

Therapeutic expansion of CD4⁺FoxP3⁺ regulatory T cells limits allergic airway inflammation during pulmonary fungal infection

Running title: Treg cell expansion in fungal airway inflammation

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ABSTRACT

Allergic asthma can be frequently caused and exacerbated by sensitization to ubiquitous fungal allergens associated with pulmonary mucus production, airway hyperresponsiveness and bronchial constriction, resulting in a complex disease that is often difficult to treat. Fungal infections are frequently complicated by the development of a type 2 immune response that prevents successful elimination of the fungal pathogen. Furthermore, production of type 2 cytokines triggers allergic airway inflammation. Following intranasal infection of BALB/c mice with the fungus *Cryptococcus neoformans*, we recently described a more pronounced type 2 immune response in the absence of regulatory T (Treg) cells. To determine whether Treg cell expansion is able to suppress type 2-related fungal allergic inflammation, we increased Treg cell numbers during pulmonary *C. neoformans* infection by administration of an interleukin (IL)-2/anti-IL-2 complex. Expansion of Treg cells resulted in reduced immunoglobulin (Ig) E production and decreased allergic airway inflammation including reduced production of pulmonary mucus and type 2 cytokines as well as production of immunosuppressive cytokines such as IL-10 and transforming growth factor (TGF)- β 1. From our data we conclude that Treg cells and/or their suppressive mediators represent potential targets for therapeutic intervention during allergic fungal airway disease.

INTRODUCTION

Diseases caused by respiratory mycoses, ranging from invasive fungal infection to severe asthma with fungal sensitization (SAFS), affect millions of people worldwide. SAFS is characterized by bronchial constriction in response to an inhaled allergen that is worsened by exposure to the airborne fungi resulting in a disease course that is often difficult to treat with standard asthma therapies (Ghosh *et al.*, 2013, Knutsen *et al.*, 2012). Experimental pulmonary infection of mice with the ubiquitous fungal pathogen *Cryptococcus neoformans* can be used as a model of an allergic bronchopulmonary mycosis (Arora *et al.*, 2005, Chen *et al.*, 2007, Hernandez *et al.*, 2005, Muller *et al.*, 2007). Depending on the mouse strain and infection dose used, infections with *C. neoformans* can be either cleared, persist with stable fungal burdens in the lungs, or progress leading to dissemination of the pathogen into the central nervous system (Coelho *et al.*, 2014). Immune responses against *C. neoformans* are shaped by different CD4 T helper (Th) cell subsets. In experimental infection it has been shown that a Th1 immune response is essential for control of *C. neoformans* infection (Decken *et al.*, 1998, Hoag *et al.*, 1997, Kawakami *et al.*, 2000), whereas a Th2 immune response contributes to disease pathology and is associated with development of allergic airway inflammation (Arora *et al.*, 2005, Hernandez *et al.*, 2005, Muller *et al.*, 2007, Piehler *et al.*, 2011). Experiments using a low inoculum of the moderate virulent strain 1841 leading to a more chronic course of pulmonary cryptococcosis allow the study of different effector cells causing allergic bronchopulmonary mycosis (Muller *et al.*, 2007, Piehler *et al.*, 2011, Piehler *et al.*, 2015). The immunopathology attributing to fungus-associated allergic pulmonary disease is characterized by mucus-producing goblet cells, robust eosinophilia and development of Th2-mediated immune response that orchestrates the production of cytokines like interleukin (IL)-4, IL-5, and IL-13 (Becker *et al.*, 2015, Ghosh *et al.*, 2013).

The regulatory subset of CD4⁺ Th cells, namely regulatory T (Treg) cells, is crucial for maintaining immune homeostasis and preventing aberrant activation of the immune system thereby minimizing collateral damage and immunopathology (Sakaguchi *et al.*, 2008). Treg cell suppressor activity includes contact-dependent inhibitory cell surface receptors, direct lysis, secretion of inhibitory cytokines, and competition for growth factors (Miyara *et al.*, 2007, Vignali *et al.*, 2008). We could recently show that Treg cells are involved in protection against *C. neoformans* as their absence results in a more pronounced Th2 immune response (Schulze *et al.*, 2014). Selective Th2 suppression by depletion of Treg cells was further confirmed in an independent study (Wiesner *et al.*, 2015). Interestingly, this group recently demonstrated that Treg cells use IRF4 and CCR5 to co-localize with and suppress Th2 cells (Wiesner *et al.*, 2016). Different from these Treg depletion strategies, it was now the aim of this study to investigate whether IL-2-driven expansion of Treg cells after *C. neoformans* infection could limit Th2 immune responses resulting in reduced allergic airway inflammation. Using IL-2/anti-IL-2 complex treatment we wished to selectively target Treg cells at a time point when Treg cells, but not other activated T cells, express the high-affinity subunit α (i.e. CD25) of the IL-2 receptor. Additionally, in this study different immune-modulating mediators were analysed in a kinetic manner to identify potential suppressive mechanisms that are regulated during pulmonary cryptococcosis.

MATERIALS AND METHODS

Mice

Female BALB/c mice (8-10 weeks) were obtained from a breeding colony from Janvier (Janvier Labs, France) and kept under specific pathogen-free conditions. Sterile food and water were given *ad libitum*. Concordantly with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations (FELASA) accreditation board, mice were periodically tested negative for pinworms and other endo- and ectoparasites. The protocols of this study were approved by the Animal Care and Usage Committee of the Landesdirektion Sachsen, Leipzig, Germany (permission no. L24-9168.11/14/1 (TVV 01/11) and LDD24-5131/276/56 (TVV 52/14)).

