

Plasmodium yoelii infection of BALB/c mice results in expansion rather than induction of CD4⁺ Foxp3⁺ regulatory T cells

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doi:10.1111/imm.12602

Received 21 December 2015; revised 22 February 2016; accepted 24 February 2016.

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Summary

Recently, we demonstrated elevated numbers of CD4⁺ Foxp3⁺ regulatory T (Treg) cells in *Plasmodium yoelii*-infected mice contributing to the regulation of anti-malarial immune response. However, it remains unclear whether this increase in Treg cells is due to thymus-derived Treg cell expansion or induction of Treg cells in the periphery. Here, we show that the frequency of Foxp3⁺ Treg cells expressing neuropilin-1 (Nrp-1) decreased at early time-points during *P. yoelii* infection, whereas percentages of Helios⁺ Foxp3⁺ Treg cells remained unchanged. Both Foxp3⁺ Nrp-1⁺ and Foxp3⁺ Nrp-1⁻ Treg cells from *P. yoelii*-infected mice exhibited a similar T-cell receptor V β chain usage and methylation pattern in the Treg-specific demethylation region within the *foxp3* locus. Strikingly, we did not observe induction of Foxp3 expression in Foxp3⁻ T cells adoptively transferred to *P. yoelii*-infected mice. Hence, our results suggest that *P. yoelii* infection triggered expansion of naturally occurring Treg cells rather than *de novo* induction of Foxp3⁺ Treg cells.

Keywords: parasitic protozoan; regulatory T cells; rodent.

Introduction

CD4⁺ Foxp3⁺ regulatory T (Treg) cells are well-known key players in the maintenance of immunological homeostasis and there is an increasing body of evidence that Treg cells also control immune responses during infectious diseases. Elevated frequencies and/or numbers of Treg cells were detected in mice infected with parasites^{1,2} and viruses³ as well as in humans suffering from *Helicobacter pylori* infection.⁴ Likewise an increase in the number of CD4⁺ CD25⁺ Foxp3⁺ Treg cells was observed in the peripheral blood of patients infected with *Plasmodium falciparum*,^{5,6} the parasite that causes severe malaria. Most recently, we detected elevated Treg numbers in spleens of BALB/c mice infected with *Plasmodium yoelii*,⁷ a well established experimental mouse model for studying *Plasmodium* infection at the blood stage. Strikingly, depletion of Treg cells from *P. yoelii*-infected mice resulted in a more efficient T-cell response accompanied by significantly reduced parasitaemia, suggesting that Foxp3⁺ Treg cells interfere with anti-malarial immune responses.⁷

Besides naturally occurring thymus-derived Foxp3⁺ Treg (nTreg) cells a heterogeneous population of induced Treg (iTreg) cells exists that arises from naive CD4⁺ CD25⁻ T cells within the periphery in several experimental settings.^{8–10} Hence, the iTreg repertoire derives from conventional T cells, whereas nTreg cells were selected by high-affinity interactions within the thymus.^{11,12} It was assumed that nTreg cells mainly recognize self-antigens, acting as important regulators of autoimmune responses. In contrast iTreg cells seem to respond to foreign antigens, so playing a crucial role in mucosal immune tolerance and chronic allergic reactions. However, they might also be generated in response to self-antigens and have been suggested to collaborate with nTreg cells to achieve optimal regulation.¹³ Until now, it is difficult to study the phenotype and function of iTreg cells versus nTreg cells due to the lack of a single specific feature of either cell type in particular during ongoing immune responses. However, a better understanding of the origin of Treg cells would be helpful to develop specific therapeutics to modulate the number and function of

Abbreviations: iTreg, induced Treg; Nrp-1, neuropilin-1; nTreg, natural Treg; p.i., post-infection; TCR, T-cell receptor; Treg cell, regulatory T cell; TSDR, Treg-specific demethylation region

Treg cells by interfering with pathways that are involved in their expansion or conversion from conventional T cells, respectively.

Helios, a member of the Ikaros family of transcription factors, was proposed for discriminating nTreg from iTreg cells,¹⁴ but its utility as an nTreg marker has been questioned due to its inconsistent expression on iTreg cells in distinct immune settings.^{15,16} Most recently, an additional marker molecule for distinguishing thymus-derived nTreg cells from iTreg cells has been described: the surface receptor neuropilin-1 (Nrp-1).^{17,18} Whereas the majority of Foxp3⁺ nTreg cells expresses Nrp-1 on their surface,¹⁹ Foxp3⁺ Treg cells converted under homeostatic conditions have been suggested to lack Nrp-1 expression.¹⁸

Analysis of epigenetic modifications within the *foxp3* locus has also been proposed to be helpful for discriminating thymus-derived nTreg cells with stable Foxp3 expression from peripherally induced Treg cells, which often show an unstable Foxp3 expression. DNA demethylation at a conserved intronic CpG-rich region, the Treg-specific demethylated region (TSDR) was correlated with stable Foxp3 expression,²⁰ but seems to be dispensable for initiation of Foxp3 expression.²¹ Accordingly, stable thymus-derived nTreg cells display a fully demethylated TSDR, whereas the TSDR of CD4⁺ CD25⁻ non-Treg cells and *in-vitro*-induced Treg cells with unstable Foxp3 expression have been described as being heavily methylated.²²

In our previous study we observed an increase in Foxp3⁺ Treg cells during *P. yoelii* infection of BALB/c mice⁷ that interferes with an effective anti-parasitic immune response but the origin of these Treg cells is still unclear. For the development of therapeutic approaches that modulate Treg responses during malaria infection, it is critical to better understand the characteristics and origin of these immunosuppressive T cells. Here, we provide evidence that *P. yoelii* infection results in an expansion of thymus-derived nTreg cells rather than peripheral induction of Foxp3⁺ Treg cells at least at early time-points during infection.

