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IL-13 Induces Disease-Promoting Type 2 Cytokines, Alternatively Activated Macrophages and Allergic Inflammation during Pulmonary Infection of Mice with Cryptococcus neoformans

Uwe Müller,* Werner Stenzel,† Gabriele Köhler,‡ Christoph Werner,§ Tobias Polte,∥ Gesine Hansen,¶ Nicole Schütze,* Reinhard K. Straubinger,* Manfred Blessing,* Andrew N. J. McKenzie,∥ Frank Brombacher,∥ and Gottfried Alber∥‡

In the murine model of Cryptococcus neoformans infection Th1 (IL-12/IFN-γ) and Th17 (IL-23/IL-17) responses are associated with protection, whereas an IL-4-dependent Th2 response exacerbates disease. To investigate the role of the Th2 cytokine IL-13 during pulmonary infection with C. neoformans, IL-13-overexpressing transgenic (IL-13Tg*), IL-13-deficient (IL-13−/−), and wild-type (WT) mice were infected intranasally. Susceptibility to C. neoformans infection was found when IL-13 was induced in WT mice or overproduced in IL-13Tg* mice. Infected IL-13Tg* mice had a reduced survival time and higher pulmonary fungal load as compared with WT mice. In contrast, infected IL-13−/− mice were resistant and 89% of these mice survived the entire period of the experiment. Ag-specific production of IL-13 by susceptible WT and IL-13Tg* mice was associated with a significant type 2 cytokine shift but only minor changes in IFN-γ production. Consistent with enhanced type 2 cytokine production, high levels of serum IgE and low ratios of serum IgG2a/IgG1 were detected in susceptible WT and IL-13Tg* mice. Interestingly, expression of IL-13 by susceptible WT and IL-13Tg* mice was associated with reduced IL-17 production. IL-13 was found to induce formation of alternatively activated macrophages expressing arginase-1, macrophage mannose receptor (CD206), and YM1. In addition, IL-13 production led to lung eosinophilia, goblet cell metaplasia and elevated mucus production, and enhanced airway hyperreactivity. This indicates that IL-13 contributes to fatal allergic inflammation during C. neoformans infection.


The opportunistic pathogenic yeast Cryptococcus neoformans has become a major health problem for immunocompromised patients, especially AIDS patients, in the last two decades. In particular, the C. neoformans variants of the serotype A (C. neoformans var. grubii) and serotype D (C. neoformans var. neoformans) are of importance for these people (1). Dry feces of birds that contain the organisms can be inhaled and are a source of infection (2, 3). From the respiratory tract C. neoformans can disseminate into the body when the immune system lacks specific control mechanisms (4, 5). C. neoformans is able to invade the brain, where it causes severe meningocerebralitis (6, 7). The cells and factors of the immune system that help the host to control and eliminate this facultative intracellular yeast are still under investigation in human patients and in particular in murine infection models.

CD4+ T cells that produce proinflammatory cytokines such as IFN-γ (8) are important for the induction of mechanisms which control cryptococcal growth and dissemination (9). Furthermore, the formation of granulomata serves as a major anti-cryptococcal control mechanism (10, 11). Besides CD4+ T cells these granulomata contain macrophages, NK cells, and CD8+ T cells (10). Phagocytic cells can engulf cryptococci (12, 13) and eliminate them by the production of reactive oxygen and in mice by producing nitrogen intermediates such as NO (14). The uptake of cryptococci is supported by complement factors that bind to the yeast surface (15–17) and by opsonizing Abs that are specific for Ags of their capsule (18–20). Effector cells such as NK cells and CD8+ T cells are potent perforin/granulysin producers that destroy C. neoformans-infected cells and C. neoformans directly (21, 22). Therefore, mice that lack T cells or NK cells are more susceptible to C. neoformans infection than wild-type (WT) mice (9, 23–25). The same phenomenon was observed in humans, where HIV-infected patients with advanced AIDS symptoms who have reduced numbers of CD4+ T cells are susceptible to cryptococcosis, whereas immunocompetent people are resistant (26). Several groups have shown that a protective CD4+ T cell immune reaction

References

1. Institute of Immunology, College of Veterinary Medicine, University of Leipzig, Leipzig, Germany; 2. Institute of Neuropathology, Medical Faculty, University of Cologne, Cologne, Germany; 3. Gerhard Domagk Institute for Pathology, University of Münster, Münster, Germany; 4. Division of Allergy and Pulmonology, Department of Pediatrics, Martin-Luther-University, Halle-Wittenberg, Halle, Germany; 5. Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom; and 6. Institute of Infectious Disease and Molecular Medicine, University of Cape Town, Cape Town, South Africa

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5 Abbreviations used in this paper: WT, wild type; p.i., postinfection; aaMph, alternatively activated macrophage; caMph, classically activated macrophage; iNOS, inducible NO synthase; Tg, transgenic.

