

Synergistic blockade of TIGIT and PD-L1 increases type-1 inflammation and improves parasite control during murine blood-stage *Plasmodium yoelii* non-lethal infection

Rebecca S. Dookie,¹ Ana Villegas-Mendez,¹ Antonn Cheeseman,¹ Adam P. Jones,¹ Ruben Barroso,¹ Jordan R. Barrett,² Simon J. Draper,² Chris J. Janse,³ Jane L. Grogan,⁴ Andrew S. MacDonald,¹ Kevin N. Couper¹

AUTHOR AFFILIATIONS See affiliation list on p. 15.

ABSTRACT Pro-inflammatory immune responses are rapidly suppressed during blood-stage malaria but the molecular mechanisms driving this regulation are still incompletely understood. In this study, we show that the co-inhibitory receptors TIGIT and PD-1 are upregulated and co-expressed by antigen-specific CD4⁺ T cells (ovalbumin-specific OT-II cells) during non-lethal *Plasmodium yoelii* expressing ovalbumin (PyNL-OVA) blood-stage infection. Synergistic blockade of TIGIT and PD-L1, but not individual blockade of each receptor, during the early stages of infection significantly improved parasite control during the peak stages (days 10–15) of infection. Mechanistically, this protection was correlated with significantly increased plasma levels of IFN- γ , TNF, and IL-2, and an increase in the frequencies of IFN- γ -producing antigen-specific T-bet⁺ CD4⁺ T cells (OT-II cells), but not antigen-specific CD8⁺ T cells (OT-I cells), along with expansion of the splenic red pulp and monocyte-derived macrophage populations. Collectively, our study identifies a novel role for TIGIT in combination with the PD1-PD-L1 axis in regulating specific components of the pro-inflammatory immune response and restricting parasite control during the acute stages of blood-stage PyNL infection.

KEYWORDS *Plasmodium*, malaria, CD4⁺ T cell, immune regulation, checkpoint molecules

CD4⁺ T cells are essential for parasite control during blood-stage malaria; however, there is accumulating evidence in both murine models and humans that CD4⁺ T cells become functionally suppressed during infection with blood-stages of different *Plasmodium* species, displaying temporally reduced protective capacities (1–3). The suboptimal activation, differentiation, and functioning of CD4⁺ T cells during blood-stage malaria are believed to prevent the generation of sterile immunity to *Plasmodium* parasites, sustain chronic infection, and increase the rate of parasite reinfection (1–3). Thus, there is significant interest in understanding the pathways responsible for CD4⁺ T cell suppression during blood-stage malaria, to develop therapies to enhance protective anti-parasite immunity.

Co-inhibitory receptors are upregulated on activated CD4⁺ T cells and exert important immunoregulatory activities during many different infections and diseases, including during blood-stage malaria (4–8). During *P. falciparum* and *P. vivax* infections, human CD4⁺ T cells express regulatory receptors including PD-1 and CTLA-4, and their blockade *in vitro* improves CD4⁺ T cell effector activities (9–11). Similarly, individual or coordinate inhibition of PD-1, CTLA-4, BTLA, and LAG-3 re-invigorates CD4⁺ T cell effector function and improves anti-parasite control *in vivo* during various different murine malaria models (12–16). Conversely, however, blockade of specific co-inhibitory molecule activities can also exacerbate inflammation during blood-stage malaria, contributing to morbidity and

Editor Jeroen P. J. Saeij, University of California Davis, Davis, California, USA

Address correspondence to Kevin N. Couper, kevin.couper@manchester.ac.uk.

The authors declare no conflict of interest.

See the funding table on p. 15.

Received 6 August 2024

Accepted 3 September 2024

Published 26 September 2024

Copyright © 2024 American Society for Microbiology. All Rights Reserved.

mortality (17, 18). Thus, there is an urgent requirement to resolve the unique, redundant, and synergistic protective and pathogenic roles of different co-inhibitory receptors, to refine approaches for therapeutically targeting CD4⁺ T cell function during blood-stage malaria.

The co-inhibitory receptor T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT) is a member of the Poliovirus receptor (PVR) family that is expressed on various cell types, including effector T cells, regulatory T cells (Tregs), and natural killer cells (NK cells) (19–23). TIGIT functions within a complex receptor/ligand network, where it competes with the co-activating receptor CD226 (and the less characterized receptors CD96 and CD112R) for binding to the ligands CD155, CD112, and CD113, which are expressed on APCs and non-immune cells (24, 25). As well as competitively inhibiting CD226 function (20), activation of TIGIT (upon binding to CD155) inhibits TCR signaling and MAP-kinase and NF- κ B pathways, directly limiting T cell and NK cell activity (26, 27). TIGIT-CD155 interactions can also indirectly regulate effector T cell responses through increasing IL-10 and downregulating IL-12 production by dendritic cells (23), and by enhancing the suppressive activity of specialized Foxp3⁺ regulatory T cell subsets, which preferentially inhibit Th1 and TH17 cell responses (21, 28). Evidence in various models suggests that TIGIT acts primarily in cooperation with PD-1 to suppress T cell effector functions, with TIGIT co-expressed with PD-1 on exhausted T cells (20, 29). Thus, synergistic TIGIT and PD-1 co-blockade are significantly more effective during cancer and infection than corresponding mono-therapy (24, 25, 30). We have previously shown that TIGIT is highly expressed on CD4⁺ T cells during PyNL blood-stage infection, and is elevated on suppressive Th1-IL-10 producing cell populations compared with IFN- γ -producing Th1 cells (31). TIGIT is also co-expressed with PD-1 on activated *P. falciparum*-specific CD4⁺ T cells (32). However, the functional importance of TIGIT in controlling CD4⁺ T cell responses during blood-stage malaria has not yet been addressed.

In this study we have investigated the role of TIGIT in suppressing CD4⁺ T cell responses during PyNL blood-stage infection. We show that TIGIT is co-ordinately expressed with PD-1 on Th1 and Tfh cells during PyNL infection. Co-blockade of TIGIT and PD-L1 significantly improved parasite control during a blood-stage PyNL-OVA infection, which was correlated with increased IFN- γ production by antigen-specific OT-II cells, but not OT-I cells, and expansion of splenic macrophage compartments. Although the co-blockade of TIGIT and PD-L1 also significantly increased Tfh cell GC B cell and plasmablast responses, treatment did not significantly influence the level of anti-Merozoite Surface Protein-1 19 kDa (MSP-1₁₉) antibody. Collectively, our results show that TIGIT and PD-1 synergistically impair parasite clearance and limit pro-inflammatory γ -1 responses during blood-stage PyNL infection.

MATERIALS AND METHODS

Mice and infections

Male 7- to 9-week-old C57BL/6 mice (CD45.2⁺) were purchased from Charles River or Envigo, UK. RAG-1 OT II x CD45.1⁺Pep3 mice (OT-II) and RAG-1 OT-I x CD45.1⁺Pep3 mice (OT-I), both on a C57BL/6 background, were bred at UoM. The RAG-1 OT-II mice used in this study were engineered with the OT-II T cell Receptor (TCR) transgene on the Y chromosome (33), limiting all adoptive transfer experiments to male mice. All mice were maintained in specific pathogen-free conditions in individually ventilated cages. Mice were infected with non-lethal *Plasmodium yoelii* expressing ovalbumin [PyNL-OVA (15)]. In this transgenic parasite line, ovalbumin is fused to mCherry and the *ova-mCherry* transgene is under the control of the constitutive and strong *hsp70* promoter. Cryopreserved PyNL-OVA parasites were passaged once in C57BL/6 mice before experimental mice were infected via intravenous (i.v.) injection of 10⁴ parasitized red blood cells (pRBCs). The course of infection was monitored by assessing peripheral parasitemia via microscopic examination of Giemsa-stained thin blood smears.

T cell purification and adoptive transfer

Spleens were isolated from male RAG-1 OT II $\times$ Pep3 mice or RAG-1 OT I $\times$ Pep3 mice and homogenized through a 70- μ m strainer (BD Biosciences) before RBCs were lysed by incubation in RBC lysis buffer (BD Biosciences). OT-II cells and OT-I cells were isolated from the resultant single-cell splenocyte suspensions using anti-CD4 or anti-CD8 conjugated microbeads (Milenyi Biotec), respectively, according to the manufacturer's instructions (purity was typically >90%). 1×10^6 OT II cells were individually transferred or co-transferred with 1×10^5 OT I cells into male C57BL/6 via i.v. injection 1 day prior to infection with PyNL-OVA pRBC (as described above).

In vivo TIGIT and PD-L1 blockade experiments

Mice received 250 μ g of α -PD-L1 (10F.9G2) (BioXcell) and/or 250 μ g of α -TIGIT [10A7; (20)] antibodies (Abs) every other day from day 5 of infection via intraperitoneal (i.p.) injection. Control mice received 250 μ g of Rat IgG (Sigma Aldrich) via i.p. injection.

Flow cytometry

Spleens were removed from naïve and malaria-infected mice. For T cell analysis, spleens were homogenized through a 70- μ m strainer and incubated with RBC lysis buffer (Thermo Fisher) to generate an RBC-free, single-cell suspension. For analysis of myeloid cells, spleens were cut into small pieces and incubated with HBSS containing 2 mg/mL collagenase D 155 (Sigma Aldrich) and 50 KU/mL Dnase (Sigma Aldrich) for 30 min at 37°C prior to homogenization through a 70- μ m strainer and RBC lysis. Absolute live-cell counts were calculated by trypan blue exclusion cell viability assay (Sigma). Splenocytes were surface stained for 25 min at 4°C with anti-mouse antibodies, depending on the panel and cell types analyzed: CD45.1 (A20), CD4 (RM4-5), CD8a (53-6.7), CD44 (IM7), PD-1 (RMPI-30), TIGIT (GIGD7), CXCR5-biotin (L138D7), CD25 (PC61) B220 (RA3-6B2), CD19 (6D5), GL7 (GL7), CD38 (90), CD138 (281-2), IgD (11-26c-2a), CD45 (30F1), Ly6C (HK1.4), F4/80 (BM8), MHC II (M5/114.15.2), CD80 (16-10A1), CD169 (3D6.112), PD-L1 (10F9G2), CD11c (N418), CD11b (M1/70), NK1.1 (PK136), and CD64 (X54-5/7.1). CD3 (17A2), CD19 (6D5), B220 (RA3-6B2), and Ly6G (1A8) were used to generate a lineage gate for innate/myeloid cell analysis. Surface staining was done in the presence of FcR block (2.4G2, BioXcell). For streptavidin staining, the cells were stained with surface antibodies, washed, and subsequently incubated for 10 min at room temperature (RT) with streptavidin BV510.

For intracellular staining, the cells were incubated with Foxp3 fixation/permeabilization buffer (eBioscience) for 30 min at 4°C. The cells were then stained with the following anti-mouse antibodies: T-bet (4B10), Foxp3 (FJK-16s), and Ki-67 (SolA15) for 30 min. For granzyme B staining, the cells were additionally fixed in 2% PFA prior to incubation with Foxp3 fixation/permeabilization buffer. For intracellular detection of IFN- γ (XMG1.2) and TNF α (MP6 XT22), the cells were stimulated *ex vivo* for 4 h at 37°C with 200 ng/mL PMA (Sigma), 1 μ g/mL ionomycin (Sigma) and Brefeldin A (1,000 $\times$) (eBioscience).

