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IL-10 Is Required for Development of Protective Th1 Responses in IL-12-Deficient Mice upon Candida albicans Infection

Antonella Mencacci,* Elio Cenci,* Giuseppe Del Sero,* Cristina Fé d’Ostiani,* Paolo Mosci,* Giorgio Trinchieri, † Luciano Adorini, ‡ and Luigina Romani2*

IL-12 is both required and prognostic for Th1 development in mice with Candida albicans infection. To delineate further the physiologic role of IL-12 in antifungal immunity, mice deficient for this cytokine were assessed for susceptibility to C. albicans infections, and for parameters of innate and adaptive immunity. IL-12-deficient mice were highly susceptible to gastrointestinal infection or to reinfection and showed elevated production of Candida-specific IgE and IL-4 and defective production of IFN-γ. The failure to mount protective Th1 responses occurred despite the presence of an unimpaired innate antifungal immune response, which correlated with unaltered IFN-γ production, but defective production of, and responsiveness to, inhibitory IL-10. IL-10 or IL-12 neutralization increased the innate antifungal resistance in wild-type mice. However, in IL-12-deficient mice, treatment with exogenous IL-12 or IL-10 impaired IL-4 production and increased resistance to infection, through a negative effect on the CTLA-4/B7-2 costimulatory pathway. These results confirm the obligatory role of IL-12 in the induction of anticanidial Th1 responses, and indicate the existence of a positive regulatory loop between IL-12 and IL-10 that may adversely affect the innate antifungal response, but is required for optimal costimulation of IL-12-dependent CD4+Th1 cells. The Journal of Immunology, 1998, 161: 6228–6237.

Helper T cells play a central role in regulating immune responses to the fungus Candida albicans by providing critical cytokine-mediated activation and deactivation signals to fungal effector cells (1–3). In experimental models of candidiasis, protection correlates with the generation of CD4+Th1 cells producing IL-2 and IFN-γ (4) and requires the concerted action of several cytokines (5–8), including IL-12 (9–11). Susceptibility to infection correlates with the presence of CD4+Th2 cells producing IL-4 and IL-10 (4, 12–14) that have been implicated in inhibiting the development of protective Th1 cells (15) and in opposing the IFN-γ-mediated activation of fungicidal macrophages (16).

Among factors contributing to CD4+Th development in mice with C. albicans infection, the initial handling of fungal pathogen by cells of the innate immune system appears to play a major role (17–19). The instructive role of the innate immune system in the adaptive immune response may operate at different levels (20, 21). In candidiasis, regulation of the early fungal burden (22), cytokine production including IL-10 and IL-12 (17–19), and expression of costimulatory molecules (8) are possible pathways through which the innate immune system may control CD4+Th immunity. Thus, qualitative or quantitative defects of antifungal effector and immunomodulatory functions of phagocytic cells result in the development of anticanidial Th2, rather than Th1, responses (17–19). Conversely, potentiation of innate antifungal immunity by neutralization of inhibitory cytokines, such as IL-10, leads to the development of protective Th1 responses in otherwise susceptible mice (13).

IL-12 is both required and prognostic for CD4+Th1 development in mice with candidiasis, being rapidly produced after infection in both resistant and susceptible mice (9–11). In genetically resistant mice, neutralization of IL-12 (9) or IFN-γ (5) prevents development of protective Th1 responses, consistent with the important role of IL-12-induced IFN-γ in activating host antifungal effector cells (23) and CD4+Th1 lymphocytes (5). In susceptible mice, IL-12 is negatively regulated by IL-4 and IL-10, and IL-12-dependent activation of CD4+Th1 lymphocytes occurs upon neutralization of these cytokines (9). However, exogenous IL-12 fails to increase resistance of susceptible mice to systemic or gastrointestinal C. albicans infection and to effectively oppose Th2 cell differentiation driven by IL-4/IL-10 (9, 11). These data indicate the existence of complex immunoregulatory circuits underlying the activity of IL-12 in candidiasis (11).

Production of IL-10 by T (24) and non-T (17–19) cells is induced by IL-12. IL-10 being a potent inhibitor of IL-12 (25). The importance of this negative regulatory loop between IL-10 and IL-12 is underlined by observations in IL-10-deficient mice infected with Toxoplasma gondii in which overproduction of IL-12 results in a toxic lethal syndrome (26). Thus, IL-10 appears to be a critical component of the host’s immune response to infectious agents (27). The finding that IL-12 induces production of IL-10 by T cells (9) as well as by neutrophils (18) in mice with candidiasis led us to investigate whether early induction of IL-10 by IL-12 could represent a major mechanism underlying the capacity of IL-12 to affect innate and adaptive antifungal immunity.

