expressing TIGIT in mice at week 24 after control (Con), low dose (LD), medium dose (MD), or HD infection. (B) Percentage of liver NK cells expressing TIGIT in mice at week 2, 12 or 24 postinfection (5-6 mice per group). (C) Percentage of $CD69^+$, $CD44^+$, or $CD226^+$ among $TIGIT^+$ or $TIGIT^-$ liver NK cells in mice at week 24 after HD infection (4 mice per group). (D) Representative flow cytometry plot and percentage (6 mice per group) of granzyme B and CD107a expression by $TIGIT^+$ or $TIGIT^-$ liver NK cells in mice at week 24 after HD infection. (E) Representative flow cytometry plot and percentage (6 mice per group) of IFN-γ and TNF-α secretion by $TIGIT^+$ or $TIGIT^-$ liver NK cells in mice at week 24 after HD infection. (F) Percentage of liver NK cells coexpressing TIGIT with PD-1, LAG3, 2B4, or TIM3 in mice at week 24 afrer HD infection (4-6 mice per group). All data are presented as mean ± SD and are representative of at least two independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$. Abbreviations: APC, allophycocyanin; Cy5.5, cyanine 5.5; FSC, forward scatter; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein.

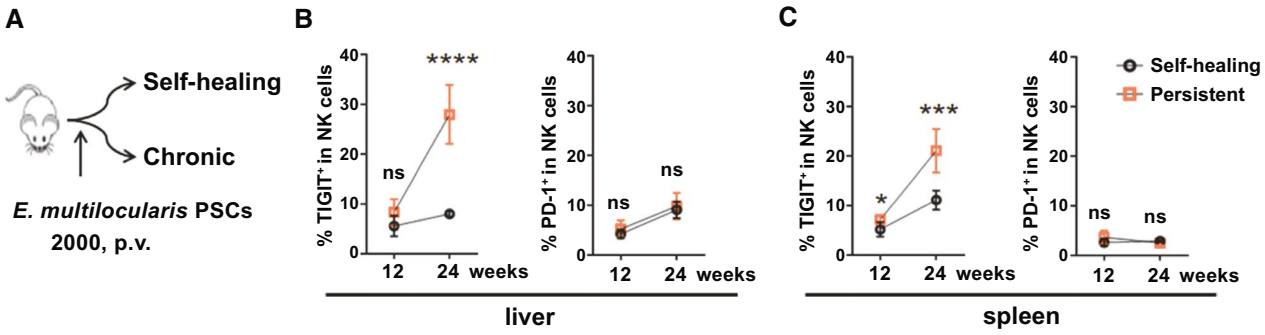

FIG. 4. High TIGIT expression correlates to persistent *E. multilocularis* infection in the long term. (A) C57BL/6 mice were infected with high-dose PSCs. (B) Percentage of liver NK cells expressing TIGIT or PD-1 in self-healing or persistent mice at week 12 or 24 after HD infection (5-6 mice per group). (C) Percentage of splenic NK cells expressing TIGIT or PD-1 in self-healing or persistent mice after 12 or 24 weeks of HD infection (5-6 mice per group). All data are presented as mean ± SD and are representative of at least two independent experiments. $^{*}P < 0.05$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s., $P > 0.05$. Abbreviations: PSCs, protoscoleces; p.v., portal vein.

(as indicated by the expression of CD69, CD44, and CD226; Supporting Fig. S1C). Furthermore, splenic TIGIT^{+} NK cells showed significantly impaired degranulation (Supporting Fig. S1D) and cytokine secretion (Supporting Fig. S1E). And, last, splenic TIGIT^{+} NK cells were mostly PD-1 negative, 2B4 negative, LAG3 negative, and TIM3 negative. Taken together, these findings indicated that high TIGIT expression was associated with functional exhaustion of NK cells in both liver and spleen in *E. multilocularis*–infected mice.

Because only a proportion of mice could eliminate the parasite during *E. multilocularis* infection in our model, we were interested to know whether TIGIT plays a role in the persistency of infection (Fig. 4A). Very interestingly, we found that mice with persistent chronic infection expressed a higher level of TIGIT compared to self-healing mice in both liver and spleen after 24 weeks of infection (Fig. 4B,C). In contrast, expression of PD-1 on NK cells showed no differences between the two groups in both liver and spleen (Fig. 4B,C), suggesting that up-regulation of TIGIT might result in the persistency of *E. multilocularis* infection by impairing NK cell functions.

To further prove the role of TIGIT on NK cells during *E. multilocularis* infection, we infected wild-type (WT) or TIGIT$^{-/-}$ mice with HD infection for 24 weeks. We found that TIGIT deficiency inhibited liver metacestode growth (Fig. 5A). Lesion weight and lesion number were significantly decreased in TIGIT-deficient mice compared to those of WT mice (Fig. 5B). TIGIT deficiency also prevented liver injury, given that both serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were decreased in TIGIT-deficient mice (Fig. 5C). Furthermore, a significantly increased level of IFN-γ was produced by liver NK cells from TIGIT-deficient mice compared to WT mice; however, no difference in the production of IFN-γ was observed between splenic NK cells from these two mouse strains (Fig. 5D). In addition, a higher level of IFN-γ was observed in liver CD8^{+} T cells from TIGIT-deficient mice, but not in splenic CD8^{+} T cells or liver and splenic CD4^{+} T cells (Supporting Fig. S2A,B).

