IL-23 Enhances the Inflammatory Cell Response in Cryptococcus neoformans Infection and Induces a Cytokine Pattern Distinct from IL-12

Melanie A. Kleinschek, Uwe Muller, Scott J. Brodie, Werner Stenzel, Gabriele Kohler, Wendy M. Blumenschein, Reinhard K. Straubinger, Terrill McClanahan, Robert A. Kastelein and Gottfried Alber

_J Immunol_ 2006; 176:1098-1106; doi: 10.4049/jimmunol.176.2.1098 http://www.jimmunol.org/content/176/2/1098

**References**

This article cites 42 articles, 27 of which you can access for free at: http://www.jimmunol.org/content/176/2/1098.full#ref-list-1

**Subscription**

Information about subscribing to _The Journal of Immunology_ is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
IL-23 Enhances the Inflammatory Cell Response in Cryptococcus neoformans Infection and Induces a Cytokine Pattern Distinct from IL-12

Melanie A. Kleinschek,* Uwe Muller,† Scott J. Brodie,‡ Werner Stenzel,§ Gabriele Kohler,§ Wendy M. Blumenschein,|| Reinhard K. Straubinger,* Terrill McClanahan,|| Robert A. Kastelein, and Gottfried Alber2*

IL-23, a heterodimeric cytokine composed of the p40 subunit of IL-12 and a novel p19 subunit, has been shown to be a key player in models of autoimmune chronic inflammation. To investigate the role of IL-23 in host resistance during chronic fungal infection, wild-type, IL-12− (IL-12p35−/−), IL-23− (IL-23p19−/−), and IL-12/IL-23− (p40-deficient) deficient mice on a C57BL/6 background were infected with Cryptococcus neoformans. Following infection, p40-deficient mice demonstrated higher mortality than IL-12p35−/− mice. Reconstitution of p40-deficient mice with rIL-23 prolonged their survival to levels similar to IL-12p35−/− mice. IL-23p19−/− mice showed a moderately reduced survival time and delayed fungal clearance in the liver. Although IFN-γ production was similar in wild-type and IL-23p19−/− mice, production of IL-17 was strongly impaired in the latter. IL-23p19−/− mice produced fewer hepatic granulomata relative to organ burden and showed defective recruitment of mononuclear cells to the brain. Moreover, activation of microglia cells and expression of IL-1β, IL-6, and MCP-1 in the brain was impaired. These results show that IL-23 complements the more dominant role of IL-12 in protection against a chronic fungal infection by an enhanced inflammatory cell response and distinct cytokine regulation. The Journal of Immunology, 2006, 176: 1098–1106.

Interleukin 23 is a member of the IL-12 family of cytokines and is composed of the p40 subunit of IL-12 and is a novel p19 subunit (1). IL-23 and IL-12 are both produced by activated APCs (2, 3) and their receptors share a common subunit IL-12Rβ1, complemented by IL-12Rβ2 for IL-12 (4) and IL-23R for IL-23 (5). Due to those common features, it was assumed that these cytokines would have mostly overlapping effects. The role of IL-12 in maintenance of Th1 responses by induction of IFN-γ in effector T cells and NK cells is well-characterized and important in host defense (6). It turned out, however, that IL-23 has very distinct effects (7, 8). Until now, in two models of T cell-mediated chronic autoimmune inflammation, experimental autoimmune encephalomyelitis (EAE) (9) and collagen-induced arthritis (10), IL-23 rather than IL-12 was demonstrated to be the critical cytokine in the development of chronic inflammation. Recently, a characteristic function of IL-23 was shown to be the promotion of IL-17 production by a distinct T cell subset (11, 12). IL-17 is a potent inflammatory cytokine stimulating mainly macrophages and endothelial cells to produce factors contributing to local inflammation (13, 14). Mice specifically lacking IL-23 or IL-17 show an impaired Ag-specific cellular immune response (15, 16). In a very recent report, the IL-23/IL-17 axis was shown to be important for regulation of granulopoiesis by induction of G-CSF (17).

