

CD4⁺FoxP3⁺ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection

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The opportunistic fungal pathogen *Cryptococcus neoformans* causes lung inflammation and fatal meningitis in immunocompromised patients. Regulatory T (Treg) cells play an important role in controlling immunity and homeostasis. However, their functional role during fungal infection is largely unknown. In this study, we investigated the role of Treg cells during experimental murine pulmonary *C. neoformans* infection. We show that the number of CD4⁺FoxP3⁺ Treg cells in the lung increases significantly within the first 4 weeks after intranasal infection of BALB/c wild-type mice. To define the function of Treg cells we used DEREK mice allowing selective depletion of CD4⁺FoxP3⁺ Treg cells by application of diphtheria toxin. In Treg cell-depleted mice, stronger pulmonary allergic inflammation with enhanced mucus production and pronounced eosinophilia, increased IgE production, and elevated fungal lung burden were found. This was accompanied by higher frequencies of GATA-3⁺ T helper (Th) 2 cells with elevated capacity to produce interleukin (IL)-4, IL-5, and IL-13. In contrast, only a mild increase in the Th1-associated immune response unrelated to the fungal infection was observed. In conclusion, the data demonstrate that during fungal infection pulmonary Treg cells are induced and preferentially suppress Th2 cells thereby mediating enhanced fungal control.

Keywords: *Cryptococcus neoformans* · Pulmonary fungal infection · Regulatory T (Treg) cells · Th2 immunity



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Introduction

Cryptococcus neoformans is a globally distributed, opportunistic fungal pathogen. Primary infection most commonly occurs via inhalation of spores or infectious propagules which encounter alveolar macrophages or dendritic cells in the lung and trigger

an immune response [1, 2]. In immunocompromised patients, the inability to control the infection by macrophages readily leads to systemic dissemination with subsequent development of fatal meningoencephalitis [3]. As a consequence, cryptococcal meningitis accounts for the majority of worldwide deaths from HIV-related fungal infections [4, 5].

It has been well established that the efficient control of *C. neoformans* requires a T helper cell (Th)1-immune response [6, 7]. Mice lacking the Th1-type cytokines interferon- γ (IFN- γ) or interleukin (IL)-12 show a decreased survival rate [8, 9].

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In contrast, the loss of hallmark Th2-type cytokines such as IL-4 and IL-13 (or their common IL-4 receptor) confers protection and promotes survival [10–13]. Less is known regarding the role of Th17-type responses during cryptococcosis. However, Th17 cells tend to be of limited importance in cryptococcosis [14–16] compared with other fungal infections where Th17 immunity is crucial [17]. Different kinetic analyses of Th cell development during cryptococcal infection showed that both Th1 and Th2 immune responses can be simultaneously induced [14, 15, 18]. Experimental infection of mice with *C. neoformans* leads to an increasing detrimental Th2 immune response resulting in death of the animals although the Th1 response is still present. Another subset of Th cells has been characterized by the expression of the transcription factor forkhead box P3 (FoxP3), i.e. regulatory T (Treg) cells [19, 20]. Treg cells play an important role in regulating the homeostasis of the immune system and mutations in the FoxP3 gene result in autoimmunity both in humans and mice [21, 22]. In addition, Treg cells have been shown to limit pathogen-specific T-cell responses in acute [23–26] as well as in chronic infections [27, 28]. So far, the role of CD4⁺FoxP3⁺ Treg cells during cryptococcal infection is unknown. Also, it is unknown whether Treg cells would target all of the Th subpopulations known to be induced by *C. neoformans* infection.

Here we show for the first time that Treg cells are essential to suppress Th2-type inflammatory responses and thereby control fungal growth during pulmonary *C. neoformans* infection.

Results

CD4⁺FoxP3⁺ regulatory T cells increase early during pulmonary infection with *C. neoformans*

Experimental pulmonary infection with the opportunistic fungal pathogen *C. neoformans* revealed a protective role of Th1 and Th17 immunity, while a Th2-biased immune response is detrimental [29]. The role of Treg cells during cryptococcal infection is hitherto unknown. To determine the role of Treg cells during pulmonary cryptococcosis, we infected wild-type BALB/c mice intranasally with *C. neoformans* and characterized Treg cells in the lung at different time points postinfection. Using flow cytometry we found a significant increase in the relative and absolute numbers of CD4⁺FoxP3⁺ Treg cells during the first 4 weeks of infection which remained elevated for the duration of this study (Fig. 1). These findings show that pulmonary Treg cells from *C. neoformans*-infected mice increase and may have an important function during fungal infection.

Strong allergic inflammation and decreased control of *C. neoformans* in the absence of Treg cells

We analyzed the potential impact of Treg cells on the progression of pulmonary cryptococcosis using DEREg (DEpletion of REgulatory T cells) mice. In DEREg mice CD4⁺FoxP3⁺ Treg cells can

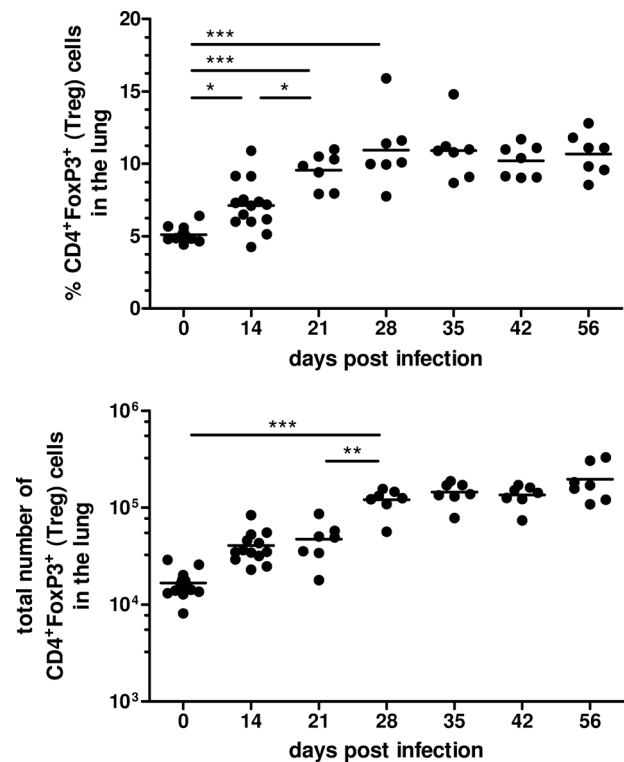


Figure 1. CD4⁺FoxP3⁺ Treg cells in the lungs of *C. neoformans*-infected mice increase in their relative and total number. BALB/c wild-type mice were intranasally infected with 500 colony forming units (CFU) of *C. neoformans* 1841 and sacrificed at the indicated time points postinfection. CD4⁺FoxP3⁺ Treg cells were identified by flow cytometric analysis of isolated lung leukocytes using a live cell and CD4⁺ T cell gate. Each data point represents one animal from one of two independent kinetic experiments. In each experiment at least seven mice per time point have been studied with partially overlapping time points (see day 0 and 14 postinfection). Statistical analysis was performed using one-way ANOVA with a Bonferroni test **p* < 0.05, ***p* < 0.01, and ****p* < 0.001.

be depleted by intraperitoneal administration of diphtheria toxin (DT) [30]. To delay the early increase of pulmonary Treg cells (see Fig. 1) we started treatment of infected DEREg mice at 7 dpi, i.e. at a time when both Th1- and Th2-immune responses have already started. We treated *C. neoformans*-infected DEREg mice as depicted in Figure 2A four times with DT (arrows), whereas control DEREg mice received PBS. After the last DT injection, peripheral Treg cell recovery took up to 14 days to fully reach the frequency seen in PBS-treated DEREg mice (Supporting Information Fig. 1). To take account of potential DT-dependent side-effects that are not related to Treg cell depletion we also treated infected WT mice with DT [31, 32].