Culture of *Cryptococcus neoformans* and infection

For all experiments mice were infected with *C. neoformans* serotype D strain 1841. This strain was originally isolated from the cerebrospinal liquor of an AIDS patient (F. Hoffmann-La Roche Ltd, Basel, Switzerland) and kept frozen in 10% skim milk at -80°C. For infection the fungus was prepared as described elsewhere (Decken *et al.*, 1998). Briefly, *C. neoformans* was cultured overnight in Sabouraud dextrose (SAB) medium with gentle shaking at 30°C. After 18 h the fungus was harvested, washed twice with PBS and the cell number was determined. Prior to infection the fungal suspension was adjusted to 2.5×10^4 cells/mL in PBS. Mice were anesthetized with a 1:2 mixture of 10% (w/v) ketamine and 2% (w/v) xylazine (both Ceva Tiergesundheit, Germany) and subsequently intranasally infected with 500 colony forming units (CFU) of *C. neoformans*. For kinetic analysis BALB/c mice were sacrificed at different time points including 7, 14, 21, 35, 42, and 56 day post infection (dpi). To verify expression levels

under homeostatic conditions naïve BALB/c mice served as controls (0 dpi). Infected mice were monitored daily for survival and morbidity.

Preparation of IL-2/anti-IL-2 complex

The immunocomplex was derived by incubating 1 µg mouse IL-2 recombinant protein (eBioscience, Germany) with 5 µg anti-mouse IL-2 antibody clone JES6-1 (eBioscience, Germany) in a total volume of 100 µL PBS for 30 minutes at 37°C. Subsequently, 100 µL of the IL-2/anti-IL-2 complex were given intraperitoneally. Mice were treated for three consecutive days (Webster *et al.*, 2009) at day 7, 8, and 9 post infection. A control group received an appropriate volume of PBS.

Blood monitoring

To assess expansion of Treg cells, blood was collected by submandibular puncture for at least two times with a minimal regeneration time of one week. From each group (n=14 mice/group) four to five animals were bled at six different time points. Two to three droplets of blood from each mouse were collected in 1 mL PBS + 2 mM EDTA and mixed gently to avoid agglutination. After washing, the pellets were dissolved in PBS and carefully layered onto 1 mL of Bicol separating solution (Biochrom, Germany, $\rho = 1.077$ g/mL) creating a sharp interface. Following density gradient centrifugation (400 x g, 30 minutes, RT, acceleration 1, brake 0) the interface containing lymphocytes was transferred in a new tube with PBS. Afterwards, cells were stained for flow cytometric analysis.

Preparation of single cell suspension from lung and determination of fungal burden

Mice were sacrificed at indicated time points and lung lobes were removed aseptically. For isolation of leukocytes lung tissue was milled and digested in the presence of DNase IV (30 µg/mL, Sigma Aldrich, Germany) and Collagenase A (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 minutes. By passing lung tissue through a 100 µm cell strainer (BD Biosciences, Germany) a single cell suspension was obtained that was treated with Gey's solution for erythrocyte lysis. Determination of fungal burden in the lung was done by serial 1:5 dilutions of 50 µL lung homogenate that was adjusted to 1 mL with PBS. Two consecutive dilution steps were chosen and incubated on SAB plates at 30°C for 72 h prior to counting colonies of *C. neoformans*. For further analysis leukocytes were enriched by Percoll (GE Healthcare Europe, Germany) density gradient centrifugation (400 x g, 20 minutes, RT, acceleration 1, brake 0). Leukocytes were resuspended in 70% Percoll and layered under 30% Percoll each diluted in RPMI1640 to respective percentage. Enriched leukocytes from the interface were recovered in IMDM medium (PAA, Germany) supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin (PAA, Germany) and 10% heat-inactivated fetal bovine serum (Gibco, Germany). For analysis of cytokine production cells were seeded at 1×10^6 cells/well in 96-well flat bottom plates (BD Biosciences) and stimulated in total for 6 h with phorbol-12-myristate-13-acetate (40 ng/mL, Enzo Life Sciences, Germany) and ionomycin (1 µg/mL, Sigma Aldrich, Germany). To block cytokine release from the cells 5 µg/mL brefeldinA and 25 µM monensin (both Sigma Aldrich, Germany) were added to the culture for the last 4 h.

Flow cytometry

Cells stimulated in the presence of brefeldinA and monensin were harvested and stained with fixable viability dye eFluor®506 (eBioscience, Germany) for discrimination of dead cells

according to the manufacturer's protocol. To avoid unspecific Fc binding cells were incubated with anti-CD16/32 antibody (BioLegend, Germany) prior to staining for flow cytometric analysis. The following antibodies were used: PerCP-Cy5.5 anti-mouse FoxP3 (FJK-16s, eBioscience), FITC anti-mouse FoxP3 (FJK-16s, eBioscience), FITC anti-mouse T1/ST2 (DJ/8, MD Biosciences), Alexa Fluor® 700 anti-mouse CD4 (RM4-5, BioLegend), eFluor660 anti-mouse GATA-3 (TWAJ, eBioscience), PE-Cy7 anti-mouse IL-4 (BVD6-24G2, eBioscience), APC anti-mouse IL-5 (TRFK5, BioLegend), FITC anti-mouse IL-13 (eBio13A, eBioscience), Pacific Blue anti-mouse IFN- γ (XMG1.2, eBioscience), and PE anti-mouse T-bet (eBio4B10, eBioscience). Surface staining was performed for 15 minutes on ice. For intracellular staining the FoxP3/Transcription Factor Staining Buffer Set from eBioscience was used as recommended by the manufacturer (eBioscience, Germany). The corresponding isotype controls were purchased from eBioscience or BioLegend. Cells were acquired with a BD LSR Fortessa™ flow cytometer (Beckton Dickinson, Germany) and data were analysed using FlowJo 7.6.5 (Treestar Inc., Ashland, USA) 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. A scoring system was used to quantify the mucus production and inflammation. Mucus score 0 = no PAS-positive bronchial epithelial cells can be detected, 1 =

single PAS-positive bronchial epithelial cells are found, 2 = clusters of mucus producing bronchial epithelial cells are detected.

RT-qPCR and QuantiGene® Plex analysis

The accessory lung lobe was used for all mRNA expression analysis either by QuantiGene® Plex assay or classical RT-qPCR analysis. Lung tissue was snap frozen in liquid nitrogen and stored at -80°C until further usage. QuantiGene® 2.0 10-plex assay was purchased from eBioscience and performed in accordance to the manufacturer instructions (eBioscience, Germany).

