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The inflammatory response against *Cryptococcus neoformans* is regulated by eosinophilic granulocytes and the interleukin-4/interleukin-4 receptor axis

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Abbreviations

C. neoformans	*Cryptococcus neoformans*
e.g.	exempli gratia
HIV	human immunodeficiency virus
i.e.	id est
IFN-γ	interferon-γ
Ig	immunoglobulin
IL	interleukin
IL-4Rα	interleukin-4 receptor alpha chain
SNP	single nucleotide polymorphism
Th cell	T helper cell
TNF-α	tumor necrosis factor-alpha

1 Introduction

We are permanently confronted with pathogens. Therefore, a network of manifold defense strategies has evolved such as physico-chemical barriers at skin and mucosal sites, bactericidal enzymes, and antimicrobial peptides. Besides these mechanical and chemical barriers, a complex system of cells and soluble factors is central in controlling interaction with microorganisms – the immune system. For a better understanding a simplified scheme divides this system in vertebrates into an innate and an adaptive part, each represented by distinct cells which communicate by direct interactions via cell-cell contacts over a short range and by soluble factors (i.e. cytokines and chemokines) over larger distances. These immune cells harbor a large arsenal of effector mechanisms to eliminate pathogens and kill infected cells. In addition, cells and factors exist that connect between the innate and adaptive parts of the immune system. These interactions between the different parts of the immune system are important for control and eradication of aggressive pathogens. Thus, the early recognition of invading pathogens is an eminent mechanism to control the infection and for the development of pathogen-specific immune responses that can provide enhanced quality of further defense reactions. Together, all these parts effectively provide protection and fatal pathogen-induced disease occurs rarely under normal circumstances. However, changes of these homeostatic conditions can empower microbes for infection and disease progression that would be otherwise effectively controlled - so called opportunistic pathogens.

1.1 *Cryptococcus neoformans*

The yeast-like organism *Cryptococcus neoformans* (*C. neoformans*) usually resides in the environment and different varieties can be readily isolated from soil and trees (LIN and HEITMAN 2006) and can also be found in humans and animals, e.g. dogs and cats (DUNCAN et al. 2005). Cryptococci not only gained scientific interest for their unique nature such as their capsule (DOERING 2009) amongst many more properties (STEENBERGEN et al. 2001; CASADEVALL et al. 2003), but some species also for their characteristics as opportunistic pathogens (LITVINTSEVA and MITCHELL 2009). The identification of the infective form is still under investigation and some recent studies point to spores (GILES et al. 2009; VELAGAPUDI et al. 2009; LITVINTSEVA and MITCHELL 2009; BOTTS and HULL 2010), but it is commonly accepted that *C. neoformans* is usually acquired by inhalation (Fig. 1) of contaminated soil or pigeon guano (LIN and HEITMAN 2006). Once inside the lung, the fate of cryptococci can be divergent, depending on their variety and the immune status of the host (LORTHOLARY et al. 2004; LI and MODY 2010). They can be contained stably in the lung with no signs of disease or disseminate throughout the host's body (Fig 1.) with neurotropic preferences (CHRETIEN et al. 2002) where they commonly cause fatal meningoencephalitis (MITCHELL and PERFECT 1995; KARSTAEDT et al. 2002).

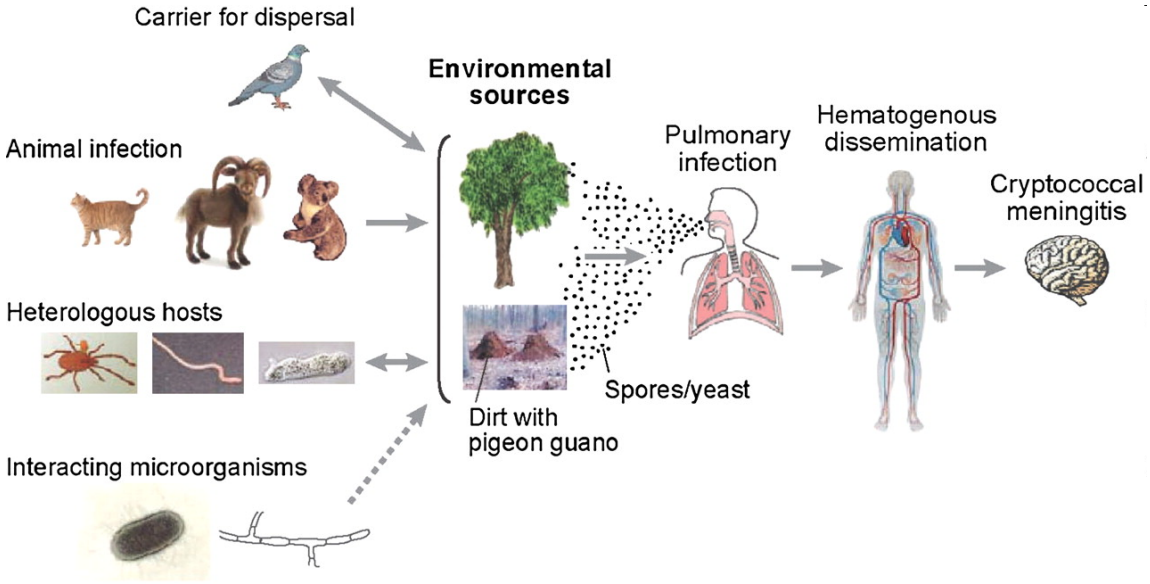

Figure 1: Infection with *Cryptococcus neoformans*.
Infective stages of *Cryptococcus neoformans* are widely found in the environment, e.g. in droppings of pigeons. They are usually acquired by inhalation. On the left several hosts are shown, on the right the possible outcome of the infection in humans is depicted. The figure is adopted from LIN and HEITMAN (2006).

Progress in epidemiological analysis and the use of molecular diagnostic revealed that the related *C. gattii* (formerly known as *C. neoformans* var. *gattii*) even tends to infect humans with no impairment of the immune system (FRASER et al. 2005; MA et al. 2009; CARRICONDE et al. 2011), whereas var. *grubii* and var. *neoformans* are associated with disease in immunosuppressed patients, e.g. resulting from corticosteroid treatment and especially HIV infection. In most cases the patients were latently infected, with reactivation of the cryptococci the immune suppression (DROMER et al. 1992; SAHA et al. 2007). According to this, *C. neoformans* gained additional attention with the emergence of the AIDS pandemic rendering affected humans highly susceptible (MITCHELL and PERFECT 1995) and research still holds on (BOULWARE et al. 2010). In 2010 about 680,000 HIV^+ patients died of cryptococcal meningitis (OLSZEWSKI et al. 2010) and even intervention with antifungal therapy in cases of acute meningoencephalitis results in 20% 3-month-mortality (LORTHOLARY et al. 2006; DROMER et al. 2007). This may account in part for resistance development due to the necessity of long term therapies (KELLY et al. 1994; LAMB et al. 1995; VENKATESWARLU et al. 1997).

1.2 Veterinary aspects of cryptococcosis

Besides humans, a broad range of domestic and free living animals also can be infected by *C. neoformans* but direct transmission between animals and humans has not been described so far (FAGGI et al. 1993). From an epidemiological point of view, pigeons are believed to represent a major distributor (Fig. 1) especially in urban areas since *C. neoformans* is readily isolated from cloaca and dried droplets of the birds (SWINNE-DESGAIN 1974; ROSARIO et al. 2005; ROSARIO et al. 2010). For free living animals there are reports about amoebae (STEENBERGEN et al. 2001),

amphibians (SEIXAS et al. 2008), reptilians (HOUGH 1998), marsupials (KROCKENBERGER et al. 2003), cheetahs (MILLWARD and WILLIAMS 2005), seals and toothed whales (STEPHEN et al. 2002a) acquiring cryptococcal infection. This list is not complete but it clearly emphasizes that *C. neoformans* infection is not limited to a special host species (Fig. 1). *C. neoformans* also reached the scope of veterinary clinicians and microbiologists since feline cryptococcosis represents the cat's most common systemic mycosis worldwide (FLATLAND et al. 1996; GERDS-GROGAN and YRELL-HART 1997; MCGILL et al. 2009). Besides cats, also dogs (MALIK et al. 1995), horses (SCOTT et al. 1974; TEUSCHER et al. 1984; CHO et al. 1986; CHANDNA et al. 1993) goats (CHAPMAN et al. 1990), sheep (LEMOS et al. 2007), and alpacas (GOODCHILD et al. 1996) are reported to be vulnerable to infection with *C. neoformans*. Of regional economic interest are cases of mastitis and also pneumonia occurring in cattle herds (GALLI and SOCCI 1969; PAL and MEHROTRA 1983). The highest incidence among animals of veterinarian interest is found in koalas mainly caused by *C. gattii* that may be related to their life-style on eucalypt trees, a plant which is associated with this fungus (BOLLIGER and FINCKH 1962; KROCKENBERGER et al. 2002). Animal species which have been used for experimental studies with *C. neoformans* include typical laboratory animals such as rats and mice, but also rabbits. It is still unknown why certain species are more resistant than others. It is likely that the type of the T helper (Th) cell response is important for resistance or susceptibility, as was shown for different mouse strains. Mouse strains that develop a Th1 response during cryptococcal infection remain healthy (though persistently infected), whereas animals developing a Th2 response become susceptible and succumb to the infection (KOGUCHI and KAWAKAMI 2002). In addition, morphological differences such as anatomy of nasal cavities may account for more cases of rhinosinusitis, especially in horses, dogs, and cats than affection of lungs in humans. As mentioned before, pigeons are suspected to spread *C. neoformans* but birds themselves are affected very rarely which might be a function of their higher body temperature as compared to mammals and other animals. However, some case reports exist, describing disease with dissemination in psittacines (MALIK et al. 2003; RASO et al. 2004) and occasional disease in other avian species (MALIK et al. 2003).

Until now, many reports from animal species are case studies and are not based on molecular phenotyping of fungi – epidemiological research has just started following events such as the outbreak in Vancouver Island, Canada (STEPHEN et al. 2002; LESTER et al. 2004; HOANG et al. 2004; DUNCAN et al. 2006; MACDOUGALL et al. 2007; DATTA et al. 2009) or Australia (CARRICONDE et al. 2011). As for human cryptococcosis, therapy of *Cryptococcus*-infected animals often includes long term treatment with antifungals with the risk of relapse or re-infection (O'BRIEN et al. 2006).

1.3 T helper cells and cytokines in cryptococcosis

Despite of the medical significance of cryptococcosis in humans especially in sub-Saharan Africa and animal patients in Canada and Australia, the immune response of the host against cryptococci is only incompletely understood. Gaps in knowledge about the protective immunity to *C. neoformans* together with immunosuppressive properties of cryptococci have prevented the development of concepts for vaccination of patients so far. One major field of the present research is pathogen recognition by cells of the immune system and the resulting inflammatory response that is mediated by interleukins amongst other factors (TAKEDA et al. 2003; GEIJTENBEEK and GRINGHUIS 2009; ROMANI 2011). Since interleukins can have profound effects on their target cells, a fine-tuned control is of outstanding importance for the host to avoid persistent inflammation and life-threatening cell damage. Keeping the balance between tissue damage and pathogen eradication requires an adequate and pathogen-dependent response. Due to the enormous heterogeneity of pathogens, responses can be quite different in their cytokine patterns. Among the many different cell types that are able to synthesize cytokines, Th cells are central in regulation of adaptive immunity and the orchestration of the whole immune response (innate and adaptive) against a specific pathogen (SPRENT and SURH 2002). Since Th cells are able to secrete a broad spectrum of cytokines, they are traditionally referred to several Th cell subsets, e.g. Th1, Th2, and Th17 according to their cytokine signature (ZHU et al. 2010; MURPHY and STOCKINGER 2010).

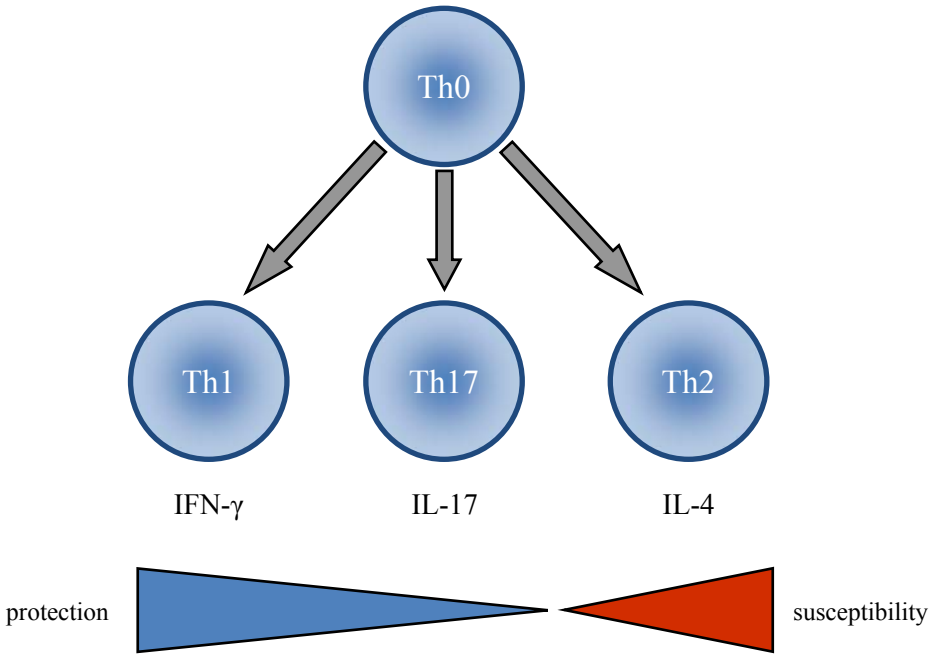

Figure 2: Established T helper cell subsets and their role in cryptococcosis.
Starting from the naive T helper (Th) 0 cell stage, different subsets of Th cells develop. From left to right: Th1 cells produce the signature cytokine interferon (IFN)-γ, Th17 cells secrete the signature cytokine interleukin(IL)-17, and from Th2 cells IL-4 is derived. In cryptococcosis Th1 and Th17 cells mediate protection, Th2 cells confer susceptibility.

Earlier studies on experimental cryptococcosis tested the requirement for helper and cytotoxic T cells and could show that while they are both necessary for protection, Th cell are more important (LIM and MURPHY 1980; MODY et al. 1990; HUFFNAGLE et al. 1991; HUFFNAGLE et al. 1994). Previous studies report that Th1-biased responses are of benefit in cryptococcosis (Fig. 2). IFN-γ (KAWAKAMI et al. 1999; CHEN et al. 2005; ARORA et al. 2005; ARORA et al. 2011), IL-12 (KAWAKAMI et al. 1996; DECKEN et al. 1998; BEENHOUWER et al. 2001; KLEINSCHEK et al. 2006), IL-18 (KAWAKAMI et al. 2000), TNF-α (AGUIRRE et al. 1995; HOAG et al. 1997; HERRING et al. 2002) and also the more recently discovered Th17 cytokines confer control of *C. neoformans* (KLEINSCHEK et al. 2006; VOELZ et al. 2009). On the other hand, Th2 responses (Fig. 2) have been linked to susceptibility in *C. neoformans* infection (HOAG et al. 1995; HERNANDEZ et al. 2005; MÜLLER et al. 2007; JAIN et al. 2009). IL-4 is known for its central role in Th2 responses (PAUL and OHARA 1987) and can even induce Th2 immunity in absence of other major Th2 cytokines like IL-5, IL-9 and IL-13 (FALLON et al. 2002). IL-4 exerts several effector functions, namely growth and divison of B cells and furthermore the antibody isotype switch in these cells towards human immunoglobulin (Ig) G4 and murine IgG1 (SIDERAS et al. 1985) and IgE (COFFMAN et al. 1986; KUHN et al. 1991). IL-4 also induces the alternative activation of macrophages (GORDON 2003). It is involved in Th cell growth and activation, Th2 polarization/differentiation (KUHN et al. 1991; KOPF et al. 1993). Besides that, IL-4 inhibits production of Th1 cytokines IFN-γ and IL-12 (MOSMANN et al. 1986; PFEIFER et al. 1987; SWAIN et al. 1991; SZABO et al. 1997). All these features contribute to disease development in cryptococcosis (KOGUCHI and KAWAKAMI 2002). It is therefore an attractive molecule for research because it has been also known for years that distinct cells besides Th cells can produce IL-4 (GESSNER et al. 2005; CHEN and KUNG 2007; LEVESON-GOWER et al. 2011) but these have not been identified in cryptococcosis yet.

1.4 The eosinophilic granulocyte in cryptococcosis

Eosinophilic granulocytes belong to the myeloid lineage of haematopoietic cells and seem to be very old cells from an evolutionary point of view – there is evidence for ‘comparable’ ameboid granulated cells already in invertebrates while only vertebrates harbor cells that are referred to be “true” eosinophilic granulocytes (LEE et al. 2010). Studies done on human eosinophils and in laboratory animals revealed a broad range of effector molecules secreted by these granulocytes ranging from toxic compounds, lipid mediators and chemokines to a wide array of interleukins – reviewed by ROTHENBERG and HOGAN 2006; TRIVEDI and LLOYD 2007, and SHAMRI et al. 2011. Despite these multiple effector molecules and the resulting implications under homeostatic as well as pathophysiological conditions, these cells were treated for a long time simply as tissue-destructing cells (GLEICH et al. 1973; WASSOM and GLEICH 1979; FILLEY et al. 1982; HOGAN et al. 2008) dealing with pathogens that were not ingestible by phagocytes (KLION and NUTMAN 2004). Recent

reports demonstrate that release of toxic compounds and degranulation by eosinophils is not found in experimental asthma (DENZLER et al. 2000; DENZLER et al. 2001). Augmentation of symptoms in asthmatics and laboratory animals asthma models is often contributed to the actions of eosinophils (TRIVEDI and LLOYD 2007), e.g. by amplification of Th2 cell action (RUMBLEY et al. 1999; MATTES et al. 2002). Under physiological conditions, eosinophils are present in several organs and trafficking is mainly facilitated by eotaxins and IL-5-dependent signalling. This is not exclusive as REESE et al. (2007) demonstrated that chitin, second most occurring polysaccharide in the world and common cell wall component of fungi, also found in *C. neoformans*, can drive recruitment of eosinophils to the lungs.

As pointed out earlier, cryptococcosis proceeds deleteriously under Th2 circumstances. Therefore eosinophils gained some attention by *in vitro* (FELDMESSER et al. 1997; FELDMESSER et al. 1998; FELDMESSER et al. 2001) and *in vivo* studies (HUFFNAGLE et al. 1994; HUFFNAGLE et al. 1998). This indicated some role for Th cells in eosinophil recruitment by use of anti CD4 antibodies (HUFFNAGLE et al. 1994a) and involvement of eosinophils in leukocyte influx to the lungs by neutralizing IL-5 (HUFFNAGLE et al. 1998). Inhibition of eosinophil recruitment to the lung by engagement of an activation molecule (OX-40) on Th cells mediating IFN-γ synthesis by the Th cell was also found (HUMPHREYS et al. 2003). In the cryptococcosis model a series of reports on eosinophils have been observed in susceptible mice but this cell type was not analyzed for a regulatory function (OLSZEWSKI et al. 2000; ARORA et al. 2005; CHEN et al. 2007; MÜLLER et al. 2007; OSTERHOLZER et al. 2009; JAIN et al. 2009).

