From the Institute of Immunology

Faculty of Veterinary Medicine, University of Leipzig

Analysis of lung epithelial cell regulation and possible involvement in immunity upon pulmonary cryptococcosis

Inaugural-Dissertation

to obtain the degree of a

Doctor medicinae veterinariae (Dr. med. vet.)

from the Faculty of Veterinary Medicine

University of Leipzig

Submitted by

Laura Heyen

from Berlin, Germany

Leipzig, 2017

Mit Genehmigung der Veterinärmedizinischen Fakultät der Universität Leipzig

Dekan:	Professor Dr. Walter Brehm
Betreuer:	Professor Dr. Gottfried Alber
Gutachter:	Prof. Dr. Gottfried Alber Institut für Immunologie Veterinärmedizinische Fakultät, Universität Leipzig
	Prof. Dr. Alexander Dalpke Department für Infektiologie Medizinische Mikrobiologie und Hygiene Universitätsklinikum Heidelberg

Tag der Verteidigung: 4. April 2017

Meiner Familie

Table of contents

Abbreviations

AIDS	acquired immunodeficiency syndrome
BALF	bronchoalveolar lavage fluid
CD	cluster of differentiation
C. gattii	*Cryptococcus gattii*
C. neoformans	*Cryptococcus neoformans*
CNS	central nervous system
COPD	chronic obstructive pulmonary disease
DMEM	Dulbecco's modified eagle's medium
DNA	desoxyribunucleic acid
e.g.	exempli gratia
Ep-CAM	epithelial cell adhesion molecule
HDM	house dust mite
HIV	human immunodeficiency virus
ICAM-1	intercellular adhesion molecule 1
i.e.	id est
IFN-γ	interferon gamma
Ig	immunoglobulin
IL	interleukin
ILC2	innate lymphoid cell type 2
KLRG1	killer cell lectin-like receptor subfamily G member 1
MHC	major histocompatibility complex
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa B
RAO	recurrent airway obstruction

RNA	ribonucleic acid
RT-qPCR	real-time reverse transcription quantitative polymerase chain reaction
SP-C	surfactant protein C
TGF-β	transforming growth factor beta
Th cell	T helper cell
Th1 cell	T helper cell type 1
Th2 cell	T helper cell type 2
TLR	toll-like receptor
TNF-α	tumor necrosis factor alpha
Treg	regulatory T cell
WT	wild-type

1 Introduction

1.1 *Cryptococcus neoformans*

Cryptococcus represents a yeast-like fungus with a worldwide distribution. The fungal genus comprises at least 70 known *Cryptococcus* species (FONSECA et al. 2010), which can be preferentially found in avian excreta especially from pigeons, in soil, water and on decaying wood in the environment (CASADEVALL and PERFECT 1998). Due to the cosmopolitan presence of *Cryptococcus*, humans and animals are frequently exposed to the fungus (Figure 1). The majority of *Cryptococcus* species is not able to survive in mammalian tissue (CASADEVALL and PERFECT 1998). But *C. gattii* and *C. neoformans* represent the most important pathogenic *Cryptococcus* species for humans and animals causing the disease cryptococcosis (KWON-CHUNG et al. 2014). *C. gattii* and *C. neoformans*, initially considered as one homogenous agent, are two closely related species, whereby *C. neoformans* can be further classified into two varieties (*C. neoformans* var. *grubii* and *C. neoformans* var. *neoformans*) (HEITMAN et al. 2011). Pathogenicity of *C. neoformans* and *C. gattii* is based on specific virulence factors that facilitate survival and dissemination of the fungus in the infected host. The major virulence traits imply in particular: a polysaccharide capsule, growth at 37°C, production of enzymes and melanin in the fungal cell wall (KRONSTAD et al. 2011).

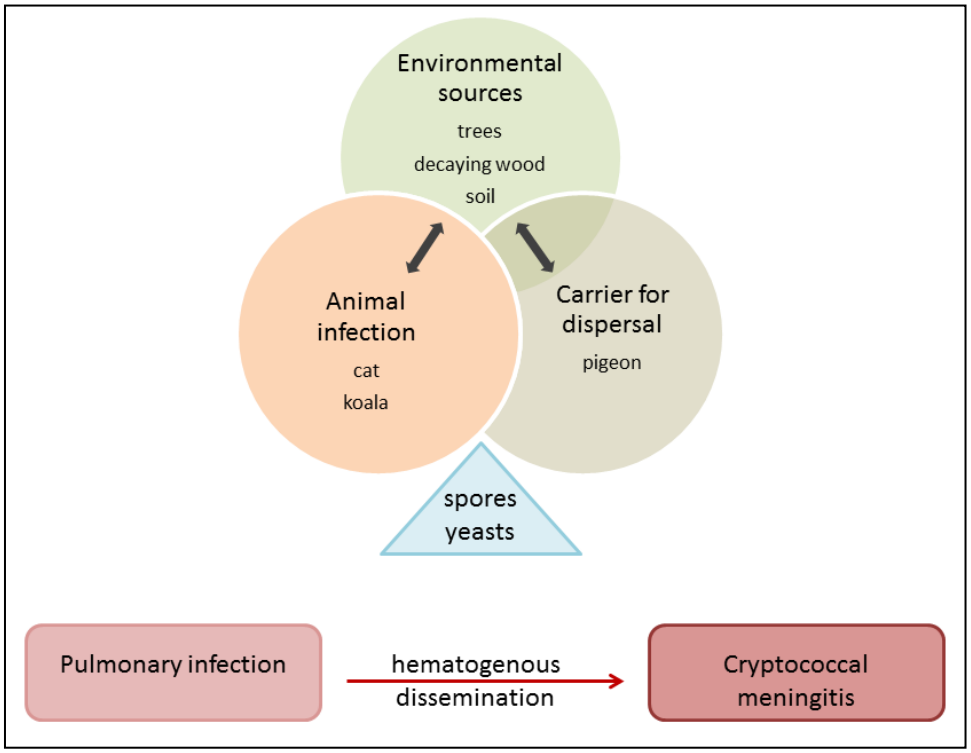


Figure 1: Infection cycle of *C. neoformans*

The fungus *C. neoformans* can survive in the environment and is able to infect both animals and humans. Uptake of spores or desiccated yeasts by inhalation establishes a pulmonary infection. If the host becomes immuno-compromised the fungus is able to disseminate hematogenously, causes systemic infection leading to infection of the CNS and other organs as well.

In contrast to *C. gattii*, which is characterized as a primary pathogen (HEITMAN et al. 2011; KRONSTAD et al. 2011), *C. neoformans* has a facultative pathogenicity and possesses the tendency to infect in particular individuals with a compromised immune system (CASADEVALL and PERFECT 1998). Initial infection primarily develops after desiccated cryptococcal yeast cells or spores are inhaled (VELAGAPUDI et al. 2009). Asymptomatic pulmonary infection occurs in immunocompetent individuals. However, when the immune system becomes compromised, pulmonary cryptococcosis with features of allergic airway inflammation (MÜLLER et al. 2007; PIEHLER et al. 2016) arises, can spread systemically, affect the CNS and develop into a life-threatening meningitis (VOELZ and MAY 2010) (Figure 1). This is the reason why patients infected with HIV have an increased susceptibility to *C. neoformans* infection (MITCHELL and PERFECT 1995) and represent the majority of cryptococcosis cases since the AIDS epidemic 1980 (CASADEVALL and PERFECT 1998). Cryptococcal meningitis represents the most common meningitis form among patients with HIV infection in sub-Saharan Africa (VELTMAN et al. 2014) and approximately 600.000 patients die per year due to cryptococcal meningitis worldwide (PARK et al. 2009). The development of both pulmonary and subsequent systemic cryptococcosis is based on a detrimental Th2 cell related immune response by the host (MÜLLER et al. 2012; MÜLLER et al. 2007; STENZEL et al. 2009).

1.2 Cryptococcosis in veterinary medicine

Cryptococcus is able to provoke disease in a variety of species like amphibians, reptiles, birds, monotremes and in particular mammals (HEITMAN et al. 2011). But it is considered for all vertebrate species that encounters with *C. neoformans* and *C. gattii* cause severe disease only under specific conditions. Although cryptococcosis occurs rather sporadically in general veterinary medicine, outbreaks in the form of mastitis in dairy cattle, sheep and goat herds have been recognized several times (BARO et al. 1998; PAL and MEHROTRA 1983; SPANAMBERG et al. 2008; SORRELL et al. 1996). Moreover, cryptococcosis is considered as the most frequent systemic mycosis of cats throughout the world (O'BRIEN et al. 2004; PENNISI et al. 2013), and appears often in several wildlife species (KROCKENBERGER et al. 2003; VAUGHAN et al. 2007). Due to anatomic differences, manifestation of primary cryptococcosis as mycotic rhinosinusitis or mastitis is common in many animal species in contrast to human patients (HEITMAN et al. 2011). Nevertheless, disseminated disease with infestation of the CNS either hematogenously or via the cribriform plate, with early involvement of the olfactory bulbs and optic nerves, has been additionally described.

In the recent past a multispecies outbreak of cryptococcosis caused by *C. gattii* in British Columbia (Canada, 2000-2001) occurred and involved primarily animals as cats, dogs, porpoises, ferrets and llamas but also humans (STEPHEN et al. 2002; HEITMAN et al. 2011). Until now a zoonotic potential of *Cryptococcus* has not been described, nevertheless, considering the “one health”-concept

veterinarians should be aware of this fungus, which could cause serious mycoses for both humans and animals.

1.2.1 Cases and symptoms of cryptococcosis in selected animal species

Cryptococcosis in cats and dogs is more common in North America, Australia and Japan, than in Europe (HEITMAN et al. 2011). Comprehensive studies in Australia revealed, that *C. neoformans* and *C. gattii* act as primary pathogens in cats and found out that clinical cryptococcosis has been most frequently diagnosed at young male cats of specific breeds like siamese, birman and ragdoll (O'BRIEN et al. 2004; TORIBIO et al. 2009). Whereas the nasal cavity was the most common primary site of infection (O'BRIEN et al. 2004), also cutaneous lesions could be seen (MALIK et al. 2006). Dissemination of infection to the CNS occurred generally at a late phase of disease (O'BRIEN et al. 2004). Canine cryptococcosis occurred especially among young-adult dogs of large-breeds, i.e. boxer, German shepherd, and dalmatian. Whereas the nasal cavity of dogs was often colonized with *C. neoformans*, clinical cryptococcosis frequently developed into a systemic infection with CNS involvement (O'BRIEN et al. 2004). Although some reports have been published that cats and dogs with diagnosed cryptococcosis had a history of diseases resulting in immunosuppression, e.g. infection with the feline immunodeficiency virus or steroid treatment, animal immunodeficiency is not clearly combined with enhanced susceptibility to cryptococcosis as in human beings (DUNCAN et al. 2006). Only a limited proportion of cats with persistent feline immunodeficiency virus infection had an increased propensity to develop cryptococcosis, but almost certainly, long term treatment with corticosteroids resulted in progression of disease (HEITMAN et al. 2011). With the administration of fluconazole or itraconazole for uncomplicated cases and amphotericin B and flucytosine for severe cases, clinical cryptococcosis in cats and dogs can be treated successfully (O'BRIEN et al. 2006).

Compared to dogs also ferrets seem to have an increased susceptibility for developing cryptococcosis primarily in the upper respiratory tract due to their domicile and behavior close to soil and dirt (MALIK et al. 2002).

Because of the high body temperature birds are usually only colonized by *Cryptococcus* but provoke widespread dissemination of the fungus via their faeces. Only few cases of clinical cryptococcosis have been reported. Primarily the respiratory tract of male birds, both immunocompetent and –compromised, were infected mostly by *C. gattii* and were difficult to treat (MALIK et al. 2003). In contrast to birds, clinical cryptococcosis in koalas is frequently diagnosed and a significant cause of death, probably due to their lower body temperature. Most of the cases were caused by *C. gattii* and affected initially the respiratory tract. After systemic dissemination of the pathogen, the CNS was often involved (KROCKENBERGER et al. 2003). Serological studies revealed that prevalence of

subclinical disease in koalas was directly correlated with the extent of cryptocccal presence in their habitat (KROCKENBERGER et al. 2002).

1.3 T cell immune response upon infection with *Cryptococcus neoformans*

A T cell-mediated immune response of the host is required for protection against *C. neoformans* (HILL and HARMSEN 1991; HUFFNAGLE et al. 1994; HUFFNAGLE et al. 1991). Thus, cryptococcosis occurs in particular in individuals with a compromised T cell function, like for instance AIDS patients. An intact T cell defense is primarily characterized by the secretion of cytokines and chemokines for recruitment and activation of phagocytic cells like macrophages and neutrophils to diminish and kill *C. neoformans* (HUFFNAGLE et al. 1994). Although $CD8^+$ T cells also contribute to host defense against cryptococcosis (MODY et al. 1994), $CD4^+$ T cells are of major importance regarding both host protection and cryptococcal pathogenesis (Figure 2).

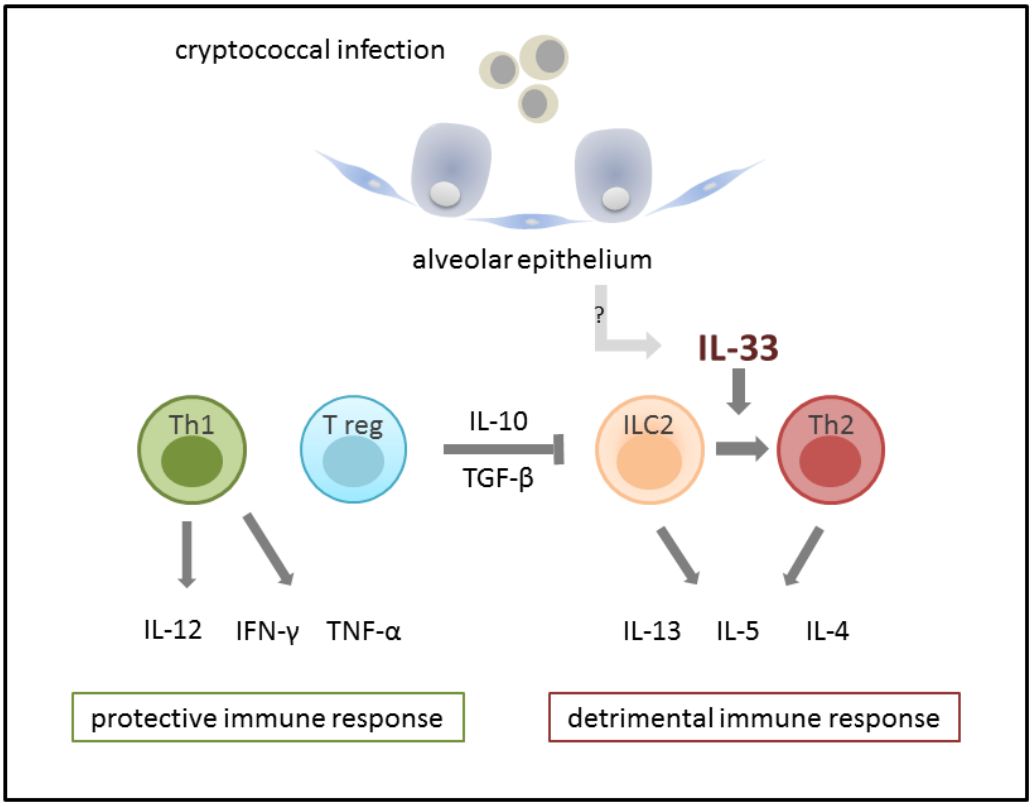


Figure 2: T cell immunity in response to cryptococcal infection

Domination of cytokines produced by ILC2 and Th2 cells (IL-4, -5, -13) over cytokines expressed by Th1 and Treg cells (IL-12, IFN-γ, TNF-α, IL-10, TGF-β) results in fungal growth and dissemination instead of cryptococcal clearance and host protection.

The cytokines produced by Th1 cells (e.g. IL-12, IFN-γ and TNF-α) (KAWAKAMI et al. 1996; CHEN et al. 2005; HUFFNAGLE et al. 1996) and Treg cells (e.g. IL-10, TGF-β) (SCHULZE et al. 2014; SCHULZE et al. 2016) are required for clearance of the organism. In contrast, cytokines secreted by Th2 cells, such as

IL-4, -5, -13, lead to fungal growth and systemic dissemination. During pulmonary cryptococcosis for instance IL-4 and IL-13 cause induction of B cell derived IgE, enhanced mucus production and an increase of alternatively activated macrophages with diminished pathogen killing function (MÜLLER et al. 2007; PIEHLER et al. 2013; MÜLLER et al. 2012; GOERDT et al. 1999; ARORA et al. 2005). Moreover, it is of interest to note that Treg cells are protective by selectively suppressing Th2 cells (SCHULZE et al. 2014). Consequently an imbalance of the Th cell-mediated immune response expressed by the predomination of Th2 over Th1 cytokines results in exacerbation of cryptococcal disease (KOGUCHI and KAWAKAMI 2002; DECKEN et al. 1998). Th2 cytokines derive not only from Th2 cells but also from early progenitor cells named ILC2. Both cell types are regulated by the cytokine IL-33 and upregulate its receptor (T1/ST2) during pulmonary cryptococcosis. IL-33 is induced upon cryptococcosis, acts as a chemotactic factor for Th2 cells (KOMAI-KOMA et al. 2007) and activates the Th2 cytokine production (SCHMITZ et al. 2005). *In vivo* it has been already shown that T1/ST2 deficiency upon cryptococcal infection showed reduced $CD4^+$ T cells, IL-4 and IL-13 levels, decreased mucus production and diminished fungal burden in the lung in comparison to WT mice (PIEHLER et al. 2013). Thus, IL-33 is an important initiator of detrimental Th2-mediated immunity during cryptococcosis. But the cellular source of this crucial cytokine *in vivo* has not been defined yet. The role of innate and adaptive immune cells in host immunity following cryptococcal infection was the focus of several detailed studies. However, so far relatively little is known about how pulmonary epithelial cells get generally affected by the pathogen and are potentially involved in initiation of the immune response via humoral factors or cellular interactions.

1.4 Allergic airway inflammation: pulmonary cryptococcosis and asthma

It is well established that lung epithelial cells recognize and respond to a variety of inhaled pathogens and allergens due to their exposed position. As a critical first defense epithelium controls in particular allergic sensitization and is therefore an important player in inflammatory and immune responses (HALLSTRAND et al. 2014; LAMBRECHT and HAMMAD 2012).

As already mentioned symptoms of pulmonary cryptococcosis, i.e. increased mucus production, induction of Ig E, accumulation of eosinophils and alternatively activated macrophages, are based on a Th2-mediated immune response and resemble features of allergic airway inflammation. Asthma is also related to this pulmonary allergic context. Although it was realized that different pathogenetic phenotypes of asthma exist, the Th2 lymphocyte-controlled inflammation occurs commonly in half of asthmatic patients (BAGNASCO et al. 2016). Interestingly, several studies suggest that specific fungal infections are able to contribute to the pathogenesis of asthma. For instance it was early established that fungal colonization with *Aspergillus* can result in asthmatic symptoms represented by the allergic type of broncho-pulmonary aspergillosis (HINSON et al. 1952). Furthermore, in human BALF

of asthmatic patients a significant increased IgA reactivity to proteins of *Aspergillus fumigatus* and *C. neoformans* in comparison to non-asthmatics was found (GOLDMAN et al. 2012).

Grahnert et al. for instance observed a tendency of enhanced *Cryptococcus*-specific IgG antibodies in sera of asthma patients of an urban German area (GRAHNERT et al. 2015). Likewise it has been demonstrated in an animal model that chronic and/or persistent cryptococcal infection can promote development of asthma (GOLDMAN et al. 2006).

The mouse model, which was used for the following study, represents a chronic infection model for fungus-induced type 2 cytokine-dependent allergic airway inflammation and is therefore particular suited for the analysis of epithelial cell function not only in pulmonary cryptococcosis but in allergic airway inflammation in general.

A more detailed understanding of how respiratory epithelium regulates pulmonary immune function will provide important and novel insights into the pathogenesis of not only pulmonary cryptococcosis but several widely disseminated allergic airway diseases.

1.5 The first contact site of *C. neoformans* infection: lung epithelial cells

As already mentioned, infection with *C. neoformans* occurs mainly via inhalation. Consequently the respiratory tract and more precisely lung epithelial cells represent the first contact site for the fungal pathogen with the host.

In vitro C. neoformans is able to adhere to a human lung epithelial cell line (A549) and has been also shown to be internalized (MERKEL and SCOFIELD 1997) with subsequent direct epithelial cell damage (BARBOSA et al. 2006). Further studies identified glucuronoxylomannan for encapsulated and mannoprotein 84 for acapsular *C. neoformans* strains to mediate adherence to A549 (BARBOSA et al. 2006; BARBOSA et al. 2007; TEIXEIRA et al. 2014). Furthermore, acapsular *C. neoformans* showed higher adherence and internalization to A549 than capsular cryptococcal strains (CHOO et al. 2015). Phospholipase B1, a secreted virulence factor from *C. neoformans* (serotype A, H99), is described to increase fungal adhesion to A549 (GANENDREN et al. 2006), whereas *C. neoformans* secreted antigens downregulate ICAM-1 expressed by A549 (MERKEL and SCOFIELD 2000). Additionally, incubation of normal human bronchiolar epithelial cells and A549 with acapsular *C. neoformans* results in epithelial IL-8 secretion (GUILLOT et al. 2008; BARBOSA et al. 2007). IL-8 represents an important chemokine especially for neutrophils (KUNKEL et al. 1991), which infiltrate the lung upon cryptococcal infection *in vivo* (KAWAKAMI et al. 1999). Moreover, a mouse lung epithelial cell line (MLE-12) showed upregulation of IL-33 mRNA after stimulation with the more virulent cryptococcal strain H99 (FLACZYK et al. 2013).

In summary, so far *in vivo* studies focusing on epithelial cells during cryptococcosis are missing, but could provide a better understanding of function and reactions of pulmonary epithelial cells upon

cryptococcal infection as well as the missing link between pathogen recognition and host defense initiation.

1.6 Aims of study

T cell-mediated immunity in response to cryptococcal infection is necessary for effective defense and prevention of cryptococcosis. But the dominance of an IL-33-dependent Th2-associated immune response leads to specific conditions in the infected host which rather favor fungal growth and dissemination than clearance of the pathogen.

This study aimed to analyze *in vivo* the function of lung epithelial cells in Th2 immunity upon cryptococcal infection. First, it is analyzed whether epithelium represents the cellular source of IL-33 in pulmonary cryptococcosis. Then the effects of cryptococcal infection on epithelial adhesion molecule (E-cadherin), chemokine (CXCL15) and surfactant (SP-C) production are examined. The last part focuses on expression of ligands and receptors which might facilitate direct cellular interaction between epithelial and immune cells.

2. Results

2.1 publication:

Lung epithelium is the major source of IL-33 and is regulated by IL-33-dependent and IL-33-independent mechanisms in pulmonary cryptococcosis

This work has been published in:

FEMS Pathogens and Disease

74 (2016) ftw086

Author's contribution:

Laura Heyen:
- experimental design
- practical experimental work
- data acquisition
- data analysis and interpretation
- manuscript writing

Uwe Müller:
- assistance with practical experimental work

Sabine Siegemund:
- assistance with practical experimental work

Bianca Schulze:
- assistance with practical experimental work

Martina Protschka:
- assistance with practical experimental work

Gottfried Alber:
- assistance with experimental design and data interpretation
- support to manuscript writing

Daniel Piehler:
- experimental design
- assistance with practical experimental work, data analysis and interpretation
- support to manuscript writing

Pathogens and Disease, 74, 2016, ftw086

doi: 10.1093/femspd/ftw086
Advance Access Publication Date: 4 September 2016
Research Article

RESEARCH ARTICLE

Lung epithelium is the major source of IL-33 and is regulated by IL-33-dependent and IL-33-independent mechanisms in pulmonary cryptococcosis

Laura Heyen, Uwe Müller, Sabine Siegemund, Bianca Schulze[†], Martina Protschka, Gottfried Alber[*] and Daniel Piehler[‡]

Institute of Immunology/Molecular Pathogenesis, Center for Biotechnology and Biomedicine, College of Veterinary Medicine, University of Leipzig, An den Tierkliniken 11, D – 04103 Leipzig, Germany

[*]**Corresponding author:** Institute of Immunology, College of Veterinary Medicine, University of Leipzig, An den Tierkliniken 11, D – 04103 Leipzig, Germany. Tel +49-341-9738328; Fax +49-341-9738147; E-mail: alber@rz.uni-leipzig.de
[†]**Present address:** Microbial Immunology, Leibniz-Institute for Natural Product Research and Infection Biology - Hans-Knöll-Institute (HKI), Jena, Germany.
[‡]**Present address:** Vaxxinova GmbH, Leipzig, Germany.
One sentence summary: Lung epithelium represents a significant source of IL-33 in pulmonary cryptococcosis and is regulated in an IL-33-dependent but also IL-33-independent manner.
Editor: Willem van Eden

ABSTRACT

Inhalation of the fungus *Cryptococcus neoformans* (*C. neoformans*) results in pulmonary cryptococcosis associated with IL-33-dependent type 2 immunity. Lung epithelium represents the initial contact site of infection. The role of IL-33 in type 2 immunity has been analyzed, but the source of this cytokine and its effect on lung epithelial cell function in pulmonary cryptococcosis remained unclear. In mice infected with *C. neoformans*, we identified alveolar type 2 epithelial cells as major source of IL-33. On both, IL-33-positive and IL-33-negative epithelial cells, IL-33 receptor expression was detectable. Therefore, we studied the role of IL-33 receptor expression for IL-33 synthesis during fungal infection on lung epithelial cells and found no auto-/paracrine IL-33 induction. Next, the effect of IL-33 on epithelial E-cadherin expression, a cell-to-cell adhesion molecule, was analyzed. Fungal infection resulted in E-cadherin downregulation in an IL-33-dependent manner on pulmonary epithelial cells both at the single-cell and at the population level. On the other hand, epithelial cells from infected mice upregulated surfactant protein C (SP-C) and CXCL15 mRNA production together with but independently of IL-33. In conclusion, lung epithelium represents a significant source of IL-33 in pulmonary cryptococcosis and is regulated in an IL-33-dependent but also IL-33–independent manner.

Keywords: IL-33; *Cryptococcus neoformans*; pulmonary infection; airway epithelial cells; mouse

INTRODUCTION

The opportunistic fungal pathogen *Cryptococcus neoformans* (*C. neoformans*) is ubiquitously distributed. Cryptococcal infection primarily develops after spores or desiccated yeast cells are inhaled (Velagapudi *et al.* 2009). Consequently, lung epithelial cells represent one of the first contact sites but also the first barrier to *C. neoformans*.