Cell-mediated immunity is an important feature in immunity against intracellular infections. Previously, we and others have reported that mice lacking the p40 subunit of IL-12/IL-23 are more susceptible to chronic infection with intracellular pathogens than mice lacking the p35 subunit of IL-12 (18–22). In the murine model of infection with Cryptococcus neoformans, we found that IL-12p35−/− and p40-deficient mice develop a Th2 response associated with elevated susceptibility (18). This pointed to the essential role of IL-12 in immunity to C. neoformans. Nevertheless, IL-12p35−/− mice were still able to form granulomata during cryptococciosis, whereas granuloma formation in mice lacking the p40 subunit was greatly impaired (18). In light of these data and the demonstrated role of IL-23 in cellular immune responses in various noninfection in vivo models (9–11, 15), a contribution of IL-23 to resistance in C. neoformans infection is conceivable.

C. neoformans is an encapsulated yeast-like, facultative intracellular organism and causes meningocencephalitis in immunodeficient hosts. It is the most common lethal fungal infection in AIDS patients with CNS disease (23). IL-12, IFN-γ, TNF-α, and inducible NO synthase play a central role in protective immunity to C. neoformans (18, 24–27). T cells and macrophages are the predominant cell types that characterize a granulomatous inflammatory response associated with protection against C. neoformans infection in patients as well as in mouse models (23).

In the present study, we investigate the contribution of IL-23 to host defense in chronic C. neoformans infection by reconstitution...
of infected p40-deficient mice with recombinant murine IL-23 (rmIL-23), and by infection of mice specifically lacking IL-23 while still able to produce IL-12 (IL-23p19−/−; mice; Ref. 9). The results demonstrate that IL-23 enhances the inflammatory cell responses and thus complements the more dominant role of IL-12 in protective immunity to *C. neoformans*.

**Materials and Methods**

**Mice**

*C. neoformans*, strain 1841, serotype D, was used as described previously (18). The acapsular *C. neoformans* strain CAP67, serotype D, was used as it was found to have better stimulatory capacities than a highly virulent strain (M. A. Kleinschek and G. Alber, unpublished observations and Ref. 31). Splenocytes were either stimulated with viable (10⁶ cells/ml) or heat-killed (10⁶ cells/ml) *C. neoformans* organisms. After a 48-h incubation at 37°C in a humidified CO₂ atmosphere, supernatants were taken and stored at −20°C until assayed for cytokines and chemokines.

For intracerebral cytokine staining, splenocytes were prepared and plated as described above and stimulated with plate-bound anti-CD3 plus soluble anti-CD28 (1 μg/ml) in the presence of Golgi-plug (according to BD Pharmingen’s Cytofix/Cytoperm Plus kit instructions) for the final 4 h. This was followed by surface staining with anti-CD4 (FITC-labeled), permeabilization with Cytofix/Cytoperm buffer, and intracellular cytokine staining using anti-IFN-γ (allophycocyanin-conjugated). Cells were analyzed with a FACSCalibur flow cytometer. All Abs and staining buffers were purchased from BD Biosciences.

**Cytokines, chemokines, IgE**

Splenocyte culture supernatants, sera, and brain homogenates were assayed using the Beadlyte Mouse Multicytokine Detection System 2 and additional IL-17 and MCP-1 beadsets, following the manufacturer’s recommendations (Upstate Biotechnology), including modification for the use of the high biotin-containing RPMI 1640 medium. When serum samples or supernatants of organ homogenates were assessed, the protocol was modified for the analysis of sera according to the manufacturer’s recommendations. Briefly, 50 μl of the diluted standards or samples were added to each well with the addition of the cytokine capture Ab beads. After a 2-h incubation, plates were subjected to a vacuum manifold to control for high biotin in the medium. Reporter solution was added and incubated for 1.5 h. Diluted streptavidin-PE solution was added to each well, and plates were incubated for 30 min. After stopping the reaction, plates were subjected to vacuum and after addition of assay buffer, read on the Luminex (100) machine which was programmed with specific bead-signature numbers representing the cytokines assayed.