The effects of Treg cell depletion on the inflammatory response were analyzed by histopathological examination of lung tissue (Fig. 2B). Prominent foamy macrophages and stronger pulmonary eosinophilia were seen in Treg cell-depleted DEREg mice where eosinophils formed clusters (Fig. 2B, circles) in contrast to control groups with only single eosinophils (Fig. 2B, black arrow heads). In addition, elevated epithelial mucus production was detected by PAS staining (Fig. 2B, insets). Quantification of pulmonary mucus

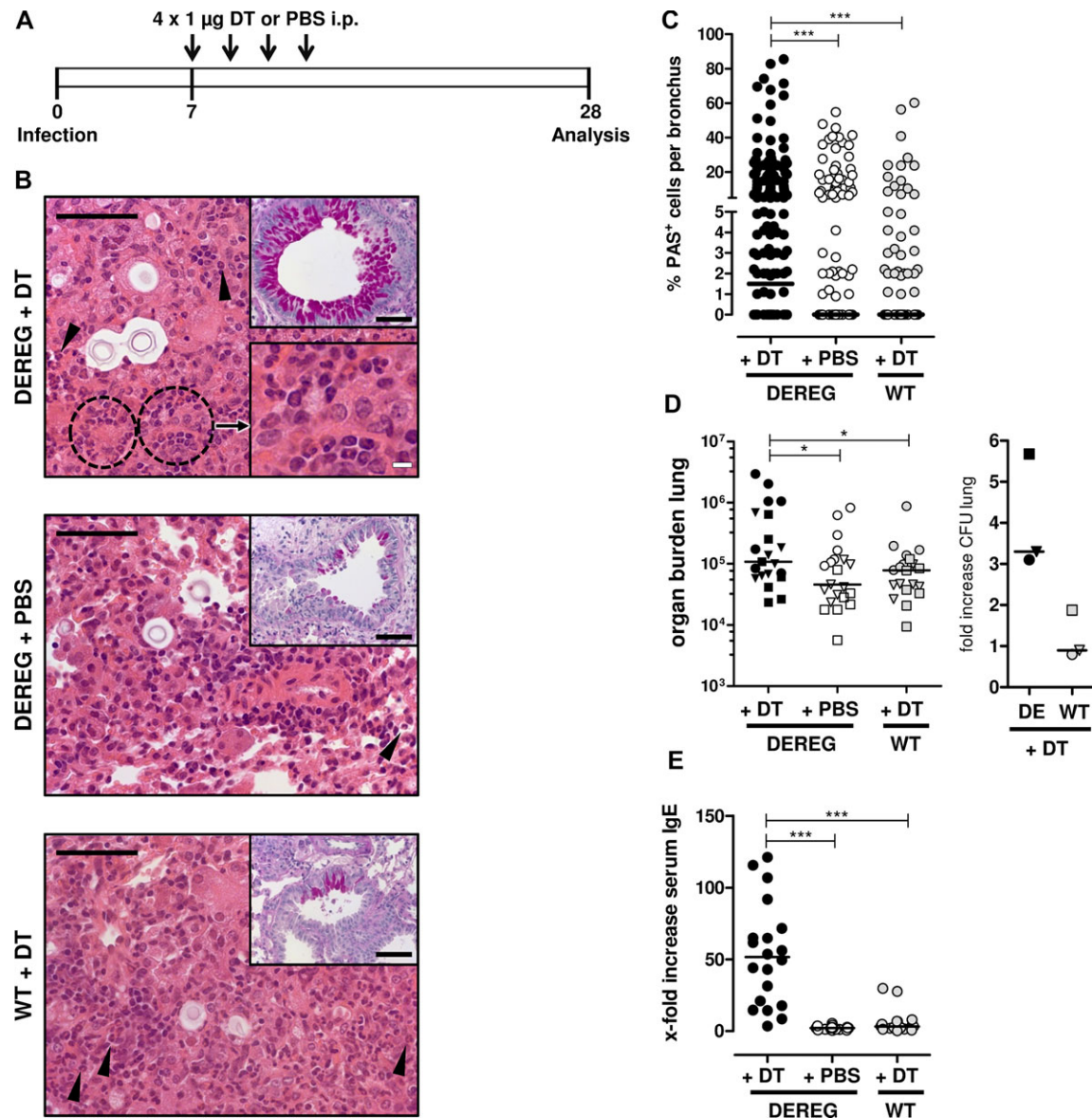


Figure 2. Increased allergic inflammation, higher fungal burden, and elevated IgE production following Treg cell depletion in *C. neoformans*-infected mice. (A) Schematic representation of the experimental set-up. DEREG mice were intranasally infected with 500 CFU of *C. neoformans* 1841. CD4⁺FoxP3⁺ Treg cell depletion was achieved by intraperitoneal (i.p.) administration of 1 μ g diphtheria toxin (DT) at 7, 9, 11, and 13 days postinfection (dpi). (B) Lung slices were obtained from mice 28 dpi and stained with H&E and periodic acid Schiff reagent (PAS) (black scale bar 100 μ m). Lung sections show inflammatory foci in Treg cell-depleted DEREG mice as well as in the control groups. Sites of inflammation containing single eosinophils (arrowheads) are shown. Groups of eosinophils (circles) were found in the absence of Treg cells (top panel), whereas only single eosinophils (arrowheads) can be found in the control groups (middle and bottom panel). An enhanced image showing one group of eosinophils is added in the lower right part of the top panel (white scale bar 10 μ m). Mucus production visualized by PAS staining is depicted in the upper right part of each picture (40x objective, scale bar 100 μ m). Each section is representative of 20–21 mice/group, representative of three independent experiments performed. (C) Mucus production was analyzed by quantification of PAS⁺ bronchial epithelial cells. Each symbol represents the percentage of PAS⁺ cells per bronchus. Ten bronchi per mouse from at least two different regions of the lung were counted, 20–21 mice/group, representative of three independent experiments performed. ***p < 0.001 (Mann-Whitney U-test). (D) Mice were treated as described in (A), sacrificed 28 dpi and the fungal growth in the lung was examined. As control an additional group of DEREG mice was treated with PBS. To control for potential toxic side-effects of DT, WT mice were treated with DT. For analysis of fungal lung burden related groups from three independent experiments (6–7 mice/group in each experiment) were pooled. Thus, each different type of symbol (circle, square, and triangle) represents a different experiment. The graph shows the medians as following pooling the single experiments (n = 20–21 mice/group) nonparametric analysis by Mann-Whitney U-test was required (although in each of the three individual experiments some groups passed the KS normality test). On the right part x-fold increase in fungal lung burden was determined from the medians of each single experiment normalized to the PBS-treated DEREG control group (DE: DEREG). Mann-Whitney U-test was used to determine statistical significance, *p < 0.05. (E) Mice were bled submandibular prior to infection and serum was analyzed for total IgE concentration. After infection and treatment with DT or PBS (see scheme in (A)) mice were sacrificed at 28 dpi and total IgE in the serum was determined by ELISA. The x-fold increase from individual mice (n = 20–21 mice/group pooled from three independent experiments performed) dividing the values from 28 dpi by those from preinfection is shown. Statistical analysis was done using Mann-Whitney test, ***p < 0.001.

production by counting PAS⁺ bronchial epithelial cells revealed a highly significant difference between DT-treated DERE mice and control groups (Fig. 2C). Together, the observed shift to allergic pulmonary inflammation indicates a pronounced Th2-biased immune response in Treg cell-depleted DERE mice.

To determine the role of Treg cells in fungal control we analyzed the organ burden following *C. neoformans* infection. Depletion of Treg cells resulted in a threefold higher fungal load in the lungs of DT-treated DERE mice compared with DT-treated WT control mice 28 days postinfection (dpi) (Fig. 2D). Fungal dissemination from lung to brain did not occur at 28 dpi (data not shown) as expected for this time point of analysis with the strain 1841 of *C. neoformans* used [33].

Since the histopathological analysis of lung tissue (Fig. 2B and C) pointed toward an elevated Th2-associated immune response in Treg cell-depleted DERE mice, IgE production as another effector function indicative of a Th2 bias was measured. Intranasal infection with *C. neoformans* induced an increase of serum IgE at 28 dpi in PBS-treated DERE mice and control DT-treated WT mice (data not shown). However, IgE serum concentration was elevated at a significantly higher degree (about 50-fold) after Treg cell depletion in DERE mice (Fig. 2E).

In conclusion, these data demonstrate that the transient absence of Treg cells during infection with *C. neoformans* results in a series of Th2-dependent effector functions such as pronounced allergic pulmonary inflammation, impaired fungal growth control, and significantly elevated IgE production.

Transient loss of Treg cells in pulmonary cryptococcosis promotes a dominant increase of Th2 cells

Besides IL-4-dependent IgE production, recruitment of eosinophils to the site of infection is known to depend on IL-5, and IL-13 has been found to be critical for mucus production by bronchial epithelial cells [34]. To analyze the underlying regulatory mechanisms for the observed Th2-dependent effector functions, we analyzed CD4⁺ T cells for expression of the Th2-associated transcription factor GATA-3 [35] and the Th2-related cytokines. Within the pulmonary leukocytes a significantly higher frequency of GATA-3⁺ Th2 cells in Treg cell-depleted DERE mice compared with the control groups was present (Fig. 3A and B). Consistent with expression of GATA-3, intracellular staining of IL-4 and IL-5 in pulmonary leukocytes exhibited analogous results underpinning a stronger Th2 response in Treg cell-depleted DERE mice (Fig. 3C). Increased amounts of IL-4, IL-5, and IL-13 in the supernatant of pulmonary leukocytes were measured by ELISA (Fig. 3D) corroborating a consistent pattern of significantly higher Th2 responses in the absence of Treg cells.