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depends on a Th1 response (8, 9, 11, 27). IL-12 has been demonstrated to be essential for induction of such Th1 responses (11, 28–30). Mice which lack the ability to produce IL-12 or other members of the IL-12 family (e.g., IL-23) (31) showed reduced survival times when compared with WT mice (11).

Th2 responses are regulated by IL-4 and IL-13 (32–34). These two closely related cytokines (35, 36) share common functions such as the regulation of macrophages (37) and up-regulation of MHC class II molecules (38). IL-4 and IL-13 mediate their effects via a complex receptor system. The specific high-affinity receptors for IL-4 and IL-13 contain the IL-4Ra-chain (39) in combination with the γ chain or the IL-13Ra1/2 chains. Unlike IL-4 that can directly induce the differentiation of naïve CD4+ T cells into Th2 cells (type 1 receptor), IL-13 acts on human B cells, macrophages, respiratory epithelial cells and smooth muscle cells but not on T cells (type 2 receptor) (34). IL-4 and IL-13 also display other unique features (40–43). For example, IL-13 is important for the expulsion of gastrointestinal helminths from the gut, but IL-4 is not (33). Furthermore, the induction of allergic inflammation, e.g., asthma is associated with IL-13 and only to a much lower degree with IL-4 (42, 44, 45).

Both, IL-4 and IL-13 were found to induce development of alternatively activated macrophages (aaMph) (46). In experimental studies using the intracellular pathogen Leishmania major (47, 48), the switch from classically activated macrophages (caMph) to aaMph could be associated with development of susceptibility. These macrophages do not produce reactive nitrogen intermediates but are characterized by elevated levels of arginase-1, expression of IL-4R, mannose receptor, and YM1 induced by IL-4 or IL-13 (46). In aaMph arginase-1 successfully competes with inducible NO synthase (iNOS) for arginine, the substrate by which iNOS produces NO and other reactive nitrogen intermediates. Following infection of IFN-γ−/− mice with C. neoformans high numbers of aaMph were found that could be associated with susceptibility in cryptococcosis (49).

IL-4 has been shown to be responsible for susceptibility during murine cryptococcosis (11, 50, 51). However, the role of IL-13 in pathogenesis of C. neoformans infection has not been characterized yet. This study elucidates the role of IL-13 in pulmonary (with early local infection of the lung and late systemic infection) cryptococcosis. IL-13-deficient mice (IL-13−/−) and IL-13-overexpressing transgenic (Tg) mice (IL-13Tg+) demonstrate profound effects of IL-13 on the pathogenesis of murine cryptococcosis.

### Materials and Methods

#### Mouse

Six to 10-wk-old female WT, IL-13−/− (33) as well as IL-13Tg+ (52) mice on 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 Use Committee of the Regierungspräsidium Leipzig. The murine IL-13 transgene was generated using the human CD2 locus control region to facilitate IL-13 expression. The cells were stimulated in medium alone as a control, with 5 ng/ml Con A, or with 1 × 10^6/ml heat-inactivated acapsulated C. neoformans strain CAP67. After 72 h, the peritoneal cells were harvested and analyzed for production of several cytokines and NO.

For immunohistochemistry, 10-μm frozen sections were prepared in a serial fashion (15 transversal sections per lung on four consecutive levels). The following anti-mouse Abs were used for staining procedures: iNOS rabbit anti-mouse Abs (Alexis), CD206 (mannose receptor) rat anti-mouse rabbit anti-mouse Abs (Serotec, YM1 (eosinophil chemotactic factor-L) goat anti-mouse Ab (R&D Systems).

Histopathological analysis of lung sections was done by Elastica von Gieson staining. To study recruitment of granulocytes, the tissue sections were stained with naphthol AS-D-chloroacetate.

### Digestion of spleen

The spleens were passaged through a 100-μm mesh in PBS to obtain a single-cell suspension. Thereafter erythrocytes were lysed using Gey’s solution and cells counted using a hemocytometer. Single cells were resuspended at 5 × 10^6/ml in Iscove’s (containing glucose, glutamine) medium supplemented with 1% FCS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were stimulated in medium alone as a control, with 5 μg/ml Con A, or with 1 × 10^6/ml heat-inactivated acapsulated C. neoformans strain CAP67. After 72 h, the supernatant was harvested and analyzed for production of several cytokines and NO.

For immunohistochemistry, 10-μm frozen sections were prepared in a serial fashion (15 transversal sections per lung on four consecutive levels). The following anti-mouse Abs were used for staining procedures: iNOS rabbit anti-mouse Abs (Alexis), CD206 (mannose receptor) rat anti-mouse Ab (Serotec, YM1 (eosinophil chemotactic factor-L) goat anti-mouse Ab (R&D Systems). Histopathological alterations were microscopically evaluated on H&E and immunostained lung sections.

Another part of the lung and the other organs were fixed in neutral-buffered formalin and embedded in paraffin. Sections were stained with H&E and immunostained lung sections.