The IgE concentration was determined by sandwich ELISA using the capture Ab R32-72 (BD Pharmingen) and a biotinylated anti-mouse IgE Ab (BD Pharmingen; R35-118) for detection after incubation with peroxidase-labeled streptavidin (Southern Biotechnology Associates) and tetramethyl benzidine as substrate (KPL).

**Quantitative RT-PCR**

Total RNA was prepared from frozen brain tissue samples using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. Five micrograms of total RNA was reverse-transcribed with oligo (dT)15 (Roche) and random hexamers (Promega) following elimination of genomic DNA by DNaseI digestion (Ambion). Cytokine-, chemokine- and cell marker-specific mRNA was measured using real-time quantitative PCR (ABI 7500) with SYBR Green PCR Mastermix (Applied Biosystems) as described previously (11). Gene expression levels were normalized to expression of the housekeeping gene ubiquitin for each sample; relative expression levels of infected mice were further normalized to naive controls and compared between groups as x-fold up-regulation.

**Statistical analysis**

The Mann-Whitney rank sum test was used for comparisons of two groups. When comparing three or more groups, Kruskal-Wallis statistics followed by Dunn’s posttest was performed. Survival proportions were displayed using the Kaplan-Meier method; statistical differences were assessed by the log rank test. Median survival times were compared using a paired *t* test. In either case, GraphPad Prism software was used (GraphPad Software). Statistical significance was defined to be based on a *p* value <0.05.

**Results**

*Treatment with rIL-23 is protective in *C. neoformans* infection in the absence of IL-12*

We previously reported that p40-deficient mice (which lack all p40-dependent IL-12 family members) show a higher susceptibility to infection with *C. neoformans* than IL-12p35−/− mice (lacking only IL-12) (18). Therefore, we addressed the question if the
elevated susceptibility in p40-deficient mice was due to the lack of IL-23, a heterodimer of p40 and p19 (1). Wild-type (WT), IL-12p35−/−, and p40-deficient mice were infected with the C. neoformans serotype D strain 1841 used previously (18). The lack of IL-12 (IL-12p35−/− mice) is associated with a significantly reduced survival time (Fig. 1; p < 0.01) consistent with our previous report (18). In addition, the combined lack of IL-12 and IL-23 (p40-deficient mice) results in an even shorter survival time (Fig. 1). rmIL-23 or PBS was administered to p40-deficient mice beginning 2 days before infection, continued daily until day 20 p.i. and twice a week thereafter. The administration of rmIL-23 resulted in a significantly prolonged survival period of p40-deficient mice as compared with their PBS-treated littermates (Fig. 1; p < 0.001). IL-23 treated p40-deficient mice reached survival periods comparable to those of IL-12p35−/− mice (Fig. 1). Besides these effects on survival, rmIL-23 treatment resulted in a significantly reduced fungal load in liver and brain of p40-deficient mice at day 8 after infection with C. neoformans (data not shown). Our findings demonstrate a protective effect of rmIL-23 on the outcome of infection in IL-12/IL-23-deficient mice, strongly suggesting that IL-23 is the responsible molecule for the higher resistance of IL-12p35−/− mice compared with p40-deficient mice. Moreover, reconstitution of p40-deficient mice with exogenous IL-23 showed that the protective activity of rmIL-23 is independent of endogenous IL-12.