Asymptomatic respiratory infection occurs in immunocompetent individuals but can spread systemically and develop

Received: 14 July 2016; **Accepted:** 1 September 2016

into a life-threatening meningitis when the immune system becomes compromised (Voelz and May 2010). This detrimental infection development is favored by type 2-biased immunity (Müller *et al.* 2007). Interleukin (IL)-33 can be an amplifier of the type 2 immune response as it promotes activation of IL-5 and IL-13-producing type 2 innate lymphoid cells (ILC2s) (Piehler *et al.* 2015) and subsequent T helper (Th) 2 cells (Spits and Cupedo 2012). In mice, IL-33 is induced in the lung during cryptococcal infection (Flaczyk *et al.* 2013) and its receptor T1/ST2 is upregulated on Th2 cells (Piehler *et al.* 2013). T1/ST2 deficiency in mice (T1/ST2$^{-/-}$) results in prolonged survival upon infection with *C. neoformans* and demonstrates that IL-33 signaling supports growth and systemic dissemination of the fungal pathogen (Flaczyk *et al.* 2013; Piehler *et al.* 2013). It has been observed *in vivo* in humans (Moussion, Ortega and Girard 2008) and in mice (Pichery *et al.* 2012) that lung epithelial cells constitutively express IL-33 and T1/ST2 (Hardman, Panova and McKenzie 2013), but the cellular source(s) for this cytokine and its impact on epithelial cells during cryptococcal infection *in vivo* are unknown.

A key feature of epithelial cells *in vivo* is barrier integrity, and E-cadherin is an important adhesion molecule contributing to tight epithelial adherent junctions (van Roy and Berx 2008). Besides its structural function, E-cadherin is also considered to regulate signaling pathways in epithelial cells (Qian *et al.* 2004). Next to barrier integrity, surfactant production by pulmonary epithelial cells is of striking importance for physiological function of the lung. Surfactant keeps surface tension low and is exclusively produced and secreted by alveolar epithelial cells type 2 (King 1982), whereas type 1 epithelial cells, representing a more frequent subpopulation, do not contribute to production of surfactants. How IL-33 induced upon pulmonary cryptococcosis, affects these lung epithelial cell functions is unknown.

Epithelial cells are not only important for maintenance of homeostasis in the naïve organism, but also influence the nature of an immune response to infection with certain pathogens (Whitsett and Alenghat 2015). IL-8 and CXCL1 secretion by normal human bronchiolar epithelial cells (NHBEs) has been observed in response to acapsular *C. neoformans* (Guillot *et al.* 2008). IL-8 represents an important chemotactic factor especially for neutrophils (Kunkel *et al.* 1991) that are part of the infiltrating leukocytes in the lung upon cryptococcal infection *in vivo* (Kawakami *et al.* 1999). In the murine lung, the neutrophil-targeted chemokine has been identified as lungkine (CXCL15) (Rossi *et al.* 1999; Chen *et al.* 2001), but its cellular source during pulmonary cryptococcosis has not been examined yet.

In this study, we identified the cellular source of IL-33 upon pulmonary cryptococcosis and further investigated how IL-33 regulates lung epithelial E-cadherin expression, surfactant and chemokine production.

MATERIAL AND METHODS

Mice

Mice were maintained under specific pathogen-free conditions, according to the guidelines authorized by the Animal Care and Usage Committee of the 'Landesdirektion Sachsen' (accredition no. 24–9168.11/18/35). Sterile food and water were given *ad libitum*. Wild-type (WT) BALB/c mice were purchased from Janvier (Janvier, France) and crossed with IL-33 citrine reporter mice (*IL33*$^{cit/cit}$) on BALB/c background (kind gift of Dr A. N. J. McKenzie, LMB Cambridge, UK). The *IL33*$^{cit/cit}$ is characterized by the insert of the citrine gene directly downstream of the ATG start codon of IL-33 (Hardman, Panova and McKenzie 2013). Therefore, the production of fluorescent citrine in cells, detectable by flow cytometry and immunofluorescence microscopy, reports indirectly the expression of IL-33 mRNA. For the experiments 8- to 18-week-old female *IL33*$^{cit/+}$, *IL33*$^{cit/cit}$ and WT littermates were used. BALB/c mice deficient for T1/ST2 (T1/ST2$^{-/-}$) (Townsend *et al.* 2000) were kindly provided by Prof. Dr T. Kamradt, Friedrich-Schiller-University, Jena, Germany. Infected mice were monitored daily for survival and morbidity. Mice were euthanized with CO_2 and every effort was made to minimize pain and suffering.

Cryptococcus neoformans and infection of mice

Cryptococcus neoformans strain 1841 (serotype D) was isolated from an HIV patient (Decken *et al.* 1998). It was kept as a frozen stock in 10% skim milk and was grown in Sabouraud dextrose medium (2% glucose, 1% peptone; Sigma Aldrich, Munich, Germany) overnight on a shaker at 30°C. Cells were harvested and washed twice with sterile phosphate-buffered saline (PBS). After counting the fungal cells in a hematocytometer, the culture was resuspended at a concentration of 2.5×10^4 ml^{-1} in PBS for intranasal infection. Mice were anesthetized by an intraperitoneal injection with a 1:1 mixture of Ketamine (100 mg ml^{-1}; Ceva Tiergesundheit, Düsseldorf, Germany) and Xylazine (20 mg ml^{-1}; Ceva Tiergesundheit), and received intranasally 20 μl containing 500 colony-forming units of *C. neoformans*, in order to establish a chronic infection model closely resembling cryptococcal pathogenesis under real conditions.

Preparation of pulmonary cells, fungal analysis of lung burden

For lung digestion and preparation of pulmonary cells, lungs were perfused using sterile 0.9% sodium chloride solution. After removal, the Lung Dissociation Protocol (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed using the Lung Dissociation Kit mouse (Miltenyi Biotec) and the gentleMACS Dissociator (Miltenyi Biotec). Red blood cells were lysed with Gey's solution. For the determination of fungal lung burden, 50 μl of lung single-cell suspension was serially diluted and plated on Sabouraud dextrose agar plates. After the incubation period of 72 h at 30°C colonies were counted.

Flow cytometry

To exclude dead cells from analysis Fixable Viability Dye eFluor 780 (eBioscience, Frankfurt, Germany) was used prior to specific antibody staining. For surface staining, cells were incubated with Tru Stain fcX (anti-mouse CD16/32, clone 93, BioLegend, Fell, Germany) to avoid unspecific Fc binding. Staining antibodies were added for a 15-min incubation period at 4°C in the dark and afterwards cells were fixed with 2% paraformaldehyde (Serva, Heidelberg, Germany) for 20 min at 4°C in the dark. For immunophenotyping, stained lung cells were acquired on a BD LSRFortessa II (BD Biosciences). Sorting of viable epithelial cells was performed with BD FACSAria III (sort of epithelial cell adhesion molecule (Ep-CAM)$^+$ citrine$^{+/-}$ cells from *IL33*$^{cit/+}$ mice: purity 95%; sort of Ep-CAM$^+$ cells from WT and *IL33*$^{cit/cit}$ mice: purity 90%). The following antibodies were utilized: Alexa Fluor 647 anti-CD326 (Ep-CAM, clone G8.8, BioLegend), Alexa Fluor 700 anti-CD45 (clone 30-F11, eBioscience), biotinylated anti-T1/ST2 (clone DJ/8, MD Biosciences, Zürich, Switzerland), PE anti-CD324 (E-cadherin, clone DECAM-1, BioLegend), Brilliant Violet 421 anti-SiglecF (clone E50-2440, BD Biosciences, Heidelberg, Germany), Brilliant Violet 605 anti-CD11b

(clone M1/70, BioLegend) and APC anti-CD11c (clone N418, BioLegend). Cells labeled with biotinylated antibodies were further stained with PerCp eFluor 710 streptavidin (eBioscience). Suitable isotype antibodies were used from BioLegend and eBioscience. The analysis was performed with DIVA 6.1.3 and FlowJo X0.7 (Treestar Inc., Ashland, OR) software.

In all flow cytometric analyses, cells of interest were gated on single live cells. For the characterization of epithelial cells, dead, $CD45^+$ cells and doublets were excluded. Ep-CAM^+ cells were analyzed for citrine, T1/ST2 and E-cadherin expression.

Enzyme-linked immunosorbent assay

Supernatants from homogenized accessory lung lobes were analyzed for total IL-33 protein levels according to the manufacturer's protocol using R&D Systems IL-33 DuoSet Enzyme-Linked Immunosorbent Assay (ELISA) kit (R&D Systems GmbH, Wiesbaden, Germany).

RNA isolation, reverse transcription (RT) and real-time PCR analysis

FACS-sorted cells were resuspended in peqGOLD TriFast (Peqlab, Erlangen, Germany) and stored at –80°C. After extraction with chloroform, RNA was precipitated with isopropanol, washed twice with 70% ethanol and resuspended in DEPC water (all: Roth, Karlsruhe, Germany). In order to eliminate genomic DNA, RNA was treated with RNase-free DNase I (2.5 units per sample; Thermo Fisher Scientific GmbH, Schwerte, Germany) in the presence of RiboLock RNase inhibitor (20 units per sample; Thermo Fisher Scientific GmbH, Schwerte, Germany). Afterwards RNA transcription to complementary DNA (cDNA) with High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) using a blend of random/ oligo (dT) 18 primer was performed according to manufacturer's instruction. RT-qPCR was processed in duplicates with the iTaq Universal SYBR® Green Supermix (BIO-RAD, München, Germany) in an iCycler iQ5 (BIO-RAD, München, Germany). The following primers were used for RT-qPCR: mm_hprt_for: 5′-GTTGGATACAGGCCAGACTTTGTTG-3′ and mm_hprt_rev: 5′- GATTCAACTTGCGCTCATCTTAGGC-3′ (GenBank Accession No. NM_013556; positions 660–684 and 822–798); mm_sp-c_for: 5′-AGCAAAGAGGTCCTGATGGA – 3′ and mm_sp-c_rev: 5′-CTCATCTCAAGGACCATCTCAGTA–3′ (GenBank Accession No. NM_011359.2; positions 51–70 and 253–230); mm_cxcl15_for: 5′- CTAGGCATCTTCGTCCGTCC–3′ and mm_cxcl15_rev: 5′-TTGGGCCAACAGTAGCCTTC–3′ (GenBank Accession No. NM_0113339 positions 83–102 and 299–280) and mm_IL-33_for: 5′- GGAAAGAACCCACGAAAAGA-3′ and mm_IL-33_rev: 5′-TGTACTCAGGGAGGCAGGAG-3′ (GenBank Accession No. NM_001164724.1; positions 268–287 and 455–436).

Immunofluorescence microscopy

Lobes of lung tissue were incubated in 4% PFA solution overnight and were transferred into a 30% sucrose solution for at least one additional day, followed by incubation in prewarmed (37°C) GSEM (15% sucrose, 7.5% gelatin (pig), 1xPBS) at 37°C for 30 min. Lung lobes were then embedded, frozen in Tissue Freezing Medium (Jung, Nussloch, Germany) at –80°C, and sectioned at –20°C. After blocking with 10% normal donkey serum (United States Biological, Salem, MA) in PBS with 0.1% Tween (PBST, Roth, Karlsruhe, Germany) for 1 h at room temperature, cryosections were stained with Alexa Fluor 647-conjugated anti-CD326 rat IgG2a (Ep-CAM, clone G8.8, BioLegend; 1:200) in 10% normal donkey serum with PBST and the negative control was incubated in PBST. After washing, the sections were incubated with Cy3-conjugated goat anti-rat IgG (Jackson ImmunoResearch Laboratories, Inc., USA; 1: 250) in 10% normal donkey serum with PBST for 1 h at room temperature. The slides were washed and sealed with Aqua Poly/Mount (Polysciences, Warrington, PA) and a cover slip. After incubation for at least 1 h at 4°C, the slides were analyzed with a fluorescence microscope (Olympus BX40, Olympus, Hamburg, Germany) with an XM10 camera (Olympus) and the CellSens Dimension software version 1.3 (Olympus).

Statistical evaluation

Statistical analysis, evaluation and graphical representation were done with the Graph Pad Prism 5.01 software (Graphad Software Inc., San Diego, USA). Normality was determined by the Kolmogorov–Smirnov test. Equally distributed values were analyzed by one-way ANOVA followed by Dunnett's Multiple Comparison post test, not equally distributed values with the Dunn's Multiple Comparison test, respectively, in order to compare different time points in the kinetic study. Differences between two groups with normally distributed data were analyzed by unpaired *t*-test. The Mann–Whitney U-test was performed in order to compare nonparametric data. Statistical significance was determined for *P*-values < 0.05. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

RESULTS

Alveolar epithelium represents the major source of IL-33 during cryptococcal infection

Recently, it has been shown that IL-33 is induced in response to infection with different serotypes of *C. neoformans* and plays a crucial role in the initiation of type 2 immunity (Flaczyk *et al.* 2013; Piehler *et al.* 2015). Here, we analyzed the progression of pulmonary IL-33 protein expression in BALB/c mice infected intranasally with 500 colony-forming units of *C. neoformans* strain 1841 (serotype D) for 49 days. Analysis of lung homogenates revealed a significant increase in IL-33 protein production (Fig. 1A). However, the cellular source of IL-33 *in vivo* has not yet been identified for pulmonary cryptococcosis. Therefore, we infected IL-33 citrine reporter mice ($IL33^{cit/+}$ (Hardman, Panova and McKenzie 2013)) with *C. neoformans* and characterized $citrine^+$ cells in the lung at different time points post infection. Citrine fluorescence is a surrogate for IL-33 mRNA expression (Hardman, Panova and McKenzie 2013). *In situ* we confirmed both the constitutive expression of IL-33 mRNA in lungs of naïve mice and its infection-dependent induction and upregulation by using immunofluorescence microscopy of cryosections of lung tissue (Fig. 1B). Furthermore, we demonstrated that mainly alveolar epithelial cells were $citrine^+$, whereas citrine fluorescence in bronchiolar epithelial cells was less detectable (Fig. 1B). The flow cytometric analysis of $citrine^+$ cells in the lung of $IL33^{cit/+}$ mice at different time points post infection in comparison to WT mice showed induction of citrine in primarily nonhematopoietic $CD45^-$ lung cells, whereas $CD45^+$ cells were involved to a lower degree (Fig. 2A and B). When citrine production was compared between $CD45^-$ and $CD45^+$ lung cells at 70 days post infection (dpi), we found ~7-fold more $CD45^-$ $citrine^+$ cells (mean 75 300 ± 18 611) per whole lung than $CD45^+$ $citrine^+$ cells (mean 11 500 ± 4354) (Fig. 2B). By analyzing the expression of epithelial cell adhesion molecule (Ep-CAM), a transmembrane

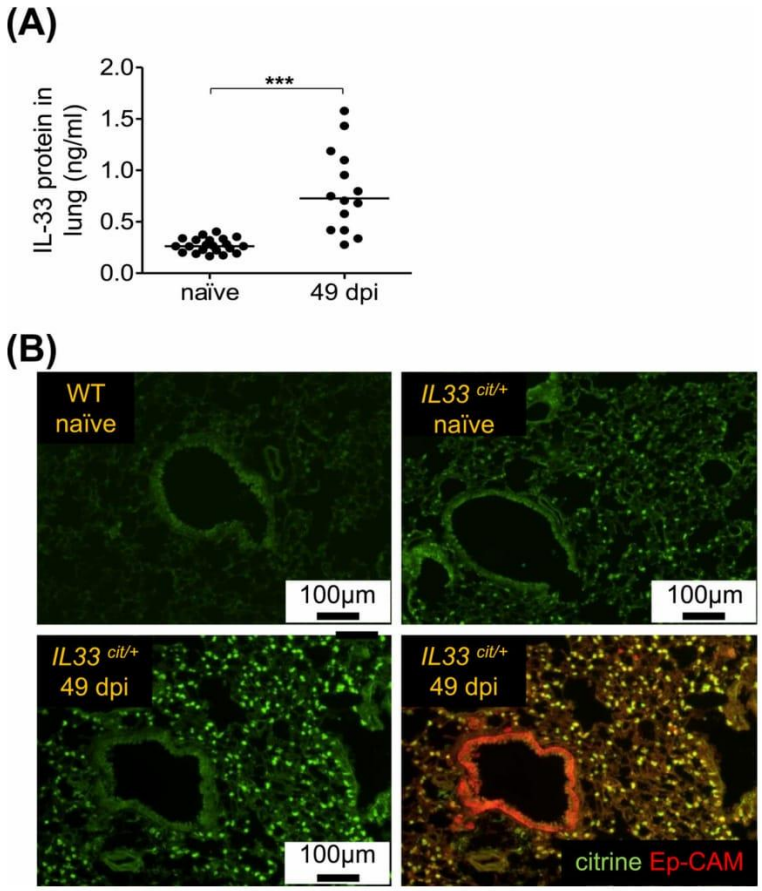


Figure 1. Pulmonary IL-33 protein, citrine$^+$ alveolar epithelial cells and the intensity of citrine expression increase upon cryptococcal infection. *IL33*$^{cit/+}$ mice were infected intranasally with *C. neoformans* and analyzed 49 days post infection (dpi). (**A**) IL-33 protein in supernatant of homogenized accessory lung lobe was determined by ELISA with a detection limit at 0.06 ng/ml (left panel). Statistical analysis was performed using two-tailed unpaired *t*-test $^{***}P < 0{,}001$. Each data point represents one animal and is from two independent experiments pooled here, each with $n = 6$–7 mice per time point. (**B**) Immunofluorescence images of cryosections of lung tissue from naïve wild-type (WT) (top left) and from naïve (top right), infected (bottom) *IL33*$^{cit/+}$ mice, from representative micrographs are shown (10×/0.30 Ph1 magnification). An immunofluorescence image (bottom right) showing double-staining with anti-epithelial cell adhesion molecule (Ep-CAM) (Cy3/red) and with citrine (green) demonstrates a majority of Ep-CAM$^+$citrine$^+$ (red + green = yellow) alveolar epithelial cells.

glycoprotein that is expressed by most epithelia (Martowicz, Seeber and Untergasser 2016), we identified one prominent CD45$^-$ citrine$^+$ population as epithelial cells (Fig. 3A and B). Further analysis of sorted Ep-CAM$^+$ citrine$^+$ cells showed high expression of surfactant protein C (SP-C) mRNA, in contrast to Ep-CAM$^+$ citrine$^-$ cells (Fig. 1, Supporting Information), and were therefore identified as type 2 alveolar epithelial cells (King 1982). Ep-CAM$^+$ epithelial cells represented the main citrine$^+$ cell population at every time point examined post infection (Fig. 3B). Moreover, we observed that the mean fluorescence intensity (MFI) of citrine in epithelial cells increased until 49 dpi (Fig. 3C), indicating enhanced levels of IL-33 mRNA at the single cell level. Thus, even though Ep-CAM$^+$ citrine$^+$ cells are only a small fraction of all Ep-CAM$^+$ cells in the lung (Fig. 2, Supporting Information), they represent a major cellular source of IL-33 during pulmonary cryptococcosis. A more detailed analysis of hematopoietic citrine$^+$ cells revealed enhanced IL-33 mRNA levels in sorted exudative macrophages (SiglecF$^+$ CD11c$^+$ CD11b$^+$) of infected WT mice (Fig. 3, Supporting Information). This is indicative of the involvement of exudative macrophages in IL-33 production by leukocytes upon crypotococcal infection (Osterholzer *et al.* 2011). Alveolar macrophages (SiglecF$^+$ CD11c$^+$ CD11b$^-$), a more frequent macrophage subpopulation in the lung, however, did not seem to be involved (Fig. 2, Supporting Information). Similarly, lung Ly6G$^+$Ly6C$^+$ neutrophils did not express citrine either (data not shown). Taken together, we demonstrated by flow cytometry and immunofluorescence microscopy the induction of IL-33 mRNA expression as well as elevated IL-33 protein levels in the lung following cryptococcal infection and identified type 2 lung epithelial cells as the major IL-33-producing pulmonary cells.

IL-33 production by pulmonary Ep-CAM$^+$ epithelial cells upon infection with *Cryptococcus neoformans* is independent of epithelial IL-33 receptor expression

It is well established that the IL-33 receptor T1/ST2 is expressed by the main effector cells of type 2 immunity, namely ILC2s (Neill *et al.* 2010), Th2 cells (Piehler *et al.* 2013) and eosinophils (Oboki *et al.* 2011). Upon cryptococcal infection of WT mice, the constitutively low frequency of pulmonary CD4$^+$ T cells expressing

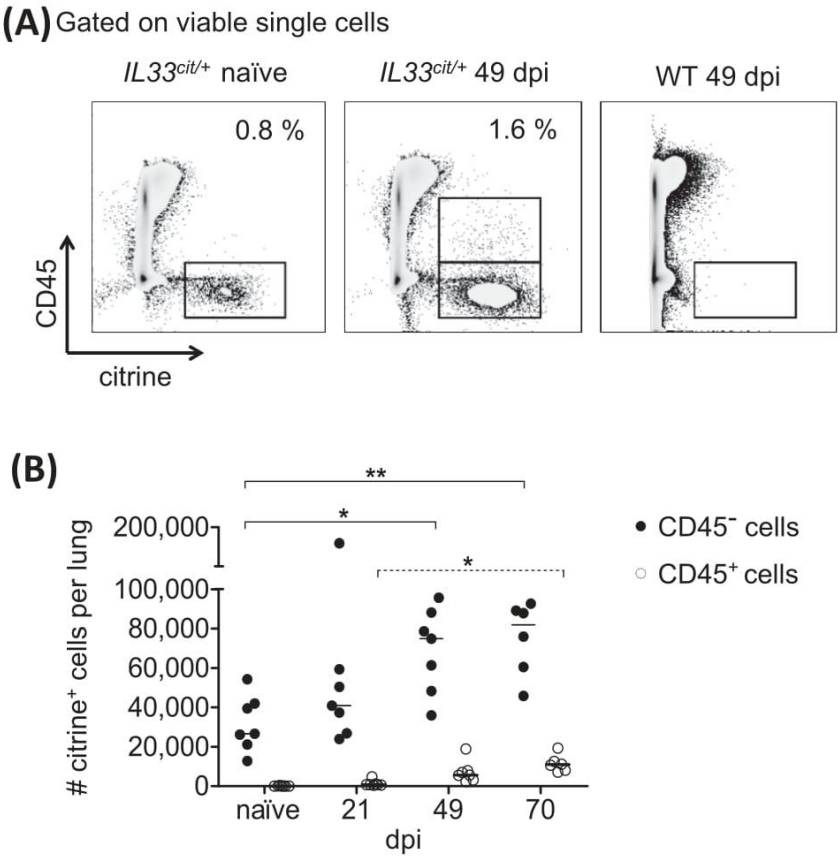


Figure 2. CD45$^-$ cells represent the main citrine$^+$ cell population until 70 days post cryptococcal infection (dpi). *IL33*$^{cit/+}$ and WT mice were infected intranasally with *C. neoformans* and analyzed at indicated time points. (**A**) Representative flow cytometry plots of pulmonary viable, single cells gated on CD45 and citrine are depicted. (**B**) Total numbers of CD45$^-$citrine$^+$ and CD45$^+$citrine$^+$ cells from *IL33*$^{cit/+}$ mice are shown. Each data point represents one animal and is from one of two independent experiments each with $n = 6$–7 mice per time point. One-way ANOVA with Dunn's Multiple Comparison Test was performed to determine statistical significance $^*P < 0.05$, $^{**}P < 0.01$.

T1/ST2 increases significantly (Piehler *et al.* 2013). Interestingly, for epithelial cells, a constitutive frequency of Ep-CAM$^+$ T1/ST2$^+$ cells (mean 23%, ± 17.6) in naïve WT mice (Fig. 4A) and no increase during pulmonary cryptococcosis at 49 dpi but rather a reduced frequency was observed (Fig. 4A, left and middle panel). Hardman, Panova and McKenzie 2013 also described T1/ST2 expression by epithelial cells and showed that IL-33 application promotes its own expression in epithelial cells *in vivo*. This implies that IL-33 may be able to affect its own cellular source in an auto-/paracrine manner. To determine the function of T1/ST2 on epithelial cell IL-33 induction in our model, we infected mice deficient for T1/ST2 (T1/ST2$^{-/-}$) with *C. neoformans*. T1/ST2$^{-/-}$ and WT mice had similar IL-33 protein levels 49 dpi in whole lung tissue (Fig. 4A, right panel), arguing against a role of T1/ST2 for the induction of IL-33. When we analyzed epithelial T1/ST2 expression in homozygous IL-33 citrine reporter mice (*IL33*$^{cit/cit}$ mice deficient in IL-33 protein production), we found, similar frequencies of Ep-CAM$^+$ T1/ST2$^+$ cells upon infection as in WT mice (data not shown). Moreover, T1/ST2 was expressed not only by Ep-CAM$^+$ citrine$^+$ but also by Ep-CAM$^+$ citrine$^-$ cells (Fig. 4B). This indicates that T1/ST2 expression is not regulated by IL-33. In summary, our observations argue against an auto-/paracrine feedback of IL-33 on its receptor expressed on lung epithelium during pulmonary cryptococcosis.