### Conclusion

The role of IL-13 in murine cryptococcosis is demonstrated in this study. IL-13 is important for the expulsion of gastrointestinal helminths from the gut, but IL-4 is not. Furthermore, the induction of allergic inflammation, e.g., asthma is associated with IL-13 and only to a much lower degree with IL-4. Both, IL-4 and IL-13 were found to induce development of alternatively activated macrophages (aaMph) (46). In experimental studies using the intracellular pathogen Leishmania major (47, 48), the switch from classically activated macrophages (caMph) to aaMph could be associated with development of susceptibility. These macrophages do not produce reactive nitrogen intermediates but are characterized by elevated levels of arginase-1, expression of IL-4R, mannose receptor, and YM1 induced by IL-4 or IL-13 (46). In aaMph arginase-1 successfully competes with inducible NO synthase (iNOS) for arginine, the substrate by which iNOS produces NO and other reactive nitrogen intermediates. Following infection of IFN-γ−/− mice with C. neoformans high numbers of aaMph were found that could be associated with susceptibility in cryptococcosis (49). IL-4 has been shown to be responsible for susceptibility during murine cryptococcosis (11, 50, 51). However, the role of IL-13 in pathogenesis of C. neoformans infection has not been characterized yet. This study elucidates the role of IL-13 in pulmonary (with early local infection of the lung and late systemic infection) cryptococcosis. IL-13-deficient mice (IL-13−/−) and IL-13-overexpressing transgenic (Tg) mice (IL-13Tg+) demonstrate profound effects of IL-13 on the pathogenesis of murine cryptococcosis.
solution and cells were counted using a cell counter (Vi-CELL XR; Beckman Coulter). To differentiate between lung cells and cryptococci, the cells were counted twice after an incubation period of 10 min in aqua dest. In aqua dest, the lung cells are lysed but not the cryptococci. To gain the number of lung cells, the number of the second count was subtracted from the first one.

The recruitment of cells was analyzed by flow cytometry (FACSCalibur; BD Biosciences). The cells were stained for leukocytes (leukocyte common Ag CD45, clone HI30; BD Biosciences), Th cells (CD4, clone H129.19; BD Biosciences), CTLs (CD8, clone 53-6-7; BD Biosciences), B cells (CD19, clone RA3-6B2; Caltag Laboratories), granulocytes (Gr-1, clone RB6-8C5; BD Biosciences), dendritic cells (CD11c, clone HL3; BD Biosciences), and macrophages (Gr-1+/CD11b+, clone M1/70.15; Caltag Laboratories).

**Cytokine and Ab analysis**

Cytokine concentrations were determined by sandwich ELISA systems with unlabeled capture Abs and labeled detection Abs. To determine the concentration of IL-4, mAb 11B11 was used as the capture Ab and biotin-labeled BVD6-24G2 (BD Pharmingen) was used as the detection Ab followed by incubation with peroxidase-labeled streptavidin. To determine the concentration of IFN-γ, mAb AN18 was coated as the capture Ab and peroxidase-labeled XMG1.2 was used in the detection step. The concentrations of IL-13, IL-5, TNF-α, and IL-17 were detected with the R&D Systems DuoSet kits.

The total serum IgE concentration using a murine IgE standard (BD Pharmingen) was determined with R32-72 (BD Pharmingen) as capture Ab and a biotinylated anti-mouse-IgE Ab (R35-118; BD Pharmingen) was used for detection by incubation with peroxidase-labeled streptavidin. Total serum IgG1 and IgG2a were detected with unlabeled polycional goat antihuman IgG1 or IgG2a (Southern Biotechnology Associates) as capture Abs, respectively. Biotinylated polycional goat anti-mouse IgG1 and IgG2a (Southern Biotechnology Associates) were used for detection by incubation with peroxidase-labeled streptavidin. For quantification of total serum IgG1 and IgG2a we used murine IgG1 and IgG2a standards (Southern Biotechnology Associates).

**Measurement of respiratory function of the lung**

The respiratory lung function of intranasally infected mice was measured in a plethysmographic chamber (model PLT URN MS; emka Technologies) for freely moving animals. The pressure inside the chamber was measured by a differential pressure transducer connected to an amplifier (model AC264) and was continuously monitored through software (XA version 1.565). Changes in box pressure are differentiated to give a pseudoflow signal, which is then dependent experiments are shown. Significant differences are shown as indicated. Pooled data from four independent experiments are shown. Significant differences are shown as indicated.

**Statistical analysis**

The statistical significance of differences between experimental groups of animals was determined using the log-rank test for survival analysis, the one-tailed Mann-Whitney U test for organ burden and FACS analysis, and the two-tailed Mann-Whitney U test for cytokine and isotype levels as well as for the IgG2a to IgG1 ratio and airway hyperreactivity.