Endogenous IL-23 contributes to resistance to C. neoformans infection in the presence of IL-12

We then wished to define the role of endogenous IL-23 in immunity against C. neoformans. Therefore, IL-23p19−/− mice that specifically lack IL-23, but in contrast to p40-deficient mice, are still able to produce IL-12, were infected with C. neoformans. In five independently performed experiments survival was monitored. Comparing median survival periods of IL-23p19−/− and WT mice obtained in all five experiments, IL-23p19−/− mice showed a significantly reduced median survival time (p < 0.05; Fig. 2A). Statistics performed individually on each of the five experiments show a significantly reduced survival time of IL-23p19−/− in two experiments (*, p < 0.05); the other three experiments (○), including the depicted one (■), showed a trend for reduced survival times in IL-23p19−/− mice. * p < 0.05, n = 8–10 mice for each experiment. Median survival times were compared using paired t test; survival curves were compared by log rank test as described in Materials and Methods.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.

Unaltered Th1/Th2 balance, but impaired IL-17 expression in infected IL-23p19−/− mice

Next, we wished to study the basis for the increased susceptibility of IL-23p19−/− mice to C. neoformans infection. Protection in this liver burden, whereas the fungal load in livers of IL-23p19−/− mice was similar to organ burden observed at day 21 p.i. (p < 0.05; Fig. 3A). At later time points no differences in liver burden were observed (data not shown) indicating that the lack of IL-23 leads to a delayed clearance of C. neoformans organisms in the liver. The fungal load of the brain was comparable between both genotypes at both time points (Fig. 3B). Brain burdens tended to differ at day 49 after infection (without statistical significance) but were similar thereafter (data not shown). These data indicate that even in the presence of IL-12, endogenous IL-23 contributes to resistance to C. neoformans, particularly at later time points.
infection model is shown to be strongly dependent on cellular immune mechanisms, underlining the importance of Th1 promoting IL-12 effects (23, 32). Following infection with *C. neoformans*, IL-23p19−/− mice show similar serum levels of IgE compared with WT controls at day 21 and 49 p.i. (Fig. 4A). Furthermore, both IL-23p19−/− and WT mice had comparable amounts of circulating IFN-γ (Fig. 4B). In contrast, p40-deficient mice that additionally lack IL-12 show a clear Th2 shift by elevated serum IgE levels (p < 0.05) and no detectable circulating IFN-γ (Fig. 4, A and B).

In addition, when we assessed T cell functions at day 21 p.i. by restimulation of splenocytes of *C. neoformans*-infected mice with either a polyclonal stimulus (anti-CD3) or Ag-specific stimuli such as viable (vCn) and heat-killed (hkCn) *C. neoformans* organisms, WT and IL-23p19−/− responded similarly with pronounced IFN-γ production. In contrast, IFN-γ expression in p40-deficient mice was significantly reduced after Ag-specific stimulation (Fig. 4C).

The data demonstrate that the lack of IL-23 does not alter the Th1/Th2 balance in *C. neoformans* infection but leads to a significantly impaired IL-17 production.
Defect in granuloma formation in the livers of infected IL-23p19−/− mice

Protective immunity in C. neoformans infection is characterized by mononuclear cell recruitment to the site of infection leading to the formation of granulomata in most tissues (23). Previously, we reported an impaired ability of p40-deficient mice to form granulomata when compared with IL-12p35−/− mice (18). Because both genotypes of mice used in those studies lack IL-12, we now were interested in assessing the individual contribution of IL-23 for the granulomatous response in cryptococcal infection. Therefore, we compared granuloma formation in the liver between infected IL-23p19−/− and WT mice by light microscopy.

As described above, liver burdens differed between the genotypes at day 35 p.i. but not at day 21 p.i. (Fig. 3). For this reason, we normalized the number of granulomata to the liver fungal burden by calculating the ratio between the mean number of granulomata per ×100 field and the CFU in the liver for each animal. On day 21 after infection with C. neoformans, no difference between WT and IL-23p19−/− mice could be found, whereas at day 35 p.i. WT mice had a significantly elevated normalized number of liver granulomata as compared with IL-23p19−/− mice (Fig. 5). No significant differences were seen in terms of granuloma size in livers of infected WT and IL-23p19−/− mice (data not shown). These findings provide evidence for a role of IL-23 in granuloma formation even in the presence of IL-12.

