Protective role of regulatory T cells in host defence of pulmonary *Cryptococcus neoformans* infection

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There are in fact two things,
science and opinion;
the former begets knowledge,
the latter ignorance.

Hippokrates von Kós (um 460 v. Chr. – 370 v. Chr.)

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BIBLIOGRAPHISCHE DARSTELLUNG

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Protective role of regulatory T cells in host defence of pulmonary *Cryptococcus neoformans* infection

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Dissertation

95 Seiten, 263 Literaturangaben, 19 Abbildungen, 2 Tabellen

Pilzinfektionen zählen heute zu den häufigsten tödlichen sekundären Erkrankungen bei Patienten mit einem geschwächten Immunsystem. Zu dieser Gruppe gehören z.B. mit dem humanen Immundefizienz-Virus (HIV) infizierte Menschen und ebenso mit Immunsuppressiva behandelte Patienten, wie z.B. Transplantatempfänger. Der opportunistische fungale Erreger *Cryptococcus neoformans* infiziert jedes Jahr über eine Million Menschen und belegt in Afrika Platz vier der Todesursachen von HIV-Patienten. Die Behandlung der Kryptokokkose mit Antimykotika ist möglich, jedoch weisen die bisher zur Verfügung stehenden Therapeutika zum Teil starke Nebenwirkungen auf. Aufgrund der bislang unzureichenden Behandlungsmöglichkeiten liegt ein wichtiger Forschungsschwerpunkt in der Untersuchung der Interaktion des Pilzes mit dem Immunsystem des Wirtes, um neue therapeutische Ansatzpunkte zu identifizieren. In bisherigen Arbeiten standen dabei vor allem die an der Kryptokokkose zentral beteiligten $CD4^+$ T-Helferzellen im Vordergrund. Es ist bekannt, dass T-Helferzellen vom Typ1 (Th1-Zellen) zu einer protektiven Immunantwort führen, wohingegen Th2-Zellen mit unkontrolliertem Wachstum und Dissemination des Erregers assoziiert sind. Im Gegensatz zu den bekannten Funktionen der Th1- und Th2-assoziierten Immunantwort in der Kryptokokkose ist die Rolle einer weiteren Teilpopulation der $CD4^+$ T-Helferzellen, der sogenannten regulatorischen T (Treg)-Zellen, bislang noch nicht untersucht. Treg-Zellen hemmen vor allem überschießende Immunantworten und verhindern dadurch Gewebeschädigungen, werden aber auch mit der Toleranz gegenüber verschiedener Umwelt- und Nahrungsmittelantigenen assoziiert. Zunächst sollte im intranasalen murinen Infektionsmodell die Entwicklung der Treg-Zellen in der Lunge charakterisiert werden. In einem Zeitraum von drei Wochen nach Infektion wurde ein Anstieg an pulmonalen Treg-Zellen beobachtet. Zur Klärung der Frage nach der Funktion der Treg-Zellen wurde zunächst eine Depletionsanalyse durchgeführt. Diese zeigte, dass es bei Abwesenheit von Treg-Zellen in der Frühphase der Infektion zu einer gesteigerten Th2-Immunantwort kommt, die mit einer starken allergischen Entzündungsreaktion in der Lunge korreliert ist. Umkehrstudien, in denen Treg-Zellen mittels IL-2/anti-IL-2 Komplexbehandlung expandiert wurden, bestätigten eine direkte Suppression der Th2-assoziierten Entzündungsreaktion durch Treg-Zellen und lassen somit auf eine protektive Rolle der Treg-Zellen in der pulmonalen Kryptokokkose schließen.

ABBREVIATIONS

Ab	antibody
APC	antigen presenting cell
AM	alveolar macrophage
CD	cluster of differentiation
cDNA	complementary deoxyribonucleic acid
CFU	colony forming units
CTL	$CD8^+$ cytotoxic T lymphocytes
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DC	dendritic cell(s)
DEREG	depletion of regulatory T cell
dpi	days post infection
DT	diphtheria-toxin
Ebi3	Epstein Barr virus induced gene-3
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FELASA	federation of laboratory animal science associations
FoxP3	*forkhead box P3*
GATA-3	GATA-binding protein 3
GFP	green fluorescent protein
GITR	glucocorticoid-induced tumour necrosis factor receptor
GXM	glucuronoxylomannan
H&E	hematoxylin and eosin
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPRT	hypoxanthine-guanine-phosphoribosyl transferase 1
HRP	horseradish peroxidase
ICOS	inducible T-cell co-stimulator
ICOS-L	inducible T-cell co-stimulator ligand
IDO	indoleamine 2,3-dioxygenase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
ILC2	group 2 innate lymphoid cells

IMDM	Iscove's modified Dulbecco's medium
i.n.	intranasal
i.p.	intraperitoneal
IPEX	immunodysregulation, polyendocrinopathy and enteropathy, X-linked
IRF	interferon regulated factor
MDSC	myeloid derived suppressor cells
mRNA	messenger-ribonucleic acid
NK cells	natural killer cells
PBS	phosphate buffered saline
PMA	phorbol 12-myristate 13-acetate
pTreg cell	peripheral-derived Treg cell
*q*RT-PCR	quantitative reverse transcriptase polymerase chain reaction
RA	retinoic acid
RORγt	retinoic acid-related orphan receptor
SAB medium	Sabouraud dextrose medium
STAT	signal transducer and activator of transcription
T-bet	T-box-expressed-in-T-cells
TCR	T cell receptor
TGF-β	transforming growth factor-β
Th cell(s)	T helper cell(s)
Tr1 cells	type-(1)-regulatory T cells
Treg cells	regulatory T cells
TSDR	Treg cell-specific demethylated region
TSLP	thymic stromal lymphopoietin
tTreg cells	thymic-derived Treg cell
WT	wild-type

1. INTRODUCTION

1.1 THE IMMUNE SYSTEM AND INFECTIONS

Protection from infectious agents and the damage they cause is fundamental to the body and is mediated by the immune system which has evolved both innate and adaptive mechanisms to deal with invading pathogens. Under homeostatic conditions not all foreign substances cause an immune response, even if they have the capacity. This state of unresponsiveness of the immune system is known as immunological tolerance and can be classified into central and peripheral tolerance [1]. Deficits in either central or peripheral tolerance can cause autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis and potentially contribute to asthma, allergy and inflammatory bowel disease [1,2]. In contrast, in the course of an immune response due to foreign antigens different effector mechanisms including the activation and recruitment of inflammatory cells to the site of infection have evolved [3]. These immune responses can eliminate or slow down the spread of the pathogen but can also cause severe inflammation and collateral tissue damage [4]. To prevent infection-induced immunopathology or to simply stop an ongoing immune response a variety of suppressor mechanisms, including the production of anti-inflammatory cytokines by cells of the innate immune system [5,6] or the generation of antigen-specific regulatory T cells [7-10] is induced.

1.2 REGULATORY T (TREG) CELLS

The presence of regulatory immune cells controlling effective immunity and avoiding autoimmunity as well as immune pathology has been postulated for more than three decades. However, today's knowledge of their functional properties is far from complete [11]. To date, different subsets of multiple immune regulators with overlapping functions have been described, e.g. myeloid derived suppressor cells (MDSC) [12,13], regulatory T and B cells [14,15]. In early publications the presence of a suppressive subset of T cells was usually demonstrated indirectly due to methodological limitations associated with doubts whether regulatory T (Treg) cells really exist. In 2001, with the identification of FoxP3 (*forkhead box P3*), as a reliable lineage marker, the existence of $FoxP3^+$ $CD4^+$ regulatory T cells was proven and allowed for insights into their suppressive mechanisms and biology [16,17]. The loss of FoxP3, e.g. by spontaneous loss-of-function mutation in mice (known as *scurfy* mice), results in fatal autoimmunity with uncontrolled excessive $CD4^+$ T cell activation [16,18]. Although FoxP3 was proven to be an individual marker of murine Treg cells, its role in human Treg cells is less straightforward as many activated (non-regulatory) human T cells also express FoxP3 [17,19-23]. Nevertheless, humans lacking functional FoxP3 develop early in infancy a severe autoimmune disease, the immunodysregulation, polyendocrinopathy and enteropathy, X-linked (IPEX) syndrome [24,25]. In light of the broad detrimental effects of this disease a simple medication is impossible. Immunosuppressive therapy combined with bone marrow transplantation have been shown to be of benefit in some cases [25].

1.2.1 DIFFERENT SUBSETS OF TREG CELLS

There are different subsets of Treg cells described in literature (Fig. I). In the thymus naturally occurring $FoxP3^+CD4^+CD25^+$ Treg (tTreg) cells develop, characterised by a diverse T-cell-receptor (TCR) repertoire that is specific for self-antigens [26,27]. Another subset of Treg cells can be generated in the periphery with certain cytokine environments from undifferentiated naïve peripheral $CD4^+$ T cells (pTreg cells) during inflammatory processes [28-30]. That inducible regulatory T cell population include T helper (Th)3 cells that express FoxP3 and secrete both inhibitory cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β [31-33]. In contrast, T regulatory 1 (Tr1) cells, which belong functionally to the group of pTreg cells, secrete IL-10 but lack the expression of the key transcription factor FoxP3 [30,31,34].

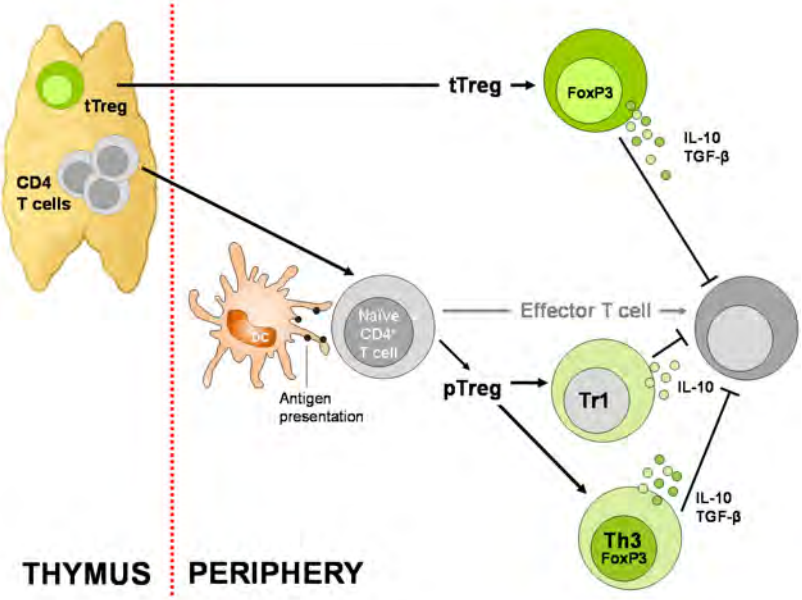


Figure I. Development of regulatory T (Treg) cells
Treg cells can develop either in the thymus (tTreg cells) as an antigen-primed and functionally mature T cell subpopulation or from naïve conventional $CD4^+$ T cells in the periphery under certain conditions (pTreg cells). While tTreg cells can secrete different cytokines that act suppressive on effector T cells, pTreg cells are more heterogeneous. Th3 cells express FoxP3 and can secrete interleukin (IL)-10 as well as the transforming growth factor (TGF)-β. In contrast, T regulatory 1 (Tr1) cells secrete only IL-10 and are shown to lack the expression of the transcription factor FoxP3.

A number of markers such as neuropilin-1, Helios or glucocorticoid-induced tumour necrosis factor receptor (GITR) have been described to be differently expressed on tTreg and pTreg cell subsets [35-37]. However, none of these markers is exclusive for Treg cells [38]. Therefore, distinguishing between tTreg and pTreg cells during infection is often not possible. Moreover, there is evidence that the cytokine signals which polarize conventional T helper cells also have an impact on the functional specification of Treg cells [39-42]. For example, the interferon regulated factor (IRF) 4, a transcription factor that is required for the differentiation of Th2 and Th17 cells, is essential for the control of Th2-driven autoimmunity by Treg cells [42]. This concept was extended by observations that T-bet, a transcription factor essential for the differentiation of Th1 cells, can be up-regulated by Treg cells to promote suppression of Th1 responses *in vivo* [40]. Although GATA-binding protein 3 (GATA-3), that is essential for Th2 development, is not required for Treg cell development, GATA-3-deficient Treg cells were shown to be defective in their ability

to control inflammatory responses with Th2 and Th17 cell involvement [43-45]. Taken together, Treg cells differ in their phenotype dependent on the disease setting, inflammatory status of the local environment, and their anatomical localization where they can use different suppressive mechanisms [46].

1.2.2 MECHANISMS OF TREG CELL FUNCTION

Treg cells are important to control peripheral tolerance by a variety of mechanisms, which from a functional perspective, can be grouped into four basic modes of action (Fig. II) [47]. In general, Treg cells can act by cell-contact-dependent as well as cell-contact-independent mechanisms [48,49]. Cell-contact-independent mechanisms include the secretion of cytokines, like IL-10 and TGF-β [28,50], that act as suppressive mediators. Different *in vivo* studies in allergy and asthma models revealed an important role for IL-10 and TGF-β in disease control indicating that both, naturally occurring tTreg cells and induced antigen-specific pTreg cells, produce suppressive cytokines [28,51-53]. The cytokine IL-35 was most recently described to play a role in Treg cell function. This new member of the IL-12 heterodimeric cytokine family is formed by pairing the Epstein-Barr virus-induced gene 3 (Ebi3) and p35. IL-35 is required for maximal suppressive activity as Treg cells deficient for Ebi3 or p35 had significant reduced regulatory activities [54,55]. Additional, as a cell-contact-dependent mechanism membrane-tethered TGF-β can suppress effector T cells [47]. Another cell-contact-dependent mechanism for Treg cell-mediated suppression of effector T cells is the modulation of the maturation and/or function of dendritic cells (DCs) which are required for the activation of effector T cells. It has been shown that the interaction of cytotoxic T lymphocyte-associated antigen (CTLA)-4, which is constitutively expressed on Treg cells [48,56], with CD80 and/or CD86 on DCs results in production of suppressive indoleamine 2,3 dioxygenase (IDO) [57,58]. IDO is the rate-limiting enzyme of the tryptophan catabolism, causing depletion of tryptophan and results thereby in limited growth of effector T cells. It was further demonstrated that the use of CTLA-4 specific blocking antibodies or CTLA-4-deficient Treg cells is associated with reduced suppression of effector T cells by DCs [59,60]. As Treg cells express high levels of the high-affinity IL-2 receptor (CD25) they consume local IL-2 and therefore starve dividing effector T cells in need of IL-2 as survival factor [49,61]. Another mechanism has been described in which Treg cells contribute to cytolysis of effector T cells [62,63]. Primarily, secretion of granzymes had long been considered to be a special mechanism of natural killer (NK) cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) [64]. However, it has been shown that Treg cells kill B cells, NK cells as well as CTLs in a granzyme B-dependent and partially perforin-dependent manner [65,66]. For human naturally occurring Treg cells the expression of granzyme A has been reported [63]. In summary, Treg cells are able to use different mechanisms for effective suppression of effector T cells, B cells, NK cells, DCs or CTLs. For example in tumour microenvironment Treg cells have been shown to use cytolytic mechanisms [65], whereas in settings of infection down-modulating cytokine responses are more common [67-70].

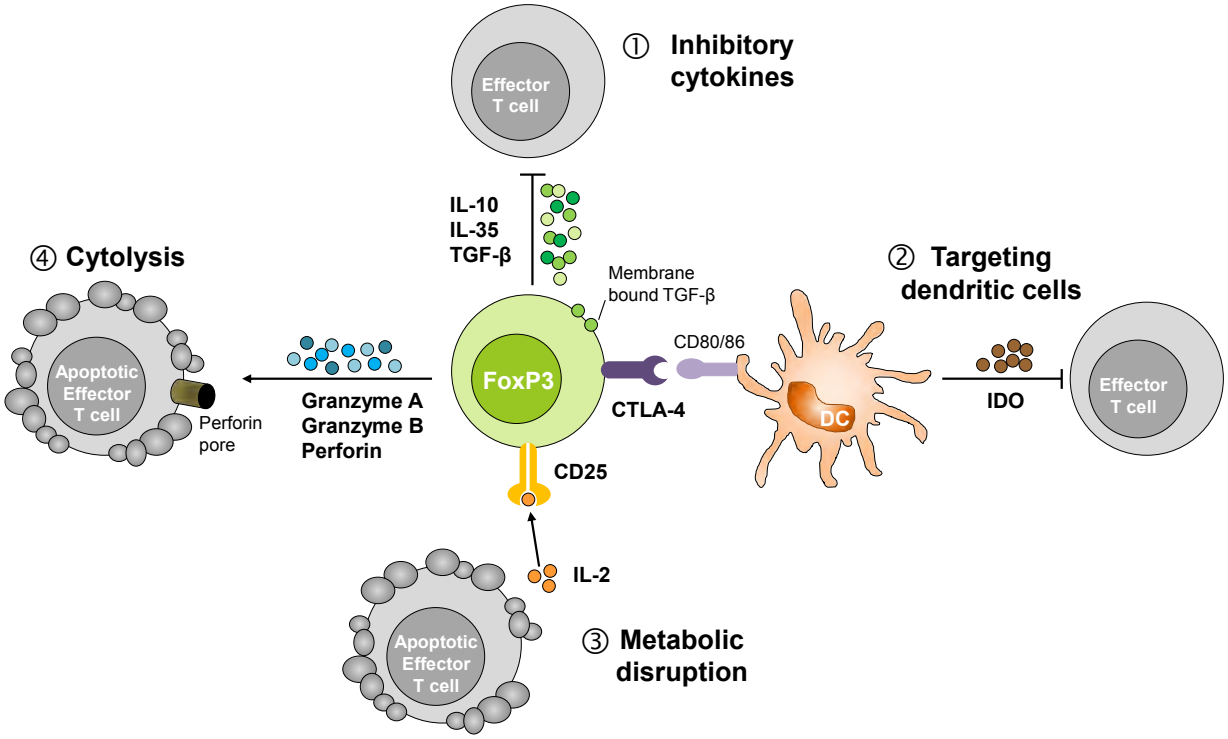


Figure II. Basic inhibitory mechanisms of Treg cells
Schematic overview of the four main mechanisms used by Treg cells for suppression of effector T cell responses. ① Inhibitory cytokines like interleukin (IL)-10, IL-35 and transforming growth factor (TGF)-β can be secreted by Treg cells to suppress effector T cells in a cell-contact-independent manner. Additionally, cell-contact-dependent mediated suppression of effector T cells can occur by membrane-tethered TGF-β on Treg cell surfaces. ② Interaction of Treg cells via cytotoxic T lymphocyte-associated antigen (CTLA)-4 with CD80/86 on dendritic cells (DCs) results in secretion of indoleamine 2,3-dioxygenase (IDO) a suppressive mediator of effector T cell responses. ③ Metabolic disruption includes snatch of IL-2 by Treg cell high-affinity receptor CD25 inducing cytokine-deprivation-mediated apoptosis of effector T cells. ④ Treg cells have the ability to induce granzyme A- and granzyme B-dependent as well as perforin-dependent killing of effector T cells.

1.2.3 TREG CELLS IN CLINICAL APPLICATION

Treg cells play crucial roles in immunopathologies, such as allergy or in IPEX syndrome. In Crohn's disease and other settings of autoimmunity transfer or expansion of Treg cells can be used therapeutically to limit the inflammatory responses [71-73]. Other clinical trials are also focused on techniques that expand Treg cells, e.g. in settings of organ transplantations as infiltration of Treg cells results in stable transplantation tolerance [74,75]. There are *in vivo* as well as *in vitro* strategies available allowing for clonal expansion of functionally stable antigen-specific Treg cells [74]. Conversely, developing strategies to inhibit Treg cell functions may enhance immunotherapy to cancer and help to promote resolution of several chronic infections [76]. Overall, Treg cells are potential targets for clinical use as they are naturally present in the circulation. However, further research is needed to identify specific surface markers for discrimination of Treg cells from conventional T cells [77]. Taken together, to manipulate Treg cell progression (both positively and negatively) would open up new therapies for treating autoimmunity, promoting transplantation tolerance, enhancing cancer immunotherapy, or resolving chronic infections.