**Results**

**Effect of IL-13 on survival and organ burden in pulmonary cryptococcosis**

To elucidate the role of IL-13 in the pathogenesis of pulmonary cryptococcosis, we infected WT, IL-13Tg$, and IL-13$^{+/−}$ BALB/c mice intranasally with 500 CFU of the highly virulent C. neoformans strain 1841 (11, 31) and observed the animals for a period of 150 days. WT and IL-13Tg$^+$ mice were susceptible with a median survival time of 81 and 72 days p.i., respectively (Fig. 1A). The difference in the survival time of susceptible WT and IL-13Tg$^+$ mice was highly significant ($p = 0.0026$). In contrast, 89% of IL-13$^{+/−}$ mice were resistant to the cryptococcal infection ($p < 0.0001$ comparing WT and IL-13$^{+/−}$ mice). These findings were paralleled by the organ burden of the primary infected organ, the lung (Fig. 1B). IL-13Tg$^+$ mice showed a significantly elevated fungal burden in the lung on day 60 p.i. in comparison to WT animals ($p = 0.0083$). In agreement with their elevated survival time, IL-13$^{+/−}$ mice had significantly lower fungal pulmonary burden than WT and IL-13Tg$^+$ mice ($p < 0.0001$) (Fig. 1B). Dissemination of C. neoformans from the lung to other organs occurred, e.g., to spleen, and similarly as in lungs, higher fungal organ burden was found there in the presence of IL-13 (data not shown).

C. neoformans infection induces production of IL-13 that drives a pronounced type 2 cytokine and Ab isotype profile

Elevated levels of IL-4 and IL-13 were found in lungs of susceptible genotypes on day 60 p.i., indicating C. neoformans-induced production of type 2 cytokines (Fig. 2A). Resistant IL-13$^{+/−}$ mice showed IL-4 levels in the lung that were comparable to levels found in lungs of the susceptible strains, indicating IL-4-independent but IL-13-dependent mechanisms for susceptibility to C. neoformans infection (Fig. 2A). However, IL-13$^{+/−}$ mice produced only minor levels of IL-4 and IL-5 by ex vivo stimulated splenocytes as compared with WT and especially IL-13Tg$^+$ mice (Fig.
The Con A-induced splenocyte response of these susceptible strains was characterized by pronounced production of IL-4, IL-13, and IL-5, indicating a type 2 cytokine bias that is enhanced by IL-13 (Fig. 2B). In response to *C. neoformans* Ag, IL-4 production by splenocytes of WT and IL-13Tg/Tg mice was comparable but IL-13 secretion much higher in IL-13Tg/Tg mice. Thus, reduced survival time and higher organ load in IL-13Tg/Tg mice shown in Fig. 1 were found to be associated with elevated production of IL-13 by ex vivo restimulated splenocytes. In splenocytes of naive mice, the production of these cytokines was marginal (data not shown), which demonstrates that the observed type 2 bias in WT and especially IL-13Tg/Tg mice is specifically induced by *C. neoformans* infection.

The cytokine profile of lung and spleen was reflected in the Ab isotype profiles of the examined genotypes. The genotypes with high type 2 cytokine levels developed high levels of IgE on day 60 p.i. (top) and compared with serum of naive mice (bottom). Data from four independent experiments, the same as in Fig. 1B, are shown. Significant differences are shown as indicated.

**FIGURE 2.** In the presence of IL-13 a Th2 phenotype in intranasal cryptococcosis is induced. A, WT, IL-13−/−, and IL-13Tg+ mice were intranasally inoculated with *C. neoformans* (500 CFU/mouse) and sacrificed on day 60 p.i. The production of IL-4, IL-13, and IL-5 by lung cells was analyzed by ELISA. Data from two independent experiments are pooled. B, Splenocytes of infected mice were restimulated ex vivo for 72 h and examined for the production of IL-4, IL-13, and IL-5 by ELISA. In comparison splenocytes of naive mice were analyzed (data not shown). Splenocytes of the mice were pooled (5 × 10⁶/ml) and stimulated in triplicates with ConA or heat-killed *C. neoformans* (hk Cn) for 72 h. Results from one representative experiment of two are shown. C, Serum of WT, IL-13−/−, and IL-13Tg+ mice was analyzed for total IgE and IgG1 levels on day 60 p.i. (top) and compared with serum of naive mice (bottom). Data from four independent experiments, the same as in Fig. 1B, are shown. Significant differences are shown as indicated.
60 p.i. in the serum (Fig. 2C), whereas only some IL-13−/− mice showed induction of IgE. However, the IgE-producing IL-13−/− mice did not develop higher pulmonary fungal loads than IgE-negative IL-13−/− mice (data not shown). IgE production was induced by *C. neoformans* infection because naive mice did not develop detectable serum IgE (Fig. 2C) except for IL-13Tg+ mice, which showed low production of IgE. A similar type 2 cytokine-dependent isotype profile is seen in the IgG1 levels of the different genotypes. Resistant IL-13−/− mice develop significantly lower IgG1 levels than susceptible wild-type and IL-13Tg+ mice after infection with *C. neoformans* (Fig. 2C).