For RT-qPCR snap frozen samples were homogenized in peqGOLD TriFast solution (Peqlab, Germany) using the Precellys Ceramic Kit 1.4 mm (Peqlab, Germany) at 5000 rpm for 15 seconds in a Precellys®24 homogenisator (Peqlab, Germany). Thereafter, RNA was isolated and transcribed to cDNA with the High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Germany) using a blend of random/oligo(dT)₁₈ primer according to manufacturer's instruction for further analysis. Duplicates were used for RT-qPCR with the iTaq™ Universal SYBR® Green Supermix in an iCycler iQ™5 (both: Bio-Rad, Germany). The murine house-keeping gene hypoxanthine phosphoribosyl transferase 1 (*hprt*) was used as reference gene to calculate expression levels of the target genes using the $2^{(-\Delta\Delta Ct)}$ method (Livak *et al.*, 2001, Pfaffl *et al.*, 2001). The following primers were used: mm_hprt_for: 5'-GTTGGATACAGGCCAGACTTTGTTG-3' and mm_hprt_rev: 5'-GATTCAACTTGCGCTCATCTTAGGC-3' (GenBank Accession No. NM_013556; positions 660-684 and 822-798); mm_gob5a_for: 5'-AGCGTCACAGCCTTGATT-3' and mm_gob5a_rev: 5'-GGACGTGGTGGGTTTCATTCT-3' (GenBank Accession Nr. NM_017474; position 1907-1924 and 2175-2156); mm_muc5ac_for: 5'-GCTGAGCAAGCCTTGTGAC-3' and mm_muc5ac_rev: 5'-CACCATGATCTCCGTCTGC-3' (GenBank Accession Nr. NM_010844;

position 1371-1389 and 1503-1485); mm_ccl11_for: 5'- GCTCACGGTCACTTCCTTCA-3' and mm_ccl11_rev: 5'- CTTGAAGACTATGGCTTTCAGGGT-3' (GenBank Accession Nr. NM_011330; position 168-187 and 330-307).

Enzyme-Linked Immunosorbent Assay (ELISA)

Total IgE levels in mouse sera were analysed using a sandwich ELISA system. Plates (NUNC™, Thermo Scientific, Denmark) were coated with goat anti-mouse IgE unlabeled antibody prior to serum incubation. All IgE components were purchased from Southern Biotech (BIOZOL, Germany). To calculate concentrations a standard curve containing serial dilutions of purified mouse IgE was used. Thereafter, rat anti-mouse IgE-HRP conjugate was added for detection in combination with the TMB Microwell Peroxidase System (KPL, Germany) resulting in colorimetric development. For cytokine analysis supernatants from stimulated lung cells were used. Murine IL-10 (eBioscience, Germany), total TGF-β1 and latent TGF-β1 (both: BioLegend, Germany) were analysed in accordance to the manufactures protocol.

Statistical analysis

For evaluation of statistical analysis and graphical representation the Graph Pad Prism 5.01 software (GraphPad Software Inc., San Diego, USA) was used. The Kolmogorov-Smirnov test was used to test for normality. Normally distributed data are presented with the mean whereas non-parametric data are depicted with the median. One-way ANOVA with a Bonferroni post hoc test was used in order to compare different time points in the kinetic study. Differences between two groups were analysed by Student's t-test if the data were normally distributed. The Mann-Whitney U-test was used in order to compare nonparametric data. Statistical significance was considered for p -values < 0.05 . $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

RESULTS

Expansion of Treg cells in fungal infection reduces serum immunoglobulin (Ig)E levels

In a former study we reported that depletion of Treg cells resulted in stronger allergic inflammation, increased frequencies of GATA-3⁺ Th2 cells, and elevated fungal lung burden (Schulze *et al.*, 2014). On the basis of the Treg depletion data we hypothesized that expansion of Treg cells early in pulmonary *C. neoformans* infection might contribute to disease control. It has been shown that treatment with anti-IL-2 monoclonal antibody clone JES6-1, used in this study, preferentially amplifies Treg cells by blocking the IL-2 binding site of the medium-affinity IL-2R $\beta\gamma$ receptor (expressed on CD8⁺ T cells and NK cells), whereas the binding site of the high-affinity IL-2R $\alpha\beta\gamma$ receptor on Treg cells remains exposed (Boyman *et al.*, 2006, Letourneau *et al.*, 2010). In different studies the successful expansion of Treg cells in several tissues by treatment with the IL-2/anti-IL-2 complex has been shown (Webster *et al.*, 2009, Wiesner *et al.*, 2015). To choose a suitable time point for selective Treg cell expansion without affecting the Th2 cell population, a kinetic analysis of pulmonary CD4⁺ Th cells, expressing CD25 and FoxP3 (Treg cells) or T1/ST2 (Th2 cells) was performed (Fig. 1A). These data revealed that the majority of CD4⁺CD25⁺ T cells, responsive for the treatment with IL-2/anti-IL-2 complex (Webster *et al.*, 2009) co-expressed FoxP3 (~80%), allowing for selective expansion of Treg cells one week after infection (Fig. 1B). ~~It has been shown that treatment with anti-IL-2 monoclonal antibody clone JES6-1, used in this study, preferentially amplifies Treg cells by blocking the IL-2 binding site of the medium-affinity IL-2R $\beta\gamma$ receptor (expressed on CD8⁺ T cells and NK cells), whereas the binding site of the high-affinity IL-2R $\alpha\beta\gamma$ receptor on Treg cells remains exposed (Boyman *et al.*, 2006, Letourneau *et al.*, 2010). In different studies the successful expansion of Treg cells in several tissues by treatment with the IL-2/anti-IL-2 complex has been shown (Webster *et al.*, 2009, Wiesner *et al.*, 2015).~~ To test this treatment procedure in our model, BALB/c mice were