1.3 FUNGAL PATHOGENS

Fungal infections contribute substantially to human morbidity and mortality, yet the impact of fungi as human pathogens is often overlooked. To date, more than 100,000 fungal species have been described; however, only a small portion causes disease in humans [78]. Most common among fungal diseases are superficial infections of the skin and nails that affect ~25% of the general population worldwide [79]. Mucosal infections of the oral and genital tracts are also common, in fact, 50 to 75% of women in their childbearing years suffer from at least one episode of vulvovaginal candidiasis [80]. In contrast to superficial infections invasive fungal infections have a much lower incidence though, they are of greater concern as they are associated with unacceptably high mortality rates of about one and a half million every year (summarized in table 1) [81].

Table 1. Statistics of the most opportunistic invasive mycoses worldwide

Disease (most common species)	Estimated life-threatening infections/year worldwide	Mortality rates (% in infected populations)
Opportunistic invasive mycoses		
Cryptococcosis (*Cryptococcus neoformans*)	> 1,000,000	20-70
Candidiasis (*Candida albicans*)	> 400,000	46-75
Aspergillosis (*Aspergillus fumigatus*)	> 200,000	30-95
Pneumocystis (*Pneumocystis jirovecii*)	> 400,000	20-80
Mucormycosis (*Rhizopus oryzae*)	> 10,000	30-90

The epidemiological data presented here are adapted from Brown *et al.,* 2012 (ref. 86) and are largely extrapolated from a few studies that have been performed. As fungal infections are often misdiagnosed and most fungal diseases need no official notification to the Center for Disease Control and Prevention (CDC), the calculations done here may significantly underestimate the true burden of invasive fungal diseases.

In about 90% of all reported fungal-related deaths one of these four fungal species is involved: *Cryptococcus*, *Candida*, *Aspergillus,* and *Pneumocystis*. Invasive mycoses have emerged as live-threatening diseases, establishing a pressing need for more research in this field to facilitate the development of better diagnostic tests, therapies, and vaccines for prevention [81].

1.3.1 *CRYPTOCOCCUS NEOFORMANS*

Cryptococcus neoformans is globally distributed and can be found particularly in soil and avian habitats, as the main ecological niche of this pathogen are the pigeon droppings [86,113]. Primary infection with *C. neoformans* most commonly occurs after inhalation of spores or infectious propagules which encounter alveolar macrophages or dendritic cells in the lung and trigger an immune response [82,83]. Whereas in healthy individuals professional phagocytes such as macrophages, DCs, and neutrophils are responsible for

clearing the infection, in individuals with a compromised immune system, both local and systemic spread of the fungus can occur due to the inability of effective control [78]. As the fungus has a preference for invading the central nervous system development of fatal meningoencephalitis is often the consequence. Data presented by Park [84] estimated the annual global burden of cryptococcal meningitis to reach nearly one million cases, with more than 620,000 deaths in sub-Saharan Africa accounting for the majority of worldwide deaths from HIV-related fungal infections.

1.3.2 IMMUNITY TO *C. NEOFORMANS*

Upon infection, cells of the innate immune system rapidly recognize *C. neoformans* by a variety of Toll-like receptors [85-87], mannose receptors [86], β-glucan receptors [88,89], and resulting together with the complement system in activation of innate immune responses. Different cell types can detect cryptococcal antigens and products, e.g. professional antigen-presenting cells (APCs) including DCs [86,87,90-93] and macrophages [94-99], but also natural killer (NK) cells [100-103], as well as endothelial [104] and epithelial cells have been described. Macrophages, dendritic cells, NK cells and neutrophils have been shown to effectively kill *C. neoformans;* however, most pathogenic strains can rapidly upregulate factors that promote their growth in the presence of an intact innate immune response [105-111]. Thereby, it has been shown that innate immune responses alone are insufficient to protect the host in the absence of adaptive immunity [106, 112]. However, cells of the innate immune system provide signals necessary for the development of the adaptive immune response [106-110,112] (Fig. III). Additionally, in different studies it has been demonstrated that efficient control of *C. neoformans* infection depends on the relation of Th1- and Th2-type responses [106,108,113,114]. It has been shown that a Th1-type response is essential to control infection [115-120] as decreased survival was found among mice missing the Th1-type cytokines interferon-γ (IFN-γ) and IL-12 [116,121]. In contrast, depletion of the Th2-type cytokines IL-4 and/or IL-13 has been shown to be associated with increased survival [115,122]. Besides the mentioned functions of Th1- and Th2-type responses, other subsets of the adaptive immune response might play a role in fungal clearance as well. For example, some studies suggest that the Th17 arm of the immune response might contribute to anti-cryptococcal protection [118,123-126]. Although a lot of work has been done to characterise cytokine dependence and Th cell involvement, understanding of immunity to cryptococcosis is still incomplete and needs further investigations. Because prior to this dissertation there was nothing known about the role of Treg cells in pulmonary cryptococcosis, the aim of this study was a detailed characterisation.

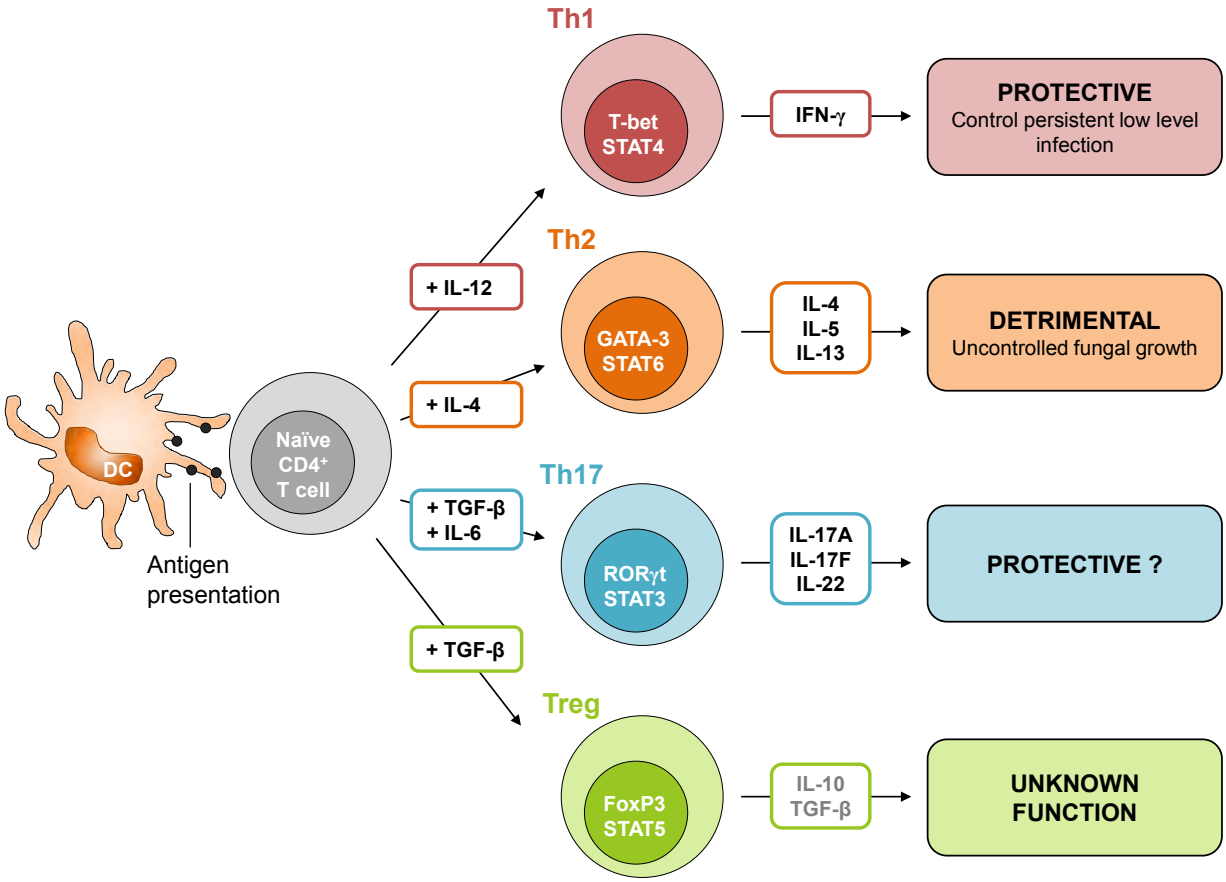


Figure III. Development of *C. neoformans* specific immune response after antigen presentation
Overview of T helper cell-associated immune responses to cryptococcal infection including transcription factors, signal transducer and activator of transcription (STAT) and cytokines expressed. After antigen presentation by dendritic cells naïve $CD4^+$ T helper cells start to differentiate depending on the cytokine environment in Th1, Th2, Th17 or Treg cells. Production of interferon (IFN)-γ results in a protective immune response against the fungi leading to controlled fungal growth but also to persistent low level infection. In contrast, secretion of IL-4, IL-5, and/or IL-13 in order of a Th2-type immune response results in uncontrolled fungal growth and dissemination into the central nervous system. There are hints that Th17-related immunity contribute to protection from cryptococcosis. Prior to this dissertation the role of Treg cells in this fungal infection was unclear.

1.4 TREG CELLS IN INFECTION

Homeostasis of the immune system depends on a balance between the responses that control infection and responses that prevent inflammation and autoimmune diseases. Treg cells are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. However, by avoiding collateral tissue damage Treg cells might also limit host-protective responses leading to pathogen survival and persistent infection [3,77,127,128]. Table 2 summarizes the impact of Treg cells on the host response in viral, parasite, bacterial, and fungal infection models. Using transgenic mice that allow *in vivo* Treg cell ablation based on FoxP3 expression, protective roles for Treg cells against viral pathogens (e.g. herpes simplex virus 2, lymphocytic choriomeningitis virus, West Nile virus) have been uncovered. By contrast, for bacterial and mycobacterial infections experimental manipulation of $FoxP3^+$ T cells revealed detrimental roles of Treg cells in host defence. This variability of the impact of Treg cells in

host defence against infection is probably related to functional plasticity in Treg cell suppression that might differ with the type of pathogen.

Table 2. Impact of Treg cells on the host defence in mice

Class	Pathogen	Impact of FoxP3$^+$ Treg cells	Manipulation and effects on immunopathology	Ref
Virus	*Herpes simplex virus*	protective	Treg cell ablation accelerates mortality and increases viral load	[129]
	West Nile virus	protective	Treg cell ablation increases viral load and mortality	[130]
	Lymphocytic choriomeningitis virus	protective	Treg cell ablation increases viral load	[129]
Parasite	*Plasmodium berghei*	protective	Treg cell expansion protects against severe disease and reduces pathogen burden that is reversed by Treg cell ablation	[131]
	Plasmodium berghei	no effect - detrimental	Treg cell ablation from baseline levels has no impact on survival or pathogen burden, or presence of Treg cells results in parasite expansion by limiting effector T cell responses	[131-133]
	Schistosoma mansoni	protective	Treg cell transfer leads to control of liver pathology and supports host survival	[134]
	Leishmania major	detrimental	Treg cell transfer favours parasite persistence in resistant strains	[10,135]
	Plasmodium yoelii	protective	Treg cell ablation favours uncontrolled expansion of parasite, leading to death of the host	[136]
	Toxoplasma gondii	protective	Treg cell survival by IL-2/anti-IL-2 complex treatment as infection results in apoptosis of FoxP3$^+$ Treg cells	[137]
	Heligmosomoides polygyrus	no effect	Treg cell ablation does not change the pathogen burden	[138]
Bacteria	*Listeria monocytogenes*	detrimental	Treg cell expansion results in increased bacterial load that it reversed by Treg cell ablation	[139]
	Salmonella enterica	detrimental	Treg cell expansion during pregnancy results in increased bacterial burden, Treg cell ablation accelerates bacterial clearance and effector T cell activation	[139,140]
	Helicobacter hepaticus	no effect - protective	Treg cell transfer prevents *H. hepaticus*-induced intestinal inflammation but has no effect on bacterial colonization	[141]
	Helicobacter pylori	detrimental	Treg cell ablation increases antigen-specific T–cell proliferation resulting in immunopathology	[142]
Mycobacteria	*Mycobacterium tuberculosis*	detrimental	Treg cell depletion reduces pathogen burden whereas adoptive transfer of Treg cells blunts effector cell expansion and increases pathogen load	[143,144]
Fungi	*Candida albicans*	protective	Treg cell co-transfer enhances fungal clearance	[145,146]
	Pneumocystis carinii	detrimental	Treg cell co-transfer increases pathogen load	[147]

Summary of Treg cell functions in the host defence against different infection. Listed are results from different mouse models using transfer studies of Treg cells and genetically manipulated Treg cells, as well as models in which Treg cells were depleted or expanded. Table adapted from [3,38,128].

During fungal infections, both inflammation and immune tolerance in the respiratory or gastrointestinal mucosa were shown to be controlled by different Treg cell subsets [148]. In a model of *Pneumocystis carinii*

infection transfer of $CD4^+CD25^-$ effector T cells into infected T- and B-cell deficient *Rag2*$^{-/-}$ mice resulted in reduced pathogen load but also in severe lung inflammation and fatal wasting disease. Co-transfer of $CD4^+CD25^+$ Treg cells in those mice prevented lung inflammation but resulted also in increased fungal load [147]. Similarly, for infection with *Candida albicans* the absence of $CD4^+CD25^+$ Treg cells was shown to increase inflammatory pathology [145]. Moreover, in a model of oral *C. albicans* infection in mice, Treg cells enhanced IL-17 cytokine production in effector T cells, which markedly enhanced fungal clearance and recovery from infection [146]. In *Histoplasma capsulatum* infection decreased Treg cell numbers in the lung were found to be associated with selective increase of Th17 cytokines [149]. For *Aspergillus fumigatus* infection an essential role for induced Treg cells was assumed in the induction of protective Th1 immune responses including T regulatory 1 (Tr1) cells [150]. To sum this up, as the function of Treg cells differs widely between different fungal infection models, it was not possible to predict their function in host response toward *Cryptococcus neoformans* infection.

1.5 AIMS OF THE STUDY

Invasive fungal infections become more and more prominent as they are correlated with high mortality and morbidity rates in immunocompromised patients worldwide. Therefore, invasive mycoses have emerged as live-threatening diseases, establishing a pressing need for more research in this field including definition of the underlying host-pathogen-interaction mechanisms. *Cryptococcus neoformans* is one of the four main occurring fungal pathogens. The course of cryptococcal disease depends on the kind of adaptive immune responses. In this context, induction of Th1 cells has been shown to be related with a protective immune response, whereas Th2 cells were associated with detrimental disease outcome. So far, nothing is known about the role of regulatory T (Treg) cells. The aim of this study was to define the role of Treg cells in host response to pulmonary *C. neoformans* infection. Analyses using a well-established mouse model of pulmonary cryptococcosis were performed to:

- Characterise the development of Treg cells in the course of pulmonary infection,
- Define the function of Treg cells under conditions of Treg cell depletion,
- Define the function of Treg cells under conditions of Treg cell expansion, and
- Initially characterise Treg cell activation and effector mechanisms by addressing the following questions:
    - Which suppressive mechanisms are used by Treg cells during pulmonary cryptococcosis?
    - How do Treg cells become activated during cryptococcal infection?
    - What are the cellular interaction partners?

2. RESULTS

2.1. T HELPER CELL DEVELOPMENT IN THE COURSE OF *C. NEOFORMANS* INFECTION

Beside the known functions of T helper (Th) cell subsets in pulmonary cryptococcosis the detailed course of Th cell polarisation has not been described yet. Therefore, in a kinetic analysis the T helper cell development in response to pulmonary cryptocccosis was analysed (Fig. IV; Bianca Schulze, unpublished data). BALB/c mice were intranasally infected with the serotype D strain of *C. neoformans* 1841 and at different time points post infection Th cell populations were studied by intracellular staining of their key transcription factors. Flow cytometric analysis revealed that the Th2-related as well as the Th17-related immune responses increased with ongoing infection. In contrast, the Th1 immune response increased during the first three weeks after infection with elevated frequencies until eight weeks post infection. Overall, in this mouse model at later time points the Th2-related immune response strongly dominated the other immune responses, resulting in death of BALB/c mice at around ten weeks post infection [122].

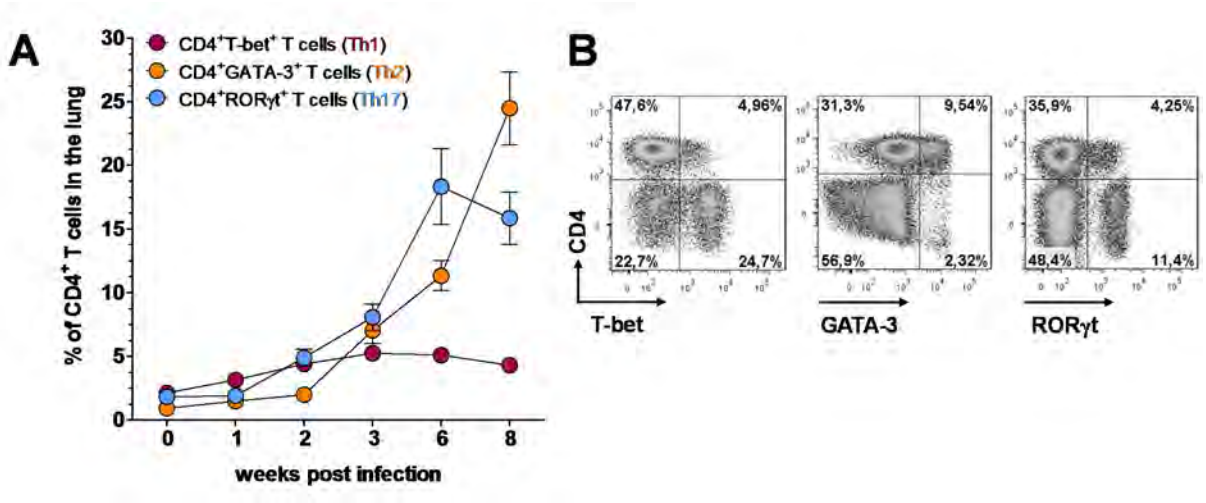
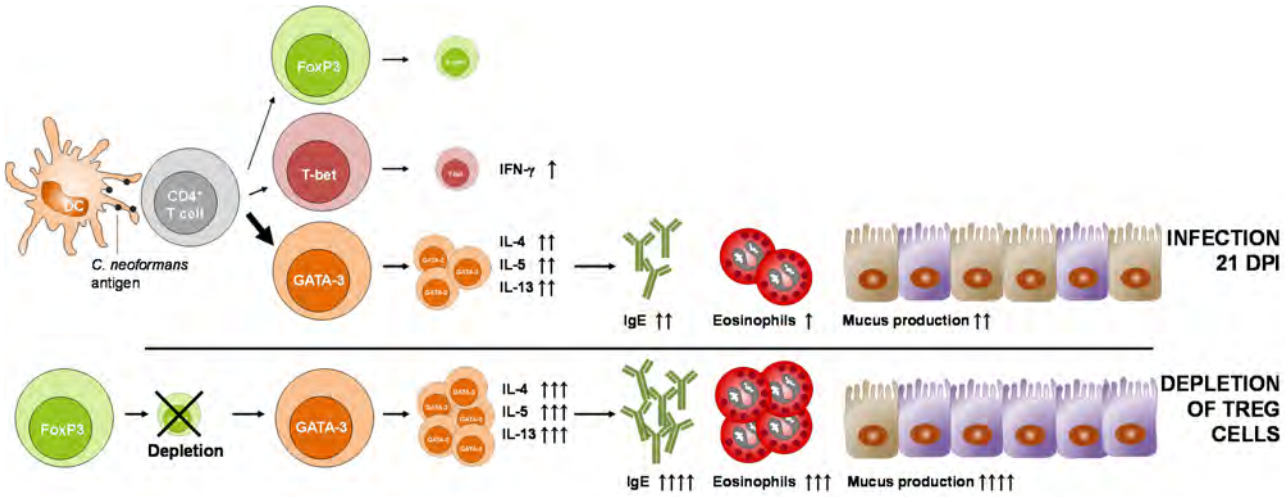


Figure IV. T helper cell development in the lung of *C. neoformans* infected BALB/c mice
Mice were intranasally infected with 500 colony forming units of *C. neoformans* and sacrificed at indicated time points. Lung leukocytes were isolated and single cell suspension was prepared. (A) Flow cytometric analysis of the key transcription factors allowed for differentiation of T helper cell subsets. Graph shows the mean ± SEM. (B) Representative plots showing T helper cell subsets in the upper right quadrant from one animal at 56 days post infection. Data shown here are from one experiment with seven mice per time point.

