

RESEARCH ARTICLE

The immunoregulatory role of CD4⁺FoxP3⁺CD25[−] regulatory T cells in lungs of mice infected with *Bordetella pertussis*

Michelle M. Coleman¹, Conor M. Finlay¹, Barry Moran¹, Joseph Keane², Pádraic J. Dunne¹ & Kingston H.G. Mills¹

¹Immune Regulation Research Group, School of Biochemistry and Immunology, Trinity College Dublin, Dublin, Ireland; and ²St. James's Hospital and Trinity College Dublin, Dublin, Ireland

Correspondence: Pádraic J. Dunne, Phase I Immunology, Trinity Centre for Health Sciences, St. James's Hospital, Dublin 8, Ireland.
Tel.: +353 1 896 2478; fax: +353 1 677 2086;
e-mail: padraic.dunne@tcd.ie

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Abstract

The identification of regulatory T (Treg) cells was originally based on CD25 expression; however, CD25 is also expressed by activated effector T cells. FoxP3 is a more definitive marker of Treg cells, and CD4⁺FoxP3⁺CD25⁺ T cells are considered the dominant natural Treg (nTreg) population. It has been suggested that certain CD4⁺FoxP3⁺ Treg cells do not express CD25. In this study, we used a murine model of respiratory infection with *Bordetella pertussis* to examine the role of Treg cells in protective immunity in the lung. We first demonstrated that CD4⁺FoxP3⁺CD25[−] cells are the dominant Treg population in the lung, gut and liver. Pre-activated lung CD4⁺FoxP3⁺CD25[−] cells suppressed CD4⁺ effector T cells *in vitro*, which was partly mediated by IL-10 and not dependent on cell contact. Furthermore, CD4⁺FoxP3⁺CD25[−]IL-10⁺ T cells were found in the lungs of mice at the peak of infection with *B. pertussis*. The rate of bacterial clearance was not affected by depletion of CD25⁺ cells or in IL-10-deficient (IL-10^{−/−}) mice, but was compromised in CD25-depleted IL-10^{−/−} mice. Our findings suggest that IL-10-producing CD4⁺FoxP3⁺CD25[−] T cells represent an important regulatory cell in the lung.

Introduction

It is well established that regulatory T (Treg) cells can be broadly divided into thymically derived natural (n) Treg (Sakaguchi *et al.*, 1995) and inducible (i) Treg cells (Hafler *et al.*, 1997; Roncarolo *et al.*, 2001; McGuirk *et al.*, 2002; Tran *et al.*, 2007). Depletion of or defects in nTreg cells can lead to the emergence of a wide variety of autoimmune disease and debilitating tissue destruction, as exemplified in *Scurfy* mutant mice (Ono *et al.*, 2006), and immune dysregulation, polyendocrinopathy, enteropathy and X-linked syndrome in humans (Patel, 2001). A mutation in the gene encoding the transcription factor, forkhead box protein 3 (FoxP3), underlies both phenotypes. Intracellular FoxP3 expression is currently regarded as the definitive marker for cells with regulatory function in mice and humans (Fontenot *et al.*, 2003, 2005).

Activated human T cells transiently express FoxP3, making it difficult to differentiate between Treg and activated T cells during an immune response (Allan *et al.*,

2007). The search for a common, stable marker for Treg cells has proved elusive, forcing researchers to use a battery of surface and intracellular markers to identify a growing number of Treg subsets. Chief of these surface markers has been CD25, the alpha chain of the IL-2 receptor (Sakaguchi *et al.*, 1995; Asano *et al.*, 1996; Suri-Payer *et al.*, 1998), which is constitutively expressed on nTreg cells (Sakaguchi *et al.*, 1995). However, CD25 is an activation marker for both murine and human CD4⁺ T cells and therefore does not represent a definitive marker for Treg cells (Asano *et al.*, 1996). Despite this, a combination of FoxP3 and CD25 expression is now widely used to detect, analyse and purify Treg cells.

Despite the fact that the majority of Treg cells express surface CD25 (particularly nTreg cells), subsets of CD4⁺FoxP3⁺ cells that lack CD25 expression have also been shown to have suppressive function *in vitro* and *in vivo* (Alyanakian *et al.*, 2003; Ono *et al.*, 2006). Furthermore, it has been postulated that Treg cells lacking CD25 might accumulate in aged mice and humans within memory T cell

subsets (Nishioka *et al.*, 2006). Interestingly, it has been shown, using FoxP3^{gfp+} mice, that CD4⁺FoxP3^{gfp+}CD25^{low} cells accumulated in the lungs of uninfected as well as chronically *Mycobacterium tuberculosis*-infected mice (Fontenot *et al.*, 2005). Finally, it was demonstrated that Treg cells from the lung parenchyma of mice infected with respiratory syncytial virus express less CD25 than their counterparts in the draining lymph nodes (Fulton *et al.*, 2010).

The Gram-negative bacterium *Bordetella pertussis* is the causative agent of whooping cough, a severe and sometimes lethal respiratory disease in infants. Protective immunity is mediated by a combination of innate and adaptive immune responses (Mills, 2001; Yeh, 2003). However, studies in a mouse model have shown that the respiratory infection persists because of immune suppressive responses induced by the pathogen. We have previously demonstrated that IL-10-secreting Treg cells are induced during *B. pertussis* infection and that these cells are capable of suppressing protective Th1 cells (McGuirk *et al.*, 2002). However, the role of FoxP3⁺ Treg cells in this infection has received little attention.

The aim of this study was to examine the role of FoxP3⁺ Treg cells in immunity to *B. pertussis* in mice. We demonstrate that the majority of FoxP3⁺ T cells in the lungs of naive mice do not express surface CD25. CD4⁺FoxP3⁺CD25⁻ T cells isolated from lungs had to be pre-activated to suppress CD4⁺ effector T cells *in vitro*, and this suppression was not dependent on cell contact, but did require IL-10. In addition, there was a significant increase in the numbers of IL-10-secreting CD4⁺FoxP3⁺CD25⁻ T cells in the lungs throughout the course of infection with *B. pertussis*. Depletion of CD25⁺ cells significantly reduced the rate of bacterial clearance following *B. pertussis* challenge, but only in IL-10-defective (IL-10^{-/-}) mice. This CD25⁻ Treg subset represents an important regulatory arm in the maintenance of tolerance in the lung.

Materials and methods

Animals

Specific pathogen-free Balb/c and C57BL/6 mice were purchased from Harlan UK Ltd. C57BL/6 IL-10^{-/-} mice were purchased from Jackson Laboratory. Animal experiments and maintenance were approved and regulated by the university ethics committee and the Irish Department of Health (licence number 3803).