**IL-13 has only a minor role in IFN-γ regulation but inhibits IL-17 production**

In contrast to the differential production of the type 2 cytokines IL-4 and IL-13, only low levels of IFN-γ were found in lung homogenates. However, pulmonary expression of IFN-γ did not differ between the resistant IL-13−/− mice and the susceptible WT and IL-13Tg+ mice (data not shown). In contrast, ex vivo restimulated splenocytes of day 60-infected mice (but not naive mice) secreted IFN-γ (Fig. 3A). Resistant and susceptible genotypes showed comparable production of IFN-γ with a slight increase of Th1 cytokines produced by the resistant IL-13−/− mice. The small increase in the Ag-specific secretion of IFN-γ by splenocytes derived from IL-13−/− mice apparently was not associated with elevated serum IgG2a (Fig. 3B). The Th1-dependent isotype IgG2a was induced by *C. neoformans* infection for all genotypes in the same manner (Fig. 3B). The IgG2a to IgG1 ratio, a marker for type 1 to type 2 responses, was found to be significantly higher for IL-13−/− mice than for the susceptible genotypes, indicating a type 1 bias for infected IL-13−/− mice (*p < 0.013* WT vs IL-13−/− mice) (Fig. 3C). Comparing isotype levels of *C. neoformans*-infected mice with naive mice shows that it is mainly the type 2 cytokine-dependent isotypes IgE and IgG1, which are regulated by IL-13 (Fig. 2C).

Recently we showed that an IL-23-dependent Th17 response contributes to protection against *C. neoformans* infection (31). Thus, we were interested in characterizing the production of IL-17A by ex vivo restimulated splenocytes of resistant vs susceptible mouse strains. High amounts of IL-17A were produced by Con A- or Ag-stimulated spleen cells from resistant IL-13−/− mice but only low IL-17A production was found in splenocyte supernatants from susceptible WT and IL-13Tg+ mice upon infection with *C. neoformans* (Fig. 3A). Again, Ag-specific production of IL-17A by ex vivo restimulated splenocytes was marginal in naive mice (data not shown).

**Pulmonary type 2 inflammatory response, lung eosinophilia, goblet cell metaplasia, and elevated mucus production in the presence of IL-13**

The inflammatory response was analyzed histologically at day 60 p.i. Lung eosinophilia was detected in WT and with an even higher frequency in IL-13Tg+ mice but not at all in IL-13−/− mice (Fig. 4, A–C, open arrow). It is evident in the photomicrographs that the extent of the pulmonary inflammatory response was stronger in WT (Fig. 4, A and D) and IL-13Tg+ (Fig. 4, C and F) mice than in IL-13−/− mice (Fig. 4, B and E). In WT mice, a nodular inflammatory reaction comprising neutrophils, macrophages, eosinophils, and few lymphocytes was observed. *C. neoformans* was detectable inside of macrophages and multinucleated giant cells.
but also extracellular intra-alveolar proliferation of cryptococci was found in WT mice. In marked contrast, cryptococci were found to be widely distributed in the alveoli of IL-13Tg/H11001 mice (Fig. 4, C and F) associated with frequent massive intra-alveolar lesions. The diffuse distribution of cryptococci in the lung of IL-13Tg/H11001 mice was accompanied by the appearance of several eosinophils, some neutrophils and macrophages with intracytoplasmic yeasts, and few lymphocytes (Fig. 4, C and F). Only a minimal inflammatory infiltrate was found in lungs of IL-13/H11002 mice (Fig. 4, B and E). All naive control mice revealed no inflammatory infiltrate in the lungs (Fig. 4, G–I).

Consistent with the histopathological analysis of lung sections, FACS staining of single lung leukocytes of day 60-infected mice revealed a similar pattern (Fig. 5A). The proportion of granulocyte, macrophage, and dendritic cell populations was elevated in susceptible WT and IL-13Tg+ mice, whereas IL-13Tg+ mice showed a significantly smaller Th cell population than WT and especially IL-13−/− mice (Fig. 5A). The number of total leukocytes was elevated in susceptible WT and IL-13Tg+ mice, whereas a smaller leukocyte population was observed in the lungs of resistant IL-13−/− mice 60 days p.i. (Fig. 5B). Elevated absolute numbers of granulocytes, macrophages, and dendritic cells were detected in the presence of IL-13 (Fig. 5B). Interestingly, we observed a more moderate inflammatory response with respect to granulocytes and macrophages in the lungs of C. neoformans-infected IL-13−/− mice as compared with IL-4−/− mice pointing to distinct activities of IL-13 vs IL-4 in cryptococcosis (data not shown). When comparing lung leukocyte counts from mice infected for 60 days vs naive mice, we found no differences in the numbers of the various leukocyte subpopulations of IL-13−/− mice consistent with the observed minimal inflammatory infiltrate in this genotype at this time point (Fig. 4, B and E), but a more than 2-fold increase in the infected susceptible genotypes as compared with naive WT and IL-13Tg+ mice (data not shown).