Additionally, Th1 and Th17 cells were analyzed by their signature transcription factors and cytokines. Lung cells from Treg cell-depleted DERE mice showed increased Th1-associated expression of T-bet and IFN- γ in CD4⁺ Th cells (Supporting Information Fig. 2). Consistent with elevated Th1 cells in the absence of Treg cells, elevated IFN- γ production in the lung and increased

IgG2a serum concentrations during *C. neoformans* infection were found in DT-treated DERE mice compared with the control groups (Supporting Information Fig. 2). This 11-fold increase in serum IgG2a of Treg cell-depleted DERE mice and a fourfold increase in both control groups (Supporting Information Fig. 2D) showed a significant but smaller influence of Treg cells on production of IgG2a during pulmonary cryptococcosis compared with IgE production (Fig. 2E). By contrast, Th17-associated ROR- γ t expression and IL-17A production were not altered after Treg cell depletion (data not shown). Altogether our data demonstrate a highly elevated Th2-associated immune response upon transient depletion of Treg cells, whereas Th1-associated immunity is only mildly affected.

In order to obtain more insight into the observed preferential Treg-dependent regulation of Th2 cells compared with Th1 cells, we additionally analyzed the influence of Treg cell depletion under homeostatic conditions. Therefore, a separate cohort of non-infected DERE mice was treated with DT and compared with infected DT-treated or PBS-treated DERE mice. Interestingly, Treg cell-depleted naïve and infected DERE mice had a comparable increase in the population of T-bet⁺ Th1 cells (Fig. 4B). As the levels of Th1 cells were higher independent of fungal infection, it appears that Treg cells regulate Th1 cell expansion already during homeostasis with no further effect by infection with *C. neoformans* (Fig. 4B). In contrast to the results obtained for Th1 cells, Treg cell-depleted infected DERE mice had significantly higher proportions of GATA-3⁺ Th2 cells than Treg cell-depleted naïve DERE mice (Fig. 4A). In addition, the proportion of GATA-3⁺ Th2 cells is higher than the percentage of the T-bet⁺ Th1 cells (Fig. 4A and B). This suggests that in the absence of Treg cells, Th2 cells rapidly expand in response to *C. neoformans*.

In conclusion, these data demonstrate that the transient depletion of Treg cells during infection with *C. neoformans* leads to a preferential and strongly increased Th2 cell subpopulation thereby promoting Th2-dependent susceptibility.

Discussion

Fungal infections can result in severe life-threatening diseases in immunocompromised patients [36]. As the number of immunocompromised individuals has increased in recent decades due to the HIV pandemic in underdeveloped countries and medical services have improved in the Western world that are associated with immunosuppression (e.g. organ transplantation and cancer treatment), the incidence of invasive fungal infections such as candidiasis, cryptococcosis, and aspergillosis has also increased [37]. Despite the benefit of antifungal drugs, the emergence of drug-resistant fungal strains and serious side-effects of antifungal agents limit their therapeutic use. In conclusion, there is a significant medical need for additional therapies such as adjunct or sole immunotherapies [3, 38].

It was shown that a dominant Th1-related immune response is associated with protective immunity against fungi, whereas Th2-related immunity is detrimental [36, 39, 40]. Treg cells

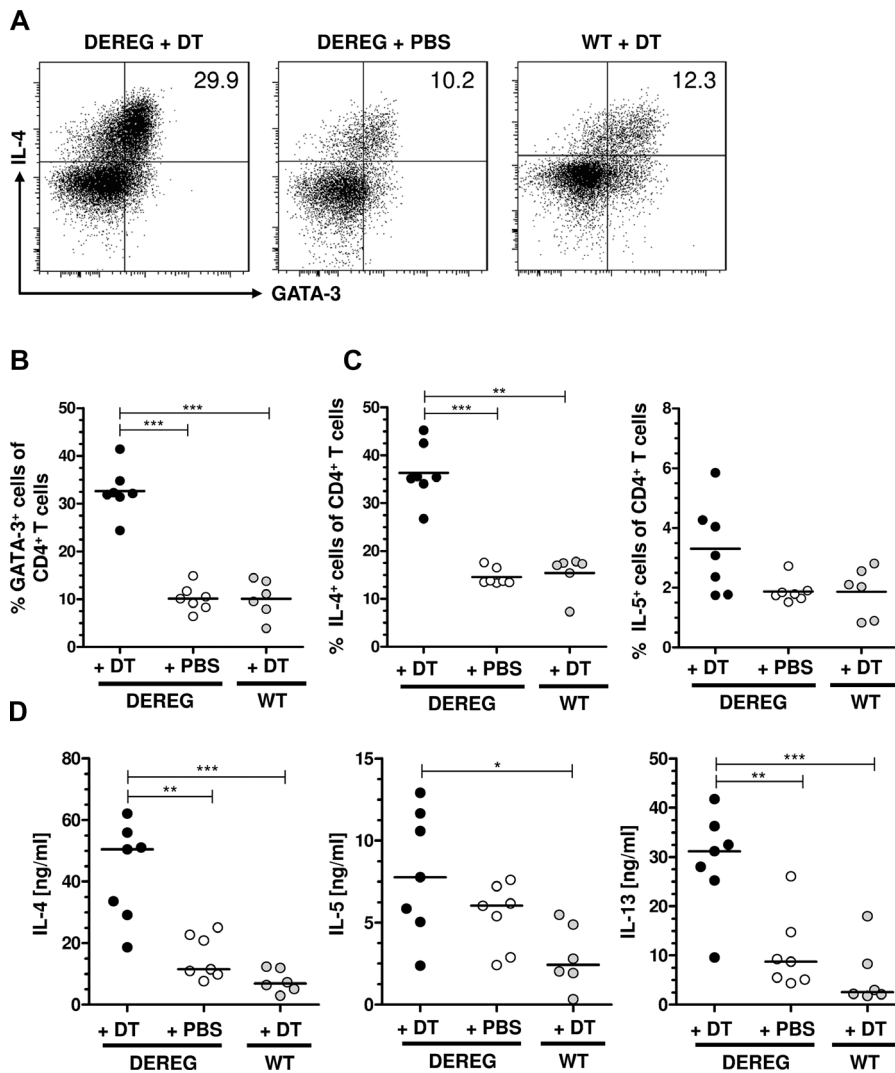


Figure 3. Increased frequency of GATA-3⁺ Th2 cells producing IL-4, IL-5, and IL-13 upon Treg-cell depletion. Leukocytes from lung tissue were isolated at 28 dpi and stimulated with phorbol myristate acetate and ionomycin for 4 h. For flow cytometric analyses of cytokine production, lung leukocytes were additionally incubated in the presence of brefeldin A and monensin. Representative dot plots ($n=6-7$ mice/group) from flow cytometric analysis are depicted in (A). In all flow cytometric analyses cells of interest were gated on live cells and CD4⁺ T cells prior to cytokine and transcription factor analysis. Numbers in the upper right quadrant show the portion of Th2 cells which express the transcription factor GATA-3 and additionally produce IL-4. (B) GATA-3 as well as (C) IL-4- and IL-5-producing Th cells were analyzed by flow cytometry. (D) The Th2-related cytokines IL-4, IL-5, and IL-13 secreted from stimulated lung leukocytes were quantified by ELISA. Cells were incubated in the absence of brefeldin A and monensin during stimulation with phorbol myristate acetate and ionomycin for 4 h. Shown is one representative of three independent experiments with six to seven mice per group. Student's t-test (B–D: IL-4) and Mann-Whitney-test (C and D: IL-5, D: IL-13) were used to determine statistical significance. ** $p < 0.01$, *** $p < 0.001$.

may restrain exuberant immune reactions limiting collateral immunopathology but therefore also dampen the efficiency of protective immunity which can lead to immunoevasion of the fungus [41, 42]. In a model of fungal infection using *Pneumocystis carinii*-infected mice it was shown that Treg cells prevent excessive tissue damage during pathogen clearance [43]. In contrast, in a model of gastrointestinal candidiasis, elimination of *Candida albicans* was prevented by IL-10- and TGF- β -producing Treg cells, whereas in a model of pulmonary aspergillosis Treg cells function to prevent allergy to the fungus [44, 45]. Altogether, the function of Treg cells may vary greatly depending on the site of infection and the fungal organism studied.

