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Incomplete depletion and rapid regeneration of Foxp3+ regulatory T cells following anti-CD25 treatment in malaria-infected mice

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Abstract

Investigation of the role of regulatory T cells (Treg) in model systems is facilitated by their depletion using anti-CD25 antibodies, but there has been considerable debate about the effectiveness of this strategy. Here, we have compared the depletion and repopulation of CD4⁺CD25⁺Foxp3⁺ Treg in uninfected and malaria-infected mice using 7D4 and/or PC61 anti-CD25 antibodies. We find that numbers and percentages of CD25^{hi} cells, but not Foxp3⁺ cells, are transiently reduced after 7D4 treatment whereas treatment with PC61 - alone or in combination with 7D4 (7D4+PC61) - reduces, but does not eliminate, Foxp3⁺ cells for up to two weeks. Importantly, all protocols fail to eliminate significant populations of CD25⁻Foxp3⁺ or CD25^{low}Foxp3⁺ cells, which retain potent regulatory capacity. By adoptive transfer we show that repopulation of the spleen by CD25^{hi}Foxp3⁺ cells results from re-expression of CD25 on peripheral populations of CD25⁻Foxp3⁺ but not from conversion of peripheral Foxp3⁻ cells. CD25^{hi}Foxp3⁺ repopulation occurs more rapidly in 7D4-treated mice than in 7D4+PC61-treated mice, reflecting ongoing clearance of emergent CD25⁺Foxp3⁺ cells by persistent PC61 antibody. However, in 7D4+PC61-treated mice undergoing acute malaria infection, repopulation of the spleen by CD25⁺Foxp3⁺ cells occurs extremely rapidly, with malaria infection driving proliferation and CD25 expression in peripheral CD4⁺CD25⁻Foxp3⁺ cells and/or conversion of CD4⁺CD25⁻Foxp3⁻ cells. Finally, we reveal an essential role for IL-2 for re-expression of CD25 by Foxp3⁺ cells after anti-CD25 treatment and observe that TGF- β is required - in the absence of CD25 and IL-2 - to maintain splenic Foxp3⁺ cell numbers and a normal ratio of Treg:non-Treg cells.

Keywords

Rodent; T cells; parasitic protozoan; spleen and lymph nodes; immune regulation

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Introduction

Regulatory T cells (Treg) play an essential role in controlling immune responses during autoimmune and infectious diseases. Treg are characterised by their surface expression of CD4 and high levels of the high affinity IL-2 receptor α chain (CD25^{hi}) in conjunction with CTLA-4, CD45RB, and/or GITR (Reviewed 1-3). However these markers are also expressed on activated and effector CD4 T cells. The forkhead family transcription factor, Foxp3, is a lineage specific differentiation factor that is intrinsically linked to the regulatory capacity of natural Treg (4-8). Foxp3⁺ cells are principally found within the CD4⁺CD25⁺ population but the association is not absolute (5, 9); CD4⁺CD25⁻Foxp3⁺ cells are found in significant numbers in normal mice and have been reported to be functionally suppressive (5, 9), which is in agreement with previous studies demonstrating regulatory properties of CD4⁺CD25⁻ T cells (10-12).

CD4⁺CD25⁺Foxp3⁺ Treg originate from the thymus (natural or endogenous Treg) in response to CD28 signalling (3, 13) or can be generated *in vitro* and in the periphery from CD4⁺CD25⁻ cells which encounter antigen in the context of TGF- β (14-22) and/or CTLA-4 (23). These peripherally generated Treg display similar regulatory and suppressive characteristics to natural Treg and are able, for example, to suppress autoimmune disease, graft-versus-host disease and allergic lung responses via TGF- β and cell contact-dependent mechanisms (15, 19, 24). IL-2 is also critically required for the maintenance of Treg and has been postulated to be important for their generation in the periphery (3, 25, 26). Homeostasis may be a powerful trigger for Treg differentiation in that peripheral Treg also develop in response to lymphopenia (27) and the number of Treg appears to be regulated by the number of IL-2 producing cells (28). However, Treg also differentiate in response to inflammation, whether auto-immune or infectious in origin (29-39) and may be generated as part of a normal immune response following antigen presentation by mature dendritic cells (40-42). Whether Treg differentiation in these conditions is primarily a result of an increased T effector cell to Treg ratio, due to expansion of the effector T cell population, or is a response to specific inflammatory stimuli, is unclear and detailed studies of the kinetics and function of effector and regulatory T cell populations in different disease settings are required to elucidate this.

Since Foxp3 is not expressed at the cell surface, until recently the only way to deplete Treg was to administer anti-CD25 antibodies. However, different depletion strategies appear to be more or less effective at depleting Foxp3⁺ cells and, as recently documented, the effects of anti-CD25 treatment can be misleading since, despite the apparent depletion of CD25^{hi} cells, significant numbers of Foxp3⁺ cells remain (44-48). Furthermore, there is an ongoing controversy regarding the extent to which anti-CD25 treatment abrogates Treg activity (47, 48).

Here we have compared three different protocols for depletion of CD25⁺ cells and find that a combination of IgM (7D4) and IgG (PC61) antibodies leads to rapid and sustained abrogation of CD25 expression but only up to 40% reduction in numbers of splenic CD4⁺Foxp3⁺ cells. We also find that splenic Treg repopulation occurs principally from peripheral CD4⁺ cells, rather than from thymic emigrants, and results from both differentiation of CD25⁻ cells and re-expression of CD25 on Foxp3⁺ cells that transiently down-regulated CD25 in the presence of anti-CD25 antibody. Interestingly, the effective duration of Treg depletion following administration of anti-CD25 antibodies was very much reduced during malaria infection. This suggests that the utility of anti-CD25 depletion regimes is determined by the extent of subsequent effector and regulatory T cell activation, and, moreover, that inflammation may be a more powerful signal than a disturbed Treg:non-

Treg ratio for inducing differentiation of Treg. Finally we demonstrate that the regeneration of CD25⁺Foxp3⁺ cells in the periphery is not dependent upon TGF- β signalling.

Materials and Methods

Mice and parasites

C57BL/6 (Ly5.2) and C57BL/6 (Ly5.1) mice were bred in house or purchased from Harlan (Oxford, UK) and used at 7-9 wks of age. C57BL/6 Foxp3-GFP knockin mice (49) were bred in house at the National Institutes of Health (NIH), Bethesda, USA. Cryopreserved *Plasmodium yoelii* 17X (non-lethal) parasites were thawed and passaged once *in vivo* before being used to infect experimental animals. All infections were initiated by intravenous injection of 1×10^4 parasitised red blood cells and parasitemia was monitored daily by examination of Giemsa-stained thin smears of tail blood.

To deplete CD25⁺ cells, mice were given a single intraperitoneal (i.p.) injection of either 7D4 (0.75mg; Rat IgM, Bioexpress Inc, USA), PC61 (0.75mg; Rat IgG1, Bioexpress Inc, USA) or 7D4 plus PC61 (0.25mg 7D4 plus 0.75 mg PC61) on day 0. To investigate the importance of TGF- β and IL-2 in driving the repopulation of CD25⁺Foxp3⁺ cells, 7D4 (0.5mg) was administered on day 0 together with anti-TGF- β (0.25mg; 1D11, Mouse IgG1, Bioexpress Inc, USA) on days 0,1,2,3,4,6,7 and 8 or with anti-IL-2 (0.5mg; JES6-5H4, Rat IgG2b, Bioexpress Inc, USA) on days 0,3, 6 and 8. When anti-CD25 depletion was combined with malaria infection, a single dose of either 7D4 (0.75mg) or 7D4+PC61 (0.25mg+0.75mg) was administered i.p. on day 0 relative to *P. yoelii* infection.

Flow cytometry

The extent of Treg cell depletion and the dynamics of Treg repopulation were evaluated by flow cytometric analysis of splenic mononuclear cells; cells were permeabilised with 0.1% Saponin in PBS prior to intracellular staining for Foxp3. Antibodies used were anti-CD4-PERCP (RM4-5; IgG2a; BD Pharmingen), anti-Foxp3-APC or FITC (FJK-16s; IgG2a; Insight Biotechnology Ltd, UK), anti-CD25 (PC61)-APC (IgG1; Insight Biotechnology Ltd) and streptavidin conjugated anti-CD25 (7D4;IgM; Insight Biotechnology Ltd), together with biotin-PE. Isotype control antibodies were: Rat IgG2a (clone R35-95), Rat IgG1 (clone A110-1) and Rat IgM (clone R4-22). Flow cytometric acquisition was performed using a BD FACSCalibur (Becton Dickinson) and analysed with Flowjo (Tree Star, Inc, USA) software.

Purification of CD4⁺ CD25⁻ cells

Splenic Ly5.1⁺CD4⁺ cells were enriched by MACS (Miltenyi Biotec. Inc, USA) positive selection using anti-CD4-conjugated midiMACS beads, according to the manufacturer's instructions. For adoptive transfer experiments, 5×10^6 CD4⁺ lymphocytes were transferred to individual congenic Ly5.2⁺ recipient mice. In some experiments the selected CD4⁺ cells were stained with anti-CD4 and anti-CD25 fluorochrome labelled antibodies and CD4⁺CD25⁻ cells were sorted by flow cytometry using a BD FACSvantage (Becton Dickinson). Cells were checked for purity using a FACSCalibur and were routinely found to be >99% CD4⁺CD25⁻. For use in adoptive transfer experiments 2×10^6 CD4⁺CD25⁻ cells were then adoptively transferred to congenic Ly5.2⁺ recipient mice.