In addition, goblet cell metaplasia was found in WT (Fig. 4D) and IL-13Tg+ (Fig. 4F) but not in IL-13−/− (Fig. 4E) mice. Pulmonary fibrosis was analyzed by Elastic van Gieson staining for collagen but it was neither evident in lungs of the highly susceptible IL-13Tg+ and WT mice nor in the resistant IL-13−/− mice.
Pulmonary hyperreactivity in the presence of IL-13

To study lung function in C. neoformans-infected mice, we examined naive and infected mice under resting conditions and increasing methacholine concentrations. We found that under resting conditions day 60-infected WT and IL-13−/− mice had a pulmonary resistance similar to naive mice (Fig. 7A). However, already in the resting state susceptible IL-13Tg mice on day 60 p.i. exhibited a significantly increased pulmonary resistance as compared with naive and even to the other infected mice strains (in particular as compared with infected WT mice) (Fig. 7A). This result is consistent with the high infection burden found in the lungs of the IL-13Tg mice (Fig. 1B). Upon methacholine challenge naive animals of each genotype responded with a small dose-dependent increase in airway resistance (Fig. 7B). Infected IL-13−/− mice showed a mild increase in pulmonary resistance upon methacholine challenge, which was significantly lower than the methacholine responses observed in both, infected WT and IL-13Tg mice (Fig. 7B). These findings are consistent with high fungal burden and elevated mucus production in lungs of C. neoformans-infected WT and IL-13Tg mice.

Discussion

Previous experimental work from our group has shown that long-term survival of mice infected with the highly virulent C. neoformans strain 1841 can be either achieved by continuous administration of the Th1-inducing IL-12 or by suppression of the Th2-inducing cytokine IL-4 (11). In the present study we show an additional strategy allowing for long-term survival following C. neoformans infection: the suppression of IL-13 as another Th2 cytokine.

Indirect evidence for a potential role of IL-13 in cryptococcosis was provided by other earlier studies. Previously, treatment of C. neoformans-infected mice with anti-IL-4 mAb resulted in enhanced IFN-γ production and prolonged survival of mice (50). However, despite of the IL-4 mAb treatment IL-4 levels in bronchoalveolar lavage fluid remained elevated. Unchanged IL-4 synthesis upon neutralization of IL-4 might be an indication for IL-4-independent and potentially IL-13-dependent Th2 differentiation (50). Induction of IL-13 during cryptococcosis was reported in a few recent studies (49, 55, 56). However, the functional role of IL-13 during cryptococcosis has not been directly addressed.

We have approached the functional characterization of IL-13 in C. neoformans infection by analyzing IL-13 gene-deficient mice and IL-13-overexpressing mice in comparison to WT mice. Our results demonstrate for the first time a role of IL-13 in susceptibility during cryptococcal infection: IL-13 expression leads to greatly reduced resistance as evidenced by reduced survival and higher pulmonary fungal burden, goblet cell metaplasia, mucus production, and elevated airway hyperreactivity. Moreover, our results point to several mechanisms by which IL-13 negatively

(data not shown). Minimal fibrosis was detectable only in the intralveolar cryptococcosis regions but not in the peribronchial or interstitial lung tissue of IL-13Tg mice (data not shown).

Development of lung aaMph expressing arginase-1, mannose receptor (CD206), and Ym1 in the presence of IL-13

IFN-γ-dependent activation of macrophages for fungicidal activation is essential for resistance against C. neoformans (14, 54). In lung macrophages of WT (Fig. 6A) and IL-13Tg mice (Fig. 6C), phagocytosed cryptococci were found. In addition, IL-13Tg mice showed strong extracellular multiplication of the yeast (Fig. 6C). In contrast, macrophages of IL-13−/− mice apparently had cleared most cryptococci at day 60 p.i. (Fig. 6B). This indicates insufficient killing mechanisms in the presence of IL-13. Recently, pulmonary macrophages of Cryptococcus-infected susceptible IFN-γ−/− mice were shown to be alternatively activated (49). Therefore, lung sections of naive and day 60-infected WT, IL-13−/−, and IL-13Tg mice were stained for markers of caMph vs aaMph. Upon infection of susceptible WT and IL-13Tg mice with C. neoformans high numbers of enlarged lung macrophages expressing arginase-1 (Fig. 6, D–F), CD206 (Fig. 6, G–I), Ym1 (Fig. 6, J–L) were detected. In addition, consistent with the observed low-level production of IFN-γ in WT and (IL-13Tg mice also expression of iNOS was found in these susceptible mouse strains (data not shown). As could be expected, the resistant genotype IL-13−/− with its strikingly reduced pulmonary inflammatory response (see Fig. 4, B and E) had only few and weakly stained lung macrophages expressing CD206, and almost no cells positive for Ym1 or arginase-1 (Fig. 6, E, H, and K). Similarly, the frequency of iNOS expressing cells was minimal in lungs of IL-13−/− mice infected for 60 days (data not shown). However, 2 wk earlier in the course of infection (a time point with higher fungal burden in lungs of IL-13−/− mice as compared with day 60), some expression of iNOS in lungs of IL-13−/− mice was detectable (data not shown) suggesting increasing control of the pathogen by IL-13−/− mice with time and Ag load-driven iNOS expression. In summary, a high number of lung macrophages expressing arginase-1 together with CD206 and Ym1 in WT and IL-13Tg mice indicate development of aaMph in the presence of IL-13.

