Interleukin 18 Restores Defective Th1 Immunity to Candida albicans in Caspase 1-Deficient Mice

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Received 22 February 2000/Returned for modification 1 May 2000/Accepted 20 June 2000

Caspase 1, formerly designated interleukin 1β (IL-1β)-converting enzyme, processes pro-IL-1β and pro-IL-18 to yield active cytokines that play a pivotal role in inflammation and cell activation. We show here the effect of caspase 1 deficiency on the inflammatory and adaptive immune responses to the fungus Candida albicans. Caspase 1 deficiency did not affect susceptibility to primary systemic infection with the fungus, as revealed by survival and fungal growth. However, Th1-mediated resistance to reinfection was greatly impaired in caspase 1-deficient mice, and this correlated with low-level production of IL-12 and gamma interferon. Early in infection, production of these cytokines and that of tumor necrosis factor alpha, IL-6, and, interestingly, IL-1β occurred normally in caspase 1-deficient mice, while that of IL-18 was severely impaired. Exogenous administration of IL-18, more than IL-12, restored the Th1-mediated resistance to the infection. We conclude that, while caspase 1 is not indispensable for release of mature IL-1β in candidiasis, the caspase 1-dependent production of IL-18 may represent an important and novel pathway for the expression of sustained Th1 reactivity to the fungus.

Caspases, an expanding family of cysteine proteases with a substrate specificity for aspartic acid, play pivotal roles in inflammation and mammalian apoptotic cell death (9, 11). The prototype, caspase 1, or interleukin 1β (IL-1β)-converting enzyme, participates in the cellular export of some proinflammatory cytokines, thus having a prominent role in inflammation. Precursors of both IL-1β (8, 50) and IL-18 (16, 17, 23, 50) undergo proteolytic cleavage by caspase 1, permitting the activated cytokines to exit the cells. Peptide inhibitors of caspase 1 block IL-1β and IL-18 release from activated macrophages in vitro (17). Caspase 1-deficient mice fail to exhibit elevated levels of IL-1β, IL-18, or other proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α), IL-1α, or IL-6, following lipopolysaccharide (LPS) challenge. They are also resistant to LPS toxicity (24). However, other enzymes, in addition to caspase 1, are able to cleave pro-IL-1β and generate biologically active molecules (13, 53), including bacterial (4, 19) and fungal (3) enzymes. Thus, it appears that caspase 1 is not always indispensable for release of active IL-1β, but it is necessary for the production of bioactive mature IL-18 (12, 13). Like IL-12, IL-18 promotes gamma interferon (IFN-γ) production by Th1 and natural killer cells in both mice and humans (reviewed in references 10 and 31) and increases Th1 resistance to infections (5, 20, 35, 52, 56). Unlike IL-12, however, IL-18 by itself is unable to induce IFN-γ (22) and to drive Th1 development (37).

Previous studies showed that IL-12-induced IFN-γ production is essential for resistance to Candida albicans (28, 35, 38, 39, 46), the most frequently isolated fungal pathogen of humans (30). In mucosal colonization and systemic infection of mice with the fungus, Th1 cells mediate phagocyte-dependent protection and are the principal mediators of acquired protective immunity. In contrast, production of inhibitory cytokines such as IL-4 and IL-10 by Th2 cells and high levels of immunoglobulin E are associated with disease progression (28, 35, 38, 39, 46). Th2-like reactivity is frequently observed in patients with Candida-related pathology, such as in symptomatic infections (14, 35) and allergy (2). Th1-type responses may thus characterize the carriage of saprophytic yeast and the resistance to disease seen in healthy humans, whereas Th2 responses may be associated predominantly with pathology.

In murine candidiasis, Th1 differentiation requires the combined effects of different cytokines in the relative absence of counterregulatory cytokines, such as IL-4 and IL-10, which are, per se, necessary and sufficient to drive Th2 polarization (25). Deficient IFN-γ (6), transforming growth factor β (48), IL-6 (43), and TNF-α (26) responses could each block the induction of protective immunity; however, only IL-12 was both required and prognostic for the development of protective Th1 responses to Candida (44, 45).

Because IL-18 synergizes with IL-12 for induction of Th1 cell development (37, 49), in the present study we used caspase 1-deficient mice to assess (i) the patterns of proinflammatory and Th cytokine production in C. albicans infection and (ii) the effect of exogenous IL-18 on IL-12 and IFN-γ production and resistance to the infection.