Bordetella pertussis respiratory challenge model

Mice were challenged by aerosol exposure to live *B. pertussis* and *B. pertussis* CFU in lungs determined at intervals

after infection as described (McGuirk & Mills, 2002). Briefly, virulent *B. pertussis* BP338 was grown at 36 °C in Stainer–Scholte liquid medium. Bacteria from a 48-h culture were resuspended at a concentration of approximately 2×10^{10} CFU mL⁻¹ in physiological saline containing 1% casein. The challenge inoculum was administered to mice over a period of 15 min (0.5 mL min⁻¹) with a nebulizer in a sealed container within a class 3 exhaust-protected cabinet. This was followed by a rest period of 10 min.

Depleting and blocking antibodies

Hybridoma cells were obtained from the ATCC that secrete the rat anti-mouse IgG₁ clone PC61 (anti-CD25). The depleting antibody was purified from hybridoma supernatants using a MAbTrap affinity chromatography kit (GE Healthcare). The optimal concentration of antibody required to deplete > 95% of the relevant target cells was determined by titration. Briefly, mice were injected i. p. with increasing concentrations of depleting antibody. The spleens of these mice were examined for CD25 and FoxP3 expression by flow cytometric analysis on 24, 48 h and 7 day postinjection. A rat anti-mouse horseradish peroxidase (HRPN) IgG₁ was used as the isotype-matched control (Bioxcell). An alternative anti-CD25 antibody specific for a different epitope on the CD25 molecule (7D4; eBioscience) was used to examine CD25 depletion. 10 µg mL⁻¹ of either anti-IL-10 receptor (R) (BD Pharmingen) or neutralizing anti-IL-10 antibody (BD Pharmingen) was used to block IL-10 signalling *in vitro*.

Cell isolation and purification

Infiltrating leucocytes were isolated from lungs by digesting lung tissue in PBS containing 1 mg mL⁻¹ Collagenase D (Roche) plus 0.2 µM DNase (Sigma) for 1 h at 37 °C. The same procedure was used to isolate cells from liver tissue with the exception that Collagenase IV (Sigma) was used instead of Collagenase D. Colon tissue was digested as described (Weigmann *et al.*, 2007). Briefly, colon tissue was incubated in HBSS (Biosera) containing 5 mM EDTA (Sigma) and 1 mM DTT (Sigma) for 20 min at 37 °C and passed through a 100-µm cell strainer. The tissue was then digested using 0.05 g of Collagenase D (Roche), 0.05 g of DNase I (Sigma) and 0.3 g of Dispase II (Roche) digestion solution in 100 mL of PBS for 20 min at 37 °C. Erythrocytes were lysed by incubating cell suspensions in ammonium chloride lysis buffer at 37 °C for 5 min.

Flow cytometry

Cells were prepared from digested spleens, lungs, colons, cervical lymph nodes (CLN) or thoracic lymph nodes

(TLN) of naive mice. Cells were stained with antibodies directed against CD3, CD4, CD25, CD69, ICOS, GITR, CTLA-4, CD127, CD45RB, CD44, CD40L, CD73, CD39, FoxP3, IL-10, IL-17 and IFN- γ (eBioscience). The appropriate flow cytometry controls were applied throughout and included the use of unstained tissue-matched controls, fluorescent isotype-matched irrelevant control antibodies, fluorescence-minus one (FMO) controls and the addition of Fc block (eBioscience) to prevent non-specific antibody binding. Labelled cells were acquired using a CyAn flow cytometer (Beckman Coulter). Instrument compensation and cell subset gates were set based on unstained, isotype and FMO controls, which were all based on viable forward vs. side scatter lymphocyte gates. For intracellular cytokine staining, cells were isolated and activated with either 2 $\mu\text{g mL}^{-1}$ of immobilized anti-CD3 (R&D systems) plus irradiated APC or with specific heat-killed *B. pertussis* and incubated for 5 days at 37 °C. Cells were subsequently reactivated with 1 ng mL⁻¹ PMA (Sigma) and 1 $\mu\text{g mL}^{-1}$ ionomycin (Sigma) and incubated for 5 h in the presence of 2 $\mu\text{g mL}^{-1}$ of brefeldin A (Sigma). After washing, these cells were fixed and permeabilized using a FoxP3 buffer set (eBioscience) kit and incubated at room temperature in the presence of relevant antibody.

Cell sorting

Cells were isolated from digested lungs and stained with rat anti-mouse CD4 PE (eBioscience), CD45RB FITC (eBioscience) and CD25 PE-Cy5 (eBioscience) and sorted using a MoFlo cell sorter (DakoCytomation). nTreg cells, FoxP3⁺CD25⁻ and naive effector CD4⁺ T cells were sorted based on the relevant expression of CD4, CD25 and CD45RB. Lack of CD45RB expression is widely used as a surrogate for FoxP3 expression because it is not possible to sort live cells from Balb/c mice on the basis of intracellular FoxP3 (Powrie *et al.*, 1994).

Suppression assay

CD4⁺CD25⁻CD45RB^{low} (0.1×10^5) cells were pre-activated with 1 $\mu\text{g mL}^{-1}$ immobilized anti-CD3 (R&D systems) and irradiated splenocytes (0.5×10^6) in the presence of 20 U mL⁻¹ IL-2 for 5 days and then washed and reactivated in the presence of CD4⁺CD25⁻CD45RB^{high} effector T cells (0.1×10^5) using 10 $\mu\text{g mL}^{-1}$ immobilized anti-CD3 and irradiated APC (0.5×10^6). IL-10 and IFN- γ concentrations were determined in supernatants 48 h later by ELISA (R&D systems), while proliferation was determined by tritiated thymidine incorporation (Amersham) after 72 h. Suppression by

carboxyfluorescein diacetate succinimidyl ester (CFSE) loss was determined by labelling freshly isolated CD4⁺CD25⁻CD45RB^{high} effector T cells with 0.5 μM CFSE (Molecular Probes). Pre-activated CD25⁻ Treg cells were co-cultured with CFSE-labelled effector T cells (1 : 1) and activated in the presence of 10 $\mu\text{g mL}^{-1}$ immobilized anti-CD3 and 5×10^5 irradiated APC for 5 days. Cells were subsequently stained with antibodies directed against surface, CD4 and CD25 as well as intracellular FoxP3, and CFSE loss was determined by flow cytometry.

Ex vivo intracellular cytokine secretion by antigen-specific T cells

Heat-killed *B. pertussis* was generated as follows: virulent *B. pertussis* (BP338) were grown in Stainer–Scholte liquid medium for 48 h at 37 °C. The cultures were centrifuged and resuspended at 1×10^{10} bacteria per mL in 1% casein and subsequently heated to 65 °C for 30 min. *Bordetella pertussis*-challenged mice were injected with 4×10^9 heat-killed *B. pertussis*, at various time points post challenge, and sacrificed by cervical dislocation 4 h later. Leucocytes were isolated from digested lungs and incubated overnight at 37 °C in complete medium, supplemented with 2 $\mu\text{g mL}^{-1}$ of brefeldin A (Sigma). After washing, these cells were fixed and permeabilized using permeabilization buffers as part of the FoxP3 staining kit (eBioscience) and incubated at room temperature in the presence of the relevant antibody.