In vitro suppression assay

Using CD4⁺ T cell midiMACS isolation kits (Miltenyi Biotec. Inc), CD4⁺ T cells were negatively selected from spleens of Foxp3-GFP knockin mice which had been treated 3 days previously with 0.75 mg 7D4, 0.25 7D4 plus 0.75mg PC61 or PBS. The selected cells were stained with fluorochrome labelled anti-CD4 antibody and CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻

cells were sorted by flow cytometry using a BD FACS Aria (Becton Dickinson). Sorted cell populations were routinely >99% pure. Separately, splenocytes from C57BL/6 mice were depleted of T cells using anti-Thy1.2 midiMACS beads (Miltenyi Biotec, Inc) and irradiated at 8000Rads for use as antigen presenting cells. CD4⁺Foxp3⁻ cells (50,000/well) were cultured with antigen presenting cells (100,000/well) and anti-CD3 (BD Biosciences 0.5µg/ml) in the presence of varying numbers of CD4⁺Foxp3⁺ cells derived from either anti-CD25 antibody treated or control (PBS treated) mice. The cells were incubated at 5% CO₂ 37°C for 50 hours before the addition of methyl-³H-thymidine (MP Biomedicals, Solon, OH, USA) at 1.0 µCi/well for a further 15hrs. The cells were then harvested and proliferation was determined by measuring thymidine incorporation by liquid scintillation counting.

Statistical analysis

Statistical significance was determined using Student's t test for paired or unpaired data, as appropriate.

Results

An effective strategy for long term depletion of CD4⁺CD25^{hi} cells

Depletion of CD25⁺ cells in mice has typically been accomplished using either the IgM anti-CD25 antibody 7D4 (50) or the IgG1 anti-CD25 antibody PC61 (51,52). To optimize a depletion regime for CD25⁺ cells we directly compared the efficacy of the two antibodies by administering a single dose of antibody intraperitoneally on day 0 and assessing the proportions and absolute numbers of CD4⁺CD25⁺ splenocytes over a period of 13 days. We also tested the effects of combining the two antibodies to determine if this would improve either the rate or the duration of depletion of CD25⁺ cells.

Figure 1a shows a representative example of the flow cytometric analysis of CD25 expression on untreated splenic lymphocytes or on splenic lymphocytes 3 days after treatment with 7D4 alone, PC61 alone or after combined treatment with 7D4 and PC61. Since unlabelled anti-CD25 antibody used for cell depletion might prevent binding of the fluorochrome-labelled antibody used for detection - leading to overestimation of the efficiency of the depletion strategy - we compared the numbers of remaining CD25⁺ cells using either PC61 (upper row of plots) or 7D4 (lower row of plots) as the detecting antibody. Preliminary *in vitro* experiments confirmed that unlabelled 7D4 did not interfere with binding of labelled PC61 and vice versa (data not shown).

Treatment with 7D4 alone led to a marked reduction in the proportion of CD25^{hi} cells but a sizeable population of CD25^{low} cells remained; this was apparent whether CD25⁺ cells were detected with PC61 or 7D4. By comparison, treatment with PC61 alone or combined treatment with 7D4 and PC61 and subsequent staining with labelled PC61 led to an apparently much more effective removal of CD25⁺ cells. However, when the cells were analysed using labelled 7D4, a significant population of CD25^{low} cells was detected in animals treated either with PC61 alone or with 7D4+PC61. Thus, treatment with either 7D4, PC61 or 7D4+PC61 effectively reduced the proportion of CD25^{hi} cells within 3 days but all three treatments were less effective at removing CD25^{low} cells. Moreover, the efficacy of depletion was overestimated when PC61 antibody was used for both depletion and detection, presumably due to masking of surface expressed CD25 by persisting PC61 antibody. On the contrary, masking did not appear to be a problem when labelled 7D4 antibody was used for CD25 detection; 3 days after 7D4 treatment similar numbers of CD25⁺ cells were detected using either labelled PC61 or labelled 7D4 (data not shown but provided for review). Thus, for future experiments labelled 7D4 was used to monitor the kinetics of the CD25⁺ cell population following PC61 or 7D4+PC61 treatment.

Treatment with 7D4 led to a rapid but transient decrease in the percentage of CD25⁺ cells, with the proportion of CD25⁺ cells being minimal 3 days after treatment, but increasing again very rapidly from day 7 onwards (Fig 1b). In contrast, treatment with PC61 alone led to a slower decline in the frequency of CD25⁺ cells, but CD25⁺ cell numbers remained low for at least 13 days. Treatment with 7D4 plus PC61 was more effective than treatment with either 7D4 or PC61 alone, and led to a rapid and sustained reduction in the frequency of CD25⁺ cells (Figure 1b).

Three days after treatment with 7D4, PC61 or 7D4 plus PC61, all remaining CD25⁺ cells expressed low levels of CD25 (Figure 1c). In animals treated with 7D4 alone, repopulation by CD25⁺ cells was associated with gradual up regulation of CD25 with cells initially expressing intermediate levels of CD25, but by day 13 approx 20% of the CD25⁺ cells were CD25^{hi}. In contrast, in mice treated with PC61 or 7D4+PC61, the few returning CD25⁺ cells expressed only low or intermediate levels of CD25 for the duration of the experiment.

Depletion of CD4⁺CD25^{hi} cells reduces the numbers of, but fails to eliminate, CD4⁺Foxp3⁺ cells

Although antibodies to CD25 have routinely been used to deplete Treg, CD25 is an imprecise marker for regulatory T cells. Therefore, we evaluated the effectiveness of anti-CD25 administration in depletion of Treg by determining the numbers of cells expressing the Treg-specific transcription factor Foxp3 at several time points after anti-CD25 treatment. Figure 2a shows representative examples of the intracellular Foxp3 staining profiles 3 days after treatment with 7D4 alone, PC61 alone or 7D4+PC61.

In mice treated with 7D4 alone there was a slight (albeit statistically significant, $P < 0.05$) reduction in the percentage of CD4⁺ Foxp3⁺ cells which was maintained for at least 13 days (Figure 2b). Treatment with 7D4+PC61 resulted in much more effective depletion of Foxp3⁺ cells, with an approximate 40% reduction in both the frequency (Figure 2b) and absolute number (data not shown) of Foxp3⁺ splenocytes by day 3 post-treatment; again this was highly reproducible and was maintained for up to 13 days. Treatment with PC61 alone appeared to be most effective at depleting Foxp3⁺ cells, with an approximate 60% reduction in Foxp3⁺ cells by day 7 post infection. However, PC61 acted more slowly than 7D4+PC61 and was less effective at depleting Foxp3⁺ cells in the first week post-treatment and the advantage of PC61 over 7D4+PC61 was lost by 13 days post-treatment. In accordance with the data shown in Figure 1, the majority of the remaining Foxp3⁺ cells were initially CD25⁻ or CD25^{low} (Figure 2c). However, in 7D4-treated mice, but not PC61 treated mice or 7D4+PC61-treated mice, 75 % of Foxp3⁺ cells had re-expressed intermediate or high levels of CD25 by day 13 (Figure 2d). Thus, in agreement with recently published data (44, 46), we found that treatment of mice with 7D4 alone leads to a short term reduction in the numbers of CD25^{hi} cells but has minimal impact on numbers of splenic Foxp3⁺ cells. Furthermore, although PC61 is eventually as effective as 7D4+PC61 at depleting Foxp3⁺ cells, it is rather slow acting. Thus, we show for the first time, that a rapid and long-term reduction in numbers of CD25⁺ cells, combined with rapid and sustained depletion of Foxp3⁺ cells, can be achieved by administration of 7D4 combined with PC61. We are confident that the more rapid decline in CD25⁺ and Foxp3⁺ cells in animals treated with 7D4+PC61 is due to a synergistic effect of the two antibodies - rather than simply an effect of higher total dose of anti-CD25 antibody - since dose-response experiments show that the total dose of antibody affects only the duration of the effect not the rate of onset (data not shown but provided for review). It is important to note however that at no time were Foxp3⁺ cells completely eliminated by any of the treatment protocols.

Splenic repopulation by CD4⁺CD25⁺Foxp3⁺ T cells after anti-CD25 treatment occurs from a peripheral population of mature CD4⁺ T cells

The data presented thus far indicate that anti-CD25 treatment depletes a proportion of Foxp3⁺ Treg and causes CD25 expression to be down-regulated on any Foxp3⁺ cells that remain. The subsequent reappearance of CD4⁺CD25⁺Foxp3⁺ cells in 7D4 treated mice might thus be due to repopulation by newly generated thymic Treg, *de novo* generation of CD25⁺Foxp3⁺ cells in the periphery or re-expression of CD25 on the persisting Foxp3⁺ population. To determine which of these mechanisms might account for Treg repopulation, Ly5.1⁺ CD4⁺ T cells were purified and adoptively transferred into congenic Ly5.2⁺ C57BL/6 mice and then CD25⁺ cells were transiently depleted in the recipient mice with a single dose of 7D4 antibody. 7D4 treatment was chosen for this study as its effects are rapid and short-lived, reducing the potential for residual antibody to complicate the assessment of repopulation. The gating strategy is shown in Fig 3a. Approx 5% of the Ly5.1⁺ CD4 cells were Foxp3⁺ both before (left plot) and immediately after (right plot) transfer.