Doublets and dead cells were excluded from all analyses using forward and side scatter properties and LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Life Technologies). All antibodies were acquired from eBioscience/ThermoFisher or BioLegend. Samples were acquired on a Fortessa (BD Systems, UK) and all analysis was performed using FlowJo Software (Treestar Inc, OR, USA). Samples from PyNL-infected mice were combined to generate fluorescence minus one (FMO) control samples.

Quantification of plasma cytokines

Plasma isolated from naïve or malaria-infected mice was stored at -80°C until further use. The concentration of IFN- γ , TNF, IL-10, and IL-2 was measured by a Cytometric Bead Array (CBA) mouse Th1/Th2/TH17 cytokine kit (BD Biosciences), according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay to measure PyNL-specific IgG

Enzyme-linked immunosorbent assays (ELISAs) to measure MSP-1₁₉-specific IgG levels in plasma were performed as previously described (15). Briefly, Nunc MaxiSorp plates (Fisher), previously coated and incubated (at 4°C overnight) with 2 µg/mL *P. yoelii* MSP-1 19 kDa (MSP-1₁₉) protein in Dulbecco's PBS (DPBS) (Sigma), were washed with PBS/T [PBS with 0.05% Tween-20 (Sigma)] and blocked at room temperature (RT) for 1 h using 5% milk (sigma) in PBS/T. Following washing, sera samples were plated in duplicate (diluted in PBS/T) in a threefold dilution series and the plate was incubated for 2 h at RT. After washing, goat anti-mouse IgG-AP (Sigma) was added to the plate (at 1:1,000 dilution), incubated for 1 h at RT, and plates were washed and development buffer [PnPP (Thermo) dissolved in Diethanolamine substrate buffer (Pierce)] was added to each well and plates read.

Statistical analysis

Statistical analysis was performed in GraphPad Prism (GraphPad Software, USA). When data from two combined experiments were represented graphically, comparisons between two or more groups were performed using a two-way ANOVA with Tukey's test, to account for potential inter-experimental variability affecting analyses. Significant inter-experimental variability was detected in some analyses (not annotated in graphs). When data from one experiment (of *n* number of experiments) were presented, data were first analyzed using the Shapiro–Wilk normality test. Depending upon distribution of data, two group comparisons were carried out using an unpaired *t* test or a Mann–Whitney test and more than two group comparisons using a one-way ANOVA with Tukey's Test or Kuskall–Wallis with Dunn's test. Results were considered significant when *P* < 0.05. Statistical analyses performed in each figure are outlined in figure legends.

RESULTS

Expression of TIGIT on leukocyte populations during PyNL infection

TIGIT can be expressed on multiple immune cell types during infection and disease (19–23). Therefore, to examine the various potential immunoregulatory roles of TIGIT during blood-stage malaria we first characterized its expression on different splenic leucocyte populations during blood-stage infection with *PyNL* parasites expressing ovalbumin in blood-stages (*PyNL*-OVA). On day 15 of infection [relating to peak parasitemia: (34)], TIGIT was significantly upregulated on an apparent subpopulation of CD4⁺ T cells, generally upregulated on CD8⁺ T cells and NK cells, but was not upregulated on B cells, with TIGIT MFI measured on total cell populations (Fig. 1A and B). Interestingly, CD4⁺ T cells exhibited the highest fold increase in TIGIT expression on day 15 infection (increase in expression above level on corresponding cells in uninfected mice), compared with other examined cell populations (Fig. 1C). TIGIT was rapidly upregulated on endogenous polyclonal CD4⁺ T cells by day 5 of infection, with levels increasing slightly between day 5 and the peak of infection on day 15 (Fig. 1D through F). Thus, TIGIT is rapidly expressed to potentially regulate CD4⁺ T cell responses during blood-stage *PyNL* infection.

Having identified that endogenous polyclonal CD4⁺ T cells upregulate TIGIT during infection, we next assessed the expression of TIGIT on antigen-specific CD4⁺ T cells. OT-II cells were rapidly activated in male mice during *PyNL* infection, with high levels of IFN-γ production and Ki-67 expression on day 5 of infection (Fig. S1). However, effector (CD44⁺) OT-II cells failed to sustain effector functions, with significant reduction in IFN-γ and Ki67 expression by day 9 of *PyNL*-OVA infection (Fig. S1). Relating to the functional decline of effector OT-II cells, TIGIT was highly expressed on OT-II T-bet⁺ and PD-1⁺CXCR5⁺ Tfh cells (gating strategy shown in Fig. S2) by day 5 of *PyNL*-OVA infection (Fig. 1G and H). Although TIGIT was stably maintained on T-bet⁺ OT-II cells up to day 15 of infection, TIGIT levels peaked on Tfh OT-II cells on day 5 of infection before declining on day 15 of infection (Fig. 1G and H). We did not observe induction of Foxp3 in OT-II T cells (Fig. S2), so we were unable to examine the level of TIGIT on antigen-specific OT-II Treg cells

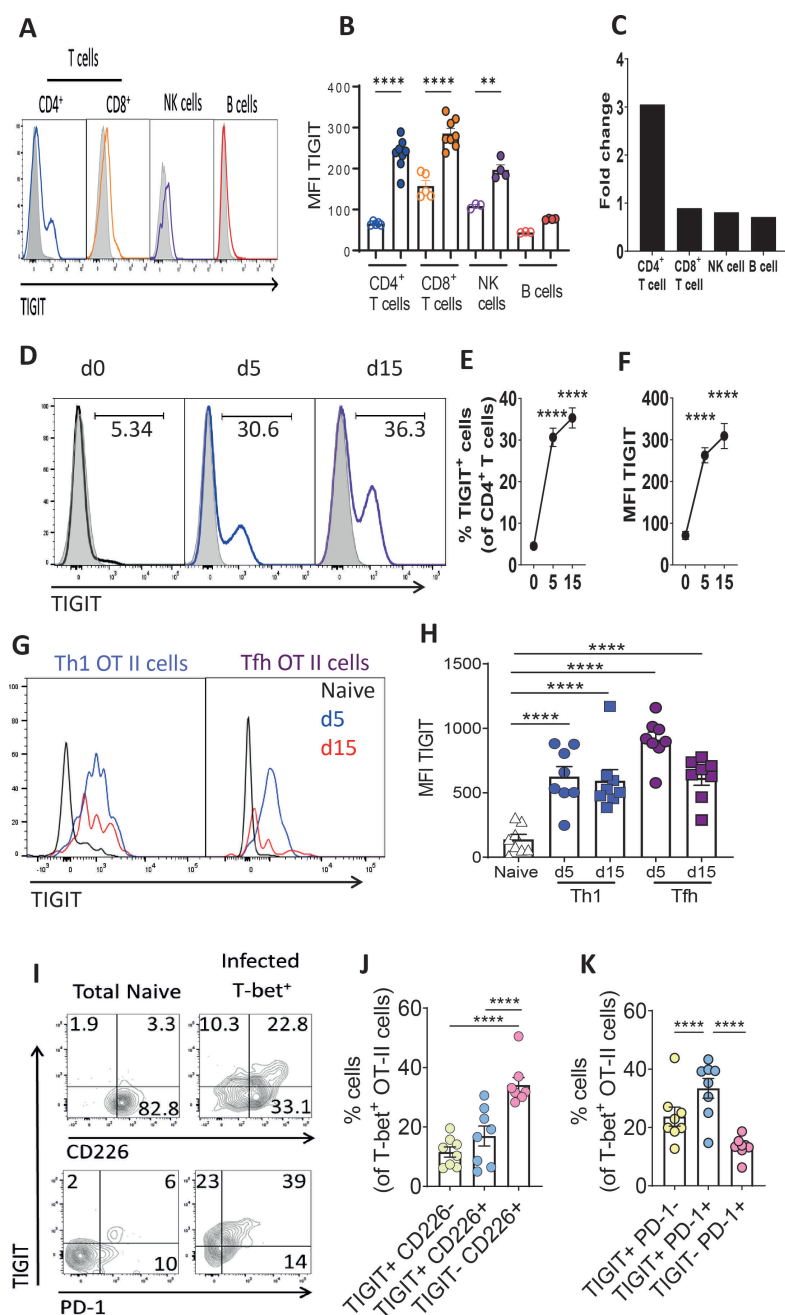


FIG 1 TIGIT is highly expressed in combination with PD-1 on antigen-specific CD4⁺ T cells during *PyNL*-OVA infection (A–F) C57BL/6 mice were infected (i.v.) with 1×10^4 *PyNL*-OVA pRBC. Spleens were removed on (A–C) days 0 and 15 of infection and (D–F) days 0, 5, and 15 of infection. (A) Representative histogram showing TIGIT expression on gated splenic immune cell populations on day 15 (colored lines) compared with day 0 (shaded line) of infection. (B) Mean fluorescence intensity of TIGIT expression on splenic immune populations from naïve (open circles) and infected (D15) mice (filled circles). (C) Fold change in MFI expression of TIGIT on splenic immune cells on day 15 of infection relative to expression on immune cells in naïve mice. (D) Representative histograms showing TIGIT expression by gated CD4⁺ T cells on days 0, 5, and 15 (colored lines) compared with FMO (shaded line). (E) The percentages of splenic CD4⁺ T cells expressing TIGIT and (F) The mean fluorescence intensity of TIGIT expression by splenic CD4⁺ T cells on days 0, 5, and 15 of infection. (G–K) 1×10^6 CD45.1⁺ OT-II cells were adoptively transferred into C57BL/6 mice prior to infection with *PyNL*-OVA pRBC. Spleens were removed on days 0, 5, and 15 of infection. (G) Representative histograms showing TIGIT expression by Tbet⁺ (Th1) OT-II (Continued on next page)

Fig 1 (Continued)

and PD1^{hi}CXCR5^{hi} (Tfh) OT-II CD44⁺ T cells during infection, compared with OT-II cells from naïve mice. (H) Mean fluorescence intensity of TIGIT expression on Th1 and Tfh OT-II CD44⁺ T cells on days 5 and 15 of infection, compared with expression by naïve OT-II cells. (I) Representative dot plots and (J and K) calculated percentages of T-bet⁺ Th1 OT-II CD44⁺ T cells expressing (J) TIGIT and CD226 and (K) TIGIT and PD-1 on day 15 of infection. Results are combined from two independent experiments ($n = 4$ per group per experiment; total $n = 8$). Bars represent mean $\pm$ SEM. $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.001$ [(B, H, J and K), two-way ANOVA with Tukey's multiple comparison test; (E and F), one-way ANOVA with Tukey's multiple comparison test].

during the course of PyNL-OVA infection. Collectively, these data imply that TIGIT may play a role in controlling antigen-specific CD4⁺ T cell responses during blood-stage PyNL-OVA infection.