2 Aims of study

Interleukin-4 (IL-4) promotes the induction and maintenance of T helper (Th) 2 responses. In pulmonary infection with the fungal pathogen *Cryptococcus neoformans* Th2 responses are associated with uncontrolled growth of the pathogen. The Th2 response results in the development of disease symptoms typically found in asthma. Finally Th2-induced morbidity in the murine model of cryptococcosis leads to death of infected mice. So far, the source(s) of IL-4 in cryptococcosis are unidentified although there is strong evidence for IL-4 producing Th2 cells in the literature. Thus, the first part of the presented work aimed to analyze the time course of IL-4 production during infection with *C. neoformans*. With the aid of these kinetic data the following analyses should clarify the identity of the IL-4 producing cell(s). Finally, the contribution of non-Th2 cell-derived IL-4 to pulmonary pathology should be examined.

The answers to these described tasks represent a follow-up to an earlier study in pulmonary cryptococcosis. The outstanding role of IL-4 in cryptococcosis was known already and our initial research focused on the question if the degree of pathology is a function of the magnitude of consumable IL-4. We answered this question by performing experiments using an *in vivo* system with gradual IL-4 signalling.

3.1.1 Preface to manuscript no. 1

Available data dealing with the function of T helper (Th) cell subsets and cytokine responses in murine cryptococcosis show that Th1 related cytokines IFN-γ, (CHEN et al. 2005; ARORA et al. 2005; ARORA et al. 2011), IL-12 (HOAG et al. 1997; DECKEN et al. 1998; KAWAKAMI et al. 2000; BEENHOUWER et al. 2001) and TNF-α (AGUIRRE et al. 1995; HERRING et al. 2002; HERRING et al. 2005) provide the basis for protection. Besides the Th1 cytokines Th17 cells are also associated with beneficial outcome (KLEINSCHEK et al. 2006; ARORA et al. 2011). On the other hand, several studies have shown that interleukin (IL) -4 and other Th2 related cytokines are involved in the detrimental progress of experimental murine cryptococcosis (HERNANDEZ et al. 2005; MÜLLER et al. 2007; STENZEL et al. 2009) but so far the identity of innate IL-4-producing cells remained elusive.

Major results

- ❖ Upon pulmonary infection with *C. neoformans*, IL-4 production is not found before six weeks of infection.
- ❖ The production of immunoglobulin E is strictly linked to the late onset of IL-4 synthesis.
- ❖ Eosinophilic granulocytes and T helper cells increase in lung parenchyma concomitantly.
- ❖ Eosinophilic granulocytes and Th cells are the sources of IL-4 in pulmonary cryptococcosis.
- ❖ Depletion of eosinophils reveals that they are a non-redundant IL-4 source and significantly shape the cytokine profile and inflammatory response of Th cells.

Conclusions

Th cells and eosinophilic granulocytes are concomitantly recruited to lungs in a strictly time-dependent manner and represent the major if not sole sources of pathology-driving IL-4. The loss of eosinophil-derived IL-4 cannot be compensated and reveals a regulatory influence of eosinophils on Th cell cytokine profile and the recruitment of leukocytes to lungs.

The mechanism(s) responsible for the induction of IL-4 production not before six weeks of infection remain(s) to be elucidated.

Animal experiments

The mice used for the work in the publication “Eosinophils Contribute to IL-4 Production and Shape the T-Helper Cytokine Profile and Inflammatory Response in Pulmonary Cryptococcosis.” are part of the record 24-9168.11 (TVV 16/09) (Landesdirektion Leipzig).

3.1.2 Eosinophils promote Th2 responses in cryptococcosis

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Immunopathology and Infectious Diseases

Eosinophils Contribute to IL-4 Production and Shape the T-Helper Cytokine Profile and Inflammatory Response in Pulmonary Cryptococcosis

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Susceptibility to infection with *Cryptococcus neoformans* is tightly determined by production of IL-4. In this study, we investigated the time course of IL-4 production and its innate cellular source in mice infected intranasally with *C. neoformans*. We show that pulmonary IL-4 production starts surprisingly late after 6 weeks of infection. Interestingly, in the lungs of infected mice, pulmonary T helper (Th) cells and eosinophils produce significant amounts of IL-4. In eosinophil-deficient ΔdblGATA mice, IL-33 receptor–expressing Th2s are significantly reduced, albeit not absent, whereas protective Th1 and Th17 responses are enhanced. In addition, recruitment of pulmonary inflammatory cells during infection with *C. neoformans* is reduced in the absence of eosinophils. These data expand previous findings emphasizing an exclusively destructive effector function by eosinophilic granulocytes. Moreover, in ΔdblGATA mice, fungal control is slightly enhanced in the lung; however, dissemination of *Cryptococcus* is not prevented. Therefore, eosinophils play an immunoregulatory role that contributes to Th2-dependent susceptibility in allergic inflammation during bronchopulmonary mycosis. ***(Am J Pathol 2011, 179:733–744; DOI: 10.1016/j.ajpath.2011.04.025)***

Cryptococcus neoformans is a facultative intracellular pathogen that is acquired by inhalation of spores and/or desiccated yeasts and leads to latent pulmonary infection in immunocompetent humans.[1] The development of cryptococcal meningitis occurs mainly in immunocompromised HIV-1–infected patients, most likely by reactivation of latent pulmonary *C. neoformans* infection.[2] It is estimated that 504,000 HIV-1–infected patients die every year from cryptococcal meningitis in sub-Saharan Africa,[3] which surprisingly exceeds the annual death rate of tuberculosis-associated HIV cases. Resistance against *C. neoformans* primarily involves monocytic effector mechanisms.[4–6] In this context, T helper (Th) cells are central regulatory players with profound effects. Whereas IL-12–dependent Th1 responses are protective, with an additional contribution by IL-23–dependent Th17 responses,[7–9] Th2 cells producing IL-4, IL-13, and IL-5 are detrimental.[10,11] Studies[12–14] that used i.v. inoculation examined the traversal of the blood-brain barrier by *C. neoformans* and led to the conclusion that transmigration can occur with intracellular and extracellular fungi. In case of bronchopulmonary infection, dissemination seems to rely more on Th2 cytokines. This allergic Th2-driven inflammation represents the immunopathological pathway promoting disease by allowing cryptococci to grow inside the lung and finally enabling dissemination to the brain, ultimately causing fatal meningoencephalitis.[15] This sequela is accompanied by development of IL-4/IL-13–dependent alternatively activated macrophages, suggesting that those cells may be involved in dissemination. Alternatively activated macrophages are found only in susceptible mice[15] and show significantly reduced control of intracellular growth.[5] In addition, IL-13–dependent mucus production by goblet cells, IL-4–dependent IgE production, IL-5–dependent eosinophilia, and functional pulmonary impairment can be found; these features

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are also typically described in asthma.[16–18] Studies[10,11,19] of pulmonary and cerebral cryptococcosis in IL-4–, IL-13–, IL-4 receptor α–, and IL-4/IL-13–deficient mice or mice treated with anti-IL-5 convincingly provide a basis for future immunotherapies by targeting one or several of these Th2-associated molecules. However, it is unclear when IL-4 production starts after pulmonary infection. In addition, potential innate immune cell(s) producing IL-4 and thereby promoting Th2 initiation and/or Th2 maintenance remain to be identified. Therefore, in this study, we aimed to define the following: i) the onset and time course of IL-4 production, ii) the IL-4–producing innate cell type(s) supporting Th2 development, and iii) the immunological and phenotypic consequences of innate IL-4 production in pulmonary cryptococcosis. Our results indicate that eosinophilic granulocytes are a significant source of IL-4, with distinct regulatory consequences in murine cryptococcosis.

Materials and Methods

Mice

Female wild-type (WT) mice (Janvier, Le Genest Saint Isle, France), 4get mice[20] (provided by André Gessner, Clinical Microbiology and Immunology, Erlangen, Germany), and ΔdblGATA mice[21] (provided by Achim Hoerauf, Institute of Medical Microbiology, Immunology und Parasitology, Bonn, Germany), aged 6 to 10 weeks, on a BALB/c background were maintained in an individually ventilated caging system under specific pathogen-free conditions and in accordance with the guidelines approved by the Animal Care and Usage Committee of the Landesdirektion Leipzig. Sterile food and water were given ad libitum. The mice were tested periodically for pathogens, in accordance with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations accreditation board. All mice had negative test results for pinworms and other endoparasites and ectoparasites.

C. neoformans *and Infection*

Encapsulated *C. neoformans*, strain 1841, serotype D, was kept as a frozen stock in skim milk and was grown in Sabouraud dextrose medium (2% glucose and 1% peptone; Sigma, Deisenhofen, Germany) overnight on a shaker at 30°C. Cells were washed twice in sterile PBS, resuspended in PBS, and counted in a hematocytometer. Inocula were diluted in PBS to a concentration of 2.5×10^4/mL for intranasal (i.n.) infection. Mice were infected by i.n. application of 20-μL volumes containing 500 colony-forming units (CFUs). Before infection, mice were anesthetized i.p. with a 1:1 mixture of 10% ketamine (100 mg/mL; Ceva Tiergesundheit, Düsseldorf, Germany) and 2% xylazine (20 mg/mL; Ceva Tiergesundheit).

Leukocyte Preparation for Flow Cytometry and CFU Enumeration

Infected mice were monitored daily for survival and morbidity. After sterile removal of the brain from sacrificed mice, half was processed for histological examination and the remaining half was processed for determination of organ burden (CFU). After homogenization in 1-mL PBS with an Ultra-Turrax (T8; IKA-Werke, Staufen, Germany), serial dilutions of the homogenates were plated on Sabouraud dextrose agar plates and colonies were counted after an incubation period of 48 hours at 30°C. After sterile removal, lungs from sacrificed mice were minced and digested for 30 minutes at 37°C in RPMI 1640 medium supplemented with collagenase (Roche Diagnostics Deutschland GmbH, Mannheim, Germany), 100 μmol/L sodium pyruvate, and DNase IV (Sigma-Aldrich, Steinheim, Germany). After passage through a 100-μm nylon mesh (BD Biosciences, Heidelberg, Germany), filtrate was resuspended in 1-mL RPMI 1640 medium (PAA Laboratories, Pasching, Austria); and 50 μL was taken for CFU enumeration. Serial dilutions were plated on Sabouraud dextrose agar plates, and colonies were counted after an incubation period of 48 hours at 30°C. Remaining filtrate was resuspended in 70% Percoll (GH Healthcare Biosciences AB, Uppsala, Sweden) and layered under 26% Percoll. Leukocytes were recovered from interphase, washed with Iscove's modified Dulbecco's medium (PAA Laboratories), and counted in trypan blue (Fluka Chemie AG, Buchs, Switzerland). For surface staining, 1×10^5 to 2×10^5 cells were used; and for intracellular cytokine staining, 1×10^6 cells were acquired.

Flow Cytometry

Purified cells were adjusted to 5×10^6/mL in Iscove's modified Dulbecco's medium and stimulated either 6 hours with ionomycin (1 μg/mL; Sigma-Aldrich) and phorbol 12-myristate 13-acetate (PMA) (40 ng/mL; Alexis Corporation, Lausen, Switzerland) or 22 hours with specific antigen. For the accumulation of cytokines, brefeldin A (5 μg/mL; Sigma-Aldrich), was added for the last 4 hours. The acapsular *C. neoformans* serotype D strain CAP67 (provided by Dr. Bettina Fries, Albert Einstein College of Medicine, Bronx, NY) was used as a specific stimulus (1×10^7 cryptococci/mL, termed C.n. antigen) for restimulation of pulmonary leukocytes from *C. neoformans*–infected mice. The CAP67 strain has better restimulatory capacities than the highly virulent strain 1841. It was cultured and maintained in the same manner as strain 1841; before use, it was heat inactivated at 60°C for 1 hour.[22]

First, Near-IR Dead Cell Stain (Invitrogen, Darmstadt, Germany) was used to ensure discrimination and exclusion of dead cells during analysis. Second, cells were fixed with 2% paraformaldehyde (Serva, Heidelberg, Germany) for 20 minutes on ice. When intracellular staining was performed, permeabilization was included by using fluorescence-activated cell sorting buffer (ie, PBS containing 3% heat-inactivated fetal calf serum and 0.1% sodium azide) containing 0.5% saponin (w/v; Serva).

Cells were incubated for 15 minutes on ice with FcR block (2×10^6 μg cells/L; purified from 2.4G2 hybridoma supernatant) and rat serum (Sigma-Aldrich) to avoid unspecific staining. Antibodies (Abs) and FcR block for intracellular staining were diluted in fluorescence-activated cell sorting buffer containing 0.5% saponin (w/v; Serva). For specific stainings, the following Abs were used: anti-CD4-PerCP-Cy5.5 (RM4-5; eBioscience, Frankfurt, Germany); anti-interferon (IFN)-γ–fluorescein isothiocyanate (XMG1.2; eBioscience); anti-IL-4–allophycocyanine (APC) (11B11; Biolegend, Fell, Germany); anti-IL-17-PE-Cy7 (eBio17B7; eBioscience); anti-Siglec-F (E50-2440; BD Biosciences) biotinylated, following standard procedures; anti-Siglec-F-PE (E50-2440; BD Biosciences); anti-F4/80-PE-Cy7 (BM8; eBioscience); anti-CD11c–APC (N418; eBioscience); anti-CD154-PE (MR1; Miltenyi Biotec, Bergisch Gladbach, Germany); and anti-T1/ST2–fluorescein isothiocyanate (MD Biosciences, Zürich, Switzerland). Appropriate isotype Abs were all from eBioscience, except for anti-IL-4 from Biolegend. Cells labeled with biotinylated Abs were further stained with streptavidin-PerCp (eBioscience). Cells were acquired on a BD FACS Calibur using CellQuest software version 3.0.1 and BD FACS CANTO II using DIVA version 6.1.1 and FlowJo version 7.6.1 (Treestar Inc., Ashland, OR) software for analysis.

IL-4 Secretion Assay

An IL-4 secretion assay (Miltenyi Biotec) was performed according to manufacturer's instructions. Percoll (GH Healthcare) purified pulmonary leukocytes were stained with anti-CD4–fluorescein isothiocyanate (RM4-5; eBioscience) and, afterward, with anti-fluorescein isothiocyanate MicroBeads (Miltenyi Biotec). Cells were then applied to an MS column (Miltenyi Biotec) to separate $CD4^+$ and $CD4^-$ cells. Both fractions were stimulated for 2 hours with ionomycin (1 μg/mL; Sigma-Aldrich) and PMA (40 ng/mL; Alexis Corporation). After stimulation, cells were applied to an IL-4 secretion assay (Miltenyi Biotec) and were additionally stained with anti-CD11c–APC (N418; eBioscience), anti-CD3-biotin (145-2C11; eBioscience), and biotinylated anti-Siglec-F (E50-2440; BD Biosciences). Cells labeled with biotinylated Abs were further stained with streptavidin-PerCp (eBioscience).

Cytokine ELISA

Cytokine concentrations were determined by sandwich enzyme-linked immunosorbent assay (ELISA) systems with unlabeled capture Abs and labeled detection Abs. To determine the concentration of IL-4, monoclonal Ab (mAb) 11B11 was used as the capture Ab and biotin-labeled BVD6-24G2 (BD Biosciences) was used as the detection Ab, followed by incubation with peroxidase-labeled streptavidin (Southern Biotechnology Associates, Birmingham, AL). IFN-γ was captured by mAb AN-18 and detected by peroxidase-labeled mAb XMG1.2. The concentration of IL-17 was detected with the R&D Systems Duoset kit (R&D Systems GmbH, Wiesbaden, Germany).

Histopathological Analysis

Lung samples were processed for histological analysis, as previously described.[11]

IHC

Lung samples were processed for histological analysis, as previously described.[15] In brief, the accessory lobe of the lung was sterilely removed, mounted on thick filter paper with Tissue Tek optimal cutting temperature compound (Miles Scientific, Naperville, IL), snap frozen in isopentane (Fluka, Neu-Ulm, Germany) precooled on dry ice, and stored at -80°C. For immunohistochemistry (IHC), 10-μm frozen sections were prepared in a serial fashion (30 transversal sections on six consecutive levels per lung). Glucuronoxylomannan immunostaining was performed using mAb 18B7 (provided by Dr. Arturo Casadevall, Albert Einstein College of Medicine, New York, NY). The mAb 18B7 was biotinylated (Sigma-Aldrich) before use, and lung slides were incubated in a secondary step with ExtrAvidin peroxidase (Sigma-Aldrich). The peroxidase reaction product was visualized using 3,3′-diaminobenzidine (Sigma-Aldrich) as the chromogene and H_2O_2 as the cosubstrate.

Serum Immunoglobin Measurement

Total serum IgG1, IgG2a, and IgE levels were analyzed, as described earlier.[11] For determination of *C. neoformans*–specific serum IgE, the following capture ELISA was developed. ELISA plates (Nunc GmbH & Co KG, Langenselbold, Germany) were coated with 5 μg/mL rat–anti-mouse IgE mAb (R35-72; BD Biosciences) in carbonate buffer (pH 9.5) overnight at 4°C. To prevent unspecific binding, plates were washed and blocked with 5% (w/v) skim milk (Sigma-Aldrich) in PBS for 3 hours at room temperature. After washing, serum samples were diluted 1:10 in blocking buffer containing 0.1% (v/v) Tween 20 (Karl Roth AG, Karlsruhe, Germany) and added in duplicate for 2 hours at room temperature. Plates were washed again and biotinylated (Sigma-Aldrich). *C. neoformans* 1841D homogenate (5 μg/mL) was incubated for 2 hours at room temperature. This step was followed by incubation with peroxidase-labeled streptavidin (Southern Biotechnology Associates), diluted 1:4000 in Tween 20 containing blocking buffer, for 45 minutes at room temperature for detection. Plates were washed, and the TMB Microwell peroxidase system (KPL, Gaithersburg, MD) was used as a substrate for the final colorimetric reaction. The reaction was discontinued after 2 hours by adding 1 mol/L H_3PO_4, and ODs were read using a Spectra-max 340 ELISA reader (Molecular Devices GmbH, Ismaning, Germany) at 450 nm, with background subtraction at 630 nm. Wells incubated without serum samples but with all other reagents were used as plate blank. To control the specificity of this ELISA, we added a monoclonal IgE isotype control (C38-2; BD Biosciences) specific for the hapten trinitrophenyl that we also used as a standard for the total IgE quantification after coating with IgE mAb (R35-72; BD Biosciences) and blocking. After an-

other incubation with blocking buffer, plates were either incubated with anti-mouse IgE–horseradish peroxidase (23G3; Southern Biotechnology Associates) or biotinylated *C. neoformans* homogenate. Plates incubated with biotinylated homogenate were additionally incubated with peroxidase-labeled streptavidin (Southern Biotechnology Associates). Development was performed with the TMB Microwell peroxidase system (KPL). This confirmed that even high concentrations of anti-trinitrophenyl IgE (eg, 20 μg/mL) do not bind to biotinylated *C. neoformans* homogenate nonspecifically. Specificity was further confirmed by using serum samples from naïve BALB/c WT instead of anti-trinitrophenyl IgE. The median OD_{450} was 0.015.

Statistical Analysis

The one-tailed Mann-Whitney *U*-test was performed to determine the significance of differences in kinetic analyses of 4get mice and between WT and ΔdblGATA mice. Data are presented as the mean ± SEM. The level of confidence for significance was $P < 0.05$.