IL-33 leads to downregulation of E-cadherin expression by lung epithelial cells in pulmonary cryptococcosis

Regulation of the expression of the epithelial adhesion molecule E-cadherin during cryptococcal infection has not been studied yet. We could confirm constitutive expression of E-cadherin on Ep-CAM$^+$ cells in naïve WT mice (mean 45%, ± 11.3) (Fig. 5A). Upon cryptococcal infection, we found a decrease of Ep-CAM$^+$ E-cadherin$^+$ cells (mean 32%, ± 10.8) as well as a reduced E-cadherin expression per cell (Fig. 5A). As E-cadherin promotes cell-to-cell adhesion, these data point to reduced barrier integrity of the lung epithelium following fungal infection. In order to examine whether the regulation of E-cadherin depends on IL-33, we analyzed E-cadherin expression in *IL33*$^{cit/cit}$ mice that are deficient in IL-33. Interestingly, we found a significantly lower MFI of E-cadherin in naïve *IL33*$^{cit/cit}$ in comparison to WT mice (Fig. 5A, right panel), suggesting a homeostatic role of IL-33 for E-cadherin expression. Upon cryptococcal infection (which is associated with IL-33 production, see Fig. 3), the frequency of Ep-CAM$^+$ E-cadherin$^+$ cells and the MFI of E-cadherin expression decreased in WT mice (Fig. 5A). In contrast, in *IL33*$^{cit/cit}$ mice no differences of the MFI and the frequency of Ep-CAM$^+$ E-cadherin$^+$ cells upon cryptococcal infection was detectable (Fig. 5A). Consistent with these data further analysis of naïve

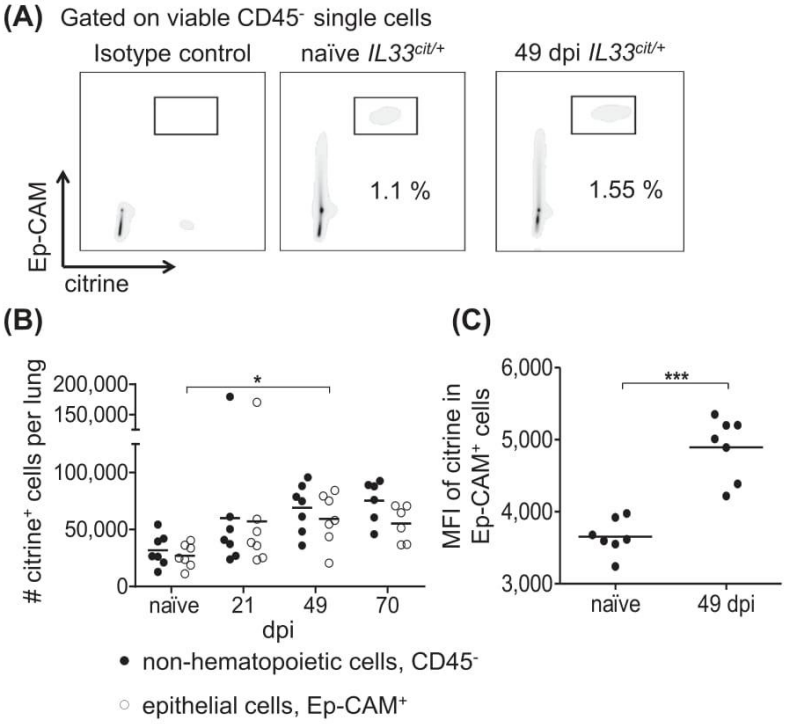


Figure 3. Pulmonary citrine$^+$ epithelial cells increase during cryptococcal infection. *IL33*$^{cit/+}$ mice were infected intranasally with *C. neoformans* and analyzed at indicated time points. (**A**) Representative flow cytometry plots of viable, CD45$^-$, single cells gated on Ep-CAM and citrine are shown. (**B**) Total numbers of CD45$^-$citrine$^+$ and Ep-CAM$^+$citrine$^+$ cells at indicated time points after cryptococcal infection are depicted. Statistical significance was determined using one-way ANOVA with Dunn's Multiple Comparison Test $^*P < 0.05$. (**C**) Flow cytometric analysis of citrine mean fluorescent intensity (MFI) expressed by epithelial cells are shown. Statistical analysis was performed using unpaired two-tailed *t*-test $^{***}P < 0.001$. Each data point represents one animal from one out of two independent experiments each with $n = 6–7$ mice per group.

and infected *IL33*$^{cit/cit}$ mice revealed that the majority (mean 90.5%, ± 10) of Ep-CAM$^+$ citrine$^+$ cells also expressed E-cadherin, whereas the majority of Ep-CAM$^+$ citrine$^-$ cells was E-cadherin$^-$ (Fig. 5B). Taken together, our observations suggest that cryptococcal infection leads to E-cadherin downregulation in an IL-33-dependent manner.

Infection with *Cryptococcus neoformans* induces enhanced CXCL15 and SP-C transcription by lung epithelial cells mainly in IL-33 producing cells but independently of IL-33

CXCL15 represents the murine chemokine for neutrophil granulocyte recruitment in the lung (Kunkel *et al.* 1991; Rossi *et al.* 1999; Chen *et al.* 2001). It has been demonstrated *in vivo* that neutrophils accumulate in the lung upon infection with *C. neoformans* (Kawakami *et al.* 1999) and that neutropenic mice show an increased survival after pulmonary cryptococcal infection (Mednick *et al.* 2003). These observations suggest a role of neutrophils in pulmonary cryptococcosis but the cellular source(s) of chemokines able to recruit neutrophils have not been characterized yet. We observed a high constitutive expression of CXCL15 mRNA in pulmonary epithelial cells of WT and *IL33*$^{cit/cit}$ mice, which, upon fungal infection, increased significantly (Fig. 6A, left panel). This indicates that CXCL15 is regulated independently of IL-33. However, the induction of CXCL15 mRNA was restricted exclusively to Ep-CAM$^+$ citrine$^+$ cells (Fig. 6A, right panel). This identifies IL-33-producing epithelial cells (i.e. type 2 alveolar epithelial cells) also as an important cellular source for CXCL15 during pulmonary cryptococcosis.

In addition to CXCL15, IL-33-positive epithelial cells also produce SP-C. The role of SP-C upon infection with *C. neoformans* has not been examined yet. We observed *in vivo* a significant induction of SP-C mRNA in pulmonary epithelial cells in WT mice upon pulmonary cryptococcosis but not in *IL33*$^{cit/cit}$ mice suggesting homeostatic regulation of SP-C by IL-33, (Fig. 6B, left panel). Moreover, we identified in *IL33*$^{cit/+}$ mice that exclusively Ep-CAM$^+$ citrine$^+$ cells upregulate SP-C mRNA upon infection (Fig. 6B, right panel; Fig. 3D). Taken together these results reveal that, even though upon cryptococcal infection CXCL15 and SP-C are induced in epithelial cells independently of IL-33, it is the IL-33-expressing epithelial cell that is also a potent producer of CXCL15 and SP-C. Thus, type 2 alveolar epithelial cells appear to actively contribute to the immune response during pulmonary cryptococcosis.

DISCUSSION

In this study, we characterized the cellular source of IL-33 and defined IL-33-dependent and IL-33-independent regulation of pulmonary epithelial cells upon cryptococcal infection. We found that (i) type 2 alveolar epithelial cells are the primary producers of IL-33, (ii) IL-33 production is independent of IL-33 receptor expression on lung epithelial cells, (iii) E-cadherin expression by Ep-CAM$^+$ lung epithelial cells decreases upon infection in an IL-33-dependent manner and (iv) the induction of

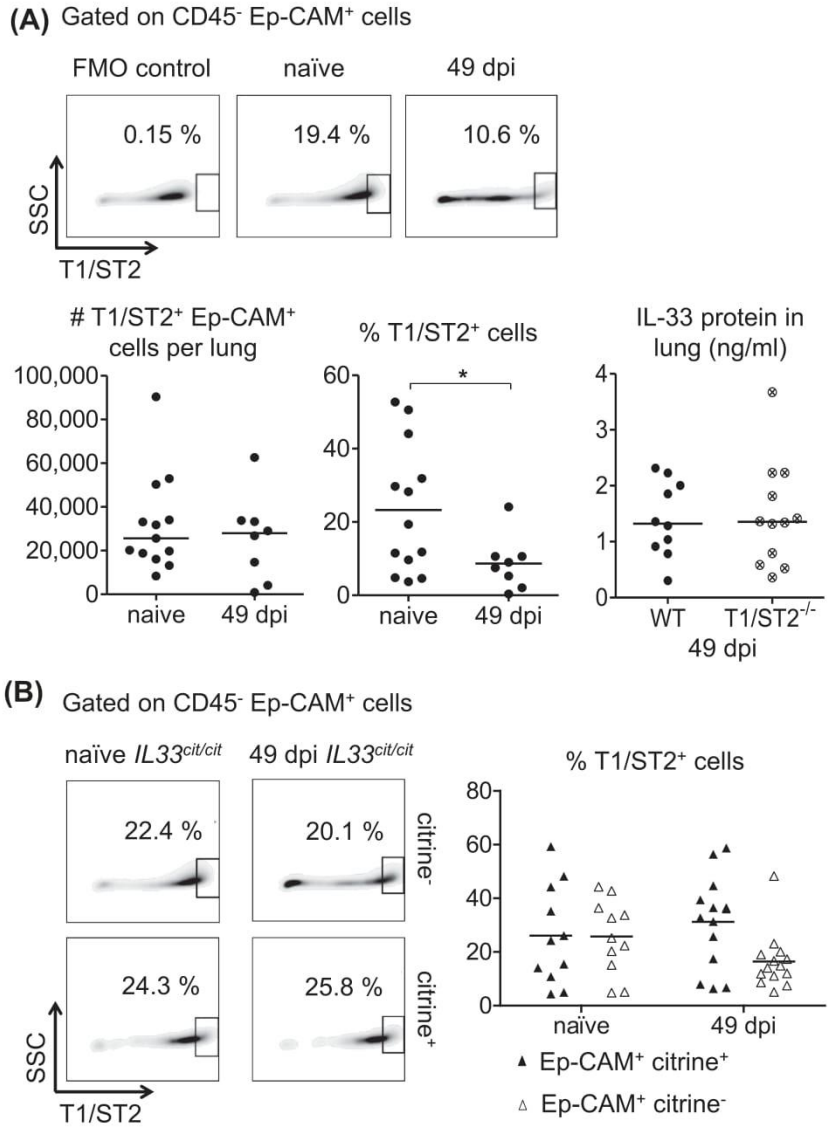


Figure 4. T1/ST2 expression by epithelial cells during cryptococcal infection is independent of epithelial IL-33 production. Mice were infected intranasally with *C. neoformans* and analyzed at indicated time points. (**A**) Representative flow cytometry plots of viable, CD45$^-$, single, Ep-CAM$^+$ cells of wild-type (WT) mice gated on T1/ST2 and compared to the Fluorescence-Minus-One (FMO) control are shown. Frequencies (left panel) and total numbers (central panel) of Ep-CAM$^+$T1/ST2$^+$ cells in WT mice upon cryptococcal infection are displayed. IL-33 protein in supernatant of homogenized accessory lung lobe from wild-type (WT) or T1ST2$^{-/-}$ mice was determined by ELISA with a detection limit at 0.06 ng/ml (right panel). (**B**) Viable, CD45$^-$, single, Ep-CAM$^+$, citrine$^+$ or citrine$^-$ cells from *IL33*$^{cit/cit}$ mice (deficient for IL-33 protein synthesis) were gated and expression of T1/ST2 was analyzed. Representative flow cytometry plots and T1/ST2 expression frequencies after infection are shown. Each data point represents one animal from two independent experiments each with $n = 4$–7 mice per group. Statistical analysis was performed using unpaired two-tailed t-test $^*P < 0.05$.

chemokine and surfactant production by Ep-CAM$^+$ lung epithelial cells is IL-33-independent. The results from our study should shed light on the pathogenesis of fungal allergic inflammation and may enable early therapeutic opportunities of intervention.

IL-33, belonging to the IL-1 family of cytokines, is induced upon infection with *C. neoformans* and plays a crucial role in immunopathology during cryptococcosis (Flaczyk *et al.* 2013; Piehler *et al.* 2013). Susceptibility to cryptococcal infection has been characterized as type 2 mediated (Hernandez *et al.* 2005; Müller *et al.* 2007; Müller *et al.* 2008). However, the initiation and maintenance of type 2-biased immunopathology is not clearly understood. Here, we identified type 2 alveolar epithelial cells, representing only 5% of the pulmonary epithelium, as major cellular source of IL-33 *in vivo* during cryptococcal infection. T1/ST2- expressing ILC2s and Th2 cells are the primary sources of the type 2 cytokines IL-5 and IL-13 during pulmonary cryptococcosis (Piehler *et al.* 2013). Thus, our results suggest lung epithelial cell involvement in type 2-dependent inflammation during infection with *C. neoformans*. However, our study reveals a significant increase of IL-33 mRNA and protein only after 49 dpi.

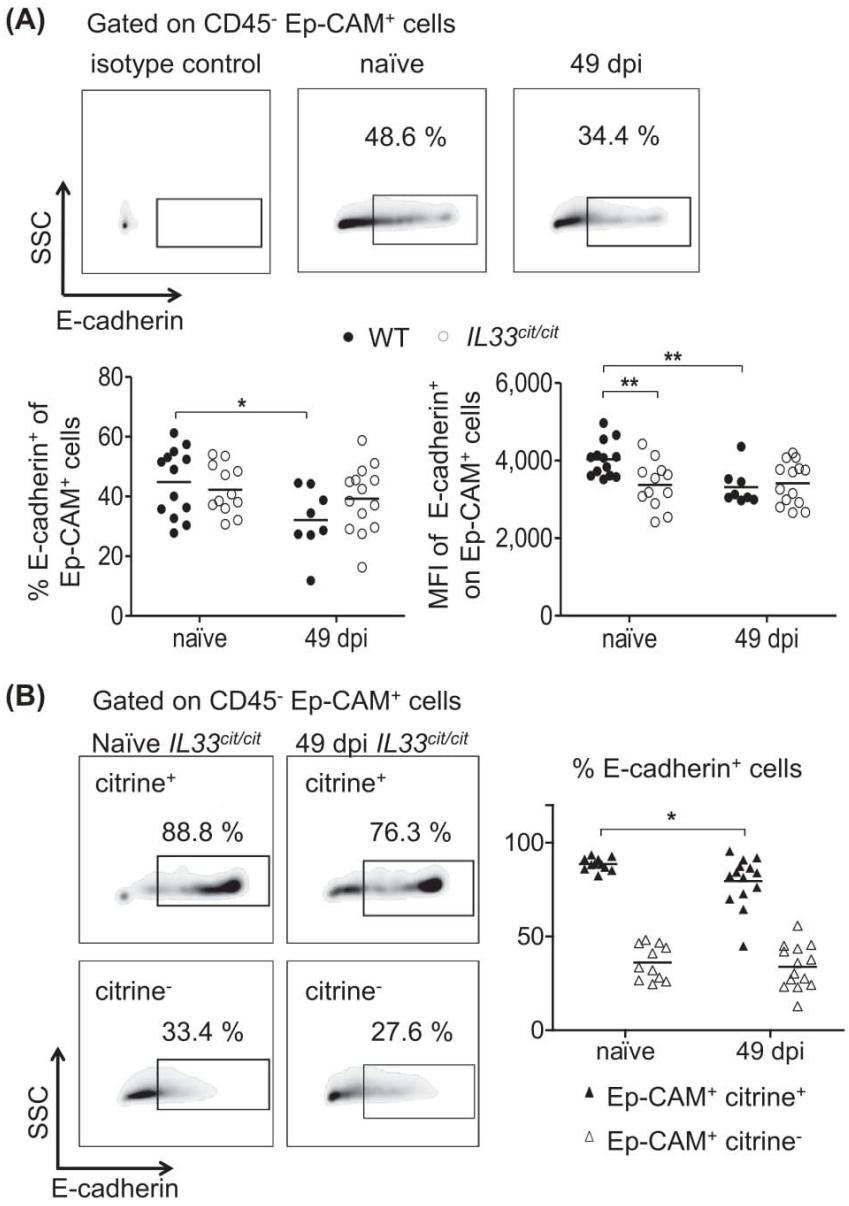


Figure 5. Decreased E-cadherin expression by epithelial cells during cryptococcal infection. Wild-type (WT) and *IL33*$^{cit/cit}$ mice were infected intranasally with *C. neoformans* and analyzed at 49 dpi. (**A**) Representative flow cytometry plots of viable, CD45$^-$, single, Ep-CAM$^+$ cells of WT mice gated on E-cadherin are shown. The data are from one out of two independent experiments. The frequencies of viable, CD45$^-$, single, Ep-CAM$^+$, E-cadherin$^+$ cells per lung (left panel) and analysis of MFI of E-cadherin expressed by epithelial cells (right panel) of WT and *IL33*$^{cit/cit}$ mice upon cryptococcal infection are depicted. (**B**) Viable, CD45$^-$, single, Ep-CAM$^+$, citrine$^+$/citrine$^-$ cells were gated and expression of E-cadherin was analyzed; representative flow cytometry plots and frequencies are shown. Statistical significance was determined by using two-tailed unpaired *t*-test $^{***}P < 0.001$. Each data point represents one animal from two independent experiments, each with $n = 4$–7 mice per group. Statistical significance was determined by using two-tailed unpaired *t*-test $^{*}P < 0.05$, $^{**}P < 0.01$.

In previous studies, we already demonstrated an essential role of IL-33 at earlier time points in pulmonary cryptococcosis as induction of IL-13, produced by IL-33-dependent ILC2 occurs already 21 dpi (Piehler *et al.* 2015). Taking into account that IL-13 has been described as the most capable inducer of IL-33 mRNA in human lung epithelial cell lines (Christianson *et al.* 2015), one could hypothesize that enhanced IL-13 production by ILC2 and Th2 cells probably leads in turn to a significant increase and in particular maintenance of already constitutively high IL-33 levels at later time points of cryptococcal infection.

We demonstrated T1/ST2 expression after cryptococcal infection not only by IL-33-positive but also IL-33-negative lung epithelial cells. Pneumocytes constitutively express T1/ST2 (Hardman, Panova and McKenzie 2013) and this expression enables upregulation of IL-33 in an auto-/paracrine manner during allergic lung inflammation (Hardman, Panova and

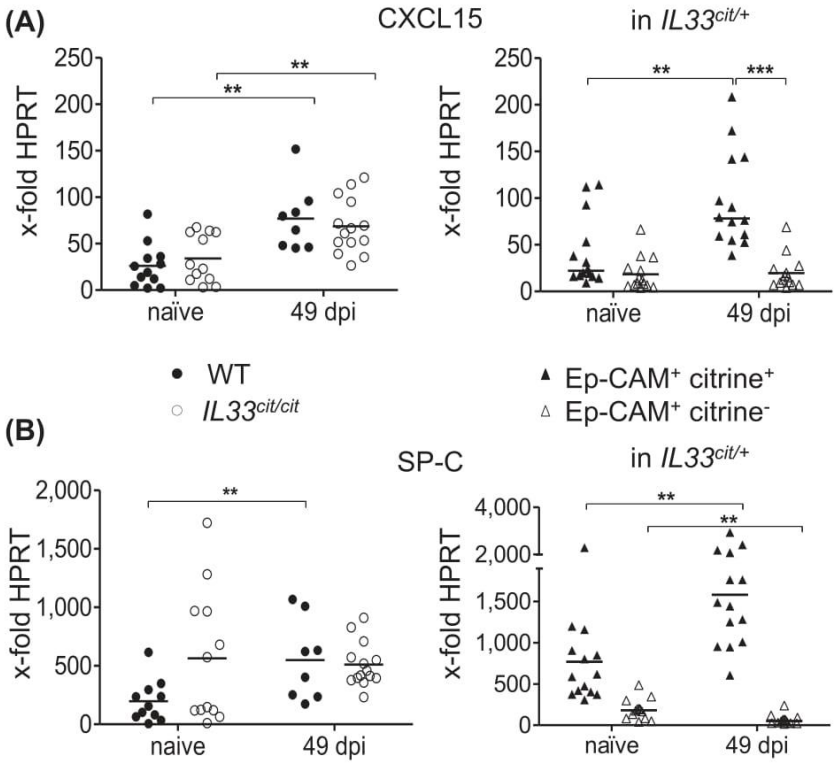


Figure 6. Induction of CXCL15 and SP-C in IL-33$^+$ epithelial cells during cryptococcal infection. Mice were infected intranasally with *C. neoformans* and analyzed 49 dpi. (**A**) Expression of CXCL15 mRNA was measured by RT-qPCR in sorted Ep-CAM$^+$ cells of wild-type (WT) and *IL33*$^{cit/cit}$ mice (left panel). CXCL15 mRNA expression was determined in sorted Ep-CAM$^+$ and citrine$^+$ or citrine$^-$ cells in *IL33*$^{cit/+}$ mice (right panel). (**B**) Expression of SP-C mRNA was measured in sorted Ep-CAM$^+$ cells of WT and *IL33*$^{cit/cit}$ mice (left panel). Note: for comparison data presented in Fig. 1, Supporting Information (49 dpi, Ep-CAM$^+$ citrine$^{+/-}$) are re-shown in the right panel. For naïve WT mice one value was excluded after determination as an outlier by Grubbs' test. SP-C (surfactant protein C) mRNA expression was measured in sorted Ep-CAM$^+$ citrine$^+$/citrine$^-$ cells in *IL33*$^{cit/+}$ mice by RT-qPCR (right panel). Two-tailed unpaired t-test and two-tailed Mann Whitney test (for nonparametric distributed data) $^{**}P < 0.01$, $^{***}P < 0.001$ were performed to determine statistical significance. Each data point represents one animal and is from two independent pooled experiments each with $n = 4$–7 mice per group. For both genes, the relative expression was calculated relative to mouse house-keeping gene hypoxanthine phosphoribosyl transferase 1 (HPRT).

McKenzie 2013; Christianson *et al.* 2015). In our infection model, we observed no difference of IL-33 protein levels between infected WT and T1/ST2$^{-/-}$ mice. Therefore, we conclude that IL-33 does not regulate its own production in fungal pulmonary infection.

In this study, we provide first evidence that IL-33 has an impact on epithelial E-cadherin expression in particular under inflammatory conditions. We examined the effect of cryptococcal infection on E-cadherin expression by lung epithelial cells *in vivo* and found a decrease. As E-cadherin is necessary for epithelial integrity, our results might indicate epithelial barrier disruption by cryptococcal infection. Barrier disruption is combined with subsequent increased permeability, although appropriate *in vitro* experiments to prove this suggestion have not been included in this study. It has been likewise demonstrated *in vitro* that the fungus *Candida albicans* is able to degrade E-cadherin of mucosal epithelium in order to invade mucosal tissue (Villar *et al.* 2007). It is additionally conceivable that enhanced barrier permeability facilitates innate host defense mechanisms performed by dendritic cells next to the epithelium (Hammad and Lambrecht 2008). However, we found decreasing E-cadherin expression only in WT but not in IL33$^{cit/cit}$ mice infected with *C. neoformans*, suggesting an indirect and/or direct regulation of adhesion molecule expression by IL-33. The Th2 cytokines IL-4 and IL-13 were shown *in vitro* to induce downregulation of junctional components including E-cadherin in human bronchiolar epithelial cells (Saatian *et al.* 2013), representing a possible indirect impact by IL-33. Direct adhesion molecule impairment caused by IL-33 has been described until now only for vascular endothelial cadherin in human endothelial cell culture (Chalubinski *et al.* 2015). It is known that E-cadherin represents not only a cell-to-cell adhesion protein but is additionally involved in regulation of signaling. In this context, it has been described that decreased E-cadherin expression results in increased epidermal growth factor receptor-dependent signaling (Qian *et al.* 2004), which in turn leads to Th2 cell-promoting activity by human lung epithelial cells (Heijink *et al.* 2007). Together, our results might be indicative of an IL-33-dependent impaired barrier integrity and enhanced immune activation.

A further indication for infection-induced enhanced immune-related epithelial activity could be CXCL15. Our study demonstrated for the first time *in vivo* that lung epithelial cells upregulate CXCL15 mRNA after *C. neoformans* infection and support *in vitro* studies showing IL-8 induction in human A549 cells and NHBE (Barbosa *et al.* 2007; Guillot *et al.* 2008). Although CXCL15 mRNA production was mainly restricted to IL-33 positive epithelial cells, the production itself was independent of IL-33. Kawakami *et al.* described an increase of lung leukocytes

comprising mainly neutrophils post cryptococcal infection of mice (Kawakami *et al.* 1999). This recruitment might be based on epithelial cell-derived chemokine production. However, it remains to be shown whether neutrophils support host defense or favor fungal dissemination during cryptococcal infection. *In vitro* studies revealed effective killing of cryptococcal cells by neutrophils (Miller and Mitchell 1991), whereas *in vivo* studies showed inconsistent results (Graybill *et al.* 1997; Mednick *et al.* 2003). Nevertheless, an initial recruitment of phagocytic immune cells next to the barrier due to epithelial-derived chemokines is very likely.

Also pointing to enhanced lung epithelial cell activity upon pulmonary cryptococcosis was a significant upregulation of epithelial SP-C mRNA together with, but independent of IL-33. In contrast to our findings, SP-C levels in pulmonary infection of mice with the fungal pathogens *Aspergillus fumigatus* or *Pneumocystis carinii* were decreasing (Atochina *et al.* 2000; Haczku *et al.* 2001). In pulmonary cryptococcosis surfactant proteins A and D (SP-A and SP-D) were shown to bind to *C. neoformans* cells (Walenkamp *et al.* 1999; Geunes-Boyer *et al.* 2009). SP-D-protected fungi from macrophage killing (Geunes-Boyer *et al.* 2009) and consequently enhanced susceptibility to pulmonary cryptococcal infection (Geunes-Boyer *et al.* 2012; Holmer *et al.* 2014). SP-C is known to be able to bind to CD14 (Augusto *et al.* 2003), a Toll-like receptor (TLR)-associated pattern recognition receptor which is mainly expressed by macrophages, monocytes and neutrophils. In consequence, the interaction of SP-C with CD14 results in increased binding of CD14 to LPS (Augusto *et al.* 2003). *In vitro*, CD14 also serves as a receptor for glucuronoxylomannan (Barbosa *et al.* 2007), the major component of the cryptococcal capsule. CD14 is necessary for efficient phagocytosis and subsequent killing of cryptococcal yeast cells *in vivo* (Yauch *et al.* 2004) and mice deficient for CD14 showed reduced survival after cryptococcal infection in comparison to WT mice (Yauch *et al.* 2004). Thus, increased SP-C production by lung epithelial cells may probably result in enhanced recognition and phagocytosis of cryptococcal cells.

In summary, our study identified type 2 alveolar epithelial cells as major IL-33 producing cell population in pulmonary cryptococcosis and further investigated regulation of lung epithelium by IL-33. We demonstrated type 2 alveolar epithelial activation upon cryptococcal infection by IL-33-independent increase of CXCL15 and SP-C production and IL-33-dependent downregulation of E-cadherin.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSPD online.

ACKNOWLEDGEMENTS

We thank Dr A.N.J. McKenzie for providing the IL-33 citrine reporter mice and Prof. Dr T. Kamradt for allocating T1/ST2$^{-/-}$ mice. We are grateful to R. Voigtländer and L. Mittag from the research facility of the Max Planck institute for evolutionary anthropology for excellent mouse care. We thank Dr M. Gericke from the Institute of Anatomy, Medical Faculty, University of Leipzig for support with immunofluorescence microscopy. For technical assistance, we like to thank S. Lehnert. Dr A. Dalpke is thanked for critically reading this manuscript. Flow cytometry was performed at the Core Unit Flow Cytometry (CUDZ) of the College of Veterinary Medicine, University of Leipzig.

FUNDING

This work was supported by the Deutsche Forschungsgemeinschaft (German Research Foundation) [PI 1066/1-1 to D.P.] and L. Heyen was supported by the PhD program from the Hanns Seidel Foundation, Germany.

Conflict of interest. None declared.

REFERENCES

Atochina EN, Beers MF, Scanlon ST *et al. P. carinii* induces selective alterations in component expression and biophysical activity of lung surfactant. *Am J Physiol Lung C* 2000;**3**:L599–609.

Augusto LA, Synguelakis M, Johansson J *et al.* Interaction of pulmonary surfactant protein C with CD14 and lipopolysaccharide. *Infect Immun* 2003;**1**:61–7.

Barbosa FM, Fonseca FL, Figueiredo RT *et al.* Binding of glucuronoxylomannan to the CD14 receptor in human A549 alveolar cells induces interleukin-8 production. *Clin Vaccine Immunol* 2007;**1**:94–8.

