Combinatorial Tim-3 and PD-1 activity sustains antigen-specific Th1 cell numbers during blood-stage malaria

Dookie, R.S.; Villegas-Mendez, A.; Kroeze, H.; Barrett, J.R.; Draper, S.J.; Franke-Fayard, B.M.; ... ; Couper, K.N.

Citation
Dookie, R. S., Villegas-Mendez, A., Kroeze, H., Barrett, J. R., Draper, S. J., Franke-Fayard, B. M., … Couper, K. N. (2020). Combinatorial Tim-3 and PD-1 activity sustains antigen-specific Th1 cell numbers during blood-stage malaria. *Parasite Immunology*, *42*(9). doi:10.1111/pim.12723

Version: Publisher's Version
License: Creative Commons CC BY 4.0 license
Downloaded from: https://hdl.handle.net/1887/3184355

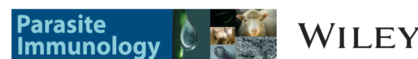
Note: To cite this publication please use the final published version (if applicable).

Check for updates

Received: 20 January 2020 | Revised: 10 April 2020 | Accepted: 14 April 2020

DOI: 10.1111/pim.12723

ORIGINAL ARTICLE



Combinatorial Tim-3 and PD-1 activity sustains antigen-specific Th1 cell numbers during blood-stage malaria

Rebecca S. Dookie[1] | Ana Villegas-Mendez[1] | Hans Kroeze[2] | Jordan R. Barrett[3] | Simon J. Draper[3] | Blandine M. Franke-Fayard[2] | Chris J. Janse[2] | Andrew S. MacDonald[1] | Kevin N. Couper[1]

[1]Faculty of Biology, Medicine and Health, The Lydia Becker Institute of Immunology and Inflammation, University of Manchester, Manchester, UK

[2]Leiden malaria group, Department of Parasitology, Leiden University Medical Center, Leiden, The Netherlands

[3]The Jenner Institute, University of Oxford, Oxford, UK

Correspondence
Kevin N. Couper, Faculty of Biology, Medicine and Health, The Lydia Becker Institute of Immunology and Inflammation, University of Manchester, Manchester, UK.
Email: kevin.couper@manchester.ac.uk

Funding information
This work was supported by the MRC (MR/L008564/1) to KNC. SJD is a Jenner Investigator and a Wellcome Trust Senior Fellow [106917/Z/15/Z].

Abstract

Aims: Co-inhibitory receptors play a major role in controlling the Th1 response during blood-stage malaria. Whilst PD-1 is viewed as the dominant co-inhibitory receptor restricting T cell responses, the roles of other such receptors in coordinating Th1 cell activity during malaria are poorly understood.

Methods and Results: Here, we show that the co-inhibitory receptor Tim-3 is expressed on splenic antigen-specific T-bet$^+$ (Th1) OT-II cells transiently during the early stage of infection with transgenic *Plasmodium yoelii* NL parasites expressing ovalbumin (*P yoelii* NL-*OVA*). We reveal that co-blockade of Tim-3 and PD-L1 during the acute phase of *P yoelii* NL infection did not improve the Th1 cell response but instead led to a specific reduction in the numbers of splenic Th1 OT-II cells. Combined blockade of Tim-3 and PD-L1 did elevate anti-parasite IgG antibody responses. Nevertheless, co-blockade of Tim-3 and PD-L1 did not affect IFN-γ production by OT-II cells and did not influence parasite control during *P yoelii* NL-*OVA* infection.

Conclusion: Thus, our results show that Tim-3 plays an unexpected combinatorial role with PD-1 in promoting and/ or sustaining a Th1 cell response during the early phase of blood-stage *P. yoelii* NL infection but combined blockade does not dramatically influence anti-parasite immunity.

KEYWORDS
CD4$^+$ T cells, co-inhibitory receptors, immunoregulation, malaria, T cell exhaustion

1 | INTRODUCTION

Effector CD4$^+$ T cells coordinate protective immunity during blood-stage malaria by promoting parasite phagocytosis and antibody production.[1,2] However, it is now evident that effector CD4$^+$ T cell function is strongly regulated during the course of blood-stage malaria by the activity of co-inhibitory receptors, which are upregulated on activated CD4$^+$ T cells as infection progresses.[3-7] The expression of co-inhibitory molecules (including PD-1, CTLA-4 and LAG-3), dampens effector CD4$^+$ T cell cytokine production and proliferation, impairs memory T cell formation and promotes infection chronicity.[8,9] Consequently, there is currently major interest in

The peer review history for this article is available at https://publons.com/publon/10.1111/pim.12723.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.
© 2020 The Authors. *Parasite Immunology* published by John Wiley & Sons Ltd

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

resolving the independent and overlapping roles of different co-inhibitory receptors in regulating T cell function during malaria, to develop new strategies to reinvigorate effector CD4$^+$ T cell responses and treat chronic malarial disease.[7-10]

Tim-3 is a co-inhibitory cell surface molecule that is widely expressed by innate and adaptive leucocyte populations.[11,12] Tim-3 exerts pleiotropic regulatory effects on APCs and NK cells, inhibiting activation, phagocytosis, cytokine production and cytotoxicity.[11,12] In effector CD4$^+$ Th1 and CD8$^+$ T cells, Tim-3 limits T cell receptor (TCR) signalling and promotes cellular apoptosis.[13,14] Consequently, Tim-3 has been shown to inhibit T cell responses during cancer and various chronic bacterial and viral infections.[15-17] Interestingly, despite the identified direct inhibitory activities of Tim-3, it has been suggested that the molecule may act predominantly in synergy with PD-1 to repress effector T cell responses.[11] Indeed, combined blockade of Tim-3 and PD-1 has been shown to be significantly more effective at restoring T cell effector function than individual Tim-3 or PD-1 blockade in chronic viral infection.[18,19] At present, the role of Tim-3 in regulating protective CD4$^+$ T cell immune responses during blood-stage malaria is unclear.[9,20-23] Moreover, the potential synergistic interaction between Tim-3 and PD-1 in regulating effector CD4$^+$ T cell responses during malaria has not yet been addressed.

In this study, we identify that antigen-specific effector CD4$^+$ T cells only transiently express Tim-3 during the early phase of T cell activation during blood-stage *P yoelii* NL infection. Interestingly, we show that instead of improving the strength of the antigen-specific T-bet$^+$ Th1 response, combinatorial blockade of Tim-3 and PD-L1 significantly reduced the frequencies and numbers of antigen-specific Th1 cells during the early phase of blood-stage *P yoelii* NL infection. Nevertheless, combined PD-L1 and Tim-3 blockade did not impact overall IFN-γ production by antigen-specific CD4$^+$ T cells and did not alter parasite control. Thus, our data suggest that Tim-3 does not act as a negative co-inhibitory molecule in combination with PD-1 to influence Th1 cell responses, but may play a minor role in promoting/sustaining the Th1 cell population during the early stages of blood-stage malaria.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

All animal work at the University of Manchester 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). All animal work at Leiden Medical Center was approved and performed under a licence from the Animal Experiments Committee of the Leiden University Medical Center (DEC 12042). All experiments were performed in accordance with the Experiments on Animals Act (Wod, 2014), the applicable legislation in the Netherlands in accordance with the European guidelines (EU directive no. 2010/63/EU).

2.2 | Mice

Male 7-week-old C57BL/6 mice (CD45.2$^+$) were purchased from Charles River, UK, and female 6-7-week-old OF1 mice were purchased from Charles River, NL. RAG-1 OT-II x Pep3 (CD45.1$^+$) mice on a C57BL/6 background[24] were bred at the University of Manchester. All mice were maintained in specific-pathogen-free conditions in individually ventilated cages.