Percentages of CD4⁺CD25 (PC61)⁺ and CD4⁺Foxp3⁺ spleen cells were determined prior to 7D4 treatment and 4 and 12 days after treatment (Figure 3b). In agreement with the data shown above, 7D4 treatment had little effect on the percentage of Foxp3⁺ cells (middle plot), but significantly reduced the percentage of both endogenous (Ly5.2⁺) and donor (Ly5.1⁺) CD4⁺CD25⁺ splenocytes on day 4 (left plot). Consequently, 4 days after 7D4 treatment the percentage of Foxp3⁺ which co-expressed CD25⁺ was markedly reduced in both the donor Ly5.1⁺ and host Ly5.2⁺ populations (right plot). However, by 12 days post treatment, the majority of both Ly5.1⁺ and Ly5.2⁺ Foxp3⁺ cells were CD25⁺ and these cells now accounted for the same proportion of the CD4⁺ T cell population as in untreated mice. Thus, it appears that the majority of the Foxp3⁺CD25⁺ cells that repopulate the spleen after anti-CD25 treatment are derived from a peripheral pool of mature CD4 cells rather than from recent thymic emigrants.

To determine whether the CD4⁺CD25⁺Foxp3⁺ T cells which repopulate the spleen after anti-CD25 treatment are derived from mature Treg that have simply transiently down-regulated surface expression of CD25 or whether they derive from a previously CD25⁻ negative population, CD4⁺CD25⁻ splenocytes were purified from Ly5.1⁺ mice and transferred into congenic Ly5.2⁺ mice which were treated, 12 hours later, with either 7D4 or PBS (as a control). The purity of the sorted population is shown in Figure 3c; less than 1% of the sorted Ly5.1⁺ cells expressed CD25 and approx 3.5% expressed Foxp3. The adoptive transfer process itself led either to modest up regulation of CD25 or to preferential survival of CD25⁺ cells since four days after adoptive transfer approx 5% of the transferred cells were both Foxp3⁺ and CD25⁺ (Figure 3d, PBS control group). However, 7D4 treatment of the recipient mice did not lead to up regulation of Foxp3 in the donor cells. Thus, these data suggest that reappearance of CD4⁺CD25⁺Foxp3⁺ cells in 7D4 treated mice is not due to *de novo* generation of CD25⁺Foxp3⁺ cells from peripheral CD25⁻Foxp3⁻ cells.

CD4⁺CD25⁺Foxp3⁺ cells transiently down-regulate, and then re-express, CD25 following anti-CD25 antibody treatment

To determine whether cells which down-regulate CD25 after anti-CD25 treatment can subsequently re-express CD25, we treated Ly5.1⁺ mice with 7D4 (or PBS as a control) and 3 days later transferred purified CD4⁺CD25⁻ cells to recipient Ly5.2⁺ mice. As expected, the CD25⁻ cells purified from mice treated 3 days earlier with 7D4 contained significantly more Foxp3⁺ cells (approx 8%) compared with CD25⁻ cells purified from PBS control treated mice (approx 3%) (Figure 3e). Five days after transfer, the frequencies of transferred Ly5.1⁺ cells that were Foxp3⁺ or CD25⁺ were significantly higher in cells derived from 7D4-treated mice than in cells derived from control mice (Figure 3f). These results indicate that the

CD4⁺CD25⁻Foxp3⁺ cells that appear following anti-CD25 treatment are the precursors of the CD4⁺CD25⁺Foxp3⁺ cells which reappear some days after treatment.

Taken together, the data presented in Figure 3 strongly suggest that CD4⁺Foxp3⁺ cells which are not removed by anti-CD25 treatment, transiently down-regulate CD25 and then re-express CD25 once anti-CD25 antibody is removed from the system.

CD25⁻ CD4⁺Foxp3⁺ cells from anti-CD25-treated mice maintain regulatory function in vitro

We have shown that, in vivo, anti-CD25 treatment fails to eliminate a significant number of Foxp3⁺ cells (Fig 2). To determine whether or not the remaining Foxp3⁺ cells were functionally inactivated, as has recently been suggested from in vivo studies (44-48), we tested the ability of CD4⁺Foxp3⁺ cells isolated from Foxp3-GFP knockin mice (that had been pre-treated 3 days previously with 7D4, 7D4+PC61 or PBS) to suppress the proliferation of purified naïve CD4⁺Foxp3⁻ cells in vitro. As expected, both 7D4 and 7D4 + PC61 administration effectively neutralised CD25 expression on CD4⁺Foxp3-GFP-expressing cells, but failed to deplete the majority of the Foxp3-GFP⁺ cells (Fig 4a). CD25⁻ CD4⁺Foxp3⁺ cells obtained from anti-CD25-treated mice were unable to proliferate following anti-CD3 stimulation (similar to CD25⁺CD4⁺Foxp3⁺ cells from control treated mice, results not shown) but suppressed effector T cell (Teff) proliferation to a comparable extent as the CD25⁺ CD4⁺Foxp3⁺ cells derived from PBS-treated mice, with 80-90% suppression of tritiated thymidine uptake at a Treg:Teff ratio of 1:1 (Figure 4b). Although the suppressive function of CD4⁺Foxp3⁺ cells from anti-CD25-treated mice appeared to be higher than that of CD4⁺Foxp3⁺ cells from control mice at lower Treg:Teff ratios, the differences between the groups were not significant, suggesting that the cells are equally, but no more, suppressive. Thus, these results demonstrate that CD4⁺Foxp3⁺ cells derived from anti-CD25 antibody treated mice maintain regulatory potential in vitro and, on a cell per cell basis, are as regulatory as natural CD4⁺Foxp3⁺ cells from normal mice. This observation that CD4⁺Foxp3⁺ cells from anti-CD25-treated mice retained their suppressive capacity in vitro and inhibited proliferation of naïve non-Treg cells, was somewhat unexpected given that numerous studies have shown very significant in vivo effects of anti-CD25 depletion (44,46,50,53-55). We therefore considered the possibility that anti-CD25-treatment might modulate the phenotype of effector CD4⁺ cells, making them refractive to CD4⁺Foxp3⁺-mediated suppression in vivo. Using anti-CD3 antibodies to induce cell proliferation, we found that not only do Foxp3⁻ CD4⁺ (i.e. non-Treg) cells from anti-CD25-treated mice proliferate to an equivalent extent as Foxp3⁻ cells from control mice (Fig 4c) but that they are as susceptible to inhibition by CD25⁺ CD4⁺Foxp3⁺ regulatory cells as are Foxp3⁻ cells from untreated mice (Figure 4d). Thus, the in vivo effects of anti-CD25-treatment do not appear to be due to a direct effect on Foxp3⁻ (non-Treg) cells.

Repopulation of splenic CD4⁺Foxp3⁺ cells during infection

It has been repeatedly shown that perturbing the balance of Treg to non-Treg CD4⁺ cells leads to severe inflammatory disease, even in otherwise normal mice (1-3); however the homeostatic signals that maintain this balance are unknown. One possibility is that some form of “quorum sensing” within the CD4 T cell population detects a decrease in the ratio of regulatory to non-regulatory T cells and this is sufficient to induce a proportion of cells to convert to a Treg phenotype. A potential quorum sensing signal might be an increasing concentration of inflammatory mediators, derived from incipient auto-immune responses when the number of Treg falls below that required to maintain tissue homeostasis. If so, then one might expect Treg cell numbers to increase rapidly in the presence of an acute inflammatory signal, such as infection, and, in infected anti-CD25-treated animals, repopulation of Treg would be expected to occur more quickly. To test this hypothesis, we compared rates of Treg repopulation in uninfected anti-CD25 treated mice with those in

anti-CD25 treated (or control, PBS treated) mice which were infected on the same day with the 17X strain of the rodent malaria parasite, *Plasmodium yoelii*, an infection characterised by systemic pro-inflammatory cytokine production and extensive and rapid T cell proliferation (56, 57).

In normal, uninfected, mice the ratio of CD4⁺Foxp3⁺ Treg to CD4⁺Foxp3⁻ non-Treg in the spleen was approximately 0.15 and was extremely stable over time (Figure 4a), indicative of an efficient homeostatic sensing mechanism that maintains an appropriate ratio of regulatory to effector cells in the periphery. When uninfected mice were treated with either 7D4 or 7D4 plus PC61, the ratio of Treg:non-Treg fell slightly (Figure 5a) as Foxp3⁺ cells were depleted (Figure 5b); the nadir of the ratio occurred at 2-3 days post treatment in mice receiving 7D4+PC61 and the ratio remained significantly below normal for at least 13 days, suggesting that it takes sometime for the “quorum sensing” signals to develop. In contrast, in control mice or 7D4-treated mice subsequently infected with *P. yoelii*, there was a transient but statistically significant increase in the ratio of Treg:nonTreg on day 3 pi; the ratio dropped back to pre-infection levels on day 7 pi and was below normal on day 10 pi (Figure 5c). In line with our observations (above) that 7D4 treatment has a minimal impact on numbers of Foxp3⁺ cells, numbers of CD4⁺Foxp3⁺ splenocytes did not change markedly over the first week of infection (Figure 5d), indicating that changes in the Treg:nonTreg ratio are driven by an initial decrease and subsequent expansion of non-Treg. Consistent with this, in 7D4+PC61-treated mice, where there was substantial loss of Foxp3⁺ cells, the Treg:non-Treg ratio did not change in the first few days of infection. However, in all malaria-infected animals, irrespective of whether or not they received anti-CD25 antibodies, there was a marked increase in the total numbers of Foxp3⁺ cells between 7 and 10 days post-infection (Figure 5d) but this was not sufficient to restore the normal (uninfected) Treg:nonTreg ratio (Figure 5c), which was likely due to the rapid expansion of effector T cells that occurs during infection between days 7 and 10 post-infection (results not shown). Taken together, these data indicate that in a healthy animal it takes more than 2 weeks for Treg numbers and the Treg:nonTreg ratio to be restored after effective anti-CD25 treatment whereas in animals with an acute inflammatory disease, Treg numbers double within 10 days, as the immune system attempts homeostasis. Moreover, the expansion of Foxp3⁺ cells was similar in both anti-CD25 treated and untreated animals, indicating inflammation alone is sufficient to drive this process.