PKH labelling of CD25⁻ Treg cells and subsequent adoptive transfer

CD4⁺CD25⁻CD45RB^{low} Treg cells were purified from the spleens and lymph nodes of naive Balb/c mice and subsequently labelled with 2 μM PKH67 (Sigma). Mice were injected i.p. with 5×10^5 PKH67-labelled Treg cells prior to aerosol challenge with *B. pertussis*. The lungs of infected mice were removed, and the phenotype of the labelled Treg cells was determined by flow cytometry 1, 7 and 14 days post challenge.

Statistical analysis

All statistical analysis was performed using GRAPHPAD Prism software (Version 5.0a). Functionality was compared between study groups using nonparametric tests: Mann–Whitney for comparing only two groups and Kruskal–Wallis followed by a Dunn's test for multiple comparisons comparing three or more groups.

Results

CD4⁺FoxP3⁺CD25⁻ cells are the dominant Treg cell population in the lung

A number of studies have reported that CD4⁺ T cells that lack CD25 (CD4⁺CD25⁻) isolated from secondary lymphoid tissue have suppressive activity (Annacker *et al.*, 2001; Lehmann *et al.*, 2002; Ono *et al.*, 2006); however, a functional role of such cells in the lung has yet to be determined. In this study, we isolated leucocytes from the spleen and lungs of naive mice and stained them with antibodies specific for CD4, CD25 and FoxP3. In general, between 5% and 10% of CD4⁺ T cells in the spleen

express FoxP3. In the example shown here, 6% of CD4⁺ T cells isolated from the spleen of naive mice expressed FoxP3, and the majority of these CD4⁺FoxP3⁺ cells expressed CD25 (Fig. 1a). In contrast, the majority of CD4⁺FoxP3⁺ T cells in the lung did not express CD25, when compared with CD4⁺FoxP3⁺ T cells isolated from the spleen (Fig. 1b).

We next investigated whether other mucosal tissues, particularly those associated with tolerance induction and immune homeostasis (Crispe, 2009; Hill & Artis, 2010) also contained a higher frequency of CD4⁺FoxP3⁺CD25⁻ T cells, when compared with secondary lymphoid tissues such as the spleen. Phenotypic analysis revealed that the percentage of CD4⁺FoxP3⁺CD25⁻ T cells was significantly

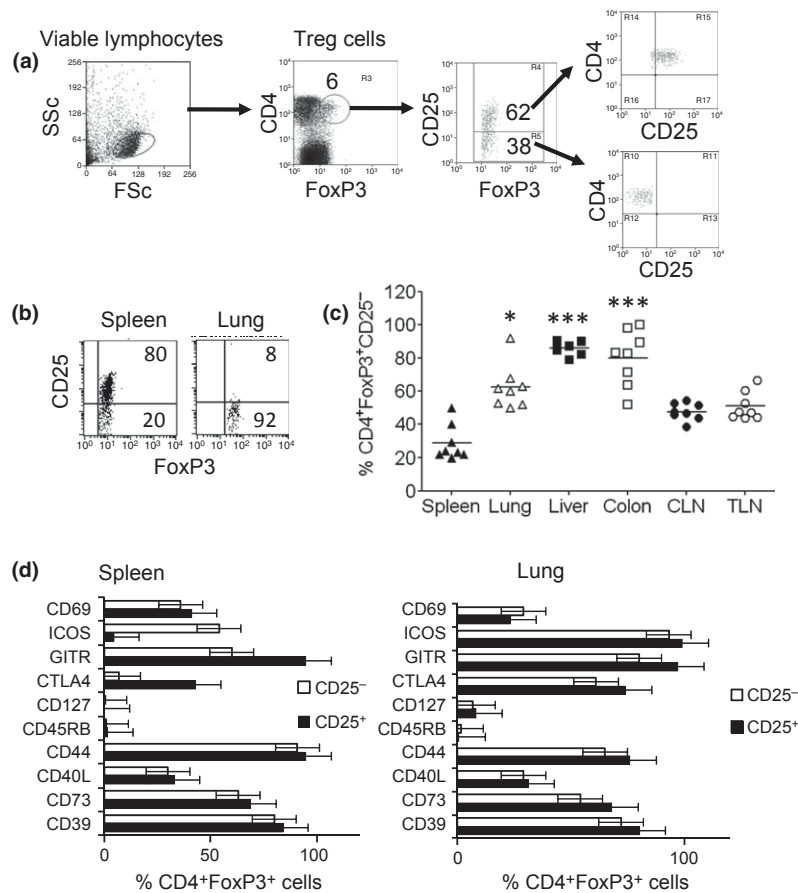


Fig. 1. The majority of CD4⁺FoxP3⁺ T cells in the lungs and other mucosal sites are CD25⁻. Lymphocytes were isolated from the digested spleen, lung, liver, colon, CLN or TLN of naive mice and labelled with monoclonal antibodies directed against CD25 and a number of other surface antigens as indicated, and intracellular CTLA-4 and FoxP3. (a) Flow cytometry gating strategy for the identification of Treg cells. Viable lymphocytes were identified by their position in a viable forward by side scatter plot. Regulatory T cells were identified as CD4⁺FoxP3⁺ cells based on the viable lymphocyte gate while CD25 expression on FoxP3⁺ lymphocytes was examined by gating on the CD4⁺FoxP3⁺ Treg gate. CD4⁺FoxP3⁺ Treg cells were subsequently compartmentalized based on CD25 expression. (b) Representative dot plots showing the percentage of CD4⁺FoxP3⁺CD25⁻ and CD4⁺FoxP3⁺CD25⁺ cells (based on the viable CD4⁺FoxP3⁺ gate) from the spleen and lungs of a naive mouse. (c) The percentage of viable CD4⁺FoxP3⁺CD25⁻ cells within the CD4⁺FoxP3⁺ gated population from spleens, lungs, colons, CLN or TLN of naive mice ($n = 8$) (main $*P < 0.05$; $***P < 0.001$ vs. the percentage of CD4⁺FoxP3⁺CD25⁻ cells in the spleen; $n = 8$). (d) Phenotypic analysis of CD4⁺FoxP3⁺ cells from the digested spleen and lungs of naive mice ($n = 15$).

greater in the liver, colon and lungs when compared with the spleen (Fig. 1c). A thorough flow cytometric analysis of CD4⁺FoxP3⁺ cells isolated from either the lungs or spleens of naive mice revealed very little difference phenotypically between CD4⁺FoxP3⁺CD25⁺ and CD4⁺FoxP3⁺CD25⁻ T cells (Fig. 1d). However, when compared with Treg cells from the spleen, CD4⁺FoxP3⁺ cells from the lung (both CD25⁺ and CD25⁻) expressed more inducible T cell co-stimulator (ICOS). Furthermore, cytotoxic T-lymphocyte-associated molecule-4 (CTLA-4) expression was higher on CD4⁺FoxP3⁺CD25⁻ cells from lung compared with the spleen. This may reflect the requirement for a regulatory phenotype to maintain tolerance in the lungs. Both CD4⁺FoxP3⁺CD25⁺ and CD4⁺FoxP3⁺CD25⁻ T cells isolated from the lung expressed canonical Treg cell markers, including glucocorticoid-induced tumour necrosis factor receptor family-related gene (GITR), and CD39 (Fig. 1d).

