Impaired Neutrophil Response and CD4$^+$ T Helper Cell 1 Development in Interleukin 6–deficient Mice Infected with *Candida albicans*

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Summary

To define the role of interleukin (IL) 6 in *Candida albicans* infection, IL-6–deficient mice were assessed for susceptibility to systemic or gastrointestinal infection, as well as for parameters of elicited T helper cell (Th) immunity. IL-6–deficient mice were more susceptible than wild-type mice to either type of infection caused by virulent *C. albicans*. In response to systemic challenge with a live vaccine strain of the yeast, IL-6–deficient mice failed to mount Th1-associated protective immunity, but the resulting Th2-biased response could be redirected to the Th1 phenotype by IL-10 neutralization. Severe impairment of the macrophage and neutrophil response to infection was observed in IL-6–deficient mice, but administration of IL-6 would increase both neutrophil response and resistance to infection. IL-6 seems to oppose the Th2-promoting role of IL-10 in candidiasis, its early regulatory activity involving effects on neutrophil function.

IL-6 is a multifunctional cytokine that regulates various aspects of the immune response, acute-phase reaction, and hematopoiesis (1). Although not produced under normal circumstances, IL-6 is abnormally produced in a number of inflammatory, immune, and infectious diseases. However, because of the pleiotropic activity of the cytokine and the complex interplay of different cytokines with similar activities (2), the specific role of IL-6 in each pathological condition has not yet been defined. In human infection with HIV (3), but also in several experimental infections, high levels of IL-6 correlate with susceptibility to infection (4, 5) and severity of the associated disease (6). Yet, IL-6 neutralization has been found to be protective in most (7, 8) but not all (9) of these experimental infections. One explanation may be the unexpected detection of high levels of IL-6 in anti-IL-6–treated mice, indicating the possible occurrence of "chaperoning" effects (7, 10). For this reason, mice with a targeted mutation should provide a more useful means of evaluating the role of IL-6 in vivo.

Candida albicans is an opportunistic fungal pathogen commonly found in the gastrointestinal (GI) tract of humans (11). Dissemination, which occurs under conditions that compromise the host immune system (12), results in a progressive disease associated with a high rate of mortality. In mice, the outcome of *C. albicans* infection is largely dependent upon the type of predominant Th cell subset (13, 14). Th1 cell responses associated with protection occur in genetically resistant mice after mouse vaccination with live vaccine strains of the yeast. Th1 responses are also detected in susceptible mice after neutralization of IL-4 (15) or IL-10 (16). These redirected responses lead to protection and onset of lasting anticandidal resistance. In contrast, unopposed Th2 cell responses are associated with susceptibility to infection and disease progression, as observed in genetically susceptible mice (17) and in resistant mice depleted of endogenous IFN-γ (18), IL-12 (19), or TGF-β (20).

In the course of candidal infections, IL-6 is rapidly detected in sera (21) and the cytokine is also produced by CD4$^+$ Th2 cells upon antigen-specific stimulation in vitro (15). In addition, overexpression of IL-6 in CAT/enhancer binding protein β-deficient mice has recently been found to correlate with development of yeast-specific Th2-type responses (22). Although pointing to a link between IL-6 production and disease outcome, these results do not clarify the functional role of IL-6 in candidal infections. In this study, IL-6–deficient mice generated by gene targeting (23), were used to assess the role of IL-6 in resistance to *Candida*. IL-6–deficient (IL-6−/−) mice were found to be

[1] *Abbreviations used in this paper:* GI, gastrointestinal; NO, nitric oxide; PCA-2, live vaccine strain of *C. albicans*; RT, reverse transcriptase.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/96/04/1345/11 $2.00
Volume 183 April 1996 1345–1355

highly susceptible to both systemic and GI infections. This susceptibility correlated with defective neutrophil function and the development of a T cell reactivity biased towards the Th2 pathway.