2.3 | Generation and genotyping of a transgenic *P yoelii* 17XNL line expressing ovalbumin OVA

To generate transgenic *P yoelii* mutant expressing ovalbumin (OVA) fused to mCherry under control of the constitutive *hsp70* promoter of *P berghei*, we generated DNA construct pL2095 (see Figure S1). First, the promoter of *hsp70* of the OVA::mCherry plasmid pL1838[25] was exchanged for the *P berghei hsp70* promoter of p286[26] amplified using primers 7652 and 7653 (5′-GGA**AGATCT**CCCGGGGTAATATTTGTTGGTGAGC and 5′-CCG**CTCGAG**GGATCCTAATTGTAATTGTAATTTATTGG) and cloned *Bgl*II-*Bam*HI/*Xho*I to remove restriction sites present in the original *hsp70* promoter region. Second, the 3′UTR of *Pbdhfr/ts* was exchanged for the 3′UTR of *Pbdhfr/ts* of pL0007[27] using restriction sites *Sph*I/*Eco*RI to introduce a *Sma*I site. Finally, the *hsp70*-OVA::mCherry expression cassette was cloned into *Sma*I-pL1847 digested plasmid[28] that has sequences targeting the 5′ and 3′ regions of the neutral *p230p* gene of *P yoelii* (PY17X_0306600). Orientation of the expression cassette (OVA-mCherry fusion under control of the *hsp70* promoter and the 3′UTR of *Pbdhfr/ts*) was analysed, and the construct was linearized using *Eco*RI sites before transfection (Figure S1).

Transfection (exp. 2510), selection and cloning of transformed parasites were performed using standard genetic modification technologies[29] using the reference "GIMO" Py17XNL parasite line 1923cl1 (GIMOPy17X; line RMgm-688; www.pberghei.eu[28]) as the parent *P yoelii* XNL parasite line. The 1923c11 line contains, as a positive/negative selectable marker (SM), the fusion gene of *hdhfr (human dihydrofolate reductase*; positive SM) and *yfcu* (*yeast cytosine deaminase and uridyl phosphoribosyl transferase*) under control of the constitutive *eef1α* promoter integrated into the silent *p230p* gene locus (PY17X_0306600).

Cloned parasite lines were obtained by the method of limiting dilution and clone 1 was used in further analyses (line *hsp70*-OVA::mCherry or *P yoelii* NL-*OVA*). Correct integration of DNA construct into the genome was verified by Southern analyses of Pulsed Field Gel (PFG)-separated chromosomes.[29] PFG-separated chromosomes of the parent line 1923cl1 were hybridized with 3′UTR *Pbdhfr/ts* probe[28] and chromosomes of the clones of 2510 with mix probe, consisting of a probe recognizing chromosome 5 and a probe against h*dhfr*[30] (Figure S1). Growth of blood stages of *P yoelii* NL-*OVA* (2510cl1 and 2510cl3) parasites was determined during the cloning period, as described previously.[29] Growth of *P yoelii* NL-*OVA*

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

parasites was similar to growth of the parent reference *P yoelii* 17XNL parasite line (data not shown).

Expression of the OVA::mCherry was analysed by fluorescence microscopy of mCherry fluorescence in blood stages, oocysts and sporozoites using a Leica DMR fluorescence microscope. In addition, expression of OVA::mCherry was analysed by immunofluorescence analysis of infected erythrocytes containing trophozoites and schizonts that were fixed on slides with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 20 minutes and permeabilized with 0.5% Triton X-100 in PBS for 10 minutes. The parasites were stained with polyclonal rabbit IgG anti-OVA antibody (kindly provided by MGM Camps, Department of Immunohematology and Blood Transfusion, LUMC), followed by Alexa Fluor FITC–anti-rabbit antibodies (Sigma). Nuclei were stained with Hoechst 33342 (2 μmol/L; Sigma) for 15 minutes. Slides were mounted in Vectashield (Vector Laboratories Inc) and examined using a DMR Leica fluorescence microscope.

2.4 | Experimental infections

Cryopreserved *P yoelii* NL-*OVA* parasites were thawed and passaged once in C57BL/6 mice before being used to infect experimental mice. Animals were infected via intravenous (iv) injection of 10^4 parasitized red blood cells (pRBC). The course of infection