MATERIALS AND METHODS

Mice. Caspase 1−/− mice were obtained as previously described (17). Briefly, chimeric mice were obtained by injection of embryonic stem cells, in which the caspase 1 gene was disrupted and replaced with a neomycin resistance cassette gene, into C57BL/6 blastocysts. The chimeric males were then mated with C57BL/6 mice. Homozygous mice with two copies of the disrupted caspase 1 gene were identified by Southern blotting of genomic DNA, and the absence of caspase 1 mRNA in caspase 1−/− mice was confirmed by reverse transcriptase PCR (RT-PCR) analysis. Homozygous mice were then interbred and used for the experiments. Animals were housed under specific-pathogen-free conditions at the breeding facilities of the University of Perugia, Perugia, Italy. C57BL/6 mice, 6 to 8 weeks old, were obtained from Charles River (Calco, Italy).
pressed as micromolar concentrations of NO2 standard Griess reaction adapted to microplates, as described previously (7). The experimental group/CFU for control cultures) day of primary or secondary infection and 1 and 3 days later.

Mice succumbing to yeast challenge didiasis. Absolute numbers of neutrophils in peripheral blood were determined by total and differential white cell counts. Quantification of yeast in the organs of infected mice was performed by a plate dilution method, using Sabouraud dextrose agar. Results were expressed as CFU per organ (mean ± standard error [SE]). Recombinant murine IL-18 (rIL-18) (R&D Systems Inc., Minneapolis, Minn.) or rIL-12 (Genetics Institute, Cambridge, Mass.) was given intraperitoneally (i.p.) at the dose of 1 µg/injection or 10 ng/injection, respectively, on the day of primary or secondary infection and 1 and 3 days later.

Purification and culture of cells. CD4⁺ T splenocytes were purified by using anti-CD4 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Briefly, 10⁶ total splenocytes were incubated with 100 µl of magnetically activated cell-sorting CD4 Microbeads for 15 min at 6°C, washed, and magnetically separated with a positive selection column (Miltenyi Biotec), according to the manufacturer’s instructions. Splenocytes were cultured by 2-h plastic adherence as previously described (6, 27, 42). Peritoneal neutrophils were collected 18 h after i.p. inoculation of aged, etodoxin-free 10% thioglycolate solution (Difco, Detroit, Mich.) as previously described (40, 41). Unfractionated splenocytes or CD4⁺ cells (10 x 10⁶/ml) were cultured in complete medium with 10% heat-inactivated C. albicans cells per ml for 48 h, before cytotoxicity measurement in culture supernatants. Irradiated splenocytes (2,000 rad) were added (10⁶/ml) to the CD4⁺ cultures as antigen-presenting cells. In selected experiments, rIL-18 (50 ng/ml) was added to the cultures of unfractionated splenocytes together with rIL-2 (100 U/ml) as previously described (18).

Cytokine assays. The levels of TNF-α, IL-6, IFN-γ, and IL-12 in culture supernatants were determined by means of cytokine-specific enzyme-linked immunosorbent assay (ELISA), using pairs of anticytokine monoclonal antibodies as previously described (6, 27, 42). The monoclonal antibody pairs used were as follows, listed by capture-biotinylated detection: TNF-α, MP6-XT22-MP6-XT3; IL-6, MP5-20F3-MP5-32C11; and IFN-γ, R4-6A2-XMG1.2 (PharMingen, San Diego, Calif.). For IL-12/70 measurement, a modified antibody-capture biossays was used (68). The levels of IL-1β and IL-18 were determined using the specific ELISA kit (R&D Systems).

Candidal assay and NO production. For the candidal assay, 5 x 10⁶ peritoneal neutrophils or splenic macrophages were incubated with 5 x 10⁶ PCA-2 cells in Costar 24-well flat-bottomed microplates (Costar, Cambridge, Mass.) for 1 or 4 h, respectively, and the number of CFU was determined as previously described (7). The percentage of CFU inhibition (mean ± SE) was determined as a percentage of colony formation inhibition = 100 – (CFU for experiment × CFU for control cultures) / 100. Nitrite concentrations, a measure of nitric oxide (NO) synthesis, was assayed in culture supernatants by a standard Griess reaction adapted to microplates, as described previously (7). The data represent the means ± SEs of quadruplicate determinations and are expressed as micromolar concentrations of NO₂⁻ per 10⁷ cells.