Consistent with the results observed in TIGIT-deficient mice, blocking TIGIT with anti-TIGIT mAb significantly reduced liver metacestode growth (Fig. 5E). Lesion weight and lesion number were significantly reduced after TIGIT blockade compared to mice treated with IgG (Fig. 5F). Yet, TIGIT blockade showed no effect on the recovery of liver injury (as indicated by the serum ALT and AST levels; Fig. 5G). Furthermore, a significantly increased level of IFN-γ was produced by liver or splenic NK cells in mice treated with anti-TIGIT mAb (Fig. 5H). Last, to further confirm the functional role of IFN-γ, we infected IFN-γ-deficient mice with *E. multilocularis*. As expected, IFN-γ deficiency promoted liver metacestode growth (Fig. 5I); furthermore, lesion weight and lesion number were significantly increased in IFN-γ-deficient mice compared to WT mice (Fig. 5J), suggesting the importance of IFN-γ during *E. multilocularis* infection. Collectively, these results indicated that TIGIT deficiency or TIGIT blockade could enhance antiparasitic immunity by reversing the

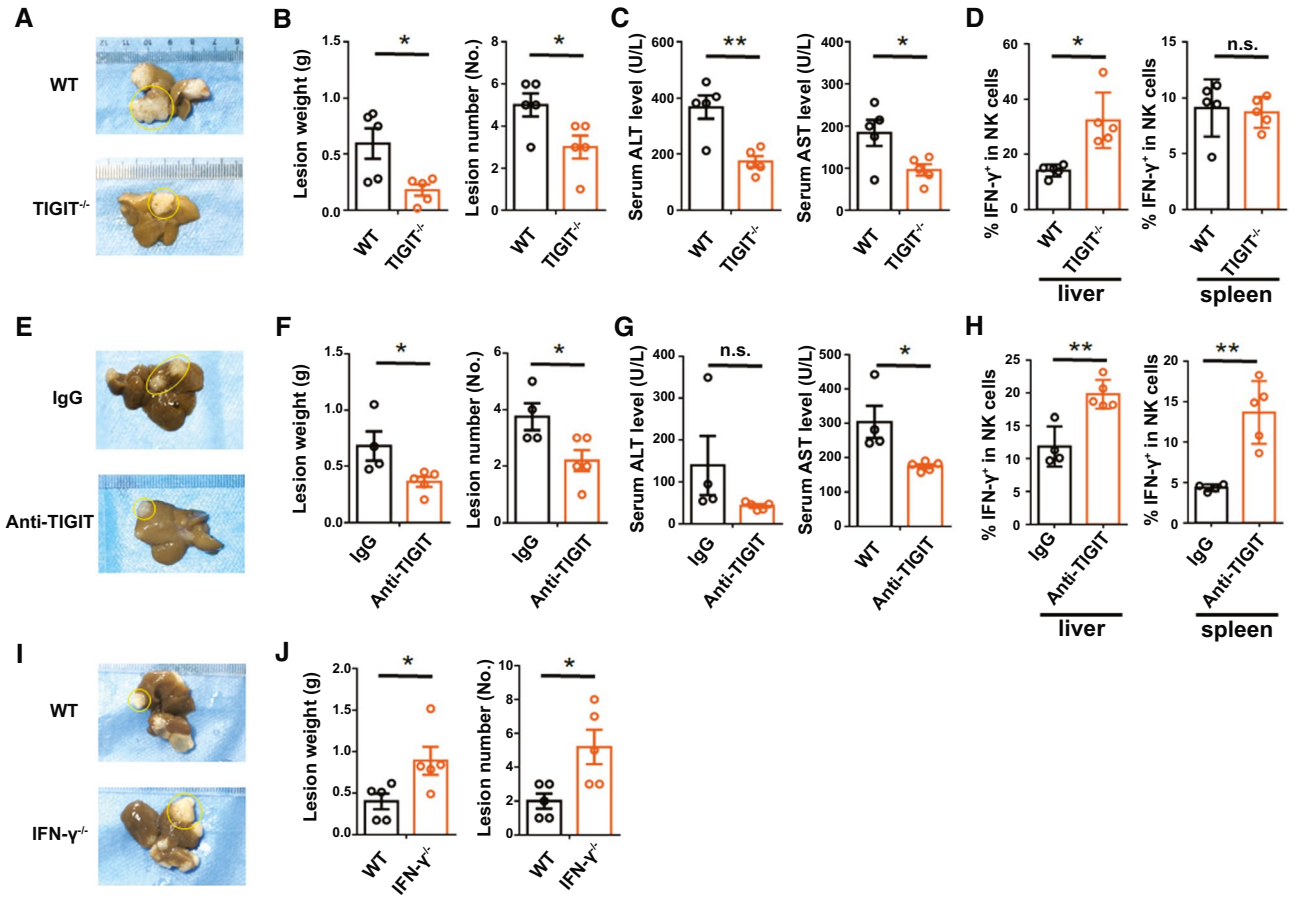

FIG. 5. TIGIT deficiency or blockade delays disease progression by inhibiting NK cell exhaustion in the mouse model of *E. multilocularis* infection. (A) Representative image of metacestode tissue in liver of WT or TIGIT$^{-/-}$ mice at week 24 after HD infection. Metacestode tissues are encircled by the yellow line. (B) Lesion weight (left) and number of infectious foci (right) in liver of WT or TIGIT$^{-/-}$ mice after 24 weeks of persistent infection (5 mice per group). (C) Serum ALT and AST concentrations of WT or TIGIT$^{-/-}$ mice after 24 weeks of persistent infection (5 mice per group). (D) Expression percentage of IFN-γ by liver (left) or splenic (right) NK cells in WT or TIGIT$^{-/-}$ mice at week 24 after HD infection (5 mice per group). (E) Representative image of metacestode tissue in liver of persistent infected mice treated with IgG or anti-TIGIT mAb for 12 weeks starting at week 12 postinfection (4-5 mice per group). (F) Lesion weight (left) and number of infectious foci (right) in liver of persistent infected mice treated with IgG or anti-TIGIT mAb for 12 weeks starting at week 12 postinfection (4-5 mice per group). (G) Serum ALT and AST concentrations of persistent infected mice treated with IgG or anti-TIGIT mAb for 12 weeks starting at week 12 postinfection (4-5 mice per group). (H) Expression percentage of IFN-γ by liver (left) and splenic (right) NK cells in the infected mice treated with IgG or anti-TIGIT mAb for 12 weeks starting from week 12 postinfection (4-5 mice per group). (I) Representative image of metacestode tissue in liver of WT or IFN-γ$^{-/-}$ mice at week 24 after HD infection. (J) Lesion weight (left) and number of infectious foci (right) in liver of WT or IFN-γ$^{-/-}$ mice at week 12 after HD infection (5 mice per group). All data are presented as mean ± SD and are representative of at least two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; n.s., P > 0.05.

exhaustion of NK cells and CD8$^+$ T cells, at least partially, by restoring their IFN-γ production.