Chalubinski M, Wojdan K, Luczak E *et al.* IL-33 and IL-4 impair barrier functions of human vascular endothelium via different mechanisms. *Vasc Pharmacol* 2015;**73**:57–63.

Chen SC, Mehrad B, Deng JC *et al.* Impaired pulmonary host defense in mice lacking expression of the CXC chemokine lungkine. *J Immunol* 2001;**5**:3362–8.

Christianson CA, Goplen NP, Zafar I *et al.* Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J Allergy Clin Immun* 2015;**1**:59–68.e14.

Decken K, Köhler G, Palmer-Lehmann K *et al.* Interleukin-12 is essential for a protective Th1 response in mice infected with *Cryptococcus neoformans*. *Infect Immun* 1998;**10**:4994–5000.

Flaczyk A, Duerr CU, Shourian M *et al.* IL-33 signaling regulates innate and adaptive immunity to *Cryptococcus neoformans*. *J Immunol* 2013;**5**:2503–13.

Geunes-Boyer S, Beers MF, Perfect JR *et al.* Surfactant protein D facilitates *Cryptococcus neoformans* infection. *Infect Immun* 2012;**7**:2444–53.

Geunes-Boyer S, Oliver TN, Janbon G *et al.* Surfactant protein D increases phagocytosis of hypocapsular *Cryptococcus neoformans* by murine macrophages and enhances fungal survival. *Infect Immun* 2009;**7**:2783–94.

Graybill JR, Bocanegra R, Lambros C *et al.* Granulocyte colony stimulating factor therapy of experimental cryptococcal meningitis. *J Med Vet Mycol* 1997;**4**:243–7.

Guillot L, Carroll SF, Badawy M *et al. Cryptococcus neoformans* induces IL-8 secretion and CXCL1 expression by human bronchial epithelial cells. *Respir Res* 2008;**9**:9.

Haczku A, Atochina EN, Tomer Y *et al.* Aspergillus fumigatus-induced allergic airway inflammation alters surfactant homeostasis and lung function in BALB/c mice. *Am J Resp Cell Mol* 2001;**1**:45–50.

Hammad H, Lambrecht BN. Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma. *Nat Rev Immunol* 2008;**3**:193–204.

Hardman CS, Panova V, McKenzie ANJ. IL-33 citrine reporter mice reveal the temporal and spatial expression of IL-33 during allergic lung inflammation. *Eur J Immunol* 2013;**2**:488–98.

Heijink IH, Kies PM, Kauffman HF *et al.* Down-regulation of E-cadherin in human bronchial epithelial cells leads to epidermal growth factor receptor-dependent Th2 cell-promoting activity. *J Immunol* 2007;**12**:7678–85.

Hernandez Y, Arora S, Erb-Downward JR *et al.* Distinct roles for IL-4 and IL-10 in regulating T2 immunity during allergic bronchopulmonary mycosis. *J Immunol* 2005;**2**:1027–36.

Holmer SM, Evans KS, Asfaw YG *et al.* Impact of surfactant protein D, interleukin-5, and eosinophilia on Cryptococcosis. *Infect Immun* 2014;**2**:683–93.

Kawakami K, Shibuya K, Qureshi MH *et al.* Chemokine responses and accumulation of inflammatory cells in the lungs of mice infected with highly virulent *Cryptococcus neoformans*: effects of interleukin-12. *FEMS Immunol Med Mic* 1999;**4**:391–402.

King RJ. Pulmonary surfactant. *J Appl Physiol Respir Environ Exerc Physiol* 1982;**1**:1–8.

Kunkel SL, Standiford T, Kasahara K *et al.* Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. *Exp Lung Res* 1991;**1**:17–23.

Martowicz A, Seeber A, Untergasser G. The role of EpCAM in physiology and pathology of the epithelium. *Histol Histopathol* 2016;**4**:349–55.

Mednick AJ, Feldmesser M, Rivera J *et al.* Neutropenia alters lung cytokine production in mice and reduces their susceptibility to pulmonary cryptococcosis. *Eur J Immunol* 2003;**6**:1744–53.

Miller MF, Mitchell TG. Killing of *Cryptococcus neoformans* strains by human neutrophils and monocytes. *Infect Immun* 1991;**1**:24–8.

Moussion C, Ortega N, Girard J. The IL-1-like cytokine IL-33 is constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*: a novel 'alarmin'? *PLoS ONE* 2008;**10**:e3331.

Müller U, Stenzel W, Köhler G *et al.* IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with *Cryptococcus neoformans*. *J Immunol* 2007;**8**:5367–77.

Müller U, Stenzel W, Köhler G *et al.* A gene-dosage effect for interleukin-4 receptor alpha-chain expression has an impact on Th2-mediated allergic inflammation during bronchopulmonary mycosis. *J Infect Dis* 2008;**11**:1714–21.

Neill DR, Wong SH, Bellosi A *et al.* Nuocytes represent a new innate effector leukocyte that mediates type-2 immunity. *Nature* 2010;**7293**:1367–70.

Oboki K, Nakae S, Matsumoto K *et al.* IL-33 and Airway Inflammation. *Allergy Asthma Immunol Res* 2011;**2**:81–8.

Osterholzer JJ, Chen GH, Olszewski MA *et al.* Chemokine receptor 2-mediated accumulation of fungicidal exudate macrophages in mice that clear cryptococcal lung infection. *Am J Pathol* 2011;**1**:198–211.

Pichery M, Mirey E, Mercier P *et al.* Endogenous IL-33 is highly expressed in mouse epithelial barrier tissues, lymphoid organs, brain, embryos, and inflamed tissues: *in situ* analysis using a novel Il-33-LacZ gene trap reporter strain. *J Immunol* 2012;**7**:3488–95.

Piehler D, Eschke M, Schulze B *et al.* The IL-33 receptor (ST2) regulates early IL-13 production in fungus-induced allergic airway inflammation. *Mucosal Immunol* 2015;**9**:937–49.

Piehler D, Grahnert A, Eschke M *et al.* T1/ST2 promotes T helper 2 cell activation and polyfunctionality in bronchopulmonary mycosis. *Mucosal Immunol* 2013;**2**:405–14.

Qian X, Karpova T, Sheppard AM *et al.* E-cadherin-mediated adhesion inhibits ligand-dependent activation of diverse receptor tyrosine kinases. *EMBO J* 2004;**8**:1739–48.

Rossi DL, Hurst SD, Xu Y *et al.* Lungkine, a novel CXC chemokine, specifically expressed by lung bronchoepithelial cells. *J Immunol* 1999;**9**:5490–7.

Saatian B, Rezaee F, Desando S *et al.* Interleukin-4 and interleukin-13 cause barrier dysfunction in human airway epithelial cells. *Tissue Barriers* 2013;**2**:e24333.

Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. *Annu Rev Immunol* 2012;**30**:647–75.

Townsend MJ, Fallon PG, Matthews DJ *et al.* T1/ST2-deficient mice demonstrate the importance of T1/ST2 in developing primary T helper cell type 2 responses. *J Exp Med* 2000;**6**:1069–76.

van Roy F, Berx G. The cell–cell adhesion molecule E-cadherin. *Cell Mol Life Sci* 2008;**23**:3756–88.

Velagapudi R, Hsueh Y, Geunes-Boyer S *et al.* Spores as infectious propagules of *Cryptococcus neoformans*. *Infect Immun* 2009;**10**:4345–55.

Villar CC, Kashleva H, Nobile CJ *et al.* Mucosal tissue invasion by *Candida albicans* is associated with E-cadherin degradation, mediated by transcription factor Rim101p and protease Sap5p. *Infect Immun* 2007;**5**:2126–35.

Voelz K, May RC. Cryptococcal interactions with the host immune system. *Eukaryot Cell* 2010;**6**:835–46.

Walenkamp AM, Verheul AF, Scharringa J *et al.* Pulmonary surfactant protein A binds to *Cryptococcus neoformans* without promoting phagocytosis. *Eur J Clin Invest* 1999;**1**:83–92.

Whitsett JA, Alenghat T. Respiratory epithelial cells orchestrate pulmonary innate immunity. *Nat Immunol* 2015;**1**:27–35.

Yauch LE, Mansour MK, Shoham S *et al.* Involvement of CD14, toll-like receptors 2 and 4, and MyD88 in the host response to the fungal pathogen *Cryptococcus neoformans in vivo*. *Infect Immun* 2004;**9**:5373–82.

2.2 unpublished work:

Pulmonary epithelial cells – distinct *in vitro* responses to *Cryptococcus neoformans* and possible cellular interactions with lung immune cells *in vivo*

This work is unpublished.

Author's contribution:

Laura Heyen:

- experimental design
- practical experimental work
- data acquisition
- data analysis and interpretation
- manuscript writing

Daniel Piehler:

- experimental design

Uwe Müller:

- assistance with practical experimental work
- support to manuscript writing

Gottfried Alber:

- assistance with experimental design and data interpretation
- support to manuscript writing

Pulmonary epithelial cells – distinct *in vitro* responses to *Cryptococcus neoformans* and possible cellular interactions with lung immune cells *in vivo*

Laura Heyen[1], Uwe Müller[1], Bianca Schulze[1, 2], Gottfried Alber[1,*], and Daniel Piehler[1, 3]

[1] Institute of Immunology/Molecular Pathogenesis, Center for Biotechnology and Biomedicine, College of Veterinary Medicine, University of Leipzig, Leipzig, Germany

[2] present address: Microbial Immunology, Leibniz-Institute for Natural Product Research and Infection Biology - Hans-Knöll-Institute (HKI), Jena, Germany

[3] present address: vaxxinova GmbH, Leipzig, Germany

Keywords:
Cryptococcus neoformans, pulmonary infection, airway epithelial cells, pulmonary inflammation, mouse

ABSTRACT

Recently we demonstrated increased IL-33, CXCL15 and SP-C expression by type 2 alveolar epithelial cells during pulmonary cryptococcosis. In order to characterize more detailed the mechanisms of these *in vivo* findings, *in vitro* experiments were performed. Primary pulmonary Ep-CAM$^+$ cells, including a 10-fold enrichment of epithelial cells, were stimulated with *C. neoformans* and production of IL-33, SP-C and CXCL15 was analyzed. Only enhanced CXCL15 and SP-C transcription was found in response to *in vitro* incubation with *C. neoformans* indicating a need for other cells in the form of molecular mediators or direct cellular contact for increased production of IL-33. To define the cell types potentially interacting with pulmonary epithelium via epithelial E-cadherin and CD200, expression of CD103 and KLRG1 as well as CD200R on different pulmonary immune cells during cryptococcal infection were analyzed *in vivo* by flow cytometry. Decreasing frequencies of CD103$^+$ dendritic cells but an increasing proportion of CD103$^+$ CD4$^+$ including FoxP3$^+$ CD4$^+$ and CD103$^+$ KLRG1$^+$ CD4$^+$ cells were found. CD200R was primarily expressed by alveolar macrophages and revealed a reduction at the single cell level upon pulmonary cryptococcosis. In conclusion, this study provides evidence for direct epithelial upregulation of CXCL15 and SP-C by *C. neoformans*, and reveals a high potential of epithelial cells to interact with lung immune cells upon pulmonary cryptococcosis.

INTRODUCTION

Recently we demonstrated that lung epithelial cells get activated during pulmonary cryptococcosis. In particular, we revealed increased IL-33, CXCL15 and SP-C expression by type 2 alveolar epithelial cells (HEYEN et al. 2016). IL-33 is induced upon cryptococcal infection and represents a cytokine, which promotes Th2 immunity (PIEHLER et al. 2015; SPITS and CUPEDO 2012) and thus growth and systemic dissemination of the fungal pathogen (FLACZYK et al. 2013; PIEHLER et al. 2013). CXCL15 (lungkine) is the neutrophil-targeted chemokine in the murine lung (ROSSI et al. 1999; CHEN et al. 2001), whereas SP-C, besides three other proteins, belongs to pulmonary surfactant, which is necessary for downregulation of surface tension in the lung (KING 1982). Especially CXCL15 and IL-33 production result in subsequent recruitment and activation of immune cells such as T cells and neutrophils, respectively (KOMAI-KOMA et al. 2007; CHEN et al. 2001). In order to analyze the conditions of epithelial regulation and activation in more detail *in-vitro* experiments have been performed.

Besides humoral factors like IL-33 and CXCL15 probably direct cell-to-cell contact enables epithelial involvement in immune defense during cryptococcal infection. *In vivo*, we analyzed already epithelial E-cadherin expression (HEYEN et al. 2016), which is an important adhesion molecule (VAN ROY and BERX 2008) but, moreover, allows interaction with CD103 and KLRG1 expressing immune cells like T cells and dendritic cells (GRÜNDEMANN et al. 2006). Whereas CD103 expression by T cells enables their mucosal recruitment (SCHÖN et al. 1999), $KLRG1^+$ T cells show no proliferation anymore but effective cytokine production, e.g. IFN-γ and TNF-α in the murine lung upon infection with *Mycobacterium tuberculosis* (REILEY et al. 2010). Furthermore, it was demonstrated *in vitro* that interaction of KLRG1 expressed by $CD8^+$ T cells with E-cadherin results in inhibition of T cell proliferation and cytolytic activity (GRÜNDEMANN et al. 2006). Furuhashi *et al*. analyzed the function of CD103 expressing dendritic cells in an ovalbumin cell culture system and demonstrated that $CD103^+$ dendritic cells induce a Th1 immune response, whereas T cell priming by $CD11b^+$ but $CD103^-$ dendritic cells results in enhanced Th2 cytokine production (FURUHASHI et al. 2012). This has been additionally confirmed in mice infected with *C. neoformans*, where in particular $CD103^-$ $CD11b^+$ dendritic cells were necessary for Th2 cell priming (WIESNER et al. 2015). Another direct epithelial - immune cell interaction could be mediated by the transmembrane glycoprotein CD200 and its receptor CD200R, which is primarily expressed by myeloid cells (WRIGHT et al. 2000). *In vivo* studies characterized CD200-CD200R signaling as inhibitory and anti-inflammatory (HOEK et al. 2000) and consequently as an essential part of maintenance of homeostasis.

In this study we analyzed *in vitro* in more detail the conditions for epithelial IL-33, CXCL15 and SP-C upregulation and examined *in vivo* possible ligand-receptor mediated interactions between epithelial and immune cells upon intranasal cryptococcal infection.

MATERIAL AND METHODS

Mice

In accordance with the guidelines authorized by the Animal Care and Usage Committee of the "Landesdirektion Sachsen" (accredition no. 24-9168.11/18/35) mice were kept under specific pathogen-free conditions in an individually ventilated caging system. WT BALB/c mice were purchased from Janvier (Janvier, France) and 8- to 18-week-old female WT littermates were utilized for the experiments. Sterile water and food were given *ad libitum*.

C. neoformans and infection of mice

C. neoformans strain 1841 (serotype D) represents a clinical isolate from a HIV patient (DECKEN et al. 1998) and was kept as frozen stock in 10% skim milk. It proliferated in Sabouraud dextrose medium (2% glucose, 1% peptone; Sigma Aldrich, Munich, Germany) overnight at 30°C. After harvesting the cells were washed twice with sterile phosphate-buffered saline (PBS). The fungal cells were counted in a hematocytometer and the culture was resuspended to a concentration of $2.5x10^4$ ml^{-1} in PBS. Before infection mice were anesthetized by an intraperitoneal injection with a 1:1 mixture of Ketamine (100 mg ml^{-1}; Ceva Tiergesundheit, Düsseldorf, Germany) and Xylazine (20 mg ml^{-1}; Ceva Tiergesundheit). Afterwards they received intranasally 20 µl containing 500 colony-forming units of *C. neoformans.*

Preparation of pulmonary cells

Lungs were perfused with sterile 0.9% sodium chloride solution. For lung digestion and preparation of a single pulmonary cell suspension the Lung Dissociation Protocol (Miltenyi Biotec, Bergisch Gladbach, Germany) was performed using the Lung Dissociation Kit mouse (Miltenyi Biotec) and the gentleMACS Dissociator (Miltenyi Biotec). Gey`s solution was utilized for lysis of red blood cells.

Flow cytometry

For the exclusion of dead cells from analysis Fixable Viability Dye eFluor 780 (eBioscience, Frankfurt, Germany) was used. In order to avoid unspecific Fc binding, cells were incubated with Tru Stain fcX™ (anti-mouse CD16/32, clone 93, BioLegend, Fell, Germany). After specific staining antibodies were added for 15 minutes, cells were fixed with 2% paraformaldehyde (Serva, Heidelberg, Germany) for 20 minutes at 4°C in the dark. The following antibodies were used: Alexa Fluor 647 anti-CD326 (Ep-CAM, clone G8.8, BioLegend), Alexa Fluor 700 anti-CD45 (clone 30-F11, eBioscience), Brilliant Violet 421 anti-SiglecF (clone E50-2440, BD Biosciences, Heidelberg, Germany), APC anti-CD11c (clone N418, BioLegend), PacBlue anti-CD4 (clone RM4-5, BioLegend), biotinylated anti-CD103 (clone M290, BD Biosciences), PE anti-KLRG1 (clone 2F1/KLRG1, BioLegend), and APC anti-CD3 (clone 145-2C11,

eBioscience). Cells labeled with biotinylated antibodies were further stained with PerCp eF 710 Streptavidin (eBioscience). Suitable isotype antibodies were used from BioLegend and eBioscience. The analysis was performed on a BD LSRFortessa II (BD Biosciences) with DIVA 6.1.3 and FlowJo X0.7 (Treestar Inc., Ashland, OR) software.

Cells of interest were gated on living single cells. For the characterization of epithelial cells, $CD45^+$ cells were excluded and Ep-CAM^+ cells were analyzed for CD200 expression. For the characterization of macrophages, $CD45^-$ cells were excluded and $SiglecF^+$, $CD11c^+$ cells were analyzed for Ep-CAM, CD103, E-cadherin and CD200R expression. For the characterization of dendritic cells, $CD45^-$ cells were excluded and $SiglecF^-$, $CD11c^+$ cells were analyzed for Ep-CAM, CD103, E-cadherin and CD200R expression. Among $SiglecF^-$ 75% of $CD11c^+$ cells express MHC II (data not shown). For the characterization of T cells, $CD45^-$ cells were excluded and $CD4^+$ cells were analyzed for CD103 and KLRG1 expression.

Primary Ep-CAM^+ cell culture

Primary cultures of lung epithelial cells were prepared as follows. After exsanguinating the mouse 2 ml of 37°C prewarmed dispase (Corning, USA) were inserted via a 22 G cannula through a hole in the trachea followed by 0.5 ml liquefied Agarose (Biozym, Oldendorf, Germany) (1%). After the lung was cooled down with ice-cold PBS, the whole tissue was put in 1 ml dispase and was incubated for 6 minutes at 37°C. Afterwards, the lung tissue was minced in a GentleMACS C tube (Miltenyi Biotec, Bergisch Gladbach, Germany) supplemented with 5 ml DMEM including 2 % FKS, 1% P/S (GE Healthcare, Buckinghamshire, UK) and 80 µl DNase (Sigma Aldrich, Schnelldorf, Germany) on a GentleMACS (Miltenyi Biotec) running the program m_lung_1.02. The resulting cell suspension was passed through a 100 and 40 µm nylon mesh and centrifuged at 300xg for 10 minutes. Afterwards a magnetic positive selection using biotinylated anti-Ep-CAM (eBioscience, Frankfurt, Germany) and MagniSort Streptavidin Positive Selection Beads (eBioscience) was performed. The resulting cell suspension was seeded on Geltrex™ (gibco®) coated wells of 96 well plates in DMEM supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) (2.5 µg/ml; Peprotech) and recombinant human keratinocyte growth factor (KGF) (10 ng/ml; Peprotech). Prior stimulation the cells adhered in a humidified incubator (37°C, 5% CO_2) for 48 h. The cells were incubated with *C. neoformans* (multiplicity of infection 2) for 48h and with phorbol 12-myristate 13-acetate (PMA) (40 ng/ml) and polyinosinic: polycytidylic acid (poly (I:C)) (100 µg/ml) for the last 6h.

The purity of primary cell culture was determined by flow cytometry using PE/Cy7 labeled Streptavidin (BioLegend). The relative proportion of Ep-CAM^+ cells was 90 %. Among all Ep-CAM^+ cells we found 50-66% $CD45^-$ cells, representing lung epithelial cells, and 33-50% $CD45^+$ cells (Figure 1). Thus, primary lung epithelial cells were seeded at a 10-fold higher concentration to the cell culture

system in comparison to an unsorted pulmonary single cell suspension. Ep-CAM^{+} CD45^{+} cells were identified as alveolar macrophages (SiglecF^{+} CD11c^{+}) and dendritic cells (SiglecF^{-} CD11c^{+} MHCII^{+}) (supporting information Figure 1).

RNA isolation, reverse transcription (RT) and real-time PCR analysis

Cultivated epithelial cells were resuspended in peqGOLD TriFast (Peqlab, Erlangen, Germany) and stored at -80°C until RNA isolation. Following extraction with chloroform, RNA was precipitated utilizing isopropanol, washed two times with 70% ethanol and resuspended in diethylpyrocarbonate water (all: Roth, Karlsruhe, Germany). For elimination of genomic DNA RNA was treated with RNase-free DNase I (2.5 units per sample; Thermo Fisher Scientific GmbH, Schwerte, Germany) in the presence of RiboLock RNase inhibitor (20 units per sample; Thermo Fisher Scientific GmbH, Schwerte, Germany). RNA transcription to complementary DNA (cDNA) was processed with High capacity cDNA Reverse Transcription Kit (Applied Biosystems, Darmstadt, Germany) using a blend of random/ oligo (dT) 18 primer according to manufacturer`s instruction. RT-qPCR was performed in an iCycler iQ^{TM}5 (BIO-RAD, München, Germany) with the iTaq TM Universal SYBR® Green Supermix (BIO-RAD, München, Germany) in duplicates. The following primers were used for RT-qPCR: mm_hprt_for: 5´-GTTGGATACAGGCCAGACTTTGTTG-3´ and mm_hprt_rev: 5´- GATTCAACTTGCGCTC ATCTTAGGC-3´ (GenBank Accession No. NM_013556; positions 660-684 and 822-798); mm_sp-c_for: 5`-AGCAAAGAGGTCCTGATGGA–3` and mm_sp-c_rev: 5`-CTCATCTCAAGGACCATCTCAGTA–3` (GenBank Accession No. NM_011359.2; positions 51-70 and 253-230); mm_cxcl15_for: 5`-CTAGGCATCTTCGTCCGTCC–3` and mm_cxcl15_rev: 5`-TTGGGCCAACAGTAGCCTTC–3` (GenBank Accession No. NM_0113339 positions 83-102 and 299-280) and mm_IL-33_for: 5´-GGAAAGAACCCACGAAAAGA-3´ and mm_IL-33 _rev: 5´-TGTACTCAGGGAGGCAGGAG-3´ (GenBank Accession No. NM_001164724.1; positions 268- 287 and 455-436).

Statistical evaluation

Graph Pad Prism 5.01 software (GraphPad Software Inc., San Diego, USA) was utilized for statistical analysis, evaluation, and graphical representation. Normality was determined by the Kolmogorov-Smirnov test. Normally distributed data were analyzed by unpaired t-test. In order to compare nonparametric data the Mann-Whitney U-test was performed. Statistical significance was indicated for p-values as follows: *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

RESULTS

1. *C. neoformans* directly increases SP-C and CXCL15 but not IL-33 mRNA expression in Ep-CAM$^+$ pulmonary cells *in vitro*

Recently we revealed *in vivo* that intranasal cryptococcal infection results in activation of pulmonary epithelial cells (CD45$^-$ Ep-CAM$^+$) by upregulation of epithelial IL-33, CXCL15 and SP-C mRNA (HEYEN et al. 2016). As IL-33 is a crucial initiator of subsequent detrimental Th2 immunity, lung epithelial cells can be expected to actively participate in immunity to cryptococcal infection. In order to analyze whether epithelial cell activation is based on direct fungus-epithelium interaction, we established a primary cell culture of magnetically sorted pulmonary Ep-CAM$^+$ cells.

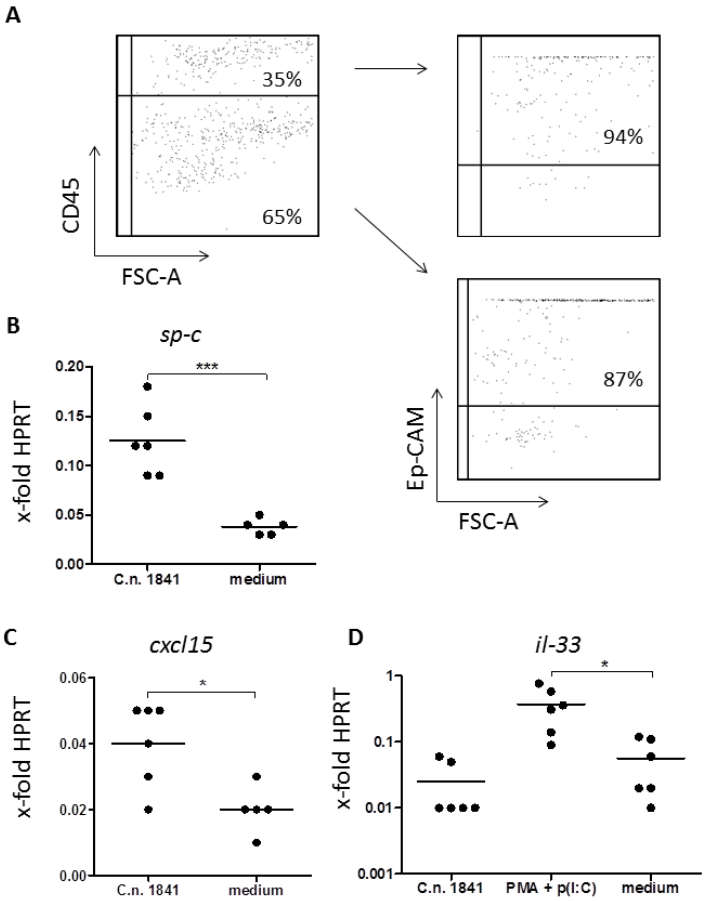


Figure 1. Isolated Ep-CAM$^+$ cells upregulate CXCL15 and SP-C but not IL-33 mRNA in response to *C. neoformans* stimulation *in vitro.* Primary Ep-CAM$^+$ lung cells were magnetically sorted. (**A**) Representative flow cytometry plots of sorted, viable, single cells gated on CD45 and Ep-CAM are shown (cut off for isotype of Ep-CAM staining is 1x10^3 (data not shown); lower border of gate). (**B-D**) Seeded Ep-CAM$^+$ cells were stimulated with *C. neoformans* strain 1841 (*C.n.* 1841) MOI 2 for 48 hours and with phorbol 12-myristate 13-acetate (PMA) and polyinosinic: polycytidylic acid (poly (I:C)) for 6 hours. Expression of SP-C, CXCL15 and IL-33 mRNA after stimulation was measured by RT-qPCR in sorted and stimulated Ep-CAM$^+$ cells. For the genes, the relative expression was calculated relative to murine house-keeping gene hypoxanthine-phosphoribosyl transferase 1 (HPRT). Each data point represents one cell culture well with seeded Ep-CAM$^+$ cells and is from 3 pooled independent experiments each with 2-3 WT mice. Two-tailed unpaired t-test was performed to determine statistical significance, *p<0.05; ***p<0.001.