TIGIT can regulate effector T cell responses directly and through antagonizing CD226-CD115 interactions (19, 24, 25, 29). Therefore, to assess the potential mechanisms of TIGIT regulation on antigen-specific CD4⁺ T cells during blood-stage PyNL-OVA infection, we investigated the co-expression of TIGIT and CD226 on T-bet⁺ OT-II cells on day 15 of infection. CD226 was highly expressed on OT-II cells from naïve mice (Fig. 1I), whereas, as previously shown (Fig. 1G and H), TIGIT was upregulated on splenic T-bet⁺ OT-II cells on day 15 of PyNL-OVA infection (Fig. 1I). Interestingly, a high proportion of T-bet⁺ OT-II cells continued to express CD226 in the absence of TIGIT on day 15 of infection (TIGIT⁻CD226⁺), and there were no significant differences in the proportions of T-bet⁺ OT-II cells expressing TIGIT singularly (TIGIT⁺CD226⁻) compared with cells concomitantly expressing both markers (TIGIT⁺CD226⁺) (Fig. 1J). This implies that TIGIT expression is not intrinsically linked with CD226 expression in activated CD4⁺ T cells, and that the CD226 pathway may not be constitutively opposed by TIGIT on antigen-specific CD4⁺ T cells during PyNL-OVA infection. Nonetheless, TIGIT was largely co-expressed with PD-1 on T-bet⁺ OT-II cells on day 15 of PyNL-OVA infection (65% of total TIGIT⁺ T-bet⁺ OT-II cells also expressed PD-1⁺) (Fig. 1I and K). This is in agreement with other studies showing that TIGIT predominantly regulates effector T cell responses in cooperation with PD-1 (20, 29, 35).

Co-blockade of TIGIT and PD-L1 significantly improves parasite control and increases antigen-specific Th1 cell function during PyNL infection

To directly examine the importance of TIGIT in regulating anti-parasite immune responses during blood-stage PyNL-OVA, we treated mice with anti-TIGIT Ab from day 5 of infection, when we observed upregulation of TIGIT on antigen-specific CD4⁺ T cells (Fig. 1D). In addition, as TIGIT was strongly co-expressed with PD-1 on T cells (Fig. 1I and K), and synergistic co-blockade of TIGIT and PD-1 has been shown to be significantly more effective than individual TIGIT inhibition in other conditions (20, 29, 30, 35), we also treated mice with anti-TIGIT in combination with anti-PD-L1 from day 5 of infection (schematic in Fig. S3A). Although individual blockade of TIGIT did not alter peripheral parasitemia during the early phase of PyNL-OVA infection (Fig. S3B), co-blockade of TIGIT and PD-L1 led to significantly reduced parasite burdens from day 11 until day 15 of PyNL-OVA infection, when the experiment was terminated (Fig. 2A). Importantly, individual blockade of PD-L1 did not alter peripheral parasitemia, implying that the improved parasite control in anti-TIGIT and anti-PD-L1 co-treated mice was not simply due to the inhibition of PD-L1 (Fig. S3B). Co-blockade of TIGIT and PD-L1 did not cause increased weight loss or anemia (Fig. 2B) or induce signs of disease up to termination of experiments on day 15 of infection, suggesting that treatment did not substantially influence malarial morbidity during blood-stage PyNL-OVA infection.

Combinatorial treatment with α -PD-L1 and α -TIGIT significantly increased plasma levels of IFN- γ , TNF, and IL-2 when measured on day 15 of infection, compared with IgG control-treated mice (Fig. 2C). Surprisingly, given our previous observation of preferential

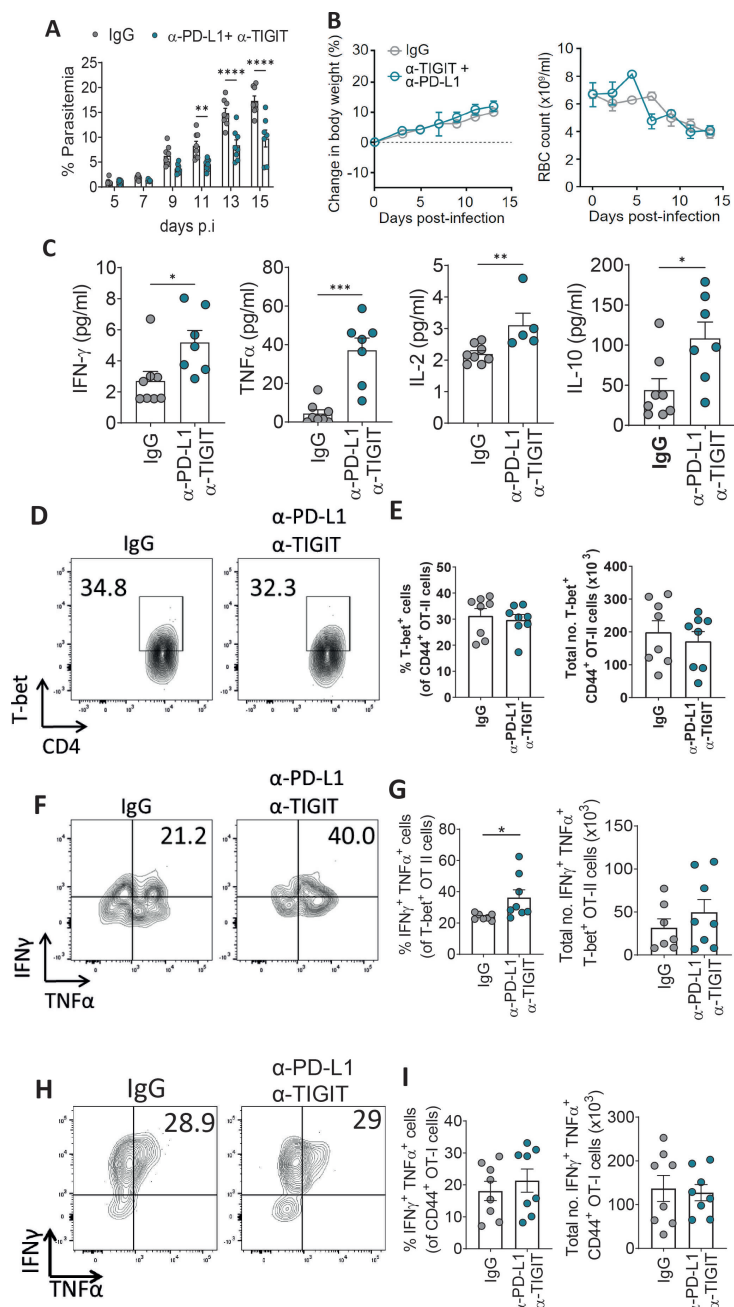


FIG 2 Co-blockade of TIGIT and PD-L1 significantly increases parasite control and augments IFN- γ responses during PyNL-OVA infection. 1×10^6 CD45.1 $^+$ OT II cells with 1×10^5 CD45.1 $^+$ OT-I cells were adoptively transferred into C57BL/6 mice prior to (i.v.) infection with 1×10^4 PyNL-OVA pRBC. Mice were either treated with control rat IgG ($n = 4$) or 250 μg α -TIGIT and 250 μg α -PD-L1 ($n = 4$) from day 5 p.i. (A) Peripheral parasitemia and (B) weight loss and RBC numbers were monitored over the course of the infection. (C) Quantification of plasma cytokines IFN- γ , TNF, IL-10, and IL-2 on day 15 p.i. (D–I) Spleens were removed on day 15 for analysis of (D–G) OT-II cells and (H–I) OT-I cells. (D) Representative flow cytometry plots showing T-bet expression by OT-II CD44 $^+$ T cells. (E) Frequencies (left) and total numbers (right) of splenic T-bet $^+$ OT-II CD44 $^+$ T cells. (F–I) Splenocytes from control and α -TIGIT and α -PD-L1-treated mice were stimulated with PMA and ionomycin and stained for IFN- γ and TNF α . (F) Representative flow cytometry plots showing IFN- γ and TNF α production by T-bet $^+$ OT-II CD44 $^+$ T cells. (G) Frequencies (left) and total numbers (right) of T-bet $^+$ OT-II CD44 $^+$ T cells co-producing IFN- γ and TNF α . (H) Representative flow cytometry plots showing IFN- γ and TNF α production by OT-I CD44 $^+$ T cells. (I) Frequencies (left) and

(Continued on next page)

Fig 2 (Continued)

total numbers (right) of OT-I CD44⁺ T cells co-producing IFN- γ and TNF α . Results are combined from two independent experiments ($n = 4$ per group per experiment; total $n = 8$). Bars represent mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.001$ [(A, B) Unpaired t test; (C, E, G, I) two-way ANOVA with Tukey's multiple comparison test].

expression of TIGIT on Th1-IL-10⁺ cells, and that TIGIT engagement can promote IL-10 production (31, 36, 37), treatment with α -TIGIT and α -PD-L1 also caused a modest increase in plasma IL-10 levels, as measured on day 15 of PyNL-OVA infection (Fig. 2C).

We next examined the cellular sources of the increased IFN- γ and TNF α production in PyNL-OVA-infected mice following anti-TIGIT and anti-PD-L1 combinatorial treatment. Although dual blockade of TIGIT and PD-L1 from day 5 of infection did not alter the frequencies or total numbers of OT-II cells expressing T-bet on day 15 of PyNL-OVA infection (Fig. 2D and E), combined blockade of TIGIT and PD-L1 led to a significant increase in the frequencies (but not total numbers) of total splenic effector T-bet⁺ OT II cells co-expressing IFN- γ and TNF α (from 20% to 30%) (Fig. 2F and G).

As CD8⁺ T cells may also produce large amounts of IFN- γ during blood-stage malaria (38, 39), and given the reported effects of TIGIT blockade on these cells in other models (20, 29), we also assessed the effect of TIGIT and PDL-1 blockade on antigen-specific CD8⁺ T cells (OT-I cells) during infection. Interestingly, despite the upregulation of TIGIT and PD-1 on antigen-specific OT-I cells during PyNL-OVA infection (Fig. S3C), co-blockade of TIGIT and PD-L1 did not significantly influence the magnitude of the antigen-specific CD8⁺ T cell (OT-I) response or the level of Granzyme B, IFN- γ or TNF α production by effector OT-I cells, when measured on day 15 of infection (Fig. S3D and E; Fig. 2H and I). Notably, OT-I cells from control IgG-treated mice continued to produce very high levels of IFN- γ on day 15 of PyNL-OVA infection (Fig. 2H). In agreement, inhibition of TIGIT and PD-L1 significantly increased IFN- γ production by endogenous T-bet⁺CD44⁺ CD4⁺ T cells, but not endogenous CD44⁺CD8⁺ T cells during infection (Fig. S3F and G). Collectively, these data show that TIGIT and the PD-L1 pathway appear to selectively moderate pro-inflammatory cytokine production specifically by CD4⁺ T cells during blood-stage PyNL-OVA infection.