Results

Pulmonary Th2 Development Occurs after 6 Weeks of Infection and Coincides with IL-4–Producing Eosinophils

Susceptibility in cryptococcosis is tightly linked with IL-4 production.[7,11] BALB/c WT mice infected i.n. with only 500 CFUs of *C. neoformans* strain 1841 show dissemination from lung to brain beginning at approximately 6 weeks after infection (data not shown), leading to death 10 weeks after infection.[7,11] This pulmonary cryptococcosis model is a long-term model compared with other published murine models.[8,23–27]

To monitor IL-4 production over time in this long-term model, IL-4 reporter mice, termed 4get mice, were infected, and enhanced green fluorescent protein (eGFP) expression, which is known to correlate with IL-4 transcription, was assessed.[20] There were constitutively $eGFP^+$ pulmonary leukocytes in naïve mice (Figures 1A and 2, A and B), as published by others.[28,29] On infection of 4get mice, the frequency of $eGFP^+$ leukocytes increased almost 10-fold, at 42 days postinfection (dpi), and stayed on this elevated level up to 70 dpi (Figure 1A). Because eGFP expression may indicate only IL-4 transcription,[20] we wanted to assess IL-4 protein production. Indeed, we found that IL-4 production in response to antigen-specific stimulation of pulmonary leukocytes with cryptococcal antigen starts to become detectable at a similarly late time point as found for eGFP expression. IL-4 was not detectable at 35 dpi (data not shown) or earlier; instead, it started to become detectable 6 weeks after infection and increased up to 70 dpi (Figure 1B). Late IL-4 expression, monitored by eGFP expression or restimulation of pulmonary leukocytes, was further confirmed by intracellular staining of IL-4 in Th cells and revealed similar results (data not shown). Consistent with the time course of IL-4 production, total and specific IgE started to increase after 42 days of infection (Figure 1, C and D). Although the sandwich ELISA for total IgE detected a median concentration of approximately 13.65 μg/mL, starting after 42 days of infection, the ELISA for specific IgE resulted in minor signals of only up to 0.099 OD_{450}, with a substrate development time of 2 hours. Thus, similar to parasite models,[30] only a minor portion of the total IgE appears to be specific for cryptococcal antigens (Figure 1D).

$CD4^+$ Th cells, and innate immune cells, have been described as cellular sources of IL-4.[29] To define the cell types producing IL-4 in pulmonary cryptococcosis, we characterized $eGFP^+$ cells in the lungs of infected 4get mice. At 70 dpi, we found elevated numbers of $eGFP^+$

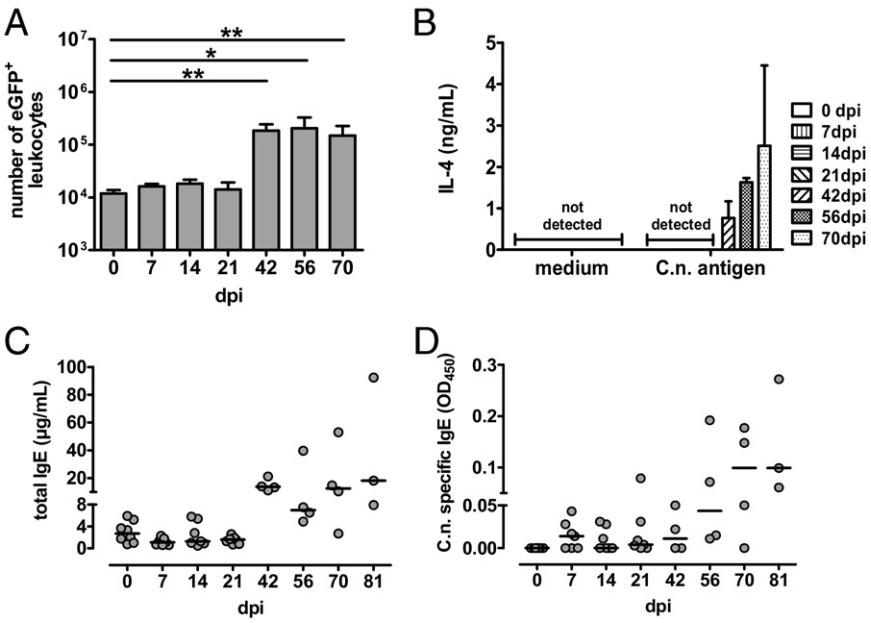

Figure 1. Coincidental accumulation of pulmonary leukocytes competent for IL-4 expression (indicated by eGFP), onset of IL-4 secretion, and increase of total and specific IgE. 4get mice were infected i.n. with *C. neoformans* 1841D. **A:** On the indicated dpi, leukocytes were isolated from lungs (n = 3 to 5 per time point), counted, and analyzed for eGFP-expression by flow cytometry. **B:** Pulmonary leukocytes were restimulated for 22 hours with C.n. antigen. IL-4 was measured by ELISA in culture supernatant. **C** and **D:** Serum samples from the same mice were examined for total IgE and *Cryptococcus*-specific IgE according to the *Materials and Methods* section. Data from two independent experiments were pooled and are expressed as the mean ± SEM. Statistical analysis was performed using the Mann-Whitney *U*-test. $^*P < 0.05$ and $^{**}P < 0.01$ comparison with naive 4get mice.

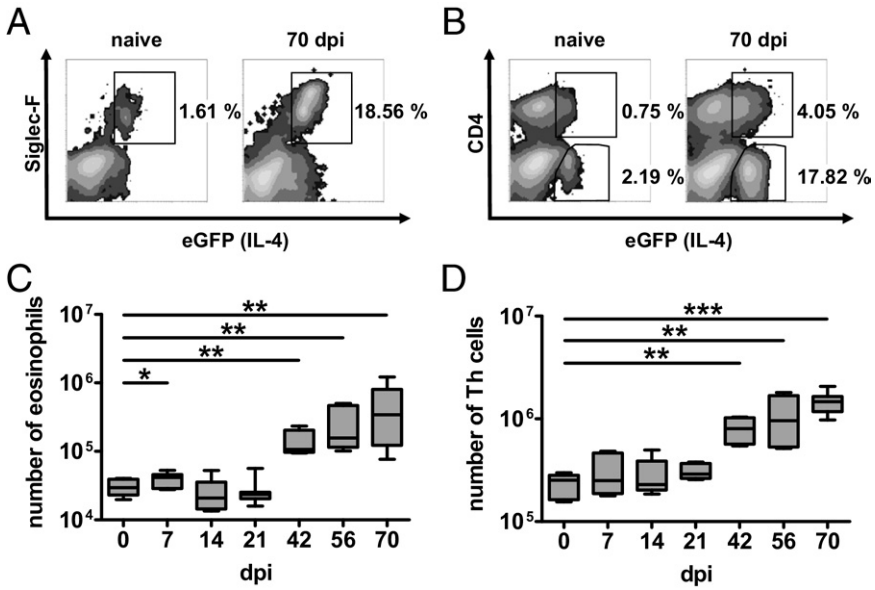

Figure 2. Th cells and eosinophils exclusively constitute eGFP$^+$ cells during pulmonary cryptococcosis. Flow cytometry was performed on pulmonary leukocytes from i.n. infected 4get mice on the indicated dpi (n = 3 to 5 per time point). **A:** CD4$^-$eGFP$^+$ cells identified by Siglec-F expression as eosinophils in representative 4get mice are shown. **B:** eGFP expression in Th cells in the same 4get mice shown in **A**. **C:** Kinetic analysis of enumerated eosinophils. **D:** Th cells at the indicated dpi are shown. Kinetic data were pooled from two independent experiments. The mean ± SEM is shown for kinetic analyses. Statistics were performed with the Mann-Whitney U-test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with naive 4get mice.

pulmonary leukocytes in both CD4$^-$ and CD4$^+$ populations (Figure 2, A and B). Interestingly, the CD4$^-$eGFP$^+$ population was identified to be Siglec-F$^+$, pointing to eosinophils (Figure 2A).[31] This prompted us to characterize the time course of recruitment of Th cells and eosinophilic granulocytes. The data shown in Figure 2, C and D, demonstrate that there is a similarly late time course of recruitment to the lung for both Th cells and eosinophils.

To directly define the cellular source(s) of pulmonary IL-4 production in cryptococcosis, we applied IL-4 secretion assays on magnetic cell sorting (MACS)-separated (Miltenyi) lung CD4$^+$ and CD4$^-$ cells to avoid cross feeding between different cells (Figure 3). Th2s represent a cell type already known to be responsible for efficient IL-4 production in cryptococcosis.[7,11] In addition to the Th cells as IL-4 sources (Figure 3C), we show that eosinophilic granulocytes (Figure 3A; further gated on Siglec-F$^+$/CD11c^{dim}) produce significant amounts of IL-4 on *Cryptococcus* infection (Figure 3B). Interestingly, a major portion of these eosinophils (ie, 17.49%) produced IL-4 constitutively after pulmonary infection with *C. neoformans* (Figure 3B, top; mean fluorescence intensity of isotype control (not shown) versus medium, 133.59 versus 330.43). This could be further enhanced (41.63% of all eosinophils) by *ex vivo* stimulation with a combination of PMA and ionomycin (mean fluorescence intensity, 784.66; Figure 3B, bottom). Together, these data demonstrate that, during pulmonary cryptococcosis, Th2 cells and eosinophils contribute to late IL-4 production at a point when IgE production is significantly increased and *C. neoformans* disseminates from the lung to the brain.

In the Absence of Eosinophils, Th2 Responses Are Reduced and Th1/Th17 Responses Are Enhanced

IL-4 is not essential for Th2 differentiation but plays a nonredundant role in the maintenance of Th2 responses.[32] We were interested in whether eosinophils

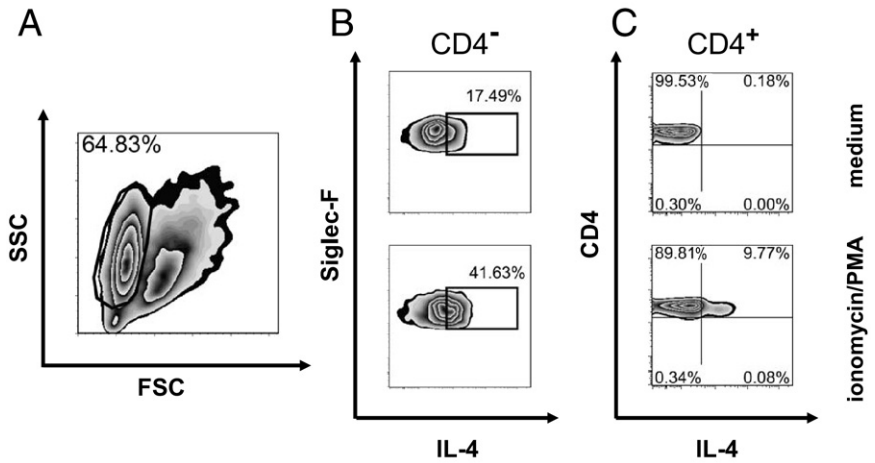

Figure 3. Both Th cells and eosinophils are the main producers of IL-4 in the lungs of infected mice. Isolated pulmonary leukocytes from i.n. infected BALB/c WT mice were positively enriched for CD4$^+$ Th cells by MACS. **A:** The remaining CD4$^-$ cells include eosinophils [gated on a side scatter (SSC)^{high}/forward scatter (FSC)^{low} plot]. Both CD4$^-$ cells (**B**) and CD4$^+$ Th cells (**C**) were either stimulated with ionomycin and PMA (**bottom**) or left untreated (**top**) before performing an IL-4 secretion assay. One of two independent experiments is shown.

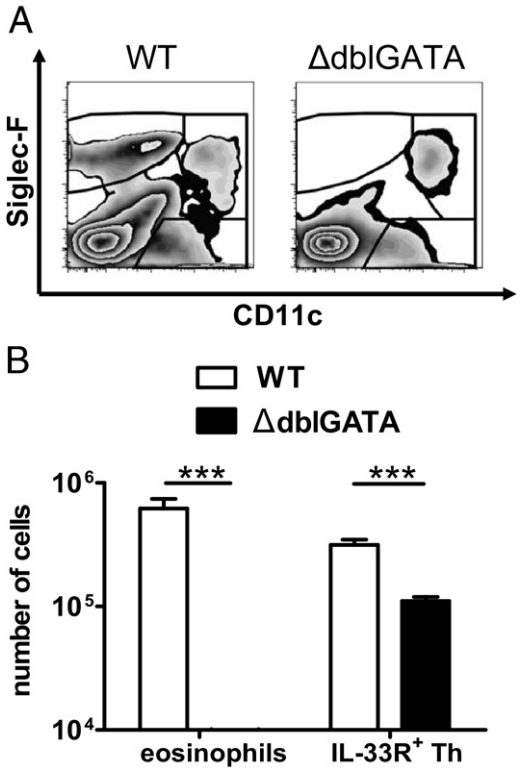

Figure 4. Mice devoid of eosinophils harbor fewer Th cells, indicated by IL-33R expression. Flow cytometry was performed on pulmonary leukocytes from i.n. infected BALB/c WT and ΔdblGATA mice at 60 dpi. **A:** The absence of eosinophils is confirmed by plots because no CD11c^{dim}/Siglec-F^{high} can be detected in the ΔdblGATA mice (**right**). A representative WT mouse is shown (**left**). **B:** Enumeration of total IL-33R$^+$ CD4$^+$ Th cells is shown, together with eosinophils. One of three independent experiments is shown (n = 6 to 7 per genotype). Values are given as the mean ± SEM. Statistical analysis was performed by using the Mann-Whitney U-test. ***$P < 0.001$.

can contribute to Th2 responses in cryptococcosis. Thus, we infected WT and eosinophil-deficient ΔdblGATA mice[21] i.n. with *C. neoformans*. The i.n. infection of WT mice led to accumulation of Siglec-F$^+$/CD11c^{dim} eosinophils in the lung, which was not the case for ΔdblGATA mice, as expected (Figure 4A). We then assessed the frequency of pulmonary Th2 cells in infected WT versus ΔdblGATA mice. In the absence of eosinophils, pulmonary Th2 cells, characterized by the expression of IL-33 receptor (IL-33R), are greatly reduced, but not completely lacking, in ΔdblGATA mice (Figure 4B). Earlier data demonstrated that the IL-33R, also termed T1/ST2, is specifically expressed on differentiated but not on naïve Th cells.[33]

Th cells are central regulators of anticryptococcal immune responses.[25,34,35] Although Th2 responses are detrimental,[11,15] Th1 and Th17 responses are protective.[7,9,25,35,36] To gain a deeper insight into the Th cytokine profile in the absence of eosinophils, we analyzed IL-4, IFN-γ, and IL-17A production by pulmonary Th cells on infection of WT and eosinophil-deficient ΔdblGATA mice. Analysis of IL-4, IFN-γ, and IL-17A in the supernatants of pulmonary leukocytes stimulated with cryptococcal antigen revealed reduced IL-4 and enhanced IFN-γ and IL-17 production by restimulated pulmonary leukocytes of ΔdblGATA versus WT mice (Figure 5, A–C). IL-4 production is substantially reduced, but not completely lacking, in pulmonary CD4$^+$ T cells from ΔdblGATA mice. This indicates a shift from Th2 to Th1/Th17 responses in the absence of eosinophils.

By direct intracellular staining of IL-4 in CD4$^+$ T cells (Figure 6), we corroborate the data shown in Figure 5, A–C, clearly demonstrating a pronounced Th2 response in *Cryptococcus*-infected WT mice. We were also able to demonstrate that only CD154$^+$ Th cells (ie, antigen-specific Th cells[37,38]) from infected mice responded with IL-4 production on stimulation with cryptococcal antigen (data not shown). Pulmonary Th cells from ΔdblGATA mice have similar proportions of Th1 cells, while they generate higher proportions of Th17 cells (Figure 6, antigen panel). A similar relative frequency of IFN-γ$^+$ Th1 cells (Figure 6) but elevated IFN-γ levels in supernatants of antigen specifically restimulated pulmonary leukocytes (Figure 5C), suggests higher IFN-γ production on a per-cell basis in Th1 cells from ΔdblGATA mice (Figure 6, C.n. antigen; IFN-γ mean fluorescence intensity, 529.30 for WT and 713.26 for ΔdblGATA). The hypothesis of a greater IFN-γ potency of ΔdblGATA Th cells on a single-cell basis is further supported by the results shown in Figure 5D (CD4$^+$ ionomycin/PMA; mean concentration of WT versus ΔdblGATA, 0.078 versus 0.835 pg/mL) because purified pulmonary Th cells were restimulated at an equal cell concentration when using this approach. Analysis of IL-4 from the same Th cells revealed comparable potency in IL-4 secretion (Figure 5E; CD4$^+$ ionomycin/PMA). Therefore, the reduced amounts of IL-4 shown in Figure 5A may result from fewer Th cells in the lungs of eosinophil-deficient mice (Figure 7B). More important, we detected a substantial amount of IL-4 in the Th-depleted fraction after ionomycin/PMA stimulation (Figure 5E; CD4$^-$ ionomycin/PMA; mean concentration of WT versus ΔdblGATA, 294 versus 0.056 pg/mL). Because ΔdblGATA mice are devoid of eosinophils (Figure 4A) and no other potential source(s) of IL-4 could be identified in this infection model, the detected IL-4 appears to depend on eosinophils in WT mice and reaches approximately one third of the Th-derived IL-4 (Figure 5E; CD4$^+$ ionomycin/PMA; mean concentration of WT versus ΔdblGATA, 890 versus 832 pg/mL). Consistent with lower IL-4 production in ΔdblGATA mice (Figure 5A), we observed substantially reduced IgE and elevated IgG2a (a marker for a Th1 response) serum levels in the absence of eosinophils (data not shown). Therefore, the presence of eosinophils contributes to enhanced fatal Th2 and reduced protective Th1 and Th17 responses.