The well-defined identification of Treg cells using the key transcription factor FoxP3 was first possible in 2003 [19, 20]. Earlier studies from the 1980s by the Murphy laboratory suggested that so-called first-order T suppressor cells (in a cascade together with second-order and third-order T suppressor cells) can inhibit cell-mediated immunity (e.g. delayed-type hypersensitivity responses) in cryptosporidiosis [46–48]. Conceivably, these studies may be considered as the earliest hints that Treg cells

may be involved in immunity to cryptosporidiosis. However, at that time the concept for characterization of today's Treg cells was not developed yet. Here we show that pulmonary CD4⁺FoxP3⁺ Treg cells increase significantly within the first 4 weeks after infection with *C. neoformans*. This may be related to enhanced proliferation or enhanced recruitment of Treg cells. It was demonstrated that glucuronoxylomannan (GXM), the major component of the capsule of *C. neoformans*, has the capability to increase the expression of TGF- β in human macrophages and dendritic cells [49, 50]. As a downstream effect, TGF- β may contribute to differentiation of pulmonary Treg cells [42] and to the initial rise of Treg cells following intranasal infection during cryptococcal infection.

To get insights into the function of Treg cells during pulmonary cryptococcosis we made use of a genetic mouse model of transient Treg cell depletion. For DEREK mice it was shown elsewhere [51, 52] that a small population of nonsuppressive eGFP⁺FoxP3⁺CD4⁺ Treg cells preferentially expand upon repeated DT treatment as a result of DT insensitivity before suppressive eGFP⁺FoxP3⁺CD4⁺ Treg cells refill their niche.

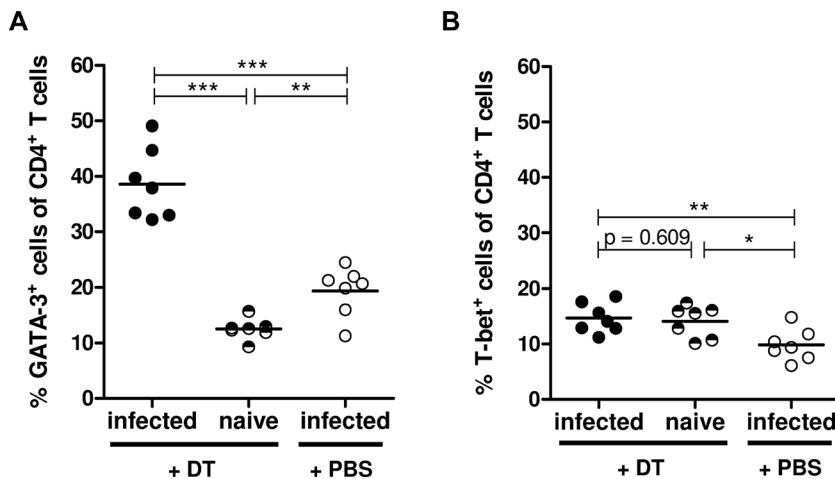


Figure 4. Significant infection-related difference in expression of GATA-3 but not T-bet in Treg cell-depleted DERE mice. Naïve and *C. neoformans*-infected DERE mice were treated with DT as described in Figure 2A. Additionally, *C. neoformans*-infected DERE mice were treated with PBS instead of DT. Leukocytes from lung tissue were isolated at 28 dpi and stimulated with phorbol myristate acetate and ionomycin in the presence of brefeldin A and monensin for 4 h. Using flow cytometry (A) Th2 and (B) Th1 cells were analyzed for the expression of their respective transcription factors by prior gating on live cells and CD4⁺ T cells. Data represent means with seven mice per group from one experiment, representative of at least three independent experiments. Unpaired Student's t-test was performed to determine statistical significance **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

During the experiments described here mice were treated four times with DT to prolong the period of absence of eGFP⁺FoxP3⁺CD4⁺ Treg cells up to 1 week. Our work demonstrates that loss of Treg cells during the second week of infection when both, Th1 and Th2 responses are ongoing [18], leads to a pronounced Th2 bias correlating with substantially higher amounts of secreted IL-4 and IL-13 in lungs and high serum IgE levels in comparison to Treg cell-competent control mice. Together with an enhanced Th2 immune response, significantly higher fungal burden was observed in animals depleted of Treg cells. From work published by several groups it is likely that a Th2-biased pulmonary response against *C. neoformans* (such as we see in Treg cell-depleted mice) will favor earlier and stronger development of meningitis [12, 13, 40]. This, however, needs to be studied at a time point later than 28 dpi, since dissemination with *C. neoformans* strain 1841 starts at about 42 dpi [33]. Although the frequency of Th1 cells, the secretion of IFN- γ , and consequently upregulation of serum IgG2a levels were also to some degree enhanced in Treg cell-depleted mice, the overall immune response was clearly Th2-biased. It remains to be shown for other strains of *C. neoformans* whether a preferential interaction of Treg cells with Th2 cells is also the case. Titration of Treg cell frequencies ranging from physiological levels to complete deficiency, demonstrated that Treg cells efficiently suppress Th2- but not Th1 cells by selective induction of apoptosis [53]. Consistent with these results our data also suggest that Th2-driven diseases may be under a tighter control by Treg cells. Of note, elevated serum IgE levels are also found in humans and mice with impaired Treg cell function [21, 22]. Whether the increase in serum IgE is due to the loss of Treg cell-dependent control of follicular T (T_{fh}) helper cell expansion and their IL-4 production or due to IL-4 expression by Th2 cells is currently under discussion [54]. In addition to the published reports, we not only show an elevated Th2 response simply caused by the absence of Treg cells (naïve DT-treated DERE mice (Fig. 4) as compared with about 3% GATA-3⁺ Th2 cells in naïve PBS-treated mice (data not shown), but provide evidence for a synergism with the development of a full-blown and detrimental Th2-driven immune response following pulmonary infection with *C. neoformans*.

Taken together, this study reveals a selective control of Th2-related immunity by Treg cells during cryptococcal infection. Our data provide an important basis for new options for the treatment of fungal infections. Different strategies for Treg cell manipulation such as expansion, activation, or enhancement of their suppressive function can be exploited therapeutically [55] to improve current treatment protocols of fungal infections.

Materials and methods

Mice

Mice (female, 8–11 weeks) were kept under specific pathogen-free conditions in accordance with the guidelines approved by the Animal Care and Usage Committee of the Landesdirektion Sachsen, Leipzig, Germany (permission no. TVV 16/13, accreditation no. 24–9168.11/18/16). For analysis of kinetic development of Treg cells during cryptococcosis BALB/c mice were purchased from Janvier (Janvier, France). For all other experiments DERE mice [30] on the BALB/c background and their wild-type littermates were used. In DERE mice a DT receptor together with an enhanced green fluorescent protein (eGFP) is expressed under the control of the FoxP3 promoter. Therefore, depletion of Treg cells can be achieved by DT application [51]. As shown elsewhere [32, 56] in naïve DERE mice a subset of FoxP3⁺ Treg cells lacking the DT receptor and GFP expression is not affected by the treatment. However, for the infection model described here even a few days of almost total ablation of Treg cells leads to a significant alteration of the immune response.

Animal study

Mice were anesthetized with a 1:2 mixture of 10% w/v Ketamine and 2% w/v Xylazine and subsequently intranasally infected with 500 colony forming units (CFU) of *C. neoformans* 1841 [18].

For kinetic analysis of pulmonary Treg cell development BALB/c mice (Janvier, France) were sacrificed 14, 21, 28, 35, 42, and 56 dpi. To verify the number of Treg cells in the lung naïve BALB/c mice were used as controls (0 dpi). For Treg cell depletion experiments DEREg-BALB/c (DEREG) mice and their WT littermate (WT) controls were used. Infected mice received intraperitoneally 1 µg of DT (Calbiochem, Merck, Germany) dissolved in PBS to a final concentration of 1 µg/100 µL at days 7, 9, 11, and 13 postinfection. As an additional control infected DEREg mice were treated with an appropriate volume of PBS. Successful depletion and rebound of Treg cells was confirmed by flow cytometric analysis of the FoxP3eGFP signal in peripheral blood leukocytes, taken by submandibular blood sampling (PBS/EDTA). We decided to administer DT four times to prolong the phase of Treg cell absence. Infected mice were monitored daily for survival and morbidity.

Leukocyte preparation and fungal burden determination

For isolation of leukocytes lungs were removed aseptically, milled and digested in the presence of DNase IV (30 µg/mL, Sigma-Aldrich, Germany) and Collagenase D (0.7 mg/mL, Roche, Germany) in RPMI1640 medium (PAA, Germany) supplemented with 1 mM sodium pyruvate (AppliChem, Germany) at 37°C while rotating for 30 min. A single cell suspension was obtained by passaging lung tissue through 100 µm cell strainer (BD Biosciences, Germany). Subsequently lysis of erythrocytes (Gey's solution) was done. Remaining cells were adjusted to 1 mL and 50 µL were used for CFU enumeration. Therefore, serial 1:5 dilutions of the lung homogenate were prepared and incubated on Sabouraud dextrose agar plates at 30°C for 72 h before colonies of *C. neoformans* were counted. Leukocytes were enriched by Percoll (GE Healthcare Europe, Germany) density gradient centrifugation and seeded at 1×10^6 cells/well in IMDM (PAA, Germany) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (PAA, Germany) and 10% heat-inactivated fetal bovine serum (Life Technologies, Germany) in 96-well flat bottom plates (BD Biosciences). For analysis of cytokine production cells were stimulated for 4 h with phorbol myristate acetate (40 ng/mL, Enzo Life Sciences, Germany) and ionomycin (1 µg/mL Sigma Aldrich, Germany). Brefeldin A (5 µg/mL, Sigma-Aldrich, Germany) and monensin (25 µM, Sigma-Aldrich, Germany) were added to the cultures when intracellular staining was performed.