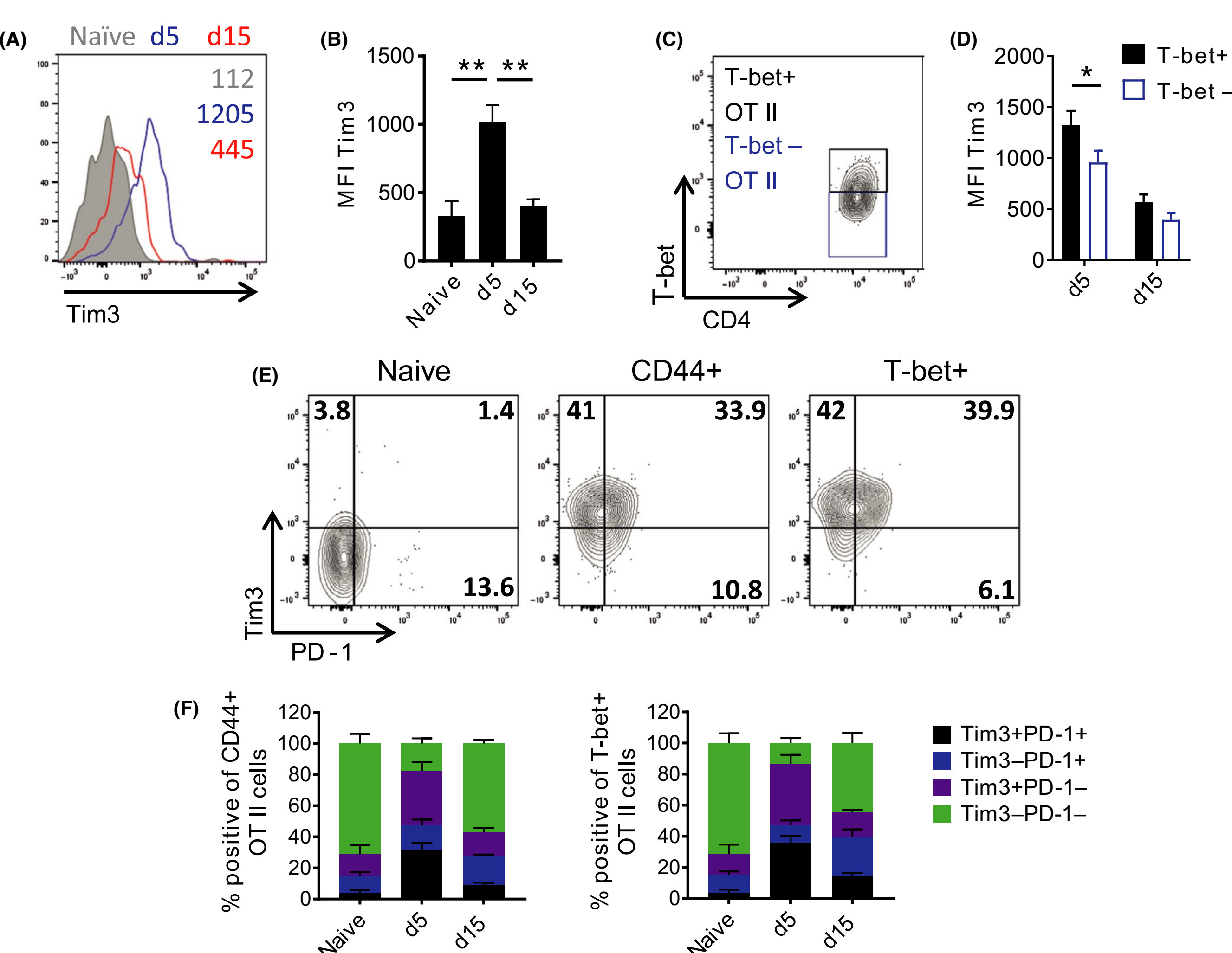


FIGURE 1 Tim-3 is transiently expressed on effector OT-II cells during *Plasmodium yoelii* NL-*OVA* infection. CD45.1$^+$ OT-II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P yoelii* NL-*OVA* pRBC. Spleens were taken at stated time points p.i and analysed by flow cytometry. A, Representative histograms and B, mean fluorescence intensity (MFI) of Tim-3 levels on effector CD44$^+$ OT-II cells during *P yoelii* NL-*OVA* infection. C, Representative flow cytometric plots showing gating of T-bet$^+$ and T-bet$^-$ OT-II sub-populations. D, MFI of Tim-3 within T-bet$^+$ and T-bet$^-$ CD44$^+$ OT-II populations on days 5 and 15 of infection. E, Representative flow cytometric plots showing Tim-3 and PD-1 expression on OT-II cells in naïve mice and on effector CD44$^+$ OT-II cells and Th1 OT-II cells on day 5 of infection. F, The percentages of Tim-3$^+$ and/or PD-1$^+$ effector CD44$^+$ OT-II cells and Th1 OT-II cells over the course of infection. Results are representative of two experiments (n = 3-4). Bars represent mean ± SEM. $^*P \le .05$, $^{**}P \le .01$ (unpaired *t* test or one-way ANOVA with Tukey's multiple comparison test)

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

was monitored every other day starting from d5 p.i by assessing peripheral parasitaemia via microscopic examination of Giemsa-stained thin blood smears.

2.5 | In vivo blockades

Mice received 250 μg of α-PD-L1 (10F.9G2) and α-Tim-3 (RMT3-23) every two days from day 5 p.i via intraperitoneal (i.p.) injection. Antibodies were obtained from BioXcell. Control mice received 250 μg of Rat IgG (Sigma Aldrich) via i.p. injection.

2.6 | $CD4^+$ T cell isolation and adoptive transfer

Spleens were isolated from RAG-1 OT-II x Pep3 mice, homogenized through a 70 μm strainer (BD Biosciences) and incubated in RBC lysis buffer (BD Biosciences) to generate an RBC-free single cell suspension. OT-II cells were isolated using anti-CD4–conjugated microbeads (Miltenyi Biotec), according to manufacturer's instructions. 1×10^6 OT-II cells were transferred into C57BL/6 via iv injection one day prior to infection with *P yoelii* NL-*OVA* pRBCs.

2.7 | Flow cytometry

Spleens were removed from naïve and malaria-infected mice at stated time points, homogenized through a 70-μm strainer and incubated with RBC lysis buffer. Absolute live cell counts were calculated by trypan blue (Sigma) exclusion cell viability assay. Splenocytes were then surface stained in flow cytometry buffer (HBSS with 2% FCS) for 25 minutes at 4°C with anti-mouse antibodies to: CD45.1 (A20), CD4 (RM4-5), CD44 (IM7), PD-1 (RMPI-30), CXCR5-biotin (LI38D7) and Tim-3 (RMT3-23). All antibody staining was done in the presence of FcR block (2.4G2, BioXcell). For detection of biotin-labelled antibody, cells were labelled with primary antibodies before being stained with streptavidin v510 for 10 minutes at room temperature (RT). For intracellular antibody staining, cells were incubated with Foxp3 fixation/permeabilization buffer (eBioscience) for 30 minutes at 4°C. Cells were subsequently stained with the following antibodies: T-bet (4B10) and Ki-67 (SolA15) for 30 minutes. For analysis of the intracellular cytokines IFN-γ (XMG1.2) and TNF-α (MP6XT22), cells were stimulated ex vivo for 4 hours at 37°C with 200 ng/mL PMA (Sigma), 1 μg/mL ionomycin (Sigma) and brefeldin A ([1000×], eBioscience). 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 obtained from eBioscience or Biolegend. Samples were acquired through the Fortessa (BD systems) and all analysis was performed using Flowjo Software (Treestar Inc). Samples from *P yoelii* NL-*OVA* infected mice were combined to generate fluorescence minus one (FMO) controls, which were used to validate the flow cytometric data.

2.8 | Enzyme-linked immunosorbent assay (ELISA)

Nunc MaxiSorp™ plates (Fisher) were coated with 50 μL/well of 2 μg/mL *P yoelii* MSP-1 19 kDa ($MSP\text{-}1_{19}$) protein[31] in Dulbecco's PBS (DPBS) (Sigma), wrapped in clingfilm and incubated at 4°C overnight (≥16 hours). Plates were then washed 6× with PBS/T (PBS with 0.05% Tween-20 (Sigma)) and blocked at room temperature (RT) for 1 hour using 5% milk (sigma) in PBS/T. Plates were again washed 6× with PBS/T.

Mouse serum samples were then diluted 1:100 in PBS/T and plated in duplicate down the plate in a 3-fold dilution series. A positive control consisting of a pool of samples was also plated in duplicate in a 3-fold dilution series. Blocking solution (5% milk in PBS/T) was added to the first and last plate columns as a blank. The plate was then incubated for 2 hours at RT before washing 6× with PBS/T. Anti-mouse IgG (whole molecule)-AP (produced in goat) (Sigma) was diluted 1:1000 in PBS/T and 50 μL/well was added to the plate, before incubation for 1 hour at RT. Development solution was prepared by diluting 5× diethanolamine substrate buffer (Pierce) to 1× in ddH_2O, and one 20 mg pNPP tablet (Thermo) was added to each 20 mL of this buffer. The plate was then washed 6× in PBS/T. 100 μL/well of development buffer was added and plates read until the positive control reached a defined endpoint titre; the endpoint titres of the test samples were then calculated.

2.9 | Statistical analysis

All statistical analysis was performed using GraphPad Prism (GraphPad Software). All data were tested for normal distributions using the Shapiro-Wilk normality test. Comparison between three or more groups was carried out using a one-way ANOVA with Tukey's test for multiple comparisons. Comparisons between two groups were carried out using an unpaired *t* test (for parametric data) or a Mann-Whitney test (for non-parametric data). Results were considered significant when $P < .05$.

3 | RESULTS

3.1 | Tim-3 is transiently expressed on antigen-specific effector $T\text{-}bet^+$ OT-II cells during blood-stage *P yoelii* NL-*OVA* infection

To examine the potential role of Tim-3 in regulating antigen-specific $CD4^+$ T cell responses during blood-stage malaria, we first examined the levels of Tim-3 on ovalbumin-specific TCR transgenic $CD4^+$ T cells (OT-II cells) during blood-stage *P yoelii* NL-*OVA* infection. This *P yoelii* NL-*OVA* line (Py-*hsp70*-OVA::mCherry) expresses ovalbumin (OVA) fused to mCherry under control of the constitutive *hsp70* promoter of *P berghei* (PBANKA_0711900; Figure S1). We have previously shown that transgenic parasites expressing OVA fused to mCherry induce strong OT-I and OT-II responses.[25] Expression of OVA::mCherry in blood and mosquito