Elevated Recruitment of Leukocytes in the Presence of Eosinophils

To study the pulmonary inflammatory response in the presence and absence of eosinophils, infected WT versus ΔdblGATA mice were analyzed at 60 dpi when significant eosinophils were present in the lungs of *C. neoformans*–infected 4get mice (Figure 2C). The pulmonary inflammatory response of infected 4get mice closely resembles WT mice (data not shown). Interestingly, more lung leukocytes were found in WT compared with

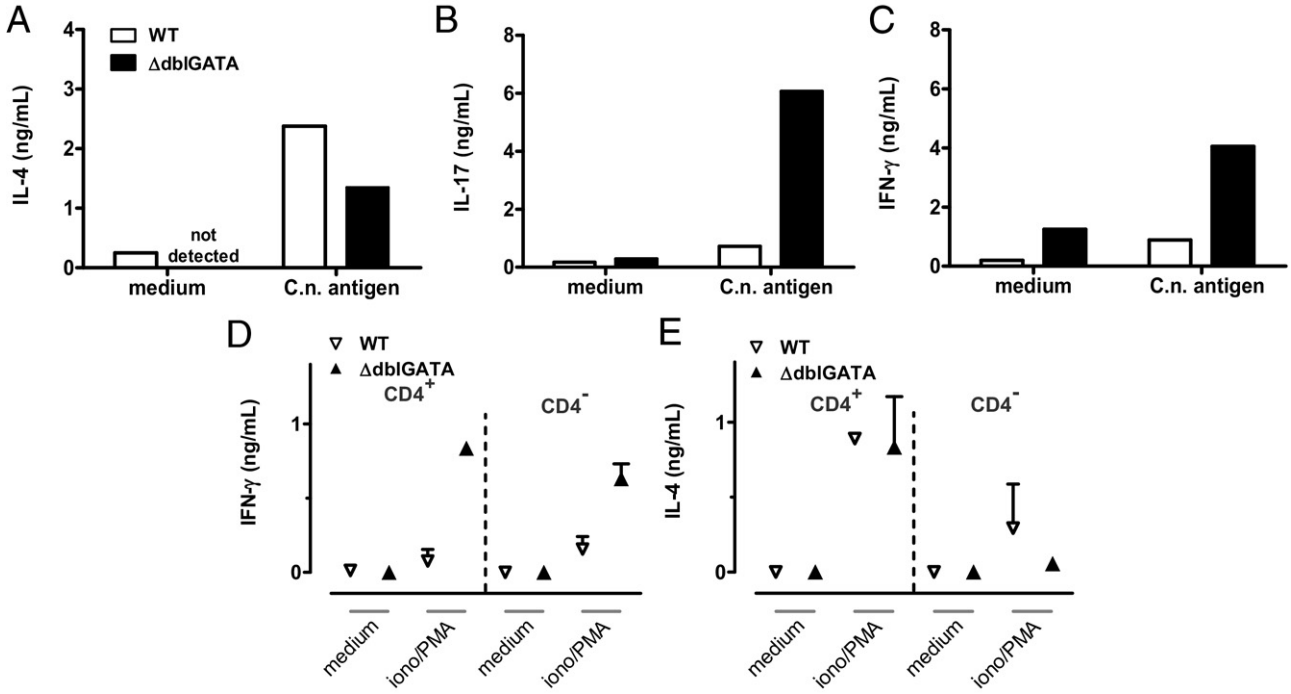

Figure 5. The absence of eosinophils promotes pronounced secretion of Th1- and Th17-associated cytokines and reduces the Th2 response. Pulmonary leukocytes were isolated from i.n. infected BALB/c WT and ΔdblGATA mice at 60 dpi. Cells were pooled per group and stimulated with C.n. antigen for 22 hours. **A** through **C:** IL-4, IL-17, and IFN-γ were measured by ELISA in culture supernatant, according to the *Materials and Methods* section. One representative of three independent experiments is shown (n = 6 to 7 per genotype). In addition, IFN-γ (**D**) and IL-4 (**E**) in the supernatant from MACS-enriched pulmonary $CD4^+$ Th cells and remaining cells either stimulated with ionomycin/PMA or left untreated for 2 hours from BALB/c WT and ΔdblGATA mice at 60 dpi are shown. Data were pooled from two independent experiments (n = 3 pooled mice per genotype and experiment).

ΔdblGATA mice (Figure 7A). An elevated frequency of total leukocytes in the presence of eosinophils corresponded with elevated numbers of Th cells in the lung (Figure 7B). We wanted to characterize the composition of other pulmonary leukocytes important in cryptococcosis. Macrophages are central effector cells that are able to direct the outcome of *C. neoformans* infection.[4,5,39,40] Alveolar and interstitial macrophages, and pulmonary dendritic cells were reduced in the absence of eosinophils (Figure 7, C–E). In summary, the data demonstrate a significant role of eosinophils in the recruitment of inflammatory cells on pulmonary infection with *C. neoformans*.

In the Absence of Eosinophils, Fungal Replication Is Reduced, but This Does Not Prevent Dissemination of C. neoformans *to the Brain*

Histopathological analysis of lungs from infected WT and ΔdblGATA mice revealed fewer and smaller foci of cryp-

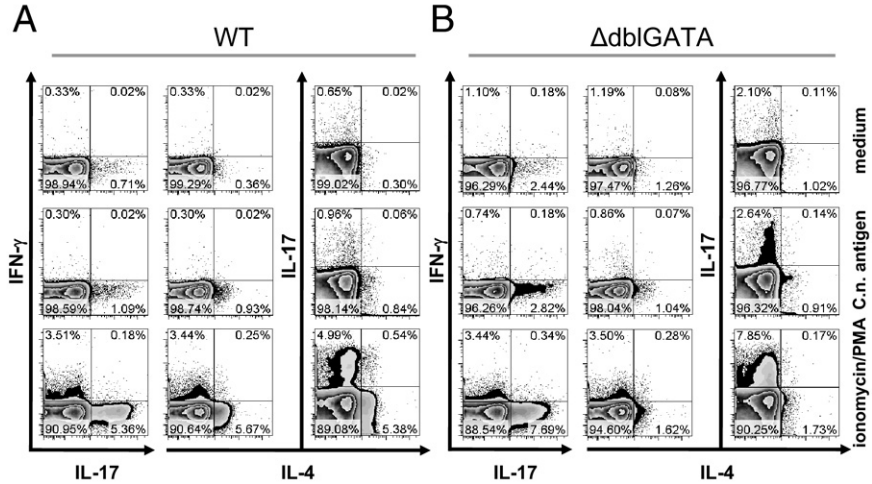

Figure 6. The Th cytokine profile is dependent on eosinophils on infection. Pulmonary leukocytes were isolated from i.n. infected BALB/c WT (**A**) and ΔdblGATA (**B**) mice at 60 dpi. Cells were pooled per group and allowed to rest for 22 hours as the negative control (**top**), stimulated with C.n. antigen for 22 hours (**middle**), or stimulated with ionomycin/PMA for 6 hours (**bottom**). Intracellular cytokine staining was performed according to the *Materials and Methods* section, and plots are gated on living $CD4^+$ Th cells. Appropriate isotype controls for staining Abs were used (data not shown), confirming the specific staining. One representative of three independent experiments is shown (n = 6 to 7 per genotype).

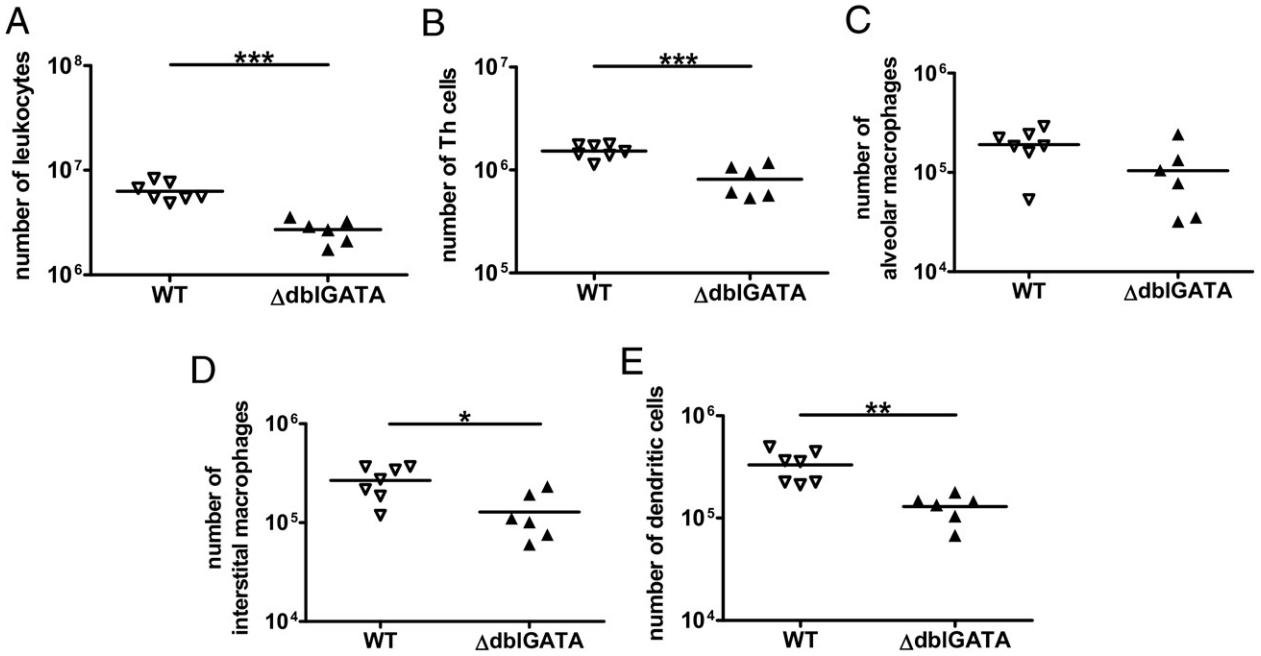

Figure 7. Recruitment of leukocytes to lung parenchyma after cryptococcal infection in the absence and presence of eosinophils. Pulmonary leukocytes were isolated from i.n. infected BALB/c WT and ΔdblGATA mice at 60 dpi. The numbers of total leukocytes (**A**), $CD4^+$ Th cells (**B**), alveolar macrophages (**C**), interstitial macrophages (**D**), and dendritic cells (**E**) are shown. One representative of three independent experiments is shown as the mean ± SEM ($n = 6$ to 7 per genotype). Statistical analysis was performed by using the Mann-Whitney *U*-test. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with BALB/c WT and ΔdblGATA mice.

tococci in the absence of eosinophils (Figure 8, A and B). Infected WT mice developed large aggregates of fungi in their lungs (Figure 8, C–F), which was also corroborated by detection of the cryptococcal capsular component glucuronoxylomannan (Figure 8, G and H). When we analyzed the total number of viable cryptococci contained in the lung, we observed substantially fewer (approximately 84-fold reduction of median fungal load) in ΔdblGATA mice compared with WT mice; however, this difference did not reach statistical significance (Figure 9A, $P = 0.0734$). In line with this finding, dissemination of *Cryptococcus* to the brain was not prevented in ΔdblGATA mice (Figure 9B, $P = 0.1375$). This indicates that the absence of eosinophils has a limited impact on protective pulmonary immunity against *C. neoformans* and does not suffice to prevent fungal dissemination.

Discussion

In this study, we report a regulatory role of eosinophilic granulocytes in cryptococcosis. Eosinophils have been mentioned before in murine models of cryptococcosis,[10,23,41–43] observed in human cryptococcosis,[44–46] and described with an emphasis on tissue damage.[41] Although *in vitro* eosinophils have phagocytosed *C. neoformans*[23] and presented cryptococcal antigens,[47] *in vivo*, no evidence for uptake of *C. neoformans* by eosinophils has been found by others[4] and in this study (data not shown). Herein, we highlight an immunoregulatory role of eosinophils that contribute to IL-4–dependent immunopathological features during murine pulmonary *C. neoformans* infection. We provide evidence for previously unrecognized features of eosinophils during bronchopulmonary infection. The protective immune response against *C. neoformans* relies on Th1-biased cellular immunity.[7,48] However, even in the presence of IFN-γ, IL-4 production has been detrimental in pulmonary cryptococcosis.[11] An exquisite role of IL-4 signaling strength has been demonstrated in our pulmonary cryptococcosis model, with a gene dosage effect of the IL-4 receptor α alleles.[18] Thus, in this report, we focus on nonprotective IL-4 production by Th cells and innate immune cells. In brief, the IL-4 competence of Th cells and eosinophils was determined by eGFP expression during 10 weeks of infection. Both cell populations show a concomitant late increase in lung parenchyma. The quantitative data obtained for IL-4 derived from Th cells and eosinophils indicate that Th2 cells are a major cellular source, followed by eosinophils as an innate cellular source of IL-4 (Figure 5E). Moreover, in cryptococcosis, eosinophils promote Th2 responses but are not essential for Th2 differentiation because we found a considerable residual frequency of $IL\text{-}33R^+$ Th2 cells in eosinophil-deficient mice (Figure 4B). Although the absence of eosinophils favors the development of a more Th1/Th17 pronounced response by modulating the Th cell cytokine secretory capacity (Figures 5, D and E, and 6), this cannot prevent dissemination of fungi, as shown by the brain cryptococcal burden (Figure 9B). Dissemination of cryptococci to the brain is only abrogated when IL-4, IL-13, or IL-4/IL-13 signaling is completely abolished.[15]

The Th2 promoting property of eosinophils has also been shown recently in a murine asthma model induced by an *Aspergillus fumigatus* extract.[49] Similar to pulmonary cryptococcosis, eosinophil-deficient ΔdblGATA mice showed reduced levels of pulmonary Th2-related cyto-

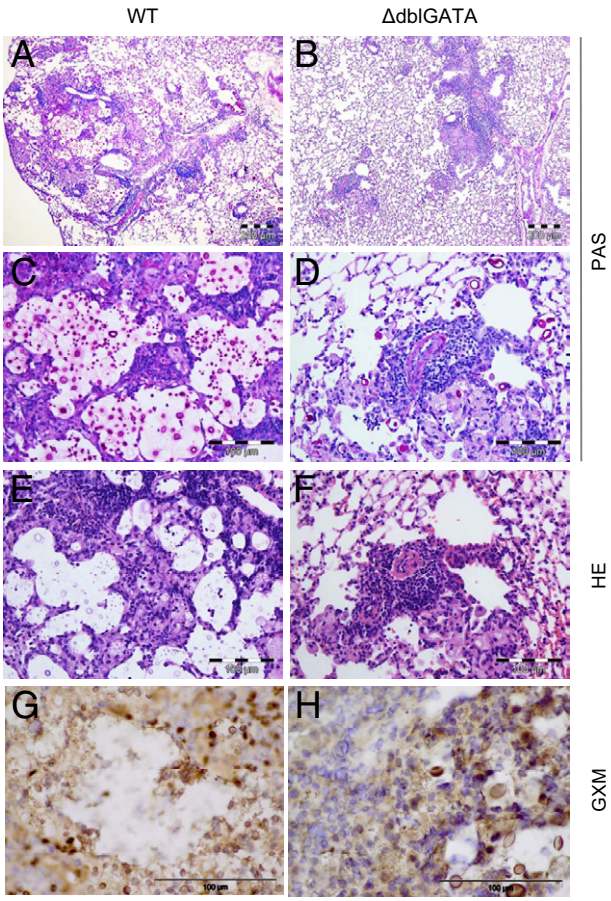

Figure 8. Lung sections from infected WT and ΔdblGATA mice indicate better fungal control in the absence of eosinophils at 60 dpi. **A** through **D:** PAS staining. Scale bars: 200 μm (**A** and **B**); 100 μm (**C** and **D**). **E** and **F:** H&E staining. Scale bar = 100 μm. **G** and **H:** IHC was performed on sections from the same mice, and glucuronoxylomannan (GXM)–containing foci are brown. Scale bar = 100 μm. ΔdblGATA mice show reduced numbers of cryptococci in the lungs (**A**–**F**) and formation of smaller foci of accumulating cryptococci and GXM compared with WT mice (**G** and **H**). There was pronounced influx of inflammatory cells in WT compared with ΔdblGATA mice.

kines and mononuclear cell recruitment.[49] The contribution of other innate immune cells (eg, basophils) potentially involved in fatal Th2 initiation in cryptococcosis remains to be tested, because basophils have been shown recently in models of parasitic disease and a protease allergen model to play an essential role in Th2 differentiation.[50–52]

In addition to well-described developmental requirements for Th1 and Th17 responses,[53,54] mechanisms of Th2 initiation are less unraveled and controversy on Th2-inducing molecules and cells continues.[32,33,54–58] Since the introduction of the Th1/Th2 paradigm, IL-4 has been tightly associated with Th2 responses[59–61]; there is clear evidence that IL-4 is indispensable for Th2 maintenance.[62] For Th2 initiation, current studies point to non-hematopoietic cells that appear to be able to support innate immune cells by secretion of chemokines[63] and novel cytokines, such as IL-33,[33] IL-25,[32,58] and thymic stromal lymphopoietin (TSLP).[64] In pulmonary cryptococcosis, airway epithelial cells and eosinophils would be candidates for cross talk between resident tissue cells and leukocytes.[65] Interestingly, we found expression of IL-33R on eosinophils in this study of pulmonary cryptococcosis (data not shown). Thus, eosinophils could be cellular targets of IL-33 produced by epithelial cells[66] and, thereby, could contribute to Th2 initiation. The definitive roles of IL-33, its cellular sources, and targets in anticryptococcal immunity remain to be defined.

Eosinophils were recognized for a long time as effector cells acting by degranulation in helminth/parasitic infections with *Trichinella spiralis*[67,68] or *Schistosoma mansoni*.[69] Eosinophils demonstrate protective mechanisms that rely on degranulation in bacterial infections[70] and release mitochondrial DNA in a unique way that clumps bacteria together.[71] In addition, antiviral effects have been reported.[72] Investigations of the role of eosinophils in fungal infection with *Candida albicans*,[73–75] *Alternaria alternata*,[76] or *C. neoformans*[10,23,41,42] were made, with a focus on their effector function. Similarly, a study[10] using anti-IL-5 treatment showed an association of eosinophil frequency with susceptibility during cryptococcosis. During the past decade, several reports[77,78] extended the function of eosinophils beyond the sole defense against nonphagotizable pathogens. Eosinophils produced various chemokines and cytokines modulating immune responses in different models.[29,49,79,80] Lee and colleagues[81] recently introduced the term LIAR (regulators of local immunity and/or remodeling/repair) for eosinophils; this term summarizes more recently discovered regulatory properties of eosinophilic granulocytes. Our data from a chronic fungal infection support the regulators of local immunity and/or remodeling/repair concept of eosinophil function. Other eosinophil-dependent factors, in addition to IL-4, that are involved in regulation of

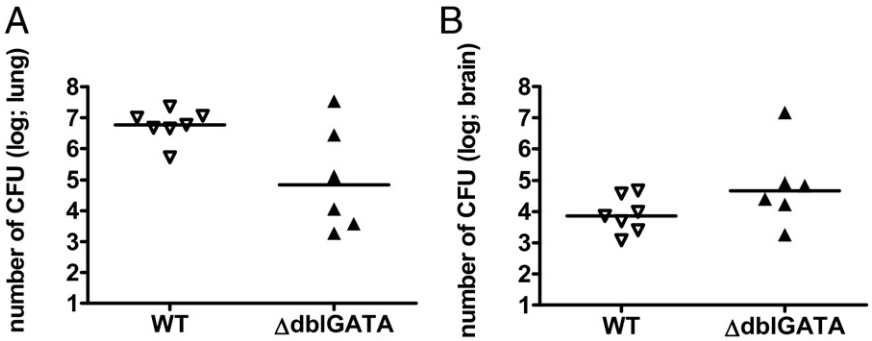

Figure 9. Organ burden in the presence and absence of eosinophils. Lung (**A**) and brain (**B**) fungal burden of WT and ΔdblGATA mice at 60 dpi was evaluated according to the *Materials and Methods* section. One representative of three independent experiments is shown as the median (n = 6 to 7 per genotype). Statistical analysis was performed by using the Mann-Whitney U-test, indicating $P = 0.0734$ for lung burden and $P = 0.1375$ for brain burden.

the Th cytokine profile and leukocyte recruitment require further investigation to enlighten the pathophysiological role that eosinophils play in cryptococcosis.

In conclusion, IL-4 production by both eosinophils and antigen-specific Th2 cells is a relatively late event in pulmonary cryptococcosis. A late and as of yet unidentified process appears to promote the onset of IL-4 production that dominates the production of otherwise protective cytokines IL-17 and IFN-γ. This suggests a cytokine hierarchy, with IL-4 on top of IFN-γ/IL-17 underlining the exquisite role of IL-4 in cryptococcosis. Therefore, it is intriguing to develop therapies antagonizing IL-4 or its receptor. Certainly, the late onset of IL-4 production by Th cells and eosinophils (shown herein) and functional studies in IL-4– or IL-4 receptor α–deficient mice, reported earlier by us,[18] make IL-4 or its receptor attractive drug targets in allergic bronchopulmonary mycosis and possibly in asthma.