Material and methods

Mice and parasites

Foxp3/eGFP mice (BALB/c) (Jackson Laboratories, Bar Harbor, ME), Thy1.1 BALB/c mice (kindly provided by Jochen Hühn) and BALB/c mice (Harlan Laboratories, Borcheln, Germany) were crossed and maintained under specific pathogen-free conditions at the Animal Facility of the University Hospital Essen. Cryopreserved *Plasmodium yoelii* 17XNL (non-lethal) infected red blood cells were passaged once through BALB/c mice before being used in experimental animals. For *P. yoelii* infection, mice were

injected intravenously with 1×10^5 infected red blood cells. Parasitaemia levels were determined by microscopic examination of Giemsa-stained blood films. All animal experiments were performed in strict accordance with the guidelines of the German Animal Protection Law, and were approved by the state authorities for Ethics in Animal Experiments of North-Rhine Westphalia, Germany.

Cell separation and adoptive transfer

Spleens were rinsed with erythrocyte lysis buffer and washed with PBS supplemented with 2% fetal calf serum and 2 mM EDTA. For isolation of CD4⁺ Foxp3⁻ T cells, CD4⁺ T cells were enriched from splenocytes isolated from Foxp3/eGFP mice by using the MACS CD4⁺ T-cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's recommendations, stained with fluorochrome-labelled CD4 antibody and sorted by using BD Aria II (BD Biosciences, Heidelberg, Germany). This process resulted in cells with a purity of 97–99%. For adoptive transfer experiments, 3×10^6 to 5×10^6 sorted CD4⁺ Foxp3⁺(eGFP⁺) or CD4⁺ Foxp3⁻ (eGFP⁻) T cells from Foxp3/eGFP reporter mice were injected intravenously into Thy1.1 BALB/c mice and infected with 1×10^5 infected red blood cells at the same day.

Antibodies and flow cytometry

Anti-CD4, anti-CD25 (BD Biosciences, Heidelberg, Germany), anti-T-cell receptor-V β (TCR-V β) antibodies (Biolegend, London, UK), anti-Helios, anti-CD90.2 (eBioscience, Frankfurt, Germany) and anti-Nrp-1 antibodies (R&D Systems, Abingdon, UK) were used as Pacific Blue, phycoerythrin, eFluor450, allophycocyanin or peridinin-chlorophyll protein conjugates. Dead cells were identified by staining with the fixable viability dye eFluor 780 (eBioscience). Flow cytometric expression analysis was performed with an LSR II instrument using DIVA software (BD Biosciences).

Methylation analysis

Quantification of methylation was performed as described recently.²³ Briefly, DNA was extracted from sorted T-cell subsets isolated from *P. yoelii*-infected Foxp3/eGFP male mice by using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and bisulphite DNA was generated using the BisulFlash DNA Modification kit (Epigentek, Farmingdale, NY). Quantitative real-time PCR was performed by using specific primers (5'-AAA TTT GTG GGG TAG ATT ATT TGT TTT TT-3' and 5'-ATC ACA ACC TAA ACT TAA CCA AAT TTT TCT-3'), specific VIC- or FAM-labelled TaqMan probes detecting methylated

progenitors (5'-ATT CGG TCG TTA TGA CGT T-3') or demethylated progenitors (5'-ATT TGG TTG TTA TGA TGT TAA T-3') and Roche TaqMan Probe Master 480 (Roche Diagnostics, Basel, Switzerland) on a Roche Light cycler 480 system.

Statistical analysis

Statistical analyses were performed with one-way analysis of variance and two-tailed Student's *t*-test as indicated with significance set at the levels of **P* < 0.05, ***P* < 0.01 and ****P* < 0.001. All analyses were calculated with GRAPH PAD PRISM 5.0 software (Graph Pad Software, La Jolla, CA).

Results

Increase in Foxp3⁺ Treg cells with reduced frequencies of Nrp-1 expressing cells at early time-points during *P. yoelii* infection

We made use of the well established *P. yoelii* infection model of BALB/c mice to carefully characterize the CD4⁺ Foxp3⁺ Treg cell population during *Plasmodium* infection. Flow cytometric analysis revealed higher percentages and numbers of Foxp3⁺ Treg cells within spleen of *P. yoelii*-infected mice at days 3 and 5 post-infection (p.i.), respectively (Fig. 1a). These cells exhibited an activated phenotype as determined by the expression level of CD25 (Fig. 1b). However, it is unclear whether this increase in Treg cells is caused by the expansion of thymus-derived nTreg cells or by the induction of iTreg cells within the periphery.