Impaired inflammatory response in brains of infected IL-23p19−/− mice

C. neoformans shows a strong tropism for the CNS, causing a sustained meningoencephalitis which subsequently leads to death (23). Therefore, it was of particular interest to investigate the inflammatory processes in the brain. Histopathological examinations of brain sections at day 49 after infection revealed a pronounced infiltration of the brain by C. neoformans organisms in both WT and IL-23p19−/− mice. Cryptococcal foci were associated with leukocyte infiltrations in both genotypes (Fig. 6, A and B). Interestingly, H&E stained brain sections revealed a diminished inflammatory response in the IL-23p19−/− mice in four individually performed experiments. To verify this observation, we characterized quality and composition of inflammatory cells by immunohistochemistry and quantified the immunostained cell populations. Immunostaining for MHC-II revealed that in WT mice C. neoformans foci were surrounded by MHC-II-positive macrophages and numerous activated MHC-II-positive microglia cells, whereas in the IL-23p19−/− mice macrophages were significantly less frequent and activation of microglial cells was markedly reduced (Fig. 6, C, D, and E). In evaluating T cell recruitment, IL-23p19−/− showed a significant reduction in CD4+ and CD8+ T cells compared with WT mice (Fig. 6, F–K). Granulocytic leukocyte infiltration of the brain was greater in infected WT vs IL-23p19−/− mice when sections were evaluated using Gr1 staining (Fig. 6).

The results from the immunohistochemical analyses were paralleled by mRNA analyses of the brains showing the degree of cellular recruitment. The levels of transcripts for the mononuclear cell markers F4/80 and CD3 were reduced ∼5-fold in brains from IL-23p19−/− mice as compared with brains from WT mice at day 21 p.i. (Fig. 7).

To characterize the functional differences in the brain inflammatory response of both genotypes, we compared the mRNA and protein expression profile of cytokines and chemokines in the brain of infected mice. At day 21 p.i., WT, but not IL-23p19−/− mice, showed increased expression of IL-1β and MCP-1 mRNA, whereas TNF-α, IL-6, IFN-γ and inducible NO synthase were not expressed at appreciable levels at this time point (Fig. 8A and data not shown). Similarly, elevated levels of MCP-1 protein were seen in brain homogenates of WT mice at day 21 p.i. Moreover, IL-1β and IL-6 protein were present in brain homogenates of WT, but not of IL-23p19−/− mice (Fig. 8B). Remarkably such changes were most prominent at day 35 p.i. than on day 21 (Fig. 8B). Although in models of autoimmunity IL-23p19−/− mice showed a markedly impaired production of TNF-α (9, 10), in the brain homogenates of C. neoformans-infected mice this defect was not that obvious (Fig. 8B). There was no difference in IFN-γ levels when comparing WT and IL-23p19−/− mice, and IL-17 was not detectable (Fig. 8B). The data depicted in Fig. 8 is representative of a trend seen in 2 independent experiments but does not reach statistical significance. Nevertheless this data suggests an important mechanism for the significantly reduced cellular infiltration in brains of infected IL-23p19−/− mice (described in Fig. 6) and points to a role of MCP-1, IL-1β, and IL-6 in IL-23-dependent inflammation. Taken together, these findings demonstrate that the absence of IL-23 is associated with an impaired inflammatory response in the brains of mice infected with C. neoformans, mainly characterized by reduced recruitment and activation of mononuclear cells.

Discussion

IL-23 has recently been described as a key player in autoimmune disease models (9, 10). In a series of in vivo experiments we aimed to explore the role of IL-23 in a chronic fungal infection. We identified a protective function of IL-23 in the absence of IL-12 by reconstitution of p40-deficient mice with rIL-23. In addition, we describe a role for endogenous IL-23 in protective immunity even in the presence of IL-12. However, in the presence of IL-12, endogenous IL-23 displays more moderate protective effects during cryptococcosis.