Flow cytometric analysis

Cells stimulated in the presence of brefeldin A and monensin were harvested, stained for flow cytometric analysis and acquired on a BD LSRFortessa (BD Biosciences). The following antibodies were used: Alexa Fluor[®] 700 anti-CD4 (clone RM4–5, BioLegend), PE anti-T-bet (eBio4B10, eBioscience), PE anti-ROR-γt (B2D, eBioscience), PE-Cy7 anti-IL-4 (BVD6–24G2, eBioscience), PE-

Cy7 anti-IL-17A (TC11–18H10.1, eBioscience), PerCP-Cy5.5 anti-FoxP3 (FKJ-16s, eBioscience), Pacific Blue anti-IFN-γ (XMG1.2, eBioscience), allophycocyanin anti-IL-5 (TRFK5, BioLegend), and eFluor660 anti-GATA-3 (TWAJ, eBioscience). The FoxP3 staining kit from eBioscience was used for intracellular staining as recommended by the manufacturer. To exclude dead cells from analysis Fixable Viability Dye eFluor[®] 506 (eBioscience) was used prior to specific antibody staining. Data were analyzed using FlowJo 7.6.5 (Treestar Inc., Ashland, OR) software.

Histopathological analysis

The upper part of the middle right lung lobe was fixed in 4% neutral-buffered formaldehyde (Carl-Roth GmbH) and embedded in paraffin. For visualization of mucus production by bronchial epithelial cells and distribution of cryptococci the sections were stained with periodic acid Schiff (PAS) reagent. An independent investigator evaluated PAS⁺ and PAS[−] bronchial epithelial cells by counting bronchial epithelial cells of at least two different lung regions with a total of 10 cross sections per mouse. Additionally, sections were stained with H&E for the detection of eosinophils and other leukocytes.

ELISA

Total IgE and IgG2a levels in the serum were analyzed as previously described [13]. For cytokine analysis supernatants from stimulated lung cells cultured in the absence of brefeldin A and monensin were used. ELISA kits were purchased and detection of IL-13 (eBioscience), IFN-γ (eBioscience), and IL-17A (R&D Systems) was done using the manufactures protocol. The amount of IL-4 was determined by use of monoclonal Ab 11B11 as a capture Ab and biotin-labeled BVD5–24G2 (BD Biosciences) as detection Ab. The same was performed for IL-5 ELISA using monoclonal Ab TRKF5 (BD Biosciences) as a capture Ab and biotin-labeled TRFK5 (BD Biosciences) for detection.

Statistical analysis

Each experiment was performed with six to seven mice per group and the data shown are from three independent experiments except indicated otherwise. Graphical and statistical analyses were performed using GraphPad Prism5.01 software (Graphpad Software Inc., San Diego, USA). Unpaired Student's *t*-test, non-parametric Mann-Whitney test and one-way ANOVA with a Bonferroni test were used to calculate the statistical significance considered for *p*-values <0.05.

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Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: DERE: DEpletion of REGulatory T cells · DT: diphtheria toxin · eGFP: enhanced green fluorescent protein · IFN- γ : interferon- γ

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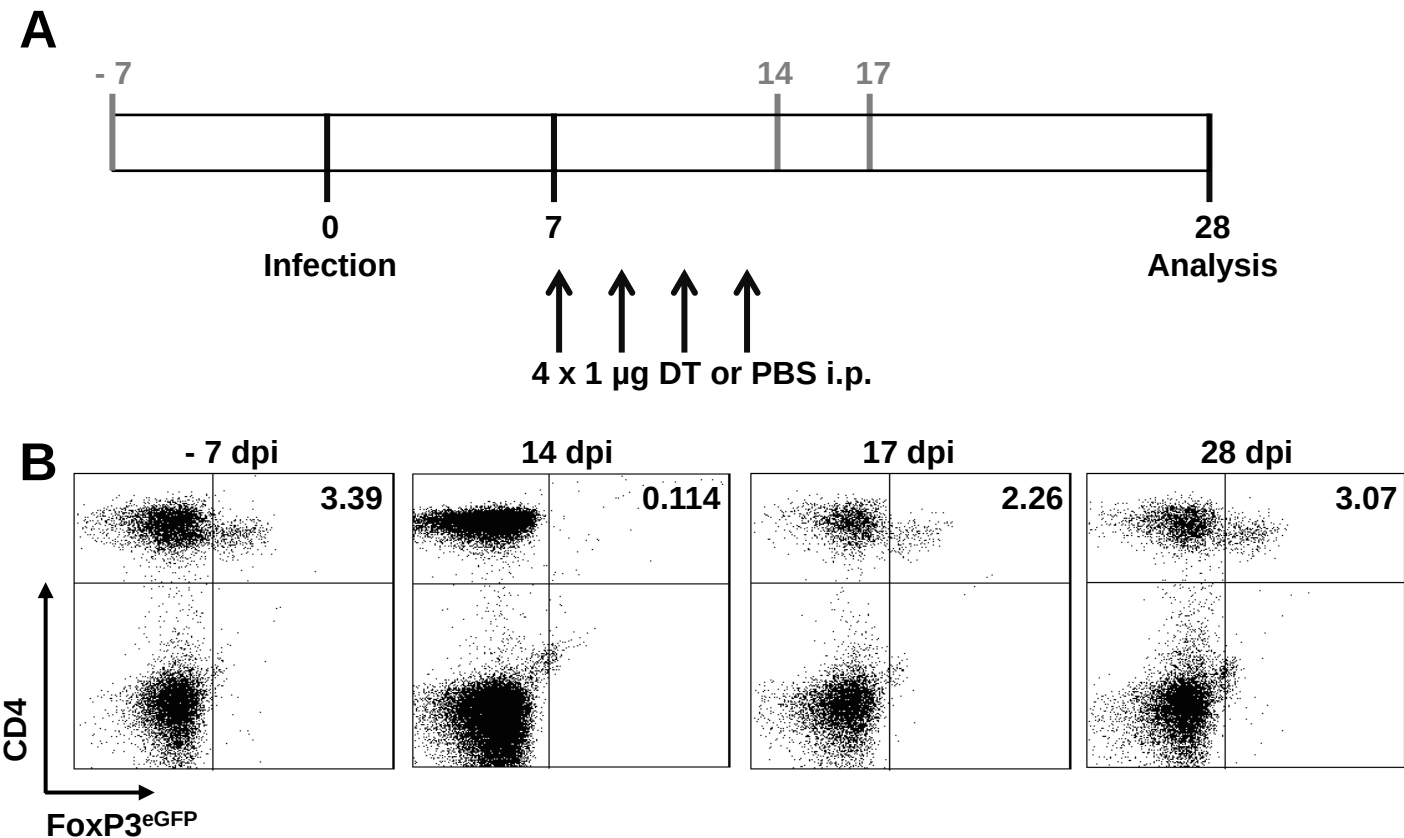
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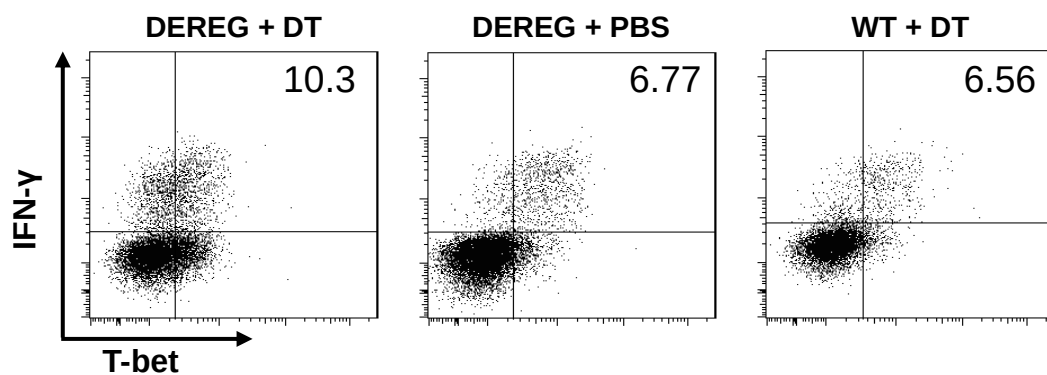
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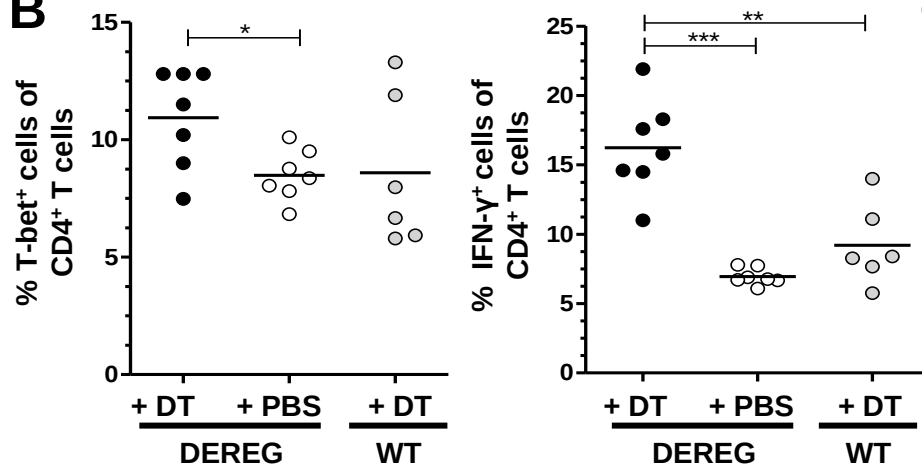
Supporting Information Figure1. Schematic representation of the experimental set-up.