infected with *C. neoformans* and treated with IL-2/anti-IL-2 complex. Successful expansion of CD4⁺FoxP3⁺ Treg cells was monitored in peripheral blood (Fig. 1C, filled circles). Data demonstrated an increase in the frequency of peripheral Treg cells starting 10 days post infection (dpi), one day after the last IL-2/anti-IL-2 complex treatment. Maximal peripheral Treg cell frequencies were detected at 13 dpi declining to numbers of PBS control eight days later. As additional control the frequencies of peripheral CD4⁺GATA-3⁺ Th2 cells were observed, revealing no changes by IL-2/anti-IL-2 complex treatment (Fig. 1C, squares). At 21 dpi mice were sacrificed and serum immunoglobulin E (IgE) levels, indicative of the manifestation of a detrimental Th2 bias in the course of cryptococcosis, were determined (Fig. 1D). Reduction of circulating Treg cells upon treatment with IL-2/anti-IL-2 complex has been previously shown to be associated with reduction of tissue-resident (e.g. pulmonary) Treg cells (Webster *et al.*, 2009, Wiesner *et al.*, 2015). As expected from our previous study (Schulze *et al.*, 2014), Treg cell expansion resulted in decreased serum IgE levels. Taken together, we found that during *C. neoformans* infection Treg cells represent the majority of CD25 expressing CD4⁺ Th cells in the lung up to 14 dpi. Thus, in our fungal infection model IL-2/anti-IL-2 complex treatment at 7 dpi allows the selective manipulation of Treg cells during an established infection.

Treg cell expansion is able to limit *C. neoformans*-infection-induced allergic airway inflammation

Depletion of Treg cells resulted in enhanced allergic inflammation with enhanced mucus production and stronger eosinophilia as shown earlier (Schulze *et al.*, 2014). Next, we analysed the impact of expansion of Treg cells on the progression of allergic bronchopulmonary mycosis. Histological analysis of lung pointed toward stronger inflammation and elevated mucus production in mice treated with PBS as compared to mice treated with IL-2/anti-IL-2 complex,

whereas similar numbers of fungi were observed (Fig. 2A). To quantify the influence of Treg cell expansion on mucus production and type 2 inflammation, a score for mucus production was done and RT-qPCR analysis of total lung tissue was performed. Scoring of mucus production revealed a significant reduction in mice treated with IL-2/anti-IL-2 complex (Fig. 2B). Scoring of pulmonary inflammation also showed a reduction by the administration of IL-2/anti-IL-2 complex, but did not reach statistical significance (data not shown). As mucus production is one of the hallmarks of allergic asthma, two independent markers, *gob-5* and *muc5ac*, were measured simultaneously by RT-qPCR. Analysis revealed a decreased allergic airway inflammation as lower mRNA levels of *gob-5* and *muc5ac* (Fig. 2C) confirmed the attenuated mucus production found in histopathology (Fig. 2A,B). Eosinophils, one of the main drivers of allergic airway inflammation (Ghosh *et al.*, 2013), were analysed by flow cytometry and appeared heterogeneous in both groups without any obvious differences in their frequency (data not shown). However, the total mRNA level of *ccl11* (*eotaxin-1*), an attractant for eosinophils, was reduced in mice treated with IL-2/anti-IL-2 complex (Fig. 2C, right graph). Furthermore, at 21 dpi the fungal lung burden and total number of lung leukocytes were determined (Fig. 2D,E) revealing no influence of Treg cell expansion on neither the fungal load nor the absolute number of leukocytes. Taken together, even after onset of fungal infection, expansion of Treg cells is able to attenuate allergic bronchopulmonary mycosis.

Expansion of Treg cells selectively inhibits Th2-associated immune responses

It has been described that the Th1 versus Th2 cell bias determines the outcome of pulmonary *C. neoformans* infection (Coelho *et al.*, 2014). For both CD4 Th cell subpopulations flow cytometric analysis was performed to measure the influence of Treg cell expansion on Th differentiation and cytokine production. As determination of total numbers of CD4 T cells in the

lung of mice from both groups did not show differences (Fig. 3A), a more detailed analysis of cytokine producing CD4 T cell subsets was done. Th1 and Th2 subsets were studied using key transcription factors (Th1: T-bet, Th2: GATA-3) in combination with their key cytokines (Th1: interferon- γ (IFN- γ) and for Th2: IL-4, IL-5, and IL-13) (Fig. 3B, C). After Treg cell expansion reduced frequencies of IL-4-, IL-5-, and IL-13-producing Th2 cells (Fig. 3B) were found. In contrast, the production of IFN- γ by Th1 cells was not affected after IL-2/anti-IL-2 complex treatment (Fig. 3C). Expansion of Treg cells resulted in selective inhibition of Th2- but not Th1-associated immune responses in pulmonary *C. neoformans* infection. As Th2-related cytokine secretion was still diminished even when circulating Treg cell frequencies had dropped to levels comparable to PBS-treated mice (Fig. 1B, 3A), analysis of Treg cell-associated suppressive cytokines was performed in the supernatant of lung leukocytes. Total amounts of IL-10 as well as TGF- β were detectable at significantly higher amounts in cultures from mice with previous Treg cell expansion (Fig. 3D). In conclusion, suppression by Treg cell expansion has a prolonged effect even when the number of Treg cells declined.