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3.2.1 Preface to manuscript no. 2

It was now clear that interleukin (IL)-4 is secreted by two distinct haematopoietic cells which were recruited to the lungs. Removal of one source, the eosinophil, from the system revealed a non-redundant contribution by eosinophils to IL-4 production. The results derived from the manuscript "Eosinophils Contribute to IL-4 Production and Shape the T-Helper Cytokine Profile and Inflammatory Response in Pulmonary Cryptococcosis" shed new light on the work previously done and emphasize the importance of IL-4 production by innate and adaptive immune cells. In brief, use was made of mice expressing the IL-4 receptor (R) alpha (α) chain either in a bi-allelic (i.e. wild-type IL-4Rα$^{+/+}$ mice) or mono-allelic fashion (i.e. first-generation F1 IL-4Rα$^{+/-}$ mice). In addition, IL-4Rα gene-deficient mice (i.e. IL-4Rα$^{-/-}$ mice) were used to characterize the phenotype of mice completely lacking IL-4Rα expression. With these earlier experiments the idea of a system of gradual pathology arose that is ultimately linked to the magnitude of total consumable IL-4.

Major results

- ❖ Infection with *C. neoformans* does not lead to up-regulation of IL-4Rα. This is in contrast to other infection models where excessive amounts of IL-4 are produced (PERONA-WRIGHT et al. 2010).
- ❖ The gradual expression of IL-4Rα, i.e. from highest in wild-type ($^{+/+}$ bi-allelic) with intermediate in F1 ($^{+/-}$ mono-allelic) and no expression in total knockout ($^{-/-}$ no allele) mice reflects pulmonary organ burden.
- ❖ Increased levels of IL-4Rα expression negatively correlate with survival.
- ❖ Pathology-associated mucus production in airways and increasing airway resistance are results of elevated IL-4Rα expression.
- ❖ The total amount of serum immunoglobulin E parallel degree of IL-4Rα expression.
- ❖ Decreased levels of IL-4Rα favour the development of a Th17 response.

Conclusions

The gradual levels of IL-4Rα, i.e. none at all, mono- or bi-allelic expression, determine susceptibility in cryptococcosis. The intermediate phenotype of mono-allelic expressing mice reveals an *il-4rα* gene-dosage-dependent effect in a fungal infection model. This distinguishes the presented model of cryptococcosis from other infection models and argues for a tightly controlled IL-4 – IL-4R pathway, since there is no *in vivo* up-regulation of the IL-4Rα.

Animal experiments

The mice used for the publication "A gene-dosage effect for interleukin-4 receptor alpha-chain expression has an impact on Th2-mediated allergic inflammation during bronchopulmonary mycosis." belong to the record 24-9168.11 (TVV 36/04) (Landesdirektion Leipzig).

3.2.2 IL-4Rα gradually regulates susceptibility in cryptococcosis

MAJOR ARTICLE

A Gene-Dosage Effect for Interleukin-4 Receptor α-Chain Expression Has an Impact on Th2-Mediated Allergic Inflammation during Bronchopulmonary Mycosis

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Interleukin (IL)–4 and IL-13 are key factors in the pathogenesis of bronchopulmonary mycosis induced in mice by infection with *Cryptococcus neoformans*. Both cytokines use the IL-4 receptor α-chain (IL-4Rα). In this study, we investigated the role played by IL-4Rα expression in susceptibility to pulmonary *C. neoformans* infection. IL-4Rα$^{-/-}$ mice were extremely resistant. To characterize the effect of IL-4Rα expression level on disease outcome, we generated IL-4Rα$^{+/-}$ first-generation (F1) mice. IL-4Rα$^{+/-}$ mice showed intermediate levels of IL-4Rα expression, in contrast to higher levels in wild-type mice and no expression in IL-4Rα$^{-/-}$ mice, indicating biallelic expression of the gene for IL-4Rα *(Il4ra)*. Concomitant with intermediate IL-4Rα expression, F1 mice showed intermediate susceptibility associated with altered Th2/Th17 cytokine production, decreased immunoglobulin E levels, and reduced allergic inflammation. This indicates a gene-dosage effect of IL-4Rα expression on susceptibility to bronchopulmonary mycosis. These data provide the basis for novel therapies antagonizing IL-4Rα in Th2-related pulmonary infection and possibly also in asthma.

The interleukin (IL)–4 receptor α-chain (IL-4Rα) is ubiquitously expressed and is part of the receptor for the Th2 cytokines IL-4 and IL-13 [1], which have been shown to be involved in the fatal outcome of experimental murine cryptococcosis [2–6]. The regulation of Th2 responses is controlled by early IL-4 production, which induces the differentiation of naive T helper cells to Th2 cells [7]. Th2 cells, along with basophils, eosinophils, mast cells, NKT, and γδ T cells, are the main producers of IL-4 and IL-13. IL-4 acts as an important factor affecting B cells, causing isotype switching that leads to the production of IgG1 or IgE, antibody classes that are important for antiparasitic defense mechanisms.

However, these mechanisms are ineffective or even disease promoting against a facultative intracellular pathogen such as *Cryptococcus neoformans*. IL-13, on the other hand, can act on smooth muscle cells and goblet cells to cause bronchoconstriction and mucus production, respectively [8–10]. It is of interest that *C. neoformans* infection can contribute to asthma development, as shown in a rat model [11]. The IL-4– and IL-13–dependent mechanisms lead to the pathogenesis of pulmonary cryptococcosis, especially by induction of alternatively activated macrophages (aaMphs) [3, 5, 12]. Thus, the IL-4 receptor is likely a key regulator in the pathogenicity of cryptococcosis, but this has not yet been proved experimentally. Downstream of the IL-4

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receptor, the signal transduction cascade is controlled by signal transducer and activator of transcription (STAT) 6 [13, 14].

In the present study, we sought to determine whether antagonism of Th2 development during pulmonary cryptococcosis by IL-4Rα ablation (i.e., using IL-4Rα$^{-/-}$ mice) has an additive or synergistic effect compared with antagonism of individual ligands (i.e., using IL-4$^{-/-}$ or IL-13$^{-/-}$ mice). Moreover, we wished to clarify whether the gene for IL-4Rα *(Il4ra)* is expressed biallelically and whether 1 allele containing functional IL-4Rα (i.e., using IL-4Rα$^{+/-}$ mice) is sufficient to induce a full or only a gradual Th2 response in cryptococcosis. Our findings unambiguously demonstrate that *Il4ra* is indeed expressed biallelically and that its expression level is critical for susceptibility to experimentally induced bronchopulmonary mycosis. Depending on the IL-4Rα expression level, there is a gradual appearance of Th2-dependent mechanisms, such as IgE production, allergic inflammation with eosinophilia, goblet cell metaplasia, mucus hyperproduction, and alternative macrophage activation after pulmonary *C. neoformans* infection.

METHODS

Mice. Female mice (6–10 weeks old) included 3 groups: wild-type (WT; IL-4Rα$^{+/+}$), IL-4Rα$^{-/-}$ mice on a BALB/c background [15], and the first generation of WT by IL-4Rα$^{-/-}$ intercrosses (IL-4Rα$^{+/-}$). They were maintained in an individually ventilated caging system under specific pathogen–free conditions and in accordance with the guidelines approved by the Animal Care and Usage Committee of the Regierungspräsidium Leipzig. Sterile food and water were given ad libitum. The mice were tested periodically for pathogens, in accordance with the recommendations for health monitoring of mice provided by the Federation of European Laboratory Animal Science Associations accreditation board.

C. neoformans *and infection of mice.* Encapsulated *C. neoformans* (strain 1841, serotype D) was kept as frozen stock in skim milk and was grown in Sabouraud dextrose medium (2% glucose and 1% peptone; Sigma) overnight on a shaker at 30°C. The mice were infected intranasally, and the acapsular *C. neoformans* serotype D strain CAP67 was used as an in vitro stimulus, both as described elsewhere [5].

Determination of survival rate and organ burden. Infected mice were monitored daily for survival and morbidity. Organ burden was determined as described elsewhere [5].

Restimulation of spleen. Splenocytes were stimulated as described elsewhere [5].

Histopathological analysis. On day 70 after infection, *C. neoformans*–infected WT, IL-4Rα$^{-/-}$, and IL-4Rα$^{+/-}$ mice as well as uninfected mice of the same genotypes were perfused intracardially with 0.9% saline while under deep CO_2 asphyxia. The lungs of the animals were removed, mounted on thick filter paper with Tissue-Tek OTC compound (Miles Scientific), snap-frozen in isopentane (Fluka) precooled on dry ice, and stored at −80°C.

For immunohistochemical analysis, 10-μm frozen sections were prepared in a serial fashion (15 transversal sections per lung on 4 consecutive levels). The YM1 (ECF-L) goat anti-mouse antibody was used to detect aaMphs (R&D Systems). Arginase-1 (BD Biosciences) immunostaining was performed using the Dako ARK peroxidase kit, in accordance with the manufacturer's protocol. Immunohistochemical analysis was performed as described elsewhere [16]. Negative controls, without application of the primary antibody, confirmed the specificity of the reactions.

Other parts of the lungs and of the other organs were fixed in neutral-buffered formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin (H&E) to estimate the extent of granulomatous lesion formation in the various organs or with periodic acid–Schiff reagent to study the distribution of cryptococci and mucus production by goblet cells in lungs, liver, spleen, and kidneys. Analyses of collagen deposition in the organs were done by elastica–van Gieson staining. To study the recruitment of granulocytes, the tissue sections were stained with naphthol AS-D-chloracetate esterase. Histopathological alterations were microscopically evaluated on H&E–stained and immunostained lung sections.

Digestion of lung tissue and analysis of lung leukocytes and blood cells. For IL-4Rα expression experiments, lungs were perfused through the right ventricle with PBS. The lungs were treated as described elsewhere [5]. IL-4Rα expression in cells was analyzed by flow cytometry (FACSCalibur; BD). The cells were stained for IL-4R (CD124; clone mIL4R-M1; BD; phycoerythrin conjugate for staining of lung cells; biotinylated M1 antibody in combination with streptavidin-allophycocyanin [BD] for blood leukocytes), T helper cells (CD4; clone H129.19; BD), cytotoxic T cells (CD8; clone 53–6.7; BD), B cells (CD45R; clone RA3-6B2; Caltag), NK cells (DX-5; clone DX5; BD), granulocytes (Gr-1; clone RB6-8C5; BD), dendritic cells (CD11c; clone HL3; BD), and macrophages (CD11c^{-}/Gr-1^{-}/CD11b^{+}; clone M1/70.15; Caltag).

Cytokine and antibody analysis. Cytokine concentrations were determined by sandwich ELISA systems using unlabeled capture antibodies and labeled detection antibodies, followed by incubation with peroxidase-labeled streptavidin if not otherwise indicated, as described elsewhere [5]. The total serum IgE, IgG1, and IgG2a concentrations in naive and infected mice were measured as detailed elsewhere [5].

Measurement of respiratory lung function. The respiratory lung function of intranasally infected mice was measured in a plethysmographic chamber (model PLT UNR MS; Emka Technologies) for freely moving animals. The pressure inside the chamber was measured by a differential pressure transducer connected to an amplifier (model AMP-B01) and continuously

monitored with IOX software (version 22.17.19). Airway hyperreactivity was examined as described elsewhere [17].

Statistical analysis. The statistical significance of differences between experimental groups of animals was determined using the log-rank test for survival analysis, the 1-tailed Mann-Whitney *U* test for organ burden and fluorescence-activated cell sorter (FACS) analysis, and the 2-tailed Mann-Whitney *U* test for cytokine, isotype levels, and airway hyperreactivity. Differences for which $P < .05$ were considered significant.

RESULTS

Susceptibility to pulmonary* C. neoformans *infection determined by IL-4Rα expression level. We and others have shown that each of the Th2 cytokines IL-4 and IL-13 is associated with susceptibility to *C. neoformans* infection [2–6]. To analyze the effect of simultaneous abrogation of IL-4 and IL-13 on *C. neoformans* infection, we used IL-4Rα$^{-/-}$ mice, which were deficient in the common receptor chain of the IL-4 and IL-13 receptor [18]. The survival of *C. neoformans*–infected IL-4– or IL-13–deficient mice was compared with that of IL-4Rα–deficient mice. In these experiments, the IL-4Rα–deficient (IL-4Rα$^{-/-}$) mice were more resistant than the ligand-deficient mice, which were significantly more resistant than the WT (IL-4Rα$^{+/+}$) mice (proportion of surviving mice in total from 3 independent experiments: for IL-4Rα$^{-/-}$ mice, 26/26; for IL-4$^{-/-}$ mice, 24/25; for IL-13$^{-/-}$ mice, 24/28). Although 100% of the IL-4Rα$^{+/+}$ mice died of the infection, the IL-4Rα$^{-/-}$ mice were completely resistant during an observation period of >200 days after infection (up to 275 days after infection) (figure 1*A*). Examination of the organ burden in lung and brain at day 70 after infection showed significantly reduced numbers of cryptococci in the lungs of the IL-4Rα$^{-/-}$ mice (figure 1*B*). In the absence of IL-4Rα expression, dissemination of *C. neoformans* to the brain is prevented (figure 1*C*). Long-term examination for >200 days after infection (up to 275 days after infection) revealed that stable control of the fungal burden in the lung is achieved in IL-4Rα$^{-/-}$ mice, but not sterile elimination (figure 1*B*).

In light of the contrasting phenotypes observed in infected IL-4Rα$^{+/+}$ versus IL-4Rα$^{-/-}$ mice, we wanted to learn whether an intermediate level of expression of IL-4Rα would have an effect on the resistance or susceptibility to pulmonary *C. neoformans* infection. Therefore, IL-4Rα heterozygous (IL-4Rα$^{+/-}$ mice) were generated. To determine the relative expression of IL-4Rα on leukocytes of naive and *C. neoformans*–infected IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ mice, we determined the median fluorescence intensity by flow cytometry, using a monoclonal antibody specific for IL-4Rα. Expression of IL-4Rα was characterized for leukocytes in lungs, blood (table 1), brain, lymph nodes, and spleen (data not shown). Interestingly, IL-4Rα expression in all examined tissues of naive IL-4Rα$^{+/-}$ mice showed an intermediate level between the high levels found in IL-4Rα$^{+/+}$ mice and

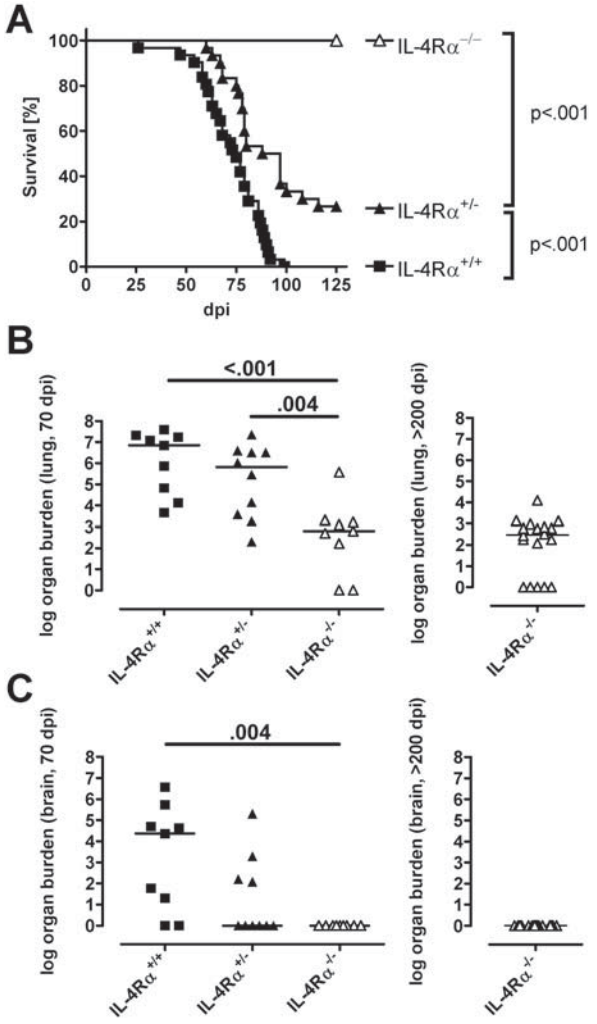

Figure 1. Resistance of homozygous and prolonged survival of heterozygous interleukin (IL)–4 receptor α-chain (IL-4Rα)–deficient mice relative to susceptible wild-type (WT) mice during pulmonary *Cryptococcus neoformans* infection. IL-4Rα$^{+/+}$, IL-4Rα$^{+/-}$, and IL-4Rα$^{-/-}$ mice were intranasally infected with 500 cfu of *C. neoformans (A)*. The survival time of mice was recorded for 150 days after infection. Although no IL-4Rα$^{-/-}$ mice died of the infection, IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ mice had median survival times of 75 and 92.5 days, respectively; 26.7% of IL-4Rα$^{+/-}$ mice survived for >150 days after infection. In one experiment the mice were examined for 272 days after infection, and 1 of 12 survived the whole period. The survival graph represents data from 3 independent experiments; the log-rank test was used for statistical analysis. Organ burdens in lungs *(B)* and brain *(C)* were analyzed on day 70 after infection and, for IL-4Rα$^{-/-}$ mice, on days 202, 246, 272, and 275 after infection as well (pooled as >200 days after infection). IL-4Rα$^{-/-}$ mice had significantly lower organ burdens in lungs and brain than did IL-4Rα$^{+/+}$ mice. IL-4Rα$^{+/-}$ mice tended to have lower organ burdens than the WT mice. IL-4Rα$^{-/-}$ mice did not completely eliminate the cryptococci but had low organ burdens even beyond 200 days after infection. There were no differences in the lung burdens of the IL-4Rα$^{-/-}$ mice between 70 and >200 days after infection. Data from 3 independent experiments were pooled. Significance was calculated with the 1-tailed Mann-Whitney *U* test. dpi, days post infection.

Table 1. Interleukin (IL)–4 receptor α-chain (IL-4Rα) expression in blood leukocytes and lung cells of naive and *Cryptococcus neoformans*–infected mice 70 days after infection.

Cell type	Median fluorescence intensity		
	IL-4Rα$^{+/+}$ mice	IL-4Rα$^{+/-}$ mice	IL-4Rα$^{-/-}$ mice
Naive PBLs			
T helper cells	53.61	32.93[a]	11.05[a,b]
Cytotoxic T cells	56.20	41.28[a]	14.67[a,b]
B cells	57.38	27.14	16.35
Macrophages	47.92	27.79	20.28[a]
Granulocytes	33.50	24.07[a]	21.46[a]
Dendritic cells	29.25	16.70	16.38[a]
NK cells	31.10	24.95	15.94
Lung cells			
Naive			
All lung cells	5.75	4.16	2.88
CD3$^+$ lung cells	6.58	3.57	1.86
CD11b$^+$ lung cells	2.94	2.56	2.32
70 days after infection			
All lung cells	4.54	3.52[a]	2.68[a]
CD3$^+$ lung cells	4.28	3.20[a]	2.24[a]
CD11b$^+$ lung cells	3.19	2.51[a]	2.81

NOTE. Heterozygous IL-4Rα–deficient mice have an intermediate level of IL-4Rα expression in blood and lung leukocytes. Although the fluorescence intensity of IL-4Rα in IL-4Rα$^{-/-}$ mice marks the minimum median fluorescence intensity and IL-4Rα$^{+/+}$ leukocytes show the maximum value of IL-4Rα expression, intermediate expression levels of IL-4Rα were found in IL-4Rα$^{+/-}$ leukocytes. For peripheral blood leukocytes (PBLs), each value represents blood analyses for 5–9 naive mice from 4 independent experiments. For lung cells, data are shown from 1 of 2 similar experiments with lung cells from naive mice and pooled data from 2 independent experiments 70 days after infection.