Blockade of TIGIT and PD-L1 does not alter anti-parasite MSP-1₁₉ antibody levels during PyNL-OVA infection

Despite our results suggesting the importance of raised IFN- γ production by Th1 cells in the enhanced parasite control observed in PyNL-infected mice following anti-TIGIT and anti-PD-L1 co-blockade, TIGIT was also highly expressed on antigen-specific Tfh cells during PyNL-OVA infection (Fig. 1G and H). TIGIT expression is also associated with the differentiation of GC-Tfh cells from precursor Tfh cells (40). Therefore, we examined if administration of anti-TIGIT and anti-PD-L1 antibodies altered the humoral response during PyNL infection.

Interestingly, administration of α -TIGIT and α -PD-L1 significantly increased the frequencies of mature GC Tfh OT II cells (PD-1^{hi}, CXCR5^{hi}) as measured on day 15 of PyNL-OVA infection, compared with IgG control treatment (Fig. 3A and B). Co-blockade also led to an increased trend in GC Tfh OT-II numbers, but this was not significant (Fig. 3B). The expression of T-bet was not significantly altered on splenic Tfh OT-II cells (gating modified from that shown in Fig. S2 to analyse T-bet expression directly in PD-1^{hi}CXCR5^{hi} cells gated from CD44⁺CD45.1⁺ OT-II cells) in anti-TIGIT and anti-PD-L1-treated mice on day 15 of PyNL-OVA infection, compared with control-treated mice (Fig. 3C). Consistent with this, combined anti-TIGIT and anti-PD-L1 treatment led to a slight but significant increase in the percentages of both GC B cells (GL7⁺CD38⁻) and plasma cells (CD138⁺CD38⁻) on day 15 of infection (Fig. 3D through F). Nevertheless, despite the increase in Tfh and B cell populations, anti-TIGIT and anti-PD-L1 co-blockade did not significantly affect the plasma titers of anti-*P. yoelii* MSP1₁₉ specific IgG antibody on day

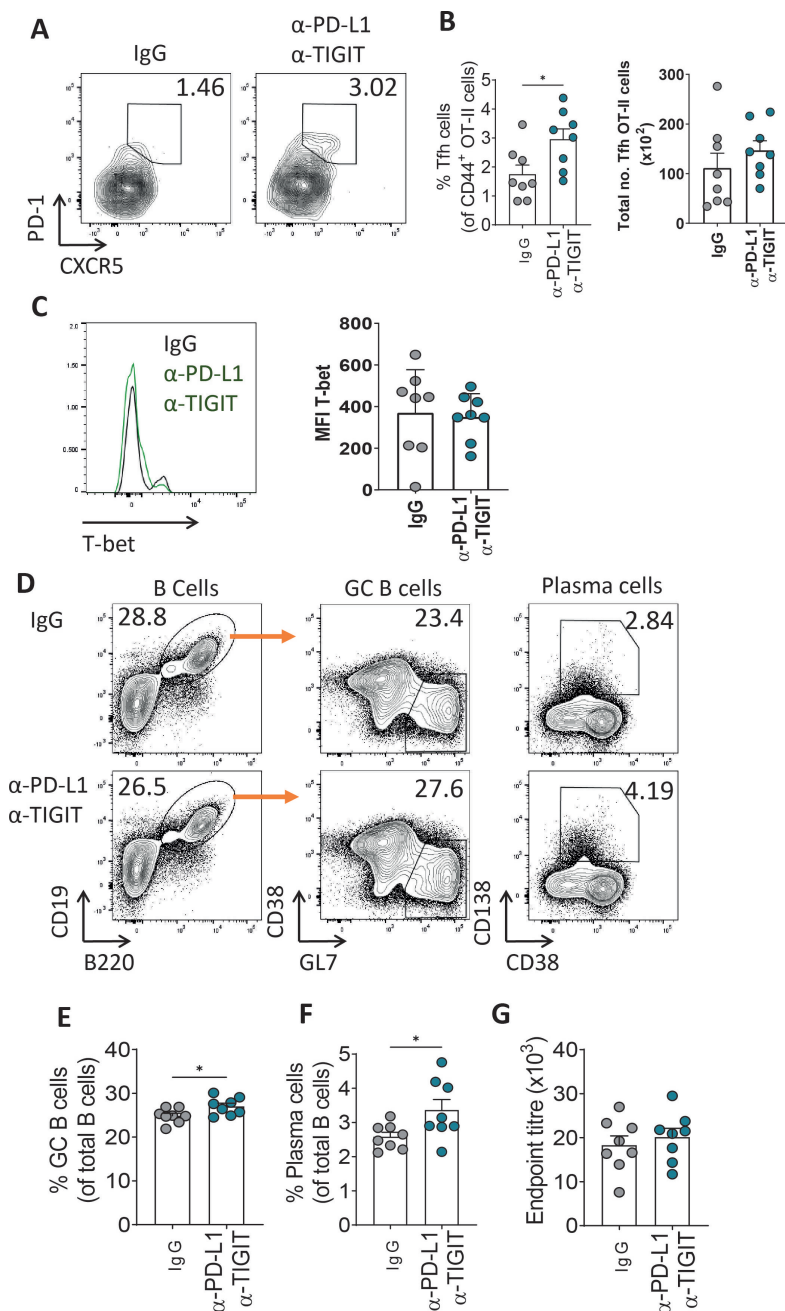


FIG 3 Co-blockade of TIGIT and PD-L1 does not increase parasite-specific antibody production during *PyNL*-OVA infection. 1×10^6 CD45.1⁺ OT-II cells were adoptively transferred into C57BL/6 mice prior to infection (i.v.) with 1×10^4 *PyNL*-OVA pRBC. Mice were either treated with control rat IgG ($n = 4$) or 250 μ g α-TIGIT and 250 μ g α-PD-L1 ($n = 4$) from day 5 p.i. Spleens were removed on day 15 of infection. (A) Representative dot plots showing identification of PD-1⁺CXCR5⁺ (Tfh) OT-II CD44⁺ cells and (B) the percentages and numbers of splenic Tfh OT-II CD44⁺ cells on day 15 of infection. (C) Representative histogram showing the expression of T-bet by Tfh OT-II CD44⁺ T cells and the mean fluorescence intensity of T-bet in gated Tfh OT-II CD44⁺ T cells on day 15 of infection. (D) Representative dot plots showing (left column) the gating of CD19⁺B220⁺ B cells, (middle column) identification of GL7⁺CD38⁺ germinal center B cells, (right column) CD138⁺CD38⁺ plasma cells. (E and F) The percentages of splenic (E) GC B cells and (F) plasma cells on day 15 of infection. (G) The end point titer of anti-MSP1₁₉ IgG in the plasma on day 15 of infection. Results are combined from two independent experiments ($n = 4$ per group per experiment; total $n = 8$). Bars represent mean $\pm$ SEM. * $P \leq 0.05$ [(B, C, E, F, G) two-way ANOVA with Tukey's multiple comparison test].

15 of PyNL-OVA infection (Fig. 3G). Consequently, combined TIGIT and PD-L1 blockade appeared to impact the numbers of canonical Tfh and GC B cells, but this did not lead to a significant improvement in antibody production against a highly immunogenic parasite antigen during blood-stage PyNL-OVA infection.

Blockade of TIGIT and PD-L1 does not alter Foxp3⁺ regulatory T cell responses during PyNL infection

TIGIT⁺ Foxp3⁺ CD4⁺ T cells have been shown to selectively inhibit Th1 cell responses (21, 28). Thus, although we did not observe induction of antigen-specific adaptive TIGIT⁺ OT-II Tregs during PyNL-OVA infection (Fig. S2), we investigated if endogenous TIGIT⁺Foxp3⁺ T cells form, or are maintained, during PyNL-OVA infection and may regulate anti-parasitic immunity. The overall level of TIGIT expression was lower on endogenous Foxp3⁺ Tregs compared with endogenous Tfh and T-bet⁺ CD4⁺ T cells on day 15 of PyNL-OVA infection, but we did identify a small population of TIGIT⁺Foxp3⁺ Tregs (Fig. 4A through C). Interestingly, the TIGIT⁺ Treg population appeared to qualitatively express comparable levels (MFI) of TIGIT as TIGIT⁺ Th1 cells, which was higher than expression by TIGIT⁺ Tfh cells (Fig. 4B). Moreover, these TIGIT⁺ Treg cells expressed higher levels of PD-1 compared with TIGIT[−] Treg cells on day 15 of PyNL-OVA infection, suggesting that TIGIT⁺ Treg cells may have a greater suppressive capacity than TIGIT[−] Treg cells (Fig. 4D). Blockade of TIGIT and PD-L1 led to a significant decrease in the frequencies of Treg cells on day 15 of infection, compared with control treatment (Fig. 4E and F). However, treatment only led to a slight non-significant reduction in the absolute numbers of Foxp3⁺ Tregs on day 15 of PyNL-OVA infection (Fig. 4F). Co-blockade of TIGIT and PD-L1 also failed to alter PD-1 expression by endogenous Foxp3⁺ Tregs, change the MFI of Foxp3, or affect the co-expression of T-bet, by the Treg cells (Fig. 4G through J). Thus, reduction in Foxp3⁺ Treg numbers or alteration in Treg phenotype, was unlikely to have significantly contributed to the elevated type 1 inflammation and improved parasite control in anti-TIGIT and anti-PD-L1-treated PyNL-infected mice.

Co-blockade of TIGIT and PD-L1 increases the number of splenic macrophages during PyNL-OVA infection

TIGIT-CD155 interactions have been shown to tolerize innate cell pro-inflammatory function (23, 41). Co-inhibition of TIGIT and PD-L1 also increased the levels of IFN- γ , a key cytokine that promotes the activation of innate cells during blood-stage malaria (42). As such, we investigated if TIGIT and PD-L1 co-blockade improved splenic APC responses during PyNL-OVA infection. In agreement with this, treatment with anti-TIGIT and anti-PD-L1 increased the frequencies of XCR1⁺ DCs, monocytes, and monocyte-derived macrophages and significantly increased the total numbers of red pulp macrophages (RPMs) and monocyte-derived macrophages on day 15 of infection, compared with IgG control treatment (Fig. 5A through C). Interestingly, TIGIT and PD-L1 blockade did not, however, alter the numbers of XCR1⁺ DCs or CD11b⁺ DCs on day 15 infections (Fig. 5A through C). As such, it is possible that the expansion of RPM and monocyte-derived macrophage populations may contribute to improved parasite control during PyNL-OVA infection after combinatorial anti-TIGIT and anti-PD-1 treatment.

DISCUSSION

In this study, we have shown that TIGIT is upregulated on antigen-specific OT-II cells during PyNL-OVA blood-stage infection, and is co-ordinately expressed in combination with PD1. Co-blockade, but not individual blockade, of TIGIT and PD-L1 markedly reduced peripheral parasitemia during infection, which was correlated with an increase in type 1 pro-inflammatory immune responses. Thus, we have identified a specific role for TIGIT, in concert with the PD-L1-PD1 pathway, in limiting anti-parasitic immune responses during blood-stage PyNL-OVA infection.