Peripheral CD4⁺CD25⁻ cells give rise to CD4⁺CD25⁺Foxp3⁺ cells during malaria infection

The rapid expansion of Foxp3⁺ cell numbers in malaria-infected mice, and the very rapid recovery of these cells in infected anti-CD25-treated mice, might be due to *de novo* production of Treg in the thymus, proliferation of existing peripheral Foxp3⁺ cells or differentiation of peripheral Foxp3⁻ cells into Treg. To explore this issue, purified CD4⁺CD25⁻ cells from Ly5.1⁺ mice were adoptively transferred into congenic Ly5.2⁺ mice (representative examples of purified, transferred cells are shown in Figure 6a), which were then infected with *P. yoelii*. Seven days after malaria infection, the number of Ly5.1⁺Foxp3⁺ cells in spleens of infected mice had increased four fold compared to numbers in uninfected mice (Figure 6b), and 60% of these cells now expressed CD25, indicating that Treg can be rapidly generated from peripheral T cells during an acute infection. In view of our previous observations ruling out significant *de novo* generation of CD25⁺Foxp3⁺ cells from peripheral CD25⁻Foxp3⁻ cells following anti-CD25 antibody administration in uninfected mice (Figure 3d), it is most probable that this increase in Treg numbers resulted from proliferation of (and up regulation of CD25 by) the small number of CD25⁻Foxp3⁺ cells in the donor cell population. However, we can not rule out the possibility that, during infection, CD25⁻ Foxp3⁻ cells may also differentiate into CD25⁺Foxp3⁺ cells.

Re-expression of CD25 by CD25⁻Foxp3⁺ cells is not dependent upon TGF- β signalling but IL-2 is essential for the maintenance of CD25⁺Foxp3⁺ cells

We have shown that following anti-CD25 treatment a significant percentage (60% or more) of Foxp3⁺ cells down-regulate CD25 and are programmed to re-express CD25 once the depleting antibody is removed from the system or when exposed to a highly pro-inflammatory environment. A number of studies have shown that peripheral CD25⁻Foxp3⁻ cells can differentiate into CD25⁺Foxp3⁺ regulatory cells in the presence of TGF- β (14-21). Furthermore, it is established that IL-2 is required for the maintenance and homeostasis of Treg (3, 25, 26). We have therefore examined the requirements for TGF- β and/or IL-2 in the re-expression of CD25 on CD25⁻Foxp3⁺ cells (Figure 7). Mice were treated with 7D4 to induce transient down regulation of CD25 on Foxp3⁺ cells and simultaneously treated with anti-IL-2, anti-TGF- β or anti-IL-2 plus anti-TGF- β . Control animals were treated with 7D4 alone, anti-IL-2 alone or anti-TGF- β alone. Representative flow cytometric data are shown, for CD4⁺ CD25⁺ cells (Figure 7a) and CD25⁺ Foxp3⁺ cells (Figure 7b) for each group either 3 or 9 days after antibody treatment. Summary data for each treatment group are shown in Figure 7c.

A significant role for IL-2 in maintaining the Treg pool was observed. Anti-IL-2 treatment alone led to a gradual decline in the percentage of both CD25⁺ and Foxp3⁺ cells, with a significant reduction of more than 50% by 9 days post-antibody administration ($P < 0.05$ compared with untreated controls). Furthermore, in mice treated with 7D4 and anti-IL-2, the usual rapid recovery of CD25⁺ and Foxp3⁺ cells, and restoration of the Treg:non-Treg ratio, was markedly delayed ($P < 0.05$ compared with 7D4 treated controls). In contrast, regeneration of CD25⁺Foxp3⁺ cells following anti-CD25 treatment did not require TGF- β signalling since, by day 9 post anti-CD25 treatment, the extent of repopulation of the spleen by CD25⁺Foxp3⁺ cells was similar in both the 7D4 and 7D4 + anti-TGF- β treated groups. Of interest, our data provide some evidence that TGF- β may play a role in maintenance of Foxp3⁺ cells in the absence of IL-2 signalling via CD25. This suggestion is supported by the finding that the percentages of Foxp3⁺ cells and the Treg:non-Treg ratio were both significantly lower 3 days after treatment in mice receiving all three antibodies (7D4, anti-IL-2 and anti-TGF- β) than in mice that received 7D4 and anti-IL-2 but not anti-TGF- β ($P < 0.05$).

Discussion

In this study we have examined, in detail, the utility of anti-CD25 depletion techniques to assess the functional importance of Treg. In agreement with other recently published studies (44-48) we have shown incomplete depletion of Foxp3⁺ regulatory cells following anti-CD25 antibody treatment and have shown that the kinetics of the splenic CD25⁺Foxp3⁺ repopulation after anti-CD25 treatment can be explained by transient down-regulation and subsequent re-expression of CD25 on Foxp3⁺ cells (48). However, we have significantly extended these observations by showing that Foxp3⁺ cell depletion can be accelerated and/or extended by co-administration of PC61 and 7D4 antibodies and that peripheral Foxp3⁻ cells fail to differentiate into Foxp3⁺ cells following anti-CD25 treatment. We have also shown that infection - in this case acute malaria infection - leads to rapid expansion of the CD4⁺CD25⁺Foxp3⁺ population in both anti-CD25 treated and untreated mice, most probably as a result of proliferation of CD25⁻Foxp3⁺ cells and their subsequent up regulation of CD25. Lastly, we have shown that purified CD25⁻Foxp3⁺ maintain regulatory capacity in vitro and that IL-2 is essential, not only for maintaining Treg numbers in the periphery in normal mice, but also for the ability of Treg to persist and repopulate after anti-CD25 treatment.

One immediate practical application of these observations is that the efficiency of Foxp3⁺ Treg depletion can be significantly enhanced by co-administration of IgM and IgG anti-CD25 antibodies and can be further improved by addition of anti-IL-2 antibodies. We observed that the 7D4 IgM antibody led to rapid but transient CD25 depletion with little or no effect on Foxp3⁺ cell numbers whereas PC61 was slower to act but eventually led to more complete and longer lasting depletion of both CD25⁺ and Foxp3⁺ cells. The combination of the two antibodies led to rapid, complete and sustained depletion/down-regulation of CD25 expression. A further important technical point is that unlabelled PC61 antibody leads to long-term blocking of the PC61 epitope such that the effectiveness of the depletion strategy will be significantly overestimated if PC61 is used for depletion and monitoring of CD25 expression. Conversely, 7D4 binding seems to be transient and 7D4 epitopes are revealed within 3 days of antibody treatment; 7D4 is thus the preferred reagent for monitoring CD25 expression after antibody treatment.

As the majority of Foxp3⁺ regulatory cells co-express CD25, previous studies using anti-CD25 depletion to ascertain the importance of Treg have assumed that the disappearance of CD25⁺ cells correlates with the depletion of CD4⁺CD25⁺ natural Treg (for example, references 50-52), we now know this is not the case. We ruled out the possibility that anti-CD25 treatment led to very transient depletion and rapid repopulation of Foxp3⁺ cells by examining Foxp3 expression 1, 2 and 3 days after antibody administration; the lowest proportion of Foxp3⁺ cells was observed 2 days after treatment with 7D4+PC61 and at no point were Foxp3⁺ cells completely eliminated. Nevertheless, numerous workers have reported significant clinical effects following anti-CD25 treatment in a variety of model systems (for example 44, 46, 50, 53-55) indicating that complete depletion of Foxp3⁺ cells is not required to alter the function of the Treg population. Kohm et al (44), who used 7D4 as their sole depleting antibody and saw a significant clinical effect despite no significant depletion of Foxp3⁺ cells, conclude that anti-CD25 treatment leads to functional inactivation of Treg due to loss of IL-2 signalling through CD25 (44). Our observation that the CD25⁻Foxp3⁺ cells that persist after anti-CD25-treatment have potent regulatory function in vitro is, therefore, initially surprising given the in vivo functional data. However, other studies have shown that Foxp3⁺ cells do not need to express CD25 to mediate suppression (5,9) and that CD25 expression on Foxp3⁺ cells is highly plastic (58). We have ruled out, as best we can, the possibility that the in vivo effects of anti-CD25 are due to a direct effect on effector T cells but in view of recent data showing that defects in certain T effector signalling pathways render them resistant to the effects of Treg (59, 60) it is still possible that some subtle and hitherto unrecognised in vivo effect of anti-CD25 antibodies may influence the activities of effector cells. Alternatively, given the suggestion that uptake of IL-2 by CD4⁺CD25⁺Foxp3⁺ Treg cells limits the availability of IL-2 for effector T cell proliferation (61, 62), it is at least theoretically possible that, in vivo, down regulation of CD25 expression on Tregs increases the availability of IL-2 to effector cells allowing them to proliferate. Such effects would not be seen in vitro since the excess anti-CD25 antibody is removed by washing of cells following separation and purification, and as we have shown in this study, CD4⁺Foxp3⁺CD25⁻ cells are programmed to rapidly up regulate CD25 expression when anti-CD25 antibody is removed.