[a] $P < .05$ for the comparison with IL-4Rα$^{+/+}$ mice (1-tailed Mann-Whitney *U* test).

[b] $P < .05$ for the comparison with IL-4Rα$^{+/-}$ mice (1-tailed Mann-Whitney *U* test).

the absent expression in IL-4Rα$^{-/-}$ mice. This argues for biallelic expression of *Il4ra*. The levels of IL-4Rα expression were especially high in lymphocytes compared with innate immune cells (table 1). No difference could be found between the IL-4Rα expression levels of IL-4Rα$^{+/-}$ and IL-4Rα$^{-/-}$ phagocytes and dendritic cells. This may be related to the detection limit of the FACS method (owing to the lower IL-4Rα expression in innate immune cells) or to monoallelic expression of IL-4Rα in phagocytes and dendritic cells in contrast to lymphocytes (table 1). Importantly, intranasal infection of mice with the highly virulent *C. neoformans* strain 1841 did not modulate IL-4Rα expression in lung cells (table 1) or blood leukocytes (data not shown).

To define the susceptibility of IL-4Rα$^{+/-}$ mice expressing intermediate levels of IL-4Rα against *C. neoformans*, we infected IL-4Rα$^{+/-}$ mice intranasally, together with IL-4Rα$^{+/+}$ and IL-4Rα$^{-/-}$ mice. Strikingly, *C. neoformans*–infected IL-4Rα$^{+/-}$ mice exhibited a significantly prolonged median survival time compared with IL-4Rα$^{+/+}$ mice (92.5 vs. 75 days after infection) (figure 1*A*). In accordance with the lower susceptibility found in infected IL-4Rα$^{+/-}$ mice, the lung and brain fungal burden of the IL-4Rα$^{+/-}$ mice was reduced (figure 1*B*). This finding indicates a gene-dosage effect of IL-4Rα expression on resistance to pulmonary infection with *C. neoformans*.

Degree of fatal Th2 induction induced by* C. neoformans *infection determined by IL-4Rα expression level. To define the mechanism(s) responsible for the partial resistance of the heterozygous IL-4Rα$^{+/-}$ mice, immune parameters were investigated. It has been shown elsewhere that development of protective Th1 versus pathological Th2 responses is critical for resistance rather than susceptibility to *C. neoformans* infection [19–22]. Interestingly, the absence of a Th2 response appears to be even more important than the magnitude of the Th1 response for the course of cryptococcosis [3, 5]. The Th2-dependent isotypes IgE and IgG1 were chosen as in vivo markers of Th2 development. Although serum IgE was significantly reduced after *C. neoformans* infection of IL-4Rα$^{+/-}$ mice compared with IL-4Rα$^{+/+}$ mice, the former group showed comparably elevated levels of IgG1 (table 2). Both IgE and IgG1 levels were significantly reduced in IL-4Rα$^{-/-}$ mice; IgE was not even detectable in infected IL-4Rα$^{-/-}$ mice. Naive mice of the IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ genotypes showed comparably low IgE levels (table 2). Thus, induction of IgE is strictly IL-4Rα dependent (table 2) and correlates with the IL-4Rα expression level in leukocytes, as shown above (table 1). Furthermore, with IgE as an important marker of fatal Th2 development during cryptococcosis, the data showed that IL-4Rα expression levels critically determine the degree of Th2-mediated susceptibility. For the Th1-related isotype IgG2a, no differences were found between the 3 genotypes. Compared with naive mice, all 3 groups showed comparably

Table 2. Serum IgE and IgG1 levels in naive and *Cryptococcus neoformans*–infected mice 70 days after infection.

Immunoglobulin	Serum concentration, μg/mL		
	IL-4Rα$^{+/+}$ mice	IL-4Rα$^{+/-}$ mice	IL-4Rα$^{-/-}$ mice
IgE			
Naive	0.97	1.42	0.00
70 days after infection	24.50	4.02[a]	0.00
IgG1			
Naive	58.04	24.07	22.75
70 days after infection	701.08	1173.94	66.83[a,b]

NOTE. Intermediate interleukin (IL)–4 receptor α-chain (IL-4Rα) expression leads to reduced IgE but comparable IgG1 levels after infection with *C. neoformans*. In infected mice, the serum levels of IgE differ significantly between these genotypes, whereas naive IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ mice show comparable levels of IgE and IgG1. Values shown are medians from 3–4 experiments with 3 mice per experiment.

[a] $P < .01$ for the comparison with IL-4Rα$^{+/+}$ mice (2-tailed Mann-Whitney *U* test).

[b] $P < .001$ for the comparison with IL-4Rα$^{+/-}$ mice (2-tailed Mann-Whitney *U* test).

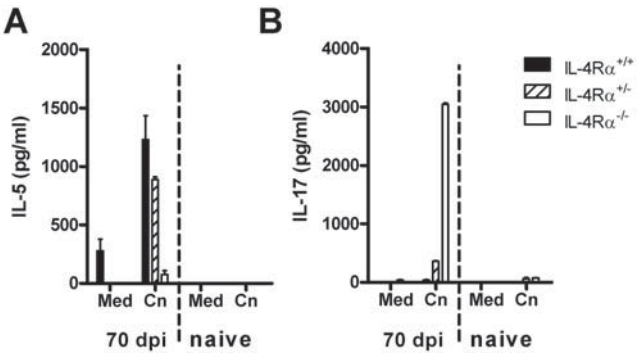

Figure 2. Dependence of antigen-specific production of Th2 *(A)* and Th17 *(B)* cytokines on the level of expression of interleukin (IL)–4 receptor α-chain (IL-4Rα). Restimulated splenocytes from naive and infected mice 70 days after infection produced *Cryptococcus neoformans*–specific IL-5 and IL-17. Although the IL-5 level increased with IL-4Rα expression, IL-17 production decreased. Data from 1 of 2 independent experiments are shown. Cn, heat-killed cryptococci (MOI of 10); dpi, days post infection; med, medium.

elevated serum IgG2a levels on day 70 after *C. neoformans* infection (data not shown).

To assess Th2 responses more directly in IL-4Rα$^{+/+}$, IL-4Rα$^{+/-}$, and IL-4Rα$^{-/-}$ mice infected intranasally with *C. neoformans,* we analyzed ex vivo cytokine production by splenocytes. Because IL-4 was consumed by cultured WT (IL-4Rα$^{+/+}$) splenocytes [23] but to a lesser degree by IL-4Rα$^{+/-}$ splenocytes and not at all by IL-4Rα$^{-/-}$ splenocytes, it was difficult to interpret antigen-specific production of IL-4. Therefore, we studied IL-5, another Th2 cytokine that has been characterized as being associated with the immunopathology of cryptococcosis [3, 5]. Antigen-specific stimulation of splenocytes with heat-killed cryptococci led to high levels of IL-5 production by splenocytes derived from susceptible IL-4Rα$^{+/+}$ mice, whereas splenocytes from partially resistant IL-4Rα$^{+/-}$ mice and totally resistant IL-4Rα$^{-/-}$ mice showed gradually reduced IL-5 production (figure 2*A*). In addition, splenocytic IL-17 production, recently shown by us to be associated with protection in *C. neoformans* infection [24], was found to be gradually elevated in partially resistant IL-4Rα$^{+/-}$ mice and totally resistant IL-4Rα$^{-/-}$ mice, compared with susceptible IL-4Rα$^{+/+}$ mice (figure 2*B*).

Nature of the pulmonary inflammatory response to* C. neoformans *infection determined by IL-4Rα expression level, which affects lung function. The pulmonary inflammatory response to *C. neoformans* infection crucially depends on the nature of the T cell response, that is, a protective Th1/Th17 versus a nonprotective Th2 response [6, 19, 24–26]. Th2 responses are associated with the appearance of eosinophils, the modulation of goblet cells, and alternative macrophage activation. In accordance with lower IL-5 production (figure 2*A*), fewer eosinophils were detected in the lungs of partially resistant IL-4Rα$^{+/-}$ mice compared with IL-4Rα$^{+/+}$ mice with pronounced cryptococcosis (figure 3*A* and 3*B*). The goblet cell metaplasia and mucus hyperproduction found in susceptible *Cryptococcus*-infected IL-4Rα$^{+/+}$ mice was strongly reduced in IL-4Rα$^{+/-}$ mice (figure 3*D* and 3*E*). No eosinophils and no alteration of goblet cells could be found in the highly resistant IL-4Rα$^{-/-}$ mice (figure 3*C* and 3*F*). Therefore, the allergic inflammatory response of the lungs reflects the IL-4Rα expression levels responsible for susceptibility against *C. neoformans* infection (figure 1 and table 1).

Alternative activation of lung macrophages is associated with a fatal course of cryptococcosis [3, 5, 12]. The immunostaining of chitinase-like YM1 and arginase-1, both markers of aaMphs, revealed only marginal expression of YM1 and arginase-1 in the lungs of infected IL-4Rα$^{-/-}$ mice but strongly enhanced expression of both in IL-4Rα$^{+/+}$ mice (figure 3*G,* 3*I,* 3*J,* and 3*L*). The number of aaMphs was found to be reduced in the heterozygous mice compared with IL-4Rα$^{+/+}$ mice (figure 3*G,* 3*H,* 3*J,* and 3*K*), again pointing to an intermediate phenotype of IL-4Rα$^{+/-}$ mice. YM1^{+} cells appeared voluminous and stained strongly positive for macrophage markers, such as F4/80 and CD11b (data not shown). The lungs of IL-4Rα$^{+/+}$ mice showed massive focal accumulations of YM1^{+} macrophages. In contrast, the distribution of YM1^{+} macrophages in IL-4Rα$^{+/-}$ mice was more diffuse, and these cells appeared smaller and more compact. These results provide evidence for IL-4Rα–dependent development of aaMphs in *C. neoformans* infection.

To see whether the IL-4Rα–dependent pulmonary inflammatory response had a functional consequence, we studied airway hyperreactivity in the 3 mouse groups. After receipt of high doses of the asthma-inducing agent methacholine, *C. neoformans*–infected IL-4Rα$^{+/+}$ mice displayed significantly elevated airway hyperreactivity compared with IL-4R α$^{-/-}$mice (figure 3*M*). IL-4Rα$^{+/-}$ mice showed a phenotype between those of WT and IL-4Rα$^{-/-}$ mice, an important hint for a critical effect of the IL-4Rα expression level on lung function.

DISCUSSION

Susceptibility to *C. neoformans* infection is critically correlated with Th2 development [2–4, 6]. In the present study, we chose to target IL-4Rα to interfere simultaneously with the fatal action of IL-4 and IL-13. Interestingly, IL-4Rα$^{-/-}$ mice were found to acquire a particularly high degree of protective immunity associated with complete control of brain infection and long-term survival. As expected, targeting the shared receptor IL-4Rα was more effective than targeting the individual ligands (especially IL-13) in protecting against *C. neoformans* infection [5]. Interestingly, IL-4Rα$^{-/-}$ mice were unable to eliminate *C. neoformans* in the lungs during a prolonged period (up to 275 days after infection) but were very well able to control the pulmonary fungal load at a stable level between 70 and 275 days after infection. This shows the impressive efficacy of antagonizing Th2 activity to prevent reactivation of *C. neoformans* infection from the

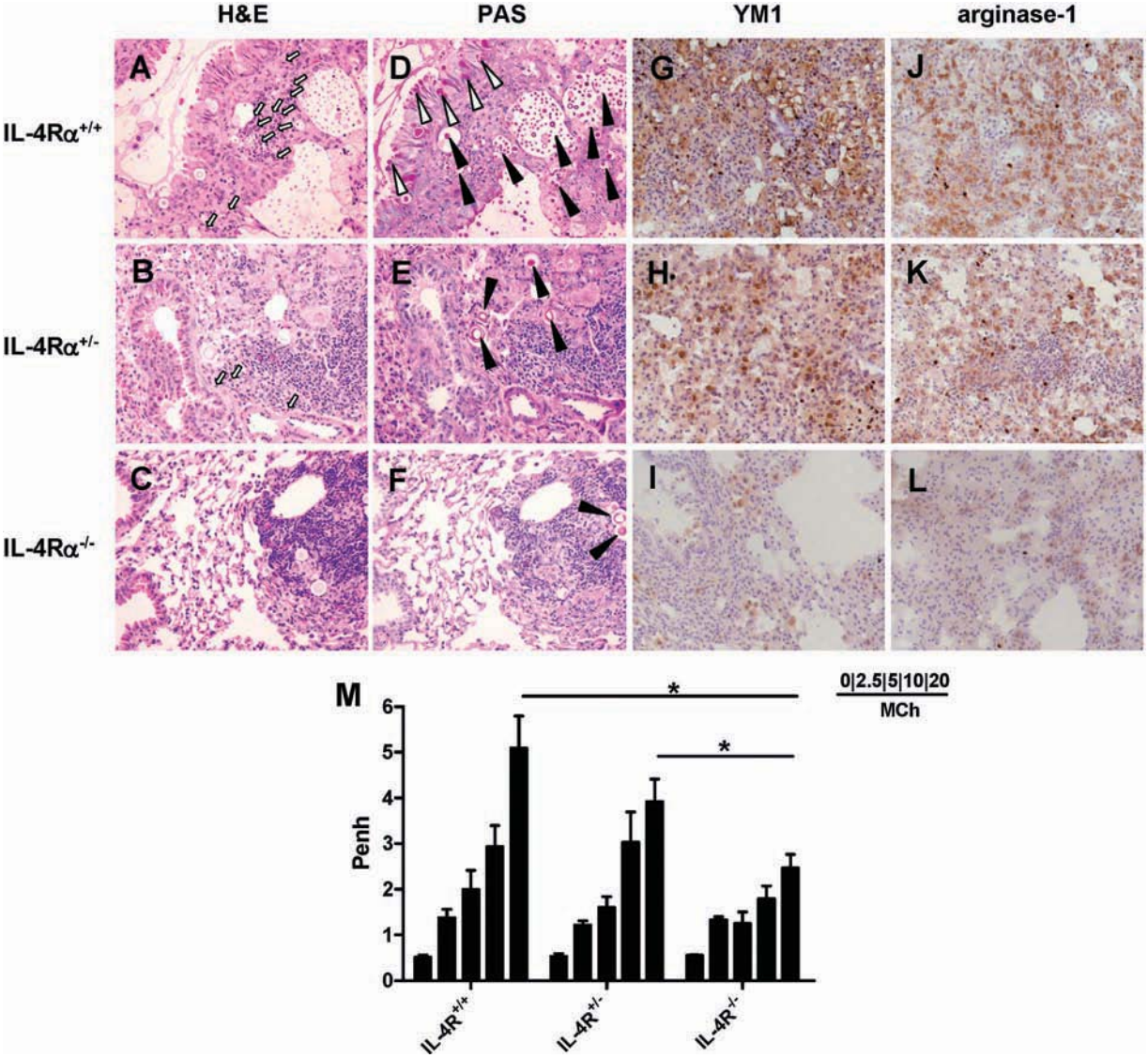

Figure 3. Reduced pulmonary eosinophilia, reduced nos. of alternatively activated macrophages (aaMphs), goblet cell metaplasia, and mucus production associated with decreased airway hyperreactivity resulting from lower expression of interleukin (IL)–4 receptor α-chain (IL-4Rα). The degree of susceptibility correlates with the nos. of eosinophils *(white arrows),* goblet cell metaplasia and mucus production *(white arrowheads),* and organ burden *(black arrowheads). A–C,* Eosinophils. Large nos. of eosinophils could be detected in the lungs of *Cryptococcus neoformans*–infected IL-4Rα$^{+/+}$ mice on day 70 after infection, whereas, in IL-4Rα$^{-/-}$ mice, no eosinophils could be detected. IL-4Rα$^{+/-}$ mice represent an intermediate phenotype; all eosinophils in the high-power field are marked *(arrows),* in contrast to the IL-4Rα$^{+/+}$ mice, for which only some of the eosinophils are marked by arrows. H&E, hematoxylin-eosin. *D–F,* Lung burden. The organ burden in the lungs correlated with the degree of susceptibility in the examined genotypes. Although IL-4Rα$^{+/+}$ mice showed large amounts of *C. neoformans*, the IL-4Rα$^{-/-}$ mice displayed only minimal nos. of fungal pathogens on day 70 after infection. The IL-4Rα$^{+/-}$ mice represented an intermediate phenotype, with reduced nos. of cryptococci. In addition, the IL-4Rα$^{+/+}$ mice displayed profound goblet cell metaplasia with mucus production, whereas IL-4Rα$^{+/-}$ mice showed only reduced and focally occurring metaplasia. In IL-4Rα$^{-/-}$ mice, no goblet cell metaplasia and mucus hyperproduction could be detected. *G–L,* aaMphs. Focal dense accumulations of large, rounded aaMphs, positive for YM1 *(G–I)* and arginase-1 *(J–L),* were detected in IL-4Rα$^{+/+}$ mice. YM1- and arginase-expressing aaMphs could also be found in IL-4Rα$^{+/-}$ mice, albeit with a more diffuse and loose distribution, but IL-4Rα$^{-/-}$ mice exhibited no significant accumulations of this cell type. Micrographs representative of 2 independent experiments are shown. *M,* Airway hyperreactivity. The airway hyperreactivity of the infected genotypes was compared between the indicated groups on day 70 after infection. Data from 2 independent experiments were pooled. MCh, methacholine (in μg/mL); Penh, enhanced pause. *$P < .05$.

lungs. Moreover, our data indicate, for the first time, a gene-dosage effect of IL-4Rα in anti-infective immunity.

The data from *C. neoformans*–infected IL-4Rα$^{-/-}$ mice do not allow us to draw conclusions on the individual contribution of IL-4 versus IL-13. Certainly, the survival rates of IL-4$^{-/-}$ mice (96%) and IL-13$^{-/-}$ mice (86% and [5]) suggest that the action of IL-4 is somewhat more important than that of IL-13 for susceptibility during cryptococcosis. However, as we have shown

recently, goblet cell metaplasia and mucus production during pulmonary cryptococcosis are IL-13 dependent [5]; this may even contribute to enhanced airway hyperreactivity (figure 3*M* and [5]). Owing to the reduced goblet cell metaplasia and mucus production found in infected IL-4Rα$^{+/-}$ mice (figure 3*D*), IL-13 definitely plays a role in susceptibility different from that played by IL-4.

The strikingly contrasting phenotypes of WT versus IL-4Rα$^{-/-}$ mice prompted us to determine whether an intermediate level of expression of IL-4Rα would also result in intermediate susceptibility. Indeed, our study describing IL-4Rα expression level–dependent immunity to *C. neoformans* infection is, to the best of our knowledge, the first example of a gene-dosage effect of IL-4Rα in antimicrobial immunity. Infection with *C. neoformans* appears to be different from a number of other infections studied, for which the level of IL-4Rα expression has not been found to be critical for IL-4/IL-13 responsiveness.