To gain further insights into the origin of Treg cells at early time-points during *P. yoelii* infection we analysed the expression of Helios and Nrp-1, both molecules proposed to identify nTreg cells, on Foxp3⁺ Treg cells by flow cytometry. As depicted in Fig. 1(c), percentages of Helios-expressing Foxp3⁺ Treg cells did not alter during *P. yoelii* infection, but we detected elevated numbers of Helios⁺ Foxp3⁺ Treg cells in infected mice compared with uninfected controls (Fig. 1c). In contrast, the frequency of Nrp-1 expressing Foxp3⁺ Treg cells significantly decreased in spleen from *P. yoelii*-infected mice at days 3, 5 and 7 p.i. in comparison to non-infected mice, whereas the absolute numbers slightly increased at early time-points during infection (Fig. 1c). Interestingly, analysis of Nrp-1 and Helios co-expression on Foxp3⁺ Treg cells revealed increasing percentages of Helios⁺ Nrp-1⁻ Foxp3⁺ Treg cells concomitant with decreasing frequencies of Helios⁺ Nrp-1⁺ Foxp3⁺ Treg cells in the course of infection (Fig. 1d). These results suggest that analysing Nrp-1 and Helios expression seems not to be sufficient to determine whether *P. yoelii* infection results in an expansion of thymus-derived nTreg cells or peripheral Treg induction.

Similar T-cell receptor-V β chain repertoire of Nrp-1⁺ and Nrp-1⁻ Foxp3⁺ Treg cells from *P. yoelii*-infected mice

To get more information about the origin of Foxp3⁺ Treg cells in *P. yoelii*-infected mice in particular with regard to Nrp-1 expression, we next analysed the TCR-V β chain repertoire on gated Nrp-1⁺ and Nrp-1⁻ Foxp3⁺ Treg cells in comparison with Foxp3⁻ T cells isolated from spleens of non-infected and *P. yoelii*-infected mice 3 and 7 days p.i. (Fig. 2a). Interestingly, similar to Foxp3⁺ Treg cells, the frequencies of most TCR-V β chains were unaffected upon infection in Foxp3⁻ T cells, suggesting a polyclonal T-cell response to a mitogen released by the parasite, as already proposed.²⁴ Nevertheless, at day 7 p.i. the frequency of Nrp-1⁺ Foxp3⁺ Treg cells with TCR-V β chain 8.1/8.2 usage was reduced, whereas the percentage of TCR-V β 11⁺ cells was increased (Fig. 2b). Similarly, we detected elevated levels of TCR-V β ⁺ Nrp-1⁻ Foxp3⁺ Treg cells in mice infected with *P. yoelii* for 7 days compared with non-infected mice (Fig. 2c), suggesting that both Foxp3⁺ Treg subsets originate from the same progenitor. Although the TCR-V β usage by Foxp3⁻ T cells was similar to Foxp3⁺ Treg cells, we detected lower frequencies of TCR-V β 7 and TCR-V β 9 and increased percentages of TCR-V β 6 and TCR-V β 11 expressing CD4⁺ Foxp3⁻ T cells at day 7 p.i. (Fig. 2d).

TSDR methylation analysis of Nrp-1⁺ and Nrp-1⁻ Foxp3⁺ Treg cells from *P. yoelii*-infected mice

Demethylation of CpGs within the TSDR located in the *foxp3* locus has been correlated with a long-term maintenance in Foxp3 expression, which is a specific feature of thymus-derived nTreg cells.²⁵ In contrast, some iTreg cell subpopulations might lose their Foxp3 expression upon activation and display a complete or at least intermediate TSDR methylation.²⁶ To analyse whether Nrp-1⁺ and Nrp-1⁻ Foxp3⁺ Treg subpopulations detected during *P. yoelii* infection differ in terms of stability in Foxp3 expression, we performed methylation analysis of the TSDR of sorted Nrp-1⁺ and Nrp-1⁻ Foxp3⁺ Treg cells as well as Nrp-1⁺ and Nrp-1⁻ Foxp3⁻ T cells isolated from non-infected and *P. yoelii*-infected Foxp3/eGFP reporter mice.

Within the Foxp3⁻ T-cell subset we did not observe any significant difference in the TSDR methylation status between Nrp-1⁺ Foxp3⁻ and Nrp-1⁻ Foxp3⁻ T cells isolated from *P. yoelii*-infected and non-infected mice (Fig. 3a). Nrp-1⁺ Foxp3⁺ Treg cells from non-infected, but also from *P. yoelii*-infected mice exhibited 1–5% methylation within the TSDR (Fig. 3b) suggesting that these cells stably express Foxp3, a typical feature of nTreg cells. Similar results were also described by Weiss *et al.*, who detected complete demethylation of the TSDR in