The improved parasite control following α -TIGIT and α -PD-L1 treatment was correlated with increased systemic IFN- γ and TNF α . In agreement with this, TIGIT and

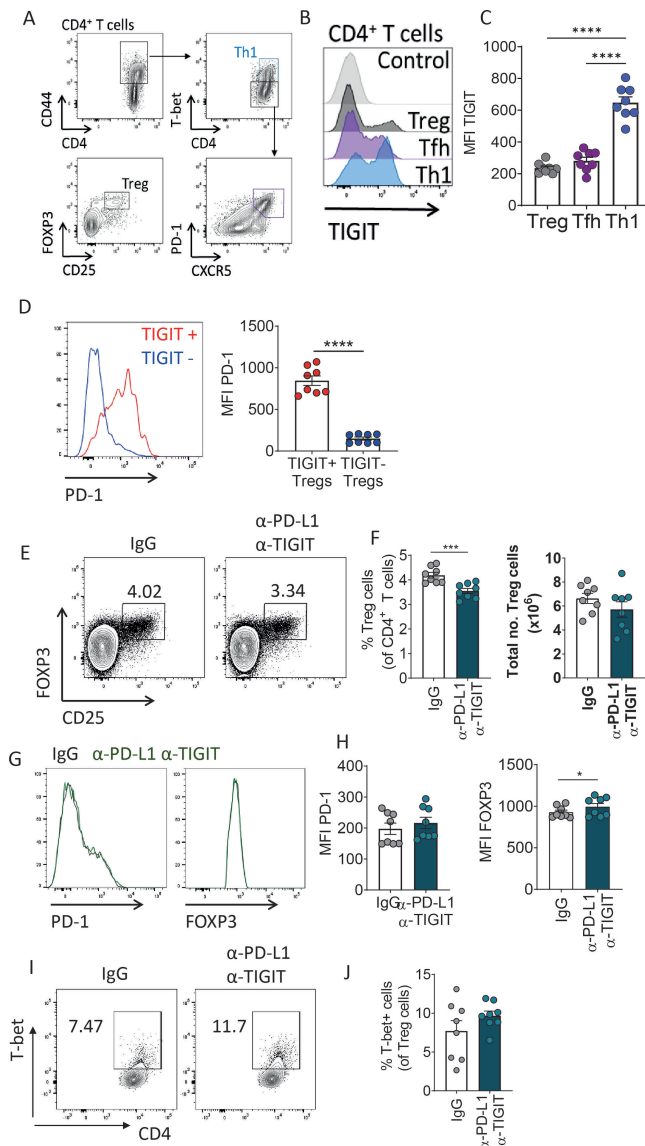


FIG 4 Co-blockade of TIGIT and PD-L1 does not remodel the Foxp3⁺ regulatory T cell compartment during PyNL-OVA infection C57BL/6 mice were infected (i.v.) with 1×10^4 PyNL-OVA pRBC. (A) Representative dot plots showing the identification of endogenous CD4⁺ T cell subsets. (B) Representative histogram showing the expression of TIGIT by splenic CD4⁺ T cell subsets on day 15 of infection. (C) The mean fluorescence intensity of TIGIT on splenic CD4⁺ T cell subsets on day 15 of infection. (D) Representative histogram showing the expression of PD-1 by TIGIT⁺ and TIGIT⁻ Tregs and the mean fluorescence intensity of PD-1 in gated splenic TIGIT⁺ and TIGIT⁻ Treg cells on day 15 of infection. (E–J) Mice were either treated with control rat IgG ($n = 4$) or 250 μ g α -TIGIT and 250 μ g α -PD-L1 ($n = 4$) from day 5 p.i. Spleens were removed on day 15 of infection. (E) Representative dot plots showing gating of Foxp3⁺CD25⁺ CD4⁺ Tregs and (F) the percentages and numbers of splenic Treg cells on day 15 of infection. (G) Representative histograms showing the expression of PD-1 and Foxp3 by gated splenic Treg cells and (H) the mean fluorescence intensity of PD-1 and Foxp3 expression by gated splenic Tregs on day 15 of infection. (I) Representative dot plots showing the expression of Tbet by gated splenic Treg cells and (J) the percentages of splenic Treg cells expressing Tbet on day 15 of infection. Results are combined from two independent experiments ($n = 4$ per group per experiment; total $n = 8$). Bars represent mean $\pm$ SEM. * $P \leq 0.05$, *** $P \leq 0.001$, **** $P \leq 0.0001$ [(C, D, F, H, J) two-way ANOVA with Tukey's multiple comparison test].

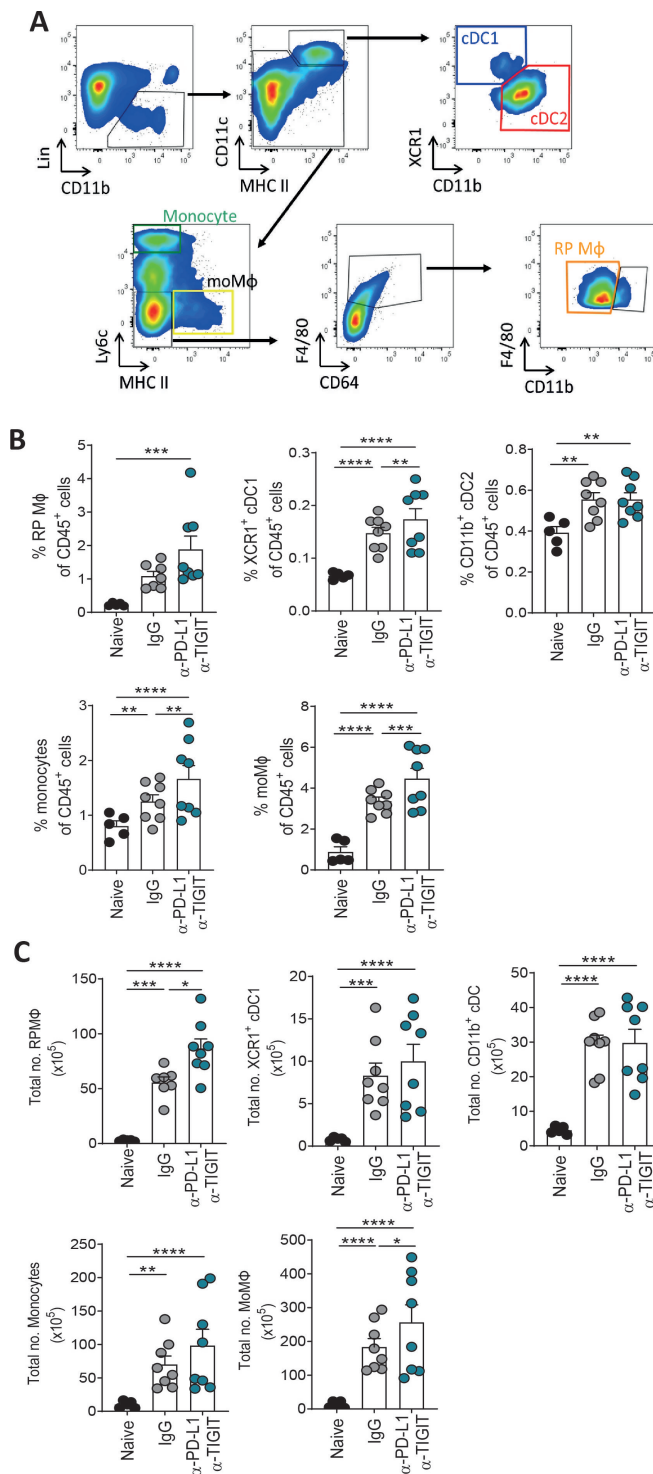


FIG 5 Co-blockade of TIGIT and PD-L1 promotes expansion of splenic red pulp macrophages during *PyNL-OVA* infection. C57BL/6 mice were infected (i.v.) with 1×10^4 *PyNL-OVA* pRBC. Mice were either treated with control rat IgG ($n = 4$) or 250 μ g α -TIGIT and 250 μ g α -PD-L1 ($n = 4$) from day 5 p.i. Spleens were removed on day 15 of infection. (A) Representative dot plots showing the identification of splenic myeloid cell and dendritic cell populations. (B) The percentages and (C) total numbers of red pulp macrophages, cDC1, cDC2, monocytes, and monocyte-derived macrophages on day 15 of infection. Results are combined from two independent experiments ($n = 4$ per group per experiment; total $n = 8$). Bars represent mean $\pm$ SEM. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$ [(B, C) two-way ANOVA with Tukey's multiple comparison test].

PD-L1 co-blockade has been shown to reactivate T cell, frequently CD8⁺ T cell, responses and promote elevated IFN- γ and TNF α production in various conditions (20–26, 29, 30). Here, we identified that TIGIT and PD-L1 co-blockade preferentially influenced CD4⁺ T cell responses, and not antigen-specific CD8⁺ T cell responses, during blood-stage PyNL-OVA infection. This was not due to negligent expression of TIGIT and PD1 on antigen-specific CD8⁺ T cells during PyNL-OVA infection, as although expression was lower than on CD4⁺ T cells, these inhibitory markers were observed on CD8⁺ T cells. Instead, this disparity potentially relates to the differing kinetics of CD4⁺ and CD8⁺ T cell exhaustion and suppression that occurs during non-lethal *Plasmodium* spp. infections within C57BL/6 mice. Although OT-II cells showed evidence of suppression by day 9 of PyNL-OVA infection, OT-I cells still maintained very high production of IFN- γ on day 15 of PyNL-OVA infection, which is consistent with observations within other studies analyzing CD4⁺ T cell and CD8⁺ T cell activities during *P. chabaudi* AS and PyNL blood-stage infections (43–45). In agreement, CD4⁺ T cell exhaustion has been reported to occur more quickly in comparison to CD8⁺ T cells during Lymphocytic choriomeningitis virus (LCMV) infection (6). Co-inhibitory receptor expression is also associated with strong CD8⁺ T cell activation, rather than suppression during acute *P. berghei* ANKA blood-stage infection (46). Thus, during the earlier stages of blood-stage PyNL-OVA infection, TIGIT and PD-1 (and co-inhibitory molecules in general) may exert stronger effects on pro-inflammatory CD4⁺ T cells rather than CD8⁺ T cells. Additional work is, however, required to confirm these hypotheses, including examining the effects of TIGIT and PDL-1 inhibition at earlier and later days of infection, rather than solely on day 15 as performed in this study, and also during infection with other *Plasmodium* spp. parasites. Furthermore, as our experiments were male-restricted and limited to C57BL/6 mice due to our use of OT-II mice, our results need to be confirmed in female mice, and in other strains of mice. Indeed, the effects of immune-checkpoint blockade during malaria can differ in C67BL/6 mice and BALB/c mice (17), male mice are generally more susceptible to blood-stage malaria than female mice (47), but female mice are more sensitive to disruption to immunoregulatory pathways during blood-stage malaria, such as seen following IL-10 inhibition (48).