Induction of suppressive mediators upon infection with *C. neoformans*

Using IL-2/anti-IL-2 complexes for expansion of Treg cells in the shown settings we were able to prove a protective role of Treg cells by selective Th2 suppression. However, as shown in Fig. 1A, the timeframe for the IL-2/anti-IL-2 complex treatment after *C. neoformans* infection is limited as 14 dpi the increased differentiation of CD25⁺ Th2 cells counteracts the selective Treg cell targeting. To exclusively expand Treg cells without affecting other Th cell subsets the IL-2/anti-IL-2 complex treatment used is not possible. Besides the option of an adoptive transfer of Treg cells, suppressive mediators might be potential targets in settings where Treg cells can not longer be exclusively manipulated. From a functional perspective suppressive properties of Treg cells

can be grouped into four basic modes of action (Vignali *et al.*, 2008). Treg cells can inhibit effector T cell responses by (i) secretion of inhibitory cytokines, (ii) cytolysis, (iii) metabolic disruption of IL-2, or (iv) in a cell-cell-contact-dependent manner directly or indirectly by activating dendritic cells via interaction of cytotoxic T lymphocyte-associated antigen (CTLA)-4 with CD80/86 leading to the secretion of indoleamine 2,3-dioxygenase (IDO). Moreover, CTLA-4 can block or even lead to removal of CD80/86 by transendocytosis (Qureshi *et al.*, 2011). To investigate the involvement of the different suppressive mechanisms, whole lung tissue of *C. neoformans*-infected BALB/c mice was analysed in a kinetic manner by QuantiGene® Plex assay technology (Supplemental Fig. 1B, C). For a better overview fungal lung burden is depicted in Supplemental Fig. 1A showing the increase in fungal load with ongoing infection. Furthermore, relative gene expression of *foxp3* supports the previously published observation that pulmonary Treg cells increase during the first 3-4 weeks of infection (Schulze *et al.*, 2014). Relative gene expression revealed an increase of mRNA levels of both analysed inhibitory cytokines (i.e. IL-10, TGF- β) as well as of CTLA-4 and IDO in the course of *C. neoformans* infection (Supplemental Fig. 1C). For granzyme B no change and for perforin only a small increase of mRNA expression late in cryptococcosis was found (Supplemental Fig. 1C) indicating that the cytolysis pathway might be regulated to a minor degree in this infection model. Taken together, several suppressive mediators which might contribute to suppressive features of Treg cells, are induced during fungal lung infection.

DISCUSSION

Allergic airway inflammation in the context of fungal sensitization is of major concern as it presents a severe clinical scenario and is difficult to treat (Knutsen *et al.*, 2012). Since fungal spores are ubiquitous in the environment it is impossible to simply avoid contact. A better understanding of the molecular mechanisms that are involved in the pathogenesis of allergic bronchopulmonary mycosis will enable the development of new treatment strategies. Immunopathology of allergic inflammation is characterized by mucus-producing bronchial epithelial cells, eosinophilia and development of Th2 cells with subsequent secretion of IL-4, IL-5, and IL-13. As Treg cells are known to limit aberrant activation of the immune system and different tools exist for their manipulation (McMurchy *et al.*, 2011, Ndure *et al.*, 2014), they might be a potential therapeutic target to prevent or reduce fungal-related airway diseases.

In a previous study we investigated the role of Treg cells in pulmonary *C. neoformans* infection (Schulze *et al.*, 2014). We found that depletion of Treg cells results in stronger allergic inflammation with increased Th2 cytokine responses and stronger fungal growth. Therefore, we concluded that Treg cells play a protective role in the onset of cryptococcosis. We decided to expand Treg cells by IL-2 at time points when CD25⁺ Treg cells were exclusive responders and Th2 development has not started yet. To this end we expanded Treg cells *in vivo* at 7 dpi by a threefold application of an IL-2/anti-IL-2 complex (Webster *et al.*, 2009). Even with this limited treatment protocol transient increase of Treg cells during an established *C. neoformans* infection results in decreased allergic airway inflammation as seen by reduced RNA levels of *gob5* and *muc5ac*, lower serum IgE levels and decreased secretion of Th2 cytokines IL-4, IL-5 and IL-13. A more extensive and prolonged treatment of infected mice with IL-2/anti-IL-2 complex as applied in other studies dealing with *Leishmania* or HSV-1 infection (Ehrlich *et al.*, 2014, Gaddipati *et al.*, 2015) may hold promise for even reducing fungal lung burden. In the study by

Wiesner and colleagues mice were infected with an inoculum of 5×10^4 CFU of a *C. neoformans* serotype A strain and treated with IL-2/anti-IL-2 complex resulting in an increase of detrimental antigen-specific Th2 cells (Wiesner *et al.*, 2015). However, infection with a low dose of 500 CFU from the *C. neoformans* serotype D strain used in our study leads to a more chronic course of infection (Muller *et al.*, 2007) with the majority (~80%) of all CD25⁺ (high IL-2 affinity receptor) cells co-expressing FoxP3 during the first 14 days post infection (see Fig. 1A). Thus, our model of fungal infection allows for the selective manipulation of Treg cells by expansion or depletion to analyse the Treg cell biology in cases of fungal infection. However, since no adoptive transfer experiments with activated Treg cells from IL-2/anti-IL-2 complex treated mice into infected recipients were performed in this study, it can not be fully ruled out that treatment with IL-2/anti-IL-2 complex also had effects on other cells which may contribute to the overall outcome. As expansion of Treg cells during an established infection may mirror chronic fungal sensitization that leads to pulmonary allergic airway inflammation, combined treatment of IL-2/anti-IL-2 complex together with antifungal agents may be used for future therapy. In the future it will be of interest to study Treg cell development in patients with cryptococcosis and unravel whether a therapeutic window exists which would allow for selective therapeutic manipulation.

As under infection conditions IL-2 responsiveness is not limited to Treg cells, other treatment strategies e.g. by neutralizing suppressive mediators of Treg cells should be investigated. In general, Treg cells are believed to use several mechanisms of immunosuppression, which from a functional perspective can be grouped into four basic modes of action (Vignali *et al.*, 2008). In this study we found that during experimental pulmonary cryptococcosis overall mRNA expression levels of mediators contributing to two Treg cell-associated suppression mechanisms were increased. This included inhibitory cytokine secretion e.g. IL-10 and TGF- β and the surface molecule CTLA-4 that might be linked directly to IDO release by immunoregulatory cells such