The FACS analysis of naïve WT mice revealed Ep-CAM expression not only by $CD45^-$ (HEYEN et al. 2016) but also by $CD45^+$ cells (Figure 1A), including macrophages and dendritic cells (supporting information Figure 1A). Thus, the primary Ep-CAM^+ cell culture included in average 33% $CD45^+$ Ep-CAM^+ cells, 66% $CD45^-$ Ep-CAM^+ cells and thereby represents a 10-fold enrichment of pulmonary epithelial cells (Figure 1A). Isolated Ep-CAM^+ cells were incubated with *C. neoformans* strain 1841 at a MOI 2. After 48 hours stimulation time CXCL15 and SP-C mRNA induction was detectable (Figure 1B+C). In contrast, incubation with *C. neoformans* does not result in enhanced IL-33 mRNA expression under the same stimulation conditions (Figure 1D), although phorbol 12-myristate 13-acetate (PMA) and polyinosinic: polycytidylic acid (poly (I:C)) stimulation revealed the potential of IL-33 mRNA upregulation by primary Ep-CAM^+ cells (Figure 1D). These results suggest direct or indirect necessity of other cell types than macrophages or dendritic cells for epithelial IL-33 upregulation. In order to verify Th2 cell involvement and IL-13 as a potent IL-33 inducer, enriched primary epithelial cells were incubated with IL-13. No induction of epithelial IL-33 mRNA was detectable (data not shown). In summary, these data are indicative for epithelial CXCL15 and SP-C but not IL-33 upregulation by direct epithelium-*Cryptococcus* interaction.

2. Prospective cell contact mediated interactions of epithelial cells with immune cells: E-cadherin - CD103, KLRG1 and CD200 - CD200R

2.1 CD103 and KLRG1 expression by $CD4^+$ T cells increases whereas dendritic cells downregulate CD103 during cryptococcal infection

Recently, we analyzed E-cadherin expression on lung epithelial cells during pulmonary cryptococcosis. Whereas Ep-CAM^+ epithelial cells, in majority alveolar cells type 2, constitutively express E-cadherin to a high degree, it is downregulated upon cryptococcal infection in an IL-33-dependent manner (HEYEN et al. 2016). E-cadherin is not only an important protein for tight barrier integrity of the epithelium but additionally enables intercellular interactions with other immune cells. E-cadherin expressed by epithelial cells is able to bind in a homophilic manner to other epithelial cells as well as in a heterophilic manner to leukocytes and has CD103 and KLRG1 as specific binding partners (VAN ROY and BERX 2008; GRÜNDEMANN et al. 2006). Flow cytometry analysis of naïve WT mice revealed E-cadherin expression as expected by pulmonary epithelial cells but also by alveolar macrophages and lung dendritic cells (supporting information Figure 1B). Next we were interested in the cell types expressing CD103 and found in naïve mice primarily $CD103^+$ dendritic cells ($CD11c^+$ $SiglecF^-$) (mean 11% ± 3.5) (Figure 2A) but also $CD103^+$ $CD4^+$ (mean 2.3% ± 0.9) (Figure 2B, right) and $CD103^+$ $CD8^+$ cells (supporting information Figure 2A). Among $CD4^+$ cells in particular Treg cells ($FoxP3^+$ $CD4^+$) were identified as $CD103^+$ (Figure 2C).

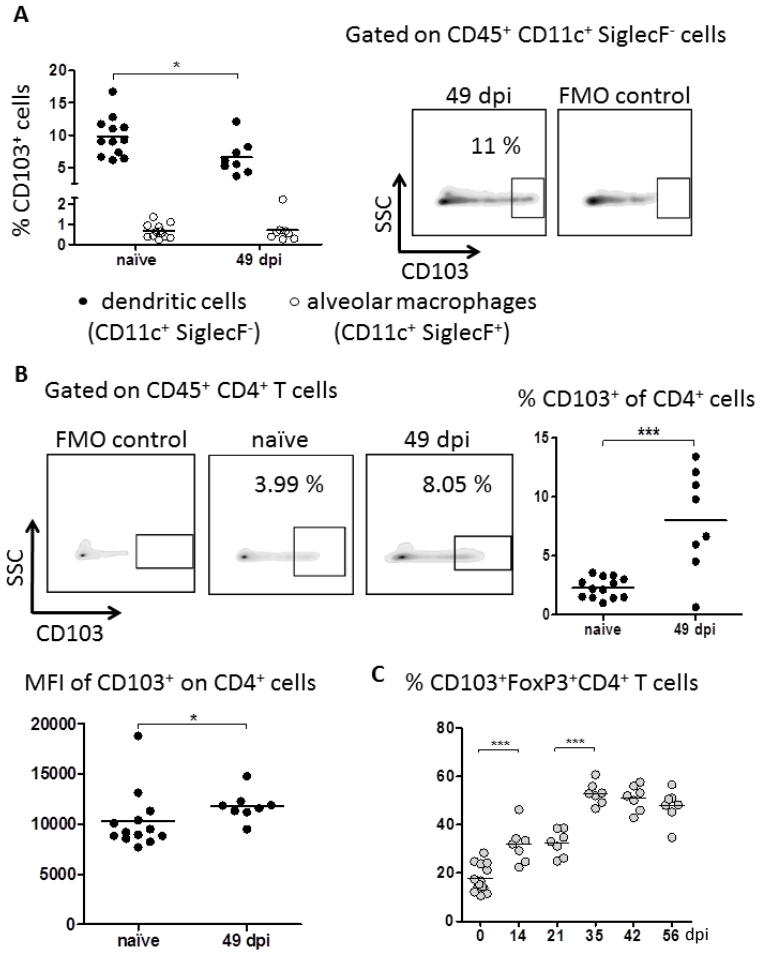


Figure 2. Expression levels of CD103 on dendritic cells, alveolar macrophages and $CD4^+$ T cells during pulmonary cryptococcosis. WT mice were infected intranasally with *C. neoformans* and analyzed 49 days post infection (dpi). (**A**) The frequencies of viable, $CD45^+$, single, $CD11c^+$, $SiglecF^{+/-}$ and $CD103^+$ cells are shown (left). Each data point represents one mouse and is from two independent experiments each with 4-6 mice. Representative flow cytometry plots of viable, $CD45^+$, single, $CD11c^+$, $SiglecF^-$ cells gated on CD103 are depicted (right). (**B**) Viable, $CD45^+$, single, $CD4^+$ cells were gated and CD103 expression was analyzed, representative flow cytometry plots are shown. Frequencies and mean fluorescent intensity (MFI) of $CD4^+$, $CD103^+$ cells are displayed. Each data point represents one mouse and is from two independent experiments each with 4-7 mice. Statistical analysis was performed using unpaired two-tailed t-test and Mann-Whitney test (for non-parametric distributed data), *p<0.05; ***p<0.001. (**C**) CD103 expression by $CD4^+FoxP3^+$ Treg cells upon cryptococcal infection is depicted. Data are from two independent experiments (7 mice per group) at different days post infection (dpi) and overlapping in day 0. Statistical analysis was performed using one-way ANOVA with a Bonferroni test ***p<0.001.

Interestingly, during pulmonary cryptococcosis the total numbers of dendritic cells per lung increased significantly (supporting information Figure 2B), whereas the percentage of $CD103^+$ dendritic cells (Figure 2A) as well as the mean fluorescent intensity (MFI) of CD103 decreased (Supporting information Figure 2C), comparable with epithelial E-cadherin (HEYEN et al. 2016). In contrast, the percentage of $CD4^+$ $CD103^+$ and $FoxP3^+$ $CD4^+$ $CD103^+$ cells revealed a 3-fold increase (Figure 2B,C). Moreover, CD103 was significantly enhanced on the single cell level of $CD4^+$ cells (Figure 2B). Alveolar macrophages as a frequent lung cell type located next to alveolar epithelium revealed a low $CD103^+$

proportion which were not found to change under disease conditions (mean 0.7% ± 0,3) (Figure 2A). Regarding KLRG1, we found also $CD4^+$ T cells to be positive but to a lower extent (mean 1.2% ± 0.8) in comparison to CD103 (supporting information Figure 3A,B). Moreover, no change of the expression level of KLRG1 upon cryptococcal infection was detectable (supporting information Figure 3A,B). Further analysis revealed not only single $CD103^+$ or single $KLRG1^+$ $CD4^+$ cells but also co-expression of both proteins on T cells (Figure 3A). During pulmonary cryptococcosis the relative proportion and total number of $CD103^+$ $KLRG1^+$ $CD4^+$ T cells increased significantly (Figure 3B). Moreover, we found that upon infection 46% (mean; ± 23) of $KLRG1^+$ $CD4^+$ cells express additionally CD103 in contrast to $CD103^+$ $CD4^+$ T cells, which show KLRG1 expression at only 13% (mean; ± 8) (supporting information Figure 3C). Thus, there are relatively more $KLRG1^+$ $CD4^+$ cells co-expressing additionally CD103 than vice versa (supporting information Figure 3C).

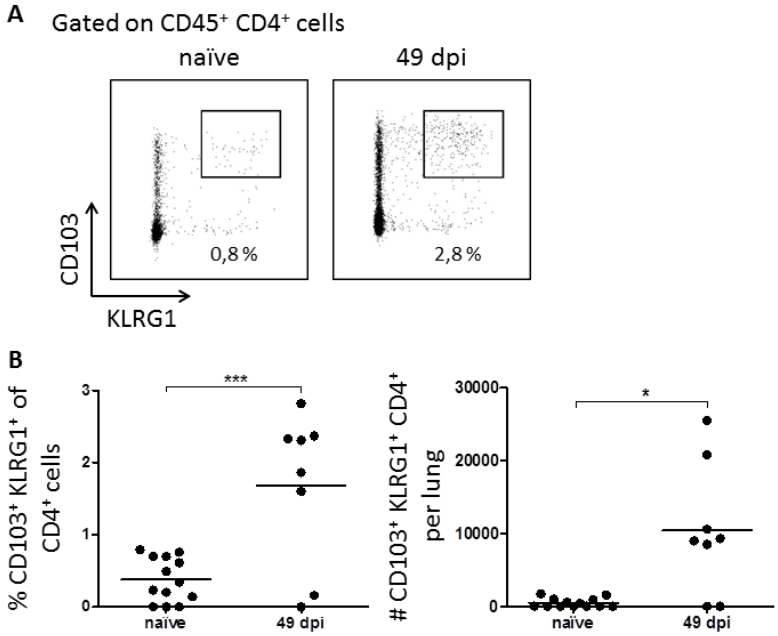


Figure 3. CD103 and KLRG1 co-expressing $CD4^+$ T cells increase upon cryptococcal infection. WT mice were infected intranasally with *C. neoformans* and analyzed 49 dpi. (**A**) Viable, $CD45^+$, single, $CD4^+$ cells were gated on CD103 and KLRG1 expression, representative flow cytometry plots are shown. (**B**) Frequencies (left) and total numbers (right) of $CD4^+$, $CD103^+$, $KLRG1^+$ cells are depicted. Each data point represents one mouse and is from two independent experiments each with 4-7 mice. Two-tailed unpaired t-test and Mann-Whitney test (for non-parametric distributed data) was performed to determine statistical significance, *p<0.05; ***p<0.001.

2.2 CD200R on alveolar macrophages and CD200 on lung epithelial cells remain highly expressed during pulmonary cryptococcosis

Besides E-cadherin we found also CD200 expression on the majority of $CD45^-$ $Ep\text{-}CAM^+$ epithelial cells in naïve mice (mean 81.4 % ± 9.06) (Figure 4A) and thereby confirmed other *in vivo* studies (JIANG-SHIEH et al. 2010). However, in contrast to E-cadherin no downregulation during pulmonary cryptococcosis but maintenance of high expression (Figure 4A), also at the single cell level (data not

shown), occurred. Further analysis in naïve mice regarding the receptor of CD200 (CD200R) revealed alveolar macrophages (mean 68 % ± 13) to a major and dendritic cells (mean 0.5 % ± 0.3) to a minor degree as $CD200R^+$ (Figure 4B upper graph). Upon cryptococcal infection the relative proportion of $CD200R^+$ alveolar macrophages remained unaffected high, whereas the percentage of $CD200R^+$ dendritic cells increased significantly (Figure 4B upper graph). In contrast, CD200R expression at the single cell level of alveolar macrophages was downregulated (Figure 4B, lower graph).

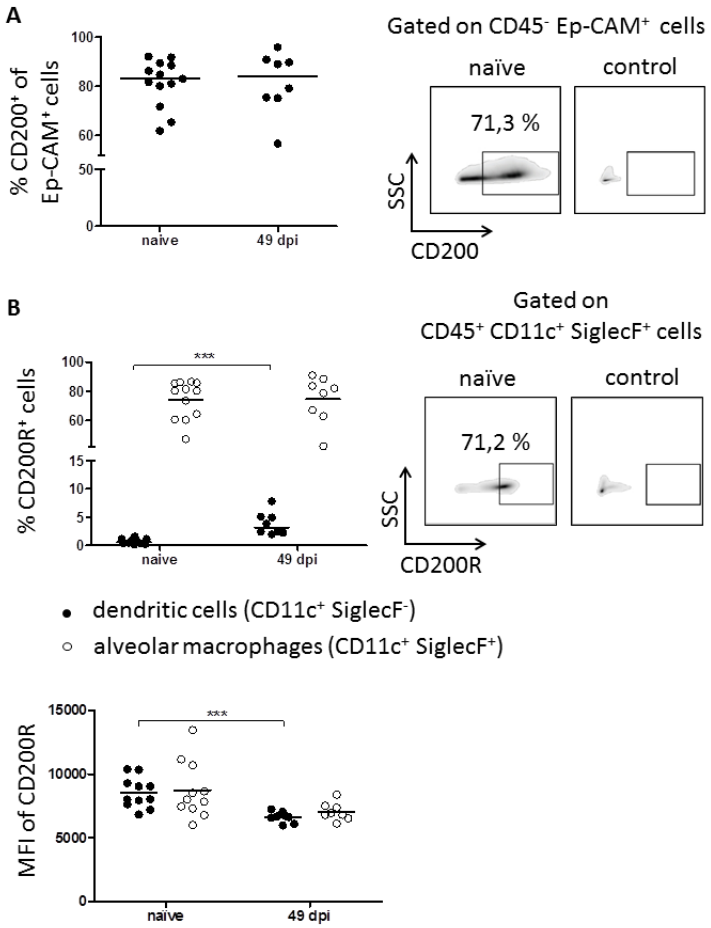


Figure 4. CD200 is highly expressed by lung epithelial cells and CD200R by alveolar macrophages in naïve and infected WT mice. WT mice were infected intranasally with *C. neoformans* and analyzed 49 dpi. (**A**) Frequencies (left) and representative flow cytometry plots (right) of viable, $CD45^-$, single, $Ep\text{-}CAM^+$ cells gated on CD200 are shown. Viable, $CD45^+$, single, $CD11c^+$, $SiglecF^{+/-}$ cells were gated and CD200R expression was analyzed; frequencies (left, upper graph), representative flow cytometry plots (right) and MFI of CD200R (left, lower graph) are depicted. Each data point represents one mouse and is from two independent experiments each with 4-6 mice. Statistical analysis was performed using unpaired two-tailed t-test, ***p<0.001.

In summary, we characterized cells expressing epithelial cell-specific ligands and receptors respectively *in vivo* indicating the potential ability of pulmonary epithelial cells to interact with T cells, alveolar macrophages and dendritic cells. Upon cryptococcal infection the interaction between $CD103^+$ dendritic cells and $E\text{-cadherin}^+$ epithelial cells seems to be downregulated. In contrast,

possible binding of $CD103^+$/$KLRG1^+$ T cells, $CD200R^+$ alveolar macrophages and $CD200R^+$ dendritic cells with epithelial cells is maintained or even upregulated during pulmonary cryptococcosis.

DISCUSSION

Recently we identified lung epithelial cells and in particular alveolar cells type 2 as major cellular source of IL-33 upon cryptococcal infection. Moreover, we demonstrated not only IL-33 but also epithelial CXCL15 and SP-C upregulation (HEYEN et al. 2016) suggesting activation and involvement of epithelial cells in immunity during pulmonary cryptococcosis. In the present study we characterized the conditions leading to increased cytokine, chemokine and surfactant production *in vitro* and analyzed further potential cellular interactions between epithelial and immune cells *in vivo*.

We revealed enhanced CXCL15 and SP-C but no IL-33 mRNA levels in enriched primary pulmonary epithelial cells in response to *C. neoformans* stimulation (strain 1841, serotype D). Interestingly, it has been demonstrated that incubation of a murine epithelial cell line (MLE-12) with *C. neoformans* (strain H99, serotype A) MOI 100 results in upregulation of IL-33 mRNA (FLACZYK et al. 2013). However, H99 represents another and more virulent serotype of *C. neoformans* (HEITMAN et al. 2011) and MOI 100 might have a stronger stimulatory or even damaging effect on epithelial cells. We saw in experiments with higher MOI that the primary epithelial cells appeared injured and revealed a reduced survival in the cell culture post stimulation (data not shown). Moreover, we think that *in vitro* incubation of epithelial cells with *C. neoformans* at a lower MOI of 2 reflects more closely the *in vivo* conditions of infection. Our results indicate that epithelial CXCL15 and SP-C induction is a result of direct *Cryptococcus*-epithelial cell interaction. At this point an influence by Ep-CAM^+ hematopoietic cells such as dendritic cells and alveolar macrophages, on epithelial cell stimulation cannot be excluded. However, epithelial interaction with lymphocytes and in particular $CD4^+$ T cells as major effector cells in immunity upon pulmonary cryptococcosis might not be essential for CXCL15 and SP-C but probably for IL-33 mRNA upregulation. In order to verify this assumption we chose IL-13, representing an important Th2 cytokine, as another stimulus for primary Ep-CAM^+ cells. Although IL-13 has been described to induce IL-33 mRNA in a human lung epithelial cell line (CHRISTIANSON et al. 2015), we could not detect an IL-33 upregulation. These results suggest that probably other Th2 cytokines such as IL-4 and/or -5 or Th1 cytokines such as TNF-α and IFN-γ, which are also described as IL-33 inducers (CHRISTIANSON et al. 2015), or even direct T cell-epithelial cell interaction might be necessary for IL-33 upregulation.

Therefore, we focused *in vivo* on possible ligands and receptors facilitating direct epithelial contact and probably signaling with T and also other immune cells upon pulmonary cryptococcosis. Recently, we revealed on lung epithelial cells E-cadherin expression, which decreases during cryptococcal infection in an IL-33-dependent manner (HEYEN et al. 2016). Here we found also E-$cadherin^+$ alveolar

macrophages and dendritic cells in naïve mice, which could facilitate presence of these cell types next or even in between the epithelial barrier through homophilic binding of E-cadherin. One possible heterophilic binding partner of E-cadherin represents CD103. We found a decreasing frequency of $CD103^+$ dendritic cells but a significant increase of total dendritic cells per lung upon infection indicating a changing phenotype and function of this cell type during pulmonary cryptococcosis. *In vitro* experiments identified CD103 expressing dendritic cells as functional primer of Th1 cells and $CD103^-$ $CD11b^+$ dendritic cells as effective primer of Th2 cells (FURUHASHI et al. 2012). Likewise it has been demonstrated in mice infected with *C. neoformans* that exclusively $CD103^-$ $CD11b^+$ dendritic cells are necessary for Th2 cell priming, which interestingly occurs not in the lymph nodes but in the lung (WIESNER et al. 2015). Consequently, a reduced proportion of CD103 expressing dendritic cells upon infection could suggest an enhanced induction of Th2 cells by increasing $CD103^-$ dendritic cells. Pulmonary dendritic cells positive for CD103 express additionally tight junction proteins like several types of claudins, which might enable this cell type to reside and act between the epithelial barrier (SUNG et al. 2006). Downregulation of CD103 could further result in reduced expression of junction proteins and consequently in enhanced dissociation of intraepithelial dendritic cells, in order to increase Th2 cell priming.

In contrast to dendritic cells, we found on $CD4^+$ T cells, in particular on Treg cells, an upregulation of CD103 during cryptococcal infection. In CD103 deficient mice a reduced accumulation of T cells has been observed (SCHÖN et al. 1999), suggesting that an increased expression of CD103 facilitates an enhanced recruitment of especially Treg cells to the site of infection. During pulmonary cryptococcosis Treg cells are induced in the lung and preferentially suppress the effects of Th2 cells associated with fungal control (SCHULZE et al. 2014; WIESNER et al. 2016). CD103 expressing T cells are able to bind to epithelial E-cadherin (CEPEK et al. 1993) but consequences of subsequent signaling have not been analyzed in detail yet. In an epithelial tumor model interaction of $CD103^+$ cytotoxic T cells with epithelial E-cadherin promotes tumor cell lysis by enhanced exocytosis of lymphocytic granules (LE FLOC'H et al. 2011).

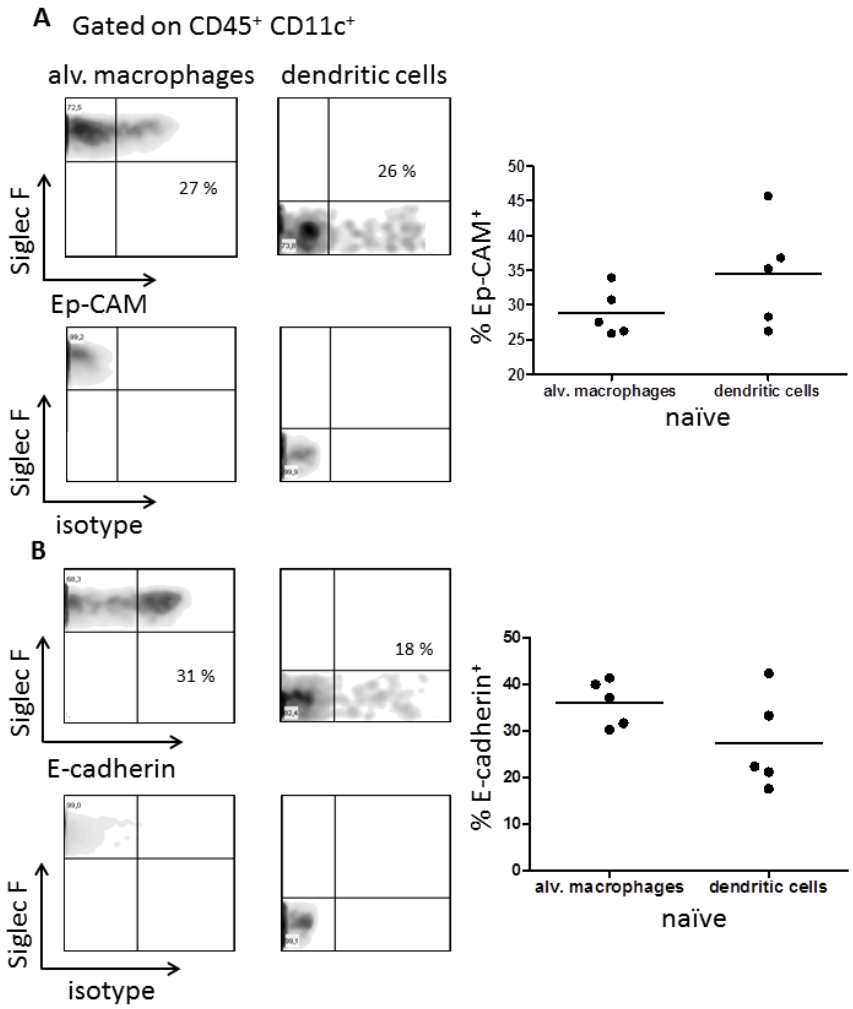
KLRG1 represents another binding partner of E-cadherin and KLRG1 expressing $CD4^+$ T cells are described as differentiated, non-proliferative but effective IFN-γ and TNF-α producers in a murine *Mycobacterium tuberculosis* model (REILEY et al. 2010). In our study we found not only a constant KLRG1 expression by $CD4^+$ T cells but also an increasing $CD4^+$ T cell population co-expressing KLRG1 and CD103 upon cryptococcal infection. In summary, these results are indicative for an enhanced activity of T cell subpopulations, including Treg cells, next to the epithelial barrier and probably suggest an increased interaction with the pulmonary epithelium during cryptococcal infection. Further confirmation respectively visualization of direct epithelial-T cell interaction *in situ* using immunofluorescence microscopy is in progress.

Another pathway of epithelial-immune cell interaction could be mediated by the ligand CD200 and its receptor CD200R. We demonstrated a constitutive high expression of CD200 by lung epithelial cells and maintenance of this expression level upon cryptococcal infection. In the lungs of rats also epithelial cells, in particular ciliated bronchiolar and alveolar cells type 2, but additionally endothelial cells have been identified as CD200 expressing cells (JIANG-SHIEH et al. 2010). However, when rats were challenged intratracheally with lipopolysaccharide (LPS) a significant downregulation of CD200 on endothelium occurred, whereas CD200 expression by epithelium remained primarily unmodified (JIANG-SHIEH et al. 2010). Obviously, this ligand plays different roles on various cell types.