We also observed that TIGIT was highly expressed by Tfh cells during PyNL-OVA infection, and that combinatorial blockade of TIGIT and PD-L1 significantly enhanced Tfh responses. This is in agreement with other studies that have shown that the CD226-CD155 axis promotes the early expansion and differentiation phase of the Tfh response (49). However, although the increased Tfh response was associated with a slight, but significant, increase in GC B cell and plasma cell responses, this did not translate to an increase in anti-MSP1₁₉ IgG titers, suggesting that, overall, TIGIT and PD-L1 co-blockade did not support enhanced humoral responses against specific and highly immunogenic parasite antigens. Consistent with our findings, increased Tfh cell numbers do not always correspond with GC B cell reactions during malaria (50). It is possible that blockade of TIGIT led to dysregulated CD226 signaling, which restricted mature GC Tfh activity and GC efficiency, due to the observed overproduction of IL-2 (49). High levels of IFN- γ may also have restricted the humoral compartment following TIGIT and PD-L1 blockade. If so, this was not correlated with the development of atypical T-bet⁺Tfh populations, previously associated with IFN- γ overproduction during malaria (50–53). Nonetheless, additional work is required to investigate if combinatorial PD-L1 and TIGIT blockade led to differences in the breadth, functionality, and quality (affinity) of anti-parasite antibodies during PyNL infection. Indeed, functional maturation and broadening of the repertoire of anti-parasite antibodies during infection, rather than overall antibody titer against the highly immunogenic MSP-1 molecule, has been shown to contribute to improved parasite control and protection during human blood-stage malaria (54), as many MSP-1₁₉ specific antibodies lack protective capacity [reviewed in (55)]. This association has also been observed in murine blood-stage infection (56). TIGIT is expressed by regulatory B cell populations (57), but its specific role in controlling antibody affinity maturation during an immune response is currently unclear.

The importance of TIGIT in influencing parasite control, at the time points examined, was only revealed following co-inhibition of PD-L1. This suggests that TIGIT exerts additive or synergistic effects with the PD1-PD-L1 pathway during blood-stage *PyNL* infection. In other models, this is due to the concomitant expression of TIGIT with PD1 on effector T cells (20, 29), as we also observed in this study, and that TIGIT and PD1 have overlapping roles in inhibiting CD226 activation to repress T cell functionality (29). Indeed, the re-activation of CD8⁺ T cell activities following TIGIT and PD1-PD-L1 inhibition has been shown to be CD226 dependent (20, 29, 35, 58). However, we did not identify strong co-expression between TIGIT and CD226 by antigen-specific T-bet⁺ OT-II cells during *PyNL*-OVA infection, which is similar to expression patterns by antigen-specific T cells during *Toxoplasma gondii* infection (59). Thus, whether TIGIT exerts suppressive activity on pro-inflammatory T-bet⁺ CD4⁺ T cells in concert with PD1 during malaria primarily through outcompeting and inhibiting the stimulatory CD226-CD155 axis, or whether TIGIT signaling and the PD1-PD-L1 axis have distinct but synergistic effects on CD4⁺ T cells is unclear. It is notable that agonistic anti-TIGIT and anti-PD-1 antibodies can directly inhibit T cell proliferation and IFN- γ production, both independent from APC interactions and the CD226-CD155 pathway (26, 60). Although we did identify a population of TIGIT⁺ Foxp3⁺ Tregs during *PyNL*-OVA infection, co-inhibition of TIGIT and PD-L1 did not substantially affect the phenotype or numbers of the Treg compartment during infection. Thus, the potential indirect effects of combinatorial blockade of TIGIT and PD-L1 on IFN- γ production and CD4⁺ T cell responses during *PyNL*-OVA blood-stage infection appear unrelated to effects on Tregs.

We have also shown that co-inhibition of TIGIT and PD-L1 led to a significant increase in the absolute numbers of RPMs and monocyte-derived macrophages but not other splenic monocytic or DC populations, during blood-stage malaria. As RPMs play an important role in the removal of pRBC (61), this data suggests that increased parasite control following combinatorial blockade may be mediated through increased phagocytosis of pRBC. Ligation of TIGIT with its ligand CD155 can directly influence innate cell responses, including macrophage polarisation toward an anti-inflammatory phenotype (41). In addition, previous studies have shown that PD-L1 blockade can increase macrophage proliferation, survival, and expression of MHC II and CD86 (62). Therefore, the observed expansion of the splenic macrophage compartment during malaria following co-blockade of TIGIT and PD-L1 could be a direct effect through inhibiting suppressive signaling pathways. Alternatively it could be indirectly mediated through the increase in IFN- γ levels. IFN- γ is a canonical stimulator of macrophages, including RPMs, and increases macrophage phagocytic and anti-microbial activities (63–65). IFN- γ also appears to exert stronger effects on macrophages than dendritic cells to promote parasite control during malaria (66), potentially contributing to the preferential increase in numbers of macrophages, but not DC populations, following blockade of TIGIT and PD-L1.

Although immune checkpoint blockade has previously been shown to increase parasite control during blood-stage malaria (13, 14, 16), during certain *Plasmodium* spp. infections and in specific strains of mice this has concomitantly led to severe immune mediated pathology and mortality (16–18). In this study, we did not observe increased weight loss or signs of anemia—which are robust measures of morbidity during nonacute episodes of blood-stage malaria (47, 67, 68)—in *PyNL*-OVA-infected mice following anti-PD-L1 and anti-TIGIT blockade. Although this suggests that combinatorial targeting the PD1-PD-L1 axis and TIGIT in male C57BL/6 mice during *PyNL*-OVA infection did not cause severe immunopathology, a limitation in our study is that our experiments were terminated on day 15 of infection. Consequently, additional research is required to assess morbidity and signs of immune-mediated pathology across the course of *PyNL* infection following PDL-1 and TIGIT dual blockade. This, along with assessment of effects of dual blockade during infection with other *Plasmodium* parasite spp., and in different strains of inbred male and female mice, is required to fully evaluate the protective and pathogenic impact of PDL1 and TIGIT inhibition on the outcome of blood-stage malaria.

In summary, we have shown that synergistic blockade of TIGIT and PD-L1 significantly improves parasite control during malaria blood-stage PyNL-OVA infection, linked with an increase in IFN- γ production. Our results have relevance to understanding the mechanisms of immunoregulation during human *P. falciparum* and *P. vivax* infections, as T cell suppression is a feature of these infections and various checkpoint blockade strategies have led to reactivation of parasite-specific CD4⁺ T cell responses *in vitro* (8–10). CD4⁺ T cells also express both PD-1 and TIGIT during blood-stage *P. falciparum* infection (32), although the expression of TIGIT by the Tr1 cell population during controlled human *P. falciparum* infection has recently been questioned (69). Whether TIGIT can synergize with other co-inhibitory receptors known to control T cell responses during malaria, such as LAG-3, CTLA-4, and BTLA, with different pairs or modules of receptors tuning distinct components of the immune response during separate phases of infection, will require further investigation. Indeed, TIGIT appears to play a larger regulatory role during acute rather than chronic stages of LCMV infection (36). Irrespective, our results provide additional insight into the immunoregulatory pathways operational during blood-stage PyNL-OVA infection, and the role of TIGIT in combination with the PD-L1 and PD-1 axis in shaping specific components of the anti-parasitic immune response during PyNL infection to limit parasite control.

ACKNOWLEDGMENTS

The authors would like to thank the late Dr. Shahid Khan from the Leiden malaria group, Leiden University Medical Center, for his critical contribution to the creation of the *P. yoelii* NL-OVA line used in this manuscript. The authors thank Dr Gareth Howell and the flow cytometry core facility at the University of Manchester for enabling the flow cytometry experiments performed in this study. The authors thank the Biological Services Facility at the University of Manchester for help with animal work.

This work was supported by the MRC (MR/L008564/1; MR/V034650/1; MR/R010099/1) to K.N.C. S.J.D. is a Jenner Investigator and held a Wellcome Trust Senior Fellowship [106917/Z/15/Z].

AUTHOR AFFILIATIONS

- ¹The Lydia Becker Institute of Immunology and Inflammation, Faculty of Biology, Medicine and Health, University of Manchester, Manchester, United Kingdom
- ²The Jenner Institute, University of Oxford, Oxford, United Kingdom
- ³Leiden Malaria Group, Center of Infectious Diseases, Leiden University Medical Center (LUMC), Leiden, The Netherlands
- ⁴Department of Cancer Immunology, Genentech, South San Francisco, California, USA

PRESENT ADDRESS

Rebecca S. Dookie, Dept. of Microbiology, Tumor and Cell Biology, Biomedicum, Karolinska Institutet, Solnavägen, Solna, Stockholm, Sweden

AUTHOR ORCIDs

Simon J. Draper  <https://orcid.org/0000-0002-9415-1357>
Kevin N. Couper  <http://orcid.org/0000-0003-4659-8960>

FUNDING

Funder	Grant(s)	Author(s)
UKRI Medical Research Council (MRC)	MR/L008564/1	Kevin N. Couper
UKRI Medical Research Council (MRC)	MR/V034650/1	Kevin N. Couper
UKRI Medical Research Council (MRC)	MR/R010099/1	Kevin N. Couper

Funder	Grant(s)	Author(s)
Wellcome Trust (WT)	106917/Z/15/Z	Simon J. Draper

AUTHOR CONTRIBUTIONS

Rebecca S. Dookie, Conceptualization, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft, Writing – review and editing | Ana Villegas-Mendez, Investigation, Methodology, Validation | Antonn Cheeseman, Investigation, Methodology | Adam P. Jones, Formal analysis | Ruben Barroso, Investigation, Methodology | Jordan R. Barrett, Formal analysis, Investigation, Methodology | Simon J. Draper, Methodology, Supervision, Writing – review and editing | Chris J. Janse, Resources, Writing – review and editing | Jane L. Grogan, Resources, Writing – review and editing | Andrew S. MacDonald, Supervision | Kevin N. Couper, Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review and editing

ETHICS APPROVAL

All animal work at the University of Manchester (UoM) was approved following local ethical review by the University of Manchester Animal Procedures and Ethics Committees and was performed in strict accordance with the U.K Home Office Animals (Scientific Procedures) Act 1986 (approved H.O. Project Licences 70/7293 and P8829D3B4).

ADDITIONAL FILES

The following material is available [online](#).

Supplemental Material

Supplemental figures (IAI00345-24-S0001.pdf). Fig. S1 to S3.

Supplemental material (IAI00345-24-S0002.docx). Supplemental figure legends.