RT-PCR. RNA extraction and amplification of synthesized cDNA from splenic adherent macrophages and purified CD4⁺ splenocytes were performed as previously described (6, 26, 27). For hypoxanthine-guanine phosphoribosyltransferase (HPRT), IL-12p0, IFN-γ, IL-4, and IL-12 receptor β2 (IL-12Rβ2), the primers and positive controls, cycles, and temperatures were as previously described (6, 26, 27). For IL-18R, the primers were synthesized using a 391 DNA synthesizer (PCR-MATE; Applied Biosystems, Foster City, Calif.). The sequences of 5′ sense primer and 3′ antisense primer were as follows: sense, 5′-ATGTTGTCGTCTCTTCCTTCCGCTG-3′, antisense, 5′-ATGTTGTCGTCTCTCTTGGCTG-3′. Each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The RPRT primers were used as a control for both reverse transcription and the PCR itself and also for comparing the amounts of products of samples obtained with the same primer. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining. PCR-assisted mRNA amplification was repeated at least twice for at least two separately prepared cDNA samples for each experiment. Data are representative of three different experiments.

Statistical analysis. Survival data were analyzed using the Mann-Whitney U test. Student’s t-test for unpaired data was used to compare the fungal growth, the cytokine and NO production, and the candidal activity. Significance was defined as P ≤ 0.05. In vivo groups consisted of four to six animals. Unless otherwise specified, the data reported were pooled from three to five experiments.

RESULTS

Course of primary and secondary C. albicans infections in caspase 1-deficient mice. Caspase 1⁻/⁻ and C57BL/6 and (SV129 x C57BL/6) mice, which have a genetic background comparable with that of the caspase 1⁻/⁻ mice, were injected i.v. with 10⁶ cells of low-virulence strain PCA-2 or 10⁵ cells of highly virulent C. albicans strain CA-6. For secondary infection, 10⁶ cells per 10⁷ cells of 14 days after primary i.v. challenge, mice were i.v. injected with 10⁶ cells of CA-6. The results (Table 1) show that survival of the primary systemic infection with either PCA-2 or CA-6 did not differ between caspase 1⁻/⁻ mice and either C57BL/6 or (SV129 x B6)F1 mice, each group of mice having survived the PCA-2 infection while similarly succumbing to the CA-6 infection. However, upon reinfection of mice surviving PCA-2 infection, C57BL/6 and (SV129 x B6)F1 mice survived the infection, but caspase 1⁻/⁻ mice did not. Quantification of fungal growth in organs in the course of the infection did not reveal major differences between mutant and wild-type mice (data not shown). Similarly, histopathological examination of the kidneys of PCA-2-infected mice revealed a slightly increased number of foci of inflammatory reaction throughout the kidney parenchyma in caspase 1⁻/⁻ mice, compared to the few lesions observed in the cortex of kidneys from (SV129 x B6)F1 mice (data not shown). Therefore, these results suggest that effector mechanisms of resistance to primary C. albicans infection were not affected in caspase 1-deficient mice, which indeed efficiently oppose infectivity in the initial stage of infection. However, caspase 1 deficiency impairs the development of acquired resistance upon primary sublethal infection.

Antifungal effector functions are unimpaired in caspase 1-deficient mice. To assess the antifungal phagocytic response in caspase 1-deficient mice upon primary infection, caspase 1⁻/⁻ and C57BL/6 mice were infected i.v. with PCA-2 and the antifungal effector functions of splenic macrophages and peritoneal neutrophils were assessed 3 days after the infection. The results (Fig. 1) indicate that both types of cells were equally activated to a candidal state in mutant and wild-type mice. Similarly, neutrophils and, to a lesser extent, macrophages produced NO upon exposure to C. albicans in vitro. We also determined the number of circulating neutrophils 2 days after infection and found that neutrophil counts increased in both types of mice upon infection, being actually higher in caspase 1-deficient mice from 361 ± 44 to 4,168 ± 380 in mutant mice.

| TABLE 1. Susceptibility of caspase 1-deficient mice to primary and secondary C. albicans infections |
|-----------------------------|---------------------|---------------------|
| Mice                        | Primary²             | Secondary³          |
|                             | C. albicans strain   | Dose (cells)        | MST¹ | D/T² | MST¹ | D/T² |
| Caspase 1⁻/⁻                 | PCA-2                | 10⁶                | >60  | 2/18 | 17   | 12/12 |
| CA-6                        | 10⁵                | 13                 | 12/12 |
| C57BL/6                     | PCA-2                | 10⁵                | >60  | 0/18 | >60  | 0/12 |
| CA-6                        | 10⁵                | 14                 | 12/12 |
| (SV129 x B6)F₁              | PCA-2                | 10⁶                | >60  | 3/18 | >60  | 0/12 |
| CA-6                        | 10⁶                | 16                 | 12/12 |