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

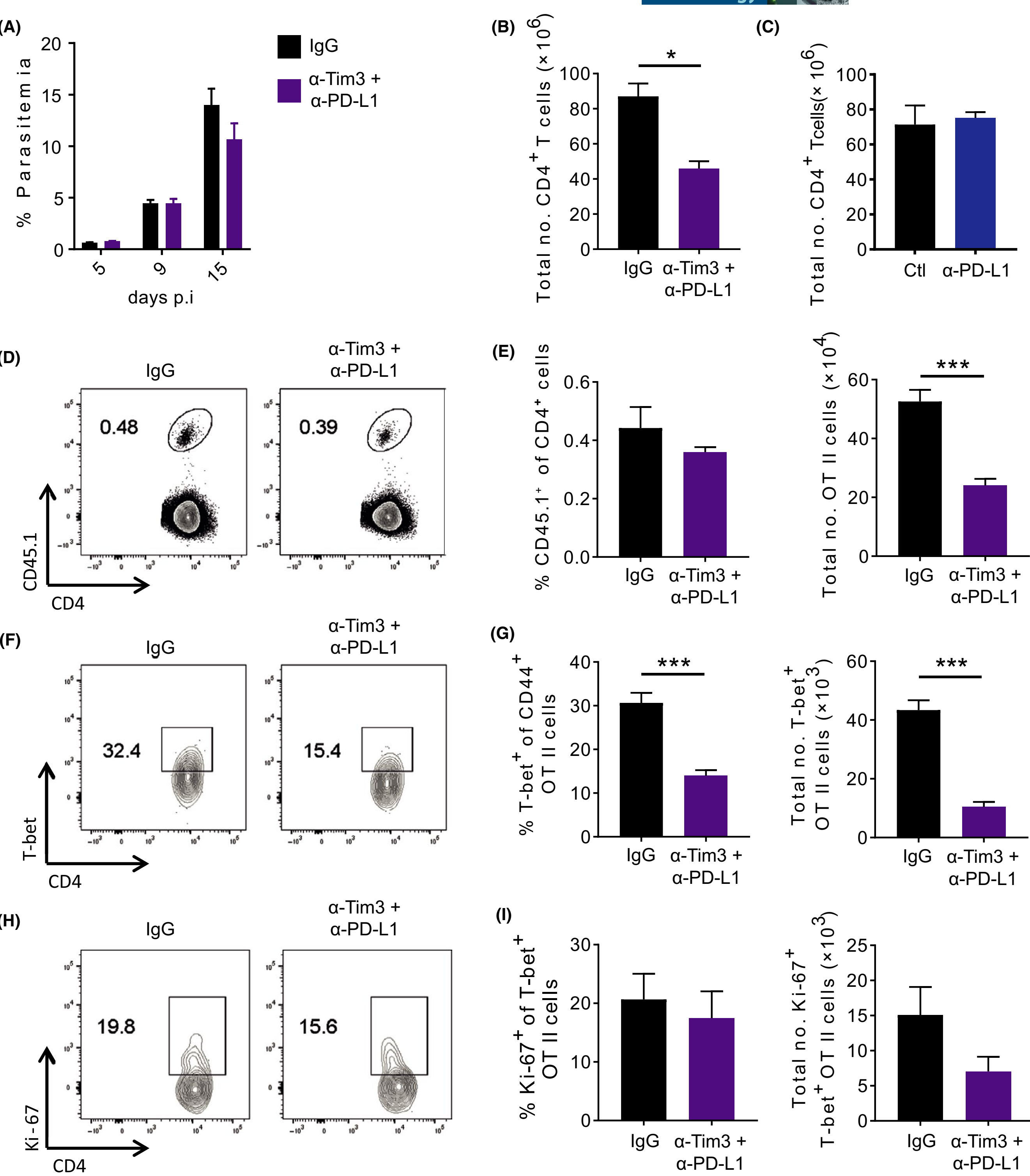


FIGURE 2 Blockade of Tim-3 and PD-L1 does not improve parasite control but reduces the Th1 response during *Plasmodium yoelii* NL-*OVA* infection. CD45.1$^+$ OT-II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P yoelii* NL-*OVA* pRBC. Infected mice were either treated with control rat IgG (n = 4) or α-Tim-3 and/ or α-PD-L1 Abs (250 µg) every 2 days starting from day 5 p.i. A, Peripheral parasitaemia during infection. B and C, Total numbers of CD4$^+$ T cells in the spleen on day 15 following (B) combined α-Tim-3 and α-PD-L1 treatment or (C) individual α-PD-L1 treatment. D, Representative flow cytometric plots, and E, percentages and total numbers of OT-II cells on day 15 of infection. F, Representative flow cytometric plots, and G, percentages and total numbers of T-bet$^+$ OT-II cells on day 15 of infection. H, Representative flow cytometric plots, and I, percentages and total numbers of Ki67$^+$ T-bet$^+$ OT-II cells on day 15 of infection. Results are representative of three independent experiments (n = 4 per group). Bars represent mean ± SEM. *$P \le .05$, ***$P \le .001$ (unpaired *t* test or Mann-Whitney test)

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

stages of *P yoelii* NL-*OVA* was shown by analysis of mCherry fluorescence and immunofluorescence analysis of OVA expression (Figure S1).

We assessed Tim-3 expression on OT-II cells on day 5 of infection, representing an early time point following T cell activation and on day 15 of infection, equivalent to peak parasitaemia.[32] Tim-3 expression was significantly increased on effector ($CD44^+$) OT-II cells during the early stage of blood-stage infection on day 5 p.i., but expression significantly decreased by day 15 p.i (Figure 1A,B). Tim-3 expression was significantly higher on effector T-bet^+ (Th1) OT-II cells, than effector T-bet^- OT-II cells on day 5 of infection, indicating that Tim-3 may preferentially regulate Th1 cell responses during the early phase of blood-stage malaria (Figure 1C,D). On day 5 of infection very few T-bet^+CD44^+ OT-II cells co-expressed GATA-3, Foxp3 or Bcl-6, confirming them as Th1 cells. On day 15 of infection a slightly larger proportion of T-bet^+CD44^+ OT-II cells co-expressed Bcl-6 but the significant majority of T-bet^+ cells remained characteristically Th1 cells (results not shown). Of the effector OT-II and Th1 OT-II cells expressing Tim-3 on day 5 of infection, approximately 50% co-expressed PD-1 (Figure 1E,F). Notably, only a small proportion effector OT-II cells and Th1-OT-II cells expressed PD-1 independently from Tim-3 on day 5 of infection (Figure 1E,F). Thus, there is high expression of Tim-3 on antigen-specific Th1 OT-II cells during the early stages of blood-stage *P yoelii* NL-*OVA* infection. The co-expression of PD-1 with Tim-3 also supports the view that Tim-3 may act in conjunction with PD-1 to regulate effector T cells during the early stages of an immune response.[11]

3.2 | Combinatorial Tim-3 and PD-L1 blockade does not influence parasite control but causes loss of Th1 OT-II cells during *P yoelii* NL-*OVA* infection

To examine the combined role of Tim-3 and PD-1 in regulating antigen-specific $CD4^+$ T cell responses and parasite control during blood-stage malaria, we treated mice with combinatorial α-Tim-3 and α-PD-L1 antibodies from day 5 of *P yoelii* NL-*OVA* infection, where maximal Tim-3 expression was observed. Blockade of Tim-3 and PD-L1 from day 5 of infection did not significantly affect peripheral parasitaemia, compared with IgG control treatment, on any examined day, until termination of experiments on day 15 of infection (Figure 2A). Surprisingly, given the perceived regulatory roles of Tim-3 and PD-1,[8-13] combinatorial α-Tim-3 and α-PD-L1 blockade led to a significant reduction in the total numbers of $CD4^+$ T cells within the spleen on day 15 of infection, compared with control IgG treatment (Figure 2B). Individual blockade of PD-L1 did not affect the total numbers of splenic $CD4^+$ T cells (Figure 2C), suggesting that the reduction in $CD4^+$ T cell numbers following combinatorial α-Tim-3 and α-PD-L1 treatment corresponded to individual or synergistic blockade of Tim-3 function.