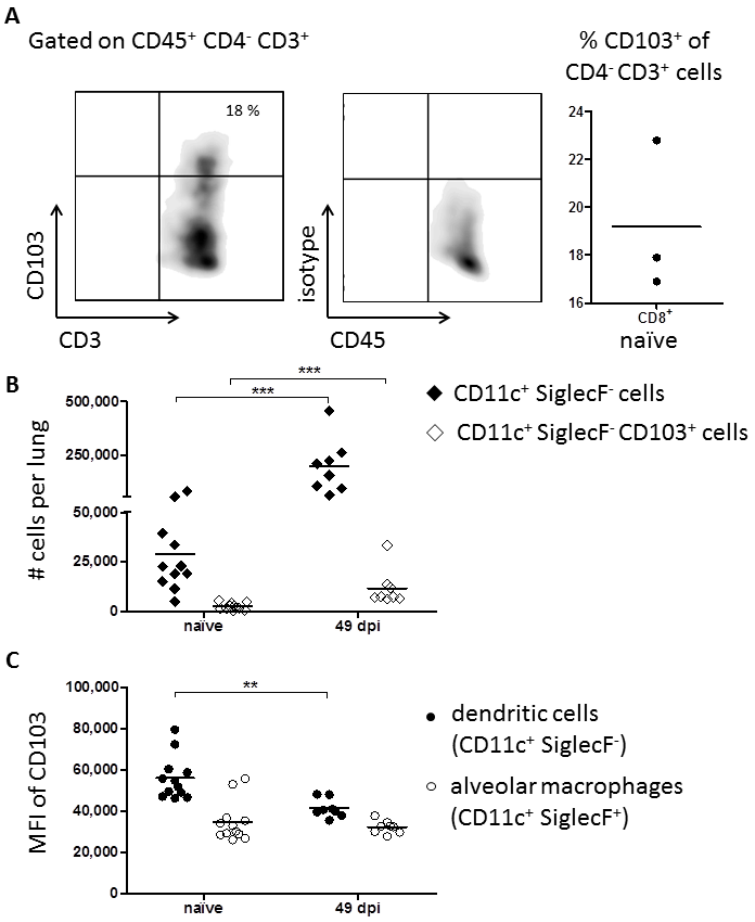
Our study identified alveolar macrophages to a major and dendritic cells to a minor degree as $CD200R^{+}$ and confirmed studies describing CD200R expression to be restricted to myeloid lineage cells (PRESTON et al. 1997). *In vivo* studies investigated the function of CD200 – CD200R interaction in the brain and characterized it as inhibitory respectively anti-inflammatory. In CD200-deficient mice increased numbers of macrophages (microglia) with an activated phenotype as well as a prompt development of experimental autoimmune encephalomyelitis were found (HOEK et al. 2000). Similar results were demonstrated with a blockade of the CD200R in rats, which revealed an exacerbation of experimental allergic encephalomyelitis (WRIGHT et al. 2000). The results of our study suggest a pronounced interaction between epithelial cells and macrophages via the CD200-CD200R pathway during pulmonary cryptococcosis. Although at late time point of infection CD200R expression decreased at the single cell level, our data are indicating that lung epithelial cells promote an inhibitory phenotype of macrophages at the site of infection which is consistent with a presumed general function of anti-inflammatory properties of pulmonary epithelial cells (WEITNAUER et al. 2016). This anti-inflammatory interaction could result in advantages but also disadvantages for the development of cryptococcal infection in the host. On the one hand macrophages are able to phagocyte and kill the fungal pathogen (BOLANOS and MITCHELL 1989; MCQUISTON et al. 2010). Consequently, increased inhibition of effective phagocytes might lead to enhanced fungal growth. On the other hand, especially alternative activated macrophages with a restricted killing ability might be used as a vehicle by the fungus in order to disseminate systemically in the host (STENZEL et al. 2009; MÜLLER et al. 2013). Thus, decreased activation of alternative activated macrophages would probably result in diminished fungal dissemination. Further *in vivo* studies with suppressed CD200 – CD200R axis upon cryptococcal infection are necessary for final functional analysis.

In summary, this study provides novel insights into activation and possible cellular interactions of lung epithelial cells in immunity upon pulmonary cryptococcosis.

SUPPORTING INFORMATION

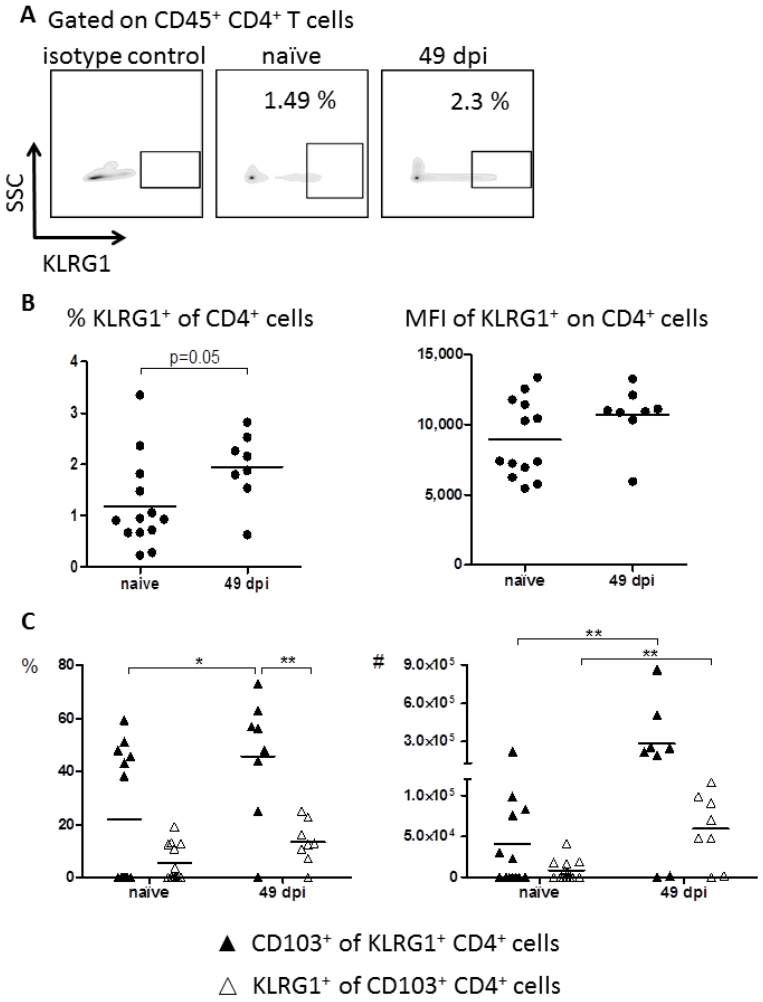


Supporting information Figure 1. Ep-CAM and E-cadherin expression by alveolar macrophages and dendritic cells in naïve WT mice. Viable, $CD45^+$, single, $CD11c^+$, $SiglecF^{+/-}$ cells were gated on (**A**) Ep-CAM and (**B**) E-cadherin; representative flow cytometry plots and frequencies are shown. Each data point represents one mouse and is from one experiment with 5 mice.



Supporting information Figure 2. (**A**) CD103 expression by $CD8^+$ cells in naïve WT mice. Viable, $CD45^+$, single, $CD3^+$, $CD4^-$ cells were gated on CD103; representative flow cytometry plots and frequencies are shown. Each data point represents one mouse and is from one experiment with 3 mice.

The total number of $CD11c^+$ $SiglecF^-$ cells and $CD11c^+$ $SiglecF^-$ $CD103^+$ cells increases, whereas the MFI of CD103 decreases during pulmonary cryptococcosis. WT mice were infected intranasally with *C. neoformans* and analyzed 49 dpi. (**B**) Total numbers of $CD11c^+$ $SiglecF^-$ cells and $CD11c^+$ $SiglecF^-$ $CD103^+$ cells per lung are displayed. (**C**) MFI of CD103 expressed by $CD11c^+$ $SiglecF^-$ and $CD11c^+$ $SiglecF^+$ cells is depicted. Each data point represents one mouse and is from two independent experiments each with 4-6 mice. Statistical analysis was performed using unpaired two-tailed t-test and Mann-Whitney test (for non-parametric distributed data), $^{**}p<0.01$; $^{***}p<0.001$.



Supporting information Figure 3. KLRG1 expression by $CD4^+$ T cells upon cryptococcal infection. WT mice were infected intranasally with *C. neoformans* and analyzed 49 dpi. Viable, $CD45^+$, single, $CD4^+$ cells were gated and CD103 expression was analyzed, (**A**) representative flow cytometry plots, (**B**) frequencies (left) and MFI (right) of $CD4^+$, $CD103^+$ cells are displayed. Each data point represents one mouse and is from two independent experiments each with 4-7 mice.

(**C**) The frequency of $KLRG1^+$ $CD4^+$ T cells co-expressing CD103 is significantly higher than the relative proportion of $CD103^+$ $CD4^+$ T cells expressing KLRG1 during pulmonary cryptococcosis. WT mice were infected intranasally with *C. neoformans* and analyzed 49 dpi. Viable, $CD45^+$, single, $CD4^+$, $KLRG1^+$ cells were gated on CD103 and viable, $CD45^+$, single, $CD4^+$, $CD103^+$ cells were gated on KLRG1; frequencies (left) and total numbers (right) are depicted. Each data point represents one mouse and is from two independent experiments each with 4-7 mice. Two-tailed unpaired t-test and Mann-Whitney test (for non-parametric distributed data) was performed to determine statistical significance, *p<0.05; **p<0.01.

ACKNOWLEDGEMENTS

We thank R. Voigtländer and L. Mittag from the research facility of the Max Planck institute for evolutionary anthropology for excellent mouse care. We are grateful to S. Lehnert for technical assistance. Flow cytometry was performed at the Core Unit Flow Cytometry (CUDZ) of the College of Veterinary Medicine, University of Leipzig.

FUNDING

This study was financially supported by the Deutsche Forschungsgemeinschaft (German Research Foundation) [PI 1066/1-1 to D.P.]. The PhD program from the Hanns Seidel Foundation, Germany supported L. Heyen.

The authors declare no conflict of interest.

Reference list

Bolanos B, Mitchell TG. Phagocytosis and killing of Cryptococcus neoformans by rat alveolar macrophages in the absence of serum. J Leukoc Biol; 1989. 6:521–528.

Cepek KL, Parker CM, Madara JL, Brenner MB. Integrin alpha E beta 7 mediates adhesion of T lymphocytes to epithelial cells. J Immunol; 1993. 8 Pt 1:3459–3470.

Chen SC, Mehrad B, Deng JC, Vassileva G, Manfra DJ, Cook DN, et al. Impaired pulmonary host defense in mice lacking expression of the CXC chemokine lungkine. J Immunol; 2001. 5:3362–3368.

Christianson CA, Goplen NP, Zafar I, Irvin C, Good JT, Rollins DR, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. J Allergy Clin Immunol; 2015. 1:59-68.e14.

Decken K, Köhler G, Palmer-Lehmann K, Wunderlin A, Mattner F, Magram J, et al. Interleukin-12 is essential for a protective Th1 response in mice infected with Cryptococcus neoformans. Infect Immun; 1998. 10:4994–5000.

Flaczyk A, Duerr CU, Shourian M, Lafferty EI, Fritz JH, Qureshi ST. IL-33 signaling regulates innate and adaptive immunity to Cryptococcus neoformans. J Immunol; 2013. 5:2503–2513.

Furuhashi K, Suda T, Hasegawa H, Suzuki Y, Hashimoto D, Enomoto N, et al. Mouse lung CD103+ and CD11bhigh dendritic cells preferentially induce distinct CD4+ T-cell responses. Am J Respir Cell Mol Biol; 2012. 2:165–172.

Gründemann C, Bauer M, Schweier O, Oppen N von, Lässing U, Saudan P, et al. Cutting edge: identification of E-cadherin as a ligand for the murine killer cell lectin-like receptor G1. J Immunol; 2006. 3:1311–1315.

Heitman J, Kozel TR, Kwon-Chung KJ, Perfect JR, Casadevall A. Cryptococcus. Washington, USA: American Society for Microbiology; 2011.

Heyen L, Muller U, Siegemund S, Schulze B, Protschka M, Alber G, et al. Lung epithelium is the major source of IL-33 and is regulated by IL-33-dependent and IL-33-independent mechanisms in pulmonary cryptococcosis. Pathog Dis; 2016. 7:ftw086

Hoek RM, Ruuls SR, Murphy CA, Wright GJ, Goddard R, Zurawski SM, et al. Down-regulation of the macrophage lineage through interaction with OX2 (CD200). Science; 2000. 5497:1768–1771.

Jiang-Shieh Y, Chien H, Chang C, Wei T, Chiu M, Chen H, et al. Distribution and expression of CD200 in the rat respiratory system under normal and endotoxin-induced pathological conditions. J Anat; 2010. 3:407–416.

King RJ. Pulmonary surfactant. J Appl Physiol Respir Environ Exerc Physiol; 1982. 1:1–8.

Komai-Koma M, Xu D, Li Y, McKenzie ANJ, McInnes IB, Liew FY. IL-33 is a chemoattractant for human Th2 cells. Eur J Immunol; 2007. 10:2779–2786.

Le Floc'h A, Jalil A, Franciszkiewicz K, Validire P, Vergnon I, Mami-Chouaib F. Minimal engagement of CD103 on cytotoxic T lymphocytes with an E-cadherin-Fc molecule triggers lytic granule polarization via a phospholipase Cgamma-dependent pathway. Cancer Res; 2011. 2:328–338.

McQuiston T, Luberto C, Del Poeta M. Role of host sphingosine kinase 1 in the lung response against Cryptococcosis. Infect Immun; 2010. 5:2342–2352.

Müller U, Stenzel W, Piehler D, Grahnert A, Protschka M, Kohler G, et al. Abrogation of IL-4 receptor-alpha-dependent alternatively activated macrophages is sufficient to confer resistance against pulmonary cryptococcosis despite an ongoing T(h)2 response. Int Immunol; 2013. 8:459–470.

Piehler D, Eschke M, Schulze B, Protschka M, Müller U, Grahnert A, et al. The IL-33 receptor (ST2) regulates early IL-13 production in fungus-induced allergic airway inflammation. Mucosal Immunol; 2015.

Piehler D, Grahnert A, Eschke M, Richter T, Köhler G, Stenzel W, et al. T1/ST2 promotes T helper 2 cell activation and polyfunctionality in bronchopulmonary mycosis. Mucosal Immunol; 2013. 2:405–414.

Preston S, Wright GJ, Starr K, Barclay AN, Brown MH. The leukocyte/neuron cell surface antigen OX2 binds to a ligand on macrophages. Eur J Immunol; 1997. 8:1911–1918.

Reiley WW, Shafiani S, Wittmer ST, Tucker-Heard G, Moon JJ, Jenkins MK, et al. Distinct functions of antigen-specific CD4 T cells during murine Mycobacterium tuberculosis infection. Proc Natl Acad Sci U S A; 2010. 45:19408–19413.

Rossi DL, Hurst SD, Xu Y, Wang W, Menon S, Coffman RL, et al. Lungkine, a novel CXC chemokine, specifically expressed by lung bronchoepithelial cells. J Immunol; 1999. 9:5490–5497.

Schön MP, Arya A, Murphy EA, Adams CM, Strauch UG, Agace WW, et al. Mucosal T lymphocyte numbers are selectively reduced in integrin alpha E (CD103)-deficient mice. J Immunol; 1999. 11:6641–6649.

Schulze B, Piehler D, Eschke M, Buttlar H von, Kohler G, Sparwasser T, et al. CD4(+) FoxP3(+) regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection. Eur J Immunol; 2014. 12:3596–3604.

Spits H, Cupedo T. Innate lymphoid cells: emerging insights in development, lineage relationships, and function. Annu Rev Immunol; 2012. 647–675.

Stenzel W, Muller U, Kohler G, Heppner FL, Blessing M, McKenzie ANJ, et al. IL-4/IL-13-dependent alternative activation of macrophages but not microglial cells is associated with uncontrolled cerebral cryptococcosis. Am J Pathol; 2009. 2:486–496.

Sung SJ, Fu SM, Rose CE, JR, Gaskin F, Ju S, Beaty SR. A major lung CD103 (alphaE)-beta7 integrin-positive epithelial dendritic cell population expressing Langerin and tight junction proteins. J Immunol; 2006. 4:2161–2172.

van Roy F, Berx G. The cell-cell adhesion molecule E-cadherin. Cell Mol Life Sci; 2008. 23:3756–3788.

Weitnauer M, Mijosek V, Dalpke AH. Control of local immunity by airway epithelial cells. Mucosal Immunol; 2016. 2:287–298.

Wiesner DL, Smith KD, Kotov DI, Nielsen JN, Bohjanen PR, Nielsen K. Regulatory T Cell Induction and Retention in the Lungs Drives Suppression of Detrimental Type 2 Th Cells During Pulmonary Cryptococcal Infection. J Immunol; 2016. 1:365–374.

Wiesner DL, Specht CA, Lee CK, Smith KD, Mukaremera L, Lee ST, et al. Chitin recognition via chitotriosidase promotes pathologic type-2 helper T cell responses to cryptococcal infection. PLoS Pathog; 2015. 3:e1004701.

Wright GJ, Puklavec MJ, Willis AC, Hoek RM, Sedgwick JD, Brown MH, et al. Lymphoid/neuronal cell surface OX2 glycoprotein recognizes a novel receptor on macrophages implicated in the control of their function. Immunity; 2000. 2:233–242.

3. Discussion

3.1 Important chronic respiratory diseases in human and veterinary medicine

The presented study analyzed the involvement of lung epithelial cells in the pathogenesis of pulmonary cryptococcosis. It was demonstrated that in particular alveolar epithelial cells type 2 are cytokine (i.e. IL-33) as well as chemokine (i.e. CXCL15) and enhanced surfactant (i.e. SP-C) producers upon infection (Figure 3). Besides expression of humoral mediators, the data suggest also direct cellular interactions between epithelial cells and immune cells, like $CD4^+$ T cells, dendritic cells or macrophages, via different ligand and receptor expression. In conclusion, this study indicates that pulmonary epithelial cells have the potential to actively participate in host immunity upon cryptococcal infection. In this discussion further aspects of epithelial involvement in the pathogenesis of clinically related respiratory diseases will be described in order to emphasize that research on this topic is rewarding, as new targets for early effective therapy might be identified.

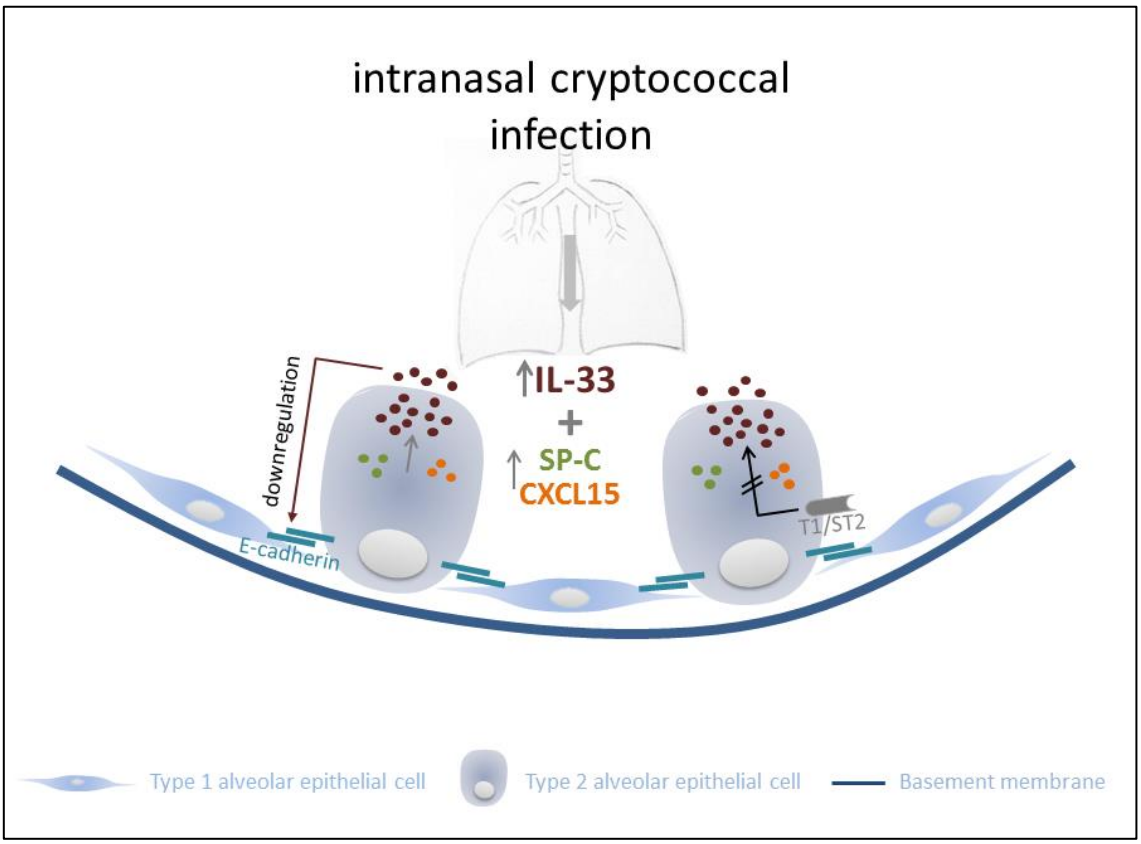


Figure 3. Lung epithelium represents a significant source of IL-33 in pulmonary cryptococcosis and is regulated in an IL-33-dependent but also IL-33-independent manner.

IL-33 is induced upon cryptococcal infection and primarily produced by type 2 alveolar epithelial cells, which upregulate the production of SP-C and CXCL15. Epithelial cells express the receptor for IL-33 (T1/ST2). E-cadherin expression is downregulated in an IL-33-dependent manner, whereas epithelial IL-33, SP-C and CXCL15 upregulation is IL-33-independent. Adapted to HEYEN et al. 2016

Besides pulmonary cryptococcosis there are further chronic airway diseases in human and veterinary medicine that reveal similar respiratory symptoms and are partly caused by Th2-associated immunopathology.

Human asthma as well as COPD have a worldwide distribution and are responsible for increasing numbers of deaths of affected patients (FANTA 2009; BARNES et al. 2015). Asthma is defined as a chronic respiratory disease which includes airway obstruction, lower airway inflammation, mucus accumulation and bronchial hyperresponsiveness (NHLBI 2007). The inflammation is characterized through the involvement of dendritic cells, Th2 cells and eosinophils (BARNES 2008). In veterinary medicine RAO describes a syndrome in horses which is characterized by the same symptoms occurring in human asthma (MCGORUM et al. 2007). RAO affects up to 50% of horses worldwide (BOWLES et al. 2002). Another syndrome very similar to human asthma is frequently diagnosed in cats (JOHNSON 2010; REINERO 2011). Feline asthma includes likewise airflow obstruction, bronchial hyperresponsiveness and airway inflammation (REINERO et al. 2009). Like in asthma inflammation dependent airway narrowing is characteristic for COPD. But the role of Th2 cells in COPD is less certain as the inflammation is especially driven by macrophages, Th1 cells and neutrophils (HOGG et al. 2004; GRUMELLI et al. 2004; BARNES 2008).

3.2 The role of epithelial cells in chronic respiratory pathologies

Pulmonary dendritic cells are known as early responders of the adaptive immune system to inhaled pathogens and environmental pollutants (HOLTZMAN et al. 2014). Consequently, this cell type has been of great scientific interest regarding initiation of chronic airway diseases. However, it became apparent that lung epithelial cells play a more central and crucial role in early pathogenesis of chronic respiratory diseases by promoting Th2 immunity through dendritic cell interaction (LAMBRECHT and HAMMAD 2014) and by activating the innate immune response at the same time (HOLTZMAN et al. 2014; LAMBRECHT and HAMMAD 2014). In the following the contribution of airway epithelial cells to the pathogenesis of chronic respiratory diseases in humans and horses will be described (Figure 4).

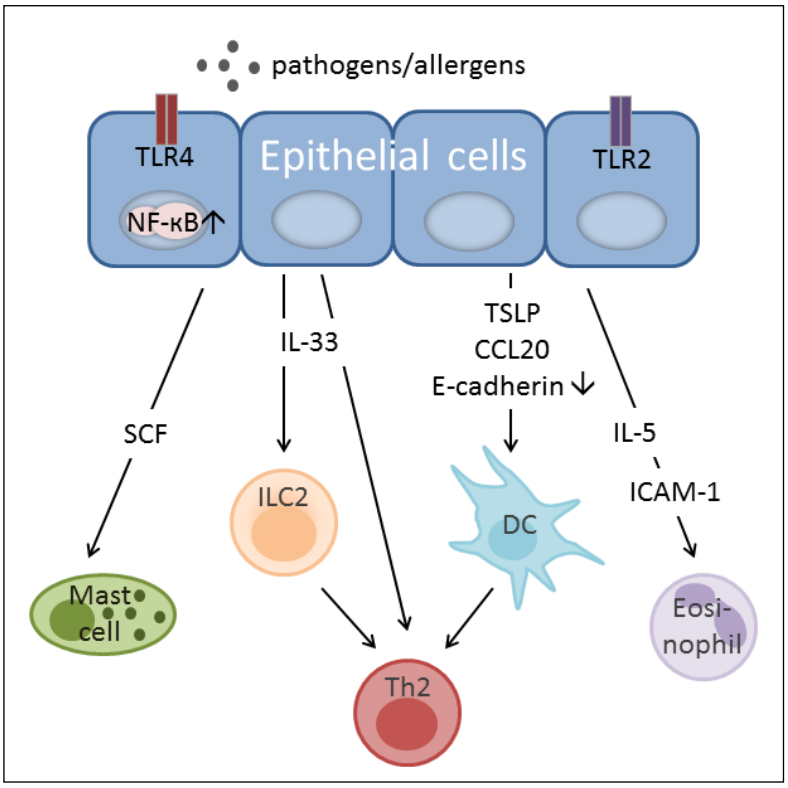


Figure 4. Involvement of lung epithelial cells in immunity driving chronic airway diseases

Inhaled pathogens/allergens can be recognized by epithelial cells through pattern recognition receptors, e.g. TLR2, 4. Epithelial cell activation results in enhanced NF-κB signaling, cytokine and chemokine production, e.g. stem cell factor (SCF), IL-33, thymic stromal lymphopoietin (TSLP), CCL20, IL-5, for recruitment and activation of several immune cells like mast cells, ILC2, Th2, dendritic cells (DC) and eosinophils.

By the expression of several pattern recognition receptors, e.g. TLR (ARMSTRONG et al. 2004; MAYER et al. 2007), RIG-I-like and NOD-like receptors (HOLTZMAN et al. 2014), lung epithelial cells recognize inhaled allergic and pathogenic agents. In an experimental mouse model it has been demonstrated that asthma development to HDM depends on epithelial TLR4 expression (HAMMAD et al. 2009). Moreover, there is evidence that epithelial C-type lectin receptor Dectin-1 is also involved in recognition of beta-glucan motifs of HDM (NATHAN et al. 2009). Allergen and pathogen recognition by epithelial cells results in epithelial activation. Enhanced transcription factor NF-κB signaling in pulmonary epithelial cells exposed to HDM has been demonstrated (OSTERLUND et al. 2009; TULLY et al. 2013). Epithelial activation in turn results in chemokine and cytokine production that recruit and activate members of the innate immune system such as dendritic cells, innate lymphoid cells, mast cells and eosinophils (LAMBRECHT and HAMMAD 2014), which finally drive airway inflammation, hyperreactivity and enhanced mucus production.

Dendritic cell migration to the epithelium can be induced by epithelial CCL20 release (THORLEY et al. 2005). It is suggested that already intraepithelial present dendritic cells can be also directly activated by E-cadherin downregulation. Under homeostatic conditions homophilic E-cadherin interaction mediates a tonic inhibitory signal to dendritic cells (JIANG et al. 2007). Further dendritic cell

activation can be induced by epithelial derived thymic stromal lymphopoietin (TSLP), which induces MHCII and I costimulatory molecules (CD40, CD80, CD86) on dendritic cells and facilitates dendritic cells to prime $CD4^+$ T cells towards a Th2-associated immune response (SOUMELIS et al. 2002; HALLSTRAND et al. 2014).