To test this hypothesis, IL-12 p40-deficient mice were assessed for pattern of cytokine production and for parameters of innate and...
acquired antifungal immunity during the course of systemic or gastrointestinal *C. albicans* infection. The results revealed a defective production of and responsiveness to IL-10, which correlated with the inability of IL-12 p40-deficient mice to mount protective anticanidal Th1 responses. This suggests the existence of an important, previously undefined, positive regulatory loop between IL-12 and IL-10, the latter being required for optimal stimulation of IL-12-induced CD4+ Th1 cells in mice with *C. albicans* infection.

**Materials and Methods**

**Mice**

Breeding pairs of homozygous IL-12 p40-deficient (IL-12 p40−/−) and control IL-12 p40+/+ BALB/c mice (28) kindly provided by Dr. J. Magram (Hoffmann-La Roche, Nutley, NJ) were bred under specific pathogen-free conditions. Mice of both sexes, 8–10 wk old, were used. IL-12 p40−/− mice were devoided of IL-12 bioactivity, as assessed by the Ab capture assay for functional IL-12 (7). Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

**Yeasts, infections, in vivo analysis, and treatments**

The origin and characteristics of the highly virulent *C. albicans* (CA-6) strain and the low virulence *C. albicans* strain and the low virulence live vaccine strain, PCA-2, used in this study have been described previously (4, 10). For infection, cells were washed twice in saline, and diluted to the desired density to be injected i.v. via the lateral tail vein in a volume of 0.5 ml or mouse or intragastrically (i.g.;3 pg/ml) of paraffin-embedded sections (3–4 μm) of formalin-fixed, paraffin-embedded tissues were stained with periodic acid-Schiff reagent and examined for histology, as described (29). Mice succumbing to yeast challenge were routinely necropsied for histopathological confirmation of disseminated candidiasis. Recombinant mouse IL-12 and polyclonal sheep anti-mouse IL-12 (Dako, Glostrup, Denmark) was given i.p. twice in saline, and diluted to the desired density to be injected i.v. via the lateral tail vein in a volume of 0.5 ml per mouse or intragastrically (i.g.;2 pg/ml) or the control fusion protein was given i.v. via the lateral tail vein the day of and 1 and 2 days after challenge (100 ng/injection for rIL-12, and 500 ng/injection for control IL-12 p40−/− mice). Mice of both sexes, 8–10 wk old, were used. IL-12 p40−/− mice were devoided of IL-12 bioactivity, as assessed by the Ab capture assay for functional IL-12 (7). Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

**Cytokine assays**

The levels of IFN-γ, IL-4, and IL-10 in culture supernatants were determined by cytokine-specific ELISAs, using pairs of anti-cytokine mAbs, as described (6, 9). The Ab pairs used were as follows, listed by capture/biotinylated detection: IFN-γ, R4-6A2/XMG12; IL-4, BVDM-1D11/ BVDM-24G2; IL-10, JES5-2A5/SX5-1 (Pharmingen, San Diego, CA). Cytokine titers were calculated by reference to standard curves constructed with known amounts of recombinant cytokines (PharMingen).

**Candidal assay and nitrite determination**

For the candidal assay, spleen-adherent macrophages or elicited peritoneal neutrophils (5 × 10⁶ cells/ml) were incubated with 10⁶ *C. albicans* cells/ml for 4 or 1 h, respectively, and the number of CFU was determined as percentage of colony formation inhibition ((CFU experimental group/CFU control cultures) × 100). Nitrite concentration, a measure of nitric oxide (NO) production, was assayed in culture supernatants by a standard Griess reaction adapted to microplates, as described (16). The data represent the means ± SE of quadruplicate determinations and are expressed as μM NO₂⁻/10⁶ cells.

**Flow cytometry**

Spleen cells were harvested, and 1 × 10⁶ cells were sequentially cultured with saturating amounts of FITC-conjugated anti-CD80 (B7-1, hamster IgG2b, mAb 16-2), and anti-IL-12 p40 mAb (B7-2) mAb (Gl3) mAb and phycoerythrin-conjugated anti-CD11b (rat IgG2b, mAb M1/70) (PharMingen). Cells were analyzed with a FACSort flow cytometer (Becton Dickinson, Mountain View, CA) using Cell Quest software for Macintosh, as described (7, 30). Nonviable cells were excluded from analysis by accepted procedures involving propidium iodide staining and narrow forward-angle light scatter gating.