Our data using a congenic cell transfer model are consistent with those of Zelenay *et al* (48), using adult thymectomy, who concluded that CD25 is rapidly re-expressed on peripheral CD25⁻Foxp3⁺ cells after anti-CD25 treatment. Importantly however, we have also shown that anti-CD25 antibody treatment does not increase the expression of Foxp3 in the pre-existing CD4⁺CD25⁻ population, thereby formally ruling out the possibility that the reappearance of classical CD4⁺CD25⁺Foxp3⁺ cells might be due to conversion of mature, peripheral, Foxp3⁻ non-Treg into Treg. The rate of reappearance of CD25⁺Foxp3⁺ cells after anti-CD25 treatment seems to depend in part on the duration of persistence of the depleting

antibody. When used on its own, 7D4 antibody seems to persist at high concentration for no more than 3 days (as demonstrated by the ability to detect CD25⁺ cells using labelled 7D4) and in this case CD25⁺Foxp3⁺ cell numbers recover and stabilise after 4 or 5 days. Similarly, the majority of CD4⁺CD25⁻ cells from anti-CD25 treated mice re-expressed CD25 within 5 days of adoptive transfer to congenic mice. On the other hand, the proportion of Foxp3⁺ cells, and the Treg:nonTreg ratio, stabilises at a marginally (but significantly) lower level in 7D4-treated than in untreated mice, suggesting that in otherwise healthy animals development/replacement of Treg is quite slow. In the case of PC61 treatment, Foxp3⁺ cell numbers and the Treg:non-Treg ratio also stabilise around day 4 after treatment but at a much lower level; this may represent the net effect of Treg repopulation being offset by continual removal of Treg by persisting PC61 antibody.

Peripheral development and *in vitro* generation of CD25⁺Foxp3⁺ natural Treg is dependent upon TGF- β signalling (14-22) and it has previously been reported that IL-2 signalling is required for the production and homeostasis of the Treg population (3,25,26). As expected, therefore, IL-2 was critically required in our study for the maintenance of CD25⁺Foxp3⁺ cells. However, our observation that anti-IL-2 antibodies potentiate the depletion of Foxp3⁺ cells by 7D4 suggests that IL-2 signalling via receptors other than CD25 can be sufficient to maintain Treg survival, and possibly function. Moreover, although rapid regeneration of CD25⁺ cells was observed in mice treated with anti-TGF- β antibodies, we observed a transient decline in Foxp3⁺ cell numbers and the Treg:non-Treg ratio in mice receiving 7D4 plus anti-TGF- β compared with mice receiving 7D4 alone, and a more marked decline in the two parameters in mice receiving 7D4 and anti-TGF- β in combination with anti-IL-2 compared with mice receiving 7D4 in combination with anti-IL-2. These completely novel observations indicate that TGF- β may play a modest role in the survival of Foxp3⁺ Treg in the absence of IL-2 signalling via CD25.

Finally, we have examined the effect of acute inflammation on the regeneration of Treg following anti-CD25 depletion. *P. yoelii* malaria infection is characterised by extensive T cell proliferation and production of pro-inflammatory mediators (54, 55) making it an ideal model in which to assess the rate of regeneration of Treg. We have observed much more rapid regeneration of CD25⁺Foxp3⁺ cells from CD25⁻ cells during the very early stage of infection, and an increase in the ratio of Treg to non-Treg, compared with uninfected mice. The period of effective CD25 depletion by 7D4+PC61 is considerably shortened during malaria infection with complete repopulation of Foxp3⁺ cells occurring between 7 and 10 days post-administration compared with greater than 15 days in uninfected mice. Although we cannot entirely rule out the possibility that this is due in part to increased consumption of PC61 *in vivo* due to up regulation of CD25 on effector cells during infection, this cannot be the sole explanation since similar expansion of Foxp3⁺ cell numbers was seen in infected but untreated mice. Rather, we suggest that rapid repopulation of Treg results from extensive proliferation of CD25⁻Foxp3⁺ cells. Although we can not discount the possibility that CD25⁻Foxp3⁻ cells differentiate into CD25⁺Foxp3⁺ cells during malaria infection, it has been reported that mature, CD25⁻ CD4⁺CD45RB^{low} cells (a population that contains Foxp3⁺ cells), but not CD4⁺CD45RB^{high} cells, preferentially differentiate into regulatory CD25⁺ T cells during lymphopenic homeostasis (27) indicating that CD4⁺CD25⁻Foxp3⁺ cells are a reservoir of regulatory T cells that may differentiate and proliferate upon perturbation of the Treg:nonTreg cell balance.

In conclusion, this study has demonstrated significant differences in the effectiveness of different strategies for depletion of Foxp3⁺ cells and has identified an improved strategy for antibody-mediated ablation of Treg. Nevertheless, Treg ablation is still incomplete, demonstrating the limitations of anti-CD25 treatment approaches for assessing the role of Treg. Furthermore, we have shown that in otherwise healthy animals, repopulation of Treg

after anti-CD25 treatment results from rapid re-expression of CD25 accompanied by a somewhat slower replacement of Foxp3⁺ cells. In contrast, in an acute inflammatory environment repopulation occurs much more quickly as the result of proliferation of peripheral Foxp3⁺ cells; this is most likely driven by inflammatory cytokines, allowing immune system homeostasis to be quickly re-established.

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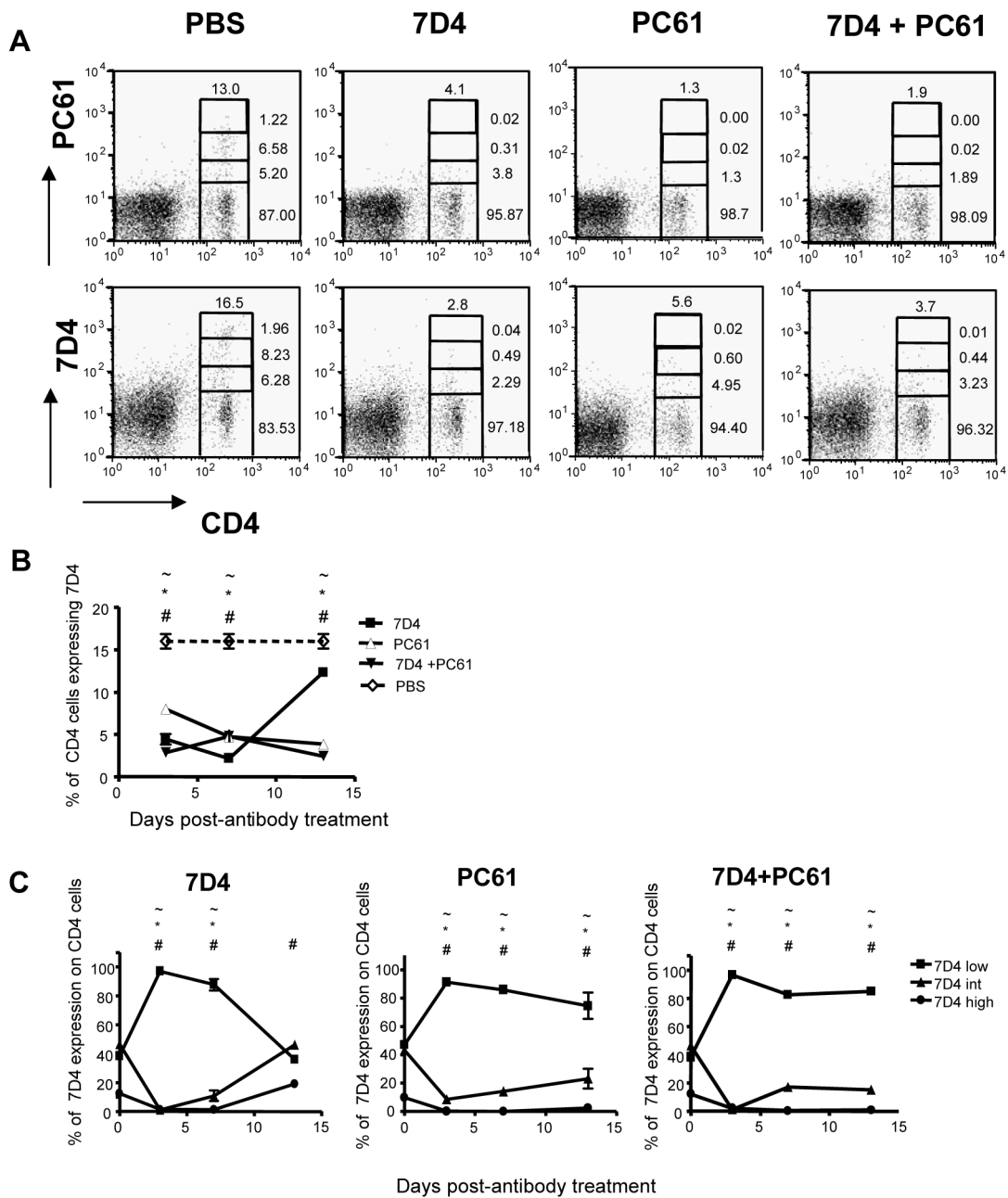