We have shown previously that p40-deficient mice are more susceptible to infection with the opportunistic pathogen C. neoformans than IL-12p35−/− mice (18). Survival times and organ burden of p40-deficient mice could not be restored by treatment with either monomeric or homodimeric p40 (18), which are produced physiologically in a large excess relative to IL-12 or IL-23 (33). In the present study, administration of rIL-23 greatly improved the survival of infected p40-deficient mice, restoring the survival period of treated mice to the level of IL-12p35−/− mice. Collectively, these data indicate that IL-23 has a role in resistance to C. neoformans infection, although IL-23 is not sufficient for complete protection in the absence of IL-12. Recently, a similar effect was attributed to IL-23 reconstitution in Toxoplasma gondii-infected p40-deficient mice (22).

FIGURE 5. Defective granuloma formation in the absence of IL-23. WT and IL-23p19−/− mice were infected with C. neoformans and the granulomatous response was quantified microscopically. Mean number of granulomata per ×100 field was determined. To compensate for differences in organ burden the ratio between the number of granulomata and the organ burden in the liver was calculated for each animal. *, p < 0.05. Data shown is pooled data of two independently performed experiments (n = 3 mice/experiment).
The shorter survival time and higher organ burden observed in C. neoformans-infected IL-23p19−/− mice compared with WT mice provide evidence for a role of endogenous IL-23 in host resistance. Interestingly, IL-23p19−/− mice infected with T. gondii did not show an altered resistance after infection (22). This may be related to the different nature of T. gondii vs C. neoformans infection and, in addition, only acute stages of infection were investigated in this study (22). Our studies of C. neoformans infection examine both early and late time points. We show that in IL-23p19−/− mice clearance of C. neoformans organisms is delayed late in infection (i.e., day 35 p.i.), presumably after initial control of the infection has been established. Consistent with this observation, impaired clearance has been documented in p40-deficient mice when compared with IL-12p35−/− mice following Francisella tularensis infection (19). In a very recent report on chronic Mycobacterium tuberculosis infection, IL-23p19−/− mice show an elevated bacterial burden in the spleen only at a late time point (34).

In the chronic phase of infection, a granulomatous inflammatory response predominantly characterized by mononuclear cells is an important feature of resistance to C. neoformans infection (23). We previously reported a defect in granuloma formation in p40-deficient mice that was not observed in IL-12p35−/− mice (18). These findings are consistent with other reports on p40-dependent, IL-12-independent defects in granuloma formation during infection with Salmonella Enteritidis or Mycobacterium bovis BCG (20, 21). Of note, in all of these studies p40-dependent granuloma formation was explored in mice lacking IL-12. In the present study, we show that the lack of IL-23 leads to impaired granuloma formation even in the presence of IL-12. These findings are supported by a recent study using a murine model of tuberculosis. Reconstitution of M. tuberculosis-infected p40-deficient mice with rIL-12 only partially restored their ability to form granulomata (35). Granulomata were fewer in IL-12-reconstituted p40-deficient mice than in WT controls suggesting that IL-12 contributes in parts to granuloma formation and that other factors such as IL-23 are likely required for a sustained granulomatous inflammatory response (35). Indeed, the higher susceptibility to disease observed in p40-deficient mice when compared with IL-12p35−/− mice was associated with an impaired granuloma formation in the former genotype (20). In mice competent for IL-12 production, however, the specific lack of IL-23 did not affect granuloma formation in M. tuberculosis infection pointing to a more crucial role of IL-12 in this infection model (34). IL-23 was shown to be responsible for the higher resistance to murine tuberculosis observed in IL-12p35−/− mice compared with p40-deficient mice and was demonstrated to induce IFN-γ in the absence of IL-12 in M.
tuberculosis infection (34). In C. neoformans infection, however, IL-12p35/−/− similarly as p40-deficient mice fail to produce IFN-γ and mount a Th2 response (18). Although neither in the presence nor in the absence of IL-12 IL-23 was shown to induce IFN-γ in C. neoformans infection, in both cases granuloma formation is impaired. Collectively these data indicate that dependent on the nature of the infectious agent IL-23 might contribute to granuloma formation by distinct means.