Although a lot of work has been done to characterise cytokines and Th cell involvement, understanding of immunity to cryptococcosis is still incomplete. For example, the role of Treg cells in pulmonary crytpococcosis has not been investigated so far. Therefore, the aim of the following studies was to characterise this population as it may yield potential therapeutical application.

2.2 CD4$^+$FOXP3$^+$ REGULATORY T CELLS SUPRESS FATAL T HELPER 2 CELL IMMUNITY DURING PULMONARY FUNGAL INFECTION

Graphical abstract:



Highlights:

- **Early increase of pulmonary Treg cells after *C. neoformans* infection**
- **Absence of Treg cells results in strong allergic inflammation associated with poorer fungal control**
- **Treg cells specifically prevent development of detrimental Th2 responses but do not modulate Th1 responses**

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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 DEREG 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

Additional supporting information may be found in the online version of this article at the publisher's web-site

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

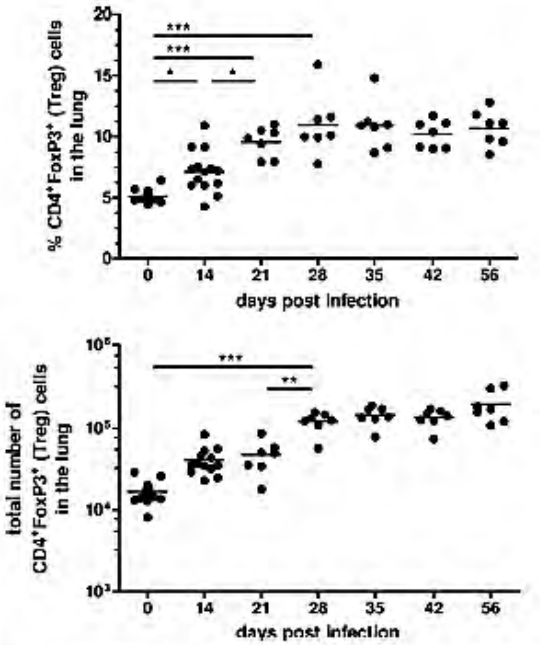

Figure 1. $CD4^+FoxP3^+$ Treg cells in the lungs of *C. neoformans*-infected mice increase in their relative and total number. BALB/c wild-type mice were intranasally infected with 500 colony forming units (CFU) of *C. neoformans* 1841 and sacrificed at the indicated time points postinfection. $CD4^+FoxP3^+$ 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

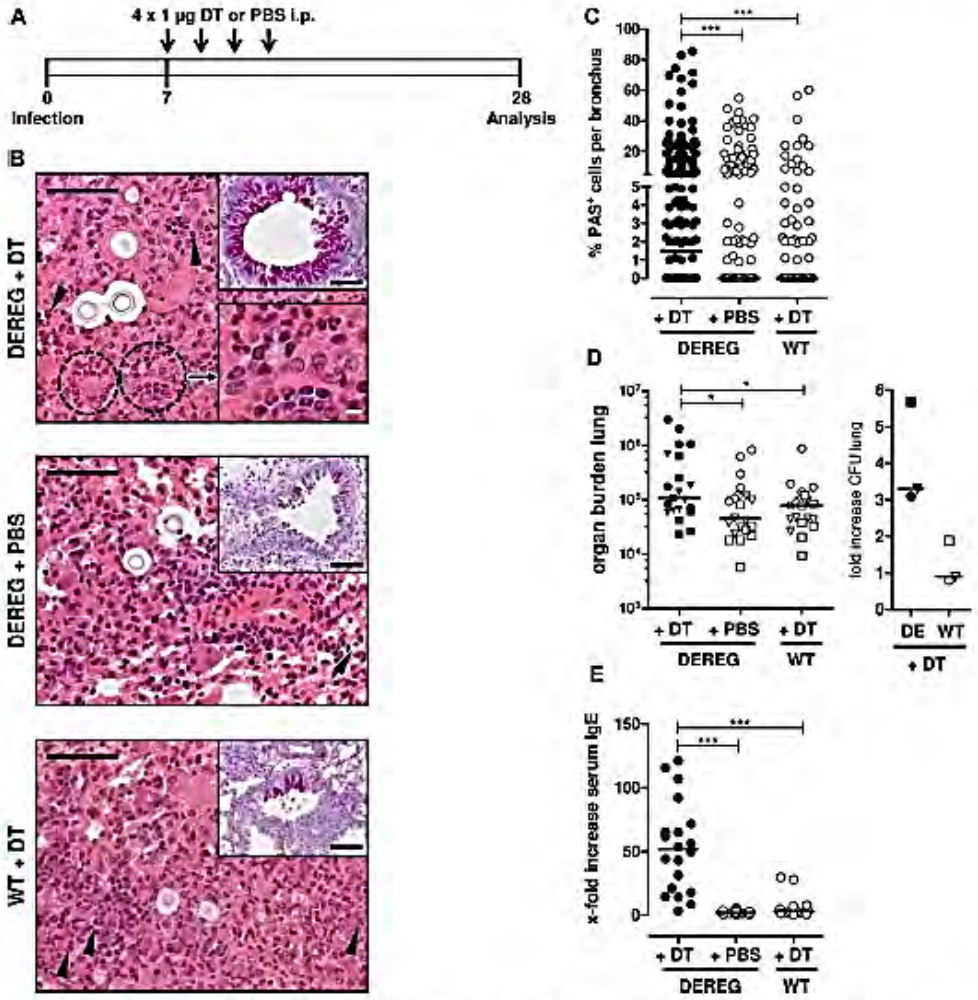

Figure 2. Increased allergic inflammation, higher fungal burden, and elevated IgE production following Treg cell depletion in *C. neoformans*-infected mice. (A) Schematic representation of the experimental set-up. DEREG mice were intranasally infected with 500 CFU of *C. neoformans* 1841. $CD4^+FoxP3^+$ Treg cell depletion was achieved by intraperitoneal (i.p.) administration of 1 µg 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 µm). 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 µm). Mucus production visualized by PAS staining is depicted in the upper right part of each picture (40x objective, scale bar 100 µm). 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 DEREG 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 DEREG 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 DEREG 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 DEREG 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 DEREG 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 DEREG 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 DEREG 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 DEREG 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 DEREG 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 DEREG mice compared with the control groups (Supporting Information Fig. 2). This 11-fold increase in serum IgG2a of Treg cell-depleted DEREG 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-γt 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 DEREG mice was treated with DT and compared with infected DT-treated or PBS-treated DEREG mice. Interestingly, Treg cell-depleted naïve and infected DEREG 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 DEREG mice had significantly higher proportions of GATA-3$^+$ Th2 cells than Treg cell-depleted naïve DEREG 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

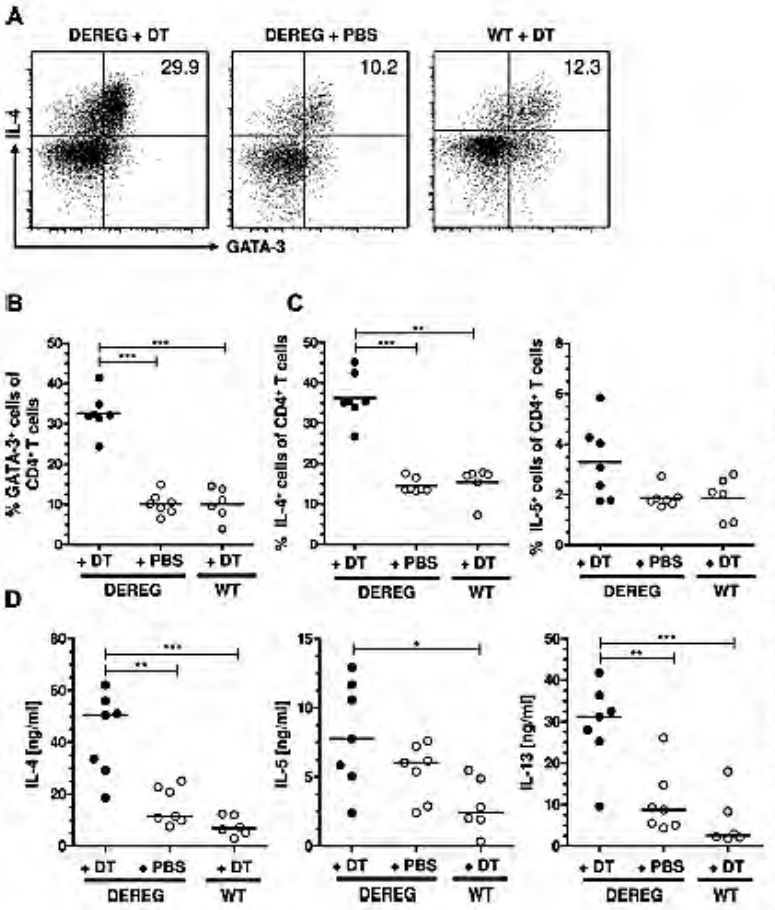

Figure 3. Increased frequency of GATA-3$^+$ Th2 cells producing IL-4, IL-5, and IL-13 upon Treg-cell depletion. Leukocytes from lung tissue were isolated at 28 dpi and stimulated with phorbol myristate acetate and ionomycin for 4 h. For flow cytometric analyses of cytokine production, lung leukocytes 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 cryptococcosis [46–48]. Conceivably, these studies may be considered as the earliest hints that Treg cells may be involved in immunity to cryptococcosis. 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 DEREG 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.

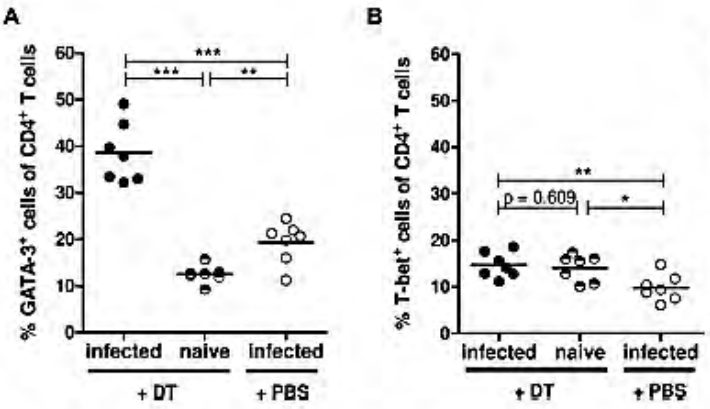

Figure 4. Significant infection-related difference in expression of GATA-3 but not T-bet in Treg cell-depleted DEREG mice. Naïve and *C. neoformans*-infected DEREG mice were treated with DT as described in Figure 2A. Additionally, *C. neoformans*-infected DEREG mice were treated with PBS instead of DT. 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. Titrating 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 (Tfh) 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 DEREG mice (Fig. 4) as compared with about 3% $GATA\text{-}3^+$ 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 DEREG mice [30] on the BALB/c background and their wild-type littermates were used. In DEREG 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 DEREG 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 Xylazin 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 optained 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, eBisocience), 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, eBisocience), 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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Abbreviations: DEREG: DEpletion of REGulatory T cells - DT: diphtheria toxin - eGFP: enhanced green fluorescent protein - IFN-γ: interferon-γ

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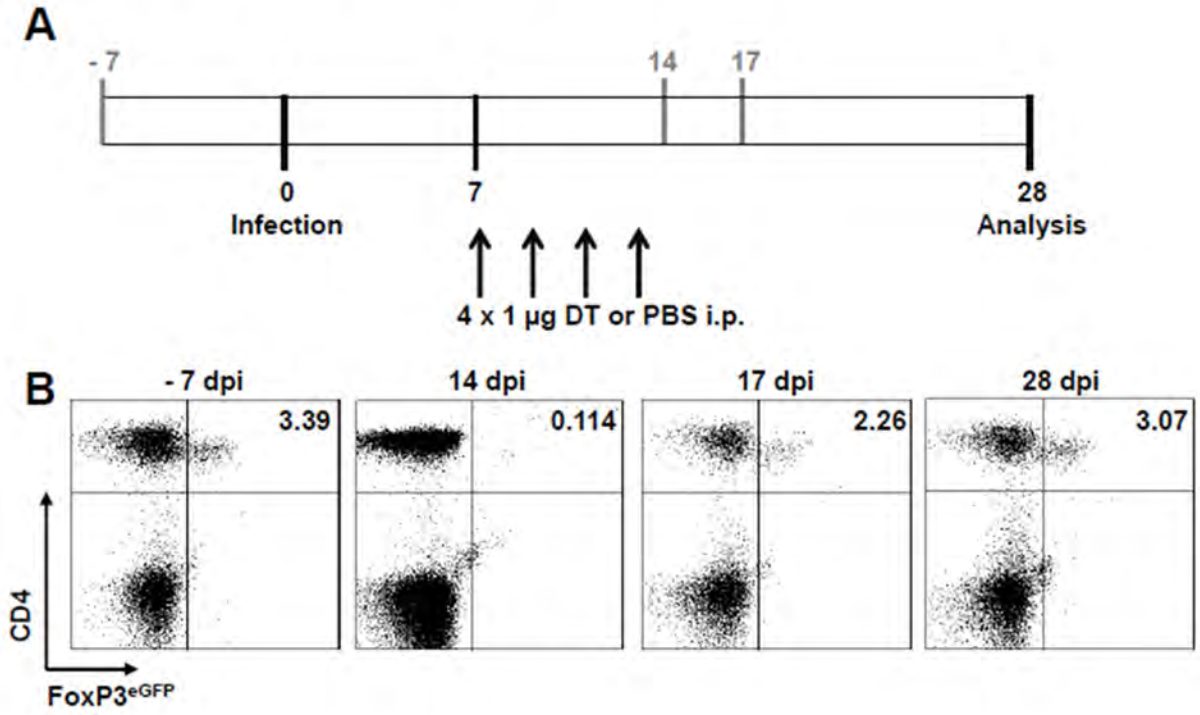
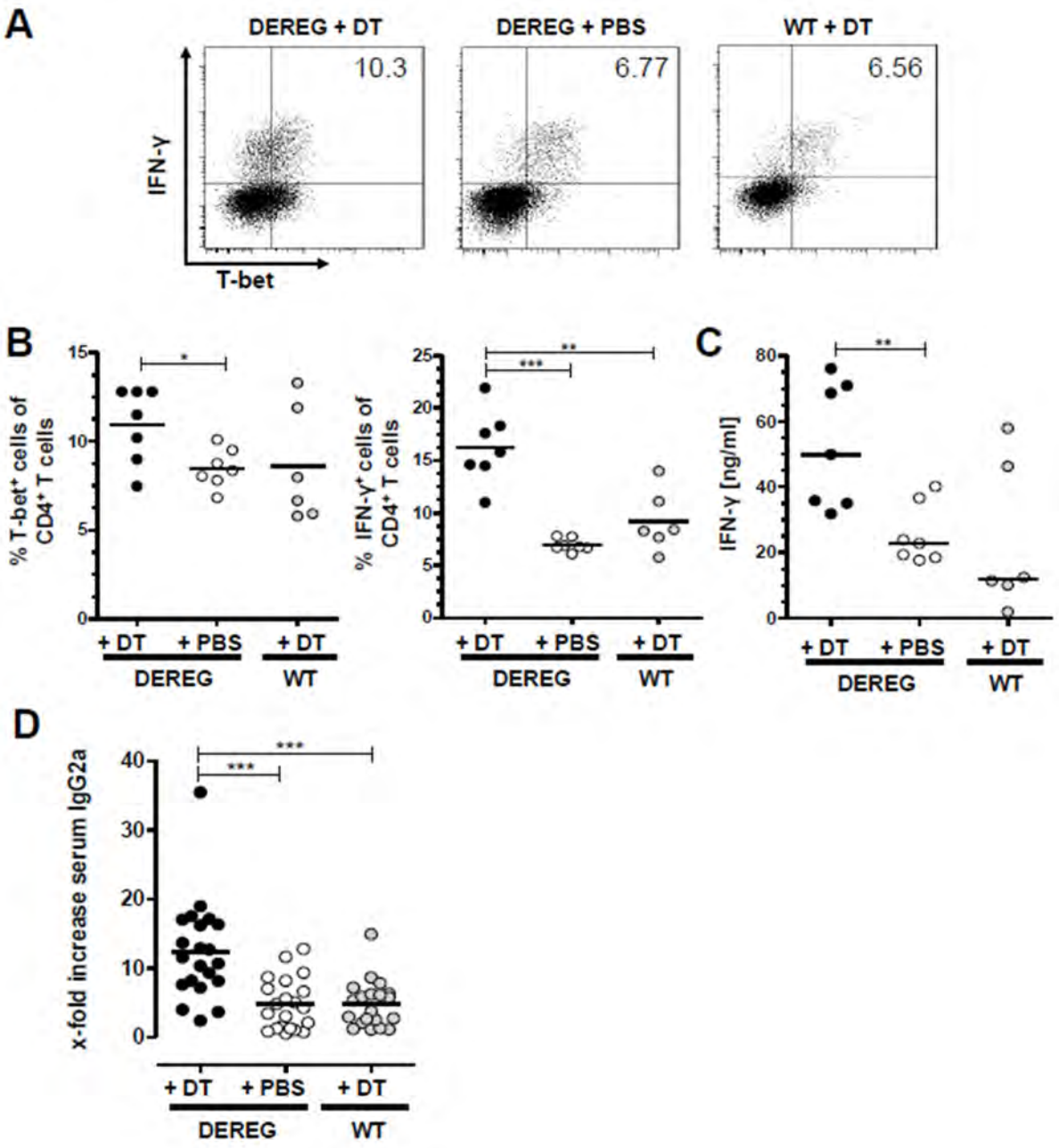
Schulze *et al.*: **Supporting Information Figure 1**



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

(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 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.

Schulze *et al.*: **Supporting Information Figure 2**

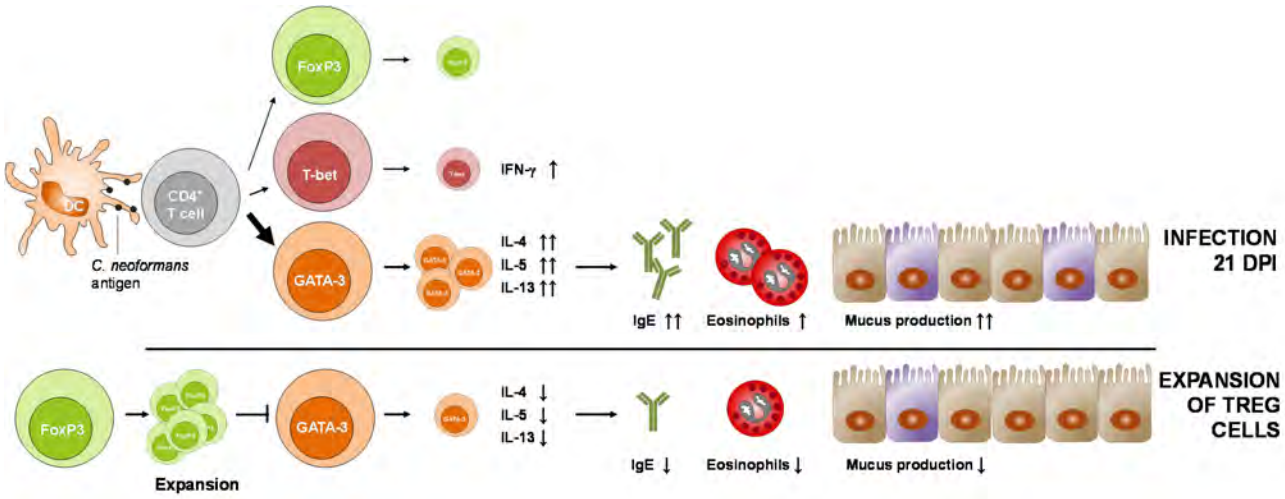


Supporting Information Figure 2. Depletion of $CD4^+FoxP3^+$ Treg cells leads to a small increase of the Th1-related immune response.