(A) Schematic representation of the experimental set-up. DEREK mice were intranasally infected with 500 CFU of *C. neoformans* 1841. CD4⁺FoxP3⁺ Treg cell depletion was achieved by intraperitoneal (i.p.) administration of 1 µg diphtheria toxin (DT) at 7, 9, 11 and 13 days postinfection (dpi). (B) Lymphocytes were isolated from blood at the indicated time points and the CD4⁺FoxP3^{eGFP} population was analyzed. FACS dot plots show representative stainings and confirm rebound of Treg cells in the blood 28 dpi. Live cells were selected prior to CD4⁺FoxP3^{eGFP} analysis. Plots are representative of 7 mice per time point.

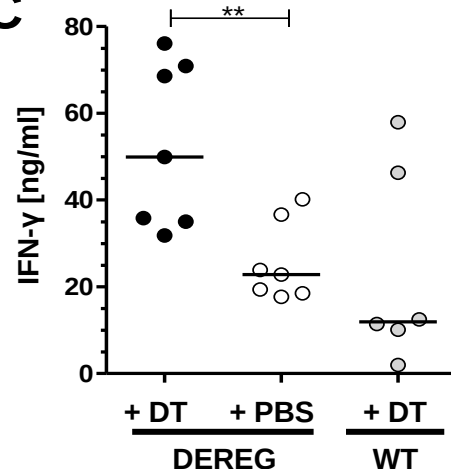
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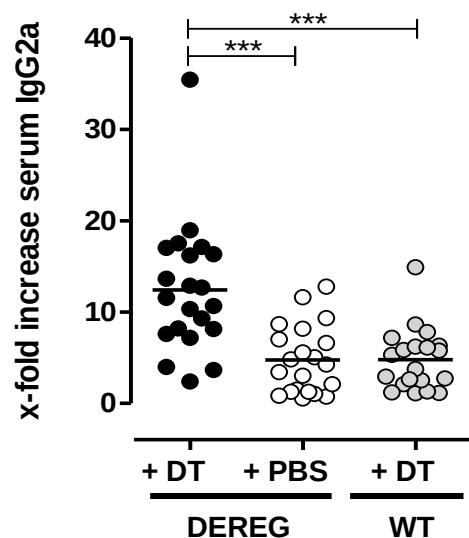
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Supporting Information Figure 2. Depletion of CD4⁺FoxP3⁺ Treg cells leads to a small increase of the Th1-related immune response.

Lung leukocytes were stimulated for 4 h with phorbol myristate acetate and ionomycin in the presence of brefeldinA and monensin. (A) Representative dot plots (n=6-7 per group) from flow cytometric analysis are depicted selecting cells first by live cell and CD4⁺ cell gate. Numbers in the upper right quadrant show percentage of Th1 cells which express the transcription factor T-bet and additionally produce IFN- γ . (B) T-bet expression as well as the intracellular production of IFN- γ among CD4⁺ Th cells was analyzed. (C) Additionally, the supernatants were analyzed for IFN- γ secretion using ELISA. Therefore, cells were stimulated for 4 h with phorbol myristate acetate and ionomycin in the absence of brefeldinA and monensin. Each dot represents one animal (n = 6-7 mice/group). One representative out of three independent experiments is shown. Student's t-test and Mann-Whitney-test were used to determine statistical significance *p<0.05, **p<0.01, ***p<0.001. (D) Total serum IgG2a concentration from *C. neoformans*-infected mice treated with DT or PBS (Fig. 2A) was measured. The x-fold increase from individual mice (n = 20-21 mice/group pooled from three independent experiments (6-7 mice per group in each experiment) dividing the values from 28 dpi by those from pre-infection is shown. Statistical significance was determined by Mann-Whitney test ***p<0.001.

CD4⁺FoxP3⁺ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection

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Handling Executive Committee member: Prof. Annette Oxenius

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 11 July 2014

Dear Dr. Alber,

Manuscript ID ejj.201444963 entitled "CD4⁺FoxP3⁺ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication.

You should also pay close attention to the editorial comments included below.

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Prof. Annette Oxenius

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Reviewer: 1

Comments to the Author

In this report Schulze et al. investigate the role of CD4⁺FoxP3⁺ T regulatory (Treg) cells during experimental murine pulmonary infection with *C. neoformans* 1841 using Balb/c and DERE mice that express a high affinity diphtheria toxin (DT) receptor-eGFP fusion protein under the control of the FoxP3 promoter.

The main findings of the paper are:

1. Treg cell numbers increase in the lung during *C. neoformans* 1841 infection of Balb/c mice.
2. Infected DERE-DT treated mice have increased lung eosinophilia, airway mucus, total serum IgE and lung fungal burden at day 28 after infection compared to DERE-PBS or wild type-DT mice.
3. Infected DERE-DT mice have a greater proportion of GATA3⁺ IL4⁺ CD4⁺ lung T cells compared to DERE-PBS or wild type-DT mice.
4. Infection of DERE-DT mice causes a significant increase in the proportion of GATA3⁺CD4⁺ T cells but not Tbet⁺CD4⁺ T cells in the lung.

Strengths:

Peer review correspondence

This is the first direct demonstration of the role of Treg cells in murine cryptococcal lung infection. The study appears to be carefully conducted with adequate sample sizes, replicates, and controls including wild type mice treated with DT and the writing style is clear.

Weaknesses:

Major points:

1. The authors state that the major cause of death from cryptococcal infection is meningitis (line 55-56); however, the current analysis at day 28 precludes any assessment of this important parameter (Line 113-115). It would be very interesting to know whether DEREK-DT mice are more susceptible to the development of meningitis at a later time point compared to controls or whether the effect of transient Treg depletion is confined to the lungs.
2. It has been stated elsewhere that the efficiency of Treg depletion does not improve with greater than 2 doses of DT due to absence of transgene expression in remaining cells (Lahl and Sparwasser 2011 *Regulatory T Cells: Methods and Protocols*, Methods in Molecular Biology, vol. 707). The current report states the rationale for the use of 4 doses of DT is to prolong Treg depletion (line 268-269), and evidence for transgene positive Treg cell rebound is provided in Supplementary Figure 1. Nevertheless, the discrepancy in the recommended and actual DT administration protocol in this study should be clarified and the emergence of transgene negative Tregs after DT should be considered in the discussion rather than the materials and methods section.

Minor points:

1. Introduction (Line 50-51): Since there is some uncertainty in the literature I would suggest the following modification "...via inhalation of spores or infectious propagules..."
2. Is there a justification for using only female mice for all experiments?
3. Figure 1: It is not clear why the sample size is larger for day 0 and day 14 compared to subsequent time points where $n=7$.
4. Figure 1: I question the use of multiple T-tests comparing day 0 to subsequent time points as an appropriate statistical test for comparison of multiple time points. Why not use ANOVA for this analysis?
5. Figure 2B: The histological characterization of lung eosinophilia must be assumed to be accurate given the magnification and background staining of the samples. Is there a way to improve the presentation of this data to allow an independent assessment by the reader?
6. Figure 2C: There appears to be an extremely large variation in the percentage of PAS+ bronchial cells in all groups yet statistical significance is indicated. The difference between groups would be clarified if a numerical mean and standard deviation/standard error of the mean were also provided for this data.