**Reverse-transcriptase PCR (RT-PCR)**

RNA extraction and amplification of synthesized cDNA from macrophages, purified CD4+ splenocytes, and elicited peritoneal neutrophils were performed as described (6, 19). For hypoxanthine-guanine phosphoribosyltransferase (HPRT) and IL-10, the cycles and temperatures were as described (8). For IL-10R, the sequences of 5′-sense primers and 3′-antisense primers were as follows: 5′-GGGAGCCGCGAGCAGCCGACGAGATGCT, 3′-TGGAGCCTGCTACGTGTCACATGAGGTCT. For IL-10R, each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The HPRT primers were used as a control for both reverse transcription and the PCR reaction itself, and also for comparing the amount of products from samples obtained with the same primer. The PCR fragments were analyzed by 1.5% agarose gel electrophoresis and visualized with ethidium bromide staining. PCR-amplified DNA was repeated at least twice for at least two separately prepared cDNA samples for each experiment. Data are representative of at least three different experiments.

**Competitive RT-PCR**

The semiquantitative competitive PCR developed by Reiner et al. (31) was performed using the competitor construct containing sequences for multiple cytokines, the primers for HPRT, IFN-γ, and IL-4 and the PCR conditions described by the authors. Briefly, aliquots of cDNA were assayed for levels of HPRT by placing serial dilutions from 1/1 to 1/40 of the experimental cDNA against a fixed concentration of the competitor construct and examining the ratio of competitor-to-wild-type band intensity after amplification with HPRT-specific primers. Adjustments were made in the amount of cDNAs needed to standardize the HPRT levels to comparable levels among all groups. Serial dilutions of these adjusted volumes of cDNA were then used to quantitate cytokine levels using a fixed concentration of competitor (3 pg/ml for HPRT and 1.5 pg/ml for IFN-γ and IL-4, 10%) phenotypes, with CD4+ T cells expressing the αβ or γδ TCR at approximately equal numbers on FACS analysis, isolated at the 40–100% interface of a discontinuous Percoll gradient (Pharmacia, Uppsala, Sweden), were cultured (5 × 10⁶ cells/ml) in the presence of irradiated (2000 cGy) splenocytes and Con A (10 μg/ml). Cultures of splenic adherent macrophages and purified peritoneal neutrophils, collected 18 h after i.p. inoculation of aged, endotoxin-free 10⁵ thioglycolate-suscetible diet (Difco, Detroit, MI), were conducted as described (17, 19), by incubating 5 × 10⁶ cells/ml in the presence of 400 U/ml IFN-γ and 40 ng/ml LPS (Sigma) or 5 × 10⁶ *C. albicans* cells/ml. Cytokine levels were quantitated in supernatants collected after 48 h (for lymphocytes) or 24 h (for neutrophils), as described (17, 19).

**Abbreviations used in this paper:** i.g., intragastrically; HPRT, hypoxanthine-guanine phosphoribosyltransferase; NO, nitric oxide.
Table I. Course of *C. albicans* infection in IL-12 p40<sup>−/−</sup> and IL-12 p40<sup>+/+</sup> mice

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Route</th>
<th>MST&lt;sup&gt;1&lt;/sup&gt;</th>
<th>D/T&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Yeast</th>
<th>Route</th>
<th>MST&lt;sup&gt;1&lt;/sup&gt;</th>
<th>D/T&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12 p40&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>CA-6 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>i.v. 9</td>
<td>8/8</td>
<td>CA-6 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>i.g. &gt;60 0/8</td>
<td>4</td>
<td>4/4</td>
</tr>
<tr>
<td>IL-12 p40&lt;sup&gt;+/+&lt;/sup&gt;</td>
<td>CA-6 10&lt;sup&gt;8&lt;/sup&gt;</td>
<td>i.v. 4</td>
<td>12/12</td>
<td>CA-6 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>i.g. &gt;60 0/8</td>
<td>23</td>
<td>4/4</td>
</tr>
</tbody>
</table>

<sup>1</sup> Mice were infected i.v. or i.g. with virulent CA-6 or low virulence PCA-2 *C. albicans*.  
<sup>2</sup> CA-6 (10<sup>6</sup>) was given i.v. 14 days after primary infection.  
<sup>3</sup> MST, median survival time (days); D/T, dead mice over total mice injected.

Results

Resistance of IL-12 p40<sup>−/−</sup> mice to primary and secondary *C. albicans* infections