TIGIT IS HIGHLY EXPRESSED ON REGULATORY NK CELLS IN *E. multilocularis* INFECTION

Human NK cells divide into two subsets: (1) CD56^{dim}CD16$^+$ NK cells that are highly cytotoxic against infected cells or tumor cells and (2) CD56^{bright}CD16$^-$ NK cells that produce cytokines.[10] Early studies have described CD56^{bright}CD16$^-$ NK cells as an "immunoregulatory" subset because of their lower capability of killing and higher production of cytokines, including immunosuppressive cytokine IL-10.[19] By analyzing CD56^{dim} and CD56^{bright} NK cell subsets in the CLT of AE patients, we found that more CD56^{bright} NK cells were infiltrated into liver lesions compared to CD56^{dim}

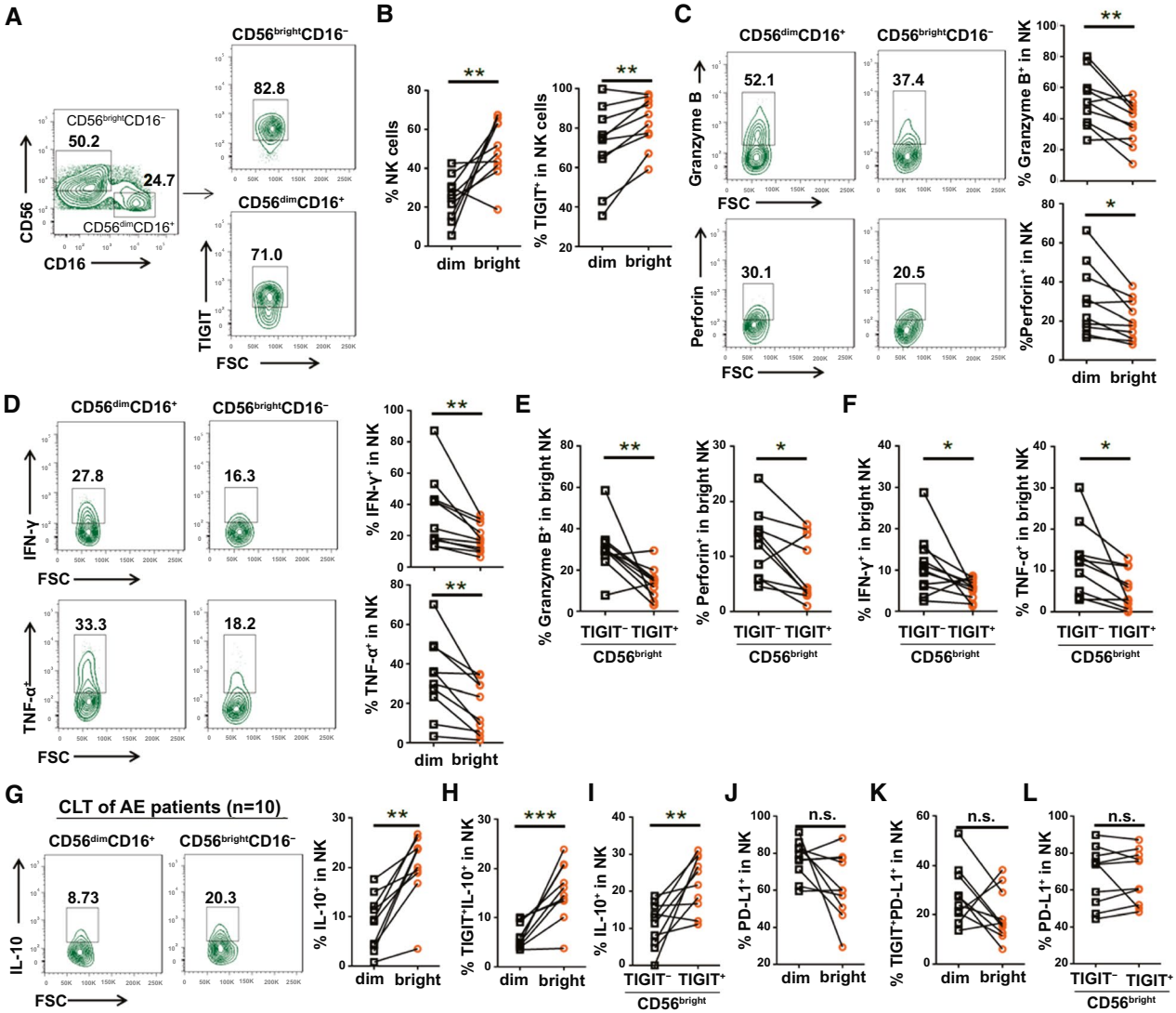

FIG. 6. Increased regulatory CD56^{bright}CD16^{-} NK cells in liver tissue of AE patients close to (CLT) the parasitic lesions. (A) Representative flow cytometry plot of TIGIT expression in CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} NK cells in CLT of AE patients. (B) Percentage of CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} NK cells or TIGIT expression in CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (C) Representative flow cytometry plot (left) and percentage (right) of granzyme B and perforin production by CD56^{dim}CD16^{+} NK cells or CD56^{bright}CD16^{-} NK cells in CLT of AE patients. (D) Representative flow cytometry plot (left) and percentage (right) of IFN-γ and TNF-α production by CD56^{dim}CD16^{+} NK cells or CD56^{bright}CD16^{-} NK cells in CLT of AE patients. (E) Percentage of granzyme B and perforin production by TIGIT^{-} or TIGIT^{+} CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (F) Percentage of IFN-γ and TNF-α production by TIGIT^{-} or TIGIT^{+} CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (G) Representative flow cytometry plot (left) and percentage (right) of IL-10 production by CD56^{dim}CD16^{+} NK cells or CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (H) Percentage of TIGIT^{+}IL-10^{+} CD56^{dim}CD16^{+} NK cells or TIGIT^{+}IL-10^{+} CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (I) Percentage of IL-10 production by TIGIT^{-} or TIGIT^{+} CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (J) Percentage of PD-L1 expression in CD56^{dim}CD16^{+} NK cells or CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (K) Percentage of TIGIT^{+}PD-L1^{+} CD56^{dim}CD16^{+} NK cells or TIGIT^{+}PD^{-}L1^{+} CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). (L) Percentage of PD-L1 expression by TIGIT^{-} or TIGIT^{+} CD56^{bright}CD16^{-} NK cells in CLT of AE patients (n = 10). All data are presented as mean ± SD. Data were analyzed using the Student *t* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$; n.s., $P > 0.05$. Abbreviation: FSC, forward scatter.

NK cells (Fig. 6A,B and Supporting Fig. S3). Based on our above results, we then analyzed the expression of TIGIT on these two NK cell subsets and discovered that $CD56^{bright}$ NK cells expressed more TIGIT than $CD56^{dim}$ NK cells (Fig. 6A,B). Furthermore, $CD56^{bright}$ NK cells in the CLT of AE patients exhibited impaired degranulation (as indicated by expression of granzyme B and perforin; Fig. 6C) and secreted a lesser amount of IFN-γ and TNF-α (Fig. 6D). $CD56^{bright}$ NK cells with surface expression of TIGIT produced less granzyme B, perforin, IFN-γ, and TNF-α (Fig. 6E,F) compared to