After initially being able to form hepatic granulomata with a frequency comparable to WT mice, at a later time point the granulomatous reaction was significantly impaired in IL-23p19−/− mice. Consistent with this data, in EAE, a mouse model of multiple sclerosis, susceptible WT and resistant IL-23p19−/− mice initially show comparable leukocyte infiltration into the brain, whereas sustained inflammation of the CNS is only seen in WT mice (9). Similarly, the magnitude of delayed-type hypersensitivity response in IL-23p19−/− mice immunized with methylated BSA was comparable to WT mice at 18 h postinduction but greatly diminished thereafter (15). In summary, such findings point to a crucial role for IL-23 in the maintenance of inflammatory processes.

Notably, the differences observed in WT vs IL-23p19−/− mice following C. neoformans infection are not as pronounced as those seen comparing IL-12p35−/− and p40-deficient mice (18). Therefore, the protective capacity of IL-23 is more apparent in the absence of IL-12, as is the case for T. gondii and M. tuberculosis infection (22, 34). It was shown that IL-12 inhibits IL-23-induced T cell production of IL-17 (12). Moreover, two reports demonstrate an enhancement of IL-23 effects in IL-12-deficient mice (IL-12p35−/− mice) in autoimmune chronic inflammation (9, 10). The subtle effects of IL-23 in the presence of IL-12 in the model of C. neoformans infection could therefore be due to suppression of IL-23 functions by endogenous IL-12/IFN-γ by a yet unknown mechanism.

FIGURE 8. Reduced expression of proinflammatory cytokines and chemokines in brains of infected IL-23p19−/− mice. WT and IL-23p19−/− mice were infected with C. neoformans. A, Total mRNA of the brain was assessed for cytokine and chemokine expression at day 21 p.i. by quantitative RT-PCR. X-fold up-regulation is shown as a ratio between infected and naive mice (pooled samples of three individual mice) of either genotype. Experiment shown is representative of two independently performed experiments.

B, Supernatants from brain homogenates were assessed for protein levels of IL-1β, IL-6, TNF-α, MCP-1, IFN-γ, and IL-17 by LUMINEX multiplexing protein assay. Experiments shown are representative of two independently performed experiments (n = 3 mice for each experiment).
C. neoformans shows a strong tropism for the CNS where it causes a sustained meningoencephalitis with subsequent death if untreated (23). Therefore, the immune response in the brain is of particular interest. Aggregates of C. neoformans accumulate in the brain of WT and IL-23p19−/− mice, eliciting an inflammatory response in both genotypes. In IL-23p19−/− mice, however, the density of inflammatory infiltrates was reduced as compared with WT mice. Interestingly, MCP-1 mRNA and protein concentrations were markedly lower in IL-23p19−/− mice. MCP-1 is a CC chemokine that mainly attracts mononuclear leukocytes (36). In pulmonary cryptococcosis, MCP-1 has been shown to be crucial for recruitment of CD4+ T cells and macrophages, production of TNF-α and IL-6, and clearance of organisms (37). Also, it has been described to be present at elevated levels in brains of mice protectively immunized against C. neoformans (38). The role of IL-23 in the maintenance of inflammatory processes might therefore partially rely on cell recruitment via induction of MCP-1.

Herein, we show that MHC-II expression in microglial cells was impaired in IL-23p19−/− mice when compared with WT controls. Also, 5-fold more MHC-II mRNA was induced in brains of WT than in IL-23p19−/− mice (data not shown). Similar observations have been described for EAE (9). Moreover, a role for IL-23 in enhancing Ag presentation has previously been shown for dendritic cells (39, 40). Because primed effector T cells are retained at the site of infection if Ag is encountered in proper MHC context, the above findings describe another IL-23-dependent mechanism leading to the maintenance of localized immune responses.