Epithelial cells promote Th2 immunity also by activating ILC2. Once stimulated by epithelial cytokines such as IL-33 (KONDO et al. 2008; BARTEMES et al. 2012), ILC2s produce in turn IL-5 and -13 and help to induce a Th2 response in naïve T cells (KLEIN et al. 2012). IL-5 is an important chemokine for eosinophil recruitment (SUR et al. 1996). In mice airway epithelial cells were identified as an additional important cellular source of IL-5 (WU et al. 2010). Furthermore, it has been revealed that also the expression of ICAM-1 enables epithelial cells to participate in eosinophil recruitment (BURKE-GAFFNEY and HELLEWELL 1998). ICAM-1 expression is significantly enhanced in bronchial epithelial cells of asthmatic patients (VIGNOLA et al. 1993). Additional crucial effector cells in respiratory diseases such as asthma are mast cells (CARROLL et al. 2002). Epithelial derived stem cell factor (SCF) supports accumulation and differentiation of mast cells (OTSUKA et al. 1998) and is increased in pulmonary epithelial cells from asthmatic patients (AL-MUHSEN et al. 2004).

Lung epithelial cells are not only initiator but also target of the immune response in airway diseases. For COPD it has been described that IL-13 is a potent stimulus for enhanced mucus production by airway epithelial cells (ALEVY et al. 2012). Moreover, epithelium from patients affected with COPD reveal increased expression of the epithelial growth factor receptor (BOER et al. 2006), which contribute to mucus hyperplasia (BURGEL and NADEL 2004) and probably to basal epithelial cell proliferation (BOER et al. 2006).

Epithelial involvement in pathogenesis of equine RAO has been the focus of several studies also. In affected horses enhanced TLR4 mRNA expression by bronchial epithelial cells was found, which was positively correlated with reinforced epithelial IL-8 production (BERNDT et al. 2007). Furthermore, increased epithelial NF-κB activity and ICAM-1 expression has been verified in the lung of horses with RAO (BUREAU et al. 2000). Likewise, epithelial cells have been identified as important chemokine producers during RAO. In restimulated primary epithelial cells from affected horses enhanced CXCL2 mRNA has been found (AINSWORTH et al. 2009). CXCL2 belongs to the CXC chemokine family and is important for neutrophil recruitment (KOBAYASHI 2008).

In conclusion, lung epithelial cells are early responder and essential controller of immunity in chronic respiratory diseases. Our results contribute to a more detailed understanding of epithelial cells as active participants in host immunity. Further profound research focusing on epithelial cells in pulmonary cryptococcosis and chronic airway diseases in general might identify new therapeutic targets for early effective intervention in respiratory immunopathology.

3.3 IL-33 in Th2-driven pulmonary diseases of humans, horses and cats

IL-33 belongs to the IL-1 cytokin family and is already constitutively present in the nucleus of primarily stromal cells (ROUSSEL et al. 2008; HARALDSEN et al. 2009) in different tissues including gut, lung, skin, lymphoid tissue, tonsils, and salivary glands (LLOYD 2010). Due to its intranuclear localization IL-33 is able to act as a transcription factor (CARRIERE et al. 2007). *In vitro* it has been demonstrated that IL-33 diminish NF-κB mediated proinflammatory gene transcription (ALI et al. 2011). Furthermore, IL-33 signals also through the extracellular receptor T1/ST2 which can be found on several cell types but strongly pronounced on Th2 cells (COYLE et al. 1999) and ILC2s (PIEHLER et al. 2016). Consequently IL-33-T1/ST2 signaling results primarily in promotion of Th2 cytokine production (SMITHGALL et al. 2008) and is therefore in particular of great interest regarding Th2-mediated pathologies. The crucial role of IL-33 in Th2-associated pulmonary cryptococcosis has been already shown in several studies (FLACZYK et al. 2013; PIEHLER et al. 2013). But also in other pulmonary Th2 type pathologies such as asthma and COPD, the relevance of IL-33 signaling has been analyzed and even partly demonstrated.

In sputum and serum of asthmatic patients increased levels of IL-33 and soluble T1/ST2 in comparison to healthy controls were found (HAMZAOUI et al. 2013; GUO et al. 2014). Moreover, the amount of IL-33 was positively correlated with disease severity (HAMZAOUI et al. 2013; GUO et al. 2014). Asthma development is not only based on specific environmental factors but is also highly heritable (ULLEMAR et al. 2016). Genome-wide association studies identified several but in particular IL-33 and T1/ST2 as asthma susceptibility loci (BONNELYKKE et al. 2014; GROTENBOER et al. 2013). In detail, eight single nucleotide polymorphisms (SNP) within the gene region of IL-33 and 15 SNPs within the gene region of T1/ST2 were found and have been reported to be associated with asthma (GROTENBOER et al. 2013).

Manifestation of RAO in horses is also a result of environmental influences, e.g. frequent exposition to dust or moldy hay, and predisposing genetic factors (MARTI et al. 1991). For instance the receptor for IL-4 was identified as a susceptibility gene for RAO (SWINBURNE et al. 2009; RACINE et al. 2011). In affected horses not only enhanced IgE levels in BALF (HALLIWELL et al. 1993; SCHMALLENBACH et al. 1998) but also increased Th2 cytokine mRNA levels (IL-4, -5, -13) (LAVOIE et al. 2001; BOWLES et al. 2002) have been found. However, there are additionally studies with different experimental methods and settings which report a combination of Th2 and Th1 immunity (GIGUERE et al. 2002; AINSWORTH et al. 2003) in affected horses. Moreover, IL-17 involvement and even IL-4 mRNA downregulation in RAO has been recently described (KORN et al. 2015), indicating several molecular phenotypes of RAO based on complex immune responses. Likewise not airway eosinophilia but neutrophilia (FAIRBAIRN et al. 1993; PIRIE et al. 2001; BOWLES et al. 2002) is more characteristic for RAO. However, in acute asthma early neutrophil accumulation occurs additionally (FAHY et al. 1995;

ORDONEZ et al. 2000), although asthma is generally described as an eosinophilic disease. Interestingly in horses with RAO it was shown that Th2 type cytokines are involved in neutrophilic inflammation, as IL-5 and IL-4 receptor expression was found on equine neutrophils (MCGORUM et al. 2007; LAVOIE-LAMOUREUX et al. 2010). In summary, clinical symptoms, genetic studies and the majority of cellular and cytokine studies in horses affected with RAO suggest a not negligible influence of Th2-mediated immunity and therefore of potential IL-33-T1/ST2 signaling in this common equine respiratory disease.

In cats it is suggested that in particular allergens are the cause of most cases of feline asthma (REINERO 2011). Serum and intradermal responses to allergens like weed and mold could be found in affected cats (MORIELLO et al. 2007). Moreover, an experimental model was established, in which HDM and Bermuda grass could reproduce the asthmatic syndrome in research cats (NORRIS REINERO et al. 2004). This study facilitated additionally to analyze in detail several parameters of induced allergic asthma in cats and demonstrated not only allergen specific IgE production in sera, eosinophilia in BALF but also significant increase of IL-4 as a Th2 cytokine in restimulated peripheral blood mononuclear cells (NORRIS REINERO et al. 2004). Although further detailed lymphocyte and cytokine analysis or rather genetic studies like in human asthmatic patients are so far missing, it is conceivable that based on the clinical and etiological similarities to human asthma, IL-33-T1/ST2 signaling might be of crucial importance also in feline asthma.

Recently a cohort study demonstrated that clinical features of asthma are shown by 15% of human patients with COPD (COSIO et al. 2016). An *in vivo* study revealed in lungs from mice and human with COPD increased IL-33 mRNA levels, increased numbers of IL-33^{+} lung epithelial cells and an association between IL-33 and IL-13 or rather mucin gene expression (BYERS et al. 2013). Furthermore, significantly elevated IL-33 as well as T1/ST2 protein levels in sera and IL-33 expression in epithelial cells, peripheral lymphocytes and neutrophils were found in affected patients (XIA et al. 2015). These data suggest that IL-33 signaling might be also important for pathogenesis of COPD.

In summary, there are several indications that IL-33 is also in other widespread Th2-driven pulmonary diseases in human and veterinary medicine of great importance. Consequently, our study which focuses on the cellular source of this crucial cytokine, contributes to a more detailed understanding of pathogenetic mechanisms of worldwide occurring respiratory diseases.

3.4 IL-33-T1/ST2 axis as therapeutic target in chronic airway diseases

For treatment of these widely disseminated diseases in both human and veterinary medicine several therapeutic agents are applied (BULLONE and LAVOIE 2015). Beside anticholinergic and β-adrenergic agents as bronchodilators, most notably corticosteroids are used, in order to promptly and reliably relieve the exaggerated immune response. However, steroid therapy has in particular in case of

systemic and chronic treatment not only anti-inflammatory impact but also undesired side effects like osteoporosis and suppressed hypothalamic-pituitary-adrenal-axis function (DAHL 2006). Moreover, it is known that corticosteroid resistance can appear as a feature of asthma and COPD (BARNES 2013; BARNES 2015; SAGLANI et al. 2013). Consequently, other and novel therapeutic agents are of significant interest.

First promising therapeutic interventions in the IL-33-T1/ST2 axis have been already tested in animal models. Application of an anti-IL-33 antibody resulted in decreased airway inflammation in a murine allergic asthma model (LIU et al. 2009). Moreover, an anti-T1/ST2 antibody as well as the soluble T1/ST2 as a decoy receptor for IL-33, represented effective inhibitors of Th2-mediated immunity in allergic respiratory mouse models (COYLE et al. 1999; HAYAKAWA et al. 2007). However, total T1/ST2 deficiency in mice exposed to allergic agents revealed inconsistent results (HOSHINO et al. 1999; SAGLANI et al. 2013), which might indicate that receptor deficiency would not result in inhibition of all IL-33-associated effects.

Furthermore, it has to be considered that IL-33 is not only a proinflammatory alarmin but has also regulatory functions, e.g. in adipose tissue (BRESTOFF et al. 2015) and the intestine (SCHIERING et al. 2014), and is therefore important for homeostasis. Thus, systemic therapeutic intervention might result in undesired side-effects. Our study identified in particular pulmonary epithelial cells as IL-33 producers upon cryptococcal infection and suggests that tissue specific blockade of IL-33 would be sufficient for improvement of pulmonary pathology. Systemic regulatory functions of IL-33 would remain unaffected. In summary, our study emphasizes IL-33 as an attractive therapeutic target, as its blockade represents not only an early interference in Th2 immunity but also the opportunity of local, steroid-sparing treatment of Th2-mediated pathologies.

3.5 Concluding remarks

Overall, the results of this work provide a more detailed understanding of lung epithelial cells as active participants in host immunity during cryptococcal infection. Clinical symptoms and pathogenic pathways of pulmonary cryptococcosis are also part of common chronic airway diseases such as asthma. Consequently, this work and further profound research focusing on lung epithelial cells in Th2-related immunopathologies might contribute to the identification of new therapeutic targets for early effective intervention in worldwide occurring respiratory diseases.

4. Summary

Laura Heyen

Analysis of lung epithelial cell regulation and possible involvement in immunity upon pulmonary cryptococcosis

Institute of Immunology, Faculty of Veterinary Medicine, University of Leipzig

Submitted in December 2016

50 pages, 17 figures, 145 references

Keywords: *Cryptococcus neoformans*, lung epithelial cells, chronic respiratory infection, T helper cell type 2 immunity

Introduction: *Cryptococcus neoformans* (*C. neoformans*) represents an opportunistic fungal pathogen with a worldwide distribution. Infection develops after inhalation of desiccated yeast cells or spores and results in an asymptomatic pulmonary infection in immunocompetent individuals. In case of a compromised immune system pulmonary cryptococcosis with features of allergic airway inflammation develops and can spread systemically, preferably into the central nervous system. The pathogenesis of cryptococcosis is mediated by a T helper cell type 2 (Th2)-associated immune response which can be induced and amplified by the cytokine interleukin (IL)-33. The role of innate and adaptive immune cells in host immunity in response to cryptococcal infection is well understood. However, relatively little is known about the role of lung epithelial cells in initiation and regulation of the immune response. Airway epithelial cells represent the first contact site for the fungal pathogen with the host and display probably an important cellular source for cytokines like IL-33.

Aims of study: The aim of this study was to provide a more detailed understanding of the potential involvement of lung epithelial cells in host immunity in response to cryptococcal infection.

Material and methods: For *in vivo* experiments female Balb/c wild-type mice, IL-33 citrine reporter mice and IL-33 receptor knock out mice were intranasally infected with *C. neoformans*, euthanized and analyzed at day 0, 21, 49 and 70 post infection (4-7 mice/group). Pulmonary cells were examined by flow cytometry and RT-qPCR. Homogenate analysis was performed with Enzyme Linked Immunosorbent Assay. Lung cell identification and analysis *in situ* was performed by immuno-fluorescence microscopy. For *in vitro* experiments and extraction of primary pulmonary cells naïve Balb/c wild-type mice were utilized. Magnetically sorted epithelial cells were cultured, stimulated with *C. neoformans* and RT-qPCR was performed for mRNA analysis. The presented results derive in

each case from 2-3 independently performed experiments. Statistical significance in case of equally distributed values was done using one-way ANOVA followed by Dunnet`s Multiple Comparison post test and two tailed unpaired t test for the comparison of two groups. One-way ANOVA followed by Dunn`s Multiple Comparison test or Mann-Whitney U-test for two groups was performed to analyze nonparametric data.

Results: Analysis of IL-33 citrine reporter mice revealed constitutive and induced IL-33 mRNA expression upon pulmonary cryptococcosis by mainly alveolar epithelial cells type 2. Although lung epithelial cells expressed the receptor for IL-33 (T1/ST2), no auto-/paracrine feedback of IL-33 on its own production in mice deficient for T1/ST2 upon cryptococcal infection was found. Instead it was demonstrated that epithelial E-cadherin, a cell-to-cell adhesion molecule, is downregulated upon pulmonary infection in an IL-33-dependent manner. Moreover, induction of CXCL15, representing a neutrophilic chemokine, and SP-C transcription upon pulmonary cryptococcosis mainly in IL-33 producing epithelial cells but independently of IL-33 was found. *In vitro* stimulation of enriched primary epithelial cells with *C. neoformans* resulted also in enhanced CXCL15 and SP-C mRNA levels indicating a direct stimulation pathway. However, *in vitro* induction of epithelial IL-33 mRNA was not detectable suggesting probably the necessity of other immune cells for upregulation of IL-33 transcription. Thus, the potential of direct cellular interaction of lung epithelial cells with other immune cells upon cryptococcal infection has been analyzed *in vivo.* CD103 and KLRG1 represent possible binding partners of E-cadherin. During infection decreasing frequencies of $CD103^+$ dendritic cells but significantly increasing frequencies of $CD103^+$ and $KLRG1^+$ $CD4^+$ T cells were found. Moreover, CD200 expression by epithelial cells and its receptor CD200R on mainly alveolar macrophages remained nearly unmodified high upon cryptococcal infection.

Conclusion: This work indicates that airway epithelial cells might contribute by humoral factors and direct cellular interactions to the immune response during pulmonary cryptococcosis.

5. Zusammenfassung

Laura Heyen

Regulation von Lungenepithelzellen und deren möglicher Einfluss auf die Immunantwort während der pulmonalen Kryptokokkose

Institut für Immunologie, Veterinärmedizinische Fakultät, Universität Leipzig

Eingereicht im Dezember 2016

50 Seiten, 17 Abbildungen, 145 Literaturangaben

Schlüsselwörter: *Cryptococcus neoformans*, Lungenepithelzellen, chronische Atemwegserkrankungen, T-Helferzellen Typ 2-assoziierte Immunität

Einleitung: *Cryptococcus neoformans* (*C. neoformans*) ist ein fungaler, opportunistischer Erreger mit einer weltweiten Verbreitung. Die Inhalation der ausgetrockneten Hefenform des Erregers oder seiner Sporen führt in immunkompetenten Individuen zu einer asymptomatischen Bronchopneumonie. Im Falle einer Immunsuppression entwickelt sich die pulmonale Kryptokokkose, die symptomatisch einer chronischen, allergischen Atemwegserkrankung ähnelt. Über das Blut kann sich der Erreger im gesamten Organismus ausbreiten und insbesondere das zentrale Nervensystem befallen. Die Pathogenese der pulmonalen und auch systemischen Kryptokokkose basiert auf einer T-Helferzellen Typ 2 (Th2) assoziierten Immunantwort des infizierten Organismus. Das Zytokin Interleukin (IL)-33 wird mit der Infektion im Organismus induziert und ist in der Lage die schädliche Th2-Immunität zu initiieren und zu verstärken. Die Rolle der Zellen des angeborenen und erworbenen Immunsystems während der Kryptokokkose ist gut untersucht. Allerdings ist bisher wenig über Lungenepithelzellen und ihren möglichen Einfluss auf die Initiierung sowie Regulation der Immunantwort bekannt. Lungenepithel ist die erste Kontaktstelle des Erregers mit dem Wirt und könnte eine wichtige zelluläre Zytokinquelle von beispielsweise IL-33 darstellen.

Ziel der Untersuchung: Ziel dieser Studie war es, die Rolle von Lungenepithelzellen in der Immunantwort während der Kryptokokkose näher zu untersuchen.

Material und Methoden: Für die *in vivo*-Versuche wurden weibliche Balb/c Wildtypmäuse, IL-33-Citrin-Reportermäuse sowie IL-33-Rezeptor-defiziente Mäuse intranasal mit *C. neoformans* infiziert, euthanasiert und an Tag 0, 21, 49 sowie 70 nach der Infektion analysiert (4-7 Mäuse/Gruppe). Die Lungenzellen wurden mittels Durchflusszytometrie und Reverser Transkriptase-Polymerase-Kettenreaktion (RT-qPCR) untersucht. Enzyme Linked Immunosorbent Assay wurde für die Untersuchung

von Lungenhomogenaten angewendet. Weitere Analysen erfolgten *in situ* mit Hilfe der Immunfluoreszenzmikroskopie. Für die *In-vitro*-Experimente wurden aus naiven Balb/c Wildtyp-mäusen primäre Lungenzellen gewonnen. Magnetisch selektierte Epithelzellen wurden kultiviert, mit *C. neoformans* stimuliert und mittels RT-qPCR analysiert. Die Daten dieser Arbeit stammen jeweils von 2-3 unabhängig voneinander durchgeführten Experimenten. Die statistische Auswertung erfolgte bei Normalverteilung mit Hilfe der einfachen Varianzanalyse mit anschließendem Dunnet's Mehrfachvergleich und beim Vergleich von nur zwei Gruppen mittels ungepaarten zweiseitigen t-Test. Für nicht parametrische Datenverteilung wurde die einfache Varianzanalyse mit anschließendem Dunn's Mehrfachvergleich oder im Falle von nur zwei Gruppen der Mann-Whitney-U-Test angewendet.

Ergebnisse: Die Analyse von IL-33-Citrin-Reportermäusen wies sowohl konstitutive als auch induzierte IL-33-mRNA-Expression während der pulmonalen Kryptokokkose hauptsächlich in Alveolarepithelzellen Typ 2 auf. Obwohl diese auch den Rezeptor für IL-33 (T1/ST2) exprimierten, konnte kein auto- bzw. parakriner Feedbackmechanismus in T1/ST2 defizienten Mäusen nach-gewiesen werden. Hingegen konnte eine Herunterregulation des epithelialen Adhäsionsmoleküls E-Cadherin in Abhängigkeit von IL-33 gezeigt werden. Weiterhin fand vorrangig in IL-33-produzierenden Epithelzellen eine verstärkte CXCL15-, ein Chemokin für neutrophile Granulozyten, und Surfactant-Protein C (SP-C)-Transkription während der Infektion mit *C. neoformans* statt. Eine IL-33-Abhängigkeit dieses Mechanismus lag nicht vor. Die Induktion von CXCL15 und SP-C konnte auch nach einer *In-vitro*-Stimulation von angereicherten Epithelzellen mit *C. neoformans* gezeigt werden und scheint damit eine Folge direkter Interaktion von dem Pilz mit den Epithelzellen zu sein. Verstärkte Transkription von IL-33 konnte hingegen unter gleichen *In-vitro*-Stimulationsbedingungen nicht induziert werden, was darauf hinweisen könnte, dass dafür das Vorhandensein von Immunzellen, wie beispielsweise T Zellen, von Nöten ist. Folglich wurde die Möglichkeit der zellulären Interaktion von Epithelzellen mit Immunzellen über Rezeptor-Ligand-Bindungen während der pulmonalen Kryptokokkose näher untersucht. CD103 und KLRG1 stellen Bindungspartner von E-Cadherin dar. Während der Infektion konnten reduzierte Frequenzen von $CD103^+$ dendritischen Zellen und ein signifikant ansteigender Anteil von $CD103^+$ und $KLRG1^+$ $CD4^+$ T-Zellen nachgewiesen werden. Weiterhin wurde die hohe Expression von CD200 auf Epithelzellen und des entsprechenden Rezeptors CD200R auf Alveolarmakrophagen während der Infektion aufrechterhalten.

Schlussfolgerung: Die Ergebnisse dieser Arbeit weisen darauf hin, dass Lungenepithelzellen während der pulmonalen Kryptokokkose die Immunantwort auf humoraler und zellulärer Ebene beeinflussen können.

6. Reference list

Ainsworth DM, Grunig G, Matychak MB, Young J, Wagner B, Erb HN, et al. Recurrent airway obstruction (RAO) in horses is characterized by IFN-gamma and IL-8 production in bronchoalveolar lavage cells. Vet Immunol Immunopathol; 2003. 1-2:83–91.

Ainsworth DM, Matychak M, Reyner CL, Erb HN, Young JC. Effects of in vitro exposure to hay dust on the gene expression of chemokines and cell-surface receptors in primary bronchial epithelial cell cultures established from horses with chronic recurrent airway obstruction. Am J Vet Res; 2009. 3:365–372.

Alevy YG, Patel AC, Romero AG, Patel DA, Tucker J, Roswit WT, et al. IL-13-induced airway mucus production is attenuated by MAPK13 inhibition. J Clin Invest; 2012. 12:4555–4568.

Ali S, Mohs A, Thomas M, Klare J, Ross R, Schmitz ML, et al. The dual function cytokine IL-33 interacts with the transcription factor NF-kappaB to dampen NF-kappaB-stimulated gene transcription. J Immunol; 2011. 4:1609–1616.

Al-Muhsen SZ, Shablovsky G, Olivenstein R, Mazer B, Hamid Q. The expression of stem cell factor and c-kit receptor in human asthmatic airways. Clin Exp Allergy; 2004. 6:911–916.

Armstrong L, Medford ARL, Uppington KM, Robertson J, Witherden IR, Tetley TD, et al. Expression of functional toll-like receptor-2 and -4 on alveolar epithelial cells. Am J Respir Cell Mol Biol; 2004. 2:241–245.

Arora S, Hernandez Y, Erb-Downward JR, McDonald RA, Toews GB, Huffnagle GB. Role of IFN-gamma in regulating T2 immunity and the development of alternatively activated macrophages during allergic bronchopulmonary mycosis. J Immunol; 2005. 10:6346–6356.

Bagnasco D, Ferrando M, Varricchi G, Passalacqua G, Canonica GW. A Critical Evaluation of Anti-IL-13 and Anti-IL-4 Strategies in Severe Asthma. Int Arch Allergy Immunol; 2016. 2:122–131.

Barbosa FM, Fonseca FL, Figueiredo RT, Bozza MT, Casadevall A, Nimrichter L, et al. Binding of glucuronoxylomannan to the CD14 receptor in human A549 alveolar cells induces interleukin-8 production. Clin Vaccine Immunol; 2007. 1:94–98.

Barbosa FM, Fonseca FL, Holandino C, Alviano CS, Nimrichter L, Rodrigues ML. Glucuronoxylomannan-mediated interaction of Cryptococcus neoformans with human alveolar cells results in fungal internalization and host cell damage. Microbes Infect; 2006. 2:493–502.

Barnes PJ. The cytokine network in asthma and chronic obstructive pulmonary disease. J Clin Invest; 2008. 11:3546–3556.

Barnes PJ. Corticosteroid resistance in patients with asthma and chronic obstructive pulmonary disease. J Allergy Clin Immunol; 2013. 3:636–645.

Barnes PJ. Therapeutic approaches to asthma-chronic obstructive pulmonary disease overlap syndromes. J Allergy Clin Immunol; 2015. 3:531–545.

Barnes PJ, Burney PGJ, Silverman EK, Celli BR, Vestbo J, Wedzicha JA, et al. Chronic obstructive pulmonary disease. Nat Rev Dis Primers; 2015. 15076.

Baro T, Torres-Rodriguez JM, Mendoza MH de, Morera Y, Alia C. First identification of autochthonous Cryptococcus neoformans var. gattii isloated from goats with predominantly severe pulmonary disease in Spain. J Clin Microbiol; 1998. 2:458–461.

Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- CD25+ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. J Immunol; 2012. 3:1503–1513.

Berndt A, Derksen FJ, Venta PJ, Ewart S, Yuzbasiyan-Gurkan V, Robinson NE. Elevated amount of Toll-like receptor 4 mRNA in bronchial epithelial cells is associated with airway inflammation in horses with recurrent airway obstruction. Am J Physiol Lung Cell Mol Physiol; 2007. 4:L936-43.

Boer WI de, Hau CM, van Schadewijk A, Stolk J, van Krieken, J Han J M, Hiemstra PS. Expression of epidermal growth factors and their receptors in the bronchial epithelium of subjects with chronic obstructive pulmonary disease. Am J Clin Pathol; 2006. 2:184–192.

Bonnelykke K, Sleiman P, Nielsen K, Kreiner-Moller E, Mercader JM, Belgrave D, et al. A genome-wide association study identifies CDHR3 as a susceptibility locus for early childhood asthma with severe exacerbations. Nat Genet; 2014. 1:51–55.

Bowles KS, Beadle RE, Mouch S, Pourciau SS, Littlefield-Chabaud MA, Le Blanc C, et al. A novel model for equine recurrent airway obstruction. Vet Immunol Immunopathol; 2002. 3-4:385–389.

Brestoff JR, Kim BS, Saenz SA, Stine RR, Monticelli LA, Sonnenberg GF, et al. Group 2 innate lymphoid cells promote beiging of white adipose tissue and limit obesity. Nature; 2015. 7542:242–246.

Bullone M, Lavoie J. Asthma "of horses and men"--how can equine heaves help us better understand human asthma immunopathology and its functional consequences? Mol Immunol; 2015. 1:97–105.

Bureau F, Bonizzi G, Kirschvink N, Delhalle S, Desmecht D, Merville MP, et al. Correlation between nuclear factor-kappaB activity in bronchial brushing samples and lung dysfunction in an animal model of asthma. Am J Respir Crit Care Med; 2000. 4 Pt 1:1314–1321.

Burgel P, Nadel JA. Roles of epidermal growth factor receptor activation in epithelial cell repair and mucin production in airway epithelium. Thorax; 2004. 11:992–996.