those without TIGIT expression. IL-10 secretion and/or PD-L1 expression are two typical signatures of regulatory NK cells; we then analyzed IL-10 production of the two NK cell subsets. Not surprisingly, we found that $CD56^{bright}$ NK cells in the CLT of AE patients secreted more IL-10 (Fig. 6G); furthermore, those $CD56^{bright}$ NK cells with TIGIT coexpression produced an even higher amount of IL-10 than those without TIGIT expression (Fig. 6H,I). No significant difference in PD-L1 expression was observed between the two NK cell subsets (Fig. 6J); furthermore, TIGIT showed no influence over PD-L1 expression on these two NK cell subsets (Fig. 6K,L). Collectively, these data indicated that $CD56^{bright}$ NK cells exhibited a more immunoregulatory function than $CD56^{dim}$ NK cells during infection, and $TIGIT^+$ $CD56^{bright}$ NK cells were probably even more "regulatory" than $TIGIT^-$ $CD56^{bright}$ NK cells.

Previous studies have indicated that murine hepatic NK cells divide into $CD49a^+CD49b^-$ and $CD49a^-CD49b^+$ NK cell subsets, among which $CD49a^+CD49b^-$ NK cells are tissue-resident NK (trNK) cells that do not circulate in the blood whereas $CD49a^-CD49b^+$ NK cells are cNK cells that circulate in the blood.[8] cNK cells are cytotoxic against infected cells or tumor cells whereas the main function of trNK cells is to produce cytokines, including IL-10, and express ligands of inhibitory receptors (so-called checkpoints) in order to negatively regulate immune response (which is similar to the $CD56^{bright}$ NK cell subset in humans). Recently, the regulatory roles of trNK cells in the liver tumor microenvironment[20] and in homeostasis by controlling liver injury of hepatic virus infection[21] have been reported. By analyzing $CD49a^-$ cNK and $CD49a^+$ trNK cell subsets in liver of *E. multilocularis*–infected mouse models, we found that the percentage and absolute number of lrNK cells were significantly increased when compared to cNK cells in the infected liver (Fig. 7A,B and Supporting Fig. S4). Interestingly, the expression of surface TIGIT was markedly increased both on cNK and lrNK cells after infection; however, the absolute number of $TIGIT^+$ lrNK cells was much higher than that of $TIGIT^+$ cNK cells in the liver (Fig. 7C). By analyzing cytokine secretion, we found that IFN-γ production by lrNK, but not cNK, cells was significantly reduced after infection, whereas TNF-α production was not changed upon infection in lrNK cells (Fig. 7D). Given that IL-10 secretion and PD-L1 expression are the two typical signatures of regulatory NK cells, we evaluated IL-10 secretion and PD-L1 expression of liver NK cell subsets. IL-10 production by lrNK cells was increased by 3-fold after infection whereas no significant change was observed in IL-10 production by cNK cells (Fig. 7E). Furthermore, lrNK cells from both healthy and infected livers expressed significantly higher PD-L1 (with greater increase after infection) than cNK cells, whereas cNK cells also increased their PD-L1 expression after infection (Fig. 7F), suggesting a regulatory characteristic of lrNK cells in the infected model. Furthermore, the role of TIGIT in liver NK cell function was carried out. $TIGIT^+$ lrNK cells produced less IFN-γ and more IL-10 and expressed more PD-L1 compared to $TIGIT^-$ lrNK cells (Fig. 7G), suggesting a critical role of TIGIT on regulatory lrNK cells during infection.