Pre-activated CD4⁺FoxP3⁺CD25⁻ T cells suppress CD4⁺ T cells in part via IL-10

CD4⁺CD45RB^{low} cells have been shown to be mainly comprised of FoxP3⁺ nTreg cells in healthy naive mice and can be used to ameliorate colitis in mice, while CD4⁺CD45RB^{high} cells represent naive effector cells capable of inducing colitis in immunocompromised mice (Powrie *et al.*, 1994; Maloy & Powrie, 2001). As almost 100% of CD4⁺CD45RB^{low} cells are FoxP3⁺, we used CD45RB as a FoxP3 surrogate marker when sorting Treg populations. A proportion of the sorted CD4⁺CD45RB^{low}CD25⁻ cells were always tested for FoxP3 expression after each sort; these sorted cells were routinely > 98% for FoxP3. Therefore, the CD25⁻ T cells sorted from either spleen or lungs were almost exclusively CD4⁺FoxP3⁺, and these cells were used in suppression assays *in vitro*.

Zelenay and colleagues have demonstrated that CD4⁺FoxP3⁺CD25⁻ Treg cells must be pre-activated prior to exerting their suppressive effect *in vitro* (Zelenay *et al.*, 2005). The authors suggest that CD25⁻ Treg cells might have a higher threshold of activation than nTreg cells (Zelenay *et al.*, 2005). In the present study, we sorted CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells from naive murine lungs and pre-activated with immobilized anti-CD3 (1 µg mL⁻¹), recombinant IL-2 (20 U mL⁻¹) and irradiated splenic APC (Supporting Information, Fig. S1). Unlike nTreg cells, CD25⁻ Treg cells were not hyperproliferative in response to exogenous IL-2 (data not shown). After 5 days, CD4⁺CD45RB^{high}CD25⁻ naive effector T cells (these effector cells do not express FoxP3) were sorted from naive murine spleens and activated with immobilized anti-CD3 (10 µg mL⁻¹) and irradiated APC in the absence or presence of pre-activated CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells. After 48 h, the superna-

tants were harvested, and the concentrations of IL-10 and IFN-γ were quantified by ELISA. Cell proliferation was determined after 72 h by ³H-thymidine incorporation. Activated CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells significantly suppressed (*P* < 0.05) the proliferation of effector T cells when co-cultured in a 1 : 1 ratio (Fig. 2a). In addition, co-culture of activated CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells with effector T cells resulted in reduced IFN-γ (*P* < 0.05) and enhanced IL-10 (*P* < 0.01) (Fig. 2a).

The suppressive capacity of CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ Treg cells, isolated from the lungs of naive mice, was confirmed by examining CFSE loss on effector T cells *in vitro* (Fig. 2b). Freshly isolated CD4⁺CD25⁻CD45RB^{high} effector T cells were labelled with 0.5 µM CFSE and subsequently co-cultured with pre-activated CD25⁻ Treg cells (1 : 1) and activated in the presence of 10 µg mL⁻¹ immobilized anti-CD3 and 5 × 10⁵ irradiated APC for 5 days. Cells were subsequently stained with antibodies directed against surface, CD4 and CD25 as well as intracellular FoxP3, and CFSE loss was determined by flow cytometry. As expected, nonactivated control effector T cells (shaded histogram; MFI 3090) did not proliferate to any great extent, whereas activated effector T cells alone (dotted line; MFI 2860) did (Fig. 2b). However, pre-activated CD25⁻ Treg cells suppressed the proliferation of activated effector T cells (solid line) when compared with activated control cells (Fig. 2b).

In these experiments, there were <1% FoxP3⁺ cells in the sorted CD4⁺CD45RB^{high}CD25⁻ naive effector T cells. While co-culture and activation of effector T cell with CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells in a 1 : 1 ratio did result in reduced proliferation, the suppressive capacity of these cells was not as potent as seen when effector T cells were co-cultured with nTreg (CD25⁺) cells. CD25⁺ nTreg cells have been shown to suppress effector T cells *in vitro* at a 1 : 4 ratio or less (Takahashi *et al.*, 1998).

Therefore, the suppression observed when sorted CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells were activated in the presence of effector T cells was unlikely to be due to contaminating nTreg cells.

ICOS expression has been associated with IL-10 secretion by T cells in the lung (Akbari *et al.*, 2002). We found that CD4⁺FoxP3⁺CD25⁻ T cells from the lung express ICOS (Fig. 1c) while co-culture of pre-activated CD4⁺FoxP3⁺CD25⁻ T cells with effector T cells promoted IL-10 production (Fig. 2a). Based on these observations, we investigated whether blocking the IL-10 receptor with a blocking antibody could reverse the suppressive capacity of CD4⁺FoxP3⁺CD25⁻ T cells *in vitro*. Pre-activated lung CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells were co-activated with freshly isolated CD4⁺CD45RB^{high}CD25⁻ effector T cells in the absence or presence of anti-IL-10 receptor