Figure 1. Anti-CD25 antibodies effectively deplete CD4CD25+ve T cells

Mice received one injection i.p. on day 0 with either 0.75mg 7D4, 0.75mg PC61, 0.25mg 7D4 in combination with 0.75mg PC61 or PBS. (a) Representative dot plots of CD25 expression on CD4⁺ cells on day 3 post administration are shown using either PC61 (upper plots) or 7D4 (lower plots) as the detecting antibody. (b) On selected days post-administration spleens were removed and depletion of CD4⁺CD25⁺ cells was determined by flow cytometry using fluorochrome labelled 7D4. Symbols represent significant differences between: ~ 7D4 treatment v/s PBS controls, * PC61 treatment v/s PBS controls, # 7D4 + PC61 treatment v/s PBS controls; P < 0.05 in all cases. (c) Levels of CD25 expression on CD4⁺CD25⁺ cells that escaped depletion. CD25^{low}, CD25^{int} and CD25^{hi} populations were

gated as shown in (a). Symbols represent significant differences between anti-CD25 treated group and control PBS treated group: \sim 7D4^{low}, * 7D4^{int}, # 7D4^{hi}; $P < 0.05$ in all cases. Live lymphocytes were gated on their forward/side scatter profiles and at least 50,000 events were acquired per sample. Groups consisted of 3 mice at each time point and the results are representative of 2 independent experiments.

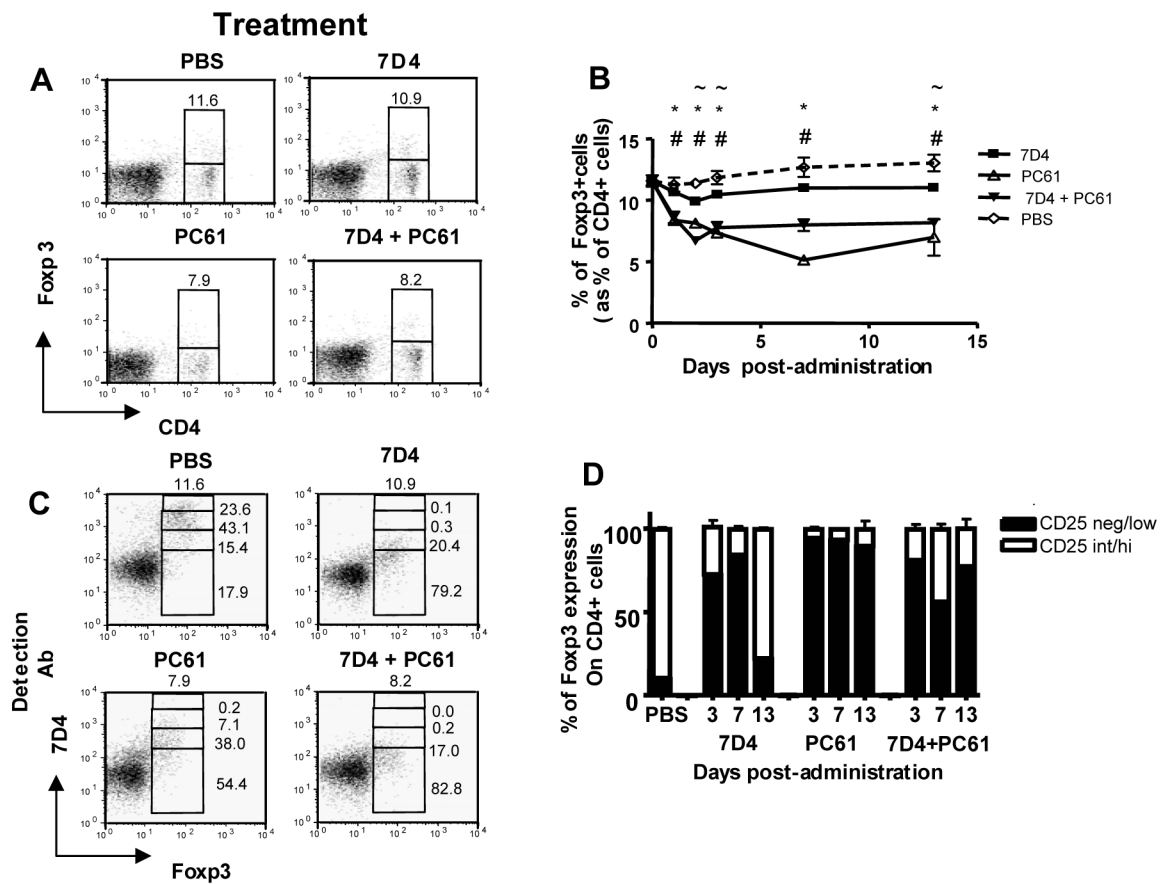


Figure 2. Anti-CD25 antibody reduces but does not eliminate CD4⁺Foxp3⁺ cells

On selected days after anti-CD25 antibody administration, spleens were removed and cells were permeabilised with 0.1% Saponin/PBS for Foxp3 detection with anti-Foxp3 fluorochrome-labelled antibody. (a) Representative dot plots showing Foxp3 expression on CD4⁺ cells 3 days after treatment with PBS, 7D4 or 7D4+PC61. (b) Percentage of CD4⁺ cells expressing Foxp3 at various time points after antibody or PBS treatment. Symbols represent statistically significant results: ~ 7D4 treatment v/s PBS controls, * PC61 treatment v/s PBS, # 7D4+PC61 treatment v/s PBS controls; P<0.05 in all cases. (c) A representative dot plot showing CD25 (7D4) expression within the CD4⁺Foxp3⁺ population from control and anti-CD25 treated mice on day 3 post-treatment. (d) Percentage of CD4⁺Foxp3⁺ cells co-expressing CD25 on selected days post-treatment. Groups consisted of 3 mice at each time point and the results are representative of 2 independent experiments

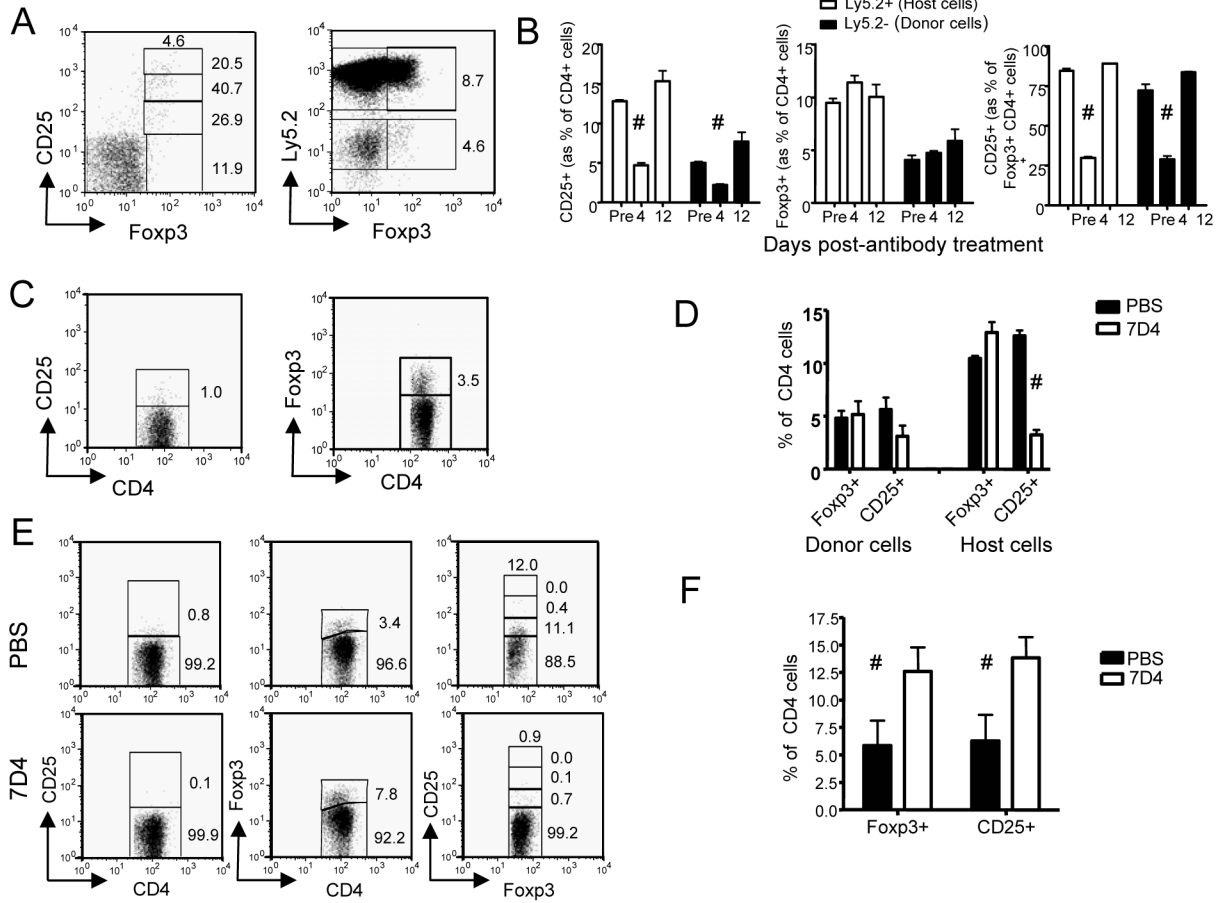


Figure 3. Peripheral generation of CD4⁺CD25⁺Foxp3⁺ cells and conversion into CD4⁺CD25⁺Foxp3⁺ Regulatory T cells