We also analyzed the phenotype and function of these two NK cell subsets in the spleen. The content of trNK was relatively low compared to cNK cells. However, after infection, the absolute number of splenic trNK, but not cNK, cells was significantly increased (Fig. 8A,B and Supporting Fig. S4). Same as observed in the liver, the expression of surface TIGIT was markedly increased both on cNK and trNK cells after infection; however, the percentage and absolute number of $TIGIT^+$ trNK cells were not significantly different from those of $TGIT^+$ cNK cells in the spleen (Fig. 8C). Regarding cytokine secretion, we found that IFN-γ production by neither trNK nor cNK cells was changed after infection, whereas TNF-α production by trNK cells was dramatically increased (Fig. 8D). IL-10 production by both trNK and cNK cells was significantly enhanced after infection, among which trNK cells produced higher IL-10 than cNK cells (Fig. 8E). Although only splenic cNK cells increased PD-L1 expression upon infection, splenic trNK cells maintained a significantly higher PD-L1 expression

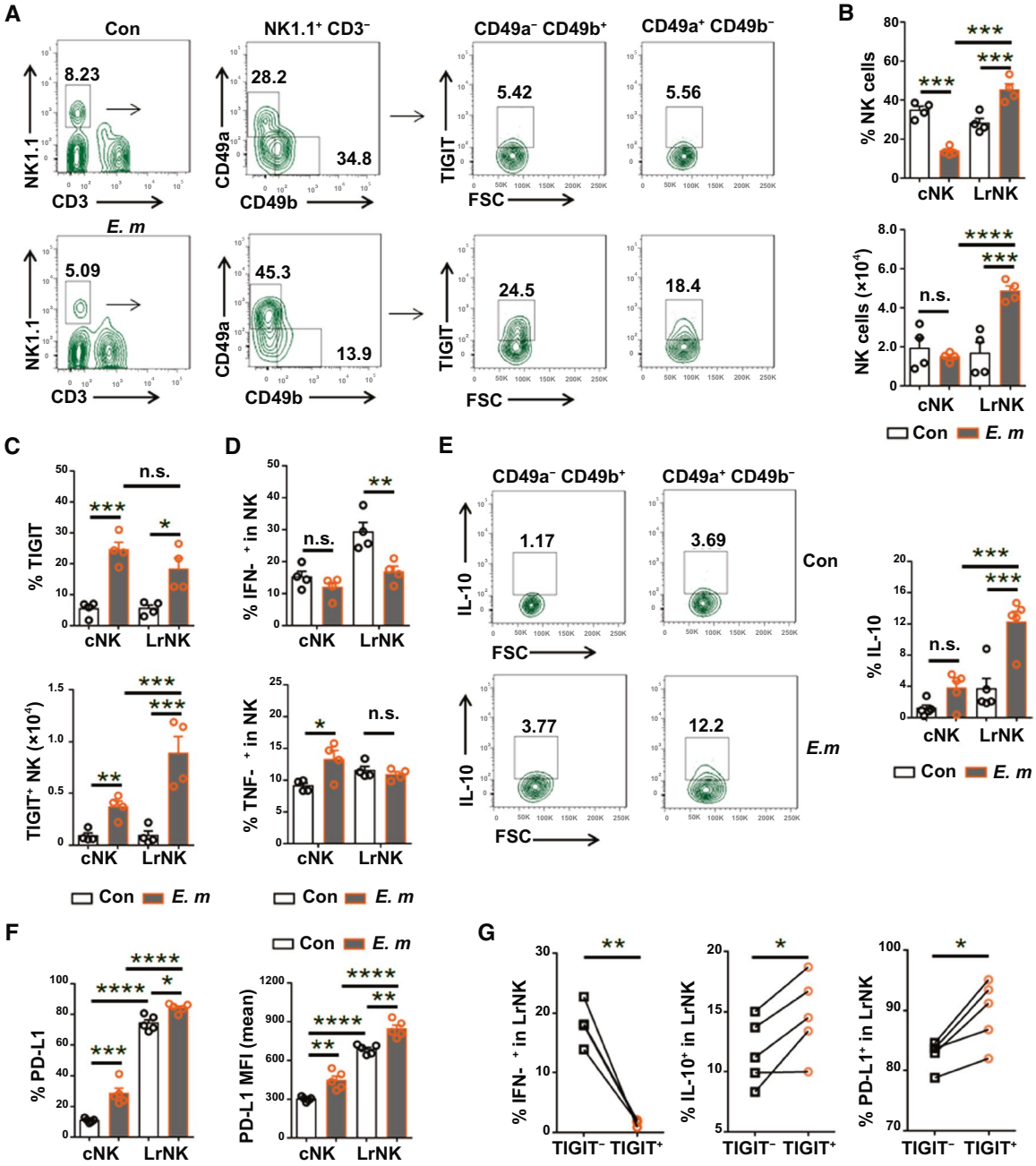

FIG. 7. Increased regulatory CD49a$^+$CD49b$^-$ NK cells in liver of *E. multilocularis*–infected mice. (A) Representative flow cytometry plot of TIGIT expression in liver CD49a$^-$CD49b$^+$ NK cells and CD49a$^+$CD49b$^-$ NK cells at week 24 after *E. multilocularis* infection. (B) Percentage and absolute number of liver CD49a$^-$CD49b$^+$ NK cells and CD49a$^+$CD49b$^-$ NK cells from mice at week 24 postinfection (4 mice per group). (C) Percentage and absolute number of TIGIT expression in liver CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells from mice at week 24 postinfection. (D) Percentage of IFN-γ and TNF-α secretion by liver CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells after PMA/ionomycin stimulation from mice at week 24 postinfection (4 mice per group). (E) Representative flow cytometry plot (left) and percentage (right) of IL-10 secretion by liver CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells after PMA/ionomycin stimulation from mice at week 24 postinfection (5 mice per group). (F) Percentage and MFI of PD-L1 expression in liver CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells from mice at week 24 postinfection (5 mice per group). (G) Percentage of IFN-γ, IL-10, and PD-L1 expression by liver TIGIT$^-$ or TIGIT$^+$ CD49a$^+$CD49b$^-$ NK cells after PMA/ionomycin stimulation from mice at week 24 postinfection (4 mice per group). All data are presented as mean ± SD and are representative of at least two independent experiments. $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$; n.s., $P > 0.05$. Abbreviations: Con, control group; *E. m*, persistent infected group; FSC, forward scatter; MFI, mean fluorescence intensity.