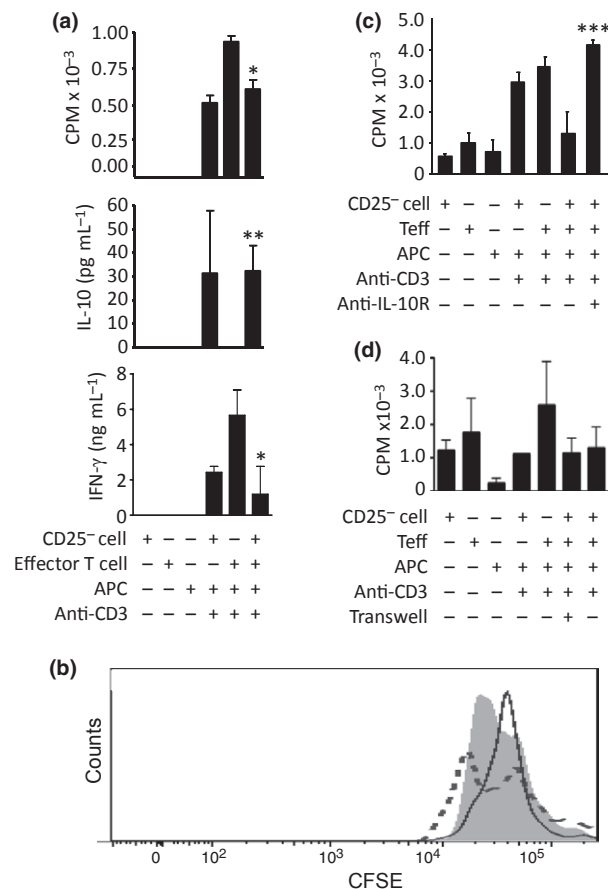


Fig. 2. Lung CD4⁺FoxP3⁺CD25⁻ T cells suppress CD4⁺ effector T cells via IL-10 *in vitro*. CD4⁺CD25⁻CD45RB^{low} cells (CD25⁻ cells) and CD4⁺CD25⁻CD45RB^{high} (effector T cell) cells were sorted from the enzymatically digested lungs of naive mice. (a) CD4⁺CD25⁻CD45RB^{low} cells (0.1×10^5) were pre-activated with $1 \mu\text{g mL}^{-1}$ immobilized anti-CD3 and irradiated splenocytes (0.5×10^6) in the presence of 20 U mL^{-1} IL-2 for 5 days, washed and reactivated with $10 \mu\text{g mL}^{-1}$ immobilized anti-CD3 and irradiated APC (0.5×10^6) in the presence of CD4⁺CD25⁻CD45RB^{high} cells (0.1×10^5). IL-10 and IFN-γ concentrations were determined in supernatants 48 h later by ELISA. Proliferation was determined by ³H-thymidine incorporation after 72 h. (* $P < 0.05$, ** $P < 0.01$ vs. anti-CD3-activated effector T cells plus irradiated APC). (b) Freshly isolated CD4⁺CD25⁻CD45RB^{high} effector T cells were labelled with $0.5 \mu\text{M}$ CFSE and subsequently co-cultured with pre-activated CD25⁻ Treg cells (1 : 1) and activated in the presence of $10 \mu\text{g mL}^{-1}$ immobilized anti-CD3 and 5×10^5 irradiated APC for 5 days. Cells were subsequently stained with antibodies directed against surface, CD4 and CD25 as well as intracellular FoxP3, and CFSE loss was determined by flow cytometry. Histogram shows CFSE loss by nonactivated control effector T cells (shaded histogram), activated effector T cells alone (dotted line) and effector T cells activated in the presence of CD25⁻ Treg cells (solid line). (c) Pre-activated CD4⁺FoxP3⁺CD25⁻ T cells were cultured with effector T cells as described in (a) with the addition of $10 \mu\text{g mL}^{-1}$ of anti-IL-10R (** $P < 0.001$ anti-IL-10R vs. cells activated in the presence of isotype control antibody). (d) Pre-activated CD4⁺CD25⁻CD45RB^{low} T cells were cultured with CD4⁺CD25⁻CD45RB^{high} effector T cells, together or separated by a transwell semi-permeable membrane, and all cells were reactivated with anti-CD3, and proliferation was determined. Results are representative of four separate experiments.

(IL-10R). Blocking the IL-10R significantly restored the proliferative capacity of effector T cells ($P < 0.001$) when compared with control cells incubated with an irrelevant isotype-matched antibody (Fig. 2c).

We next investigated whether the *in vitro* suppression observed in these experiments was cell contact mediated. Pre-activated CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells were co-incubated with effector T cells but separated via a semi-permeable membrane. Each T cell population above and below the semi-permeable membrane was activated

with immobilized anti-CD3 and irradiated splenic APC. Proliferation was measured by ³H-thymidine incorporation after 72 h. The results show that despite the absence of cell–cell contact, CD4⁺CD45RB^{low}(FoxP3⁺)CD25⁻ cells were able to suppress proliferation of effector T cells *in vitro* (Fig 2d).

Based on these findings, we propose that CD4⁺FoxP3⁺CD25⁻ T cells from the lung can suppress T cell activation *in vitro* and that this is mediated in part by IL-10. Furthermore, we believe that these cells are not