IL-12 p40<sup>−/−</sup> and IL-12 p40<sup>+/+</sup> mice were injected i.v. with 10<sup>8</sup> highly virulent *C. albicans* (CA-6) or low virulence PCA-2, or i.g. with 10<sup>6</sup> CA-6. Mice were monitored for resistance to primary and secondary infections in terms of survival (Table I) and fungal growth in the kidneys and stomach (Fig. 1). Survival to acute systemic infection with CA-6 was increased in IL-12 p40<sup>−/−</sup> mice as compared with wild-type mice, whereas survival to primary systemic infection with PCA-2 or i.g. infection with CA-6 did not differ between IL-12 p40<sup>−/−</sup> and IL-12 p40<sup>+/+</sup> mice. However, upon a lethal CA-6 challenge of survivors, IL-12 p40<sup>−/−</sup> mice either survived or showed a remarkable resistance to reinfection, while IL-12-deficient mice did not. Resistance to acute systemic infection with CA-6 correlated with a significant decrease of *Candida* growth in the target organ, the kidney (Fig. 1A). In the gastrointestinal infection, local fungal growth in the stomach did not significantly differ between IL-12 p40<sup>−/−</sup> and IL-12 p40<sup>+/+</sup> mice in the early stage of infection. However, 4 wk after infection, when this was almost cleared in wild-type mice, a remarkable fungal growth was still present in the stomachs of IL-12-deficient mice (Fig. 1B). Susceptibility to reinfection of IL-12-deficient mice surviving primary systemic or i.g. infection was associated with an increased fungal growth in the kidneys, as compared with resistant wild-type mice (Fig. 1C). Histopathologic examination of the kidneys of IL-12-deficient mice with primary or secondary systemic infection revealed lesions similar to those observed in resistant or susceptible strains of mice upon i.v. *Candida* infection (4, 14). Similarly to what was observed in resistant wild-type mice (Fig. 2, B and D), reduced fungal growth and absence of pathologic lesions characterized the kidneys of IL-12-deficient mice that were resistant to acute systemic infection with PCA-2 (Fig. 2A). In contrast, an extensive fungal growth, associated with the presence of numerous foci of inflammatory reaction (consisting mainly of polymorphonuclear cells) throughout the kidney parenchyma, was observed in IL-12 p40<sup>−/−</sup> (Fig. 2C), but not IL-12 p40<sup>+/+</sup> (Fig. 2D) mice upon reinfection. Four weeks after gastrointestinal infection, stomach sections from IL-12 p40<sup>−/−</sup> mice (Fig. 2E) revealed numerous intraepithelial abscesses consisting of a thickened keratinized outer layer that enveloped a dense aggregate of hyphae and a few infiltrating cells. Unexpectedly, erosions were present through the thickness of the gastric mucosa, characterized by epithelial necrosis, presence of numerous fungal cells, and a prominent inflammatory cell infiltrate; conspicuous signs of acanthosis and hyperkeratosis were also visible. Importantly, abscesses and mucosal infiltration were observed in the glandular region of the gastric mucosa rather than in the cardial-atrium fold, which is a major site of colonization by *C. albicans* in the murine gastrointestinal tract (29). In wild-type mice (Fig. 2F), fewer yeast cells were present in the keratinized layer, with a limited inflammatory reaction observed only at the level of the cardial-atrium fold. Altogether, these results suggest that IL-12 plays a major role in resistance to *C. albicans* infection, particularly in the gastrointestinal tract, where fungal burden and pathologic lesions are reduced in IL-12-deficient mice.

**FIGURE 1.** *C. albicans* growth in the organs of IL-12 p40<sup>−/−</sup> and IL-12 p40<sup>+/+</sup> mice. IL-12 p40<sup>−/−</sup> and IL-12 p40<sup>+/+</sup> mice were injected i.v. with virulent *C. albicans* (A, systemic infection) or i.g. (B, gastrointestinal infection) and reinjected i.v. (C, secondary infection) 14 days later. Enumeration of yeast cells recovered from the kidneys of mice with systemic infection or from the stomachs of mice with gastrointestinal infection was performed at different days after infection. In reinfected mice, yeast cell counts in the kidneys were performed 4 days after reinfection. Cumulative data from two experiments (mean ± SE, 4–6 animals per group). Vertical bars represent the upper limit of the SE. *, *p < 0.05* (IL-12 p40<sup>−/−</sup> vs IL-12 p40<sup>+/+</sup>).
data clearly indicate a two-stage control of *C. albicans* infection in IL-12-deficient mice, which efficiently oppose infectivity in the initial stage of infection, but fail to acquire resistance to it.