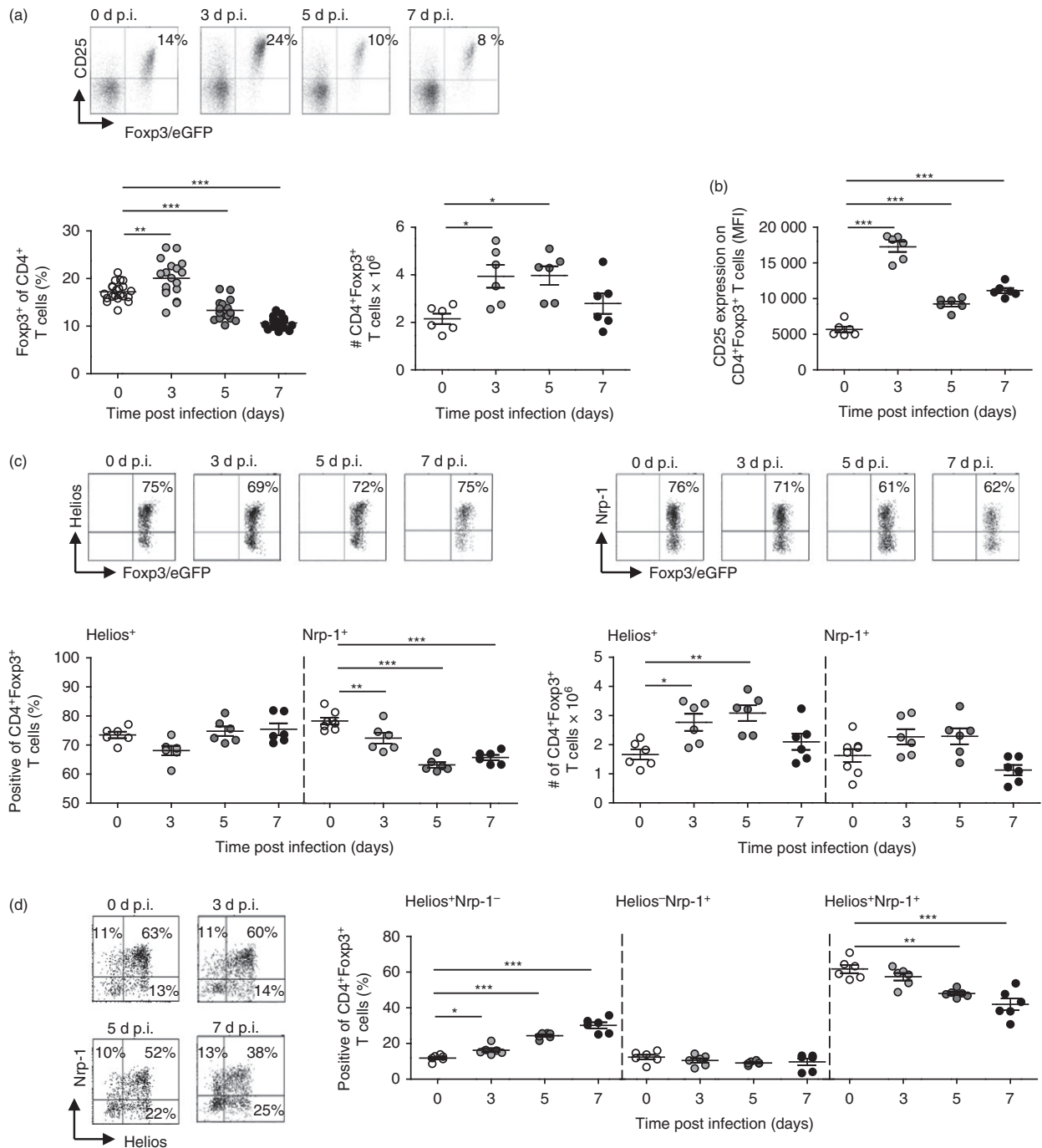


Figure 1. *Plasmodium yoelii* infection of BALB/c mice resulted in reduced percentages of neuropilin-1 (Nrp-1)-expressing Foxp3⁺ regulatory T (Treg) cells, with unaffected Helios expression. Foxp3/eGFP reporter mice were infected with 1×10^5 infected red blood cells (iRBC) intravenously. Non-infected [0 days post-infection (d p.i.)] and *P. yoelii*-infected Foxp3/eGFP mice were killed at 3, 5 and 7 d p.i. (a) The frequency (left panel) and absolute number (right panel) of Foxp3⁺ (eGFP⁺) Treg cells and (b) the mean fluorescence intensity (MFI) of CD25 expression on Foxp3⁺ Treg cells were determined on gated CD4⁺ T cells and CD4⁺ Foxp3⁺ Treg cells, respectively by flow cytometry. Representative dot plots are shown in the upper panel. (c) Helios and neuropilin-1 (Nrp-1) expression were analysed on CD4⁺ Foxp3⁺ Treg cells by flow cytometry. Representative dot plots are shown in the upper panel. Percentages and absolute numbers of Helios and Nrp-1-expressing Treg cells are summarized. (d) The frequencies of Helios⁺ Nrp-1⁻, Helios⁻ Nrp-1⁺ and Helios⁺ Nrp-1⁺ co-expressing Foxp3⁺ Treg cells were determined by flow cytometry. Representative dot plots are shown in the left panel. Results from two independent experiments with $n = 6$ mice are summarized as mean \pm SEM. Each dot represents one animal. One-way analysis of variance with Dunnett's post test was used for statistical analysis. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