FIGURE 5. An elevated number of granulocytes, macrophages, and dendritic cells in the presence of IL-13 60 days after infection with C. neoformans. A, relative distribution of leukocytes in WT, IL-13−/−, and IL-13Tg mice. Results from three independent experiments were pooled (three mice/group and experiment), *, p < 0.05; **, p < 0.01; ***, p < 0.001. B, The absolute number of lung leukocytes in WT, IL-13−/−, and IL-13Tg mice. One of two similar experiments with the total number of leukocytes as indicated is shown. Cells from three mice/group were pooled, counted (corrected for the number of cryptococci in the samples as described in Materials and Methods), and analyzed by FACS analysis. B, B cells; DC, dendritic cells; Gr, granulocytes; Leuko, total leukocytes; Mph, macrophages; Tc, CD8+ T cells; Th, CD4+ T cells.
affects lung function during cryptococcosis. These IL-13-dependent mechanisms include 1) enhanced expression of type 2 cytokine; 2) development of aaMph; 3) modulation of Th17 responses; and 4) modulation of goblet cell function.

One major mechanism of development of susceptibility to C. neoformans appears to be the IL-13-dependent enhancement of type 2 cytokine responses. As shown for leishmanial infection IL-13 is a key factor in the induction of a Th2 response even in the absence of IL-4 expression (57, 58). In our study we could detect elevated levels of IL-4, IL-13, and IL-5 in WT mice and especially in IL-13Tg mice. This type 2 cytokine profile was also evident in the increased serum IgG1 and IgE levels of the susceptible IL-13-expressing genotypes. Mechanistically IL-13-driven enhancement of Th2-dependent IgG1 and IgE must be based on indirect mechanisms because a functional receptor for IL-13 is not expressed on murine T cells (59). Together, type 2 cytokine production, generation of Th2-dependent cytokines and development of lung eosinophilia present in WT and IL-13Tg mice can be considered as processes typical of susceptibility to C. neoformans infection (55, 60). Type 2 or “allergic” inflammation was found to be enhanced in rats with chronic pulmonary C. neoformans infection (56). This recent publication points to a link between chronic exposure to cryptococcal Ags and asthma. Infected rats showed a predisposition to asthma after pulmonary infection with C. neoformans. In our infection model we could see a similar phenomenon. Susceptible IL-13-expressing WT mice showed symptoms typical of asthma after exposition to an asthma inducer only when they were previously infected with C. neoformans but not in a naive state (Fig. 7). This allergic status is supported by infection-induced elevated IgE levels.

It is noteworthy that the aforementioned markers of IL-13-driven type 2 cytokine production can be closely linked with susceptibility but apparently no direct inverse correlation is possible between IL-13 and protective Th1 development as based on production of IFN-γ or serum IgG2a (see Fig. 3). In agreement with our data, in another pulmonary C. neoformans infection model a similar observation was reported. No effect of IL-4 and IL-10 on IFN-γ induction could be found in that study (55). IFN-γ production was found to be similar between resistant and susceptible mouse strains in cryptococcosis. Thus, the cytokines more important for the course of pulmonary cryptococcosis seem to be the type 2 cytokines. It is only in the absence of these cytokines that IFN-γ is able to act in a protective manner. IL-13 has been found to down-regulate IL-12Rβ2 expression (61). We have analyzed lung mRNA expression of IL-12Rβ1 and IL-12Rβ2 (as well as IL-12p35 and p40) in the absence and presence of IL-13 expression at 60 day p.i., but found no obvious correlation with resistance or susceptibility to C. neoformans (data not shown). This is consistent with minor differences in IFN-γ production and serum IgG2a (see Fig. 3) and similar levels of lung IFN-γ in supernatants of lung homogenates (data not shown) of resistant IL-13−/− mice and susceptible WT and IL-13Tg mice at day 60 p.i. Moreover, expression of iNOS by pulmonary macrophages was found to be independent of their susceptible or resistant phenotype at day 60 p.i. (data not shown). In summary, these observations support the hypothesis that the presence of type 2 but not type 1 cytokines critically regulates immunity to pulmonary C. neoformans infection.