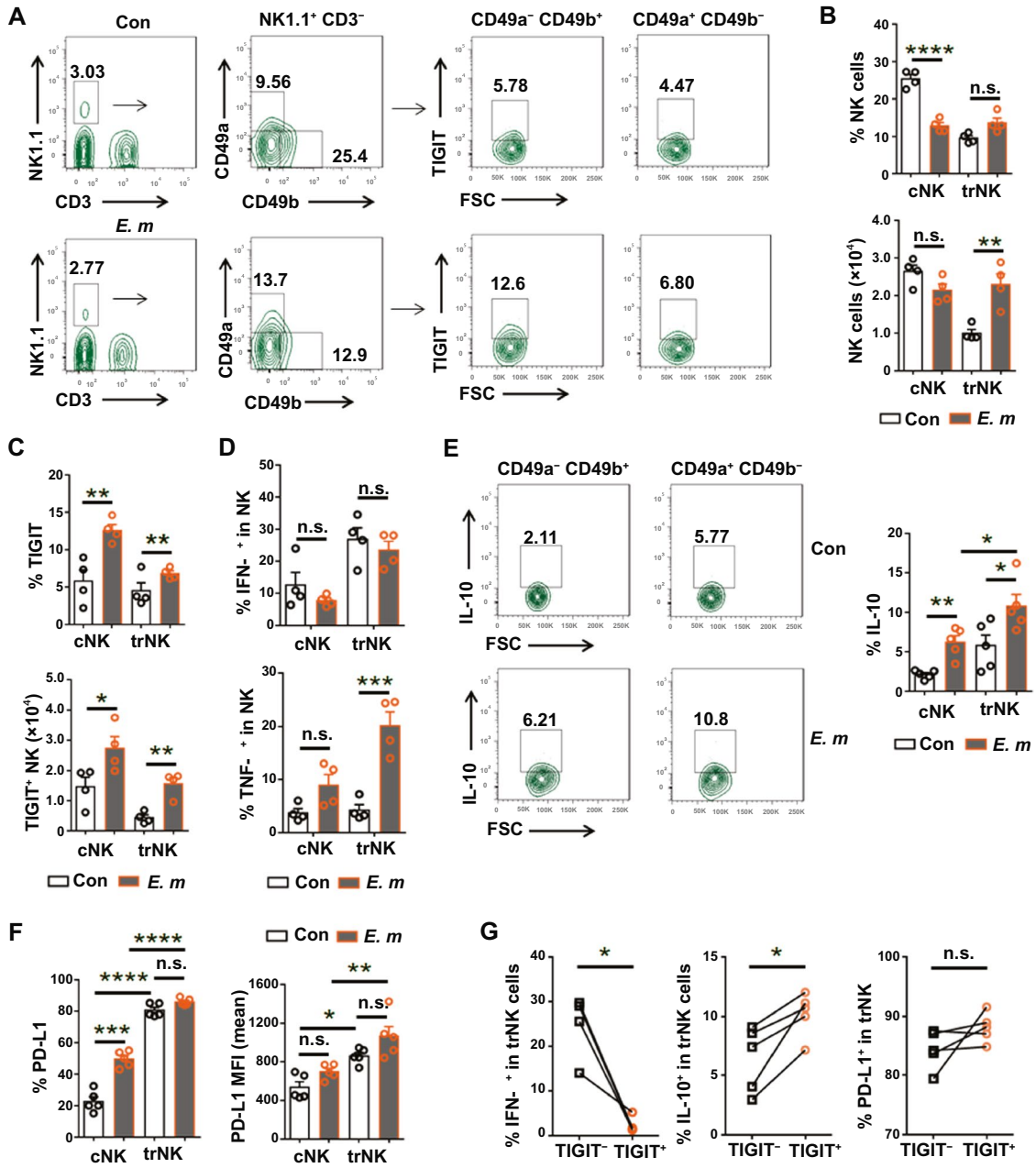

FIG. 8. Increased regulatory CD49a$^+$CD49b$^-$ NK cells in spleen of *E. multilocularis*–infected mice. (A) Representative flow cytometry plot of TIGIT expression in splenic CD49a$^-$CD49b$^+$ NK cells and CD49a$^+$CD49b$^-$ NK cells at week 24 postinfection. (B) Percentage and absolute number of splenic CD49a$^-$CD49b$^+$ NK cells and CD49a$^+$CD49b$^-$ NK cells from mice at week 24 postinfection (4 mice per group). (C) Percentage and absolute number of TIGIT expression in splenic CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells from mice at week 24 postinfection. (D) Percentage of IFN-γ and TNF-α secretion by splenic CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells after PMA/ionomycin stimulation from mice at week 24 postinfection (4 mice per group). (E) Representative flow cytometry plot (left) and percentage (right) of IL-10 secretion by splenic CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells after PMA/ionomycin stimulation from mice at week 24 postinfection (5 mice per group). (F) Percentage and MFI of PD-L1 expression in splenic CD49a$^-$CD49b$^+$ NK cells or CD49a$^+$CD49b$^-$ NK cells from mice at week 24 postinfection (5 mice per group). (G) Percentage of IFN-γ, IL-10, and PD-L1 expression by splenic TIGIT$^-$ or TIGIT$^+$ CD49a$^+$CD49b$^-$ NK cells after PMA/ionomycin stimulation from mice at week 24 postinfection (4 mice per group). All data are presented as mean ± SD and are representative of at least two independent experiments. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001; n.s., P > 0.05. Abbreviations: Con, control group; *E. m*, persistent infected group; FSC, forward scatter.

than cNK cells with or without infection (Fig. 8F), suggesting a possible regulatory role of splenic trNK cells. Same as observed in the liver, splenic TIGIT$^+$ trNK cells produced less IFN-γ and more IL-10 compared to TIGIT$^-$ trNK cells (Fig. 8G), suggesting a role of TIGIT on regulatory splenic trNK cells during infection.