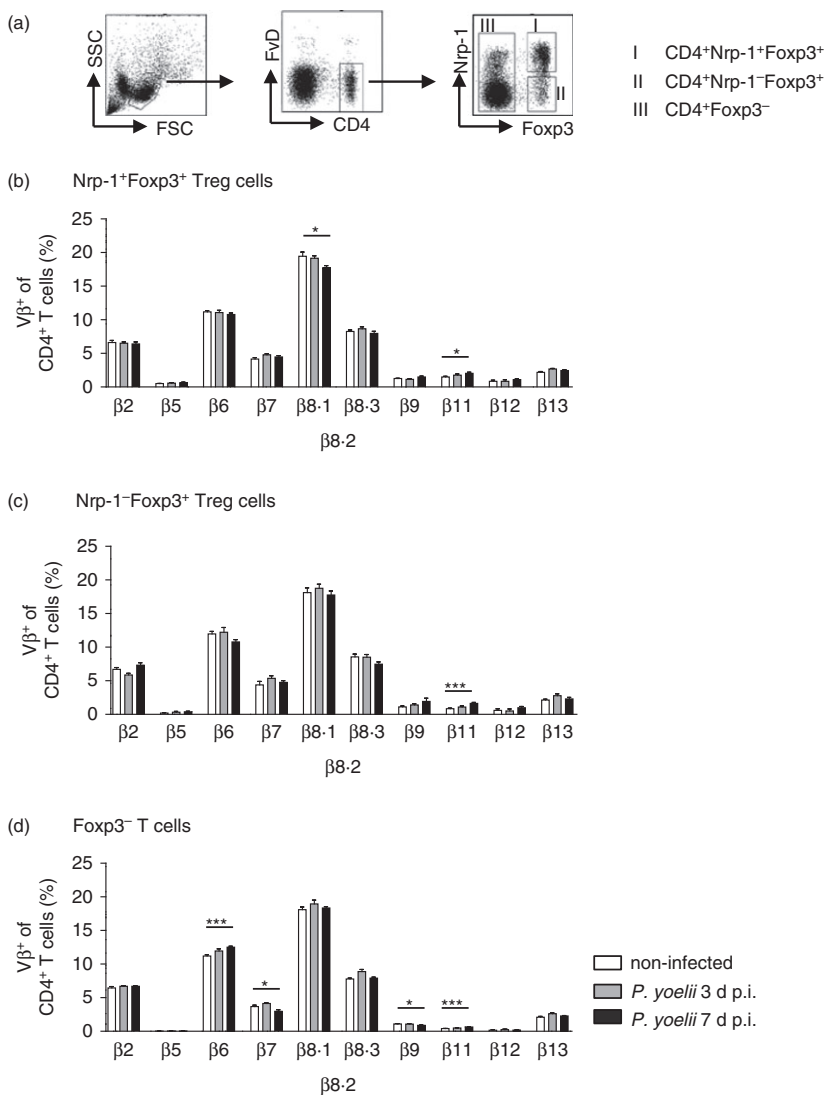


Figure 2. T-cell receptor-V β (TCR-V β) chain usage of neuropilin-1-positive (Nrp-1⁺) and Nrp-1⁻ Fcpx3⁺ regulatory T (Treg) cells at day 3 and day 7 after *Plasmodium yoelii* infection. (a) Representative dot plots illustrating the gating strategy for analysis of TCR-V β chain repertoire on different CD4⁺ T-cell subsets by flow cytometry. Percentages of (b) Nrp-1⁺ Fcpx3⁺ Treg cells, (c) Nrp-1⁻ Fcpx3⁺ Treg cells and (d) Fcpx3⁻ T cells using TCR-V β 2, TCR-V β 5, TCR-V β 6, TCR-V β 8.1/8.2, TCR-V β 8.3, TCR-V β 9, TCR-V β 11, TCR-V β 12 and TCR-V β 13 isolated from spleen of non-infected [0 days post-infection (d p.i.), white bars] and *P. yoelii*-infected Fcpx3/eGFP mice 3 days (grey bars) and 7 days p.i. (black bars). Results from two or three independent experiments with $n = 6$ to $n = 10$ mice in total are summarized as mean \pm SEM. One-way analysis of variance with Dunnett's post test was used for statistical analysis. * $P < 0.05$, *** $P < 0.001$.

Nrp-1⁺ Fcpx3⁺ Treg cells from WT mice.¹⁷ Importantly, the majority (> 90%) of Nrp-1⁻ Fcpx3⁺ Treg cells isolated from *P. yoelii*-infected mice also showed TSDR demethylation (Fig. 3b), indicating stable Fcpx3 expression in most of these cells.

Adoptively transferred Fcpx3⁻ T cells did not acquire Fcpx3 expression upon *P. yoelii* infection

Results from our Helios expression analysis, TCR-V β chain usage and TSDR methylation analysis suggest that *P. yoelii* infection resulted in an expansion of nTreg cells with stable Fcpx3 expression rather than induction of Fcpx3⁺ Treg cells from Fcpx3⁻ precursors within the periphery. To further corroborate these findings we adoptively transferred sorted Thy1.2⁺ CD4⁺ Fcpx3⁻ T cells or Thy1.2⁺ CD4⁺ Fcpx3⁺ T cells into Thy1.1⁺ BALB/c mice before *P. yoelii* infection (Fig. 4a). At days 3 and 5 p.i. Fcpx3/eGFP expression was analysed in Thy1.2⁺

splenocytes by flow cytometry. As depicted in Fig. 4 we did not detect induction of Fcpx3 expression in adoptively transferred Fcpx3⁻ T cells upon *P. yoelii* infection. Only 1% of transferred Thy1.2⁺ CD4⁺ T cells expressed Fcpx3, whereas 16–19% of endogenous Thy1.1⁺ CD4⁺ T cells in non-infected and *P. yoelii*-infected mice exhibited Fcpx3 expression, respectively (Fig. 4b,c). These results indicate that *P. yoelii* infection of BALB/c mice was not sufficient to confer Fcpx3 expression to peripheral CD4⁺ Fcpx3⁻ T cells.