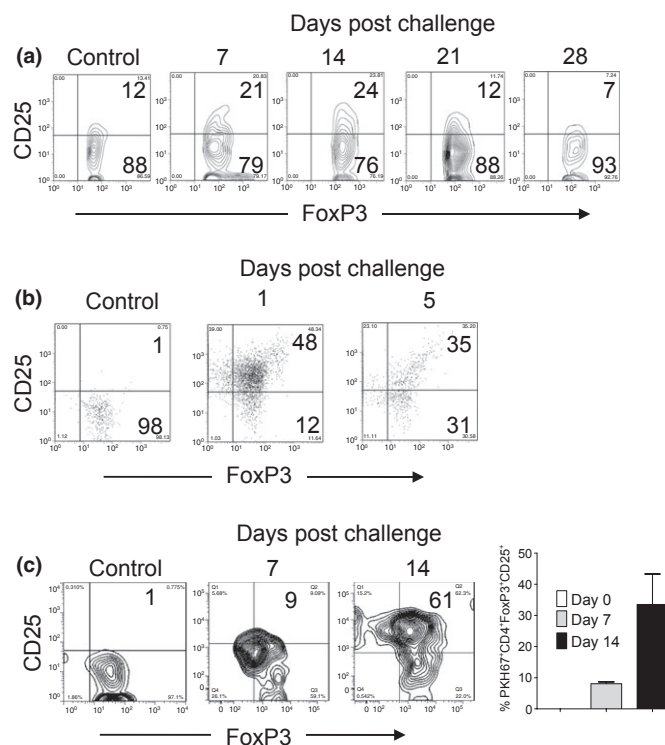


Fig. 3. CD25 upregulation on CD4⁺FoxP3⁺CD25⁻ T cells *in vitro* and *in vivo*. Mice were infected by exposure to an aerosol of live virulent *Bordetella pertussis*. Lymphocytes were isolated from the digested lungs of mice prior to challenge (control) and 7, 14, 21 and 28 days after challenge. Lymphocytes were subsequently labelled with monoclonal antibodies directed against CD4, CD25 and intracellular FoxP3. (a) Representative dot plots showing the percentage of FoxP3⁺CD25⁺ cells gated on total viable CD4⁺ T cells, at the indicated time points after challenge. (b) CD4⁺CD25⁻CD45RB^{low} cells (0.1×10^6) sorted from the digested lungs of naive mice were activated *in vitro* with $1 \mu\text{g mL}^{-1}$ immobilized anti-CD3 and irradiated spleen cells (0.5×10^6). (b) Representative dot plots showing CD25 expression on CD4⁺FoxP3⁺ T cells based on the total viable CD4⁺ T cell gate, on days 1 and 5 postactivation. (c) CD4⁺CD25⁻CD45RB^{low}(FoxP3⁺) Treg cells were purified from the spleens and lymph nodes of naive Balb/c mice and subsequently labelled with $2 \mu\text{M}$ PKH67. Mice were injected i.p. with 5×10^5 PKH67-labelled Treg cells prior to aerosol challenge with *B. pertussis*. The lungs of infected mice were removed, and the phenotype of the labelled Treg cells was determined by flow cytometry on days 1, 7 and 14 post challenge. (c) Representative dot plots show the percentage of FoxP3⁺CD25⁺ cells within the viable PKH67⁺CD4⁺ T cell gate. Bar chart displays average percentage PKH67⁺CD4⁺CD25⁺FoxP3⁺ cells values based on three mice.

nTreg cells because they require pre-activation to function, their suppressive capacity is not mediated by cell contact, but in part via IL-10, and they do not express CD25 in a quiescent state.

Lung CD4⁺FoxP3⁺CD25⁻ T cells upregulate CD25 upon activation *in vitro* and *in vivo*

We next investigated whether CD4⁺FoxP3⁺CD25⁻ T cells would remain the dominant Treg cell in the lung throughout the course of infection with *B. pertussis*. Mice were infected with *B. pertussis*, and lung cells were examined at intervals post challenge. The percentage of CD4⁺FoxP3⁺CD25⁺ cells increased to 24% by day 14 post challenge (Fig. 3a). Interestingly, the percentage of CD4⁺FoxP3⁺CD25⁺ cells returned to pre-infection levels (7%) by day 28 post challenge (Fig. 3a). The percentage of total CD4⁺CD25⁺ and CD4⁺FoxP3⁺ cells, based on the

viable CD4⁺ T cell gate, remained relatively unchanged throughout infection (data not shown). It is unclear whether CD25 expression was induced on CD4⁺FoxP3⁺CD25⁻ cells in the lung or whether the increase in the frequency of CD25⁺ T cells is as a result of infiltrating nTreg cells. Subsequent investigation revealed that CD25 expression could be induced on CD4⁺FoxP3⁺CD25⁻ cells isolated from the lungs of naive mice, following activation with anti-CD3 *in vitro* (Fig. 3b), suggesting that CD4⁺FoxP3⁺CD25⁻ might convert to CD4⁺FoxP3⁺CD25⁺ following activation during infection in the lungs.

CD4⁺CD25⁻CD45RB^{low}(FoxP3⁺) Treg cells were purified from the spleens and lymph nodes of naive Balb/c mice and subsequently labelled with $2 \mu\text{M}$ PKH67 to investigate whether CD4⁺FoxP3⁺CD25⁻ Treg cells could upregulate CD25 *in vivo*. Mice were injected i.p. with 5×10^5 PKH67-labelled Treg cells prior to aerosol

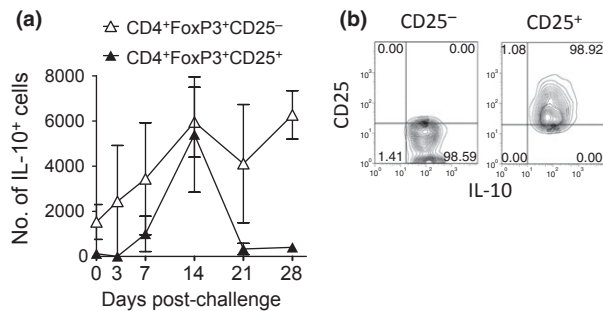


Fig. 4. Transient expression of CD25 and IL-10 by CD4⁺FoxP3⁺ T cells during infection with *Bordetella pertussis*. Mice were infected by exposure to an aerosol of live virulent *B. pertussis*. Lymphocytes were isolated from the lungs of mice prior to challenge (control) and 3, 7, 14, 21 and 28 days after challenge. (a) Antigen-specific IL-10-secreting CD4⁺FoxP3⁺CD25⁻ and CD4⁺FoxP3⁺CD25⁺ cells were quantified by *ex vivo* ICS, performed at intervals after *B. pertussis* challenge. Mice were injected i.p. with heat-killed *B. pertussis* 4 h and 7, 14, 21 and 28 day post challenge. After 4 h, leucocytes were isolated from digested lungs, and the frequency of IL-10⁺ cells was determined by flow cytometric analysis. The absolute number of cells was calculated from the total number of viable cells and the percentage of CD4⁺FoxP3⁺CD25⁻IL-10⁺ and CD4⁺FoxP3⁺CD25⁺IL-10⁺ cells within the viable lymphocyte gate. The peak percentage of CD4⁺FoxP3⁺CD25⁺IL-10⁺ T cells in the lungs of infected mice on day 14 post challenge is represented as a dot plot showing the frequency of CD25⁻IL-10⁺ and CD25⁺IL-10⁺ cells based on a viable CD4⁺FoxP3⁺ gate (b). Results are representative of three separate experiments.

challenge with *B. pertussis*. The lungs of infected mice were removed, and the phenotype of the labelled Treg cells was determined by flow cytometry on days 1, 7 and 14 post challenge. No CD4⁺FoxP3⁺CD25⁺ Treg cells were detected in the lungs of infected mice 24 h post challenge (Fig. 3c). However, the percentage of CD4⁺FoxP3⁺CD25⁺ Treg cells increased on days 7 and 14 post challenge to 9% and 60%, respectively (Fig. 3c).

IL-10-producing CD4⁺FoxP3⁺CD25⁻ cells in the lungs of mice infected with *B. pertussis*

Having shown that IL-10 may be part of the mechanism of CD4⁺FoxP3⁺CD25⁻-induced suppression *in vitro* (Fig. 2), we examined IL-10 production by CD4⁺FoxP3⁺CD25⁻ cells from the lung throughout the course of infection with *B. pertussis*. We employed an *ex vivo* intracellular cytokine staining (ICS) assay for antigen-specific cytokine production using an approach modified from that described by Jenkins and colleagues (Merica *et al.*, 2000), which involved *in vivo* re-stimulation of the T cells with specific antigen prior to ICS. Mice were injected i.p. with heat-killed *B. pertussis* 4 h post challenge and on d 3, 7, 14, 21 and 28. After 4 h, leucocytes were isolated from digested lungs, and the frequency