**IL-12-deficient mice do not develop protective antifungal CD4+ Th1 responses**

Protective acquired resistance to *C. albicans* correlates with the induction of CD4+Th1 responses (1–3). To assess the pattern of Th1 (IFN-γ) and Th2 (IL-4) cytokine production, IL-12 p40−/− and IL-12 p40+/+ mice were infected under conditions that induce protective CD4+Th1 responses, such as i.v. infection with PCA-2 or i.g. infection with CA-6, followed by reinfection with CA-6 14 days later. We found that production of IFN-γ was not significantly decreased in IL-12-deficient as compared with wild-type mice, early in infection. Comparable levels of IFN-γ were detected in culture supernatants of mitogen-stimulated splenocytes or i.e. lymphocytes from both wild-type and IL-12-deficient mice upon primary i.v. (Fig. 3A) or i.g. (Fig. 3B) infection, respectively. In contrast, IFN-γ production was severely impaired in CD4+ T splenocytes from IL-12-deficient mice upon reinfection (Fig. 3C).
In these mice, defective production of IL-2 was also observed (data not shown). IL-4 production was always present in IL-12 p40−/− mice upon primary infection (Fig. 3A, B, and C), whereas it was considerably reduced in IL-12 p40−/− mice upon secondary infection (Fig. 3C). Local production of IL-4 was comparable in IL-12 p40−/− and IL-12 p40+/− mice with gastrointestinal infection (Fig. 3B). Analysis of IFN-γ and IL-4 gene expression in CD4+ T cells by quantitative RT-PCR confirmed the results of cytokine secretion. IFN-γ gene expression was not different in wild-type and IL-12-deficient mice early in the course of primary infection, as opposed to the twofold decrease observed in CD4+ T splenocytes from IL-12-deficient mice upon reinfection (Fig. 3D). In contrast, a twofold increase of IL-4 gene expression was observed in IL-12 p40−/− mice upon primary i.v. infection, as opposed to the twofold decrease observed in IL-12 p40−/− mice upon both primary and secondary i.v. infection. Therefore, IL-12 deficiency does not impair the early production of IFN-γ in mice with C. albicans infection, but rather prevents the development of protective CD4+ Th1 responses in IL-12-deficient mice is associated with differentiation of nonprotective IL-4-producing CD4+ Th2 cells, which occurs in spite of IFN-γ production.

**Control of early C. albicans infection in IL-12-deficient mice is associated with unimpaired antifungal activity of phagocytic cells**

To evaluate the contribution of innate immunity in the ability of IL-12-deficient mice to efficiently oppose infectivity in the early stage of infection, we assessed the antifungal activities of macrophages and neutrophils under conditions of vigorous innate system activation (17), that is mice infected i.v. with virulent C. albicans 3 days earlier. Splenic macrophages and peritoneal neutrophils were assessed for candidacidal activity and production of NO. Candidacidal activity and NO production from both cell types were higher in uninfected IL-12 p40−/− than IL-12 p40+/+ mice (Fig. 4). Upon infection, the candidacidal activity continued to be elevated in IL-12-deficient mice, as opposed to the inhibition observed in wild-type mice, whereas NO production decreased in IL-12 p40−/− mice, although to a lesser extent than in IL-12
Production of and responsiveness to IL-10 are defective in IL-12-deficient mice upon C. albicans infection

The finding that IL-10 is a potent deactivating signal for fungicidal effector phagocytes (16) and is induced by IL-12 (27) prompted us to investigate whether defective IL-10 production occurred in IL-12-deficient mice. Macrophages and neutrophils, obtained as above, were assessed for IL-10 gene expression and ability to secrete NO in vitro was assessed as described in Materials and Methods. *p < 0.05 (IL-12 p40−/− vs IL-12 p40+/+).

FIGURE 4. Effector and immunomodulatory functions of macrophages and neutrophils from IL-12-deficient mice infected with C. albicans. Splenic-adherent macrophages and elicited peritoneal neutrophils were from IL-12 p40−/− and IL-12 p40+/+ mice, uninfected (0) or infected i.v. with C. albicans, 3 days before. The ability to kill yeast cells and to secrete NO in vitro was assessed as described in Materials and Methods. *p < 0.05 (IL-12 p40−/− vs IL-12 p40+/+).

Production of IL-10 and expression of IL-10 and IL-10R genes in IL-12 p40−/− and IL-12 p40+/+ mice infected with C. albicans.

A, Purified splenic macrophages and peritoneal neutrophils, obtained from uninfected (naive) or C. albicans-infected mice, at 3 days after infection with 106 CA-6 cells, were stimulated in vitro with IFN-γ (400 U/ml) and LPS (40 ng/ml) for 24 h before IL-10 determination by specific ELISA. *p < 0.05, below the detection limit of the assay. B, IL-10 and IL-10R gene expression in cells from uninfected (lane 1) or infected (lane 2) mice, as assessed by RT-PCR. N, No DNA added to the amplification mix during PCR; C, HPRT- or cytokine-, or cytokine receptor-specific controls. *p < 0.05 (IL-12 p40−/− vs IL-12 p40+/+).

FIGURE 5. Production of IL-10 and expression of IL-10 and IL-10R genes in IL-12 p40−/− and IL-12 p40+/+ mice infected with C. albicans. A, Purified splenic macrophages and peritoneal neutrophils, obtained from uninfected (naive) or C. albicans-infected mice, at 3 days after infection with 106 CA-6 cells, were stimulated in vitro with IFN-γ (400 U/ml) and LPS (40 ng/ml) for 24 h before IL-10 determination by specific ELISA. *p < 0.05, below the detection limit of the assay. B, IL-10 and IL-10R gene expression in cells from uninfected (lane 1) or infected (lane 2) mice, as assessed by RT-PCR. N, No DNA added to the amplification mix during PCR; C, HPRT- or cytokine-, or cytokine receptor-specific controls. *p < 0.05 (IL-12 p40−/− vs IL-12 p40+/+).