Discussion

In our previous study, we demonstrated that *P. yoelii* infection of BALB/c mice resulted in a significant increase in Treg cell numbers in the spleens of infected mice interfering with effective clearance of the parasite.⁷ Hence, Treg cells might represent a promising target to modulate the anti-parasitic immune response for the development

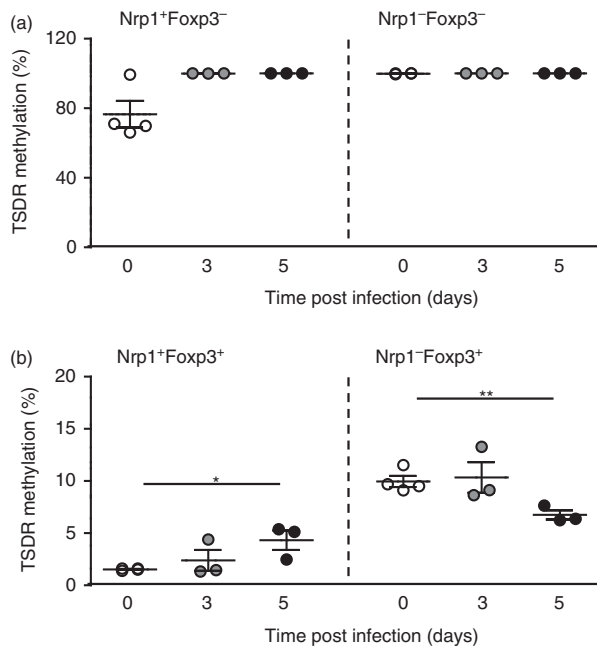


Figure 3. The majority of neuropilin-1-positive (Nrp-1⁺) and Nrp-1⁻ Foxp3⁺ regulatory T (Treg) cells from *Plasmodium yoelii*-infected mice are highly demethylated in their Treg-specific demethylation region (TSDR). (a) Nrp-1⁺ Foxp3⁻ and Nrp-1⁻ Foxp3⁻ T cells as well as (b) Nrp-1⁺ Foxp3⁺ and Nrp-1⁻ Foxp3⁺ Treg cells were isolated from spleen of non-infected Foxp3/eGFP reporter mice and *P. yoelii*-infected Foxp3/eGFP mice at days 3 and 5 post-infection (d p.i.). DNA was isolated and bisulphate treated before methylation analysis by real-time PCR. Results from three or four independent experiments with $n = 4$ to $n = 6$ mice each were summarized as mean \pm SEM. One-way analysis of variance with Dunnett's post test was used for statistical analysis. * $P < 0.05$, ** $P < 0.01$.

of immune-based therapeutics and improvement of vaccination strategies that target the blood stage of *Plasmodium* infection. However, for this purpose careful phenotypic characterization of these Treg cells is necessary. Here, we provide evidence that *P. yoelii* infection of BALB/c mice triggers expansion of thymus-derived Foxp3⁺ nTreg cells rather than *de novo* induction in the periphery.

Both Helios and Neuropilin-1 have been proposed as reliable marker molecules to discriminate nTreg cells from iTreg cells.^{14,17,18} Interestingly, the frequency of Nrp-1 expressing Foxp3⁺ Treg cells declined in *P. yoelii*-infected mice whereas the percentage of Helios⁺ Foxp3⁺ Treg cells remained unchanged, suggesting that at least one of these molecules is not suitable for identifying nTreg cells during *Plasmodium* infection.

Helios expression was originally identified to be restricted to Foxp3⁺ nTreg cells as the majority of Foxp3⁺ Treg cells in naive mice expresses the transcription factor Helios.¹⁴ However, contradictory results have been described for the expression of Helios in *in vivo* induced

Treg cells. Whereas Foxp3⁺ Treg cells induced by administration of antigen through the oral route exhibited no Helios expression,¹⁴ intravenous injection of low-dose antigen resulted in elevated Helios expression in Foxp3⁺ iTreg cells¹⁶ suggesting that the use of Helios as a specific marker for nTreg cells seems to be dependent on the *in vivo* environment.

Interestingly, we detected significantly lower percentages of Nrp-1-expressing Foxp3⁺ Treg cells in *P. yoelii*-infected mice. This might suggest that increased Foxp3⁺ Treg cells represent induced Treg cells because Nrp-1 was described as highly expressed by thymus-derived nTreg cells.^{17,18} However, similar to Helios, the usage of Nrp-1 as an nTreg marker seems to be dependent on the *in vivo* environment. Weiss *et al.* detected elevated Nrp-1 expression on Foxp3⁺ iTreg cells under inflammatory conditions¹⁷ and, most recently Petzold and colleagues described an up-regulation of Nrp-1 on Nrp-1⁻ iTreg progenitors upon TCR engagement in the presence of interleukin-2.²⁷ Hence, the reliability of Nrp-1 and Helios as marker molecules to discriminate nTreg cells from iTreg cells is still discussed controversially and seems to be dependent on the site and mode of Treg cell induction. Moreover it is unclear, whether these marker molecules define specific subpopulations of Foxp3⁺ Treg cells exhibiting different functions or whether the expression of Helios and Nrp-1 on Foxp3⁺ Treg cells is regulated based on their activation and/ or maturation. This issue has to be clarified in future experiments in the context of different types of immune responses. Interestingly, we detected elevated percentages of Helios⁺ Nrp-1⁻ Foxp3⁺ Treg cells in the course of infection in contrast to decreasing frequencies of Helios⁺ Nrp-1⁺ Foxp3⁺ Treg cells. This observation might reflect either down-regulation of Nrp-1 on Helios⁺ Foxp3⁺ Treg cells or expansion of Helios⁺ Nrp-1⁻ Foxp3⁺ Treg cells. Since Helios expression was also correlated with the activation status of CD4⁺ T cells independent of Foxp3 expression,¹⁵ the significant increase in the number of Helios-expressing cells within Foxp3⁺ Treg cells in *P. yoelii*-infected mice, might reflect the activated phenotype of Treg cells at early time-points during *P. yoelii* infection underpinned by an up-regulation of CD25 expression on Foxp3⁺ Treg cells upon *P. yoelii* infection. We also observed an up-regulation of Helios expression on Foxp3⁻ T cells in the course of *P. yoelii* infection (data not shown).