Combinatorial α-Tim-3 and α-PD-L1 blockade did not alter the overall frequencies of OT-II cells on day 15 of infection; however, due to the impact on total splenic $CD4^+$ T cell numbers (Figure 2B), treatment significantly reduced the total numbers of splenic OT-II cells, compared with mice treated with IgG control antibodies (Figure 2D,E). Interestingly, Tim-3 and PD-L1 co-blockade significantly decreased both the frequencies and total numbers of Th1 OT-II cells on day 15 of infection, compared with IgG control treatment (Figure 2F,G). Treatment did not alter the small proportion of Bcl-6^+ co-expressing cells within the T-bet^+ OT-II population, indicating the therapy did not change the composition of the T-bet^+ OT-II compartment (results not shown). Combinatorial blockade did not significantly alter Th1 OT-II cell proliferation, as measured by Ki-67 expression (Figure 2H,I), suggesting that the effects of α-Tim-3 and α-PD-L1 treatment on the frequencies and numbers of Th1 OT-II cells was not due to diminished Th1 cell proliferation.

The reduction in frequencies of Th1 OT-II cells indicated a specific detrimental impact of Tim-3 and PD-L1 co-blockade on the Th1 cell response, compared with other OT-II cell subsets. In agreement with this, the frequencies of splenic $CXCR5^+PD\text{-}1^+$ (Tfh) OT-II cells trended higher in α-Tim-3 and α-PD-L1 co-treated mice on day 15 of infection than in IgG control mice, although total cell numbers were not increased by combinatorial blockade (Figure 3A,B). PD-L1 and Tim-3 co-blockade did not influence the frequencies or numbers of germinal centre B cells (Figure 3C,D and results not shown) but combinatorial treatment did significantly increase the plasma titres of anti-*P yoelii* $MSP1_{19}$ IgG antibodies on day 15 of infection, compared with control IgG treatment (Figure 3E).

In addition, α-Tim-3 and α-PD-L1 combinatorial treatment did not alter the frequencies of T-bet^- $CXCR5^{int}PD\text{-}1^{int}$ OT-II cells, previously defined as central memory (Tcm) precursor cells,[33,34] nor the frequencies of T-bet^- $CXCR5^{lo}PD\text{-}1^{lo}$ OT-II cells on day 15 of infection (Figure 3F,G). Although Tim-3 has previously been shown to influence memory $CD4^+$ T cell development,[35] combined α-PD-L1 and α-Tim-3 treatment did not lead to an increase in CD62L expression by the $CXCR5^{int}PD\text{-}1^{int}$ or the $CXCR5^{lo}PD\text{-}1^{lo}$ OT-II subsets (Figure 3H).

Taken together, these data show that combinatorial Tim-3 and PD-L1 blockade specifically inhibited the development and/or maintenance of the Th1 OT-II cell subset, whilst increasing the anti-parasite IgG response, during the early stages of blood-stage *P yoelii* NL-*OVA* infection. Nevertheless, our co-blockade results suggest that the co-inhibitory molecules do not appear to influence parasite control during acute blood-stage malaria.

3.3 | Combinatorial Tim-3 and PD-L1 blockade does not influence overall IFN-γ production by effector OT-II cells during blood-stage *P yoelii* NL-*OVA* infection

Given the effect of α-Tim-3 and α-PD-L1 treatment on the Th1 OT-II response (Figure 2F,G), we next examined if α-Tim-3 and α-PD-L1 combinatorial treatment influenced effector OT-II pro-inflammatory function during *P yoelii* NL-*OVA* infection. Surprisingly,

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

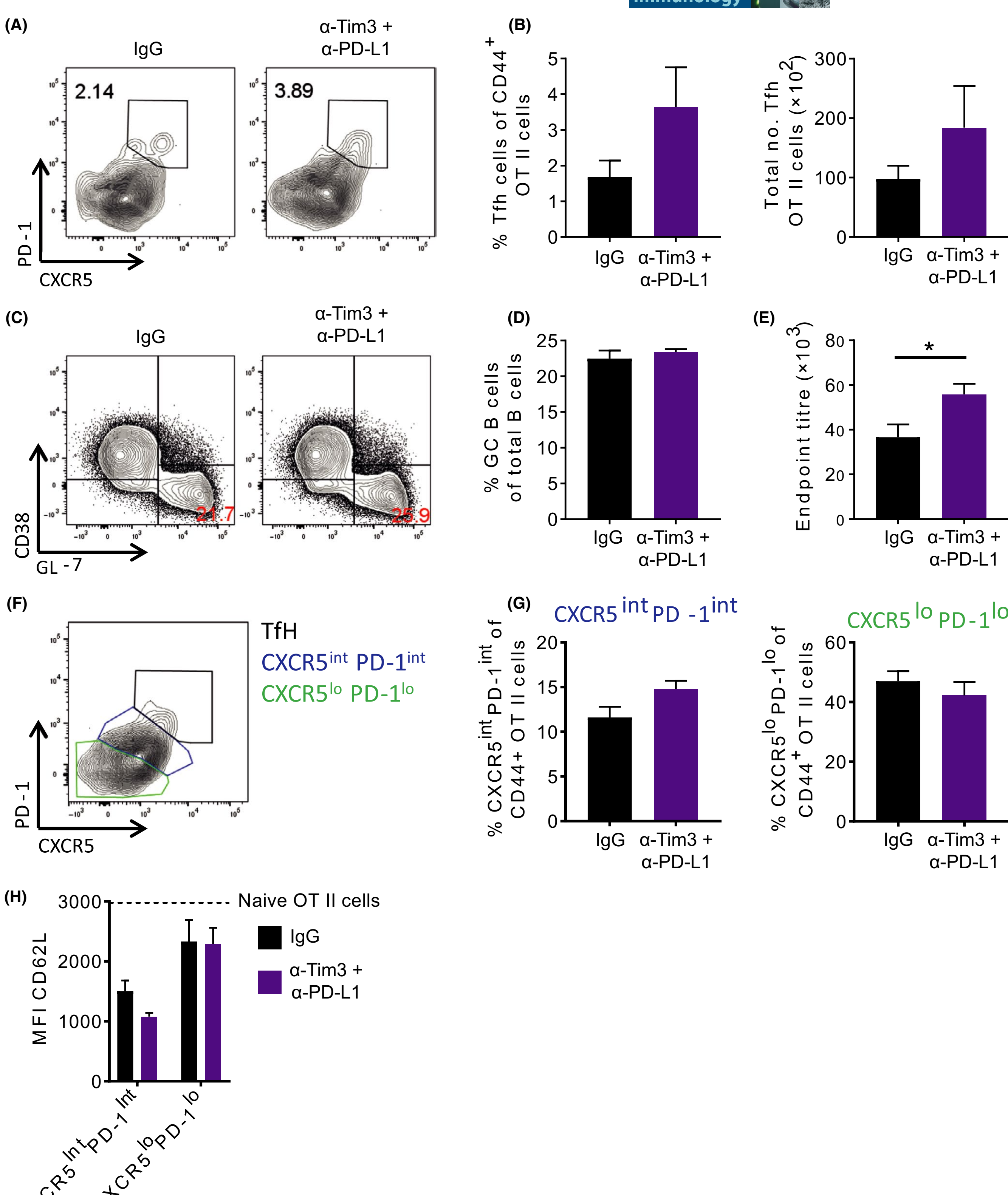


FIGURE 3 Blockade of Tim-3 and PD-L1 marginally improves the humoral response during *Plasmodium yoelii* NL-*OVA* infection. CD45.1$^+$ OT-II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *P yoelii* NL-*OVA* pRBC. Infected mice were either treated with control rat IgG or α-Tim-3 and α-PD-L1 Abs (250 μg) every 2 days starting from day 5 p.i. A, Representative flow cytometric plots, and B, percentages and total numbers of CXCR5$^+$PD-1$^+$ Tfh OT-II cells on day 15 of infection. C, Representative flow cytometric plots, and D, percentages GL-7$^+$CD38$^-$ GC B cells on day 15 of infection. E, The plasma titres of anti-MSP-1_{19} IgG on day 15 of infection. F, Representative flow cytometric plots, and G, percentages of CXCR5^{int}PD-1^{int} and CXCR5^{lo}PD-1^{lo} CD44$^+$ OT-II cells on day 15 of infection. H, The MFI of CD62L expression by CXCR5^{int}PD-1^{int} and CXCR5^{lo}PD-1^{lo} CD44$^+$ OT-II cells on day 15 of infection. Results are representative of 2-3 independent experiments (n = 4 per group). Bars represent mean ± SEM. *$P \le .05$ (unpaired *t* test)