7. Figure 2D: Why are the authors choosing to show the median for lung fungal burden? Did the authors pool all data within a group (n=20-21) and determine whether there are statistically significant differences of the mean and standard deviation/standard error of the mean among groups?

8. Figure 3C: The legend describes IL-4 and IL-13 production by Th2 cells yet the figure shows IL-4 and IL-5. In addition to correcting the legend it would strengthen the data if intracellular cytokine staining for IL-13 were included in the figure.

9. Figure 4: Is there a particular reason why box plots are used here while dot plots are shown for all other figures?

Reviewer: 2

Comments to the Author

In this study, immunology lab of Prof. Alber analyzed the role of CD4⁺FoxP3⁺ T cells in murine model of pulmonary cryptococcosis. The authors used transgenic mouse system "DEREG" to efficiently deplete CD4⁺FoxP3⁺ Treg cells by intraperitoneal administration of diphtheria toxin (DT). DT-treated WT BALB/c mice or DEREG mice treated with PBS and not DT were used as controls. In this model the authors found that the infected CD4⁺FoxP3⁺ mice developed stronger pulmonary allergic inflammation with enhanced major hallmarks of Th2 bias, elevated fungal lung burden, higher frequencies of GATA-3⁺ T helper (Th) 2 cells with elevated capacity to produce interleukin (IL)-4, IL-5 and IL-13. In contrast, only a mild increase in the Th1-associated immune response unrelated with the fungal infection was observed.

The authors concluded that their study "reveals a selective control of Th2-related immunity by Treg cells during cryptococcal infection and thus mediate enhanced fungal control".

The strengths of this paper are: 1) Novel information regarding the role of Tregs in cryptococcal infection; 2) Use of elegant model to effectively deplete Tregs; 3) High quality of data nicely linked together to provide detailed information.

The major weakness of this manuscript is the data overinterpretation and leading to a conclusion regarding selective Th2-targeted role of Tregs in cryptococcal infection. This conclusion is correct when restricted to the presented model of cryptococcosis, which is Th2-biased and non-protective as reported in multiple studies published by this group (e.g. JID 2008:198, 1714). It is not unexpected that in such model Treg would predominantly target Th2 arm of the immune response. This caveat has been not disclosed in this manuscript and therefore the general conclusion (and also the title of the manuscript) is quite misleading.

The suggested experiment to remedy this problem would be to use another strain of *C. neo* to perform (at least limited) experiment in Th1 biased system. Th1/Th2 bias in BALB/c mice infection models is dictated by the strain used for infection (Jain, I&I: 77, 2009) and Th1 biased, protective response can be generated

in response to strain 52D or other commercially available strains. The additional confirmatory experiment showing that Treg depletion has a similar Th2 promoting effect in Th1 biased model would allow to fully justify author's conclusion included in the title of the manuscript and elsewhere. Alternatively, if Th1 response is mostly enhanced in DERE mice, the conclusion would be that Treg preferentially regulate the most dominant Th-arm of the immune response in cryptococcal infection and depending on situation can be beneficial or harmful.

If these additional studies cannot be performed, the title of manuscript, discussion and other portions of manuscript should be revised so that broader audience could understand full implications and limits of this study.

Minor suggestions: The high magnification histology panels on figures 2B appear to have a strong red hue which could be a result of stain saturation, light filter or image processing. Perhaps equal amount of "electronic" color filter could be used in all 3 high power figures to correct color balance and help eosinophil to stand out on the background of purple macrophages.

First revision – authors' response – 22 July 2014

Reviewer: 1

Comments to the Author

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We thank the reviewer for raising this important comment. We have not studied a later time point. In fact, the role of Treg cells in dissemination of *C. neoformans* to the brain is intriguing but would in itself be a new project beyond this initial study focusing on the role of Treg cells in pulmonary infection (see title of manuscript). From work published by several groups it is likely that a Th2-biased pulmonary response against *C. neoformans* (such as we see in Treg cell-depleted mice) will favor earlier and stronger development of meningitis.

2. It has been stated elsewhere that the efficiency of Treg depletion does not improve with greater than 2 doses of DT due to absence of transgene expression in remaining cells (Lahl and Sparwasser 2011 Regulatory T Cells: Methods and Protocols, Methods in Molecular Biology, vol. 707). The current report states the rationale for the use of 4 doses of DT is to prolong Treg depletion (line 268-269), and evidence for transgene positive Treg cell rebound is provided in Supplementary Figure 1. Nevertheless, the discrepancy in the recommended and actual DT administration protocol in this study should be clarified and the emergence of transgene negative Tregs after DT should be considered in the discussion rather than the materials and methods section.

The reviewer is right in the point that treatment with DT at two consecutive days is enough to transiently deplete Treg cells and we thank the reviewer for the comment. Different publications described a small population of eGFP⁺FoxP3⁺CD4⁺ Treg cells that preferentially expands upon repeated DT treatment in DEREg mice as a result of DT insensitivity (Lahl and Sparwasser, 2011, In vivo depletion of FoxP3⁺ Tregs using the DEREg mouse model. Methods Mol Biol 707; Rausch *et al.*, 2009, Establishment of nematode infection despite increased Th2 responses and immunopathology after selective depletion of Foxp3⁺ cells. EJI 39: 3066-3077). As eGFP⁺FoxP3⁺CD4⁺ Treg cells were also described to be non-suppressive, we did not further study this population. Under physiological conditions suppressive eGFP⁺FoxP3⁺CD4⁺ Treg cells refill their niche after a certain period of time (Lahl and Sparwasser, 2011; Klingenberg *et al.*, 2013; own observations). Short-term DT treatment induces a Treg cell rebound after termination of DT treatment. This Treg cell rebound can be delayed by daily DT treatment of DEREg mice. For our experiments we deleted functionally suppressive Treg cells for at least one week. To avoid treating our mice every day over a period of one week in order to minimize experimental manipulation causing stress to the animals, we changed the treatment protocol toward every 48h. To control for known toxic effects we additionally used wild-type mice. This information is now integrated in the discussion section of the revised version (in addition to the information in the Material and Methods section) [lines 222-227].

Minor points:

1. Introduction (Line 50-51): Since there is some uncertainty in the literature I would suggest the following modification "...via inhalation of spores or infectious propagules..."

The authors thank the reviewer for careful reading and fully agree with the suggestion to modify the sentence [line 51].

2. Is there a justification for using only female mice for all experiments?

In our experience using groups of female mice for infection with *C. neoformans*, results in more homogenous data (and ultimately requiring lower numbers of mice and experiments). This may be related to more social stress in male vs. female mice groups and has also been observed for other infection models (Schuster and Schaub, Experimental chagas disease: the influence of sex and psychoneuroimmunological factor Parasitol Res. 2001 Dec;87(12):994-1000).

3. Figure 1: It is not clear why the sample size is larger for day 0 and day 14 compared to subsequent time points where $n=7$.

We apologize for the misunderstanding which we caused in the legend of Figure 1. Two kinetic analyses have been done ranging from 0 till 56 days post infection. At least 7 mice per time point have been studied in each experiment with partially overlapping time points (i.e. 0 and 14 days post infection). We have now rewritten the figure legend [lines 545-549].

4. Figure 1: I question the use of multiple T-tests comparing day 0 to subsequent time points as an appropriate statistical test for comparison of multiple time points. Why not use ANOVA for this analysis? Done. We changed the statistical test from Students t-test to one-way ANOVA with Bonferroni test for analysis of Figure 1.

5. Figure 2B: The histological characterization of lung eosinophilia must be assumed to be accurate given the magnification and background staining of the samples. Is there a way to improve the presentation of this data to allow an independent assessment by the reader?

Please see response to reviewer 2 (last point).

6. Figure 2C: There appears to be an extremely large variation in the percentage of PAS+ bronchial cells in all groups yet statistical significance is indicated. The difference between groups would be clarified if a numerical mean and standard deviation/standard error of the mean were also provided for this data.

We understand the concerns of the reviewer but unfortunately we are not able to show mean and standard deviation of the data as the values passed none of the normality tests which is required for the determination of the mean. For more clarity of Figure 2C the lines representing the medians have been enlarged.

7. Figure 2D: Why are the authors choosing to show the median for lung fungal burden? Did the authors pool all data within a group ($n=20-21$) and determine whether there are statistically significant differences of the mean and standard deviation/standard error of the mean among groups?

Yes, for analysis of fungal lung burden we pooled data from related groups from all three independent experiments and did a column statistic with the GraphPad Prism software. As all groups fail to pass one of the three normality tests, we used medians and performed the Mann-Whitney U-test [lines 570-573].