In contrast, our data point to an association of Th17 responses and protection in cryptococcosis. Significant T cell-derived IL-17
was detected in resistant mice as compared with susceptible IL-13 expressing mice. Thus, IL-13 expression appears to down-regulate Th17 responses. A possible link between IL-13 and IL-17 was described in a model of autoimmune inflammation of the brain (i.e., experimental allergic encephalomyelitis) (62). Mice supplemented with IL-25 produced elevated levels of IL-13 that suppressed the production of Th17 cells resulting in complete suppression of Th17-induced experimental allergic encephalomyelitis disease. Recently, we could show in an i.p. model of C. neoformans strain 1841 infection that the protective function of IL-23 is associated with IL-17 production (62). In the present pulmonary model the elevated levels of Ag-specific IL-17 produced by splenocyte cultures (Fig. 3) and lung cell cultures (data not shown) of resistant strains point to a role of Th17 responses in local pulmonary cryptococcal infection. By infecting IL-17A−/− mice intranasally with C. neoformans, we obtained evidence for a significant role of IL-17 for survival (U. Müller and G. Alber, unpublished observations). The mechanism of IL-17-dependent protection is still under investigation. Further support for a protective activity of IL-17 was provided very recently. In an allergic asthma model it could be shown that IL-17 treatment reduces pulmonary IL-5 and IL-13 production and the recruitment of eosinophils (63). Both these parameters are consistent with data from our study (e.g., elevated splenocyte IL-17 production and diminished lung eosinophilia in the absence of IL-13).

Activation of macrophages harboring cryptococci is a central effector mechanism in immunity to C. neoformans (30, 64, 65). Whereas in mice T cells and B cells only express IL-4R type 1 and therefore can only respond to IL-4, macrophages express IL-4R type 2, which can respond to IL-4 and also IL-13 (59). Therefore, both IL-4 and IL-13 have the potential to induce development of aaMph. In the present study we could detect markers specific for aaMph such as arginase-1, mannose receptor (CD206), and YM1 in macrophages of susceptible IL-13-producing strains but not in IL-13−/− mice. This demonstrates that IL-13 is able to induce the development of aaMph in C. neoformans infection and links high fungal lung burden (see Fig. 1) with insufficiently fungicidal pulmonary macrophages. As shown earlier susceptible mice such as IFN-γ−/− mice (which were found to produce IL-13) also show evidence of alternatively activated macrophages in pulmonary cryptococcosis as judged by arginase-1 vs iNOS expression and expression of eosinophilic crystalline protein YM1 (49). Development of aaMph appears to be a common feature of and maybe a marker for susceptibility to cryptococcosis. Very recently it could be shown that chitin, which is an important component of the cell wall of C. neoformans (66), is able to induce the accumulation of IL-4-producing innate immune cells, e.g., eosinophils and basophils, in tissue and mediates the activation of alternatively macrophages (67). Interestingly, in our study development of aaMph was not associated with significant fibrosis in contrast to studies looking at e.g., Schistosoma mansoni (68). The lack of a fibrotic response in our study is different from C. neoformans-infected (serotype D) IFN-γ−/− mice on a C57BL/6 background where some lung fibrosis was found by Masson’s trichrome staining of lung sections (49).

In summary, IL-13 plays an immunopathological role that adds to the fatal action of IL-4, which has been observed in studies looking at C. neoformans infection and other pathogens (11, 50, 51, 58, 61). IL-13 but not IL-4 can activate goblet cells to produce mucus that is beneficial in the gut to expulse helminths (69) but can be deleterious in experimentally induced asthma (44). By analyzing Cryptococcus-infected IL-13Tg mice together with WT and IL-13−/− mice, we could identify IL-13-specific actions. In several parameters (e.g., lung fungal burden, survival period, lung eosinophilia, inflammatory response of the lung) we found a gradual increase of these parameters in IL-13Tg−/− mice as compared with WT mice, suggesting a quantitative correlation between IL-13 production and disease severity. In addition, using IL-13Tg−/− mice and comparing these mice with IL-13−/− mice was important to us because it has been shown that the IL-13−/− mice used in this study may be partially defective in IL-4 gene expression due to disrupting a potential regulatory element of the IL-4 gene by the IL-13 gene targeting. Therefore, we determined IL-4 production in lungs and spleen cells of IL-13−/− mice and compared it with WT mice. Fig. 2A shows similar levels of IL-4 produced in lungs of infected WT and IL-13−/− mice. In contrast, IL-4 levels secreted by splenocytes of IL-13−/− mice were found to be lower than in WT mice. Also, serum IgE levels were reduced in IL-13−/− mice as compared with WT mice. Therefore, comparing WT and IL-13Tg−/− mice enabled us to draw conclusions on a specific role of IL-13 independent of IL-4. Based on this comparison, we were able to show a correlation between higher pulmonary fungal load (see Fig. 1) and elevated Ag-specific IL-13 production (see Fig. 2B), whereas IL-4 production was similar in WT and IL-13Tg−/− mice.

In our present study for the first time to our knowledge the individual contributions of the Th2 cytokine IL-13 for fatal allergic inflammation and development of aaMph during C. neoformans infection have been characterized. Future therapeutic strategies have to consider IL-13 or target the IL-13-specific receptor component IL-13Rα1 or the receptor component common for IL-13 and IL-4, IL-4Rα. Neutralizing IL-13 or blocking its receptor should be a promising therapy for long-lasting
protection during C. neoformans infection in human patients as well.

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Disclosures
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