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

combinatorial Tim-3 and PD-L1 blockade did not significantly alter the frequencies or numbers of IFN-γ$^+$ or TNF$^+$ effector OT-II cells on day 15 of infection, compared with IgG control treatment (Figure 4A,B). Indeed, Tim-3 and PD-L1 co-blockade led to a slight, non-significant, increase in the frequencies of IFN-γ producing Th1 (effector T-bet$^+$) OT-II cells, which translated to an equivalence in IFN-γ producing Th1 OT-II cell numbers in α-Tim-3 and α-PD-L1 treated mice on day 15 of infection, compared with control IgG-treated mice (Figure 4C,D). Tim-3 and PD-L1 co-blockade did not significantly influence the frequencies or numbers of TNFα-producing Th1 OT-II cells (Figure 4C,D). Combined, Tim-3 and PD-L1 blockade did not affect the level (as measured by mean fluorescence intensity) of IFN-γ produced by Th1 OT-II cells (Figure 4E). Combinatorial α-Tim-3 and α-PD-L1 treatment also failed to alter TNFα production by Th1 OT-II cells on day 15 of infection, compared with control IgG treatment (Figure 4E).

These results indicate that Tim-3 and PD-1 do not act together to regulate overall effector OT-II cell or Th1 OT-II cell pro-inflammatory responses during the early stages of blood-stage *P yoelii* NL-*OVA* infection.

4 | DISCUSSION

In this study, we have addressed the combined roles of Tim-3 and PD-1 in regulating the antigen-specific CD4$^+$ T cell response during blood-stage malaria. We have shown that antigen-specific OT-II cells express high levels of Tim-3 only transiently during the early stages of *P yoelii* NL-*OVA* infection, and that combinatorial blockade of Tim-3 and PD-L1 from day 5 of infection caused an overall deterioration, rather than an increase, in the Th1 (effector T-bet$^+$) OT-II subset when examined on day 15 of infection. Nevertheless, combined α-Tim-3 and α-PD-L1 treatment failed to significantly influence overall IFN-γ production by antigen-specific effector OT-II cells during the early stages of *P yoelii* NL-*OVA* infection and, as such, treatment did not impact blood-stage parasite control.

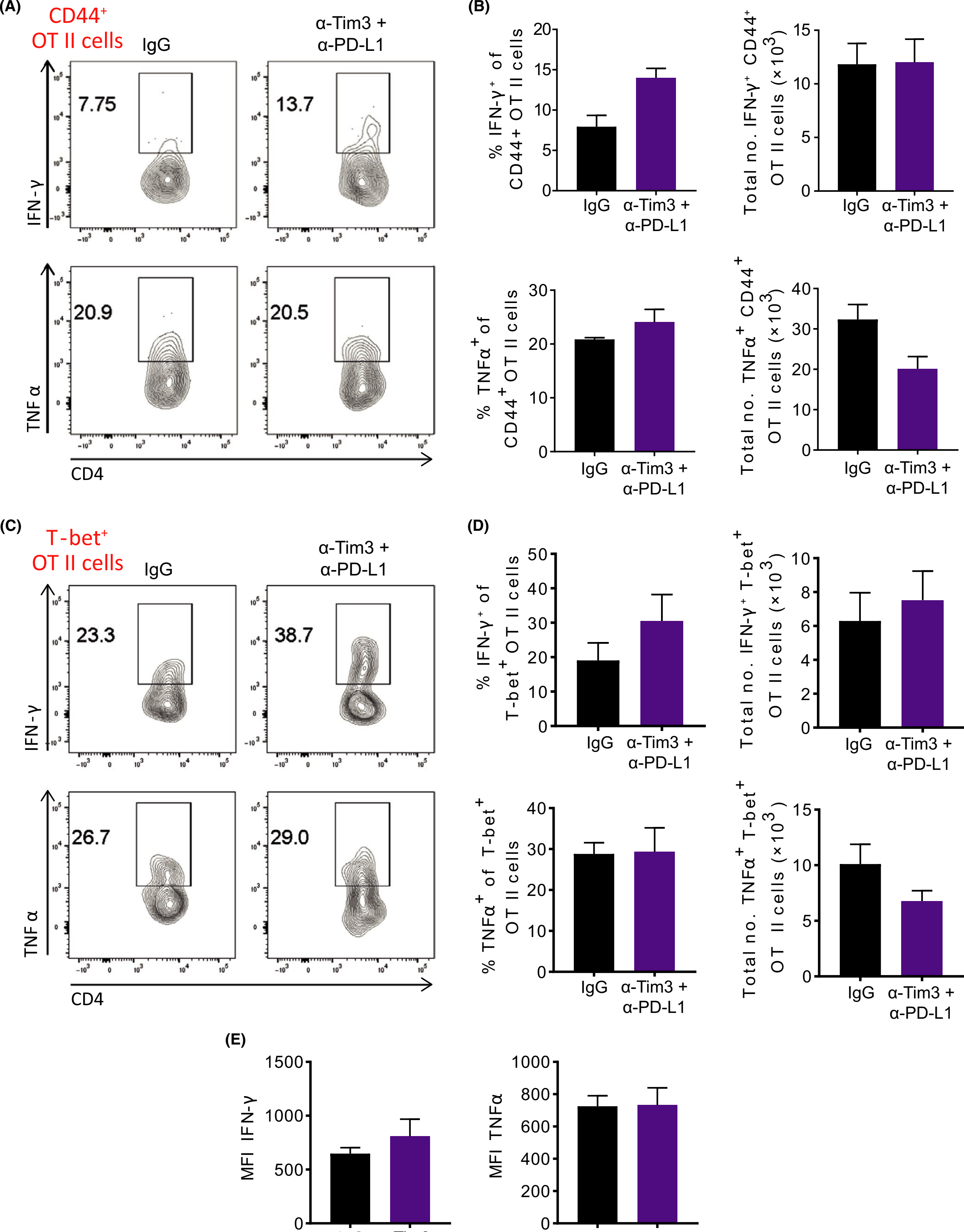
The transient expression of Tim-3 by antigen-specific CD4$^+$ T cells during infection was surprising, given that prolonged co-expression of Tim-3 and PD-1 has previously been correlated with effector T cell dysfunction during non-malarial chronic infections.[19,36] However, Hou et al[22] have also shown that Tim-3 is highly expressed by CD4$^+$ T cells on day 5 of *P berghei* ANKA infection before being subsequently downregulated as infection progresses. In contrast, Tim-3 expression continued to increase on CD8$^+$ T cells throughout the course of *P berghei* ANKA infection.[22] Consequently, it is foreseeable that the dynamics and magnitude of Tim-3 expression are different on effector CD4$^+$ T cells and CD8$^+$ T cells during blood-stage malaria. Why Tim-3 may be only transiently expressed in CD4$^+$ T cells during malaria is unclear but may relate to lack of sustained cellular stimulation during infection, which is required for maintained Tim-3 expression.[11] For example, Tim-3 expression is regulated by IL-12,[37] and IL-12R signalling is significantly restricted in effector CD4$^+$ T cells at the later stages of *Plasmodium* infection.[38]