Although infected IL-23p19−/− mice show an impaired inflammatory response in the brain, the organ burdens do not significantly differ from WT controls. Previously, we reported that IL-12 deficiency leads to severely reduced survival times after C. neoformans infection, but organ burdens at day 21 p.i. in multiple organs including the brain did not differ between IL-12p35−/− mice and resistant WT controls (18). Therefore, in our model of cryptococcosis the fungal organ burden found relatively early in the course of cryptococcal infection is not necessarily predictive for the level of resistance as determined by survival time.

We have shown that the lack of IL-23 does not affect the Th1/Th2 balance. These data are consistent with findings in other studies looking at IL-23p19−/− mice in autoimmune disease or infection models (9, 10, 22, 34) and resemble our previous finding that IL-12p35−/− and p40-deficient mice do not differ in IFN-γ production following C. neoformans infection (18). Interestingly, however, we observed a pronounced IL-17 expression in WT but no detectable IL-17 production in IL-23p19−/− mice. It is intriguing that Ag-specific production of IL-17 seems to be completely dependent on IL-23, whereas polyclonal stimulation of C. neoformans-infected splenocytes of IL-23-deficient mice led to the production of IL-23-independent IL-17, albeit to a lower level than seen in WT splenocytes after polyclonal stimulation.

Because IL-23p19−/− mice produce less IL-17 it is conceivable that IL-17 acts as a mediator of IL-23. We have observed impaired expression of myeloid cell-derived proinflammatory cytokines such as IL-6 and IL-1β during cryptococcal infection of IL-23p19−/− mice. Of note, increased expression of IL-1β and TNF-α in the brains of infected WT mice is associated with protective immunity in cryptococcosis (38). Indeed, IL-17 was shown to stimulate production of TNF-α and IL-1β in human macrophages (41). In addition, it promotes production of MCP-1 in various adherent cells (13). A protective role for the IL-23/IL-17 axis has been defined in acute Klebsiella pneumoniae infection. IL-23-induced IL-17 was shown to be crucial for neutrophil recruitment to the infected airways and subsequent clearance of pathogen (42, 43). In a model of chronic M. tuberculosis infection, IL-23p19−/− mice showed, consistent with our findings, a severe defect in Ag-specific IL-17 production that was, however, not associated with altered resistance to infection in these mice (34). In the light of the above described predominant contribution of IFN-γ to cellular responses in tuberculosis, IL-17 effects might be completely obliterated by the IL-12/IFN-γ axis. In C. neoformans infection we found IL-23-dependent expression of IL-1β, IL-6 and MCP-1 in infected brains but no detectable IL-17. Therefore, direct effects of IL-23 on macrophages could be considered. Indeed, macrophages have been shown to bear the IL-23R (5). Studies in IL-17−/− mice have to be performed to provide a deeper insight in the regulation of cellular responses by IL-23/IL-17 in C. neoformans infection.

Taken together, IL-23 complements the more dominant role of IL-12 in protection against C. neoformans by mechanisms distinct from IL-12. The IL-23 pathway is characterized by the promotion of IL-17 as well as IL-1β, IL-6, and MCP-1, all of which affect cell recruitment and maintenance of inflammatory responses.

Acknowledgments
We thank Alissa Chackerian, Daniel Cua, Martin Oft, and Eddie Bowman for helpful discussions and critically reading the manuscript, as well as Jochen Schmitz and Alexander M. Owyang for help with the animal work. Furthermore, we acknowledge Yi Chen, Sharon Osborn, and K. Brian Lee for excellent technical support, as well as our colleagues involved in breeding and maintaining the mouse colonies at DNAX Research and the Institute of Immunology, University of Leipzig.

Disclosures
The authors have no financial conflict of interest.

References