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 $^{***}p<0.001$.

2.3 INSIGHTS IN THE INDUCTION OF REGULATORY T (TREG) CELLS AND THEIR SUPPRESSIVE MECHANISMS DURING ALLERGIC INFLAMMATION IN PULMONARY FUNGAL INFECTION

Graphical abstract:



Highlights:

- **Expansion of Treg cells results in decreased allergic inflammation and reduced Th2 cytokine responses**
- **Treg cells use different suppressive mediators in *C. neoformans* infection**

Insights in the induction of regulatory T (Treg) cells and their suppressive mechanisms during allergic inflammation in pulmonary fungal infection

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ABSTRACT

The incidence of fungal infections has increased during the last years and is often associated with high morbidity and mortality, especially in cases of immunosuppression. Following infection with the opportunistic fungal pathogen *Cryptococcus neoformans* a T helper (Th)2-dominated immune response is linked to disease development. In a former study we showed that regulatory T (Treg) cells functionally contribute to the control of infection and limitation of immunopathology. Here we show that with ongoing infection lung mRNA levels of interleukin (IL)-10, transforming growth factor (TGF)-β1 as well as the cytotoxic T lymphocyte-associated antigen (CTLA)-4 and indoleamine 2,3-dioxygenase (IDO) are increased, all associated with suppressive mechanisms used by Treg cells. Moreover, we found a pronounced Treg cell activation measured by CD44 up- and CD62L downregulation. In this context upregulation of both IL-33 receptor and CD103 on Treg cells was found, both of which can contribute to crosstalk with epithelial cells. As Treg cells seem to be important for the control of cryptococcal infection, we hypothesized that their expansion could be used as an immunotherapeutic treatment approach. To test this BALB/c mice infected with *C. neoformans* were treated with an IL-2/anti-IL-2 complex to expand Treg cells. Analysis at 21 days post infection revealed lower immunoglobulin E levels in the serum as well as reduced production of key type2 cytokines, whereas the type1 cytokine interferon-γ production was not affected. Suppressive effects on Th2-associated immunity were accompanied by significantly lower pulmonary mucus production. Taken together, induction of Treg cells during pulmonary fungal infection limits bronchopulmonary allergic inflammation. Thus, Treg cells represent potential targets for therapeutical interventions.

INTRODUCTION

Regulatory T (Treg) cells are crucial for maintaining the balance of an appropriate immune response, preventing aberrant activation and minimizing collateral damage and immunopathology. Different subsets of Treg cells have been described, including a subpopulation that becomes activated in the periphery after antigen contact developing from naïve undifferentiated CD4 T cells [1,2]. A recent theory describes the expression of T helper (Th) cell subset-associated transcription factors in combination with FoxP3. This should lead to a selectively suppressing Treg cell subset that targets effector T cells expressing solely the transcription factor [2]. Up to now several markers have been described in literature to distinguish the Treg cell subpopulations including cytotoxic T lymphocyte-associated antigen (CTLA)-4, glucocorticoid-induced TNFR-related protein (GITR), glycoprotein A repetitions predominant (GARP), Helios, neuropilin-1, CD103 and inducible T-cell costimulator (ICOS). However, they often allow for distinguishing Treg cell subsets in naïve mice but as soon as immune homeostasis is disturbed these markers are less specific since effector T cells can express those markers, too [3-5]. Nevertheless, to assess Treg cell activation analysing the activation marker CD44 and the homing receptor CD62L is useful [2]. Several studies have shown that induced Treg cells highly interact with surrounding tissues especially at mucosal sides. Recently, Treg cells expressing the IL-33 receptor (IL-33R), which implies a mechanism in which secretion of the alarmin IL-33 results in prevention of excessive tissue damage by immune cells during an immune response, were described [6-8]. Furthermore, direct interaction of Treg cells with lung epithelial cells by CD103-E-cadherin interaction was shown in a study by Mock and colleagues [9]. The mechanisms involved in Treg cell suppressor activity depend on the context of the immune response and include contact-dependent inhibitory cell surface receptors, secretion of inhibitory cytokines, competition for growth factors, and direct lysis (Fig. II) [10,11].

Respiratory mycoses, ranging from invasive fungal infection to severe asthma with fungal sensitization, affect millions of people worldwide. *Cryptococcus neoformans* is a globally distributed fungal pathogen and infection is commonly associated with an impaired immune system, including people living with HIV/AIDS, cancer patients undergoing chemotherapy, and solid organ transplant recipients [12,13]. Cryptococcocal disease can result from acute infection or re-activation of latent infection causing detrimental meningoencephalitis by dissemination of the fungal pathogen to the brain [14]. CD4 Th cell subsets are critical mediators of the immune response to fungal infection. Using different settings of genetic manipulation or antibody neutralization it was shown that a Th1 immune response is essential for control of *C. neoformans* infection, whereas a Th2 immune response results in decreased survival and higher fungal burden [15-21]. Whereas the roles of Th1 and Th2 immune responses have been studied extensively, the role of Th17 immunity is less clear. However, there is evidence that the Th17 arm of immune response might contribute to anti-cryptococcal protection [18,22-25]. We could recently show that Treg cells are

involved in the protective immune response against *C. neoformans* as they selectively limit detrimental Th2 immunity [26].

In the current study we identified two possible mechanisms leading to the induction of Treg cells after pulmonary fungal infection. Furthermore, by expansion of Treg cells our findings demonstrate the potential use of those cells as therapeutic targets as they limit fungal related bronchopulmonary allergic inflammation.

RESULTS

Increased expression of different suppressive mediators with ongoing *C. neoformans* infection

Treg cells are known to have suppressive properties which can be –from a functional perspective- grouped into four basic modes of action [11]. First, by secreting inhibitory cytokines like interleukin (IL)-10, IL-35 and transforming growth factor (TGF)-β Treg cells inhibit effector T cells. Second, some studies have shown that the secretion of granzyme B and perforin by Treg cells is associated with cytolysis of effector T cells as well as of NK cells or B cells. Third, Treg cells can suppress effector T cells in a cell-cell-contact-dependent manner or indirectly by activating dendritic cells via CTLA-4-CD80/86 interaction leading to the secretion of IDO which limits effector T cell maturation and function. A fourth mechanism includes the consumption of local IL-2 by the high affinity IL-2 receptor (CD25) resulting in inhibition of dividing effector T cells which depend on IL-2 to survive. As we could show in a former study that Treg cells dampen immunopathology [26], we now examined the underlying mechanisms of suppression. To investigate principal involvement of the different suppressive mechanism, whole lung tissue was analysed in a kinetic manner by QuantiGene® Plex assay technology (Fig. 1). 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 over time. For granzyme B no change, and for perforin only a small increase of mRNA expression late in cryptococcosis was found indicating that the cytolysis pathway might be regulated to a minor degree in this infection model.

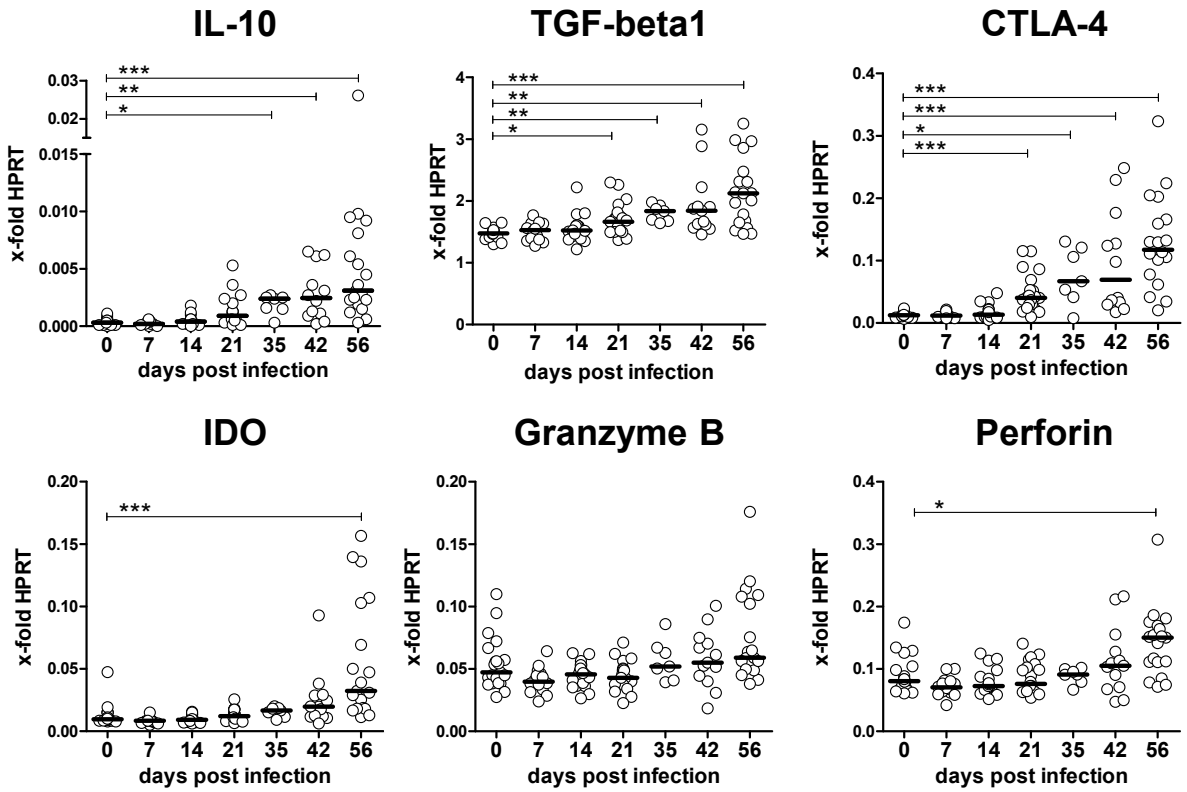

Figure 1. Induction of several inhibitory mediators during cryptococcal infection.
BALB/c mice were intranasally infected with 500 colony forming units (CFU) of *C. neoformans* strain 1841. QuantiGene® Plex assay of lung tissue was performed and expression of target genes was calculated using the house-keeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT). IDO = indoleamine 2,3-dioxygenase, IL-10 = interleukin-10, CTLA-4 = cytotoxic T lymphocyte-associated antigen 4, TGF = transforming growth factor. Shown are the pooled data from three independent kinetic experiments. In each experiment at least seven mice per time point were studied with partially overlapping time points. Statistical analysis was performed using one-way ANOVA with a Bonferroni test $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Pulmonary Treg cells become activated after fungal infection with increased ability to produce IL-10

As we found increased amounts of mRNA of molecules associated with immunosuppression in total lung tissue, we wanted to define the phenotype of pulmonary Treg cells during infection in more detail. Differentiation of naturally occurring thymus derived Treg (tTreg) cells and peripheral induced Treg (pTreg) cells is still limited and controversial, therefore we restrained our analysis on the two markers CD44 and CD62L, known to be regulated during infection (Fig. 2A) [2]. We found about 55% of pulmonary Treg cells under homeostatic conditions to express CD62L. During infection the frequency of $CD62L^+$ Treg cells dropped to 15% until 35 days post infection (Fig. 2 A, upper graph). In contrast, analysing the expression of CD44 revealed that nearly all Treg cells (~90%) become activated during infection (Fig. 2 A, lower graph) thereby representing the phenotype of pTreg cells.

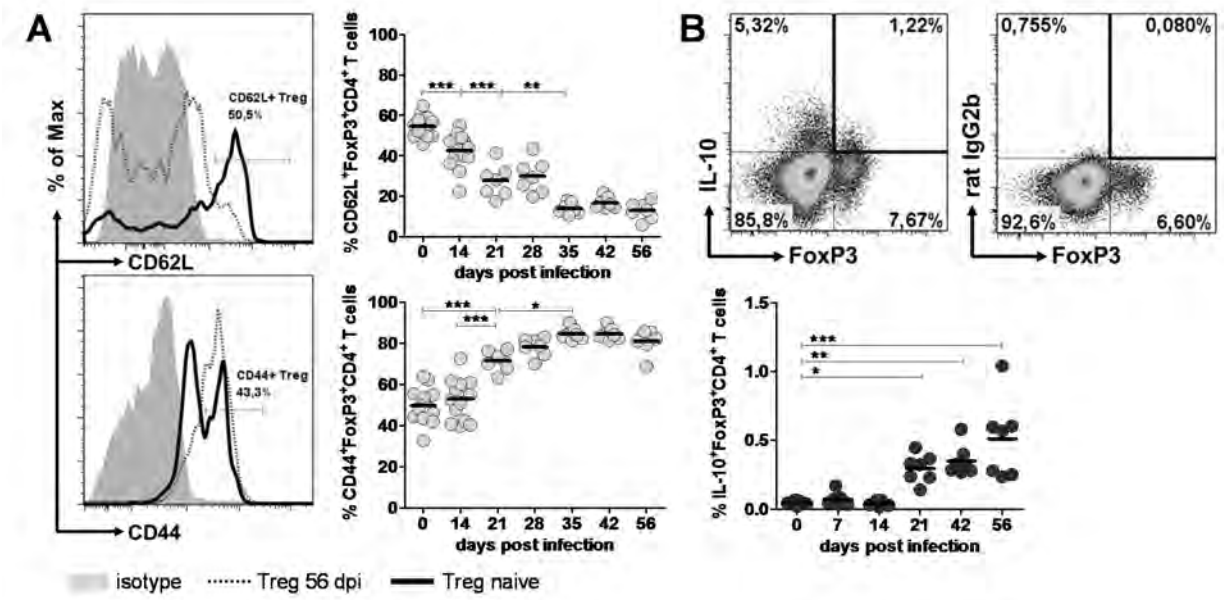

Figure 2. Treg cells become activated during pulmonary fungal infection.
BALB/c mice were intranasally infected with 500 CFU of *C. neoformans* and sacrificed at the indicated time points post infection. Naïve animals were used to show Treg cell conditions under homeostasis. (A) Activation of pulmonary Treg cells was quantified by analysing surface expression of CD44 and CD62L using flow cytometry. Representative FACS plots show expression of CD44 and CD62L from naïve control mice (black line) compared with those from 56 days post infection (dpi) (dotted line). Shown are pooled data from two independent experiments (n=7 mice per group) with partially overlapping time points (see day 0 and 14 post infection). Each data point represents one animal. (B) The ability of Treg cells to secrete inhibitory cytokine interleukin (IL)-10 was measured by flow cytometry. Therefore, isolated lung leukocytes were stimulated in presence of PMA/ionomycin for 6 h. BrefeldinA and monensin were added for the last 2 h. Representative plot for IL-10 production by Treg cells (left plot, upper right quadrant) is shown together with the fluorescence minus one (FMO) isotype control (right plot). Therefore, cells were gated for viability prior to FoxP3 and IL-10 analysis. Each data point represents one animal from one experiment with n=7 mice per group. Statistical analysis was performed using one-way ANOVA with a Bonferroni test $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Besides the characterization of surface molecules, the ability of Treg cells to secrete IL-10 during infection was investigated. As shown in Fig. 1 the mRNA expression of IL-10 was found to increase in total lung tissue. Kinetic analysis of lung leukocytes revealed that the ability of IL-10 production increased with ongoing infection (data not shown). Analysis of Treg cells revealed that they contribute to ~25% of the overall IL-10 production at 56 dpi (Fig. 2B, representative left plot showed IL-10 secretion at 56 dpi). Overall, the ability of Treg cells to secrete IL-10 significantly increased after 21 dpi (Fig. 2B, graph). Hitherto, we found that the main IL-10 producers (75%) consist of non CD4 T cells (data not shown). Taken together, we found that, after fungal infection, pulmonary Treg cells lose their CD62L and increase their CD44 expression indicating that those cells were induced and exert local suppression by e.g. increased secretion of IL-10.

Special subsets of Treg cells expressing IL-33 receptor and CD103 are preferentially induced in cryptococcosis

Recently, different groups have reported that IL-33 receptor (IL-33R)-expressing Treg cells play a role in preventing tissue damage under inflammatory conditions e.g. during inflammatory bowel disease [6-8]. Additionally, CD103 has been not only described to be an excellent marker for identifying *in vivo*-activated Treg cells [27] but has been also implicated in communication with epithelial cells via E-cadherin after tissue damage leading to proliferation of epithelial as well as Treg cells [9].

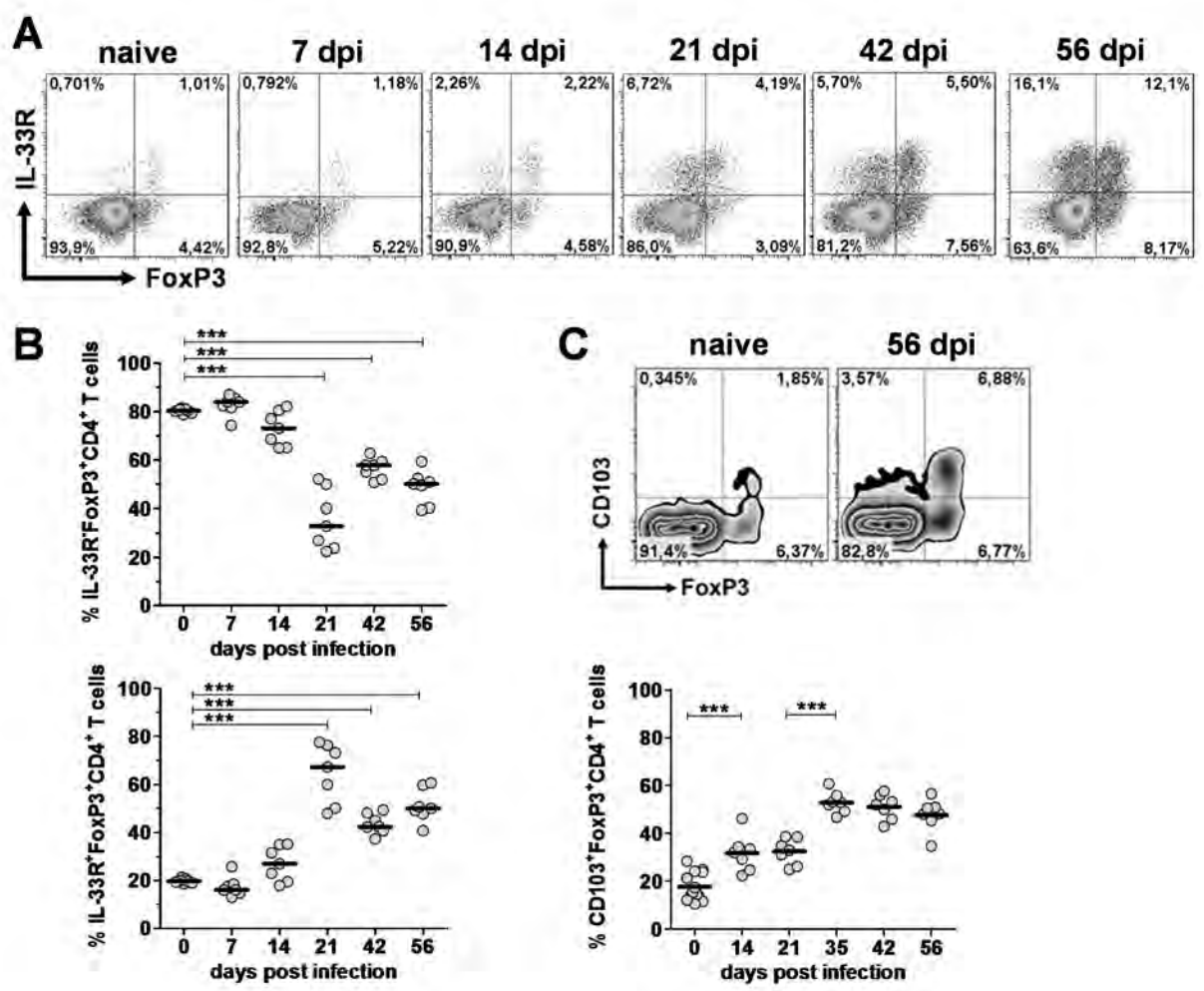

Figure 3. Increase of Treg cell subsets expressing CD103 and IL-33R during infection.
BALB/c mice were intranasally infected with 500 CFU of *C. neoformans* strain 1841 and sacrificed at indicated time points post infection. Single cell suspension from lung leukocytes was prepared and stained for flow cytometric analysis. First, cells were gated on live singlet cells. Thereafter, $CD4^+$ T cells were selected for further analysis. (A) Representative FACS plots depict the change in surface expression of the interleukin-33 receptor (IL-33R) on Treg (FoxP3) cell surface. (B) Shown is the kinetic analysis of IL-33R positive and IL-33R negative $CD4^+FoxP3^+$ Treg cells. Each data point represents one animal from one experiment with n=7 mice per group. (C) Representative plots from flow cytometric analysis depicting the differences in CD103 expression between naïve and 56 days infected mice. The graph shows the infection-dependent time course of CD103 surface expression on $CD4^+FoxP3^+$ Treg cells. Data are from two independent experiments (n=7 mice/group) with different time points of analysis and overlapping in day

0 analysis. Statistical analysis was performed using one-way ANOVA with a Bonferroni test *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

To check for an involvement of IL-33R and/or CD103 signalling in the model of cryptococcosis, BALB/c mice were infected with *C. neoformans* and pulmonary Treg cells were analysed in a time course. In naïve mice about 20% of all pulmonary Treg cells expressed the IL-33R and also 20% showed CD103 expression (Fig. 3A and Fig. 3C, representative plots). As we had not stained both markers simultaneously, we cannot conclude whether in naïve mice both markers represent different subsets of Treg cells or if those cells are double-positive. After infection frequency of $CD103^+$ Treg cells increased significantly within 14 days, whereas the IL-33R expression started to increase one week later resulting in Treg cell subsets that account for up to 50% of total Treg cells (Fig. 3). Interestingly, the majority of CD4 T cell expressing CD103 at 56 dpi were Treg cells (Fig. 3C, representative plot).
Taken together, by analysing IL-33R and CD103 expression we found an increase of both receptors on the surface of Treg cells resulting in a subpopulation of 50% that expressed IL-33R and/or CD103.