Our results showing that Tim-3 blockade, in combination with PD-1, led to a reduction in Th1 OT-II cells on day 15 of *P yoelii* NL-*OVA* infection was unexpected, given the important regulatory activity of Tim-3 in controlling effector T cell responses in various other non-malarial conditions.[11,12] Whether the positive effect of Tim-3 in sustaining Th1 cell responses during *P yoelii* NL infection is revealed only when PD-1 activity is blocked is unclear and will require additional investigation. However, of relevance to this, there is evidence that Tim-3 can directly promote T cell responses. For example, Tim-3 increased the generation of effector T cells during LCMV infection via enhancing TCR activation and influencing mTOR activity, effects that were independent of Tim-3 ligand binding.[35] Tim-3 also directly enhanced CD8$^+$ T cell responses during acute *Listeria monocytogenes* infection.[39] The contrasting pro- and anti-inflammatory effects of Tim-3 on T cell function are believed to be regulated through the phosphorylation of its intracellular domain. In the absence of ligand binding, the intracellular adaptor protein Bat3 binds to the unphosphorylated cytoplasmic tail of Tim-3 and recruits Lck to promote T cell signalling.[11,14] Thus, our data may suggest that during the early phase of blood-stage *P yoelii* NL infection, Tim-3 may act independent of its ligands to positively influence effector Th1 cell responses. However, we do not discount the possibility that combinatorial Tim-3 and PD-L1 blockade led to a transient increase in Th1 cell numbers between days 5 and 15 of infection, before the Th1 cells experienced premature/ exacerbated contraction, leading to the reduction in Th1 cell numbers observed on day 15 of infection.

The preferential expression of Tim-3 on Th1 (effector T-bet$^+$) cells may partially explain why co-blockade of Tim-3 and PD-L1 specifically affected the Th1 cell population during *P yoelii* NL-*OVA* infection whilst Tfh responses were intact/ elevated upon treatment. In support of this, there is clear evidence that different co-inhibitory and co-activating receptors can differentially affect Th1 and Tfh cell populations and function during malaria (reviewed in[40]). It is also possible that separate immunological signals, such as APC identity, chemokine receptor expression and cytokine environment, which may exert unequal effects on Th1 and Tfh cells,[40] were also modified following α-Tim-3 and α-PD-L1

FIGURE 4 Blockade of Tim-3 and PD-L1 does not influence effector CD4 + T cell or Th1 cell pro-inflammatory function during *P yoelii* NL-*OVA* infection. CD45.1$^+$ OT-II cells were adoptively transferred into C57BL/6 mice prior to infection with 10^4 *Plasmodium yoelii* NL-OVA pRBC. Infected mice were either treated with control rat IgG or α-Tim-3 and α-PD-L1 Abs (250 μg) every 2 days starting from day 5 p.i. A, Representative flow cytometric plots and B, percentages and number of CD44$^+$ effector OT-II cells producing IFN-γ or TNFα on day 15 of infection. C, Representative flow cytometric plots and D, percentages and number of T-bet$^+$ Th1 OT-II producing IFN-γ or TNFα on day 15 of infection. E, MFI of TNFα and IFN-γ production by T-bet$^+$ Th1 OT-II cells on day 15 of infection. Results are representative of two independent experiments (n = 4 per group). Bars represent mean ± SEM

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License



13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

treatment. The exact mechanisms of how Tim-3 specifically promotes/ sustains Th1 cell differentiation independently and in context of PD-1 blockade during blood-stage *P yoelii* NL infection will require additional investigation.

Notably, whilst co-blockade of Tim-3 and PD-L1 did not affect the magnitude of the Tfh population on day 15 of infection, treatment did raise the plasma titre of anti-parasitic ($MSP1_{19}$-specific) IgG. This seemingly contradictory result mirrors previous observations in IL-6 and IL-21 deficient mice, where anti-parasitic antibody responses were modified during blood-stage malaria in absence of changes in the numbers of Tfh cells.[41,42] Whether co-blockade of Tim-3 and PD-L1 increased the strength of the anti-parasite antibody response through improving the effectiveness of Tfh interaction with B cells, such as in these previous studies, will require further study. Although co-blockade of Tim-3 and PD-L1 did not influence the frequencies or numbers of GC B cells, it is unclear whether blockade influenced the functions of plasma cells or the numbers of plasmablasts during infection.

In summary, our data show that in the context of PD-1 blockade, Tim-3 is required for promoting and/ or maintaining effector T-bet$^+$ Th1 antigen-specific CD4$^+$ T cells during the early phase of *P yoelii* NL blood-stage infection. Nevertheless, as combinatorial α-PD-L1 and α-Tim-3 blockade failed to alter parasite control, our results question the importance of combinatorial Tim-3 and PD-1 activity in influencing anti-parasitic immunity, during the early acute phase of *P yoelii* NL infection.

ACKNOWLEDGEMENTS

We would like to express our thanks to Dr Shahid Khan from the Leiden malaria group, Leiden University Medical Center, for his critical contribution to the design, generation and validation of the *P yoelii* NL-*OVA* line that was used in this manuscript. Dr Khan was a highly valued and respected colleague and he will be sadly missed. We also 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. We thank the Biological Services Facility at the University of Manchester for help with animal work.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

RSD, AVM and JRB performed experiments and analysed the data. HK, BMFF, CJJ and SMK generated reagents for use in the study. SJD, ASM and KNC provided resources and supervised the work. RSD and KNC conceived the project and wrote the manuscript. CJJ, SJD and ASM revised the manuscript.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