(a and b) Ly5.1⁺ CD4⁺ cells were purified and adoptively transferred to congenic Ly5.2⁺ recipients prior to treatment with anti-CD25 (7D4) antibody. (a) Representative dot plots showing CD25 and Foxp3 expression on purified CD4⁺ cells prior to adoptive transfer (left plot) and the recovery of Foxp3⁺ Ly5.2⁺ and Ly5.2⁻ cells after adoptive transfer. (b) Percentage of CD25⁺ and Foxp3⁺, Ly5.2⁺ (host) and Ly5.2⁻ (donor) cells prior to anti-CD25 treatment (Pre), 4 and 12 days after anti-CD25 treatment.

(c and d) Ly5.1⁺ CD4⁺CD25⁻ cells were FACS purified and adoptively transferred to congenic Ly5.2⁺ recipients prior to treatment with anti-CD25 (7D4) antibody or PBS. (c) Representative dot plots showing the expression of CD25 and Foxp3 on purified CD4⁺ cells prior to adoptive transfer. (d) Percentage of CD4⁺ host and adoptively transferred (donor) cells expressing either Foxp3 or CD25, 4 days after antibody treatment with 7D4 or PBS.

(e and f) Ly5.1⁺ CD4⁺CD25⁻ cells were FACS purified from mice treated 3 days previously with 7D4 or PBS and adoptively transferred to congenic Ly5.2 recipients. (e) Representative dot plots showing expression of CD25 and Foxp3 on purified CD4⁺ cells from PBS- (top row) or 7D4- (bottom row) treated mice prior to adoptive transfer. (f) Percentage of CD4⁺ cells expressing either Foxp3 or CD25, 5 days after adoptive transfer from PBS- or 7D4-treated mice.

Groups consisted of 3-4 mice and the results are representative of 2 independent experiments. # anti-CD25 treatment v/s PBS treated controls; P<0.05 in each case.

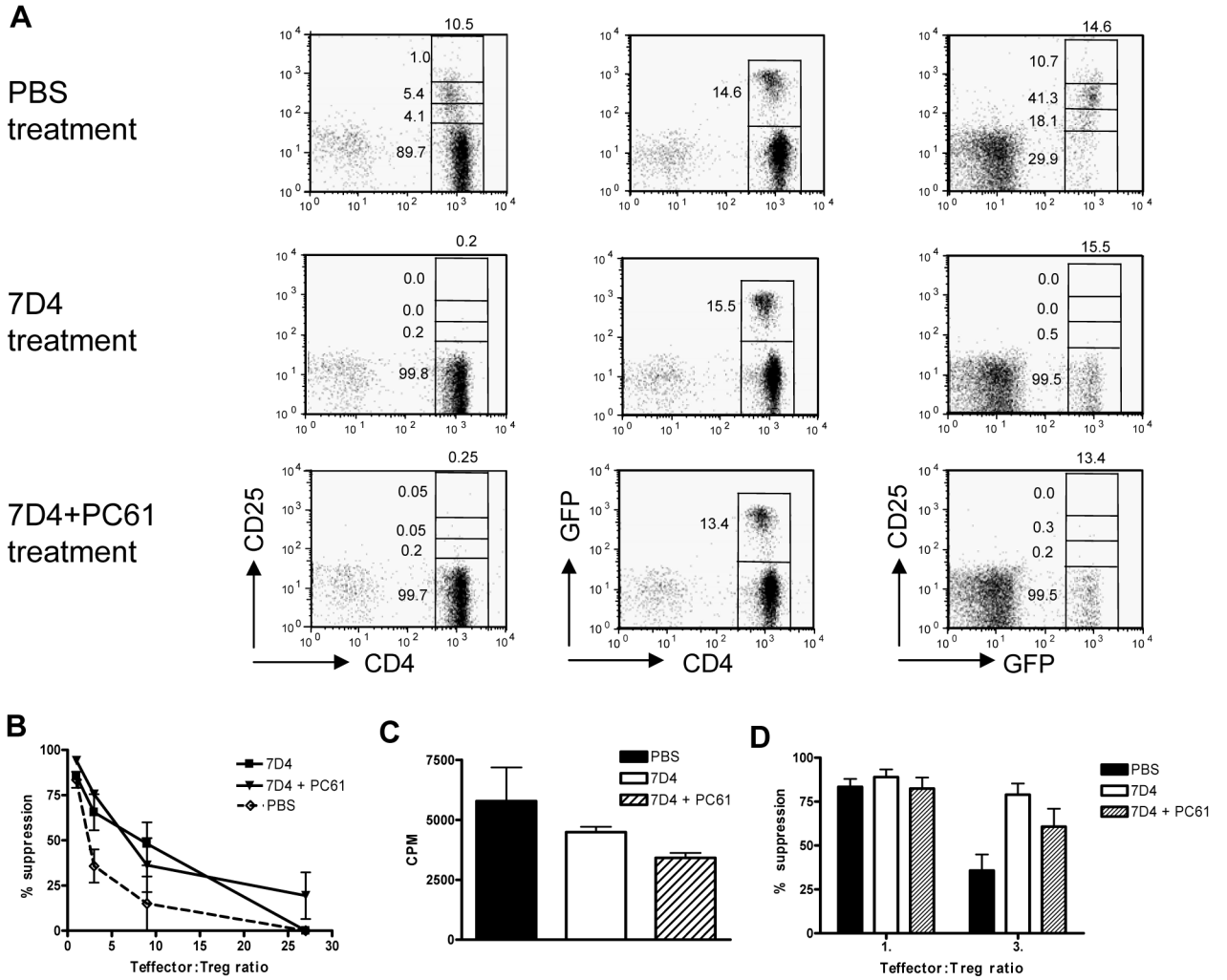


Figure 4. CD4+Foxp3+ cells retain in vitro regulatory capacity following anti-CD25 antibody administration

Foxp3-GFP knockin mice received one injection i.p. with 0.75mg 7D4, 0.25mg 7D4 plus 0.75mg PC61 or PBS. On day 3 post-injection, splenic CD4⁺GFP⁺ cells and CD4⁺GFP⁻ cells were purified from each group of mice. The expression of CD25 by CD4⁺GFP⁺ cells from anti-CD25 antibody and control treated mice was determined by flow cytometry prior to in vitro culture (a). CD4⁺GFP⁺ (Treg) cells isolated from control (PBS-treated) mice, 7D4-treated or 7D4+PC61-treated mice were co-cultured with CD4⁺GFP⁻ (non-Treg) cells from control (untreated) mice at various ratios in the presence of anti-CD3 antibody and the percentage suppression of proliferation of CD4⁺GFP⁻ (non-Treg) cells was determined (b). The proliferative capacity of GFP⁻ (non-Treg) cells derived from control (PBS-treated) mice, 7D4-treated mice or 7D4+PC61-treated mice following anti-CD3 stimulation was determined (c). Finally, the capacity of GFP⁺ (Treg) cells from control (PBS-treated) mice to suppress proliferation of GFP⁻ (non-Treg) cells from control (PBS-treated) treated, 7D4-treated or 7D4+PC61-treated mice following anti-CD3 stimulation was determined (d). The results shown are the mean +/- SEM of the group (n = 3).