Discussion

The NK cell population is one of the main actors of the immune system against intracellular viruses, tumors, and parasitic infection.[9,10,13,22] Functional deficiency of NK cells is associated with pathogen progression and contributes to disease pathogenesis.[22] A number of studies have indicated the importance of NK cells in the parasite/host interactions at different stages of echinococcosis (early and advanced).[13,14] Impaired functions of NK cells may be the key factor in the immune evasion strategy displayed by *E. multilocularis*; for example, NK cells from the peripheral blood of AE patients show lower cytotoxicity.[14] Our study showed that although more NK cells were infiltrated into the CLT of AE patients, they were functionally impaired compared to those in the DLT of AE patients.

Functions of NK cells are strictly regulated by a repertoire of activating and inhibitory receptors.[15] Available data have shown that *E. multilocularis* and its antigen inhibit the function of NK cells by up-regulating the expression of inhibitory receptors, including NKG2A, PD-1, and LAG-3.[12] We found that NK cells from the blood and CLT of AE patients expressed a higher level of inhibitory receptor TIGIT, which was reported to inhibit the activation of NK cells in tumors or chronic infection.[18,23] Our study showed that TIGIT$^+$ NK cells in the peripheral blood or CLT of AE patients were functionally exhausted with lower expression of granzyme B, perforin, IFN-γ, and TNF-α. It has been reported that exposure to *Em*-VF significantly decreases the expression of CD69 on NK cells,[11] in our study, by using the mouse model of *E. multilocularis* infection, we showed that liver and splenic TIGIT$^+$ NK cells from infected mice were less activated, as indicated by lower expression of CD69 and CD44. Furthermore, these TIGIT$^+$ NK cells were less degranulated (as indicated by perforin and granzyme B) with lower cytokine secretion (as indicated by IFN-γ and TNF-α). Together, these data suggest that NK cells accumulate and become functionally exhausted in *E. multilocularis*–infected liver, likely driven by persistent metacestode growth, during which TIGIT acts as a negative regulator of NK cells.

One issue raised in this study is how *E. multilocularis* infection induces TIGIT up-regulation on NK cells, thereby affecting the outcome of infection. Multiple lines of evidence have shown that high levels of cytokines or pathogen-derived proteins (antigen) directly or indirectly promote the up-regulation of inhibitory receptors, leading to the exhaustion of immune cells. For example, fibrinogen-like protein 2 (Fgl2) or IL-27 induce the expression of a panel of checkpoint molecules, including TIGIT, PD-1, PD-L1, and LAG-3, resulting in the dysfunction of T cells.[24,25] Studies have also reported that fatty-acid–binding protein 2 protein from *Fusobacterium nucleatum* binds directly to TIGIT to inhibit NK-cell– and T-cell–mediated tumor reactivity,[26] and that HTLV-1 bZIP factor induces the expression of TIGIT, which likely results in the impaired responses of T cells against viral antigens by enhancing IL-10 production.[27] Previous works have shown that Fgl2 and IL-27 both significantly up-regulate in liver or blood of *E. multilocularis*–infected mice or AE patients with progressive disease.[28,29] Furthermore, *Em*-VF induces the expression of inhibitory receptors (including PD-1, LAG-3, and TIM-3) on the surface of NK cells and inhibits NK cell activation and proliferation *ex vivo*.[12] Hence, these results suggest that Fgl2, IL-27, or *E. multilocularis*–derived antigens might contribute to up-regulated expression of TIGIT in NK cells during echinococcosis.

We further confirmed the reversion of NK cell exhaustion by TIGIT blockade. Addition of anti-TIGIT mAb into AE patients' PBMC culture significantly enhanced the secretion of IFN-γ and TNF-α by NK cells. Furthermore, TIGIT deficiency or blockade *in vivo* inhibited mouse liver metacestode growth, reduced liver injury, and increased the level of IFN-γ produced by liver NK cells and T cells. Our findings also showed that the proportion of TIGIT on NK cells derived from AE patients was higher than that on other lymphocytes (T cells or NKT cells; Fig. 2A), suggesting that TIGIT might be particularly important for NK cell function. One study showed that blockade of TIGIT improves adaptive immunity in a NK-cell–dependent manner in tumor models, indicating that NK cells not only exert a direct antitumor effect, but also assist the function of CTLs.[18] TIGIT

contributes to immunotolerance by inhibiting not only the immune responses mediated by T cells, but also those mediated by NK cells.[30] A recent study has reported the conversion of "exhausted NK cells" to type 1 innate lymphoid cells (ILC1s) in AE mice with PD-L1 blockade, which is involved in the inhibition of metacestode growth.[31] Overall, although we cannot exclude the role of T cells during this process, we believe that NK cells play an important role in disease progression as well, and loss or blockade of TIGIT influences disease progression through NK cells. Moreover, therapeutic strategies aiming at blocking TIGIT in tumors are being tested in various clinical trials in association with anti-LAG-3, anti-PD-1, and anti-PD-L1 (clinical trials: NCT04150965, NCT04570839, and NCT03119428) or alone (clinical trials: NCT04354246, NCT03628677). Our study suggests an opportunity of TIGIT blockade in restoring NK cell and T-cell function in AE patients.

Several studies have suggested that some NK cells play regulatory functions during infection and cancer, mainly through the production of immune-regulatory cytokines, such as IL-10, IL-13, and TGF-β,[9,32,33] or the expression of immune checkpoint molecules, such as PD-L1 and CD73.[21,34] Human NK cells divide into $CD56^{bright}CD16^{-}$ NK cells and $CD56^{dim}CD16^{+}$ NK cells; early studies have described $CD56^{bright}CD16^{-}$ NK cells as an immunoregulatory subset because of their lower cytotoxicity and higher secretion of cytokines, including immunosuppressive cytokine IL-10.[19,32] Interestingly, our study showed that these regulatory $CD56^{bright}$ NK cells were enriched in the CLT of AE patients with lower cytotoxicity (as indicated by perforin and granzyme B), lower secretion of effector cytokines (as indicated by IFN-γ and TNF-α), and higher secretion of immunosuppressive cytokine IL-10. Furthermore, TIGIT was highly expressed by $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells, and these $TIGIT^{+}CD56^{bright}$ NK cells showed lower cytotoxicity, lower effector cytokine secretion, and higher IL-10 production compared to $TIGIT^{-}CD56^{bright}$ NK cells, suggesting that the expression of TIGIT further promoted the immunoregulatory characteristic of $CD56^{bright}$ NK cells.