REFERENCES

- Kurup SP, Butler NS, Harty JT. 2019. T cell-mediated immunity to malaria. *Nat Rev Immunol* 19:457–471. <https://doi.org/10.1038/s41577-019-0158-z>
- Perez-Mazliah D, Langhorne J. 2014. CD4 T-cell subsets in malaria: TH1/TH2 revisited. *Front Immunol* 5:671. <https://doi.org/10.3389/fimmu.2014.00671>
- Kumar R, Loughland JR, Ng SS, Boyle MJ, Engwerda CR. 2020. The regulation of CD4(+) T cells during malaria. *Immunol Rev* 293:70–87. <https://doi.org/10.1111/imr.12804>
- Abel A, Steeg C, Aminkiah F, Addai-Mensah O, Addo M, Gagliani N, Casar C, Yar DD, Owusu-Dabo E, Jacobs T, Mackroth MS. 2018. Differential expression pattern of co-inhibitory molecules on CD4⁺ T cells in uncomplicated versus complicated malaria. *Sci Rep* 8:4789. <https://doi.org/10.1038/s41598-018-22659-1>
- Brandi J, Lehmann C, Kaminski LC, Schulze Zur Wiesch J, Addo M, Ramharter M, Mackroth M, Jacobs T, Riehn M. 2022. T cells expressing multiple co-inhibitory molecules in acute malaria are not exhausted but exert a suppressive function in mice. *Eur J Immunol* 52:312–327. <https://doi.org/10.1002/eji.202149424>
- Crawford A, Angelosanto JM, Kao C, Doering TA, Odorizzi PM, Barnett BE, Wherry EJ. 2014. Molecular and transcriptional basis of CD4(+) T cell dysfunction during chronic infection. *Immunity* 40:289–302. <https://doi.org/10.1016/j.immuni.2014.01.005>
- Faleiro R, Karunarathne DS, Horne-Debets JM, Wykes M. 2018. The Contribution of co-signaling pathways to anti-malarial T cell immunity. *Front Immunol* 9:2926. <https://doi.org/10.3389/fimmu.2018.02926>
- Illingworth J, Butler NS, Roetynck S, Mwacharo J, Pierce SK, Bejon P, Crompton PD, Marsh K, Ndungu FM. 2013. Chronic exposure to *Plasmodium falciparum* is associated with phenotypic evidence of B and T cell exhaustion. *J Immunol* 190:1038–1047. <https://doi.org/10.4049/jimmunol.1202438>
- Costa PAC, Leoratti FMS, Figueiredo MM, Tada MS, Pereira DB, Junqueira C, Soares IS, Barber DL, Gazzinelli RT, Antonelli LRV. 2015. Induction of inhibitory receptors on T cells during *Plasmodium vivax* malaria impairs cytokine production. *J Infect Dis* 212:1999–2010. <https://doi.org/10.1093/infdis/jiv306>
- Frimpong A, Kusi KA, Adu-Gyasi D, Amponsah J, Ofori MF, Ndifon W. 2019. Phenotypic evidence of T cell exhaustion and senescence during symptomatic *Plasmodium falciparum* malaria. *Front Immunol* 10:1345. <https://doi.org/10.3389/fimmu.2019.01345>
- Mackroth MS, Abel A, Steeg C, Schulze Zur Wiesch J, Jacobs T. 2016. Acute malaria induces PD1+CTLA4⁺ effector T cells with cell-extrinsic suppressor function. *PLoS Pathog* 12:e1005909. <https://doi.org/10.1371/journal.ppat.1005909>
- Adler G, Steeg C, Pfeffer K, Murphy TL, Murphy KM, Langhorne J, Jacobs T. 2011. B and T lymphocyte attenuator restricts the protective immune response against experimental malaria. *J Immunol* 187:5310–5319. <https://doi.org/10.4049/jimmunol.1101456>
- Butler NS, Moebius J, Pewe LL, Traore B, Doumbo OK, Tygrett LT, Waldschmidt TJ, Crompton PD, Harty JT. 2011. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage *Plasmodium* infection. *Nat Immunol* 13:188–195. <https://doi.org/10.1038/ni.2180>
- Furtado R, Chorro L, Zimmerman N, Guillen E, Spaulding E, Chin SS, Daily JP, Lauvau G. 2020. Blockade of LAG-3 in PD-L1-deficient mice enhances clearance of blood stage malaria independent of humoral responses. *Front Immunol* 11:576743. <https://doi.org/10.3389/fimmu.2020.576743>
- Dookie RS, Villegas-Mendez A, Kroeze H, Barrett JR, Draper SJ, Franke-Fayard BM, Janse CJ, MacDonald AS, Couper KN. 2020. Combinatorial Tim-3 and PD-1 activity sustains antigen-specific Th1 cell numbers during blood-stage malaria. *Parasite Immunol* 42:e12723. <https://doi.org/10.1111/pim.12723>

16. Lepenies B, Gaworski I, Tartz S, Langhorne J, Fleischer B, Jacobs T. 2007. CTLA-4 blockade differentially influences the outcome of non-lethal and lethal *Plasmodium yoelii* infections. *Microbes Infect* 9:687–694. <https://doi.org/10.1016/j.micinf.2007.02.013>
17. Hafalla JCR, Claser C, Couper KN, Grau GE, Renia L, de Souza JB, Riley EM. 2012. The CTLA-4 and PD-1/PD-L1 inhibitory pathways independently regulate host resistance to *Plasmodium*-induced acute immune pathology. *PLoS Pathog* 8:e1002504. <https://doi.org/10.1371/journal.ppat.1002504>
18. Jacobs T, Graefe SEB, Niknafs S, Gaworski I, Fleischer B. 2002. Murine malaria is exacerbated by CTLA-4 blockade. *J Immunol* 169:2323–2329. <https://doi.org/10.4049/jimmunol.169.5.2323>
19. Andrews LP, Yano H, Vignali DAA. 2019. Inhibitory receptors and ligands beyond PD-1, PD-L1 and CTLA-4: breakthroughs or backups. *Nat Immunol* 20:1425–1434. <https://doi.org/10.1038/s41590-019-0512-0>
20. Johnston RJ, Comps-Agrar L, Hackney J, Yu X, Huseni M, Yang Y, Park S, Javinal V, Chiu H, Irving B, Eaton DL, Grogan JL. 2014. The immunoreceptor TIGIT regulates antitumor and antiviral CD8(+) T cell effector function. *Cancer Cell* 26:923–937. <https://doi.org/10.1016/j.ccell.2014.10.018>
21. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, Xia J, Tan TG, Sefik E, Yajnik V, Sharpe AH, Quintana FJ, Mathis D, Benoist C, Hafler DA, Kuchroo VK. 2014. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. *Immunity* 40:569–581. <https://doi.org/10.1016/j.immuni.2014.02.012>
22. Stanietzky N, Rovis TL, Glasner A, Seidel E, Tsukerman P, Yamin R, Enk J, Jonjic S, Mandelboim O. 2013. Mouse TIGIT inhibits NK-cell cytotoxicity upon interaction with PVR. *Eur J Immunol* 43:2138–2150. <https://doi.org/10.1002/eji.201243072>
23. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D, Grogan JL. 2009. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. *Nat Immunol* 10:48–57. <https://doi.org/10.1038/ni.1674>
24. Chiang EY, Mellman I. 2022. TIGIT-CD226-PVR axis: advancing immune checkpoint blockade for cancer immunotherapy. *J Immunother Cancer* 10:e004711. <https://doi.org/10.1136/jitc-2022-004711>
25. Joller N, Kuchroo VK. 2017. Tim-3, Lag-3, and TIGIT. *Curr Top Microbiol Immunol* 410:127–156. https://doi.org/10.1007/82_2017_62
26. Lozano E, Dominguez-Villar M, Kuchroo V, Hafler DA. 2012. The TIGIT/CD226 axis regulates human T cell function. *J Immunol* 188:3869–3875. <https://doi.org/10.4049/jimmunol.1103627>
27. Joller N, Hafler JP, Brynadal B, Kassam N, Spoerl S, Levin SD, Sharpe AH, Kuchroo VK. 2011. Cutting edge: TIGIT has T cell-intrinsic inhibitory functions. *J Immunol* 186:1338–1342. <https://doi.org/10.4049/jimmunol.1003081>
28. Kurtulus S, Sakuishi K, Ngiew S-F, Joller N, Tan DJ, Teng MWL, Smyth MJ, Kuchroo VK, Anderson AC. 2015. TIGIT predominantly regulates the immune response via regulatory T cells. *J Clin Invest* 125:4053–4062. <https://doi.org/10.1172/JCI1187>
29. Banta KL, Xu X, Chitre AS, Au-Yeung A, Takahashi C, O’Gorman WE, Wu TD, Mittman S, Cubas R, Comps-Agrar L, Fulzele A, Bennett EJ, Grogan JL, Hui E, Chiang EY, Mellman I. 2022. Mechanistic convergence of the TIGIT and PD-1 inhibitory pathways necessitates co-blockade to optimize anti-tumor CD8⁺ T cell responses. *Immunity* 55:512–526. <https://doi.org/10.1016/j.immuni.2022.02.005>
30. Chu X, Tian W, Wang Z, Zhang J, Zhou R. 2023. Co-inhibition of TIGIT and PD-1/PD-L1 in cancer immunotherapy: mechanisms and clinical trials. *Mol Cancer* 22:93. <https://doi.org/10.1186/s12943-023-01800-3>
31. Villegas-Mendez A, Shaw TN, Inkson CA, Strangward P, de Souza JB, Couper KN. 2016. Parasite-specific CD4⁺ IFN- γ IL-10⁺ T cells distribute within both lymphoid and nonlymphoid compartments and are controlled systemically by interleukin-27 and ICOS during blood-stage malaria infection. *Infect Immun* 84:34–46. <https://doi.org/10.1128/IAI.01100-15>
32. Schulte S, Heide J, Ackermann C, Peine S, Ramharter M, Mackroth MS, Woost R, Jacobs T, Schulze Zur Wiesch J. 2022. Deciphering the *Plasmodium falciparum* malaria-specific CD4⁺ T-cell response: ex vivo detection of high frequencies of PD-1+TIGIT+ EXP1-specific CD4⁺ T cells using a novel HLA-DR11-restricted MHC class II tetramer. *Clin Exp Immunol* 207:227–236. <https://doi.org/10.1093/cei/uxab027>
33. Barnden MJ, Allison J, Heath WR, Carbone FR. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol Cell Biol* 76:34–40. <https://doi.org/10.1046/j.1440-1711.1998.00709.x>
34. Couper KN, Blount DG, Wilson MS, Hafalla JC, Belkaid Y, Kamanaka M, Flavell RA, de Souza JB, Riley EM. 2008. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog* 4:e1000004. <https://doi.org/10.1371/journal.ppat.1000004>
35. Weulersse M, Asrir A, Pichler AC, Lemaitre L, Braun M, Carrié N, Joubert M-V, Le Moine M, Do Souto L, Gaud G, et al. 2020. Eomes-dependent loss of the co-activating receptor CD226 restrains CD8⁺ T cell anti-tumor functions and limits the efficacy of cancer immunotherapy. *Immunity* 53:824–839. <https://doi.org/10.1016/j.immuni.2020.09.006>
36. Burton BR, Britton GJ, Fang H, Verhagen J, Smithers B, Sabatos-Peyton CA, Carney LJ, Gough J, Strobel S, Wraith DC. 2014. Sequential transcriptional changes dictate safe and effective antigen-specific immunotherapy. *Nat Commun* 5:4741. <https://doi.org/10.1038/ncomms5741>
37. Schorer M, Rakebrandt N, Lambert K, Hunziker A, Pallmer K, Oxenius A, Kipar A, Stertz S, Joller N. 2020. TIGIT limits immune pathology during viral infections. *Nat Commun* 11:1288. <https://doi.org/10.1038/s41467-020-15025-1>
38. Horne-Debets JM, Karunaratne DS, Faleiro RJ, Poh CM, Renia L, Wykes MN. 2016. Mice lacking programmed cell death-1 show a role for CD8(+) T cells in long-term immunity against blood-stage malaria. *Sci Rep* 6:26210. <https://doi.org/10.1038/srep26210>
39. Villegas-Mendez A, Greig R, Shaw TN, de Souza JB, Gwyer Findlay E, Stumhofer JS, Hafalla JCR, Blount DG, Hunter CA, Riley EM, Couper KN. 2012. IFN- γ -producing CD4⁺ T cells promote experimental cerebral malaria by modulating CD8⁺ T cell accumulation within the brain. *J Immunol* 189:968–979. <https://doi.org/10.4049/jimmunol.1200688>
40. Zhu F, McMonigle RJ, Schroeder AR, Xia X, Figge D, Greer BD, González-Avalos E, Sialer DO, Wang Y-H, Chandler KM, Getzler AJ, Brown ER, Xiao C, Kutsch O, Harada Y, Pipkin ME, Hu H. 2023. Spatiotemporal resolution of germinal center Tfh cell differentiation and divergence from central memory CD4⁺ T cell fate. *Nat Commun* 14:3611. <https://doi.org/10.1038/s41467-023-39299-3>
41. Chen X, Lu PH, Liu L, Fang ZM, Duan W, Liu ZL, Wang CY, Zhou P, Yu XF, He WT. 2016. TIGIT negatively regulates inflammation by altering macrophage phenotype. *Immunobiology* 221:48–55. <https://doi.org/10.1016/j.imbio.2015.08.003>
42. King T, Lamb T. 2015. Interferon- γ : the Jekyll and hyde of malaria. *PLoS Pathog* 11:e1005118. <https://doi.org/10.1371/journal.ppat.1005118>
43. Horne-Debets JM, Faleiro R, Karunaratne DS, Liu XQ, Lineburg KE, Poh CM, Grotenberg GM, Hill GR, MacDonald KPA, Good MF, Renia L, Ahmed R, Sharpe AH, Wykes MN. 2013. PD-1 dependent exhaustion of CD8⁺ T cells drives chronic malaria. *Cell Rep* 5:1204–1213. <https://doi.org/10.1016/j.celrep.2013.11.002>
44. Villegas-Mendez A, Khandelwal G, McGowan LM, Dookie RS, Haley MJ, George C, Sims D, Lord GM, Sinclair LV, Jenner RG, Couper KN. 2020. Exhausted CD4⁺ T cells during malaria exhibit reduced mTORc1 activity correlated with loss of T-bet expression. *J Immunol* 205:1608–1619. <https://doi.org/10.4049/jimmunol.2000450>
45. Wikenheiser DJ, Ghosh D, Kennedy B, Stumhofer JS. 2016. The costimulatory molecule ICOS regulates host Th1 and follicular Th cell differentiation in response to *Plasmodium chabaudi* chabaudi AS infection. *J Immunol* 196:778–791. <https://doi.org/10.4049/jimmunol.1403206>
46. Brandi J, Riehn M, Hadjilaou A, Jacobs T. 2022. Increased expression of multiple co-inhibitory molecules on malaria-induced CD8⁺ T cells are associated with increased function instead of exhaustion. *Front Immunol* 13:878320. <https://doi.org/10.3389/fimmu.2022.878320>
47. Cernetich A, Garver LS, Jedlicka AE, Klein PW, Kumar N, Scott AL, Klein SL. 2006. Involvement of gonadal steroids and gamma interferon in sex differences in response to blood-stage malaria infection. *Infect Immun* 74:3190–3203. <https://doi.org/10.1128/IAI.00008-06>
48. Li C, Corraliza I, Langhorne J. 1999. A defect in interleukin-10 leads to enhanced malarial disease in *Plasmodium chabaudi* chabaudi infection