Burke-Gaffney A, Hellewell PG. A CD18/ICAM-1-dependent pathway mediates eosinophil adhesion to human bronchial epithelial cells. Am J Respir Cell Mol Biol; 1998. 3:408–418.

Byers DE, Alexander-Brett J, Patel AC, Agapov E, Dang-Vu G, Jin X, et al. Long-term IL-33-producing epithelial progenitor cells in chronic obstructive lung disease. J Clin Invest; 2013. 9:3967–3982.

Carriere V, Roussel L, Ortega N, Lacorre D, Americh L, Aguilar L, et al. IL-33, the IL-1-like cytokine ligand for ST2 receptor, is a chromatin-associated nuclear factor in vivo. Proc Natl Acad Sci U S A; 2007. 1:282–287.

Carroll NG, Mutavdzic S, James AL. Distribution and degranulation of airway mast cells in normal and asthmatic subjects. Eur Respir J; 2002. 5:879–885.

Casadevall A, Perfect JR. Cryptococcus neoformans. Washington, D.C., United States of America: American Society for Microbiology; 1998.

Chen G, McDonald RA, Wells JC, Huffnagle GB, Lukacs NW, Toews GB. The gamma interferon receptor is required for the protective pulmonary inflammatory response to Cryptococcus neoformans. Infect Immun; 2005. 3:1788–1796.

Choo KK, Chong PP, Ho ASH, Yong PVC. The role of host microfilaments and microtubules during opsonin-independent interactions of Cryptococcus neoformans with mammalian lung cells. Eur J Clin Microbiol Infect Dis; 2015. 12:2421–2427.

Cosio BG, Soriano JB, Lopez-Campos JL, Calle-Rubio M, Soler-Cataluna JJ, de-Torres JP, et al. Defining the Asthma-COPD Overlap Syndrome in a COPD Cohort. Chest; 2016. 1:45–52.

Coyle AJ, Lloyd C, Tian J, Nguyen T, Erikkson C, Wang L, et al. Crucial role of the interleukin 1 receptor family member T1/ST2 in T helper cell type 2-mediated lung mucosal immune responses. J Exp Med; 1999. 7:895–902.

Dahl R. Systemic side effects of inhaled corticosteroids in patients with asthma. Respir Med; 2006. 8:1307–1317.

Decken K, Kohler G, Palmer-Lehmann K, Wunderlin A, Mattner F, Magram J, et al. Interleukin-12 is essential for a protective Th1 response in mice infected with Cryptococcus neoformans. Infect Immun; 1998. 10:4994–5000.

Duncan C, Stephen C, Campbell J. Clinical characteristics and predictors of mortality for Cryptococcus gattii infection in dogs and cats of southwestern British Columbia. Can Vet J; 2006. 10:993–998.

Fahy JV, Kim KW, Liu J, Boushey HA. Prominent neutrophilic inflammation in sputum from subjects with asthma exacerbation. J Allergy Clin Immunol; 1995. 4:843–852.

Fairbairn SM, Page CP, Lees P, Cunningham FM. Early neutrophil but not eosinophil or platelet recruitment to the lungs of allergic horses following antigen exposure. Clin Exp Allergy; 1993. 10:821–828.

Fanta CH. Asthma. N Engl J Med; 2009. 10:1002–1014.

Flaczyk A, Duerr CU, Shourian M, Lafferty EI, Fritz JH, Qureshi ST. IL-33 signaling regulates innate and adaptive immunity to Cryptococcus neoformans. J Immunol; 2013. 5:2503–2513.

Fonseca A, Fell JW, Boekhout T. *Cryptococcus* Villemin in C. P. Kurtzman, J. W. Fell, and T. Boekhout (ed.), *The Yeasts: a Taxonomic Study*, 5th ed. Amsterdam, The Netherlands: Elsevier; 2010.

Ganendren R, Carter E, Sorrell T, Widmer F, Wright L. Phospholipase B activity enhances adhesion of Cryptococcus neoformans to a human lung epithelial cell line. Microbes Infect; 2006. 4:1006–1015.

Giguere S, Viel L, Lee E, MacKay RJ, Hernandez J, Franchini M. Cytokine induction in pulmonary airways of horses with heaves and effect of therapy with inhaled fluticasone propionate. Vet Immunol Immunopathol; 2002. 3-4:147–158.

Goerdt S, Politz O, Schledzewski K, Birk R, Gratchev A, Guillot P, et al. Alternative versus classical activation of macrophages. Pathobiology; 1999. 5-6:222–226.

Goldman DL, Davis J, Bommarito F, Shao X, Casadevall A. Enhanced allergic inflammation and airway responsiveness in rats with chronic Cryptococcus neoformans infection: potential role for fungal pulmonary infection in the pathogenesis of asthma. J Infect Dis; 2006. 8:1178–1186.

Goldman DL, Li X, Tsirilakis K, Andrade C, Casadevall A, Vicencio AG. Increased chitinase expression and fungal-specific antibodies in the bronchoalveolar lavage fluid of asthmatic children. Clin Exp Allergy; 2012. 4:523–530.

Grahnert A, Muller U, Buttlar H von, Treudler R, Alber G. Analysis of asthma patients for cryptococcal seroreactivity in an urban German area. Med Mycol; 2015. 6:576–586.

Grotenboer NS, Ketelaar ME, Koppelman GH, Nawijn MC. Decoding asthma: translating genetic variation in IL33 and IL1RL1 into disease pathophysiology. J Allergy Clin Immunol; 2013. 3:856–865.

Grumelli S, Corry DB, Song L, Song L, Green L, Huh J, et al. An immune basis for lung parenchymal destruction in chronic obstructive pulmonary disease and emphysema. PLoS Med; 2004. 1:e8.

Guillot L, Carroll SF, Badawy M, Qureshi ST. Cryptococcus neoformans induces IL-8 secretion and CXCL1 expression by human bronchial epithelial cells. Respir Res; 2008. 9.

Guo Z, Wu J, Zhao J, Liu F, Chen Y, Bi L, et al. IL-33 promotes airway remodeling and is a marker of asthma disease severity. J Asthma; 2014. 8:863–869.

Halliwell RE, McGorum BC, Irving P, Dixon PM. Local and systemic antibody production in horses affected with chronic obstructive pulmonary disease. Vet Immunol Immunopathol; 1993. 3-4:201–215.

Hallstrand TS, Hackett TL, Altemeier WA, Matute-Bello G, Hansbro PM, Knight DA. Airway epithelial regulation of pulmonary immune homeostasis and inflammation. Clin Immunol; 2014. 1:1–15.

Hammad H, Chieppa M, Perros F, Willart MA, Germain RN, Lambrecht BN. House dust mite allergen induces asthma via Toll-like receptor 4 triggering of airway structural cells. Nat Med; 2009. 4:410–416.

Hamzaoui A, Berraies A, Kaabachi W, Haifa M, Ammar J, Kamel H. Induced sputum levels of IL-33 and soluble ST2 in young asthmatic children. J Asthma; 2013. 8:803–809.

Haraldsen G, Balogh J, Pollheimer J, Sponheim J, Kuchler AM. Interleukin-33 - cytokine of dual function or novel alarmin? Trends Immunol; 2009. 5:227–233.

Hayakawa H, Hayakawa M, Kume A, Tominaga S. Soluble ST2 blocks interleukin-33 signaling in allergic airway inflammation. J Biol Chem; 2007. 36:26369–26380.

Heitman J, Kozel TR, Kwon-Chung KJ, Perfect JR, Casadevall A. Cryptococcus. Washington, USA: American Society for Microbiology; 2011.

Heyen L, Muller U, Siegemund S, Schulze B, Protschka M, Alber G, et al. Lung epithelium is the major source of IL-33 and is regulated by IL-33-dependent and IL-33-independent mechanisms in pulmonary cryptococcosis. Pathog Dis; 2016. 7:ftw086

Hill JO, Harmsen AG. Intrapulmonary growth and dissemination of an avirulent strain of Cryptococcus neoformans in mice depleted of CD4+ or CD8+ T cells. J Exp Med; 1991. 3:755–758.

Hinson KFW, Moon AJ, Plummer NS. Broncho-pulmonary aspergillosis; a review and a report of eight new cases. Thorax; 1952. 4:317–333.

Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, et al. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med; 2004. 26:2645–2653.

Holtzman MJ, Byers DE, Alexander-Brett J, Wang X. The role of airway epithelial cells and innate immune cells in chronic respiratory disease. Nat Rev Immunol; 2014. 10:686–698.

Hoshino K, Kashiwamura S, Kuribayashi K, Kodama T, Tsujimura T, Nakanishi K, et al. The absence of interleukin 1 receptor-related T1/ST2 does not affect T helper cell type 2 development and its effector function. J Exp Med; 1999. 10:1541–1548.

Huffnagle GB, Lipscomb MF, Lovchik JA, Hoag KA, Street NE. The role of CD4+ and CD8+ T cells in the protective inflammatory response to a pulmonary cryptococcal infection. J Leukoc Biol; 1994. 1:35–42.

Huffnagle GB, Toews GB, Burdick MD, Boyd MB, McAllister KS, McDonald RA, et al. Afferent phase production of TNF-alpha is required for the development of protective T cell immunity to Cryptococcus neoformans. J Immunol; 1996. 10:4529–4536.

Huffnagle GB, Yates JL, Lipscomb MF. Immunity to a pulmonary Cryptococcus neoformans infection requires both CD4+ and CD8+ T cells. J Exp Med; 1991. 4:793–800.

Jiang A, Bloom O, Ono S, Cui W, Unternaehrer J, Jiang S, et al. Disruption of E-cadherin-mediated adhesion induces a functionally distinct pathway of dendritic cell maturation. Immunity; 2007. 4:610–624.

Johnson LR. Clinical canine and feline respiratory medicine. Ames, Iowa, USA: Wiley-Blackwell; 2010.

Kawakami K, Shibuya K, Qureshi MH, Zhang T, Koguchi Y, Tohyama M, et al. Chemokine responses and accumulation of inflammatory cells in the lungs of mice infected with highly virulent Cryptococcus neoformans: effects of interleukin-12. FEMS Immunol Med Microbiol; 1999. 4:391–402.

Kawakami K, Tohyama M, Xie Q, Saito A. IL-12 protects mice against pulmonary and disseminated infection caused by Cryptococcus neoformans. Clin Exp Immunol; 1996. 2:208–214.

Klein W, Roel G J, Kleinjan A, van Nimwegen M, Bergen I, Bruijn M de, Levani Y, et al. Pulmonary innate lymphoid cells are major producers of IL-5 and IL-13 in murine models of allergic asthma. Eur J Immunol; 2012. 5:1106–1116.

Kobayashi Y. The role of chemokines in neutrophil biology. Front Biosci; 2008. 2400–2407.

Koguchi Y, Kawakami K. Cryptococcal infection and Th1-Th2 cytokine balance. Int Rev Immunol; 2002. 4-5:423–438.

Komai-Koma M, Xu D, Li Y, McKenzie ANJ, McInnes IB, Liew FY. IL-33 is a chemoattractant for human Th2 cells. Eur J Immunol; 2007. 10:2779–2786.

Kondo Y, Yoshimoto T, Yasuda K, Futatsugi-Yumikura S, Morimoto M, Hayashi N, et al. Administration of IL-33 induces airway hyperresponsiveness and goblet cell hyperplasia in the lungs in the absence of adaptive immune system. Int Immunol; 2008. 6:791–800.

Korn A, Miller D, Dong L, Buckles EL, Wagner B, Ainsworth DM. Differential Gene Expression Profiles and Selected Cytokine Protein Analysis of Mediastinal Lymph Nodes of Horses with Chronic Recurrent Airway Obstruction (RAO) Support an Interleukin-17 Immune Response. PLoS One; 2015. 11:e0142622.

Krockenberger MB, Canfield PJ, Barnes J, Vogelnest L, Connolly J, Ley C, et al. Cryptococcus neoformans var. gattii in the koala (Phascolarctos cinereus): serological evidence for subclinical cryptococcosis. Med Mycol; 2002. 3:273–282.

Krockenberger MB, Canfield PJ, Malik R. Cryptococcus neoformans var. gattii in the koala (Phascolarctos cinereus): a review of 43 cases of cryptococcosis. Med Mycol; 2003. 3:225–234.

Kronstad JW, Attarian R, Cadieux B, Choi J, D'Souza CA, Griffiths EJ, et al. Expanding fungal pathogenesis: Cryptococcus breaks out of the opportunistic box. Nat Rev Microbiol; 2011. 3:193–203.

Kunkel SL, Standiford T, Kasahara K, Strieter RM. Interleukin-8 (IL-8): the major neutrophil chemotactic factor in the lung. Exp Lung Res; 1991. 1:17–23.

Kwon-Chung KJ, Fraser JA, Doering TL, Wang Z, Janbon G, Idnurm A, et al. Cryptococcus neoformans and Cryptococcus gattii, the etiologic agents of cryptococcosis. Cold Spring Harb Perspect Med; 2014. 7:a019760.

Lambrecht BN, Hammad H. The airway epithelium in asthma. Nat Med; 2012. 5:684–692.

Lambrecht BN, Hammad H. Allergens and the airway epithelium response: gateway to allergic sensitization. J Allergy Clin Immunol; 2014. 3:499–507.

Lavoie JP, Maghni K, Desnoyers M, Taha R, Martin JG, Hamid QA. Neutrophilic airway inflammation in horses with heaves is characterized by a Th2-type cytokine profile. Am J Respir Crit Care Med; 2001. 8 Pt 1:1410–1413.

Lavoie-Lamoureux A, Moran K, Beauchamp G, Mauel S, Steinbach F, Lefebvre-Lavoie J, et al. IL-4 activates equine neutrophils and induces a mixed inflammatory cytokine expression profile with enhanced neutrophil chemotactic mediator release ex vivo. Am J Physiol Lung Cell Mol Physiol; 2010. 4:L472-82.

Liu X, Li M, Wu Y, Zhou Y, Zeng L, Huang T. Anti-IL-33 antibody treatment inhibits airway inflammation in a murine model of allergic asthma. Biochem Biophys Res Commun; 2009. 1:181–185.

Lloyd CM. IL-33 family members and asthma - bridging innate and adaptive immune responses. Curr Opin Immunol; 2010. 6:800–806.

Malik R, Alderton B, Finlaison D, Krockenberger MB, Karaoglu H, Meyer W, et al. Cryptococcosis in ferrets: a diverse spectrum of clinical disease. Aust Vet J; 2002. 12:749–755.

Malik R, Krockenberger MB, Cross G, Doneley R, Madill DN, Black D, et al. Avian cryptococcosis. Med Mycol; 2003. 2:115–124.

Malik R, Norris J, White J, Jantulik B. 'Wound cat'. J Feline Med Surg; 2006. 2:135–140.

Marti E, Gerber H, Essich G, Oulehla J, Lazary S. The genetic basis of equine allergic diseases. 1. Chronic hypersensitivity bronchitis. Equine Vet J; 1991. 6:457–460.

Mayer AK, Muehmer M, Mages J, Gueinzius K, Hess C, Heeg K, et al. Differential recognition of TLR-dependent microbial ligands in human bronchial epithelial cells. J Immunol; 2007. 5:3134–3142.

McGorum BC, Dixon PM, Robinson NE, Schumacher J. Equine respiratory medicine and surgery. Philadelphia: Saunders Elsevier; 2007.

Merkel GJ, Scofield BA. The in vitro interaction of Cryptococcus neoformans with human lung epithelial cells. FEMS Immunol Med Microbiol; 1997. 3:203–213.

Merkel GJ, Scofield BA. The effects of Cryptococcus neoformans-secreted antigens on tumor necrosis factor-alpha-induced intercellular adhesion molecule-1 expression on human lung epithelial cells. FEMS Immunol Med Microbiol; 2000. 4:329–332.

Mitchell TG, Perfect JR. Cryptococcosis in the era of AIDS--100 years after the discovery of Cryptococcus neoformans. Clin Microbiol Rev; 1995. 4:515–548.

Mody CH, Chen GH, Jackson C, Curtis JL, Toews GB. In vivo depletion of murine CD8 positive T cells impairs survival during infection with a highly virulent strain of Cryptococcus neoformans. Mycopathologia; 1994. 1:7–17.

Moriello KA, Stepien RL, Henik RA, Wenholz LJ. Pilot study: prevalence of positive aeroallergen reactions in 10 cats with small-airway disease without concurrent skin disease. Vet Dermatol; 2007. 2:94–100.

Müller U, Piehler D, Stenzel W, Kohler G, Frey O, Held J, et al. Lack of IL-4 receptor expression on T helper cells reduces T helper 2 cell polyfunctionality and confers resistance in allergic bronchopulmonary mycosis. Mucosal Immunol; 2012. 3:299–310.

Müller U, Stenzel W, Kohler G, Werner C, Polte T, Hansen G, et al. IL-13 induces disease-promoting type 2 cytokines, alternatively activated macrophages and allergic inflammation during pulmonary infection of mice with Cryptococcus neoformans. J Immunol; 2007. 8:5367–5377.

Nathan AT, Peterson EA, Chakir J, Wills-Karp M. Innate immune responses of airway epithelium to house dust mite are mediated through beta-glucan-dependent pathways. J Allergy Clin Immunol; 2009. 3:612–618.

NHLBI 2007. Expert Panel Report 3 (EPR 3): Guidelines for the Diagnosis and Management of Asthma.

Norris Reinero CR, Decile KC, Berghaus RD, Williams KJ, Leutenegger CM, Walby WF, et al. An experimental model of allergic asthma in cats sensitized to house dust mite or bermuda grass allergen. Int Arch Allergy Immunol; 2004. 2:117–131.

O'Brien CR, Krockenberger MB, Martin P, Wigney DI, Malik R. Long-term outcome of therapy for 59 cats and 11 dogs with cryptococcosis. Aust Vet J; 2006. 11:384–392.

O'Brien CR, Krockenberger MB, Wigney DI, Martin P, Malik R. Retrospective study of feline and canine cryptococcosis in Australia from 1981 to 2001: 195 cases. Med Mycol; 2004. 5:449–460.

Ordonez CL, Shaughnessy TE, Matthay MA, Fahy JV. Increased neutrophil numbers and IL-8 levels in airway secretions in acute severe asthma: Clinical and biologic significance. Am J Respir Crit Care Med; 2000. 4 Pt 1:1185–1190.

Osterlund C, Gronlund H, Polovic N, Sundstrom S, Gafvelin G, Bucht A. The non-proteolytic house dust mite allergen Der p 2 induce NF-kappaB and MAPK dependent activation of bronchial epithelial cells. Clin Exp Allergy; 2009. 8:1199–1208.

Otsuka H, Kusumi T, Kanai S, Koyama M, Kuno Y, Takizawa R. Stem cell factor mRNA expression and production in human nasal epithelial cells: contribution to the accumulation of mast cells in the nasal epithelium of allergy. J Allergy Clin Immunol; 1998. 5:757–764.

Pal M, Mehrotra BS. Cryptococcal mastitis in dairy animals. Mykosen; 1983. 12:615–616.

Park BJ, Wannemuehler KA, Marston BJ, Govender N, Pappas PG, Chiller TM. Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. AIDS; 2009. 4:525–530.

Pennisi MG, Hartmann K, Lloret A, Ferrer L, Addie D, Belak S, et al. Cryptococcosis in cats: ABCD guidelines on prevention and management. J Feline Med Surg; 2013. 7:611–618.

Piehler D, Eschke M, Schulze B, Protschka M, Müller U, Grahnert A, et al. The IL-33 receptor (ST2) regulates early IL-13 production in fungus-induced allergic airway inflammation. Mucosal Immunol; 2016. 4:937–949.

Piehler D, Grahnert A, Eschke M, Richter T, Kohler G, Stenzel W, et al. T1/ST2 promotes T helper 2 cell activation and polyfunctionality in bronchopulmonary mycosis. Mucosal Immunol; 2013. 2:405–414.

Pirie RS, Dixon PM, Collie DD, McGorum BC. Pulmonary and systemic effects of inhaled endotoxin in control and heaves horses. Equine Vet J; 2001. 3:311–318.

Racine J, Gerber V, Feutz MM, Riley CP, Adamec J, Swinburne JE, et al. Comparison of genomic and proteomic data in recurrent airway obstruction affected horses using Ingenuity Pathway Analysis(R). BMC Vet Res; 2011. 48.

Reinero CR. Advances in the understanding of pathogenesis, and diagnostics and therapeutics for feline allergic asthma. Vet J; 2011. 1:28–33.

Reinero CR, DeClue AE, Rabinowitz P. Asthma in humans and cats: is there a common sensitivity to aeroallegens in shared environments? Environ Res; 2009. 5:634–640.

Roussel L, Erard M, Cayrol C, Girard J. Molecular mimicry between IL-33 and KSHV for attachment to chromatin through the H2A-H2B acidic pocket. EMBO Rep; 2008. 10:1006–1012.

Saglani S, Lui S, Ullmann N, Campbell GA, Sherburn RT, Mathie SA, et al. IL-33 promotes airway remodeling in pediatric patients with severe steroid-resistant asthma. J Allergy Clin Immunol; 2013. 3:676-685.e13.

Schiering C, Krausgruber T, Chomka A, Frohlich A, Adelmann K, Wohlfert EA, et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. Nature; 2014. 7519:564–568.

Schmallenbach KH, Rahman I, Sasse HH, Dixon PM, Halliwell RE, McGorum BC, et al. Studies on pulmonary and systemic Aspergillus fumigatus-specific IgE and IgG antibodies in horses affected with chronic obstructive pulmonary disease (COPD). Vet Immunol Immunopathol; 1998. 3-4:245–256.

Schmitz J, Owyang A, Oldham E, Song Y, Murphy E, McClanahan TK, et al. IL-33, an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and induces T helper type 2-associated cytokines. Immunity; 2005. 5:479–490.

Schulze B, Piehler D, Eschke M, Buttlar H von, Kohler G, Sparwasser T, et al. CD4(+) FoxP3(+) regulatory T cells suppress fatal T helper 2 cell immunity during pulmonary fungal infection. Eur J Immunol; 2014. 12:3596–3604.

Schulze B, Piehler D, Eschke M, Heyen L, Protschka M, Kohler G, et al. Therapeutic expansion of CD4+FoxP3+ regulatory T cells limits allergic airway inflammation during pulmonary fungal infection. Pathog Dis; 2016. 4:ftw020.

Smithgall MD, Comeau MR, Yoon BP, Kaufman D, Armitage R, Smith DE. IL-33 amplifies both Th1- and Th2-type responses through its activity on human basophils, allergen-reactive Th2 cells, iNKT and NK cells. Int Immunol; 2008. 8:1019–1030.

Sorrell TC, Brownlee AG, Ruma P, Malik R, Pfeiffer TJ, Ellis DH. Natural environmental sources of Cryptococcus neoformans var. gattii. J Clin Microbiol; 1996. 5:1261–1263.

Soumelis V, Reche PA, Kanzler H, Yuan W, Edward G, Homey B, et al. Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing TSLP. Nat Immunol; 2002. 7:673–680.

Spanamberg A, Wunder EA, JR, Brayer Pereira DI, Argenta J, Cavallini Sanches EM, Valente P, et al. Diversity of yeasts from bovine mastitis in Southern Brazil. Rev Iberoam Micol; 2008. 3:154–156.

Stenzel W, Muller U, Kohler G, Heppner FL, Blessing M, McKenzie ANJ, et al. IL-4/IL-13-dependent alternative activation of macrophages but not microglial cells is associated with uncontrolled cerebral cryptococcosis. Am J Pathol; 2009. 2:486–496.

Stephen C, Lester S, Black W, Fyfe M, Raverty S. Multispecies outbreak of cryptococcosis on southern Vancouver Island, British Columbia. Can Vet J; 2002. 10:792–794.

Sur S, Kita H, Gleich GJ, Chenier TC, Hunt LW. Eosinophil recruitment is associated with IL-5, but not with RANTES, twenty-four hours after allergen challenge. J Allergy Clin Immunol; 1996. 6:1272–1278.

Swinburne JE, Bogle H, Klukowska-Rotzler J, Drogemuller M, Leeb T, Temperton E, et al. A whole-genome scan for recurrent airway obstruction in Warmblood sport horses indicates two positional candidate regions. Mamm Genome; 2009. 8:504–515.

Teixeira PAC, Penha LL, Mendonca-Previato L, Previato JO. Mannoprotein MP84 mediates the adhesion of Cryptococcus neoformans to epithelial lung cells. Front Cell Infect Microbiol; 2014. 106.

Thorley AJ, Goldstraw P, Young A, Tetley TD. Primary human alveolar type II epithelial cell CCL20 (macrophage inflammatory protein-3alpha)-induced dendritic cell migration. Am J Respir Cell Mol Biol; 2005. 4:262–267.

Toribio JLM, Norris JM, White JD, Dhand NK, Hamilton SA, Malik R. Demographics and husbandry of pet cats living in Sydney, Australia: results of cross-sectional survey of pet ownership. J Feline Med Surg; 2009. 6:449–461.

Tully JE, Hoffman SM, Lahue KG, Nolin JD, Anathy V, Lundblad LKA, et al. Epithelial NF-kappaB orchestrates house dust mite-induced airway inflammation, hyperresponsiveness, and fibrotic remodeling. J Immunol; 2013. 12:5811–5821.

Ullemar V, Magnusson PKE, Lundholm C, Zettergren A, Melen E, Lichtenstein P, et al. Heritability and confirmation of genetic association studies for childhood asthma in twins. Allergy; 2016. 2:230–238.

Vaughan RJ, Vitali SD, Eden PA, Payne KL, Warren KS, Forshaw D, et al. Cryptococcosis in Gilbert's and long-nosed potoroo. J Zoo Wildl Med; 2007. 4:567–573.

Velagapudi R, Hsueh Y, Geunes-Boyer S, Wright JR, Heitman J. Spores as infectious propagules of Cryptococcus neoformans. Infect Immun; 2009. 10:4345–4355.

Veltman JA, Bristow CC, Klausner JD. Meningitis in HIV-positive patients in sub-Saharan Africa: a review. J Int AIDS Soc; 2014. 19184.

Vignola AM, Campbell AM, Chanez P, Bousquet J, Paul-Lacoste P, Michel FB, et al. HLA-DR and ICAM-1 expression on bronchial epithelial cells in asthma and chronic bronchitis. Am Rev Respir Dis; 1993. 3:689–694.

Voelz K, May RC. Cryptococcal interactions with the host immune system. Eukaryot Cell; 2010. 6:835–846.

Wu CA, Peluso JJ, Zhu L, Lingenheld EG, Walker ST, Puddington L. Bronchial epithelial cells produce IL-5: implications for local immune responses in the airways. Cell Immunol; 2010. 1:32–41.

Xia J, Zhao J, Shang J, Li M, Zeng Z, Zhao J, et al. Increased IL-33 expression in chronic obstructive pulmonary disease. Am J Physiol Lung Cell Mol Physiol; 2015. 7:L619-27.