The mechanism underlying the unimpaired innate antifungal response occurring in IL-12-deficient mice.

Production of IL-4 in IL-12 p40−/− mice occurs through the CTLA-4/B7-2 costimulatory pathway and is sensitive to IL-10

In mice with C. albicans infection, early production of IL-4 is associated with the expansion of a CD4+ population expressing the activated phenotype, i.e., low levels of L-selectin (Mel-14) and high levels of CD44, through the CTLA-4/B7-2 costimulatory pathway (30). To define whether blockade of this costimulatory pathway also affects IL-4 production in IL-12-deficient mice, mice were systemically infected with PCA-2, treated with CTLA4-Ig, a soluble ligand for B7 (32), and assessed 3 days later for IL-4 and IFN-γ production by CD4+ T cells. We also measured cytokine production upon short low dose treatment with either recombinant IL-10 or IL-12. Each treatment significantly reduced IL-4 and increased IFN-γ production (Table II), and this correlated with a reduced expansion of activated CD4+ T cells (data not shown).
Each treatment also increased resistance of IL-12-deficient mice to infection, as assessed by the decreased fungal growth in their kidneys. Therefore, these results indicate that early IL-4 production in IL-12-deficient mice relies on the expansion of a CD4+ population through a CTLA-4/B7-dependent pathway that is sensitive to IL-10 and IL-12.

Exogenous IL-10 inhibits up-regulation of B7-2 expression in IL-12-deficient mice upon C. albicans infection

The dependency of IL-4 production on the CTLA-4/B7 costimulatory pathway, together with the finding that IL-10 production, which is known to affect expression of B7 molecules on accessory cells (33), was reduced in IL-12-deficient mice, led us to investigate whether B7-1 and B7-2 costimulatory molecules were differentially expressed in vivo in C. albicans-infected IL-12 p40−/− and IL-12 p40+/+ mice, and were modified upon IL-10 or IL-12 administration or neutralization, respectively. Three days after infection, the expression of costimulatory molecules on splenic macrophages was assessed by flow cytometry (Fig. 7). The expression of B7-2, and to a lesser extent of B7-1, significantly increased upon infection in IL-12 p40−/− as compared with wild-type mice. However, administration of IL-10 or IL-12 inhibited the up-regulated expression of B7-2 and, interestingly, increased that of B7-1. Likewise, IL-10, and in part IL-12, neutralization resulted in an increased expression of B7-2 in IL-12 p40−/− mice. These results and those of Table II indicate that the expansion of IL-4-producing CD4+ T cells in mice with C. albicans infection is associated with up-regulated expression of B7-2 on macrophages. IL-10, and partly IL-12, inhibit the expression of B7-2, while slightly increasing that of B7-1. Interestingly, the expression of the CD40 molecule is increased upon IL-10 administration, while that of MHC class II Ag is decreased (unpublished data). These results thus suggest that IL-10, through its ability to modulate costimulatory molecules on accessory cells, may play a key role in IL-12-dependent Th1 cell development in mice with candidiasis.

**Discussion**

The present study demonstrates that development of protective anti-Candida CD4+ Th1 cell responses is impaired in IL-12-deficient mice. This confirms the obligatory role of IL-12 in the generation of antifungal Th1-dependent immunity (11, 34, 35). IL-12-deficient mice are known to be defective in IFN-γ production and type 1 cytokine responses (28, 36) and to mount a polarized Th2 cell response upon infection with *Leishmania major* (37). However, we found that production of IFN-γ was not decreased early in the course of systemic or gastrointestinal infection. This indicates that IFN-γ production in the early phase of *C. albicans* infection is relatively IL-12 independent, and that cytokines other than IL-12 induce the release of IFN-γ (38). Conversely, production of IFN-γ was greatly impaired in IL-12-deficient mice upon reinfection, and correlated with the failure to activate CD4+Th1 cells producing IFN-γ and IL-2 and with their inability to resist reinfection upon systemic or mucosal immunization. Both resistance to reinfection and resolution of the mucosal infection in murine candidiasis depend on the activation of Th1 responses (4, 29). Thus, regulation of IFN-γ production early in the course of infection may not represent a primary mechanism of IL-12 activity in *C. albicans* infection.