Expansion of Treg cells by IL-2/anti-IL-2 complex treatment limits bronchopulmonary allergic inflammation in fungal infection

In former studies we found that targeted depletion of Treg cells resulted in stronger allergic inflammation with increases in mucus production, levels of immunoglobulin (Ig) E, and frequencies of GATA-3^+ Th2 cells expressing elevated levels of interleukin (IL)-4, IL-5, and IL-13 [26]. Because Treg cells contribute to attenuation of allergic inflammation, we hypothesized that their expansion could be used as an immunotherapeutic treatment approach. Therefore, we infected BALB/c mice with *C. neoformans* and treated them with IL-2/anti-IL-2 complex one week after infection to expand Treg cells (Fig. 4A). Successful expansion of $CD4^+FoxP3^+$ Treg cells was monitored by staining peripheral blood Treg cells. 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 (Fig. 4B). Mice were sacrificed at 21 days post infection. Treg cell expansion resulted in lower serum IgE levels (Fig. 4C). Analysis of total lung tissue revealed an overall decreased allergic airway inflammation as lower mRNA levels of *gob-5* and *muc5ac* (Fig. 4D) indicate attenuated mucus production [28]. Moreover, 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. 4D, right graph). Furthermore, histological analysis of the medial lung lobe was performed using haematoxylin and eosin (H&E) as well as periodic acid Schiff reagent (PAS) staining (Fig. 4E). In summary, the histological analysis pointed toward higher inflammation and more cryptococci with stronger mucus production in mice treated with PBS (Fig. 4E), which supports the findings from qRT-PCR.

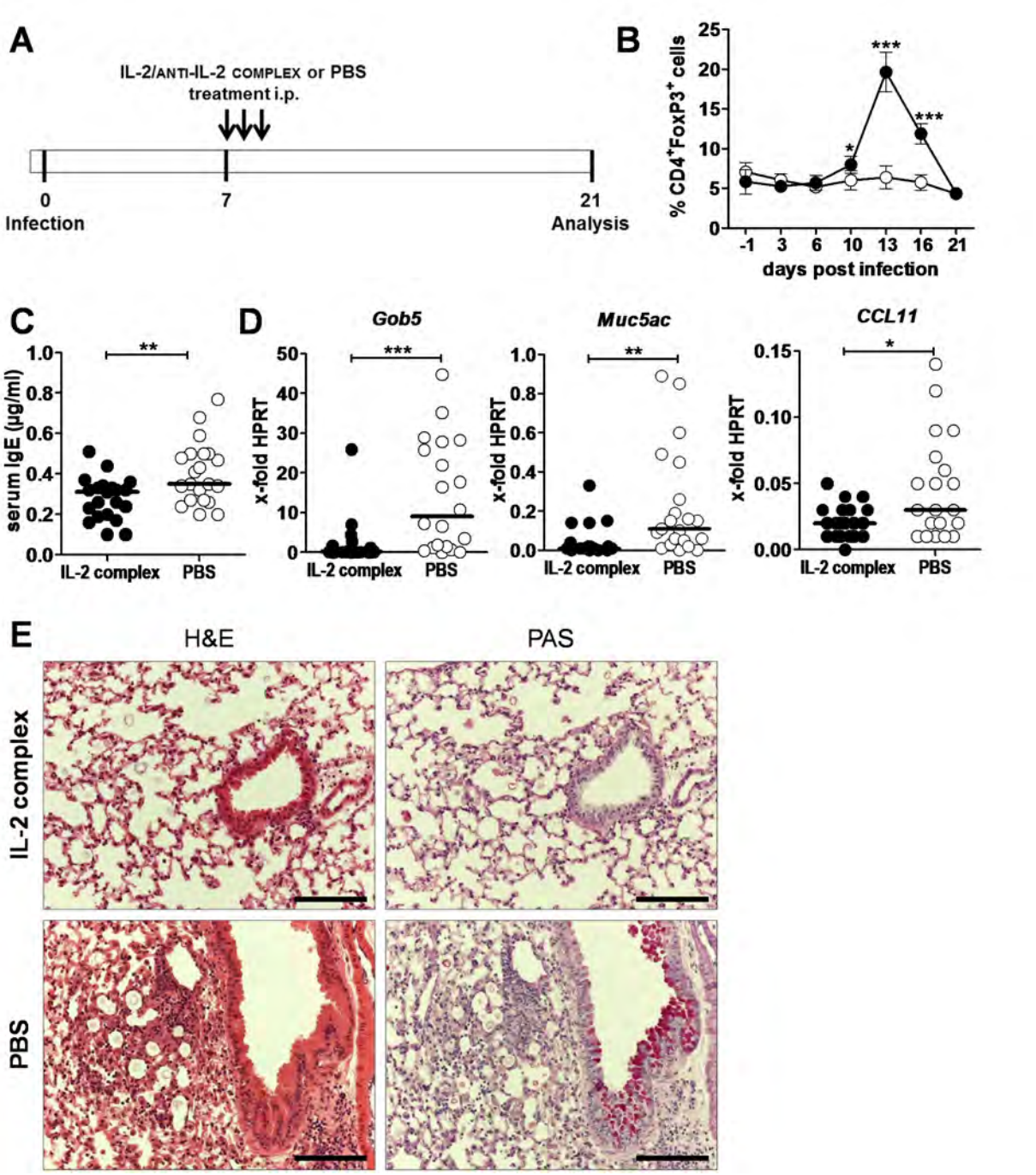

Figure 4. Expansion of Treg cells by IL-2/anti-IL-2 complex treatment in early cryptococcosis results in lower IgE production and decreased allergic inflammation

(A) Schematic representation of the experimental set-up. BALB/c mice were intranasally infected with 500 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 for three times every 24 h. Control mice received three times PBS. Mice were sacrificed at 21 dpi to study the influence of early Treg cell expansion. (B) At indicated time points mice were bled submandibularly and blood leukocytes were isolated. Single cells were stained for CD4 and FoxP3 and frequency of Treg cells was monitored by flow cytometry. Black circles represent data from IL-2/anti-IL-2 complex treated animals; white circles show data from control mice (PBS). Mean with standard deviation from at

least four to five mice per time point and group is shown. Student's t-test was performed for statistical analysis defining differences between both groups at the indicated time points. (C) Mice were sacrificed at 21 dpi and total immunoglobulin (Ig)E in the serum was determined by ELISA. (D) Mucus production was quantified in total lung tissue by qRT-PCR of *muc5ac* and *gob5* mRNA expression at 21 dpi. Additionally, the mRNA content of the eosinophil attractant *CCL11 (eotaxin-1)* was measured by qRT-PCR. Relative gene expression was calculated on mouse housekeeping gene HPRT. For C and D each data point represents one animal pooled from two independent experiments (n=21 mice per group). Statistical analysis was done using Student's t-test $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$. (E) Histological analysis was performed to visualize mucus production by periodic acid Schiff reagent staining (PAS) and cryptococci as well as surrounding leukocytes in the lung by use of haematoxylin & eosin (H&E) staining. Lung sections show inflammatory foci from one representative animal of each analysed group (magnification 400x, scale bar 100 µm).

In addition, T helper cell subsets were studied by flow cytometry using the key transcription factors (Th1: T-bet, Th2: GATA-3) in combination with the key cytokines (Fig. 5). After Treg cell expansion we found lower levels of IL-4, IL-5 and IL-13 Th2 cells (Fig. 5A). In contrast, the production of the Th1-associated cytokine interferon-γ (IFN-γ) was not affected (Fig. 5B) by IL-2/anti-IL-2 complex treatment. As Th2-related cytokine secretion was still diminished when Treg cell frequencies dropped to levels comparable to PBS-treated mice, analysis of Treg cell-associated cytokines was performed in the supernatant of lung leukocytes after PMA/ionomycin restimulation. Total IL-10 as well as two different forms of bioactive TGF-β1 were detectable (Fig. 5C).

Overall, in mice with expanded Treg cells early after infection significantly higher amounts of inhibitory cytokines were detected. However, even if the expansion of Treg cells resulted in selective suppression of Th2 immune response, those effects were too weak to reduce the fungal burden in the lung of these mice (data not shown). In summary, we demonstrated the beneficial role of Treg cells in controlling airway inflammation as *in vivo* induction of $CD4^+FoxP3^+$ Treg cells resulted in lower frequencies of Th2 cells with decreased capacity to secrete IL-4, IL-5 and IL-13, as well as reduced airway inflammation as seen by lower mucus production and decreased levels of serum IgE. Thus, our data provide a basis for future research to develop treatment methods that expand Treg cells as an immunotherapeutic approach for *C. neoformans* infection.

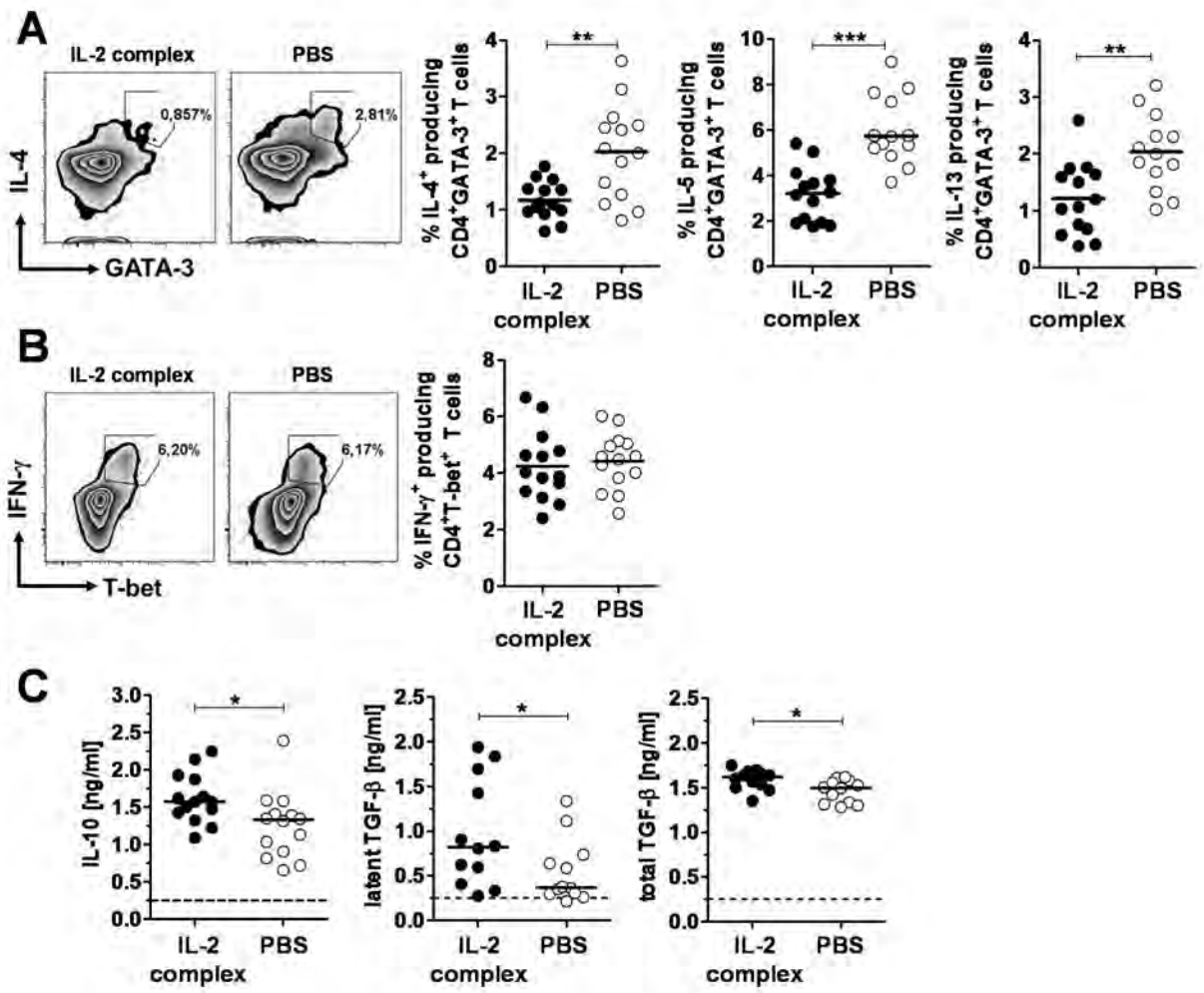

Figure 5. Expansion of Treg cells early during cryptococcosis is associated with suppression of Th2 cells and induction of inhibitory cytokine production.

BALB/c mice were intranasally infected with 500 CFU of *C. neoformans* strain 1841 and treated as described in Fig. 4A. At 21 dpi mice were sacrificed and lung leukocytes were isolated. After 6 h of restimulation with PMA/ionomycin together with brefeldinA and monensin for 2 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). (A) Th2 cells were analysed by staining for IL-4, IL-5 or IL-13 and GATA-3. (B) Determination of Th1 immune cells by analysing IFN-γ and T-bet co-expression. (C) 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. Dotted line represents the assay's detection limit. Data shown here 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 dependent on the distribution of the values $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

DISCUSSION

As fungal infections are associated with high mortality and morbidity rates, next generation anti-fungal treatment should not only block fungal growth, but should also target the host immune response. In a recent clinical trial combined treatment of IFN-γ and traditional anti-fungal therapy improved cryptococcal clearance, yet it had no significant impact on patient survival [29]. The development of new medication is focused on approaches that effectively combat lethal fungal infections by inhibiting fungal growth, dampening pathologic inflammation, and promoting beneficial anti-fungal host responses.

One of those new approaches could be the manipulation of regulatory T (Treg) cells as they are available in the periphery and a wide range of tools exist for their manipulation [30,31]. In general, Treg cells are believed to use one or more mechanisms of immunosuppression. In this study we found that during experimental pulmonary cryptococcosis overall mRNA expression levels of two Treg cell-associated suppression mechanisms were increased. That included the pathways of inhibitory cytokine secretion and crosstalk between T cells and immunoregulatory cells like dendritic cells (DCs), which highly express IDO. In contrast, mediators of the cytolysis pathway granzyme B and perforin were present in the lung with only slightly altered mRNA levels leading to the conclusion that cytolysis is not a major pathway in pulmonary cryptococcosis. Yet, as constant expression of granzmye B and perforin was found, it is likely that cytolysis takes place. Regarding to this, the group of Moody published data in which they showed that natural killer (NK) cells secrete perforin in response to *C. neoformans* [32,33]. Therefore, the slight increase in perforin mRNA expression found with ongoing infection might result from NK cell activity rather than from Treg cell responses. As total lung tissue was analysed in this study to find out which suppressive mechanisms are the most promising, activation of Treg cells and their ability to secrete IL-10 in the course of infection was investigated next. For various infections IL-10 has been found to be crucial for the control of immunopathology and that Treg cells have been involved; however, Treg cells have not been the source of IL-10 in these infection models, including *Mycobacterium tuberculosis* [34]*, Toxoplasma gondii* [35], *Leishmania major [36],* and *Trichinella spiralis* [37]. We found in our model that Treg cells are activated during pulmonary fungal infection with increased capacity to produce IL-10. Nevertheless, the amounts of IL-10 secreted by Treg cells represent only a portion of the overall IL-10 found by intracellular cytokine staining. Therefore, other cells like DCs are likely to be involved in the secretion of IL-10 as further analysis by flow cytometry revealed that most IL-10 producers are non T cells. Recently, a study revealing that blockade of IL-10 early or late in infection enhances Th1 and Th17 effector responses that promote fungal clearance was published [38]. In this model of infection with *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 [39-42]. However, in these studies the sources of IL-10 remained elusive and data about Treg cell involvement in IL-10 secretion was lacking. In contrast to our model of virulent infection, published data by Murdock and colleagues [38] present a persistent infection model in

which sustained expression of IL-10 has been measured by Luminex technology, allowing a more sensitive detection of the cytokine. The use of an IL-10R blockade resulted in decreased (not cleared) fungal burden but this blockade did not influence bronchopulmonary allergic inflammation. Earlier it was shown that *C. neoformans* can exploit the IL-10-singaling pathway to oppose protective effector mechanism by numerous virulence strategies, including expression of its polysaccharide capsule, laccase, and its interference with macrophage scavenger receptor A [41-46].

As Treg cells are important to maintain homeostatic conditions they are always in an activated state expressing moderate levels of the activation marker CD44 [47-50]. It is known that the homing receptor CD62L is required to enter secondary lymphoid tissues and is therefore expressed on Treg cells under homeostatic conditions [48,51,52]. Yet, we found only about 55% of pulmonary Treg cells under homeostatic conditions to express CD62L (Fig. 2 A, upper graph) leading to the conclusion that the remaining Treg cells might be tissue-resident Treg cells which do not need to enter the secondary lymphatic tissues and therefore lack CD62L expression. In a kinetic analysis we demonstrate that pulmonary Treg cells probably consist of two subpopulations, one with access to the lymphatic tissue and the other with tissue-homing potential. Other markers such as neuropilin-1 and Helios have been described in literature to distinguish between tTreg and pTreg cell populations [4,53]; however, we (data not shown) and others found that those markers do not prove to be reliable during infection [54-56].