as dendritic cells (DCs). Recently, a study demonstrated that blockade of IL-10 early or late in *C. neoformans* infection enhances Th1 and Th17 effector responses that promote fungal clearance (Murdock *et al.*, 2014). Using *C. neoformans* strain 52D early and sustained expression of IL-10 by leukocytes was found to be associated with fungal persistence as IL-10-deficient mice showed better fungal clearance (Beenhouwer *et al.*, 2001, Blackstock *et al.*, 1999, Guerrero *et al.*, 2008, Hernandez *et al.*, 2005). Interestingly, in our study we found significantly higher amounts of IL-10 and TGF- β in mice with previous Treg cell expansion to be correlated with decreased allergic airway inflammation and lower frequencies of Th2 cells. We therefore conclude that IL-10 production by Treg cells might contribute to selective Th2 cell suppression and not to fungal persistence as in other models of allergic inflammation and airway hyper-reactivity. Transfer of wild-type or IL-10^{-/-} Treg cells revealed IL-10-dependent suppression of Th2-driven responses to allergens *in vivo* (Rubtsov *et al.*, 2008, Vignali *et al.*, 2008). For the mediators of the cytotoxic pathway granzyme B and perforin a constant but only slightly altered mRNA expression was found in the lung with ongoing *C. neoformans* infection. As Moody *et al.* published perforin secretion by natural killer (NK) cells in response to *C. neoformans* infection (Ma *et al.*, 2004, Marr *et al.*, 2009), the increase of perforin mRNA expression might rather indicate NK cell activity than Treg cell responses.

Taken together, these experiments were focused on the modulation of an established fungal infection-induced allergic bronchopulmonary disease, with the goal of determining whether an intervention by manipulating Treg cells would have potential therapeutic value on pulmonary allergic inflammation. Indeed, we show that the *in vivo* induction of CD4⁺FoxP3⁺ Treg cells limits the development of allergic bronchopulmonary mycosis as it is associated with lower frequencies of type 2 immune cells and decreased levels of IgE. Moreover, in our study we performed a kinetic analysis of the development of potential suppressive mediators during

pulmonary *C. neoformans* infection. Thus, our data provide support for future research to study Treg function in cryptococcosis patients. Potentially treatment protocols can be developed that expand Treg cells and use suppressive mediators as an immunotherapeutic approach for *C. neoformans* infection and possibly other fungal infections leading to asthma-related airway inflammation.

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The authors declare no conflict of interest.

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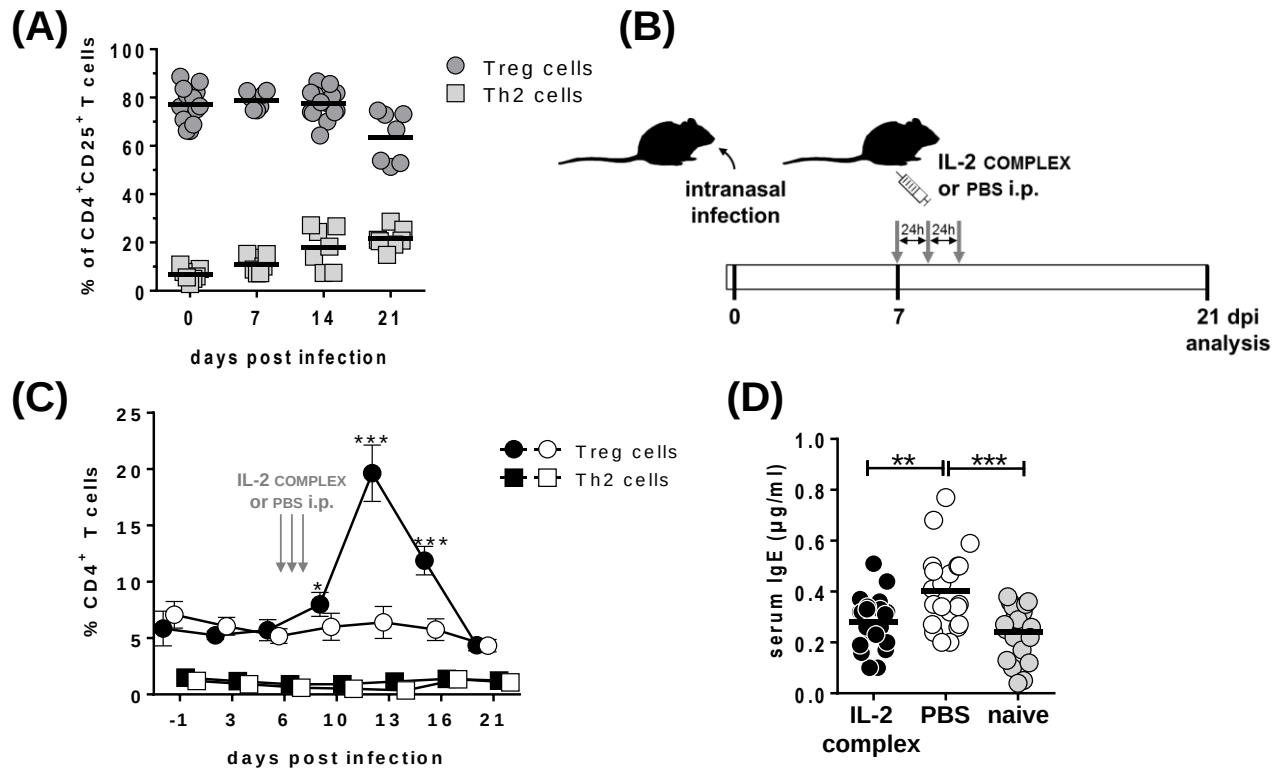


Figure 1. Expansion of Treg cells by IL-2/anti-IL-2 complex treatment in early cryptococcosis dampens serum immunoglobulin E production. **(A)** Pulmonary CD25 expressing CD4 T helper cells were analysed after *C. neoformans* infection in a kinetic manner to identify cells that respond to interleukin (IL)-2/anti-IL-2 complex (IL-2 complex) treatment. Flow cytometric analyses of CD25⁺CD4⁺ Th cells that co-express the transcription factor FoxP3 (Treg cells) or the surface molecule T1ST2 (Th2 cells) are shown. Data are from two independent experiments with n=7 mice per time point, partially overlapping in the analysis of day 0 and 14. **(B)** Schematic representation of the experimental set-up. BALB/c mice were intranasally infected with 500 colony forming units (CFU) of *C. neoformans* strain 1841. CD4⁺FoxP3⁺ Treg cells were expanded by intraperitoneal (i.p.) administration of IL-2/anti-IL-2 complex (IL-2 complex) starting seven days post infection (dpi) for three times every 24 h. Control mice received three times PBS. Mice were sacrificed at 21 days post infection to study the influence of early Treg cell expansion. **(C)** To control effective expansion of