IL-12 induces IL-10 production by T (24) and non-T (17, 19) cells. In the present study, we demonstrated the occurrence of this positive regulatory loop between IL-12 and IL-10 in mice with *C. albicans* infection. Both production of and responsiveness to IL-10 were decreased in phagocytic cells from infected IL-12-deficient mice, very likely accounting for the unimpaired innate antifungal immunity and the increased resistance of these mice to acute systemic infection. IL-10 is a potent inhibitor of NO production and candidacidal activity of IFN-γ-activated phagocytic cells (16). Both activities were indeed up-regulated in IL-12-deficient mice, although IL-12 appeared to be required for optimal production of NO by IFN-γ-activated phagocytic cells.

IL-10, but also IL-12, neutralization increased the innate antifungal resistance of wild-type mice, a finding in line with previous data showing that unphysiologic administration of either IL-10 (15) or IL-12 (9) exacerbated candidiasis in mice. However, defective IL-10 production was associated with up-regulated expression of B7 costimulatory molecules, particularly B7-2, on macrophages. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting up-regulation of B7 (33) and MHC (39) expression. It has been suggested that IL-10, by down-regulating costimulatory molecules on accessory cells, could play an indirect role in T cell activation (33, 40). We have shown previously that the effects of exogenous IL-10 in mice with candidiasis were critically dependent on the dose administered, the highest doses of IL-10 exacerbating the infection (15). In contrast, at lower doses,

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**Table II. Effect of CTLA4-Ig, IL-10, or IL-12 administration on IL-4 and IFN-γ production and resistance to infection in IL-12 p40−/− mice infected with *C. albicans***

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-4</th>
<th>IFN-γ</th>
<th>CFU</th>
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</thead>
<tbody>
<tr>
<td>None</td>
<td>69 ± 3</td>
<td>83 ± 4</td>
<td>5.3 ± 0.34</td>
</tr>
<tr>
<td>CTLA4-Ig</td>
<td>22 ± 2*</td>
<td>163 ± 3*</td>
<td>4.2 ± 0.22*</td>
</tr>
<tr>
<td>rIL-10</td>
<td>16 ± 2*</td>
<td>131 ± 3*</td>
<td>4.4 ± 0.43*</td>
</tr>
<tr>
<td>rIL-12</td>
<td>32 ± 3*</td>
<td>156 ± 5*</td>
<td>4.6 ± 0.40*</td>
</tr>
</tbody>
</table>

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* Mice were infected i.v. with 10^6 virulent yeast cells and treated with IL-10- or IL-12-neutralizing Ab.
* Anti-IL-10 mAb (100 μg/injection) was given i.p., the day before and the day of challenge; anti-mouse IL-12 IgG Ab (500 μg/injection) was given i.p. the day of challenge and the following 2 days. Enumeration of yeast cells recovered from the kidneys was performed at 3 days after infection. Vertical bars represent the upper limit of the SE. * p < 0.05 (treated vs untreated).
* Treatments were as follows: CTLA4-Ig (0.5 mg/i.v. injection) the day of infection and the following 2 days; rIL-10 (1 μg/i.p. injection) twice the day of infection and the following 2 days.
* IL-4 and IFN-γ (nanograms per milliliter) in supernatants of mitogen-stimulated CD4+ splenocytes.
* Log CFU (mean ± SE) in kidneys.
* p < 0.05 (treated vs untreated).
IL-10 paradoxically ameliorated the infection, and this was associated with a decreased production of IL-4 (15, 30). In this study, we have shown that treatment with low dose IL-10 prevents up-regulation of B7-2 expression on macrophages and concomitantly decreases IL-4 production. Interestingly, B7-1 expression was slightly increased, indicating a differential effect of IL-10 on B7-1 and B7-2 expression by accessory cells, as already suggested (40).

The engagement of appropriate costimulatory molecules is required for Ag-specific T cell activation (41) and for optimal IL-12-induced proliferation and cytokine production (42, 43).

**FIGURE 7.** Expression of B7-1 and B7-2 costimulatory molecules in MAC-1^+^ splenocytes from IL-12 p40^−/−^ and IL-12 p40^+/+^ mice infected with *C. albicans*. Spleen cells were sequentially stained with FITC-conjugated anti-CD80 (B7-1) or anti-CD86 (B7-2) mAbs and phycoerythrin-conjugated anti-CD11b mAb (MAC-1) or with an irrelevant mAb (black histogram). Cells were from uninfected mice or from mice at 3 days after i.v. infection with low virulence *C. albicans*. IL-12 p40^−/−^ mice were treated i.p. with either rIL-10 (1 µg/injection, twice the day of challenge and once 1 and 2 days later) or rIL-12 (100 ng/injection, the day of challenge and 1 and 2 days later). IL-12 p40^−/−^ mice were given anti-mouse IL-10 mAb i.p. (100 µg/injection, the day before and the day of challenge) or anti-mouse IL-12 IgG Ab (500 µg/injection, the day of challenge and the following 2 days).
Triggering of the CD28/CTLA-4 receptors on T cells by their natural ligands, B7-1 and B7-2, provides an effective costimulus for T cell activation and cytokine production. Whether B7-1 and B7-2 mediate distinct or overlapping costimulatory functions remains controversial. However, when coexpressed with B7-1 on accessory cells, B7-2 provides a predominant costimulatory signal and primes for production of IL-4 and IFN-γ.