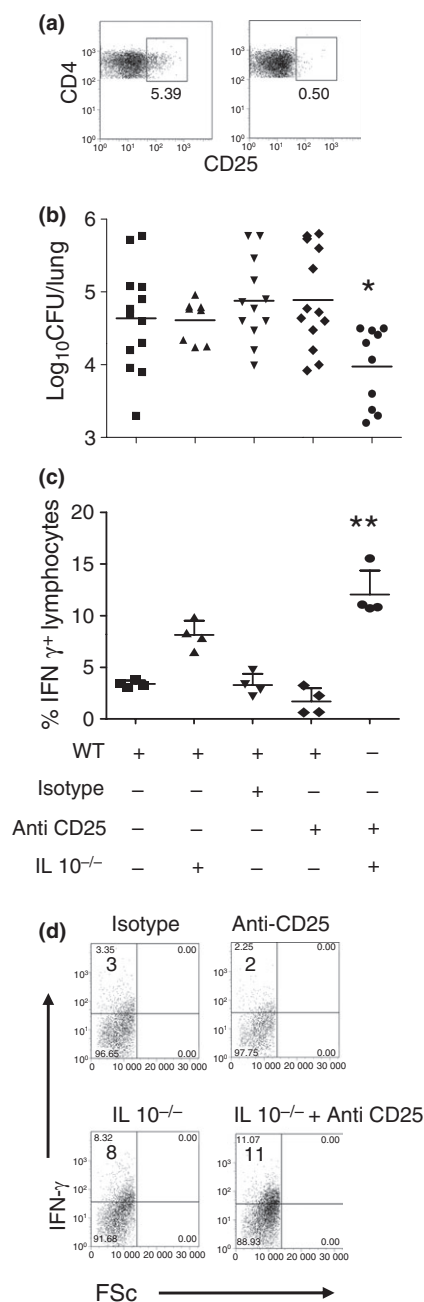
of IL-10⁺ cells was determined by flow cytometric analysis. Administration of heat-killed *B. pertussis* (i.p.) into noninfected mice had no impact on the dynamics or cytokine secretion profile of CD4⁺FoxP3⁺CD25⁻ T cells in lung when compared with treated, infected controls (data not shown). The absolute number and percentage of CD4⁺FoxP3⁺CD25⁺IL-10⁺ cells peaked by day 14 post challenge and diminished significantly thereafter (Fig. 4a and b). Conversely, the number of CD4⁺FoxP3⁺CD25⁻IL-10⁺ cells remained stable up to at least 28 days, when the experiment was terminated (Fig. 4a and b). It is clear from these results that both CD25⁻ and CD25⁺ Treg cells can produce IL-10 in the lung during bacterial infection, with the former secreting significantly ($P < 0.01$) more than the CD25⁺ Treg cells during the later stages post challenge.

Thus far, we have shown that co-culture of lung CD4⁺FoxP3⁺CD25⁻ cells with effector T cells results in enhanced IL-10 secretion and that blocking the IL-10 receptor inhibits CD4⁺FoxP3⁺CD25⁻-mediated suppression of effector T cell proliferation *in vitro*. Furthermore, CD4⁺FoxP3⁺CD25⁻ cells produce intracellular IL-10 throughout the course of infection with *B. pertussis*. These findings suggest that IL-10-secreting CD4⁺FoxP3⁺CD25⁻ cells might play a role in immune regulation in the lungs of naive and infected mice.

Depleting CD25⁺ cells in IL-10-deficient mice enhances bacterial clearance in infected mice

CD4⁺FoxP3⁺CD25⁻ T cells might act as a reservoir of quiescent Treg cells in normal naive mice (Zelenay *et al.*, 2005). If this is the case, then depletion of CD25⁺ nTreg cells prior to infection should not have a significant impact on the course of infection with *B. pertussis*. To test this hypothesis, mice were injected i.p. with 300 µg of anti-CD25 depleting antibody 24 h prior to challenge with *B. pertussis*. Injection of this antibody routinely caused a significant reduction in the percentage of CD25⁺ cells *in vivo* (Fig. 5a). CD25 depletion was confirmed using an alternative anti-CD25 antibody (7D4), specific for a different epitope to that detected by the depleting anti-CD25 (PC61). Mice were only given one injection of anti-CD25 depleting antibody so as not to interfere with infiltrating activated CD25⁺ effector T cells, which are required to clear infection.

On the basis of these results, we speculated that CD25⁺ nTreg cells and IL-10-secreting CD4⁺FoxP3⁺CD25⁻ T cells might play interchangeable, redundant regulatory roles in the lung. To address this possibility, we investigated whether CD25⁺ nTreg cells would become the dominant regulatory subset in the lung in the absence of IL-10. There was no significant difference



in bacterial burden following challenge with *B. pertussis* in mice those received an irrelevant isotype-matched control antibody or those received anti-CD25 depleting antibody (Fig. 5b). Furthermore, there was no difference in the CFU counts between IL-10^{-/-} and WT mice (Fig. 5b). In contrast, depleting CD25⁺ cells in IL-10^{-/-} mice prior to infection resulted in a significant reduction in the bacterial load ($P < 0.05$) 7 days after *B. pertussis* challenge (Fig. 5b). In addition, the percentage of total IFN-γ⁺ leucocytes was significantly higher

Fig. 5. Enhanced immunity to *Bordetella pertussis* in IL-10^{-/-} mice depleted of CD25⁺ cells. C57BL/6 wild-type (WT) or IL-10^{-/-} mice were injected i.p. with 300 μg of anti-CD25 depleting antibody or anti-HRPN isotype (IgG₁) control antibody 24 h prior to challenge with *B. pertussis*. (a) Representative dot plot of CD4⁺CD25⁺ cells in spleen isolated from a naive mouse before (left plot) and 24 h after (right plot) i.p. injection with anti-CD25 depleting antibody. CD25 expression was determined using an alternative conjugated anti-CD25 antibody (7D4) directed against a different epitope to that of the depleting antibody (PC61). (b) CFU counts were performed on the lungs of wild-type mice, anti-HRPN isotype (IgG₁) control antibody-treated wild-type, anti-CD25 antibody-treated mice, IL-10^{-/-} mice and anti-CD25-treated/IL-10^{-/-} mice 7 days after aerosol challenge with live *B. pertussis* (main $P < 0.05$; *post hoc* test $*P < 0.01$, infected CD25-depleted/IL-10^{-/-} vs. infected isotype control). (c) The percentage of IFN-γ⁺ lymphocytes based on the viable lymphocyte gate was examined in cells isolated from the CLN of infected mice 7 days after aerosol challenge with live *B. pertussis* (main $P < 0.05$; *post hoc* test $**P < 0.001$, infected CD25-depleted/IL-10^{-/-} vs. infected isotype control). (d) Representative dot plots showing total IFN-γ⁺ lymphocytes (based on the viable lymphocyte gate) of cells isolated from the CLN of infected wild-type and IL-10^{-/-} mice treated with either isotype control or anti-CD25. Representative data of three separate experiments.