Treg cells CD4⁺FoxP3⁺ Treg cells (circles) were measured in peripheral blood at indicated time points by flow cytometry. Additionally, to determine effects of Th2 cells by IL-2/anti-IL-2 complex treatment CD4⁺GATA-3⁺ T cells (squares) were monitored in the blood. Four to five mice were bled submandibularly per time point allowing each animal to rest for more than a week before taking a second sample. Filled symbols represent data from IL-2/anti-IL-2 complex treated animals; open symbols show data from PBS control mice. Mean with standard deviation is shown. Student's t-test was performed for statistical analysis defining differences between both groups at the indicated time points. **(D)** Total immunoglobulin (Ig)E in the serum of mice 21 dpi compared to naïve uninfected mice was determined by ELISA. Each data point represents one animal pooled from two independent experiments (n=21 mice per group). Statistical analysis was done using one-way ANOVA with Kruskal-Wallis test $**p < 0.01$, and $***p < 0.001$.

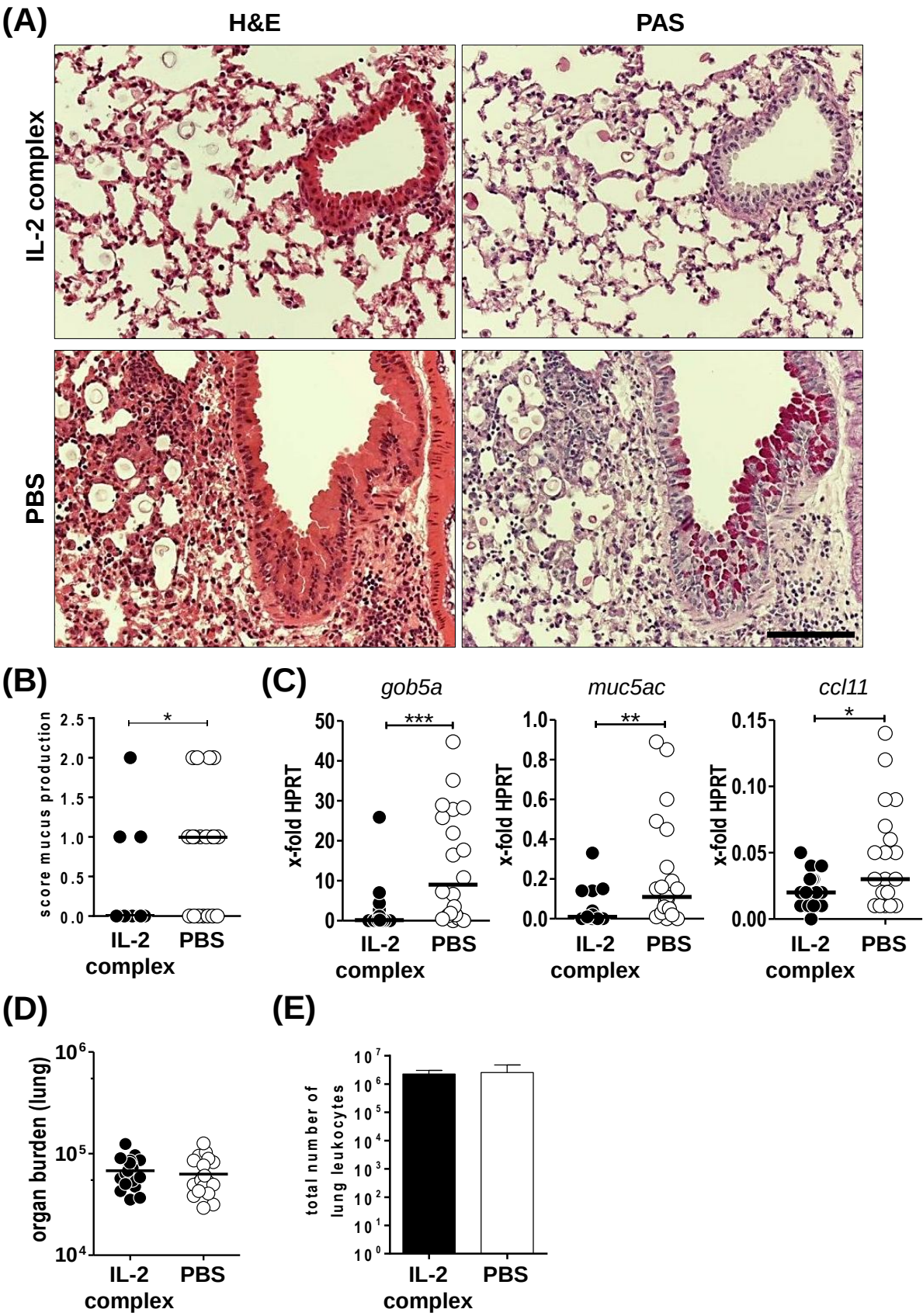


Figure 2. Treg cells contribute to control of allergic fungal airway diseases without affecting fungal burden. **(A)** Mice were treated as described in Fig. 1B and sacrificed at 21 days post infection (dpi). Histological analysis was performed to visualize mucus production by periodic acid Schiff reagent staining (PAS, right side) and cryptococci as well as surrounding leukocytes in the lung by use of hematoxylin & eosin (H&E, left side) staining. Shown are inflammatory foci from the lung sections of one representative animal of each group (magnification 400x, scale bar 100 μ m). (n=21 mice per group, two experiments in total) **(B)** Mucus production was evaluated by scoring PAS⁺ and PAS⁻ bronchial epithelial cells in at least two different lung regions with a total of 10 cross sections per mouse. Mucus score 0 = no PAS-positive bronchial epithelial cells can be detected, 1 = single PAS-positive bronchial epithelial cells are found, 2 = clusters of mucus producing bronchial epithelial cells are detected. **(C)** Mucus production was quantified in total lung tissue by RT-qPCR of *gob5* and *muc5ac* mRNA expression at 21 dpi. Additionally, the mRNA content of the eosinophil attractant *ccl11* (*eotaxin-1*) was