From the increasing expression levels of CTLA-4 and IDO in total lung tissue an interaction of Treg cells with DCs and macrophages is concluded. Furthermore, there is evidence that at mucosal sides Treg cells might be directly involved in epithelial crosstalk. Recently, in different compartments IL-33R expressing Treg cells were shown to play an important role in suppression of excessive immune responses that damage the surrounding tissue [6-8,57]. In cases of experimental cerebral malaria IL-33R$^+$ Treg cells were induced by innate lymphoid cells type 2 (ILC2) and M2 macrophages [6]. Additionally, it was recently described that after helminth infection IL-33-mediated activation of ILC2s is required for accumulation of IL-33R$^+$ Treg cells which is dependent on direct co-stimulatory interactions via ICOSL-ICOS [57]. However, mechanisms that link ILC2 activation and Treg cells remain incompletely understood. As Treg cells constitutively express the IL-33R at low frequencies it is possible that they can respond directly to IL-33 released e.g. by epithelial cells [8]. We recently found an increase in pulmonary Treg cells with ongoing *C. neoformans* infection rising between 14 and 28 days post infection (dpi) with elevated numbers until 56 dpi [26]. This is in accordance with increasing levels of IL-33 mRNA and protein in total lung tissue up to 21 dpi, identifying epithelial cells as the main source after *C. neoformans* infection (unpublished data by L. Heyen) as well as increased numbers of ILC2 (Piehler *et al.,* 2015, submitted). Analysis of IL-33R expression on Treg cell surface revealed that under homeostatic conditions only a minor percentage of the Treg cell population expressed this receptor. With ongoing fungal infection the number of IL-33R$^+$ Treg cells increases resulting in about half of the total Treg cell population being able to respond to IL-33. In

another study, it was shown that conventional mouse CD11c$^+$ DCs stimulated with IL-33 were able to secrete IL-2 which selectively expanded IL-33R$^+$ Treg cells [7]. Moreover, in those studies IL-33R expressing Treg cells have been shown to represent a more activated subset of FoxP3$^+$ cells, demonstrated to be ICOS^{high}CD44^{high} compared to the IL-33R negative Treg cell counterpart. Besides IL-33/IL-33R interaction another marker was described in literature to result in induction of Treg cells. In a study of *Leishmania major* infection accumulation of CD103-expressing Treg cells at the site of infection was found [58]. Administration of blocking antibodies against CD103 was found to be associated with decreased Treg cell numbers and decreased rates of alveolar epithelial proliferation after injury, demonstrating that Treg cells can enhance epithelial proliferation in a CD103-dependent manner [9]. As we found increasing number of CD103$^+$ Treg cells during cryptococcal infection a direct link toward crosstalk induced by lung tissue damage seems likely. In summary, in our model of experimental pulmonary fungal infection we found that both IL-33R and CD103 might be associated with the induction of pulmonary Treg cells. Together, our data point toward a more complex interaction that might include DCs, ILC2, macrophages and epithelial cell crosstalk needing further investigations.

The second part of this study was focused on the modulation of an established infection with the aim to determine whether an intervention by manipulating Treg cell numbers would be of therapeutic value. In addition to our study, another paper was published supporting our previous findings [26] that depletion of Treg cells results in stronger allergic inflammation with increased Th2 cytokine responses [59]. As we hypothesized by previous published data that Treg cells are involved in the onset of cryptococcosis thereby selectively inhibiting detrimental Th2 immune responses [26], we asked whether expansion of Treg cells would result in stronger protection of the host. Temporary increase of Treg cells *in vivo* can be achieved by application of an interleukin-2/anti-interleukin-2 (IL-2/anti-IL-2) complex [60]. Therefore, anti-IL-2 monoclonal antibody clone JES6-1 was used as this treatment has been shown to preferentially amplify Treg cells by blocking the medium-affinity IL-2Rβγ receptor (expressed on CD8$^+$ T cells and NK cells) binding sites, whereas the high-affinity IL-2R, CD25 (IL-2Rαβγ) binding site on Treg cells remained exposed [61,62]. Expansion of Treg cells in early cryptococcal infection resulted in decreased allergic airway inflammation as seen by reduced levels of *gob5* and *muc5ac,* lower serum IgE levels and a decreased secretion of Th2 cytokines IL-4, IL-5 and IL-13. Those findings are in accordance with our hypothesized model of selective suppression of Th2 immune response by Treg cells. However, the data presented here are in contrast to a recently published study by Wiesner and colleagues revealing an increase in antigen-specific Th2 cells by IL-2/anti-IL-2 complex treatment [59]. Those differences might result from the different serotypes and the amount of *C. neoformans* used in both studies. The low dose of 500 colony forming units (CFU) from the *C. neoformans* serotype D strain used in our studies leads to a more chronic course of infection allowing survival of mice up to 70 dpi. In contrast, most of the mice infected with an inoculum of $5x10^4$ CFU of the serotype A strain used in the Wiesner study succumb to the infection at about

20 dpi [59]. Therefore, in the Wiesner study the treatment time point at 7 dpi may be too late to selectively expand only Treg cells as other activated Th cell subsets, including Th2 cells, start to express CD25, too. In our model at the time point of IL-2/anti-IL-2 complex treatment more than 90% of responding CD25-expressing cells co-express FoxP3 (data not shown) which allows selective Treg cell expansion. The effects caused by Treg cell expansion were analysed 14 days after the last treatment when Treg cells reached comparable levels to the PBS group. Even if the number of Treg cells was comparable to that from the control group a lower cytokine response by Th2 cells was found. As neither the activation nor the number of Treg cells could be the reason for enhanced suppression (data not shown) supernatants from PMA/ionomycin-restimulated lung cells were analysed for IL-10 and TGF-β. Indeed, significant higher amounts of IL-10 and TGF-β were found in the group of IL-2/anti-IL-2 complex treated mice indicating a long lasting response caused by single Treg cell expansion. Moreover, in 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* [11,63]. As we found also higher levels of IL-10 this could be a promising pathway for further investigation. Nevertheless, the overall mechanism by which Treg cells can selectively suppress the Th2 immune response remains elusive and need further investigations.

Taken together, from our data we favour a model in which epithelial crosstalk with Treg cells leads to their expansion. Epithelial cells might interact with Treg cells by IL-33 secretion or CD103 interaction resulting in induction of Treg cell population with increased CD44 as well as CTLA-4 expression. Those activated Treg cells might have increased capability to secrete inhibitory cytokines such as IL-10 and TGF-β thereby directly inhibiting the detrimental Th2 response. Indirectly, Treg cells might interact by CTLA-4 with dendritic cells and macrophages leading to secretion of IDO, IL-10 and TGF-β thereby limiting effector T cell responses.

MATERIAL AND METHODS

Mice

Female BALB/c mice (8-10 weeks old) were obtained from a breeding colony from Janvier (Janvier, France) and kept under specific pathogen-free conditions. Sterile food and water were given *ad libitum*. All experiments were performed in accordance with the guidelines approved by the Animal Care and Usage Committee of the Landesdirektion Sachsen, Leipzig, Germany (permission no. TVV 01/11 and TVV 52/16).

Culture of *C. 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 (PBS) at -80°C. For infection the fungus was prepared as described elsewhere [15]. Briefly, *C. neoformans* was cultured overnight in Sabouraud dextrose (SAB) medium under gentle shaking at 30°C. After 18h the fungus was harvested, washed twice with PBS and the cell number was determined. The doubling rate was calculated to control the bioactivity of *C. neoformans* after thawing the sample. Before infection the fungal suspension was adjusted to 2.5 x 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, 28, 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 in 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 [60] at day 7, 8, and 9 post infection whereas the control group received an appropriate volume of PBS.

Blood monitoring

To check for the expansion of Treg cells, mice were bled submandibular 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 gentle to avoid agglutination. After washing, the pellets were dissolved in PBS and carefully layered onto 1 mL of Bicoll separating solution (Biochrom, Germany, ρ = 1.077 g/mL) creating a sharp interface. Following density gradient centrifugation (400 x g, 30 min, RT, acceleration 1, brake 0) the

interface containing mononuclear 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 passaging lung tissue through 100 µm cell strainers (BD Biosciences, Germany) a single cell suspension was obtained in which remaining erythrocytes were lysed by use of Gey's solution. 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. Two 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 min, RT, acceleration 1, brake 0). Therefore, remaining leukocytes were resuspended in 70% Percoll diluted in RPMI1640 and layered under 30% Percoll. 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 $1x10^6$ cells/well in 96-well flat bottom plates (BD Biosciences) and stimulated for 4 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 additional 2 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: Pacific Blue anti-mouse CD44 (clone: IM7, BioLegend), PE-Cy7 anti-mouse CD62L (MEL-14, BioLegend), Brilliant Violet 421™ anti-mouse IL-10 (JES5-16E3, BioLegend), FITC anti-mouse IFN-γ (XMG1.2, eBioscience), PerCP-Cy5.5 anti-mouse FoxP3 (FKJ-16s, eBioscience), FITC anti-mouse FoxP3 (FJK-16s, eBioscience), Alexa Fluor® 700 anti-mouse CD4 (RM4-5, BioLegend), FITC anti-mouse IL-33 receptor (DJ/8, MD Biosciences), FITC anti-mouse CD103 (2E7, eBioscience), 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.

qRT-PCR and QuantiGene® Plex analysis

The accessory lung lobe was used for all mRNA expression analysis either by QuantiGene® Plex or classical qRT-PCR 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 (eBisocience, Germany) and performed in accordance to the manufacturer instructions.

For qRT-PCR snap frozen samples were homogenized in peqGOLD TriFast solution (Peqlab, Gemany) using the Precellys Ceramic Kit 1.4 mm (Peqlab, Germany) at 5000 rpm for 15 seconds in a Precellys®24 homogenisator (Peqlab, Germany). Thereafter, messenger 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 qRT-PCR 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 [64,65]. 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) as primers. mm_Gob5a_for: 5′-AGCGTCACAGCCTTGATT-3′ and mm_Gob5a_rev: 5′-GGACGTGGTGGGTTCATTCT-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 168-178 and 330-307).

Enzyme-Linked Immunosorbend Assay (ELISA)

Total IgE levels in the mouse serum were analysed using a sandwich ELISA system. Plates (NUNC™, Thermo Scientific, Denmark) were coated with goat anti-mouse IgE unlabeled antibody was coated prior to serum incubation. 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. All IgE components were purchased from Southern Biotech (BIOZOL, Germany). 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. To check for normality the Kolmogorov-Smirnov test was used. Normally distributed data were 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 a kinetic study. Differences between two groups were analysed by Student's t-test if the data were normally distributed. Otherwise, the Mann-Whitney U-test was used in order to compare nonparametric data. Statistical significance was considered for p-values < 0.05.

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3. DISCUSSION

3.1 REGULATORY T CELLS SUPPRESS ALLERGIC INFLAMMATION IN PULMONARY CRYPTOCOCCOSIS

The aim of this thesis was to investigate the role of regulatory T (Treg) cells in pulmonary cryptococcosis. Characterization of T helper (Th) cell subpopulations was performed based on the expression of their key transcription factors (Fig. V). During pulmonary fungal infection Treg cells turned out to increase from about 5% in naïve mice to 15% in animals 56 days post infection (dpi). Treg cells were found to present the major T helper (Th) cell population until 21 dpi. Hitherto, 21 dpi turned out to be an important time point in this infection model as Th2 and Th17 immune responses started to increase (Fig. V). In both studies of Treg cell manipulation, the time point for analysis was chosen at either 21 dpi or 28 dpi. This was done as differences in the Th cell development, especially alterations in effector T cell frequencies due to lack or expansion of Treg cells are thought to be detectable.

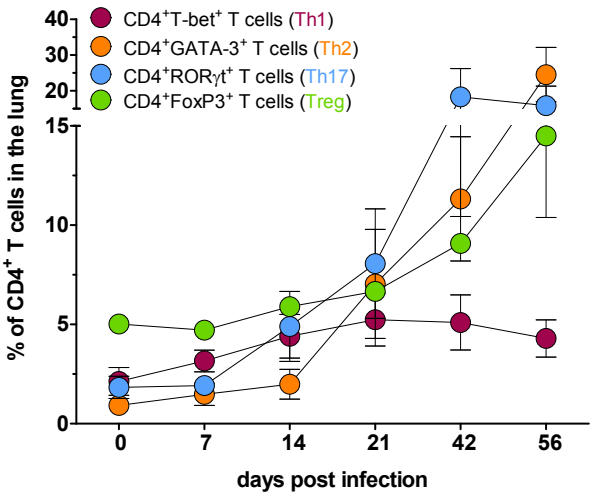

Figure V. Th cell development in the lung of *C. neoformans* infected BALB/c mice
Mice were intranasally infected with C. *neoformans* and Th cell development in the lung was analysed at indicated time points post infection. Single leukocytes were stained for the key transcription factors of each Th cell subpopulation (Th1: T-bet, Th2: GATA-3, Th17: RORγt, Treg cells: FoxP3) and measured by flow cytometry. Each time point shows the mean and standard deviation from seven mice per group.

Selective depletion of Treg cells 7 dpi revealed a protective role for Treg cells in the host response against *C. neoformans*. Due to the lack of Treg cells a strong increase in Th2 cell development was found resulting in increased fungal lung burden and stronger allergic airway inflammation [151]. This was supported by a recently published study using the serotype A *C. neoformans* strain KN99α also showing an increase in antigen-specific Th2 cells after Treg cell depletion [152]. However, that study failed to achieve the opposite effect of better fungal control by Treg cell expansion as treatment with an IL-2/anti-IL-2 complex also resulted in an increase of antigen-specific Th2 cells. Mice infected with *C. neoformans* KN99α showed a more acute disease (i.e. they died at around 20 dpi) and therefore Treg cell expansion might be too slow for sufficient suppression of Th2 cells in that model. Moreover, Th2 cells in this study were demonstrated to strongly express CD25 when mice were treated with IL-2/anti-IL-2 complex. Thus, in addition to Treg cells, Th2 cells are likely to expand in response to IL-2/anti-Il-2 complex [152]. In contrast, our kinetic analysis of Th cell development with the serotype D *C. neoformans* strain 1841 revealed that at 7 dpi Treg cells

represent more than 90% of the CD25-expressing $CD4^+$ T cells. Thus, we chose this time point for IL-2/anti-IL-2 complex treatment. With both experimental setups, i.e. depletion or expansion of Treg cells, we could correlate Treg cells with protection during *C. neoformans* infection (Fig. VI).

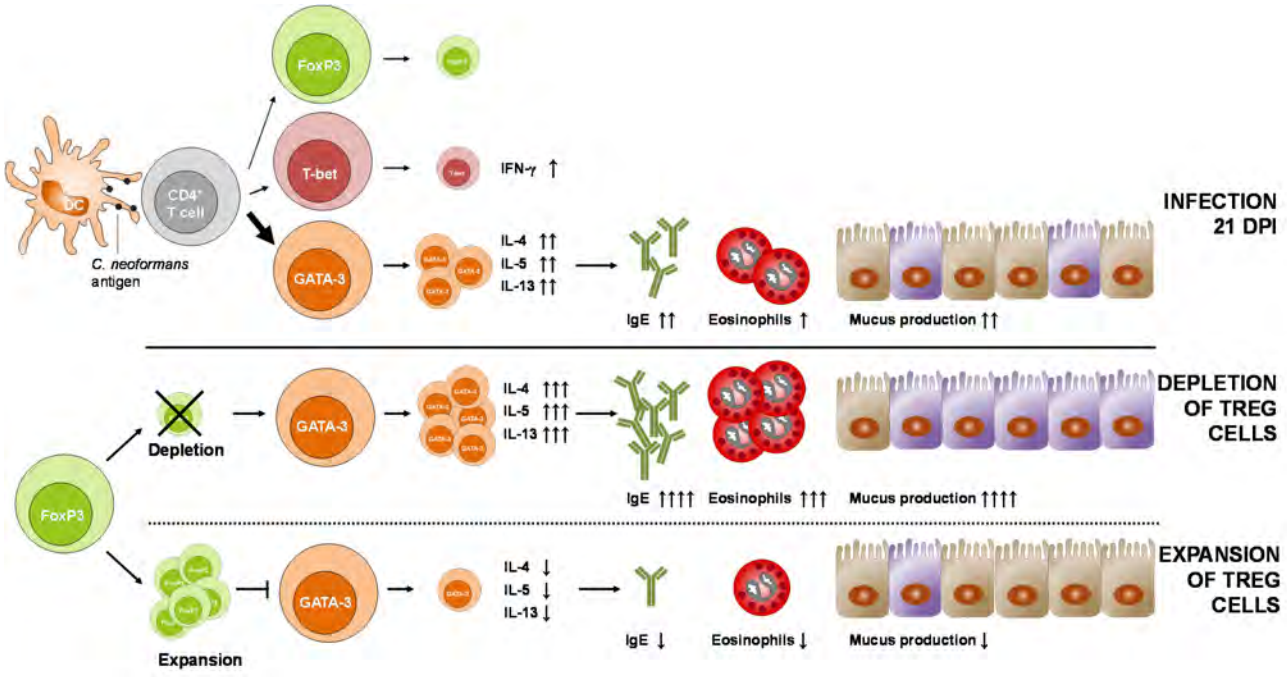

Figure VI. Manipulation of Treg cells influences control of *C. neoformans* infection
Th cell development and the influence of Treg cells on *C. neoformans* infection is shown. Following antigen presentation by dendritic cells (DCs) Th1 ($CD4^+$T-bet^+) or Th2 ($CD4^+$GATA-3^+) immune responses or Treg cells ($CD4^+FoxP3^+$) are induced. At 21 days post infection (dpi) the number of Th2 cells increases in the lung with elevated production of IL-4, IL-5, and IL-13. Moreover, eosinophils, serum IgE levels, and mucus production were increased. Depletion of Treg cells resulted in development of a detrimental Th2 immune response characterised by increased eosinophilia, serum IgE levels, as well as mucus production and could be reversed by Treg cell expansion. In contrast, neither expansion nor depletion of Treg cells influenced the infection-related Th1 immune response.

Along with these findings we conclude that Treg cells selectively suppress the detrimental arm of the immune response against *C. neoformans*. Despite decreased allergic airway inflammation of IL-2/anti-IL-2 complex treated mice, fungal burden in the lung were not reduced. Additional experiments are necessary to optimize Treg cell expansion including prolonged treatment periods and analysis of later time points as in this infection model the fungal burden in the lung does not change up to 35 dpi. Here, Treg cell expansion prior to *C. neoformans* infection was already tested (data not shown, Bianca Schulze, unpublished). In this study no significant differences between mice treated prior to infection or 7 dpi were found. Therefore, further studies should focus on experimental expansion of Treg cells shortly after infection as this is more reasonable to test for therapeutical effects of clinical relevance.