($P < 0.01$) in cells isolated from the CLN of CD25-depleted IL-10^{-/-} mice when compared with either wild-type, CD25-depleted wild-type or IL-10^{-/-} mice 7 days post challenge (Fig. 5c and d). These results indicate that both CD25⁺ nTreg cells and FoxP3⁺CD25⁺ IL-10-producing regulatory cells may have a role to play in regulating immunity in the lung.

Discussion

This study demonstrates that CD4⁺FoxP3⁺CD25⁺ T cells are the dominant FoxP3-expressing Treg cell at mucosal surfaces, including the lung. In an infection model, we demonstrate that clearance of *B. pertussis* from the lungs was enhanced by depletion of CD25 cells in IL-10^{-/-} mice. Finally, we believe that these cells are not nTreg cells because they require pre-activation to function, their suppressive capacity is not mediated by cell contact, and they do not express CD25 in a quiescent state.

We have shown that CD4⁺FoxP3⁺CD25⁺ T cells are the dominant FoxP3⁺ Treg cell not only in the lung, but also in the liver and colon of naive mice. Using a mouse model of infection with *B. pertussis*, we found that the absolute numbers of CD4⁺FoxP3⁺CD25⁺ T cells increased throughout the course of infection. There was also an increase in the absolute numbers of CD4⁺FoxP3⁺CD25⁺ T cells, but this was transient and confined to the peak of infection. Lung CD4⁺FoxP3⁺CD25⁺ T cells expressed surface ICOS, as well as expressing the definitive Treg

markers including GITR and CTLA-4. ICOS expression has been associated with IL-10-mediated suppression of murine CD4⁺ effector T cells by FoxP3⁺ Treg cells *in vitro* and *in vivo* (Kohyama *et al.*, 2004). Blocking ICOS signalling impaired this IL-10-mediated suppression (Kohyama *et al.*, 2004). Furthermore, two discrete FoxP3⁺ Treg subsets have been described in humans, based on surface ICOS expression; CD4⁺CD25⁺ICOS⁺ Treg cells suppressed APC via IL-10, while CD4⁺CD25⁺ICOS⁻ Treg-mediated inhibition of effector T cells was dependent on TGF- β (Ito *et al.*, 2008). Our study suggests that IL-10 plays a primary role in CD4⁺FoxP3⁺CD25⁻ Treg-mediated suppression. IL-10-producing CD4⁺FoxP3⁺CD25⁺ T cells were also detected in infected lungs, and it is possible that these represent activated resident CD4⁺FoxP3⁺CD25⁻ T cells, with upregulated CD25 expression. We also show here that the CD25⁻ Treg cells used in this study can upregulate CD25 expression in response to anti-CD3 *in vitro* and bacterial infection *in vivo*.

CD4⁺FoxP3⁺CD25⁻ T cells isolated from the lungs suppressed CD4⁺ effector T cells *in vitro*, which was reversed by antagonistic IL-10R antibody and was not dependent on cell contact. Furthermore, IL-10-secreting CD4⁺ T cells accumulated in the lungs of mice infected with *B. pertussis*. However, apart from a transient increase in CD4⁺FoxP3⁺CD25⁺ T cells around the peak of infection, the dominant population of Treg cells were CD4⁺FoxP3⁺CD25⁻ cells. Depletion of CD25⁺ cells using PC61 antibody has been shown to have a profound effect on host immunity to infection and tumours (Onizuka *et al.*, 1999; Quinn *et al.*, 2006). This supports the view that CD4⁺FoxP3⁺CD25⁺ plays a critical role in regulating effector and pathogenic T cell responses (Shimizu *et al.*, 1999). However, we found that depletion of CD25⁺ cells had little impact on the course of *B. pertussis* infection in the lungs. This is consistent with the reports showing that CD25 depletion had little bearing on lung bacterial burden or infection-induced pathology after intranasal challenge with *M. tuberculosis* or *Mycobacterium bovis* (Quinn *et al.*, 2006). Furthermore, CD25 depletion did not alter cytokine and chemokine secretion, neutrophil infiltration, bacterial clearance survival of mice following lung infection with *Pseudomonas aeruginosa* (Carrigan *et al.*, 2009). In addition, CD25⁺ Treg cells might not be essential to the control of parasitic infection (Couper *et al.*, 2007; Walsh *et al.*, 2007). Couper *et al.* (2007) showed a rapid proliferation of CD25⁻FoxP3⁺ cells (and subsequent upregulation of CD25) in CD25-depleted mice infected with *Plasmodium yoelii*, adding strength to the theory that depleting CD25⁺ cells does not remove the capacity for immunoregulation. Our results also showed that protective immunity in the lung was not compromised in IL-10^{-/-} mice, but interestingly, the clear-

ance of *B. pertussis* was significantly enhanced following depletion of CD25 cells in IL10^{-/-} mice. This suggests both CD25⁺ nTreg cells and IL-10-producing cells might play complimentary and redundant roles in regulating immunity to infection in the lungs.

It has been suggested that CD4⁺FoxP3⁺CD25⁻ T cells in secondary lymphoid organs and in the periphery might act as a reservoir of redundant quiescent Treg cells and that CD25 expression is merely a marker of activation (Zelenay *et al.*, 2005). There is evidence to suggest that in certain cases of autoimmunity, CD4⁺FoxP3⁺CD25⁻ Treg cells are expanded in an attempt to curtail an already out of control immune response. Phenotypic analysis of peripheral blood mononuclear cells from patients with systemic lupus erythematosus revealed increased proportions of CD4⁺FoxP3⁺CD25⁻ T cells when compared with patients with systemic sclerosis, rheumatoid arthritis or healthy controls cells (Bonelli *et al.*, 2009). These CD4⁺FoxP3⁺CD25⁻ T cells resembled Treg cells rather than activated T cells and were only partially functional in suppression assays when compared with nTreg cells (Bonelli *et al.*, 2009). In a study of multiple sclerosis, patients in remission were shown to have normal levels of nTreg cells, but relapsing patients had an increased proportion of systemic CD4⁺FoxP3⁺CD25⁻ Treg cells, and these Treg cells were able to suppress effector T cells as effectively as nTreg cells *in vitro* (Fransson *et al.*, 2010).

Our findings demonstrate that CD4⁺FoxP3⁺CD25⁻ T cells with regulatory function are the dominant FoxP3⁺ cell in the lungs of mice. These Treg cells have a higher threshold of activation than nTreg cells and might act as an ancillary regulatory arm in the lung, which work with IL-10 to maintain immune tolerance and control immunity to infection.

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Authors' contribution

P.J.D., K.H.G.M. and J.K. contributed equally to this work.

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