² Mice were infected i.v. with low-virulence PCA-2 or virulent CA-6 C. albicans.
³ CA-6 (10⁶ cells) was given i.v., 14 days after primary infection.
⁴ MST, median survival time (days).
⁵ D/T, number of dead mice over total number of mice injected.
described in Materials and Methods. Splenic adherent macrophages and elicited peritoneal neutrophils were assessed producing IFN-γ and caspase 1. The susceptibility of caspase 1-deficient mice upon infection with C. albicans was observed in caspase 1-deficient mice at levels similar to those observed for wild-type mice (Fig. 3). IL-1β was produced in wild-type mice and, interestingly, even in mutant mice. However, IL-18 could not be detected in the latter mice as opposed to wild-type mice (Fig. 3), despite the presence of the IL-18 message (data not shown). Both cytokines were detected in culture supernatants of purified neutrophils and macrophages from wild-type infected mice upon stimulation with IFN-γ and LPS in vitro (data not shown). These results suggest that, early in infection, production of proinflammatory cytokines, including IL-12 and IFN-γ, was not affected in caspase 1-deficient mice upon C. albicans infection. In contrast, a defective production of IL-18 was observed.

Exogenous IL-18 restores antifungal resistance and IFN-γ and IL-12p70 production in caspase 1-deficient mice. To assess whether IL-18 deficiency is responsible for the impaired anticandidal Th1 reactivity in caspase 1-deficient mice, exogenous rIL-18 was administered to mutant mice infected with PCA-2. The cytokine was given either at the time of the primary infection with PCA-2 or at the time of the secondary infection with CA-6. For comparison, exogenous rIL-12 was similarly administered to infected mice. Mice were monitored for resistance to the secondary infection, in terms of fungal growth in the kidneys and production of IFN-γ and IL-12. The results (Table 2) show that the fungal load was significantly decreased in mice treated with rIL-18, at the time of primary or secondary infection. Moreover, the production of IFN-γ by CD4+ T cells and that of IL-12p70 by splenocytes were significantly increased in treated, compared to untreated, mice. Similar results, although to a lesser extent, were obtained upon treatment with rIL-12 at the time of the secondary infection. The failure of earlier treatment with rIL-12 to increase Th1-mediated resistance to reinfection is a finding in line with previous results (44). In vitro, rIL-18 also increased IFN-γ and IL-12 production by antigen-activated splenocytes from caspase 1-deficient mice upon reinfection, particularly in the

![FIG. 1. Antifungal effector functions of macrophages and neutrophils from caspase 1−/− (solid bars) and caspase 1+/+ C57BL/6 (open bars) mice, uninfected or upon primary i.v. infection with C. albicans. Three days after infection, splenic adherent macrophages and elicited peritoneal neutrophils were assessed for their ability to kill yeast cells and to secrete NO in vitro. Assays were done as described in Materials and Methods. *P < 0.05, caspase 1−/− versus caspase 1+/+ mice.](http://iai.asm.org/content/175/5/5128/F1)

![FIG. 2. Cytokine and cytokine receptor gene expression in caspase 1−/− and caspase 1+/+ C57BL/6 mice upon C. albicans infection. Mice were either uninfected (lanes 1) or reinfected (lanes 2) with virulent CA-6, 14 days after the primary infection with PCA-2. Levels of IFN-γ, IL-4, IL-12Rβ2, and IL-18R mRNA (in CD4+ T splenocytes) and of IL-12p40 (in splenic macrophages) were determined by RT-PCR, 3 days after reinfec](http://iai.asm.org/content/175/5/5128/F2)
presence of IL-2. Such an increase was not observed upon antigen activation of splenocytes from nonvaccinated mice, upon infection with virulent CA-6 cells (Table 3). These results suggest that rIL-18 restores the Th1-mediated resistance of caspase 1-deficient mice to C. albicans infection, an activity that cannot be fully compensated for by exogenous rIL-12. In addition, it appears that memory rather than naive Th cells are more susceptible to the ability of IL-18 to promote IFN-γ production. Therefore, IL-18 plays an important role in maintaining sustained IFN-γ and IL-12 production in mice with C. albicans infection.

**DISCUSSION**

In the present study, the use of caspase 1-deficient mice has provided us with new insights into the cytokine-dependent regulation of immunity to C. albicans. The major findings are, firstly, that production of IL-18 is required for sustained expression of Th1 protective immunity to the fungus and, secondly, that caspase 1 activity is not necessary for the production of mature IL-1β, as it is for mature IL-18.