3.2 INDUCTION OF TREG CELLS IN PULMONARY CRYPTOCOCCOSIS

Different subsets of Treg cells exist from which the majority is generated during thymic development (thymus-derived, tTreg cells) by the selection of self-antigens. In the periphery, the Treg cell repertoire is extended by specificities against non-pathogenic foreign antigens (e.g. commensal microbiota and alimentary antigens) and microbial antigens. Peripheral-derived Treg cells (pTreg cells) are known to convert from conventional $FoxP3^{-}CD4^{+}$ T cells into $FoxP3^{+}$ pTreg cells [153-156]. To distinguish between tTreg and pTreg cell subpopulations different markers were tested. One of them, Helios, a member of the Ikaros transcription factor family seemed to be specific for tTreg cells [157,158]. However, it has been shown that Helios is also associated with T cell activation and differentiation, and under certain conditions upregulated in pTreg cells, too [155,159,160]. Under steady state conditions neuropilin-1 was reported to discriminate between tTreg and pTreg cells as only tTreg cells express that receptor [161,162]. Though, neuropilin-1 was also found to be no adequate marker in inflammatory disease models as its expression is strongly influenced by TGF-β and inflammatory environments [36,37,163]. Besides the lack of an adequate marker to distinguish between tTreg and pTreg cells, it is known that Treg cell-specific epigenetic changes are critical in the process of Treg cell specification, in regulating their plasticity, and in the establishment of a stable cell line [162,164]. Those epigenetic modifications include histone modifications, DNA methylation, microRNA, nucleosome positioning, chromatin interaction, and chromosome conformational changes [162]. Recent genome-wide analysis of human and murine conventional T cells and Treg cells revealed several regions that show different patterns of DNA methylation [165-168]. Moreover, some of the Treg cell specific changes in DNA methylation are highly stable in Treg cells, whereas others are not. Studies on methylation of CpG motifs in the *FoxP3* locus of tTreg cells identified complete demethylation within an evolutionary conserved region upstream of exon 1, named Treg cell-specific demethylated region (TSDR) which is unique to $FoxP3^{+}$ Treg cells. In contrast, *in vitro* generated induced Treg (iTreg) cells displayed only partial demethylation in spite of high FoxP3 expression [154]. Additional three conserved non-coding DNA sequence (CNS1-3) elements at the *FoxP3* locus that are linked to the regulation of size, composition and stability of the Treg cell population have been described [169]. It was found that CNS3 functionally acts to potently increase the frequencies of both tTreg and pTreg cell populations. In contrast, CNS1 was described to be superfluous for tTreg cell differentiation but is important for pTreg cell generation. Moreover, CNS2 was found to be required for FoxP3 expression in the progeny of dividing Treg cells, although it is dispensable for FoxP3 induction [169]. To sum this up, analysis of the epigenetic phenotype of sorted Treg cells during cryptococcosis would allow for differentiation of tTreg cell versus pTreg cell phenotype as flow cytometric analysis is not suitable due to the lack of markers. Another possibility would be to infect mice containing cell-specific CNS1 deletion combined with insertion of a green fluorescent protein (GFP) reporter namely $FoxP3^{\Delta CNS1\text{-}gfp}$ mice (CNS1-KO) [169]. In those mice no pTreg cells develop potentially resulting in the presence of solely tTreg cells. It is still unclear whether Treg

cell subsets share a common niche of action or division of labor occurs. Hence, to study pulmonary fungal infection by comparing Treg cells between wild-type and CNS1-KO mice would reveal additional information about recruitment, functionality and induction. Interestingly, in a study using CNS1-KO mice the absence of pTreg cells resulted in development of pronounced Th2-type pathologies at mucosal sites with hallmarks of allergic inflammation and asthma [170]. Those implications linking pTreg cells to control of mucosal Th2 inflammation are in accordance with the presented data suggesting that Treg cells in the lung were induced during *C. neoformans* infection. In the study presented here, several indicators for Treg cell induction were found, including the increase in CD44 surface expression together with the loss of CD62L on nearly all Treg cells. It has been shown that the majority of tTreg cells in lymphoid organs express the homing receptor CD62L, allowing them to recirculate through lymphoid tissues [46] as well as moderate levels of CD44 [163,170]. Additionally, IL-33R and CD103 were found to be stronger expressed on Treg cells with ongoing infection. As mRNA expression analysis of whole lung tissue revealed increased levels of CTLA-4, IDO, TGF-β, and IL-10 during infection a mechanism in which Treg cells might develop their suppressive features in cooperation with dendritic cells (DCs) or alveolar macrophages (AM) is hypothesized (Fig. VII). We suggest a model in which Treg cells are recruited at mucosal surfaces thereby interacting via their surface molecule CD103 with E-cadherin expressed by epithelial cells. As in studies by our group epithelial cells were identified as the major source of IL-33 secretion up to 21 dpi (unpublished data by L. Heyen), it seems likely that Treg cells at mucosal surfaces can be activated directly by IL-33 secretion since they express the IL-33R. Shortly after intranasal infection with *C. neoformans* an increase in Treg cell frequency was found in the lung. In this context it is conceivable that Treg cells expressing the surface molecules IL-33R and/or CD103 proliferate in response to infection associated signals. Furthermore, downstream that cascade Treg cells might start to secrete inhibitory cytokines like IL-10 and TGF-β thereby directly acting on Th cells but also on cells like DCs also capable of IL-10 and TGF-β production. This hypothesis is supported by the increased levels of CTLA-4 and IDO mRNA, that both can participate in a pathway in which Treg cells interact via CTLA-4 with CD80/86 on DCs resulting in IDO release. To determine possible interactions of Treg cells with DCs and macrophages in pulmonary cryptococcosis a CTLA-4 blocking antibody could be used at about 21 dpi. Similar to Treg cell depletion [157] CTLA-4 blockade may result in a stronger Th2 immune response.

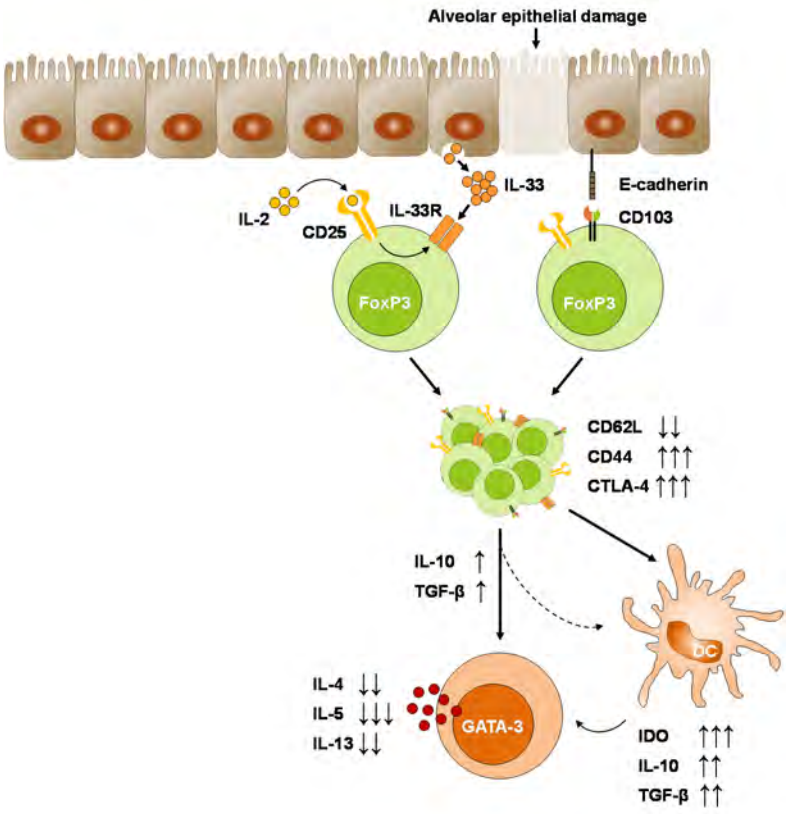

Figure VII. Hypothetical view on Treg cell function during pulmonary cryptococcosis: Treg/epithelial cell interaction selectively limits type 2 immune responses
A hypothesized model of Treg cell activation is shown. Epithelial cells stimulate Treg cells by IL-33 secretion and cell-cell-contact (CD103-E-Cadherin) resulting in CD44 as well as CTLA-4 expression and reduction of CD62L. This phenotype is characteristic for induced pTreg cells. $CD44^{+}CTLA\text{-}4^{+}FoxP3^{+}$ Treg cells secrete the inhibitory cytokines IL-10 and TGF-β. Treg cells interact with dendritic cells (DCs) by CTLA-4 inducing secretion of IDO, IL-10 and TGF-β by DCs which limits effector T cell responses.

Identification of IL-10 producers using flow cytometry revealed that Treg cells are involved in IL-10 production during cryptococcal infection. However, the majority of IL-10 producers seem to be non CD4 T cells. Over the years IL-10 production was associated with many cells of the adaptive immune system, including Th1, Th2 and Th17 cell subsets, Treg cells, CD8 T cells and B cells as well as with cells of the innate immune system such as dendritic cells, macrophages, mast cells, natural killer cells, eosinophils and neutrophils [171,172]. It was shown that mechanisms involved in IL-10 regulation are complex as IL-10 acts in paracrine and autocrine manner on many different immune cells. For example, IL-10, by acting on DCs and macrophages, has been shown to inhibit the development of Th1-type responses [171] but also to lead to the suppression of Th2 cells and allergic responses [28]. To limit the innate effector functions an additional feedback loop exists in which the autocrine inhibitory effect of IL-10 together with IL-10 produced by Th cells suppresses DCs and macrophages [28,30]. For Treg cells a positive regulatory loop was found as IL-10 enhances the differentiation of IL-10-secreting Treg cells [173]. All subsets of $CD4^{+}$ T cells have been shown to be able to produce IL-10, pointing toward a negative feedback loop ensuring that effector T cell responses do not result in immunopathology. In models of allergic airway inflammation with bronchial hyper-reactivity transfer of Treg cells resulted in selective suppression of Th2-driven responses to allergens *in vivo* by Treg cells dependent on IL-10 [53,77]. In a recent study on cryptococcal lung infection the presence of IL-10 was associated with persistent infection and dissemination of fungal

pathogen into brain [174]. Additionally, it was shown that *C. neoformans* can exploit the IL-10-signaling pathway by numerous virulence strategies to oppose protective effector mechanisms [175-181]. A detailed analysis of the role of IL-10 during pulmonary cryptococcosis could be promising as we found that at time points when Treg cell frequencies were again decrease following IL-2/anti-IL-2 complex-induced expansion, still increased levels of IL-10 can be detected in the lung. In this study we claimed that the remaining high levels of IL-10 might be the reason for the long lasting suppressive effects on Th2 cells. To study Treg cell-associated IL-10 FoxP3^{RFP}IL-10^{GFP} reporter mice could be used as IL-10 can be easily detected by flow cytometry allowing the separation of viable IL-10-producing cells for further analysis. Along with IL-10, mRNA levels of TGF-β in total lung tissue were determined. It is well known that macrophages can bind to alveolar epithelial cells by CD200/CD200R interaction resulting in release of TGF-β and retinoic acid (RA). Both factors, TGF-β as well as RA drive the local development of pTreg cells [182,183]. This could be an additional mechanism of how Treg cells are induced during fungal infection (Fig. VIII). In general, kinetic mRNA expression analysis of sorted pulmonary Treg cells from individual mice, expressing IL-33R and/or CD103, will provide insights into the suppressive and regulatory mechanisms of those cells during fungal infection. Such an experimental setting not only allows for analysis of mRNA expression of suppressive mediators like IL-10, TGF-β and indirectly of IL-35 but also for co-expression of several transcription factors like GATA-3 or RORγt. Moreover, such an analysis will reveal similarities and differences of IL-33R$^+$ and IL-33R$^-$ Treg cells. Additionally, epigenetic changes in those populations could be analysed, confirming the hypothesis of Treg cell induction in pulmonary cryptococcosis.

3.3 REGULATORY NETWORK OF GROUP 2 INNATE LYMPHOID CELLS, TREG CELLS AND TH2 CELLS

Group 2 innate lymphoid cells (ILC2) as well as CD4$^+$ Th2 cells are involved in responses to helminths and allergens [184]. Initiation of Th2 responses at mucosal sites following tissue damage, pathogen recognition or allergen exposure can occur by epithelial cell-derived cytokine release of IL-25, IL-33 and thymic stromal lymphopoietin (TSLP). Those cytokines have been shown to activate ILC2 which in turn orchestrate the Th2 response by producing IL-5 and IL-13, and to a lesser amount also IL-4 [185]. IL-25, IL-33, and TSLP can also directly activate Th2 cells that together with ILC2 secrete the cytokines IL-4, IL-5, and IL-13 beeing responsible for the accumulation of eosinophils and alternative activation of macrophages [186]. Treg cells are induced at sites of inflammation to dampen overwhelming immune responses and prevent tissue damage [77]. In settings where allergic pathology is found there is often loss of suppression e.g. due to the absence of Treg cells [187]. Analysing the effects of IL-33 on Treg cell maintenance and survival it has been found that administration of IL-33 results in expansion of systemic Treg cells which suppress the rejection of allogeneic cardiac transplants [188,189]. Furthermore, an IL-33-dependent promotion of resolution of tissue damage in models of colitis [190,191], hepatitis [192], cutaneous wounding [193],

central nervous system injury [194], and atherosclerosis [195] has been described. In several studies Treg cell expansion mediated by ILC2 was found, yet there is also evidence that in this context Treg cells suppress ILC2 development [191,196-198]. For example, in a model of experimental cerebral malaria (*Plasmodium berghei*), administration of IL-33 prevents the development of cerebral malaria leading to reduced production of inflammatory mediators [196]. Thereby, IL-33 drives the expansion of ILC2 that produce type2 cytokines IL-5 and IL-13 leading to the alternative activation of macrophages, which in turn were found to expand Treg cells. Depletion of Treg cells using the DEREG mouse model resulted in loss of resistance to cerebral malaria even when IL-33 was injected. During helminth infection IL-33-mediated activation of ILC2 is required for accumulation of IL-33R$^+$ Treg cells as they dependent on direct co-stimulatory interactions via ICOSL-ICOS [198,199]. In addition, several studies have shown that IL-33 alone can induce significant Treg cell proliferation independently of ILC2 [191,192,197]. The IL-33-ILC2-Treg cell interaction may have evolved at least in part to protect local tissues during injury or microbial invasion, especially at sites where excess or chronic inflammation is detrimental [191,198]. In our model of pulmonary cryptococcosis increasing numbers of Treg cells, ILC2 and concentrations of IL-33 were found, ([151], Piehler *et al.,* 2015, submitted, unpublished observations by L. Heyen). In this context an interaction of IL-33 with Treg cells and ILC2 seems likely. The capability of ILC2 to contribute on the one hand to Th2 development by IL-4 secretion [185] as well as macrophage and dendritic cell priming [196,197] but on the other hand also to Treg cell maturation by ICOSL-ICOS interaction suggest a complex mechanism in cryptococcosis. Recently it has been shown that fungal chitin is an important factor to enhance Th2 related cytokine production during pulmonary fungal infection [152]. Th2 cell induction has been shown to depend on the cleavage of chitin via a host-derived chitinase, chitotriosidase. In this context it is worth mentioning that in pulmonary allergy models lung epithelial cells have been found to recognize chitin fragments and produce the necessary alarmins for induction of Th2 immunity: TSLP, IL-25, and IL-33 [200-202] which are potent inducers of ILC2 proliferation and cytokine production. Besides these cytokines, ILC2 were found to constitutively express CD25 being responsive for IL-2 induced proliferation. However, treatment with IL-2/anti-IL-2 complex in a model of *C. neoformans* strain KN99α infection did not result in increased numbers of ILC2 [152].

Overall, data presented here point toward a possible induction of Treg cells at mucosal sites (Fig. VIII). On the one hand Treg cells can be induced by alveolar macrophages (AM) which secrete TGF-β and RA leading to development of Treg cells from undifferentiated CD4$^+$ T cells [183, 184]. On the other hand, activation of Treg cells might occur due to their recruitment to epithelial cell borders e.g. by CD103. This exposes Treg cells to IL-33 and TGF-β secreted by epithelial cells or AM. As both, ILC2 as well as Treg cells can respond to IL-33 secretion, both cell types might become activated. ILC2 can trigger Treg cell expansion and development by ICOSL-ICOS interaction. In a positive feedback Treg cells support their own

development by secreting the anti-inflammatory cytokine IL-10. In the course of a pulmonary *C. neoformans* infection Treg cell activation results in selective Th2 suppression.

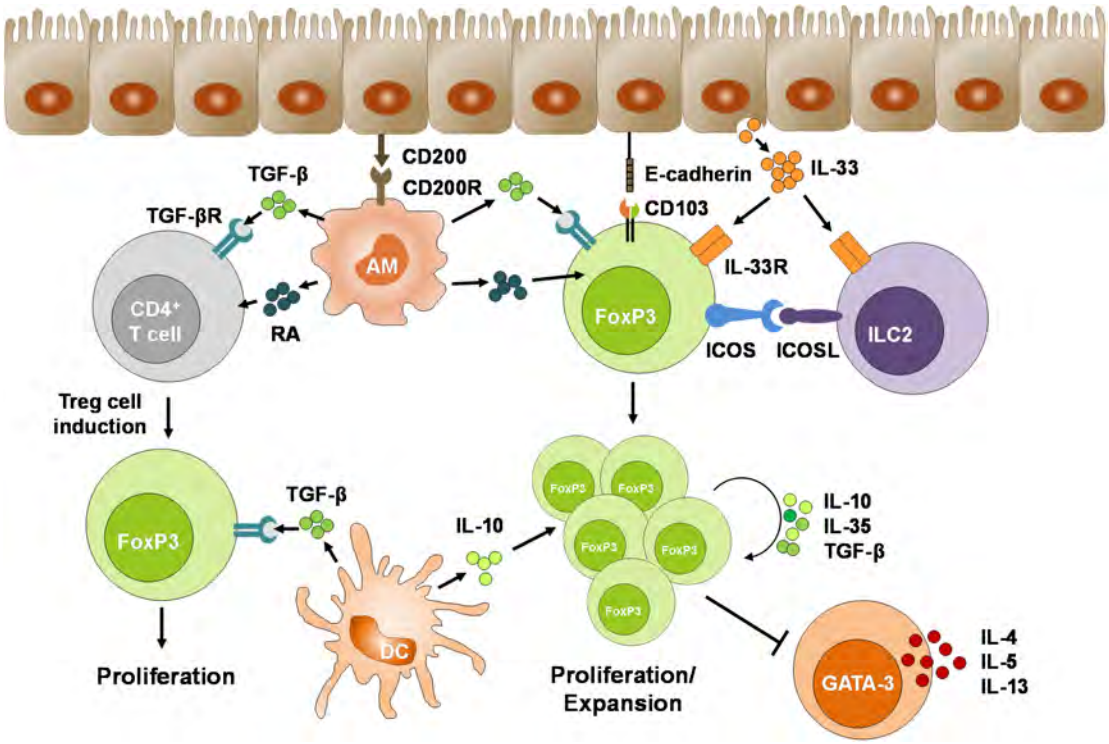

Figure VIII. Potential interactions of Treg cells at mucosal barriers
Alveolar macrophages (AM) interact with lung epithelial cells by CD200-CD200R interaction resulting in secretion of TGF-β and retinoic acid (RA) that allows Treg cell development from undifferentiated $CD4^+$ T cells. Interaction of Treg cells with epithelial cells via CD103-E-cadherin might allow for enhanced responsiveness of Treg cells to IL-33. IL-33 secretion as well as interaction of Treg cells with group 2 innate lymphoid cells (ILC2) via ICOSL-ICOS interaction might result in expansion of Treg cells which positively influence themselves by IL-10 release.

3.4 TREG CELL MODULATION AS PROPHYLACTIC STRATEGY FOR VACCINATION OR AS THERAPY IN PULMONARY CRYPTOCOCCOSIS

Development of an effective vaccine against *C. neoformans* has to overcome three important limitations as it ideally needs to (1) confer protection in persons with low $CD4^+$ T cell counts, (2) induce protection that endures during the subsequent development of immune suppression and (3) be protective against various cryptococcal strains [203]. A monoclonal antibody against *C. neoformans* capsular polysaccharide glucuronoxylomannan (GXM) was shown to be capable to reduce fungal organ burden and prolong survival in mice [204]. Many studies suggest that Treg cells can interfere with the generation of vaccine-induced immunity. Depletion of Treg cells pre-vaccination in murine models has been shown to enhance immune responses to some vaccines [205-208]. However, most of the studies investigated vaccines against established melanomas and solid tumours. In tumour immunology it is known that Treg cells are often detrimental as they suppress effective T cell responses. Therefore, it seems likely that the depletion of Treg

cells prior to tumour vaccination is a benefit for the host. In infectious diseases that also rely on Treg cells to promote escape from an effective immune response as seen in malaria, HIV, and HCV Treg cell depletion may be also an advantage for vaccine efficacy [209]. Whether Treg cells are a promising target of manipulation for an anti-cryptococcal vaccine is an open question. Our findings revealed a protective role for Treg cells in pulmonary fungal infection, yet it needs further investigations if Treg cells are potential targets for vaccination. Based on selective interaction of Treg cells with Th2 but not Th1 cells [157] activation of Treg cells during vaccination might suppress priming of a detrimental Th2 immune response while leaving Th1 development unaffected. However, for therapeutic purposes, additional experiments are needed to find out the exact mechanisms by which Treg cells suppress Th2 responses in cryptococcosis. This knowledge could contribute to improved new treatment strategies for patients already infected with the opportunistic fungal pathogen *C. neoformans*.

4. SUMMARY

Cryptococcus neoformans is an opportunistic fungal pathogen with worldwide distribution causing life-threatening disease in immunocompromised individuals. Cryptococcosis results from inhalation of fungal cells and/or spores with subsequent lung infection and pneumonia. In the absence of an effective immune response, the fungus disseminates to the brain causing cryptoccocal-meningitis. Despite the availablilty of antifungal drugs mortality is still high. Thus, new treatment strategies are needed based on a better understanding of the fungus-host interactions. So far, it has been shown that T helper (Th) 1 together with Th17-based immune responses play a key role in protective immunity against fungal infections. In contrast, development of a Th2-based immune response during infection with *C. neoformans* results in uncontrolled fungal growth and dissemination that is detrimental to the host. By now the roles of different cells belonging to either Th1- or Th2-related immune responses during the *C. neoformans* infection are well defined. However, little is known about the role of the regulatory subset of the $CD4^+$ Th cell population, namely Treg cells. Therefore, the aim of this study was to characterise Treg cells during pulmonary *C. neoformans* infection by analysing their development, functions and interactions.