measured by RT-qPCR. Relative gene expression was calculated on mouse housekeeping-gene hypoxanthine guanine phosphoribosyl transferase 1 (*hprt*). Data of two independent experiments were pooled. Each point represents one animal (n=21 mice per group, n= 7 or 14 mice/experiment). As data did not pass Kolmogorov-Smirnov test for normality, statistics were calculated using Mann-Whitney U-test * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. **(D)** Fungal lung burden as well as **(E)** total number of gradient-purified lung leukocytes were determined. (n=21 mice per group, n=7 or 14 mice/experiment, two experiments in total).

Schulze et al., Figure 3

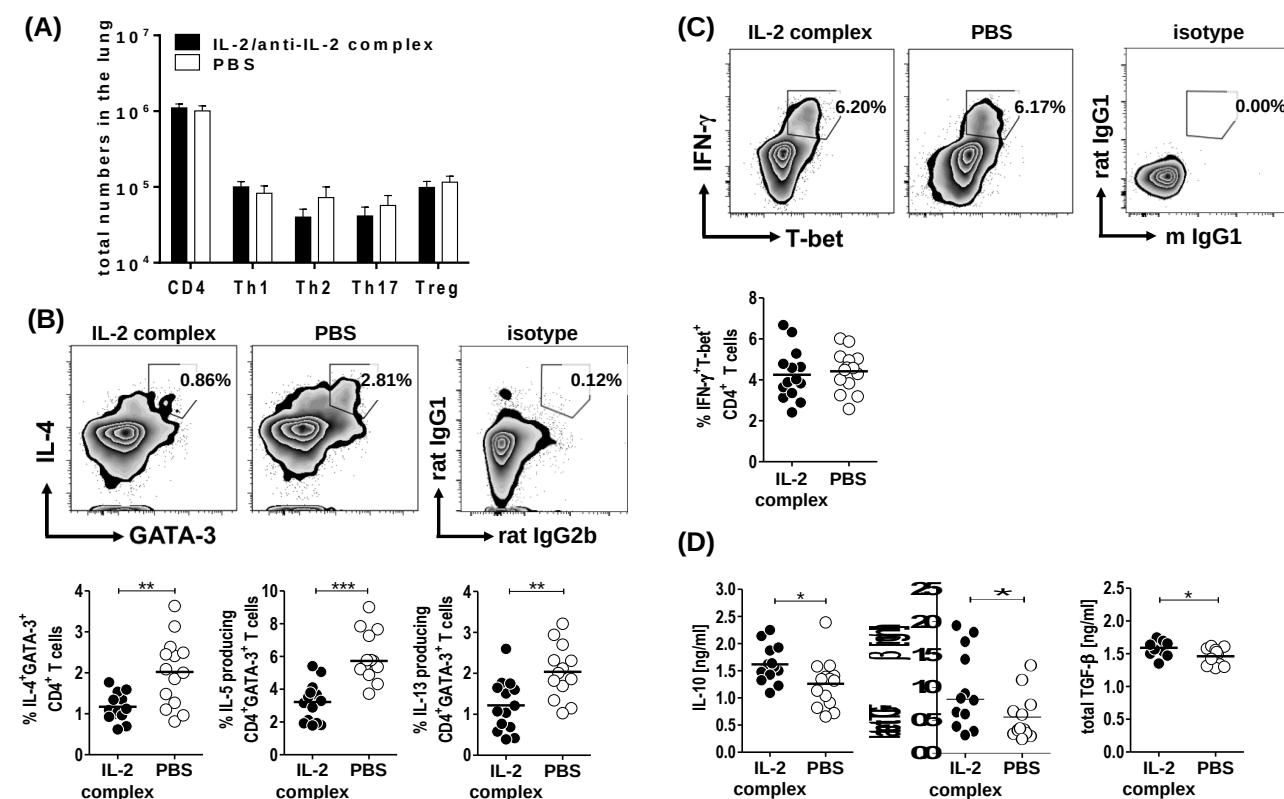


Figure 3. Expansion of Treg cells during early cryptococcosis is associated with suppression of Th2 cells and induction of inhibitory cytokine production. BALB/c mice were intranasally infected with 500 CFU of *C. neoformans* strain 1841 and treated as described in Fig. 1B with IL-2/anti-IL-2 complex. At 21 dpi mice were sacrificed and lung leukocytes were isolated. After 6 h of restimulation with phorbol-12-myristate-13-acetate (PMA)/ionomycin together with brefeldinA and monensin for the last 4 h cells were harvested and stained for flow cytometric analysis. Single viable cells were gated on CD4 prior to analysis of cytokines and transcription factors (representative plots in A and B together with the isotype control). **(A)** Total cell numbers were determined for CD4 T cells and their subsets of interest Th1, Th2, Th17 and Treg cells. **(B)** Th2 cells were further analysed by staining for IL-4, IL-5 or IL-13 and GATA-3. For IL-5 and IL-13 analysis viable single cells were gated on CD4 and GATA-3 prior to cytokine analysis. **(C)** Determination of Th1 immune cells by analysing IFN- γ and T-bet co-expression. **(D)** Supernatants from lung cell cultures after restimulation

with PMA/ionomycin for 6 h were measured by ELISA for the presence of inhibitory cytokines IL-10 and TGF- β . Two biological forms of TGF- β were analysed. Shown are from one out of two independent experiments with n=12-14 mice per group. Student's t-test or Mann-Whitney-U-test were used for statistical analysis dependent on data distribution * p < 0.05, ** p < 0.01, and *** p < 0.001.