In *Leishmania* and *Schistosoma* infection models, heterozygous IL-4Rα mice were found to have phenotypic and immunological responses similar to those in WT mice [27, 28]. It is intriguing to speculate that, in these models, higher IL-4 levels are generated, making it impossible to distinguish between IL-4Rα$^{+/+}$ and IL-4Rα$^{+/-}$ mice. Presumably, both the level of IL-4 production and the level of IL-4Rα expression together regulate the intensity of Th2-driven disease. Small changes in the level of IL-4Rα expression may be particularly limiting with low-level IL-4 production (as may be the case for *C. neoformans* infection).

As shown earlier, IL-4Rα up-regulation is dependent on the concentration of IL-4 [29]. With higher IL-4 levels produced, low or intermediate levels of IL-4Rα may be up-regulated and less limiting. It is noteworthy that we could not find modulation or even up-regulation of IL-4Rα due to *C. neoformans* infection (table 1), arguing for a low-level IL-4 system in our model. In vitro experiments need to be designed to model quantitatively the IL-4/IL-13/IL-4Rα–dependent pulmonary allergic immune response driven by *C. neoformans*.

The data from this bronchopulmonary mycosis model may have relevant implications for future therapeutic strategies against asthma and atopy. IL-4Rα polymorphism has been shown elsewhere to affect asthma development and prevalence [30–37]. It has been demonstrated that strong IL-4R–dependent signaling in newborns' monocytes and Th lymphocytes could contribute to Th1/Th2 imbalance [38]. These authors concluded that IL-4R overexpression in newborns' monocytes and lymphocytes could be an early risk marker of allergy development. In line with these findings, significantly reduced expression of IL-4Rα associated with reduced IL-4–induced signaling was found in neonatal B cells [39].

In another study, combined extended haplotypes involving IL-4, IL-13, IL-4Rα, and STAT6 were analyzed to assess the combined effect of single-nucleotide polymorphisms in the IL-4/IL-13 signaling pathway [35]. When polymorphisms in all 4 major pathway genes were combined in a stepwise procedure, the risk of high serum IgE levels increased 10.8-fold and the risk of the development of asthma increased 16.8-fold, compared with the maximum effect of any single polymorphism. Interestingly, in *Leishmania*-infected BALB/c STAT6$^{+/-}$ mice, Burgis and Gessner [40] found evidence of distinct STAT6 dosage requirements for different IL-4 functions. Their finding underscores the fact that the IL-4/IL-13 pathway is tightly regulated at different levels of the cascade. Earlier it was shown that different IL-4Rα allotypes exist in inbred mouse strains associated with different levels of IL-4–neutralizing activity [41]. Different degrees of IL-4 responsiveness may be involved in the specific phenotypes of inbred mouse strains in IL-4–dependent infections.

For therapeutic strategies against Th2-related diseases, such as asthma and atopy, our findings, together with other epidemiological data, reveal IL-4Rα to be an attractive target. It is obvious that a complete blockade would not be feasible and, as we have shown, is not necessary. Even a partial blockade could be effective for ameliorating IL-4/IL-13–driven diseases.

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4 Discussion

The plethora of microorganisms challenging us steadily requires well balanced cellular and humoral responses in order to control them and prevent disadvantages for us. This complex task is governed by distinct arms of the immune system that comprise specialized cells and soluble factors.

In general, the homeostasis of the immune system underlies manifold regulation and strongly depends on "messenger" molecules, including the big families of interleukins (IL), chemokines, growth factors etc. These essential regulatory mechanisms assure the induction of proper responses due to invading organisms and prevent excessive reactions that otherwise might be directed against the host itself harming him. The coordination of an immune response by "messenger" molecules is facilitated by specialized cells, such as T helper (Th) cells, while other cells are responsible for pathogen degradation etc. Regarding the heterogenous nature of pathogens the provoked interleukin patterns are adapted to them and are heterogenousic themselves. The host favors the eradication, or, if this is not possible, the control of the intruder. Mounting an inappropriate cytokine response can be fatal to the host and eventually results in uncontrolled pathogen replication and immunopathogenesis and finally in the death of the host. In the presented study, a fatal immunopathological response to a ubiquitous and opportunistic fungus in an experimental infection model is discussed.

IL-4 is central in detrimental Th2-driven immune responses against *Cryptococcus neoformans* (*C. neoformans*) (MÜLLER et al. 2007; STENZEL et al. 2009). The present work shows for the first time a detailed kinetic analysis of IL-4 production at the site of infection in experimental pulmonary cryptococcosis.

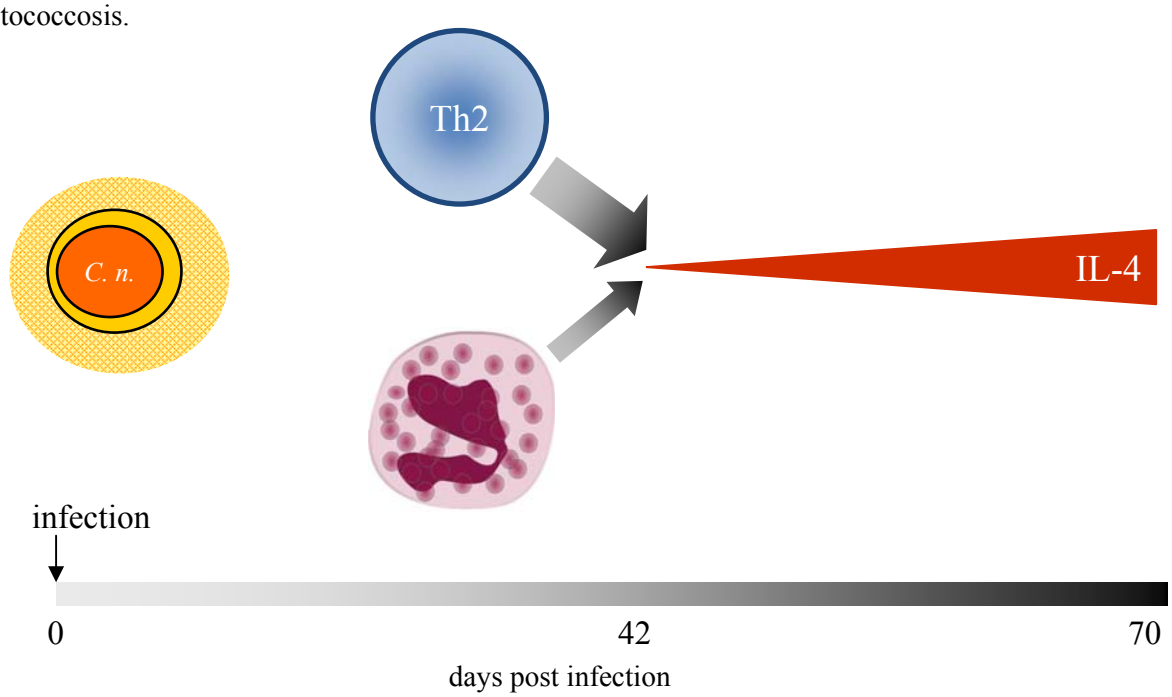

Figure 3: Interleukin-4 producing cell types in pulmonary cryptococcosis.
After pulmonary infection with *Cryptococcus neoformans* (*C. n.*) T helper 2 (Th2) cells and eosinophilic granulocytes secrete interleukin-4 (IL-4) starting six weeks after infection with increasing intensity thereafter.

Furthermore, our study provides a deeper understanding of the impact of expression levels of the IL-4 receptor (R) alpha (α) chain resulting in a gradual responsiveness to IL-4. Together, different cellular sources of IL-4 and gradual IL-4R expression were shown to be relevant for the outcome of cryptococcosis. Our analysis led to the identification of Th cells and eosinophilic granulocytes which gain IL-4 capacity relatively late, i.e. in the chronic phase of disease. No IL-4 production was detectable earlier than six weeks after intranasal infection (Fig. 3). Infected mice start to die after the onset of IL-4 production. In addition, with the onset of IL-4 production the frequencies of Th cells and eosinophils increase in the lung of infected animals. This prompted us to study their contribution to IL-4 production. IL-4 production by Th2 cells could be confirmed and, for the first time, eosinophils were identified by us as IL-4 producing regulatory cells (Fig.3).

4.1 Eosinophilic granulocytes in cryptococcosis

Over decades, eosinophils were associated with parasitic infection and tissue destruction but more recently they re-gained attention (HUMBLES et al. 2004; LEE et al. 2004; JACOBSEN et al. 2007). Earlier reports on the role of eosinophils in cryptococcosis focused on *in vitro* experiments and the investigation of their phagocytic capacity (FELDMESSER et al. 1997). Others made use of the administration of a monoclonal antibody directed against IL-5, an important factor for differentiation (CLUTTERBUCK and SANDERSON 1988), activation (WANG et al. 1989) and survival of eosinophils (SANDERSON 1990). Since IL-5 not only has an impact on eosinophils but also on other cells, these results were difficult to interpret in terms of eosinophil function. Nevertheless, a role for IL-5 has been clearly demonstrated in pulmonary cryptococcosis (HUFFNAGLE et al. 1998). With the recent generation of the ΔdblGATA-1 (YU et al. 2002) and the PHIL (LEE et al. 2004) mice which are completely lacking eosinophils, more specific studies were made possible, regarding the role of eosinophils in disease. As observed in models of allergen-induced asthma (JACOBSEN et al. 2007; JACOBSEN et al. 2008), in the absence of eosinophils Th2 cells are decreased in number and Th2-related cytokines are reduced at the pulmonary site after cryptococcal infection (Fig. 4). This may be attributed to the production of chemokines such as thymus and activation regulated chemokine (TARC) and macrophage-derived chemokine (MDC) targeting Th cells (JACOBSEN et al. 2008; SPENCER and WELLER 2010). In the presented model Th cells do not increase before day 42 at the infection site nor do they become positive for “IL-4 – competence” indicated by the expression of a green fluorescent protein in a so-called IL-4 reporter mouse. They do not secrete IL-4 in response to antigen as long as eosinophils are at basal levels (Fig. 4). This might be related to secretion of Th cell chemotactic factors as mentioned above. However, it is clearly seen in the eosinophil-deficient mouse that Th cell recruitment is not completely dependent on eosinophils as IL-4 production is also not entirely dependent on eosinophils (see Figures 5A, 5E, and 6 of the first manuscript in this thesis).

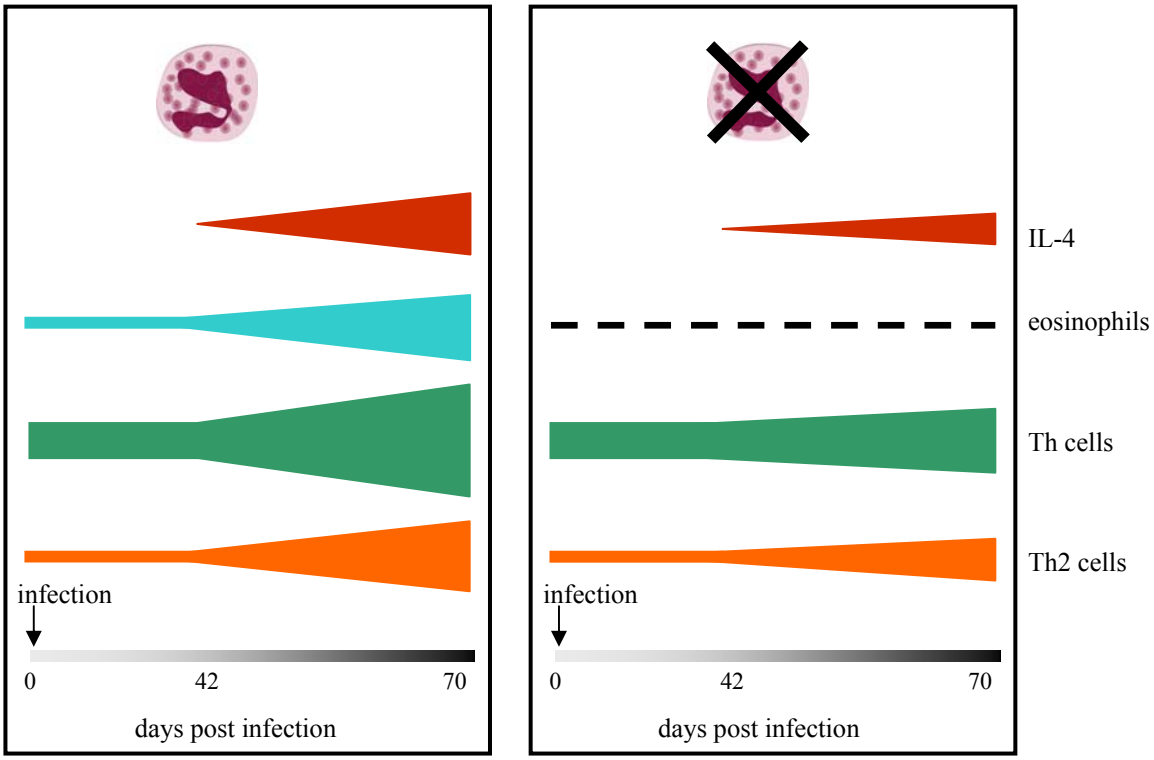

Figure 4: Effects of presence and absence of eosinophils on lung inflammation during pulmonary cryptococcosis.
Synthesis of pulmonary interleukin-4 (IL-4) starts six weeks after infection concomitantly with recruitment of eosinophils and T helper (Th) cells and increased differentiation of Th2 cells. In the absence of eosinophils (shown on the right) fewer total Th and differentiated Th2 cells are found than in the presence of eosinophils (shown on the left). In addition, less IL-4 is detectable in eosinophil-deficient mice.

It is more likely that eosinophil numbers reach a critical threshold level at the infection site according to the Local Immunity And/or Remodeling/Repair (LIAR) hypothesis of LEE et al. (2010) and act as regulators by augmenting the Th2 responses in this model of bronchopulmonary fungal infection (Fig. 4).

Until now, it is not known if eosinophils only contribute to recruitment and polarization as shown here but also directly interact with Th cells and present antigen during cryptococcosis. A recent *in vitro* study using rat eosinophils as antigen presenting cells points to a direct interaction with Th cells (GARRO et al. 2011). Therefore, it is likely that eosinophils may be one of central key players in pathogenesis of cryptococcosis by exerting multiple functions.

4.2 Implications of gradual IL-4Rα expression

In the eosinophil-deficient mouse Th2 cells are reduced and Th cells in general share another more Th1/Th17-biased cytokine profile. As they do not compensate for the reduction of IL-4 from eosinophils, it was suggestive to speculate whether IL-4 levels may regulate the degree of susceptibility. Therefore, a system with gradual IL-4Rα expression was chosen. Results from studies with a very strong Th2 bias (SABIN et al. 1996; SHINKAI et al. 2002; HERBERT et al. 2004; HOLSCHER et al. 2006) as well as *in vitro* studies (RENZ et al. 1991; PERONA-WRIGHT et al. 2010) showed that IL-4 can mediate the up-regulation of the α chain in a dose-dependent manner.

In our study with *C. neoformans*-infected mice, the amounts of IL-4 do not seem to reach the critical threshold to induce receptor up-regulation. This "low-level IL-4 production" setting with stable IL-4 receptor expression enabled us to analyze the effect of mono-allelic vs. bi-allelic IL-4R expression in comparison with complete IL-4R deficiency *in vivo* (Fig. 5).

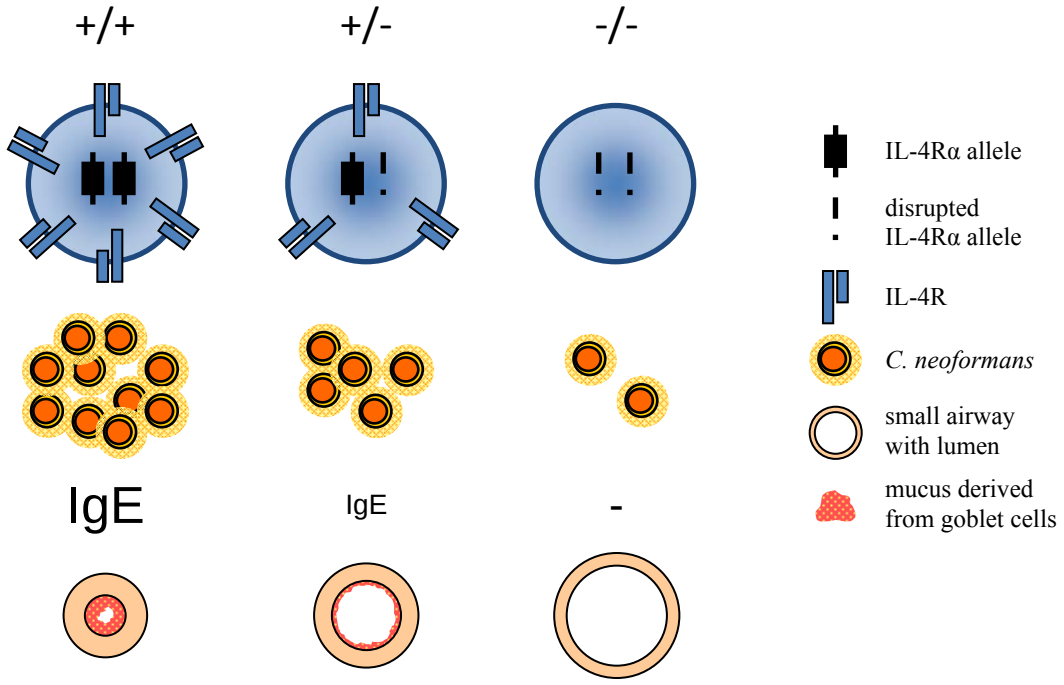

Figure 5: A gene-dosage-effect for interleukin-4 receptor α (IL-4Rα) expression determines expression levels of IL-4R α and the degree of Th2-dependent susceptibility in pulmonary cryptococcosis. From left to right: bi-allelic (+/+), mono-allelic (+/-), and complete absence (-/-) of expression of IL-4Rα. Mono-allelic and absent IL-4Rα expression enhance fungal growth control, limit immunoglobulin E (IgE) synthesis, mucus production in airways, and airway resistance (indicated by smaller lumen of the airway and thickened airway wall upon constriction after challenge with methacholin).