The main results of the study are:

1. **Pulmonary Treg cell frequencies increase after *C. neoformans* infection**
   Ex vivo analysis of pulmonary Treg cells revealed a significant increase of $CD4^+FoxP3^+$ Treg cells from 5% in naïve animals up to 10% at 21 days post infection (dpi). After this initial increase Treg cell frequencies were found to remain elevated until 56 dpi.

2. **Several suppressive mediators are expressed during fungal lung infection**
   Using QuantiGene Plex analysis suppressive mediators in total lung tissue were measured in a kinetic manner. Increasing mRNA levels of the suppressive cytokines interleukin (IL)-10 and transforming growth factor (TGF)-β were detectable. Additionally, the mRNA expression of cytotoxic T lymphocyte-associated antigen (CTLA)-4 and indoleamine 2,3-dioxygenase (IDO), both contributing to interaction of Treg cells with dendritic cells, increased over time. In contrast, for granzyme B no change and for perforin only a small increase in mRNA expression was found, indicating a less pivotal role of the cytolysis pathway in pulmonary *C. neoformans* infection.

3. **pTreg cells are primarily induced during cryptococcal infection**
   Analysis of the surface markers CD44 and CD62L allows in part for discrimination between tTreg cell and pTreg cell subpopulations. In the lung the frequency of Treg cells upregulating CD44 increased to the same degree as CD62L decreased. As naturally occurring tTreg cells express CD62L and only moderate levels of CD44 it seems likely that Treg cells found in the lung upon fungal infection are induced pTreg cells.

4. **IL-10 secretion represents one mechanism of suppressive Treg cell action during cryptococcosis**

   Intracellular cytokine staining of IL-10 together with analysis of the Treg cell key transcription factor FoxP3 revealed increasing IL-10 synthesis by Treg cells with ongoing infection. Treg cells account for ~25% of the overall IL-10 release.

5. **CD103 and IL-33R are expressed on the surface of Treg cells and might contribute to Treg cell induction**

   Flow cytometric analysis revealed upregulation of the surface molecules CD103 and IL-33 receptor (IL-33R) on Treg cells during cryptococcosis. About 50% of Treg cells express CD103 and/or IL-33R late in infection. Epithelial cells may regulate the phenotype of Treg cells by secretion of IL-33 and expression of E-Cadherin, the interaction partner of CD103.

6. **Depletion of Treg cells results in increased fungal lung burden**

   To get insights into the role of Treg cells during cryptococcal infection, Treg cells were depleted by use of DEREG (DEpletion of REGulatory T cell) mice. In DEREG mice Treg cells express the diphtheria toxin (DT) receptor under control of the *FoxP3* promoter allowing for selective depletion of Treg cells by DT administration. Depletion of Treg cells one week post infection resulted in higher fungal lung burden without affecting dissemination of the fungus to the brain.

7. **Treg cells selectively suppress detrimental Th2-related immune response in cryptococcosis**

   Depletion of Treg cells in pulmonary *C. neoformans* infection resulted in an increase of Th2 cells with enhanced IL-4, IL-5, and IL-13 production. Expansion of Treg cells suppressed the Th2-related immune response whereas the Th1 immunity was not affected.

8. **Expansion of Treg cells leads to decreased *C. neoformans*-related allergic airway inflammation**

   During pulmonary fungal infection allergic airway inflammation can be detected by mucus production, eosinophil recruitment, and IgE synthesis. Expansion of Treg cells 7 dpi resulted in decreased mucus production as indicated by lower mRNA levels of *Gob5* and *muc5ac*. mRNA analysis also revealed less amounts of the eosinophil chemoattractant *CCL11 (eotaxin)*. Additionally, decreased serum IgE levels were detected by ELISA.

9. **Suppression by Treg cell expansion lasts even when the number of Treg cells declines**

   Treg cell expansion was already reversed at the time point of analysis (21 dpi) as seen by blood monitoring. Nevertheless, higher amounts of IL-10 and TGF-β were found in supernatants of cultured lung leukocytes from mice with previous Treg cell expansion.

5. ZUSAMMENFASSUNG

Die Inzidenz invasiver Mykosen hat in den letzten Jahren zugenommen. Nicht nur mit dem *Humanen Immundefizienz*-Virus (HIV) infizierte Menschen sind suszeptibel gegenüber opportunistischen Pilzen, sondern auch Patienten, die beispielsweise im Rahmen einer Organtransplantation oder Chemotherapie eine immunsupprimierende Therapie erhalten. *Cryptococcus neoformans* gehört zu den vier am häufigsten nachgewiesenen Erregern invasiver Mykosen. Trotz der Verfügbarkeit antifungaler Medikamente sind die Mortalitätsraten nach wie vor sehr hoch. Um Ansatzpunkte für neue Behandlungsstrategien zu entwickeln, sind weiterführende Untersuchungen bezüglich der Immunantwort gegen *C. neoformans* erforderlich. Es konnte bereits gezeigt werden, dass T-Helfer-Typ 1 (Th1)- und Th17-assoziierte Immunantworten eine protektive Rolle in der Erregerabwehr von *C. neoformans* spielen. Im Gegensatz dazu wurde die Th2-assoziierte Immunantwort mit dem unkontrollierten Pilzwachstum und der Dissemination des Erregers ins zentrale Nervensystem in Verbindung gebracht. Eine wichtige Rolle in der Vermeidung von überschießenden Immunantworten und Gewebeschädigungen spielen regulatorische T (Treg)-Zellen. Bislang wurden Treg-Zellen noch nicht in der pulmonalen Kryptokokkose untersucht. Ziel dieser Arbeit war es, Treg-Zellen im Hinblick auf ihre Entwicklung, Funktion und Interaktion im Mausmodell der pulmonalen *C. neoformans* Infektion zu charakterisieren.

Die Haupterkenntnisse aus den Untersuchungen sind:

1. **Anstieg der Frequenz pulmonaler Treg-Zellen während einer *C. neoformans* Infektion**
   Die kinetische Analyse von Lungenleukozyten zeigte einen Anstieg der $CD4^+FoxP3^+$ Treg-Zellen von 5% in naiven Tieren auf 10% drei Wochen nach Infektion. Die Frequenz der Treg-Zellen nahm bis vier Wochen nach Infektion zu und blieb dann im weiteren Untersuchungszeitraum bis 56 Tage nach Infektion unverändert.

2. **Regulierung verschiedener suppressiver Mediatoren**
   Analysen der mRNA-Spiegel im Gesamtlungengewebe zu verschiedenen Zeitpunkten nach Infektion zeigten einen Anstieg in der Expression der inhibitorischen Zytokine Interleukin (IL)-10 und dem *transforming growth factor* (TGF)-β. Weiterhin wurde eine Zunahme der mRNA von *cytotoxic T lymphocid-associated antigen* (CTLA)-4 und *indoleamine 2,3-dioxygenase* (IDO) nachgewiesen. Es ist bekannt, dass die Interaktion von Treg-Zellen mit dendritischen Zellen über CTLA-4 und CD80/86 ein potentieller suppressiver Mechanismus der Treg-Zellen ist, der im weiteren Verlauf zur Sekretion von IDO führt. Für zwei untersuchte Mediatoren der Zytolyse, GranzymB und Perforin, konnte keine oder nur eine geringe Regulation der mRNA Expression im Verlauf der Pilzinfektion gefunden werden. Dies deutet auf eine geringe Rolle der Zytolyse im Krankheitsbild der Kryptokokkose hin.

3. **Während der Infektion mit *C. neoformans* werden hauptsächlich pTreg-Zellen induziert**
   Verschiedene Subpopulationen der Treg-Zellen können zum Teil anhand der Expressionsintensität der Oberflächenmoleküle CD44 und CD62L eingeteilt werden. Im Thymus gebildete Treg-Zellen (tTreg-Zellen) exprimieren CD62L mit hoher und CD44 mit mittlerer Intensität. In der Peripherie gebildete Treg-Zellen (pTreg-Zellen) sind durch fehlende CD62L und starke CD44 Expression gekennzeichnet. Im Verlauf der *C. neoformans* Infektion konnte eine Abnahme der CD62L und einer Zunahme der CD44 Expression innerhalb der Treg-Zellen gezeigt werden. Dies deutet auf einen Anstieg der pTreg Subpopulation im Infektionsverlauf hin.

4. **Die IL-10 Sekretion ist ein potentieller Mechanismus der Effektor-T-Zell-Inhibierung durch Treg-Zellen in der pulmonalen Kryptokokkose**
   Mittels durchflusszytometrischer intrazellulärer Zytokinfärbungen wurde die IL-10 Produktion durch $FoxP3^+$ Treg-Zellen untersucht. Treg-Zellen steigern ihre IL-10-Bildung und produzieren bis zu 25% des gesamten in der Lunge vorkommenden IL-10 zu späten Zeitpunkten in der Infektion.

5. **CD103 und IL-33 Rezeptor (IL-33R) werden in zunehmendem Maße mit fortlaufender Infektion auf der Oberfläche von Treg-Zellen exprimiert**
   Für die weitere Charakterisierung der Treg-Zellen wurden die Oberflächenmoleküle CD103 und IL-33R im Verlauf der pulmonalen Kryptokokkose untersucht. Während in unbehandelten Mäusen ~20% der Treg-Zellen CD103 und/oder den IL-33R exprimierten, stieg der Anteil auf bis zu 50% nach Infektion an. Die Ergebnisse deuten auf eine Induktion der Treg-Zellen durch Interaktion mit Epithelzellen hin, da diese IL-33 produzieren und durch E-Cadherin-Expression mit CD103 wechselwirken können.

6. **Die Abwesenheit von Treg-Zellen führt zu einer erhöhten Keimlast der Lunge**
   Für die Untersuchung des Einflusses der Treg-Zellen auf den Krankheitsverlauf der Kryptokokkeninfektion wurden diese zeitweise depletiert. Dazu wurde die DEREG (DEpletion of REGulatory T cell) Maus verwendet. Treg-Zellen der DEREG Maus sind genetisch so verändert, dass diese einen Diphtherie-Toxin (DT) Rezeptor unter der Kontrolle des *FoxP3* Promoters exprimieren und somit über intraperitoneale Gabe von DT zeitweise depletiert werden können. Die Abwesenheit von Treg-Zellen eine Woche nach Infektion führte zu einer erhöhten Keimlast der Lunge, wirkte sich jedoch nicht auf die Dissemination des fungalen Erregers ins Gehirn aus. Damit konnte gezeigt werden, dass die Anwesenheit von Treg-Zellen sich besonders auf die Vermehrung des Pilzes in der Lunge auswirkt.

7. **Treg-Zellen hemmen in der Kryptokokkose selektiv die Th2-Immunantwort**

   Im Fall der Treg-Zell-Depletion konnte eine stärker ausgeprägte Th2-Immunantwort beschrieben werden, die mit einer höheren Expression der Zytokine IL-4, IL-5 und IL-13 assoziiert war. Im Gegensatz dazu wurde bei der Treg-Zell-Expansion eine stark verringerte Th2-Immunantwort im Vergleich zu den Kontrolltieren gemessen. Beide Studien belegen eine direkte und selektive Hemmung der Th2-assoziierten Immunantwort durch Treg-Zellen in der pulmonalen Kryptokokkose.

8. **Die Expansion von Treg-Zellen führt zur einer Verringerung der infektionsassoziierten allergischen Entzündungsreaktion der Lunge**

   Im Verlauf der experimentellen Kryptokokkose kann eine allergische Entzündung der Lunge festgestellt werden. Diese ist geprägt durch Schleimbildung der bronchialen Epithelzellen, dem Vorkommen eosinophiler Granulozyten im Lungengewebe und zusätzlich erhöhtem Immunglobulin (Ig)E im Serum. Treg-Zellen können durch eine IL-2/anti-IL-2-Komplex-Behandlung zur Expansion angeregt werden. Eine Vermehrung der Treg-Zellen führte zu einer verringerten allergischen Entzündungsreaktion der Lunge im Vergleich zur Kontrollgruppe. Diese zeigte sich durch verringerte mRNA Expressionen der Schleimbildungs-assoziierten Gene *Gob5* und *muc5ac*. Das Chemokin *CCL11* (*eotaxin:* Vermittlung der Rekrutierung eosinophiler Granulozyten) konnte ebenfalls in deutlich geringerer Menge im Lungengewebe von Tieren mit IL-2/anti-IL-2-Komplex-Behandlung nachgewiesen werden. Weiterhin wurden stark verringerte IgE Konzentrationen im Serum von Mäusen nach Expansion von Treg-Zellen gemessen.

9. **Die suppressiven Folgen der Treg-Zell-Expansion bleiben nach deren Rückgang auf Normalfrequenz zeitweise bestehen**

   Durch Blutanalysen wurde festgestellt, dass zum Analysezeitpunkt (21 Tage nach Infektion) die Frequenz an Treg-Zellen in infizierten Mäusen mit IL-2/anti-IL-2-Komplex-Behandlung wieder vergleichbar zu Tieren der Kontrollgruppe war. Messungen der Zytokine IL-10 und TGF-β im Überstand stimulierter Lungenleukozyten ergaben jedoch signifikant erhöhte Werte dieser immunsuppressiv wirkenden Zytokine in Tieren deren Treg-Zellen zuvor expandiert wurden. Durch Sekretion dieser Zytokine bleibt die suppressive Wirkung der Treg-Zellen über den Zeitraum ihrer Expansion bestehen.

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8. CONTRIBUTION OF CO-AUTHORS

In the following the contribution of co-authors to the original publication are listed. The experimental designs were all created by me and discussed with Prof. Dr. Gottfried Alber and Dr. Daniel Piehler. The manuscript writing was done by me and corrected by all co-authors. I did all the data analysing whereby the interpretation of the results was done by me and discussed with Prof. Dr. Gottfried Alber and Dr. Daniel Piehler.
Most of the experimental work was done by me; in particular I performed the following techniques and methods when indicated in the manuscript:

- Preparation of fungi prior infection
- Infection, monitoring and dissection of mice
- Intraperitoneal achievement of diphtheria-toxin
- Determination of fungal organ burden
- Isolation and *ex vivo* restimulation of lung and blood cells
- ELISA of cell culture supernatants
- Flow cytometric cell surface and intracellular staining
- Determination of total serum IgE

Bianca Schulze

Title: **$CD4^+FoxP3^+$ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection**

Journal: European Journal of Immunology, 2014, DOI:10.1002/eji201444963

Authors: Bianca Schulze, Daniel Piehler, Maria Eschke, Heiner von Buttlar, Gabriele Köhler, Tim Sparwasser and Gottfried Alber

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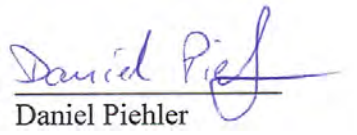
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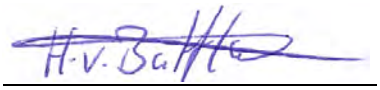
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4) Gabriele Köhler
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Gabriele Köhler

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5) Tim Sparwasser
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6) Gottfried Alber
    - Supervisor of the present thesis


Gottfried Alber

9. SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit versichere ich, die vorliegende Dissertation, soweit nicht explizit unter der Angabe zu den Beiträgen der Co-Autoren angegeben, selbstständig ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt zu haben. Dabei wurden keine unzulässigen oder andere als die angegebenen Hilfsmittel in Anspruch genommen. Direkt oder indirekt aus fremden Quellen übernommene Gedanken wurden in der Dissertation als solche kenntlich gemacht.

Ich versichere, dass Dritte von mir weder unmittelbar noch mittelbar geldwerte Leistungen für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen. Alles aus anderen Quellen und von anderen Personen übernommene Material, das in der Arbeit verwendet wurde oder auf das direkt Bezug genommen wird, wurde als solches kenntlich gemacht. Bei der geistigen Herstellung der Arbeit wurde kein Promotionsberater in Anspruch genommen.

Bislang habe ich mich keinem anderen Promotionsverfahren unterzogen. Die Dissertation wurde weder in der gegenwärtigen noch in anderer Fassung weder der Fakultät für Biowissenschaften, Pharmazie und Psychologie der Universität Leipzig noch einer anderen wissenschaftlichen Einrichtung vorgelegt.

Leipzig, den 13.08.2015

Bianca Schulze

11. PUBLIKATIONSLISTE

WISSENSCHAFTLICHE VERÖFFENTLICHUNGEN

Die Publikationen wurden in Journalen mit Begutachtungs-System eingereicht und veröffentlicht.

Aus der vorliegenden Arbeit entstammt der erste Ergebnisteil der folgenden wissenschaftlichen Veröffentlichung:

1. Schulze, B., Piehler, D., Eschke, M., von Buttlar, H., Köhler, G., Sparwasser, T., and Alber, G. „$CD4^+FoxP3^+$ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection" Eur. J. Immunol., 2014, DOI: 10.1002/eji.20144496

Weitere Veröffentlichungen:

2. Burgardt, N. I., Schmidt, A., Manns, A., Schutkowski, A., Jahreis, G., Lin, Y.J., Schulze, B., Masch, A., Lücke, C. and Weiwad, M. „Parvulin 17-catalyzed tubulin polymerization is regulated by calmodulin in a calcium-dependent manner" J Biol Chem, 2015, DOI:10.1074/jbc.M114.593228

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POSTER

1. Schulze B., Piehler D., Eschke M., Richter T., Grahnert A., and Alber, G. „Regulatory T cells in pulmonary cryptococcosis" *11th Leipzig Research Festival for Life Sciences,* 2012.

2. Schulze B., Piehler D., Eschke M., Richter T., Grahnert A., and Alber, G. „Regulatory T cells in pulmonary cryptococcosis" *5th Autumn School Current Concepts in Immunology,* 2013.

3. Schulze B., Piehler D., Eschke M., Richter T., Grahnert A., and Alber, G. „Regulatory T cells in pulmonary cryptococcosis" *Nationale Forschungsplattform für Zoonosen, Junior Scientist Zoonoses Meeting,* 2013.

4. Schulze B., Piehler D., Eschke M., Richter T., Grahnert A., and Alber, G. „Regulatory T cells in pulmonary cryptococcosis" *12th Leipzig Research Festival for Life Sciences,* 2013.

5. Schulze B., Piehler D., Eschke M., von Buttlar H., Köhler G., Sparwasser T., and Alber, G. „$CD4^+FoxP3^+$ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection“ *13th Leipzig Research Festival for Life Sciences,* 2014.

6. Schulze B., Piehler D., Eschke M., von Buttlar H., Köhler G., Sparwasser T., and Alber, G. „$CD4^+FoxP3^+$ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection“ *Gordon Research Seminar Immunology of Fungal Infections,* 2015.

7. Schulze B., Piehler D., Eschke M., von Buttlar H., Köhler G., Sparwasser T., and Alber, G. „$CD4^+FoxP3^+$ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection“ *Gordon Research Conference Immunology of Fungal Infections,* 2015.

8. Schulze B., Piehler D., Eschke M., von Buttlar H., Köhler G., Sparwasser T., and Alber, G. „$CD4^+FoxP3^+$ regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection“ *11th Spring School on Immunology,* 2015.

VORTRÄGE

1. Schulze B. Regulatory T cells in pulmonary cryptococcosis. *Doktoranden-Kolloquium des BBZ, Universität Leipzig,* 2013.

2. Schulze B. Depletion of regualtory T cells promotes fatal T helper 2 cell development in pulmonary fungal infection. *Doktoranden-Kolloquium des BBZ, Universität Leipzig,* 2014.

3. Schulze B. Depletion of $CD4^+FoxP3^+$ regulatory T cells promotes fatal T helper (Th)2 cell development in pulmonary fungal infection. *17th Meeting on T-Cells Subsets and Functions,* 2014.

4. Schulze B. Characterisation of regulatory immune cells in a model of AIDS-related *Cryptococcus neoformans*-infection. *3tes Leipziger Doktorandenforum, Veterinärmedizinische Fakultät der Universität Leipzig,* 2014.