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

REFERENCES

1. Kurup SP, Butler NS, Harty JT. T cell-mediated immunity to malaria. *Nat Rev Immunol*. 2019;19(7):457-471.
2. Perez-Mazliah D, Langhorne J. CD4 T-cell subsets in malaria: TH1/TH2 revisited. *Front Immunol*. 2014;5:671.
3. Schlotmann T, Waase I, Julch C, et al. CD4 alphabeta T lymphocytes express high levels of the T lymphocyte antigen CTLA-4 (CD152) in acute malaria. *J Infect Dis*. 2000;182(1):367-370.
4. Mackroth MS, Abel A, Steeg C, Schulze Zur Wiesch J, Jacobs T. Acute malaria induces PD1+CTLA4+ effector T cells with cell-extrinsic suppressor function. *PLoS Pathog*. 2016;12(11):e1005909.
5. Illingworth J, Butler NS, Roetynck S, et al. Chronic exposure to *Plasmodium falciparum* is associated with phenotypic evidence of B and T cell exhaustion. *J Immunol*. 2013;190(3):1038-1047.
6. Edwards CL, Ng SS, Corvino D, et al. Early changes in CD4+ T-cell activation during blood-stage *Plasmodium falciparum* infection. *J Infect Dis*. 2018;218(7):1119-1129.
7. Butler NS, Moebius J, Pewe LL, et al. Therapeutic blockade of PD-L1 and LAG-3 rapidly clears established blood-stage Plasmodium infection. *Nat Immunol*. 2011;13(2):188-195.
8. Wykes MN, Lewin SR. Immune checkpoint blockade in infectious diseases. *Nat Rev Immunol*. 2018;18(2):91-104.
9. Faleiro R, Karunarathne DS, Horne-Debets JM, Wykes M. The contribution of co-signaling pathways to anti-malarial T cell immunity. *Front Immunol*. 2018;9:2926.
10. Freeman GJ, Sharpe AH. A new therapeutic strategy for malaria: targeting T cell exhaustion. *Nat Immunol*. 2012;13(2):113-115.
11. Joller N, Kuchroo VK. Tim-3, Lag-3, and TIGIT. *Curr Top Microbiol Immunol*. 2017;410:127-156.
12. Anderson AC, Joller N, Kuchroo VK. Lag-3, Tim-3, and TIGIT: co-inhibitory receptors with specialized functions in immune regulation. *Immunity*. 2016;44(5):989-1004.
13. Zhu C, Anderson AC, Schubart A, et al. The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. *Nat Immunol*. 2005;6(12):1245-1252.
14. Rangachari M, Zhu C, Sakuishi K, et al. Bat3 promotes T cell responses and autoimmunity by repressing Tim-3-mediated cell death and exhaustion. *Nat Med*. 2012;18(9):1394-1400.
15. Gao X, Zhu Y, Li G, et al. TIM-3 expression characterizes regulatory T cells in tumor tissues and is associated with lung cancer progression. *PLoS ONE*. 2012;7(2):e30676.
16. Golden-Mason L, Palmer BE, Kassam N, et al. Negative immune regulator Tim-3 is overexpressed on T cells in hepatitis C virus infection and its blockade rescues dysfunctional CD4+ and CD8+ T cells. *J Virol*. 2009;83(18):9122-9130.
17. Monney L, Sabatos CA, Gaglia JL, et al. Th1-specific cell surface protein Tim-3 regulates macrophage activation and severity of an autoimmune disease. *Nature*. 2002;415(6871):536-541.
18. McMahan RH, Golden-Mason L, Nishimura MI, et al. Tim-3 expression on PD-1+ HCV-specific human CTLs is associated with viral persistence, and its blockade restores hepatocyte-directed in vitro cytotoxicity. *J Clin Invest*. 2010;120(12):4546-4557.
19. Jin H-T, Anderson AC, Tan WG, et al. Cooperation of Tim-3 and PD-1 in CD8 T-cell exhaustion during chronic viral infection. *Proc Natl Acad Sci USA*. 2010;107(33):14733-14738.
20. Costa PA, Leoratti FM, Figueiredo MM, et al. Induction of inhibitory receptors on T cells during *Plasmodium vivax* malaria impairs cytokine production. *J Infect Dis*. 2015;212(12):1999-2010.

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

21. Abel A, Steeg C, Aminkiah F, et al. Differential expression pattern of co-inhibitory molecules on CD4(+) T cells in uncomplicated versus complicated malaria. *Sci Rep*. 2018;8(1):4789.
22. Hou N, Zou Y, Piao X, et al. T-Cell immunoglobulin- and mucin-domain-containing molecule 3 signaling blockade improves cell-mediated immunity against malaria. *J Infect Dis*. 2016;214(10):1547-1556.
23. Huang BO, Liu M, Huang S, et al. Expression of Tim-1 and Tim-3 in *Plasmodium berghei* ANKA infection. *Parasitol Res*. 2013;112(7):2713-2719.
24. Barnden MJ, Allison J, Heath WR, Carbone FR. 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*. 1998;76(1):34-40.
25. Lin J-W, Shaw TN, Annoura T, et al. The subcellular location of ovalbumin in *Plasmodium berghei* blood stages influences the magnitude of T-cell responses. *Infect Immun*. 2014;82(11):4654-4665.
26. Kooij TW, Rauch MM, Matuschewski K. Expansion of experimental genetics approaches for *Plasmodium berghei* with versatile transfection vectors. *Mol Biochem Parasitol*. 2012;185(1):19-26.
27. de Moraes LV, Dechavanne S, Sousa PM, et al. Murine model for preclinical studies of Var2CSA-mediated pathology associated with malaria in pregnancy. *Infect Immun*. 2016;84(6):1761-1774.
28. Lin J-W, Annoura T, Sajid M, et al. A novel 'gene insertion/marker out' (GIMO) method for transgene expression and gene complementation in rodent malaria parasites. *PLoS ONE*. 2011;6(12):e29289.
29. Janse CJ, Ramesar J, Waters AP. High-efficiency transfection and drug selection of genetically transformed blood stages of the rodent malaria parasite *Plasmodium berghei*. *Nat Protoc*. 2006;1(1):346-356.
30. Salman AM, Mogollon CM, Lin JW, van Pul FJ, Janse CJ, Khan SM. Generation of transgenic rodent malaria parasites expressing human malaria parasite proteins. *Methods Mol Biol*. 2015;1325:257-286.
31. Draper SJ, Moore AC, Goodman AL, et al. Effective induction of high-titer antibodies by viral vector vaccines. *Nat Med*. 2008;14(8):819-821.
32. Couper KN, Blount DG, Wilson MS, et al. IL-10 from CD4CD25Foxp3CD127 adaptive regulatory T cells modulates parasite clearance and pathology during malaria infection. *PLoS Pathog*. 2008;4(2):e1000004.
33. Gasper DJ, Tejera MM, Suresh M. CD4 T-cell memory generation and maintenance. *Crit Rev Immunol*. 2014;34(2):121-146.
34. Pepper M, Pagan AJ, Igyarto BZ, Taylor JJ, Jenkins MK. Opposing signals from the Bcl6 transcription factor and the interleukin-2 receptor generate T helper 1 central and effector memory cells. *Immunity*. 2011;35(4):583-595.
35. Avery L, Filderman J, Szymczak-Workman AL, Kane LP. Tim-3 co-stimulation promotes short-lived effector T cells, restricts memory precursors, and is dispensable for T cell exhaustion. *Proc Natl Acad Sci USA*. 2018;115(10):2455-2460.
36. Jayaraman P, Jacques MK, Zhu C, et al. TIM3 mediates T cell exhaustion during *Mycobacterium tuberculosis* Infection. *PLoS Pathog*. 2016;12(3):e1005490.
37. Yang Z-Z, Grote DM, Ziesmer SC, et al. IL-12 upregulates TIM-3 expression and induces T cell exhaustion in patients with follicular B cell non-Hodgkin lymphoma. *J Clin Invest*. 2012;122(4):1271-1282.
38. Villegas-Mendez A, de Souza JB, Lavelle S-W, et al. IL-27 receptor signalling restricts the formation of pathogenic, terminally differentiated Th1 cells during malaria infection by repressing IL-12 dependent signals. *PLoS Pathog*. 2013;9(4):e1003293.
39. Gorman JV, Starbeck-Miller G, Pham NL, et al. Tim-3 directly enhances CD8 T cell responses to acute Listeria monocytogenes infection. *J Immunol*. 2014;192(7):3133-3142.
40. Soon MSF, Haque A. Recent Insights into CD4(+) Th Cell Differentiation in Malaria. *J Immunol*. 2018;200(6):1965-1975.
41. Pérez-Mazliah D, Ng DHL, Freitas do Rosário AP, et al. Disruption of IL-21 signaling affects T cell-B cell interactions and abrogates protective humoral immunity to malaria. *PLoS Pathog*. 2015;11(3):e1004715.
42. Sebina I, Fogg LG, James KR, et al. IL-6 promotes CD4(+) T-cell and B-cell activation during Plasmodium infection. *Parasite Immunol*. 2017;39(10):e12455.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Dookie RS, Villegas-Mendez A, Kroeze H, et al. Combinatorial Tim-3 and PD-1 activity sustains antigen-specific Th1 cell numbers during blood-stage malaria. *Parasite Immunol*. 2020;42:e12723. https://doi.org/10.1111/pim.12723

13653024, 2020, 9, Downloaded from https://onlinelibrary.wiley.com/doi/10.1111/pim.12723 by University Of Leiden, Wiley Online Library on [14/11/2022]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License