8. Figure 3C: The legend describes IL-4 and IL-13 production by Th2 cells yet the figure shows IL-4 and IL-5. In addition to correcting the legend it would strengthen the data if intracellular cytokine staining for IL-13 were included in the figure.

We apologize for this mistake. Figure legend has been now corrected [line 593]. Unfortunately we did no intracellular cytokine staining for IL-13.

9. Figure 4: Is there a particular reason why box plots are used here while dot plots are shown for all other figures?

As the reviewer assumes there is no particular reason for showing box plots instead of dot plots. We thank the reviewer for this hint and changed the figures in the revised version [Figure 4A and B] to have the figures appear more uniformly.

Reviewer: 2

Comments to the Author

In this study, immunology lab of Prof. Alber analyzed the role of CD4+FoxP3+ T cells in murine model of pulmonary cryptococcosis. The authors used transgenic mouse system "DEREG" to efficiently deplete CD4+FoxP3+ Treg cells by intraperitoneal administration of diphtheria toxin (DT). DT-treated WT BALB/c mice or DEREG mice treated with PBS and not DT were used as controls. In this model the authors found that the infected CD4+FoxP3+ mice developed stronger pulmonary allergic inflammation with enhanced major hallmarks of Th2 bias, elevated fungal lung burden, higher frequencies of GATA-3+ T helper (Th) 2 cells with elevated capacity to produce interleukin (IL)-4, IL-5 and IL-13. In contrast, only a mild increase in the Th1-associated immune response unrelated with the fungal infection was observed.

The authors concluded that their study "reveals a selective control of Th2-related immunity by Treg cells during cryptococcal infection and thus mediate enhanced fungal control".

The strengths of this paper are: 1) Novel information regarding the role of Tregs in cryptococcal infection; 2) Use of elegant model to effectively deplete Tregs; 3) High quality of data nicely linked together to provide detailed information.

The major weakness of this manuscript is the data overinterpretation and leading to a conclusion regarding selective Th2-targeted role of Tregs in cryptococcal infection. This conclusion is correct when restricted to the presented model of cryptococcosis, which is Th2-biased and non-protective as reported in multiple studies published by this group (e.g. JID 2008:198, 1714). It is not unexpected that in such model Treg would predominantly target Th2 arm of the immune response. This caveat has been not disclosed in this manuscript and therefore the general conclusion (and also the title of the manuscript) is quite misleading.

The suggested experiment to remedy this problem would be to use another strain of *C. neo* to perform (at least limited) experiment in Th1 biased system. Th1/Th2 bias in BALB/c mice infection models is dictated by the strain used for infection (Jain, I&I: 77, 2009) and Th1 biased, protective response can be generated in response to strain 52D or other commercially available strains. The additional confirmatory experiment showing that Treg depletion has a similar Th2 promoting effect in Th1 biased model would allow to fully justify author's conclusion included in the title of the manuscript and elsewhere. Alternatively, if Th1 response is mostly enhanced in DEREG mice, the conclusion would be that Treg preferentially regulate the most dominant Th-arm of the immune response in cryptococcal infection and depending on situation can be beneficial or harmful.

We apologize for causing misunderstanding of our model. In fact, the *C. neoformans* strain 1841 used for this study induces an early Th1 response in addition to Th2 induction (Grahner *et al.*, 2014, PLoS One; see Figure 4C for early IFN-gamma production and Figure 3D for early appearance to Th2 cells). It was also shown for strain 52D of *C. neoformans* (Murdock *et al.*, 2014; Szymaczak *et al.*, 2012) that both, Th1 and Th2 immune responses develop after infection. This important information is now added to the Introduction section of our manuscript [lines 65-69; 76-77] and is also referred in both the Results [lines 100-102] and the Discussion [lines 228-229, 235-237] section.

For the reviewer we confidentially provide data for a kinetic analysis ranging from 0 dpi to 56 dpi analyses [Fig. A] where it is shown that at the time point of Treg cell depletion (7 – 14 dpi) either Th subpopulation is induced. Additionally, as Fig. A demonstrates, within the first 2-3 weeks post-infection the Th1-related immune response is significantly increased compared to naïve animals (0 dpi). According to the reviewer's question it could have been expected that depletion of Treg cells promotes the most prominent Th subpopulation (i.e. between 7 and 14 days post-infection the Th1 immune response). Surprisingly, depletion of Treg cells in a period of a more prominent Th1-immune response preferentially enhances the

weaker Th2 immune response (blue and red circle in Fig. A show the relative portions of pulmonary Th1 and Th2 cells after Treg cell depletion). In addition, when comparing the percentages of Th1 and Th2 cells from naïve DEREG mice treated with DT (see manuscript Fig. 4) it is noteworthy that in the absence of Treg cells in non-infected mice both Th1 and Th2 populations are found at equal amounts.

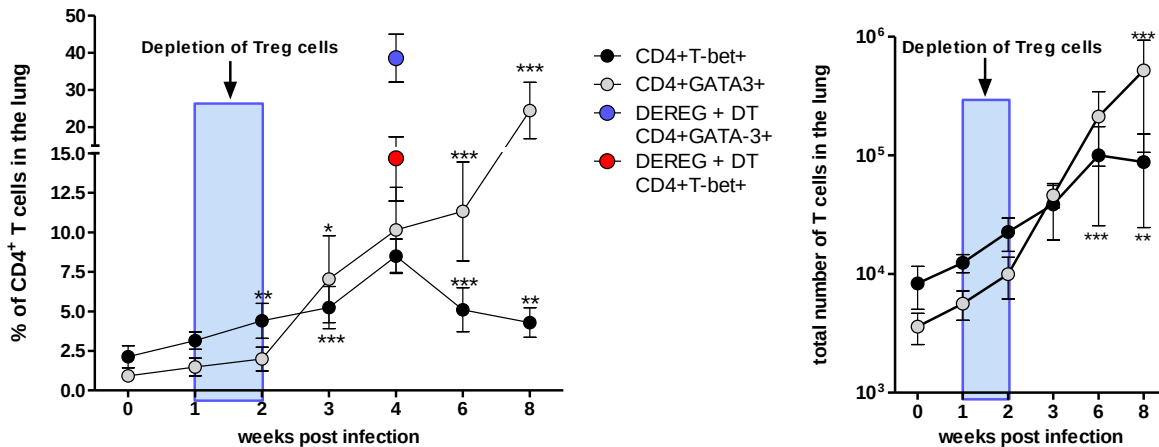


Fig. A: Kinetic analysis of pulmonary Th1 and Th2 development during *C. neoformans* infection in Balb/c wild-type mice. Mice were intranasally infected with 500 colony forming units of *C. neoformans* 1841 and sacrificed at the indicated time points. Leukocytes from lung tissue were isolated at the indicated time points and analyzed for T-bet and GATA-3 expression among living CD4⁺ T helper cells by flow cytometry. Blue box indicates the time frame where DEREG mice were treated with DT to deplete Treg cells in the experiments from this manuscript. Red and blue colored circles show the percentages of Th1 and Th2 cells after transient Treg cell depletion in infected DEREG mice (data from the manuscript). Shown is the mean $\pm$ SD from 7 mice per analyzed time point (n=1). Statistical analysis was performed by using 1way ANOVA with Dunnett's Multiple Comparison Test to examine if there is a significant increase compared to day 0.

If these additional studies cannot be performed, the title of manuscript, discussion and other portions of manuscript should be revised so that broader audience could understand full implications and limits of this study.

Please see response above.

We hope that with the additional information provided to the reviewer (see above Fig. A) and also given in the revised manuscript (introduction [lines 65-69; 76-77], results [lines 100-102], and discussion [lines 228-229] + [lines 235-237]) we have convincing data that Treg cells indeed preferentially regulate Th2 immunity in fungal infection. Moreover, from more recent data, even in noninfectious models where Th1 and Th2 cells are both present, it becomes increasingly clear that Treg cells can dominantly target Th2 cells by additional mechanisms as compared with Th1 cells (see ref. 53 of revised ms.).

Minor suggestions: The high magnification histology panels on figures 2B appear to have a strong red hue which could be a result of stain saturation, light filter or image processing. Perhaps equal amount of "electronic" color filter could be used in all 3 high power figures to correct color balance and help eosinophil to stand out on the background of purple macrophages.

The authors thank both reviewers for the advice that the resolution of eosinophils is too bad to allow independent observation. We realized that by transmitting the figures for the review we lost parts of the resolution. We now used our original high-resolution pictures and additionally added an enhanced image for eosinophils in Figure 3B, lower right part of top panel [lines 560-561, Figure legend]. We believe that the eosinophils can be easier identified now.

Peer review correspondence

Second Editorial Decision – 13 August 2014

Dear Dr. Alber,

It is a pleasure to provisionally accept your manuscript entitled "CD4+FoxP3+ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. Annette Oxenius

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