From our TCR-V β chain usage analysis one might speculate about the same origin of both Nrp-1⁺ and Nrp-1⁻ Treg cells because we detected an increase in TCR-V β 11 usage in both populations upon *P. yoelii* infection. Within the Foxp3⁻ T-cell population we also observed elevated percentages of TCR-V β 11⁺ cells in *P. yoelii*-infected mice, but additionally decreased frequencies of TCR-V β 7 and TCR-V β 9 expressing cells. Preferential deletion of T cells expressing TCR-V β 9 was already

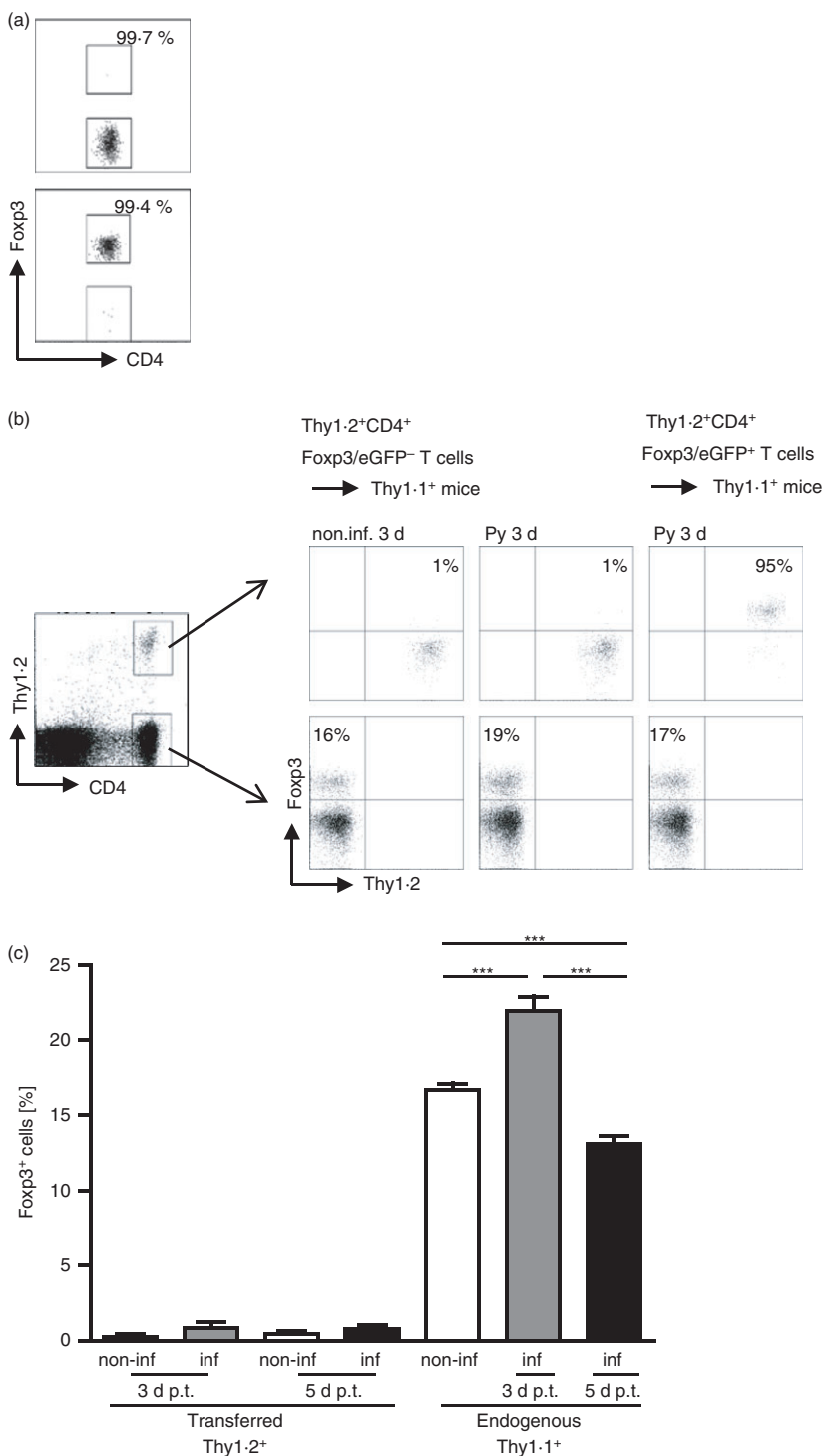


Figure 4. Adoptively transferred CD4⁺ Foxp3⁻ T cells do not acquire Foxp3 expression in *Plasmodium yoelii*-infected mice. From 3×10^6 to 5×10^6 sorted Thy1.2⁺ CD4⁺ Foxp3⁻ T cells or Thy1.2⁺ CD4⁺ Foxp3⁺ T cells from naive Foxp3/eGFP mice were injected intravenously into Thy1.1⁺ BALB/c mice before infection with 1×10^5 infected red blood cells (iRBC) or in naive, non-infected mice. At 3 and 5 days post-infection (d p.i.) mice were killed and Foxp3 expression was analysed in gated CD4⁺ Thy1.2⁺ and CD4⁺ Thy1.1⁺ T cells, respectively by flow cytometry. (a) Purity of sorted CD4⁺ Foxp3/eGFP⁻ T cells and CD4⁺ Foxp3/eGFP⁺ T cells is shown in representative dot plots. (b) Representative dot plots for either non-infected or *P. yoelii*-infected Thy1.1 mice receiving either Thy1.2⁺ CD4⁺ Foxp3⁻ T cells or Thy1.2⁺ CD4⁺ Foxp3⁺ T cells 3 d p.i. / cell transfer. (c) Data from two or three independent experiments with $n = 4$ to $n = 11$ mice in total are summarized as mean \pm SEM. Student's *t*-test was used for statistical analysis. *** $P < 0.001$.

described in *P. yoelii*-infected C57BL/6 mice and proposed to be a result of super-antigenic activity during acute infection.²⁸ However, Swardson and colleagues observed no differences in the course of *P. yoelii* infection between BALB/c mice and BALB/c.D2 mice, which express a super-antigen encoded by an endogenous retroviral gene resulting in deletion of TCR-V β 9⁺ T cells.²⁹ Moreover, TCR-V β repertoire studies in African children

suffering from malaria argue against the idea of dominant super-antigenic activities.³⁰ Therefore, further studies with regard to the TCR-V β profile and also TCR-V α chain usage by T cells during *Plasmodium* infection would be helpful to better understand elicitation and regulation of parasite-specific adaptive immune response.

Analysis of TSDR methylation of Nrp-1⁺ and Nrp-1⁻ Foxp3⁺ Treg cells as well as Nrp-1⁺ and Nrp-1⁻ Foxp3⁺

T cells from *P. yoelii*-infected mice revealed an almost complete TSDR demethylation in Foxp3⁺ Treg cells independent of Nr1p-1 expression, whereas Foxp3⁻ T cells exhibited 100% TSDR methylation upon *P. yoelii* infection. Interestingly, at day 5 p.i. the methylation status of the TSDR in Nr1p-1⁻ Foxp3⁺ Treg cells significantly decreased, which might suggest that stable Foxp3-expressing nTreg cells lose their Nr1p-1 expression in the course of infection. This would argue for an expansion of thymus-derived nTreg cells upon *P. yoelii* infection in line with our TCR-V β usage analysis. However, as we have not analysed all TCR-V β chains, we could not exclude that T-cell clones with distinct specificities are also affected during *P. yoelii* infection.