Studies performed on caspase 1 (12, 13, 17, 23, 24) or IL-18 (49, 52)-deficient mice have revealed an essential role for IL-18 in IFN-γ production in models of infection and inflammation. Despite normal (13) or even higher (52) levels of IL-12 production, reduced levels of IFN-γ were observed in caspase 1-deficient mice after stimulation with LP5 (10, 12, 13) or in IL-18-deficient mice upon infection with intracellular or extracellular pathogens (52). Although IL-18 and IL-12 exerted a synergistic effect on IFN-γ production by Th1 cells (1, 55), IL-18 also acted as an IL-12-independent regulator of IFN-γ production (21) and of cell proliferation induced by microbial stimuli (13). Indeed, in the absence of IL-18, IL-12 alone was insufficient for the induction of Th1cell expansion in vivo (49, 52). Furthermore, it has recently been demonstrated that IL-18R is selectively expressed on murine Th1, but not Th2, cells (54, 55). Therefore, although IL-18, unlike IL-12, was unable to drive Th1 cell expansion in vitro (37), these results clearly indicate that IL-18 has a direct and profound effect on the activation and development of Th1 cells in vivo. The results of the present study confirm this notion, by clearly showing that IL-18 is required for sustained production of IFN-γ and IL-12 in C. albicans infection. Acquired immunity to the fungus relied on the induction of protective Th1 cells producing IFN-γ and expressing the IL-12Rβ2. Although caspase 1-deficient IL-18 production was not required for IFN-γ production after concanavalin A stimulation (13), IL-18 sustained the expression of the IL-12Rβ2 mRNA (54). Thus, IL-18R may transmit signals that maintain antifungal Th1 development through the IL-12R complex. As in turn IL-12 up-regulates the expression of the IL-18R (55), the synergistic effect of IL-12 and IL-18 on Th1 development may rely on the reciprocal regulation of their...
receptors. However, the expression of the IL-18R gene was not impaired in caspase 1-deficient mice upon C. albicans infection, nor in IL-12-deficient mice after infection (data not shown). These findings indicate that factors other than IL-12 may regulate the IL-18R mRNA in C. albicans infection.

Production of proinflammatory cytokines, including IFN-γ and IL-12, occurred normally in caspase 1-deficient mice early in infection, a finding suggesting that the early cytokine response in C. albicans infection is relatively independent of caspase 1 processing of pro-IL-18. This observation is apparent at variance with what was observed in an experimental model of cryptococcosis, in which the protective efficacy of IL-18 alone (20) or combined with IL-12 (36) was seen early on but not at 3 to 6 weeks after infection and was dependent on IFN-γ production by stimulated NK cells (56). It appears that differences in the relative contributions of various effector mechanisms in the host defense against each fungal pathogen may determine the outcome of treatment with IL-18.

One important observation of the present study is that production of IL-18 was observed in caspase 1-deficient mice infected with C. albicans. That caspase 1 is not always required for release of active IL-18 and that the requirement for caspase 1 in IL-18 processing is stimulus dependent has already been reported (13, 53). In particular, the finding that IL-18 is produced in caspase 1-deficient mice after C. albicans infection suggests that proteinases secreted by C. albicans may play an important role in IL-18 processing, as already demonstrated (3). In this regard, it is worth mentioning that proteinases are produced by the PDA-2 C. albicans strain used in the present study, particularly during infection (F. de Bernardis, personal communication).

It has been suggested that C. albicans proteinases may contribute to the inflammatory nature of mucosal candidiasis by local activation of inflammatory IL-1β (3). In our study, caspase 1−/− mice were not overtly more susceptible than wild-type mice to gastrointestinal C. albicans infection (data not shown). This finding would suggest a nonessential role of IL-1β in the pathogenesis of mucosal candidiasis, even though local production of IL-1β was not measured in caspase 1-deficient mice with gastrointestinal infection. Instead, production of IL-1β was observed in the course of disseminated infection with low-virulence C. albicans, a finding that confirms the protective effect that IL-1β may have in infection (34).

Given the involvement of caspase 1 and IL-18 in inflammatory (15, 29) and noninflammatory (47) disease and the growing importance of IL-18 in the induction of optimal host immune defenses against pathogens (22, 31) and tumors (33), the present study provides us with important insights into the caspase 1-dependent IL-18 production in mice with candidiasis. As the experimental model adopted in the present study closely mimics the state of long-lived commensalism with the fungus and the ensuing immunity to it (35, 46), it appears that IL-18 meets the requirement of a candidate cytokine which is required for sustained expression of antifungal Th1 immunity in self-limiting infection and saprophytism.

ACKNOWLEDGMENTS

This study was supported by the National Research Project on AIDS, contract 508.33, “Opportunistic Infections and Tuberculosis,” Italy.

We thank Jo-Anne Rowe for editorial assistance. The murine rIL-12 was supplied by the Bioanalytical Sciences Department of Genetics Institute Inc., Cambridge, Mass.
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