Although the extent to which B7-1 and B7-2 may participate in Th selection in response to C. albicans awaits further clarification, the results of the present study indicate that B7-2, rather than B7-1, is required for the production of IL-4 by activated CD4+ T cells. In this respect, it is interesting to note that Candida-induced proliferation of human memory T cells is highly dependent on B7-2 expression on monocytes. In murine C. albicans infection, production of IL-4 by CD4+ T cells is critically dependent upon the CTLA-4/B7 interaction. Interference with this costimulatory pathway during infection results in a decreased IL-2-dependent IL-4 production by CD4+ T cells expressing an activated phenotype, i.e., Mel-14lowCD44high, and in the activation of CD4+ Th1 cells. In the present study, we found that blockade of this costimulatory pathway by CTLA4-Ig, IL-10, or IL-12 administration prevents the expansion of CD4+ Mel-14lowCD44high cells and results in decreased IL-4 production by infected IL-12-deficient mice. Thus, rather than opposing the Th1-promoting activity of IL-12, endogenous IL-10 appears to be required for optimal costimulation of IL-12-induced CD4+ Th1 cells.

IL-10 has been implicated in regulation of inflammation and of a wide variety of immune responses (49, 50), but many of the studies have relied upon the unphysiologic administration of exogenous IL-10. Although studies on IL-10-deficient or IL-10-overexpressing mice will definitely clarify the issue, the experimental models described in the present study would suggest that an overproduction of IL-10 may adversely affect the innate control of C. albicans infection; yet, endogenously produced IL-10 is required for development of antifungal Th1 responses. This is in line with recent findings showing that Th1-mediated resistance to L. major is unaffected in mice overexpressing IL-10 (51). The use of C. albicans strains of different virulence, thus having a different impact on the innate immune system (18), allows us to dissect the opposite effects of IL-10 in antifungal immunity. It is conceivable that the ultimate effect of IL-10 in C. albicans infections may depend on the relative contribution of innate vs specific immune responses in the control of the infection and its pathology.

IL-4 may antagonize IL-12 in Th development (11, 52). However, recent studies have revealed a more complex relationship between IL-12 and IL-4 in CD4+ Th differentiation and phenotype stability. IL-4 appears to be a component of IL-12-dependent activation of CD4+ Th1 lymphocytes (53–55): it is produced by CD4+ T lymphocytes in response to IL-12 (54), and the IL-4R is expressed on Th1 cells (54, 56). We have recently observed that endogenous IL-4 is indeed physiologically required for development and maintenance of CD4+ Th1 responses in mice with candidiasis (22, 57). Therefore, a finely regulated balance of di rective cytokines, such as IL-4, IL-10, and IL-12, rather than the relative absence of opposing cytokines, appears to be required for optimal development and maintenance of Th1 responses in mice with candidiasis. In particular, IL-12-dependent Th1 differentiation in vivo appears to occur in the presence of appropriate levels of IL-4 (22) and IL-10 (this study) in addition to other cytokines, including IFN-γ (5), TGF-β (30), IL-6 (6), and TNF-α (8). It is conceivable that IL-12 acts at different levels to favor antifungal Th1 responses. By inducing IL-10 (9, 19) and TGF-β (unpublished observation), IL-12 may limit the consequences of an exaggerated proinflammatory response, including aberrant expression of costimulatory molecules on accessory cells. At the level of CD4+ T cell priming, IL-12, rather than IFN-γ, acts as a limiting factor for optimal Th1 development (11). The occurrence of early and sustained levels of IFN-γ in Candida-driven Th2 development makes it unlikely that IL-12 plays only an indirect role in Th1 differentiation, by inducing soon after infection IFN-γ as an essential requirement for CD4+ Th1 differentiation (10). It has recently been demonstrated that IFN-γ is required for expression of the IL-12Rβ2 message in activated CD4+ Th1 cells (58). The finding that IL-12Rβ2 transcripts are similarly expressed in IL-12-deficient and wild-type mice (unpublished data) could suggest that IFN-γ is required for the induction of IL-12 responsiveness in naive CD4+ T cells, but that IL-12 is required to induce IFN-γ production by CD4+ Th1 cells.

Overall, the data in this study stress the obligatory role of IL-12 in the induction of antifungal Th1 responses. They also highlight a positive regulatory loop between IL-12 and IL-10 that negatively affects the innate antifungal response, but is required for the induction of optimal adaptive immune response to C. albicans.

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References