- in mice. *Infect Immun* 67:4435–4442. <https://doi.org/10.1128/IAI.67.9.4435-4442.1999>
49. Yasutomi M, Christiaansen AF, Imai N, Martin-Orozco N, Forst CV, Chen G, Ueno H. 2022. CD226 and TIGIT cooperate in the differentiation and maturation of human Tfh cells. *Front Immunol* 13:840457. <https://doi.org/10.3389/fimmu.2022.840457>
 50. Zander RA, Obeng-Adjei N, Guthmiller JJ, Kulu DI, Li J, Ongoiba A, Traore B, Crompton PD, Butler NS. 2015. PD-1 co-inhibitory and OX40 co-stimulatory crosstalk regulates helper T cell differentiation and anti-*Plasmodium* humoral immunity. *Cell Host Microbe* 17:628–641. <https://doi.org/10.1016/j.chom.2015.03.007>
 51. Obeng-Adjei N, Portugal S, Tran TM, Yazew TB, Skinner J, Li S, Jain A, Felgner PL, Doumbo OK, Kayentao K, Ongoiba A, Traore B, Crompton PD. 2015. Circulating Th1-cell-type Tfh cells that exhibit impaired B cell help are preferentially activated during acute malaria in children. *Cell Rep* 13:425–439. <https://doi.org/10.1016/j.celrep.2015.09.004>
 52. Ryg-Cornejo V, Ioannidis LJ, Ly A, Chiu CY, Tellier J, Hill DL, Preston SP, Pellegrini M, Yu D, Nutt SL, Kallies A, Hansen DS. 2016. Severe malaria infections impair germinal center responses by inhibiting T follicular helper cell differentiation. *Cell Rep* 14:68–81. <https://doi.org/10.1016/j.celrep.2015.12.006>
 53. Guthmiller JJ, Graham AC, Zander RA, Pope RL, Butler NS. 2017. Cutting edge: IL-10 is essential for the generation of germinal center B cell responses and anti-*Plasmodium* humoral immunity. *J Immunol* 198:617–622. <https://doi.org/10.4049/jimmunol.1601762>
 54. Dent AE, Nakajima R, Liang L, Baum E, Moormann AM, Sumba PO, Vulule J, Babineau D, Randall A, Davies DH, Felgner PL, Kazura JW. 2015. *Plasmodium falciparum* protein microarray antibody profiles correlate with protection from symptomatic malaria in Kenya. *J Infect Dis* 212:1429–1438. <https://doi.org/10.1093/infdis/jiv224>
 55. Opi DH, Kurtovic L, Chan JA, Horton JL, Feng G, Beeson JG. 2021. Multi-functional antibody profiling for malaria vaccine development and evaluation. *Expert Rev Vaccines* 20:1257–1272. <https://doi.org/10.1080/14760584.2021.1981864>
 56. Shaw TN, Inkson CA, Villegas-Mendez A, Pattinson DJ, Strangward P, Else KJ, Draper SJ, Zeef LAH, Couper KN. 2019. Infection-induced resistance to experimental cerebral malaria is dependent upon secreted antibody-mediated inhibition of pathogenic CD8⁺ T cell responses. *Front Immunol* 10:248. <https://doi.org/10.3389/fimmu.2019.00248>
 57. Xiao S, Bod L, Pochet N, Kota SB, Hu D, Madi A, Kilpatrick J, Shi J, Ho A, Zhang H, Sobel R, Weiner HL, Strom TB, Quintana FJ, Joller N, Kuchroo VK. 2020. Checkpoint receptor TIGIT expressed on Tim-1⁺ B cells regulates tissue inflammation. *Cell Rep* 32:107892. <https://doi.org/10.1016/j.celrep.2020.107892>
 58. Jin H, Ko M, Choi D, Kim JH, Lee D, Kang S-H, Kim I, Lee HJ, Choi EK, Kim K, Yoo C, Park Y. 2020. CD226hiCD8⁺ T cells are a prerequisite for anti-TIGIT immunotherapy. *Cancer Immunol Res* 8:912–925. <https://doi.org/10.1158/2326-6066.CIR-19-0877>
 59. Aldridge DL, Phan AT, de Waal Malefyt R, Hunter CA. 2021. Limited impact of the inhibitory receptor TIGIT on NK and T cell responses during *Toxoplasma gondii* infection. *Immunohorizons* 5:384–394. <https://doi.org/10.4049/immunohorizons.2100007>
 60. Suzuki K, Tajima M, Tokumaru Y, Oshiro Y, Nagata S, Kamada H, Kihara M, Nakano K, Honjo T, Ohta A. 2023. Anti-PD-1 antibodies recognizing the membrane-proximal region are PD-1 agonists that can down-regulate inflammatory diseases. *Sci Immunol* 8:eadd4947. <https://doi.org/10.1126/sciimmunol.add4947>
 61. Stevenson MM, Riley EM. 2004. Innate immunity to malaria. *Nat Rev Immunol* 4:169–180. <https://doi.org/10.1038/nri1311>
 62. Hartley GP, Chow L, Ammons DT, Wheat WH, Dow SW. 2018. Programmed cell death ligand 1 (PD-L1) signaling regulates macrophage proliferation and activation. *Cancer Immunol Res* 6:1260–1273. <https://doi.org/10.1158/2326-6066.CIR-17-0537>
 63. Lane TE, Wu-Hsieh BA, Howard DH. 1993. Gamma interferon cooperates with lipopolysaccharide to activate mouse splenic macrophages to an antihistoplasma state. *Infect Immun* 61:1468–1473. <https://doi.org/10.1128/iai.61.4.1468-1473.1993>
 64. Kurotaki D, Uede T, Tamura T. 2015. Functions and development of red pulp macrophages. *Microbiol Immunol* 59:55–62. <https://doi.org/10.1111/1348-0421.12228>
 65. Schroder K, Hertzog PJ, Ravasi T, Hume DA. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75:163–189. <https://doi.org/10.1189/jlb.0603252>
 66. Deroost K, Alder C, Hosking C, McLaughlin S, Lin JW, Lewis MD, Saavedra-Torres Y, Addy JWG, Levy P, Giorgalli M, Langhorne J. 2021. Tissue macrophages and interferon-gamma signalling control blood-stage *Plasmodium chabaudi* infections derived from mosquito-transmitted parasites. *Curr Res Immunol* 2:104–119. <https://doi.org/10.1016/j.crimmu.2021.07.002>
 67. Freitas do Rosário AP, Lamb T, Spence P, Stephens R, Lang A, Roers A, Muller W, O'Garra A, Langhorne J. 2012. IL-27 promotes IL-10 production by effector Th1 CD4⁺ T cells: a critical mechanism for protection from severe immunopathology during malaria infection. *J Immunol* 188:1178–1190. <https://doi.org/10.4049/jimmunol.1102755>
 68. Findlay EG, Greig R, Stumhofer JS, Hafalla JCR, de Souza JB, Saris CJ, Hunter CA, Riley EM, Couper KN. 2010. Essential role for IL-27 receptor signaling in prevention of Th1-mediated immunopathology during malaria infection. *J Immunol* 185:2482–2492. <https://doi.org/10.4049/jimmunol.0904019>
 69. Edwards CL, Ng SS, de Labastida Rivera F, Corvino D, Engel JA, Montes de Oca M, Bukali L, Frame TC, Bunn PT, Chauhan SB, et al. 2023. IL-10-producing Th1 cells possess a distinct molecular signature in malaria. *J Clin Invest* 133:e169299. <https://doi.org/10.1172/JCI169299>