We showed that the level of CD4⁺ Foxp3⁺ Treg cells was elevated post *P. yoelii* infection, whereas the expression of Foxp3 was not induced in adoptively transferred Foxp3⁻ T cells in mice previously infected with *P. yoelii*. These results argue against *de novo* induction of Foxp3⁺ Treg cells during *P. yoelii* infection. However, we and others detected adaptive interleukin-10 producing CD4⁺ Foxp3⁻ T cells that were generated during *P. yoelii* infection.^{7,31} These cells exhibited immunosuppressive function *in vitro* and T-cell-specific interleukin-10 deletion resulted in enhanced T-cell activation in *P. yoelii*-infected mice.⁷ Hence, our data provide evidence that *P. yoelii* infection of BALB/c mice triggers induction of interleukin-10-producing Foxp3⁻ Treg cells and expansion of pre-existing Foxp3⁺ thymus-derived nTreg cells. Similar results were also described for viral infections. Foxp3⁻ T cells did not acquire Foxp3 expression upon adoptive transfer to Friend virus-infected³² or lymphocytic choriomeningitis virus-infected mice,³³ suggesting expansion of nTreg cells rather than conversion of non-Treg cells into Foxp3⁺ iTreg cells during these viral infections.

With regard to future therapeutic interventions one might think of blocking molecules highly expressed by nTreg cells using small interfering RNA approaches or application of specific antibodies. Since we observed elevated frequencies of Helios-expressing Foxp3⁺ Treg cells and Helios⁺ Foxp3⁺ Treg cells have been described to have a high suppressive activity,³⁴ probably due to their more activated phenotype, Helios might be an attractive target to interfere with Foxp3⁺ Treg cell function during *Plasmodium* infection. However, one has to keep in mind that Helios is also up-regulated on Foxp3⁻ T cells during infection and modulating the intrinsic suppressive function of Foxp3⁺ Treg cells might also result in immunopathological side effects. Therefore, approaches to prevent expansion of nTreg cells would be a more reasonable therapeutic strategy. Our findings should foster further experiments to identify specific factors that support nTreg expansion in *Plasmodium* infection because they represent promising targets for therapeutic regulation of anti-parasitic immune responses.

Acknowledgements

We are thankful to Carolin Wevers and Sina Luppus for excellent technical assistance and Patrick Juszczak and Witold Bartosik for cell sorting. This work was supported by Deutsche Forschungsgemeinschaft (GRK1949) to W.H.

Disclosure

The authors declare no conflict of interest.

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