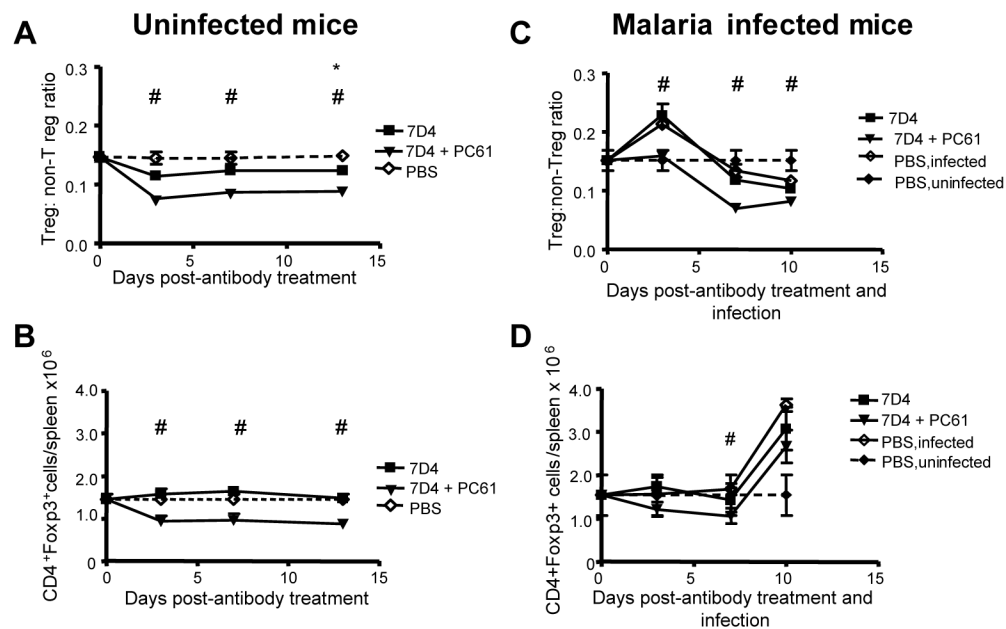


Figure 5. Repopulation of CD4⁺CD25⁺Foxp3⁺ cells is accelerated during acute malaria infection
 Mice received one injection i.p. on day 0 with 0.75mg 7D4, 0.25mg 7D4 plus 0.75mg PC61 or PBS and were concurrently infected (or not) with a non-lethal strain of *Plasmodium yoelii*. The ratio of Treg (CD4⁺Foxp3⁺) to non-Treg (CD4⁺Foxp3⁻) cells (a,c) and the absolute number of Treg (b,d) in spleens of uninfected PBS-treated mice (a,b) and malaria-infected (c,d) mice were calculated on the specified days post treatment/infection. Groups consisted of 3-4 mice and the results are representative of 2 independent experiments. Symbols indicate statistically significant differences: * 7D4 treatment v/s PBS controls, # 7D4 + PC61 treatment v/s PBS controls; P<0.05 in each case.

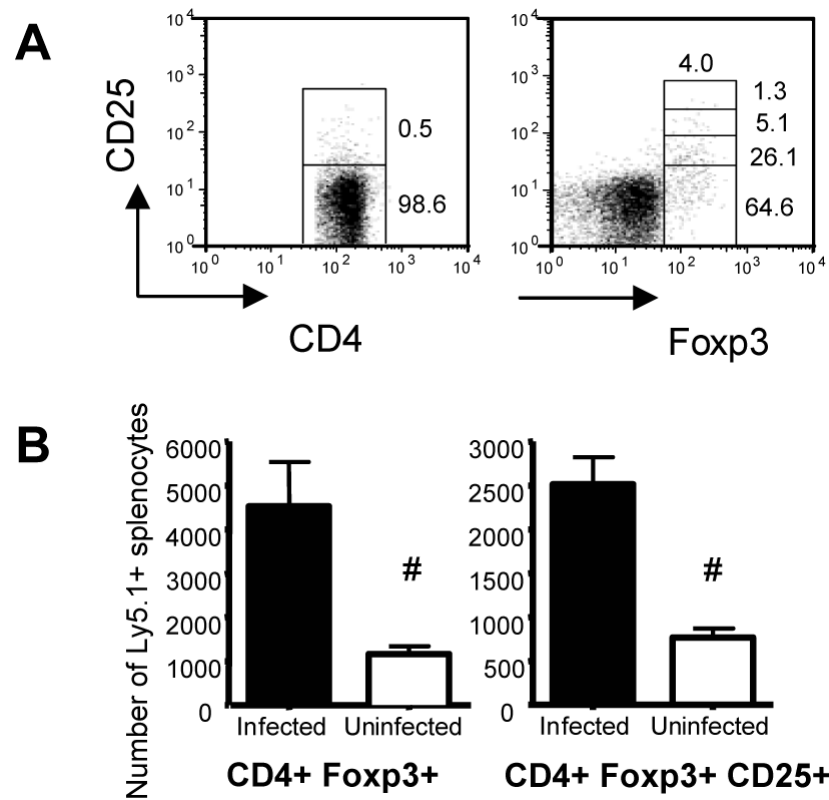


Figure 6. Peripheral expansion of CD4⁺ Foxp3⁺ Treg cells during acute malaria infection
 Ly5.1⁺ CD4⁺CD25⁻ cells were FACS purified and adoptively transferred into congenic Ly5.2⁺ recipients prior to infection with a non-lethal strain of *P. yoelii*. (a) Representative dot plots showing CD25 and Foxp3 expression on purified Ly5.1⁺ cells prior to adoptive transfer. (b) Absolute numbers of CD4⁺Ly5.1⁺ cells expressing Foxp3 (left plot) or Foxp3 and CD25 (right plot) in spleens of malaria-infected or uninfected mice, 7 days post-transfer/infection. Groups consisted of 3-4 mice and the results are representative of 2 independent experiments. # malaria infected mice v/s PBS treated controls; P<0.05.

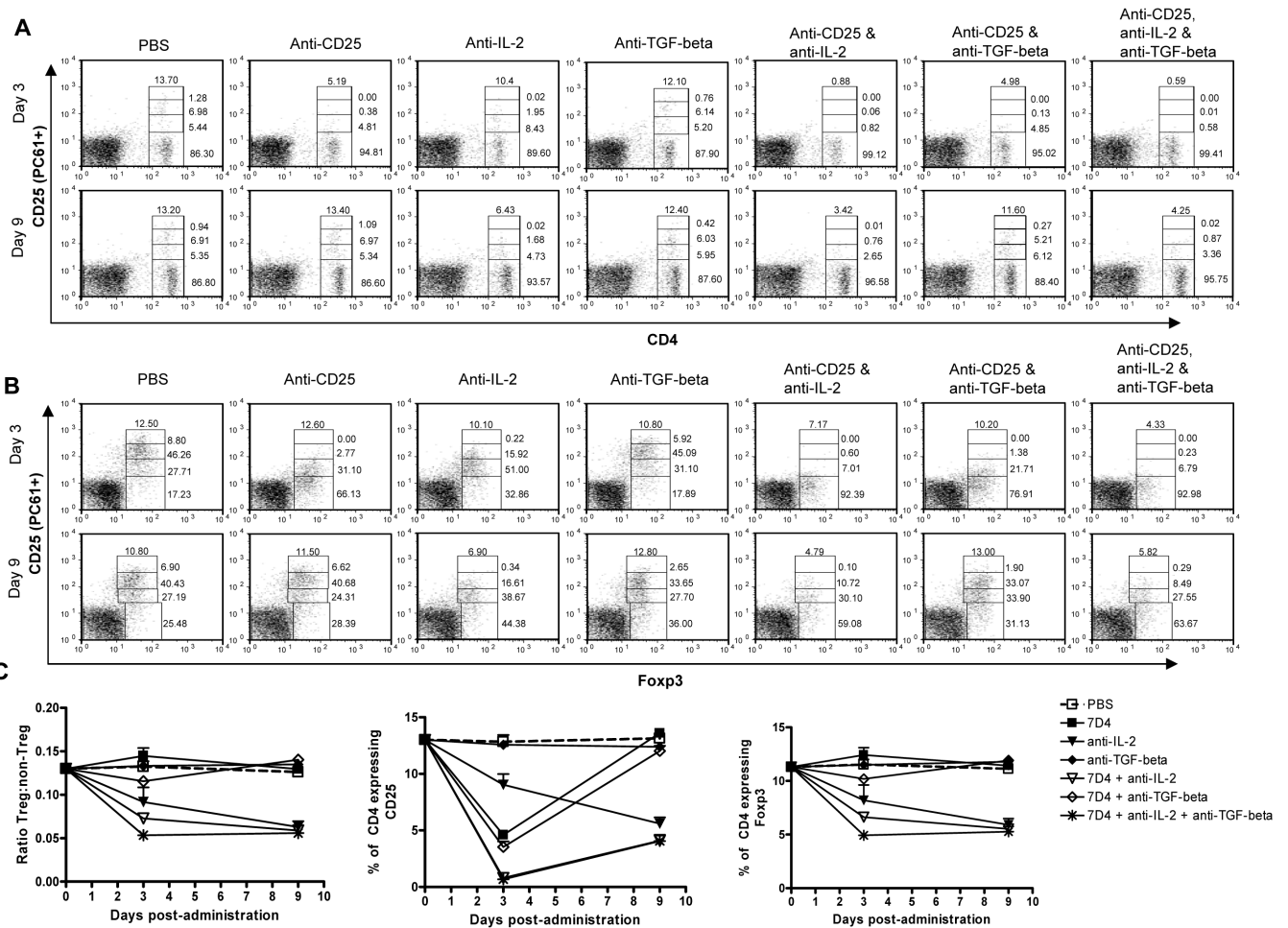


Figure 7. Effects of anti-IL-2 and anti-TGF- β on homeostasis of CD4⁺Foxp3⁺ Treg cells
 Mice received a single injection i.p. of either 0.5mg 7D4 or PBS on day 0, or received anti-IL-2 (0.5mg on days 0,3,6 and 8) or anti-TGF- β (0.25mg on days 0,1,2,3,4,6,7,8) with or without 7D4 (0.5mg Day 0). Representative dot plots showing CD25 expression on CD4⁺ cells (a) or CD25 and Foxp3 expression on gated CD4⁺ cells (b) on days 3 and 9 post antibody treatment. (c) Ratio of CD4⁺Foxp3⁺ cells to CD4⁺Foxp3⁻ cells, percentage of CD4⁺ cells expressing CD25 and percentage of CD4⁺ cells expressing Foxp3 on days 3 and 9 post antibody treatment. Detection of CD25 expression was performed using PC61 in all cases. Groups consisted of 3-4 mice at each time point.