As shown in the second paper of this thesis the stepwise increase of fungal burden, airway resistance and immunoglobulin E serum levels correlates with expression of IL-4Rα chain. This situation may reflect the situation in the human population since polymorphisms of either the *il-4rα* (BASEHORE et al. 2004; LOZA and CHANG 2007; MICHEL et al. 2010), the *il-4* or *il-13* (HOWARD et al. 2001; MICHEL et al. 2010) genes or their regulatory elements (VAN DER POUW KRAAN TC et al. 1999; HUMMELSHOJ et al. 2003) exist and may provide a comparable scenario to pulmonary cryptococcosis with different signalling intensity in this pathway. Some of the polymorphisms are correlated with increased risk of asthma development (VERCELLI 2008). IL-4Rα polymorphism has already been shown to be associated with asthma prevalence in humans (VERCELLI 2008). A single nucleotide polymorphism (SNP) of IL-4Rα was shown to decrease levels of soluble IL-4R (and thereby relatively increasing available IL-4 for uptake by membrane bound IL-4R) and is suspected to be linked to asthma development (HACKSTEIN et al. 2001). An earlier study showed that another SNP of the IL-4R (MITSUYASU et al. 1998) results in increased affinity of the receptor for IL-4 and recently another SNP has been found, also promoting signalling intensity between IL-4 and its receptor (TACHDJIAN et al. 2009). Therefore, our mouse model with gradual IL-4R expression

provides a similar situation with differences in the signal strength of the IL-4 – IL-4R pathway and may be of importance for human asthma. Interestingly, the IL-4Rα gene has also be identified as candidate gene determining susceptibility in equine recurrent airway obstruction (JOST et al. 2007). Thus, IL-4 – IL-4Rα axis could be an attractive target for blockade by antagonists as our model system highlights the outstanding role of it during the Th2-dependent susceptibility in cryptococcosis (Fig. 5). KURUP et al. (1997) showed in an *Aspergillus fumigatus* model diminished eosinophils and IgE levels by the injection of anti-IL-4 antibodies (KURUP et al. 1997). Although interesting, this approach might not be of practical relevance as half-life of anti-IL-4 antibodies is too short. To overcome this malus cytokine traps have been developed also for IL-4 and may be more efficient due to their extended halflife (ECONOMIDES et al. 2003). An IL-4 vaccine showed in an experimental model a reduction of serum IgE, eosinophil numbers in bronchoalveolar fluid as well as decreased airway inflammation (MA et al. 2007) and provided long lasting protection against IL-4 effects. IL-13 also signals through the IL-4 Type II receptor (IL-4Rα-chain + IL-13R) and also considerably contributes to airway pathology in Th2-dominated diseases (GRUNIG et al. 1998). IL-13 has been shown to negatively influence the outcome of cryptococcal infection in our model (MÜLLER et al. 2007). Therefore the α chain of the common IL-4R may be an even more valuable target than either of its ligands IL-4 and IL-13. In a murine model soluble IL-4Rα reduced mucus production, eosinophil infiltration, airway inflammation (HENDERSON, JR. et al. 2000), and airway hyper-reactivity (see second paper). In trials with human patients soluble IL-4R also exerted a positive influence on asthma symptoms (BORISH et al. 1999; BORISH et al. 2001).

4.3 Therapeutic opportunities targeting immunoglobulin E or eosinophilic granulocytes

Another interesting therapeutic target could be immunoglobulin (Ig) E. As described above the level of IL-4R expression in *C. neoformans*-infected mice is associated with different levels of IgE produced upon infection (see Table 2 in the second manuscript of this thesis and Fig. 5). The functional role of IgE in cryptococcosis is enigmatic. As shown here, the increase of IgE specific for *C. neoformans* parallels the increase of total IgE, but so far no study investigated the potential contribution to pathology or exacerbation of *Cryptococcus*-associated Th2-biased inflammation in the lungs. One recent report also found a positive correlation between airway resistance, serum IgE and virulence of a cryptococcal isolate (JAIN et al. 2009). A vaccine against receptor binding sites of the IgE molecule which was already shown in rats and mice to decrease serum levels of IgE and inhibit binding of IgE to high affinity IgE receptor in a trichosanthin model (PENG et al. 2007) hold promising results. In human asthmatics (elevated) serum IgE concentrations positively correlate with airway hyper-reactivity, exhaled nitric oxide, and numbers of sputum eosinophils (AVILA 2007). Treatment of patients with monoclonal antibodies against IgE showed good compliance and reduction of exacerbations in asthmatics, even in severe cases of the disease (CORNE et al. 1997; BUSSE 2001;

HOLGATE et al. 2005; AVILA 2007). This underlines the important role of IgE in allergic disease and the potential power of an anti-IgE therapy since it has been shown in a rat model that *C. neoformans* can even increase development of allergic responses against other non-related antigens (GOLDMAN et al. 2006).

There have been efforts also in veterinary medicine to elucidate to possible roles of IgE in various diseases but its role is still inscrutable and less explored. For example, in horses baseline IgE levels differ in a broad range and even between healthy animals and horses suffering from recurrent airway obstruction show overlap in serum levels of IgE making it impossible to distinguish between them (KUNZLE et al. 2007). Others found a correlation between horses with *A. fumigatus* induced airway pathology, but only locally, i.e. in bronchoalveolar fluid (SCHMALLENBACH et al. 1998). Until now, testing for specific reaction against a particular antigen often suffers from crude preparations that are used for a prick test and often yield false positive results (WAGNER 2009). So far, a relationship between Th2-biased airway disease and elevated serum IgE levels only seems to exist in Iceland horses (WAGNER 2009). Another species often reported with IgE-related disorders is the dog with breed predispositions in terriers, Dalmatians, and Irish setters (SCHICK and FADOK 1986). Interestingly, there is evidence for vertical transmission of allergy predisposition in dogs (SCHIESSL et al. 2003) leading to the suggestion that in Th2-biased diseases an anti-IgE therapy could be useful in these animal patients, too.

Besides the molecule-based strategies discussed here cellular targets are also of interest, since the present work underscores a role especially for eosinophilic granulocytes. Therefore, more eosinophil-directed therapeutic interventions could be considered. Although questions remain open about eosinophils in cryptococcosis regarding their role in Th polarization, *in vivo* interaction with *C. neoformans*, and other molecules involved in pathology in cryptococcosis, the presented data here and derived from mouse asthma models (JUSTICE et al. 2003; FULKERSON et al. 2006; WEGMANN et al. 2007; ZIMMERMANN et al. 2008) and human asthma studies (FLOOD-PAGE et al. 2003a; NAIR et al. 2009; HALDAR et al. 2009) suggest that eosinophil targeting may be valuable in pulmonary cryptococcosis and other respiratory-tract associated Th2-dominated diseases. By now, a major pitfall is the usage of antibodies that not only target eosinophils. Using antibodies against IL-5 (LECKIE et al. 2000; KIPS et al. 2003; FLOOD-PAGE et al. 2003b; HALDAR et al. 2009; NAIR et al. 2009), anti-Siglec-F (ZIMMERMANN et al. 2008) or chemokine receptor 3 (JUSTICE et al. 2003; WEGMANN et al. 2007) and its ligand CCL11 (DING et al. 2004) makes it difficult to interpret the role of eosinophils in studies done in humans. An alternative experimental approach in a mouse model was made by combined vaccination against IL-5 and eotaxin, leading to a drastic reduction in eosinophils in several organs in an experimental model of asthma (ZOU et al. 2010), but this study is probably also hampered by side-effects of the vaccine on other cells.

4.4 Cryptococcosis in HIV-1-infected patients

As mentioned in the introduction, especially HIV-1 infected patients are co-infected at a significantly higher degree with *C. neoformans.* Amongst the many studies undertaken to understand HIV-related pathology much of work has been spent to study the cytokines involved. Studies with peripheral blood mononuclear cells from healthy donors and HIV-1^+ patients revealed a Th1 to Th2 shift in latter ones, as IFN-γ production was strongly reduced and IL-4 increased in response to a mitogen (KLEIN et al. 1997) and also in response to various pathogens including *C. neoformans* (HARRISON and LEVITZ 1997). Viral glycoprotein 120 was found to down-regulate expression of IL-12 and its receptor (PIETRELLA et al. 2001). Therefore, it is conceivable that HIV favours cryptococcal replication and dissemination not simply by limiting Th cell numbers but also by induction of a more Th2-like milieu. This leads to the conclusion that the treatment of a HIV-1 infection might result in an improved control of *C. neoformans* thereby mitigating the clinical symptoms of an associated meningoencephalitis. However, treatment of HIV-1 patients with cryptococcosis can harbor the risk of the development of an immune reconstitution inflammatory syndrome (HADDOW et al. 2010).

4.5 Final conclusions

Interleukin (IL)-4 is tightly linked with T helper (Th) 2 responses. Our analysis of the role of IL-4 in pulmonary cryptococcosis led to the insight that it is tightly connected to susceptibility. Clinical signs of pulmonary cryptococcosis partially resemble those found in asthma, a disease also mainly driven by IL-4.

The present work identifies different IL-4 producing cells in pulmonary cryptococcosis – Th2 cells and eosinophilic granulocytes. Both cells contribute non-redundantly to IL-4 synthesis and eosinophils are shown to shift the Th cell cytokine pattern even more towards a Th2 profile. The dependency of diseases degree on several IL-4 sources was confirmed in a system of gradual expression of the IL-4 receptor (R) ranging from 0 over 50 to 100% of expression. This allowed the analysis of different amounts of consumable IL-4 and revealed a similarly reduced pathology in the presence of reduced levels of IL-4R and indirectly of IL-4 itself indicated by reduced airway hyperreactivity, mucus production, serum immunoglobulin E (IgE) concentrations, and enhanced fungal growth control.

From a clinical point of view, it is obvious that already a partial block of the IL-4-IL-4R axis provides benefit in cryptococcosis. The results imply that the IL-4-IL-4R axis and eosinophils undoubtly contribute to susceptibility. Targeting both can support an anticryptococcal therapy and ameliorate the associated Th2-biased airway hyperreactivity and probably exacerbations. The importance of available monoclonal antibodies against pathology-associated molecules (e.g. IL-4, -5, -13 or IgE) can extend conventional treatment concepts that are based solely on antifungal drugs. Therapy of *Cryptococcus neoformans* infection and associated disease should be improved with a combined intervention against the pathogen itself and against the inappropriate immune response of the host. The presented work

provides insights in the pathology of cryptococcosis and possibly in asthma, and highlights multiple targets of clinical importance.

5 Summary

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The inflammatory response against *Cryptococcus neoformans* is regulated by eosinophilic granulocytes and the interleukin-4/interleukin-4 receptor axis

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Cytokines play an important regulatory role during immune responses against pathogens. The outcome of an induced cytokine pattern is determined by many factors. It strongly depends on the nature of the pathogen and the host's ability to control the quality and strength of cytokine signals. In pulmonary infection with *Cryptococcus neoformans* T helper (Th) 1 and Th17 cell subsets and their associated cytokines confer protection, whereas a Th2-biased response with production of interleukin (IL) -4 confers susceptibility. Since inappropriate Th responses often lead to death in immunosuppressed human patients, especially HIV-1 infected patients, this work aimed to elucidate mechanisms of Th2 induction and regulation by assessing the Th2 hallmark cytokine IL-4 in an experimental model of cryptococcosis. Therefore, a kinetic study of IL-4 expression during 70 days after intranasal infection was performed in susceptible mice. The analyses included characterization of pulmonary leukocytes and Th cell cytokine profiling. IL-4 profiling revealed *Cryptococcus*-specific IL-4 production does not start as late as six weeks after infection. This unexpected finding was further validated by equal results observed in a kinetic study done in IL-4 reporter mice. Two cellular sources of IL-4 were identified: Th2 cells as expected, but also, as shown for the first time in cryptococcosis, eosinophilic granulocytes could be demonstrated to secrete IL-4.
Next, the influence of eosinophils on pulmonary inflammation and disease development was investigated using ΔdblGATA-1 mice constitutively devoid of eosinophilic granulocytes. Experiments with infected ΔdblGATA-1 mice revealed novel regulatory functions of eosinophils in cryptococcosis. In the absence of eosinophils pulmonary Th cell recruitment was significantly diminished. In addition, Th2 polarization was reduced in ΔdblGATA-1 mice as shown by reduced numbers of Th2 cells expressing the Th2-related surface marker T1/ST2 and reduced albeit not absent IL-4 production by Th cells. In addition to reduced IL-4 production, in the absence of eosinophils, Th cells with enhanced interferon-γ and IL-17 production were observed. However, control of pulmonary fungal growth was only slightly enhanced in the absence of eosinophils and dissemination of cryptococci to the brain was

unaltered. This may be related to the shared IL-4 production by not only eosinophils but also Th2 cells. Blocking more than one cellular source of IL-4 could be required to prevent immunopathology.
To test the hypothesis of gradual IL-4-dependent immunopathology, experiments were conducted using mice expressing only one allele of the IL-4 receptor (R) alpha (α) chain (+/-) instead of two (+/+). Indeed, mono-allelic expression of the IL-4Rα resulted in an intermediate expression of the IL-4R on the surface of myeloid and lymphoid cells indicating a gene-dosage effect for IL-4R expression. Infected IL-4Rα$^{+/-}$ mice displayed reduced susceptibility as compared with IL-4Rα$^{+/+}$ mice, and IL-4Rα$^{-/-}$ mice completely lacking IL-4R expression were found to be highly protected with survival for the complete time period of the experiment (i.e. up to 275 days). Reduced susceptibility found in infected IL-4Rα$^{+/-}$ mice was associated with decreased serum levels of immunoglobulin E, reduced mucus production by airway epithelia, attenuation of airway hyper-reactivity, and reduced formation of alternatively activated macrophages in lung parenchyma – pathophysiological features, which are typically found in experimental models of asthma but also in asthma of humans and animals. Since no up-regulation of IL-4R by the infection with *Cryptococcus neoformans* was found, the experimental pulmonary infection model used appears to be a very sensitive low-level IL-4 system. This work highlights the outstanding role of IL-4 and its different cellular sources as well as its receptor in cryptococcosis and provides novel insights into pathogenesis. Moreover, a cellular (i.e. eosinophils) and a molecular (i.e. IL-4R) target for treatment of this mycosis and possibly of asthma is provided.

6 Zusammenfassung

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Die Entzündungsreaktion gegen *Cryptococcus neoformans* wird von eosinophilen Granulozyten und der Interleukin-4 / Interleukin-4 Rezeptor – Achse reguliert

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Zytokine nehmen eine außerordentlich wichtige regulierende Rolle bei der Abwehr von Infektionserregern ein. Sie können dabei von sehr verschiedenen Zellen gebildet werden, eine herausragende Rolle haben dabei T-Helfer (Th) Zellen. Viele verschiedene Faktoren nehmen Einfluss auf Verlauf und Ausgang einer Immunreaktion. Große Bedeutung kommt dabei einerseits dem Typ des Erregers zu, d.h. welche Möglichkeiten und Strategien zum Überleben ihm in der Auseinandersetzung mit dem Wirt zur Verfügung stehen. Auf der anderen Seite steht die Fähigkeit des Wirtes, eine adäquate pathogenspezifische Immunreaktion zu induzieren, deren Koordination ein Zusammenspiel verschiedener Zytokine zu Grunde liegt.
Betrachtet man - wie in der vorliegenden Arbeit dargestellt - die pulmonale Infektion mit *Cryptococcus neoformans* (*C. neoformans*), so zeigt sich, dass eine Immunreaktion auf der Basis von Zytokinen, die mit einer Th1- und Th17- Reaktion assoziiert werden, mit Schutz und Kontrolle von *C. neoformans* einhergehen. Dem gegenüber steht die Möglichkeit, dass es dem Wirt nicht gelingt, den Erreger zu kontrollieren und dieser sich vermehren kann. Hier liegt häufig eine Th2-basierte Reaktion vor, die u.a. durch die Bildung des Zytokins Interleukin (IL) -4 charakterisiert ist. Besonders immunsupprimierte Patienten, darunter zu großen Anteilen HIV-infizierte Menschen, versterben häufig an den Folgen der Infektion, da sie *C. neoformans* nicht erfolgreich abwehren können und die dominierenden Zytokine einem Th2 Profil entsprechen. Die vorliegende Arbeit hatte daher das Ziel, die Mechanismen der Th2-Reaktion anhand des Schlüsselzytokins IL-4 näher in einem experimentellen murinen Modell der Kryptokokkose zu charakterisieren. Zunächst wurde in einer kinetischen Analyse die IL-4-Induktion über einen Zeitraum von zehn Wochen nach intranasaler Infektion in suszeptiblen Mäusen untersucht. Diese Analysen schlossen die Charakterisierung pulmonaler Leukozyten sowie des Th-Zell-Zytokinprofils ein. Es zeigte sich, dass bis sechs Wochen nach Infektion keine messbare IL-4-Bildung stattfindet. Dieses Resultat wurde unter Zuhilfenahme von IL-4 Reportermäusen bestätigt. Nach sechs Wochen konnten zwei IL-4-Produzenten identifiziert

werden: neben den zu erwartenden Th2-Zellen konnten eosinophile Granulozyten identifiziert werden, deren IL-4 Bildung zum ersten Mal in der experimentellen Kryptokokkose gezeigt werden konnte. Daraufhin wurde in einem nächsten Schritt der Einfluss eosinophiler Granulozyten auf die Entwicklung des Krankheitsverlaufs untersucht unter Verwendung von ΔdblGATA-1 Mäusen, die keine eosinophilen Granulozyten bilden können. Diese Untersuchungen deckten bisher unbekannte regulatorische Funktionen dieser Zellen in der Kryptokokkose auf. Ohne eosinophile Granulozyten ist die Rekrutierung von Th-Zellen in das Lungenparenchym signifikant reduziert. Weiterhin konnte unter Zuhilfenahme des Th2-Zell-assoziierten Moleküls T1/ST2 gezeigt werden, dass die Th2-Zell-Differenzierung signifikant vermindert ist. Zudem ist in Abwesenheit von eosinophilen Granulozyten die Kapazität der Th2-Zellen im Hinblick auf IL-4-Produktion reduziert, wenngleich nicht völlig aufgehoben. In Ergänzung zur reduzierten IL-4-Synthese der Th-Zellen wurde eine erhöhte Produktion von IL-17A und Interferon-γ gefunden. Dieses modifizierte Zytokinmuster hatte eine verbesserte pulmonale Kontrolle von *C. neoformans* zur Folge. Die Dissemination des Erregers in das Zentralnervensystem blieb weitestgehend unbeeinflusst. Dies liegt möglicherweise darin begründet, dass mit der Th2-Zelle noch eine IL-4-Quelle existiert, und es nicht genügt nur eine IL-4-Quelle zu blockieren, um der Immunpathologie vorzubeugen. Diese Hypothese einer einer graduellen IL-4-Abhängigkeit wurde mittles Analysen an Mäusen durchgeführt, die statt einer biallelen IL-4 Rezeptor (R) alpha (α) Kette – Expression (+/+) nur eine monoallele (+/-) Expression aufweisen. In der Tat äußerte sich die monoallele Expression in einer intermediären Expression des IL-4R auf der Oberfläche von myeloiden und lymphoiden Zellen und lässt daher auf einen Gen-Dosis-Effekt schließen. Die fehlende Aufregulation des IL-4 Rezeptors in diesem Kryptokokkenmodell spricht dabei für ein sehr sensibles System mit geringen Mengen IL-4. Infizierte IL-4Rα$^{+/-}$ Mäuse zeigten verminderte Empfindlichkeit gegenüber IL-4Rα$^{+/+}$ Mäusen, wohingegen IL-4Rα $^{-/-}$ Tiere, die keine IL-4R - Expression zeigen, sogar die komplette Versuchszeit (bis zu 275 Tage) überlebten. Die intermediäre Expression der IL-4Rα$^{+/-}$ Mäuse spiegelte sich im Vergleich zu IL-4Rα$^{+/+}$ Mäusen auch in verminderten Konzentrationen von Immunglobulin E im Serum, reduzierter Mucusproduktion der bronchialen Epithelien, herabgesetztem Atemwegswiderstand sowie abgeschwächter Differenzierung alternativ aktivierter Makrophagen im Lungenparenchym wider. Diese pathophysiologischen Erscheinungen werden typischer Weise auch in experimentellen Asthmamodellen und bei humanen Patienten mit Asthma, dessen molekulare Ursache oftmals eine Th2 Reaktion ist, gefunden.

Die vorliegende Arbeit stellt die herausragende Bedeutung von IL-4 und seiner zellulären Quellen sowie seines Rezeptors in der Kryptokokkose dar und liefert neue Erkenntnisse zur Pathogenese. Weiterhin wurden eine zelluläre (eosinophile Granulozyten) sowie eine molekulare (IL-4R) Zielstruktur identifiziert, die als therapeutische Angriffspunkte für neue Therapien bei dieser Mykose und möglicherweise auch bei Asthma dienen können.

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