Murine liver NK cells divide into $CD49a^{+}CD49b^{-}$ and $CD49a^{-}CD49b^{+}$ NK cells[8]; the former are lrNK cells (recently also called ILC1s) that produce immunosuppressive cytokines, such as IL-10, and express checkpoint ligand PD-L1 (which is similar to $CD56^{bright}$ NK cells in humans).[21,35] We and others have found that lrNK and cNK cells are developmentally, phenotypically, and functionally distinct subsets.[10] Previous studies have reported that trNK cells expressing TIGIT, PD-1, and TIM-3 are more exhausted in tumors, whereas cNK cells can still harbor the classical phenotype, therefore being activated, and mount an efficient immune response.[20,21,36] The regulatory function of trNK cells has also been reported in the immune surveillance against pathogens.[37] Consistent with such works, our study showed that lrNK cells were enriched in *E. multilocularis*–infected mice and produced more IL-10 compared to cNK cells; in addition, TIGIT was highly expressed on lrNK cells, suggesting that lrNK cells play a more important role in this infection model. $TIGIT^{+}$ lrNK cells expressed higher PD-L1 and produced more IL-10, two typical characteristics of regulatory function, compared to $TIGIT^{-}$ lrNK cells. Blocking TIGIT *in vivo* significantly down-regulated the level of IL-10 in liver tissue surrounding the lesion using immunohistochemical staining (data not shown). In accordance with our study, previous works have also indicated that IL-10-producing NK cells can limit T-cell responses during parasitic infection[38] or viral infection.[39] Furthermore, NK cells can limit DC maturation or T-cell responses through a PD-L1–dependent mechanism during anti-tumor or -viral immune responses.[21,40] Therefore, we suggest that regulatory NK cells are enriched in the CLT of AE patients or liver of *E. multilocularis*–infected mice, and sustained up-regulation of TIGIT on regulatory NK cells may maintain NK cell exhaustion in both liver and periphery, which alternatively results in impaired antiparasitic responses and *E. multilocularis* persistence. Production of IL-10 or expression of PD-L1 by $TIGIT^{+}$ NK cells contributes to the compromised immunity that is associated with *E. multilocularis*–mediated chronic inflammation.

Meanwhile, the phenotypical and functional analysis in our study showed that NK cell responses in *E. multilocularis*–infected mice were different in the liver and spleen. In both organs, the absolute number of trNK cells was significantly increased after infection; however, $TIGIT^{+}$ lrNK cells was the dominant subset in the liver, whereas $TIGIT^{+}$ cNK cells was the dominant subset in the spleen. Furthermore, lrNK cells produced less IFN-γ and expressed more PD-L1 after *E. multilocularis* infection, whereas splenic trNK cells showed no change in expression of IFN-γ or PD-L1.

However, both liver and splenic trNK cells produced more IL-10 upon *E. multilocularis* infection. Organ-specific NK cell response has been reported during infections with lymphocytic choriomeningitis virus, murine cytomegalovirus, and *Plasmodium yoelii*.[41,42] The differences in NK cell response observed in these two organs could be a result of organ microenvironment and local immune characteristics, suggesting that organ-specific strategies are important in treating these organ-specific diseases.

A previous study has reported that TIGIT deficiency or inhibition promotes antitumor and/or antiviral function of NK cells, which is characterized by increased production of effector cytokine IFN-γ.[17,18] We also showed that TIGIT deficiency *in vivo* increased the level of IFN-γ produced by liver NK cells and $CD8^+$ T cells, which may be responsible for the inhibition of metacestode growth in *E. multilocularis*–infected mice. Furthermore, NK cells also produced more IFN-γ after TIGIT blockade, providing a major source of IFN-γ that confers host protection during chronic infection.[5] However, in contrast to IFN-γ, the level of TNF-α produced by NK cells was unchanged in TIGIT-deficient mice or mice treated with anti-TIGIT mAb during *E. multilocularis* infection. In line with our results, reduced production of IFN-γ has been observed in AE patients whereas TNF-α expression has not been detected in AE patients with abortive lesions.[43] These data suggest that IFN-γ, but not TNF-α, may act synergistically with other factors to slow parasite growth or achieve curative effects. In fact, available data have indicated that IFN-γ depends on the synergism of antiparasitic drugs or other factors to induce curative effects on AE patients.[44] Its role in the inhibition of larval growth is consistent with the findings on IFN-γ-deficient mice infected with *E. multilocularis* in our study.

Altogether, our study demonstrated that TIGIT was an important potential checkpoint to mediate NK cell exhaustion in AE patients and *E. multilocularis*–infected mice. Deficiency or blockade of TIGIT inhibited liver metacestode growth partly through reversing the functional impairment of NK cells. Furthermore, the increased prevalence of $TIGIT^+$ NK cells and their IL-10 and/or PD-L1 expression were important factors governing the persistent parasitic infection during echinococcosis. The findings suggest that targeting TIGIT may be a potential immune therapeutic strategy to treat patients with AE.

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Author Contributions: Conceived and design: C.S.Z., H.W., J.L., H.Y.S., and Z.G.T.; performed the experiments: C.S.Z., H.W., J.L., X.L.H., L.H.L., W.W., D.W.L., and Y.S.; analyzed the data: C.S.Z., L.L., and Z.B.Z.; contributed to technical- or materials-related issues: L.L., H.W., T.A., and Y.M.S.; drafted and revised the manuscript: C.S.Z., D.A.V., H.Y.S., H.W., and Z.G.T.; supervised the study: Z.G.T. and H.W. All authors reviewed and approved the manuscript.

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