Materials and Methods

Mice. IL-6−/− and +/+ female mice were bred in our barrier facility, and were used by the age of 8–10 wk. The generation and genetic background of these mice have been described in detail (23). Mice were maintained in standard conditions under a 12-h light–dark cycle, and provided irradiated food (4RF21; Mucedola SRL; Settimo Milanese, Milan, Italy) and chlorinated water ad libitum. 129/SV/EV mice (24), 8–10 wk old, were a kind gift from E. Robertson (Harvard Medical School, Boston, MA) live vaccine strain of *C. albicans* and were bred at Istituto di Ricerche di Biologia Molecolare P. Angeletti. Female $CD2F_1$ mice ($H\text{-}2^d/H\text{-}2^d$), 6–8 wk old, were obtained from Charles River (Calco, Milan, Italy). 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 *C. albicans* highly virulent CA-6 strain and the live vaccine strain PCA-2 used in this study have already been described in detail (17). For infection, cells were washed twice in saline, and diluted to the desired density to be injected intravenously or intragastrically into mice. Quantification of yeast cells in organs of infected mice (four to six per group) was performed by a plate dilution method, using Sabouraud dextrose agar, and results (means ± SEM) were expressed as CFU per organ. For histology, tissues were excised and immediately fixed in formalin. Sections (3–4 μm) of paraffin-embedded tissues were stained with periodic acid-Schiff reagent and examined for histology, as described (25). Mice succumbing to yeast challenge were routinely necropsied for histopathological confirmation of disseminated candidiasis. For differential blood counts, animals were bled through the tail vein and smears were stained with May-Grünwald Giemsa reagents (Sigma Chemical Co., St. Louis, MO) before analysis. Recombinant human IL-6 was produced in *Escherichia coli* and purified to homogeneity according to published procedures, with minor modifications (26). Protein concentration was determined by analysis of the purified product. The specific bioactivity was 2×10^8 U/mg of protein as assessed by the 7TD1 cell growth assay (27). The recombinant IL-6 (10 μg) was given in 0.2 ml s.c. of PBS 4 h before and 1 d after challenge. Control mice received vehicle alone. IL-10–neutralizing antibodies (100 μg of affinity-purified mAb from SXC-1 hybridoma; American Type Culture Collection, Rockville, MD) was injected into mice 1 d before challenge. Depletion of neutrophils was performed by injecting the mice 4 h before infection with 30 μg i.p. of the affinity-purified RB6-8C5 rat IgG2b antibody, a gift from Dr. R.L. Coffman (DNAX Research Institute, Palo Alto, CA). An equivalent amount of isotype-matched antibodies (Zymed Laboratories, Inc., South San Francisco, CA) was injected into control mice. Endotoxin was removed from all solutions with Detoxi-gel (Pierce Chemical Co., Rockford, IL).

Macrophage Cultures and Nitrite Determination. Macrophages were obtained from spleen cell suspensions and nitrite concentration, a measure of nitric oxide (NO) synthesis, was determined as described (28). Briefly, plastic adherent spleen cells were incubated overnight in the presence of 100 U/ml of rIFN-γ and 10 ng/ml of LPS (Sigma Chemical Co.). Viable yeast cells were then added, and supernatants were removed after 4 h and assessed for nitrite content.

Antibody Assay. A micro-ELISA procedure was employed to quantitate total and yeast-specific antibodies in sera (IgG2a and IgE) and fecal samples (IgA) of mice (17, 25).

Cell Cultures. Unfractionated splenocytes (5×10^6/ml) were cultured in the presence of 5×10^5 heat-inactivated *Candida* cells, in RPMI 1640 medium, 10% FCS, and antibiotics. $CD4^+$ lymphocytes were positively selected from pools of spleen cells by means of a panning procedure using purified antimurine CD4 mAb GK1.5, which resulted in a >95% pure population via FACS® analysis. $CD4^+$ (5×10^6) cells were cultured in the presence of 5×10^5 irradiated (2,000 rad) splenocytes and heat-inactivated yeast cells. Cytokine measurements were performed in supernatants collected after 48 h, as described (17).

Enumeration of Cytokine-producing $CD4^+$ Cells and Cytokine Assays. The procedure used to enumerate cytokine-producing cells was based on a spot ELISA, using $CD4^+$ cells from freshly isolated splenocytes, as described previously (16, 25). Source and characteristics of the antibody reagents used in IFN-γ, IL-2, IL-4, and IL-10 ELISPOT and ELISA assays were as described (16, 19, 29). In IL-6 measurement, two-site ELISA involved the use of mAb MP5-20F3 in combination with biotinylated monoclonal MP5-32c11 (PharMingen, San Diego, CA). In the ELISPOT assays, spots were counted with the aid of a dissecting microscope, and results were expressed as the mean number of cytokine-producing cells per 10^4 cells, calculated using replicates of serial two-fold dilutions of cells pooled from four mice per group. Cytokine titers were calculated by reference to standard curves constructed with known amounts of the recombinant cytokines.

RNA Preparation and Reverse Transcriptase. $CD4^+$ lymphocytes (2×10^7 cells) or adherent macrophages from pooled spleens of three to four animals were subjected to RNA extraction by the guanidium thiocyanate-phenol-chloroform procedure, as described (30). Briefly, 3 μg of total RNA was incubated with 0.5 μg of oligo(dT) (Pharmacia, Uppsala, Sweden) for 3 min at 65°C and chilled on ice for 5 min. Each sample was then incubated for 2 h at 42°C after adding 20 U RNase inhibitors (Boehringer Mannheim, Milan, Italy), 1.5 mM deoxynucleoside triphosphates, 25 U avian myeloblastosis virus reverse transcriptase (RT; Boehringer Mannheim), and RT buffer (50 mM Tris-HCl, pH 8.3, 8 mM $MgCl_2$, 30 mM KCl, and 10 mM dithiothreitol, final concentration) in a final volume of 20 μl. cDNA was diluted to a total volume of 500 μl with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and frozen at −20°C until use. Amplification of synthesized cDNA from each sample was carried out as described previously (31). Briefly, 5 μl of cDNA was added to a reaction mixture containing 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.0 mM $MgCl_2$, 0.01% gelatin, 0.2 deoxynucleoside triphosphate, 1 μM of each primer, and 2.5 U AmpliTaq polymerase (Perkin-Elmer Corp., Hayward, CA). Each 100-μl sample was overlaid with 75 μl mineral oil (Sigma Chemical Co.) and incubated in a DNA Thermal Cycler 480 (Perkin-Elmer Corp.) for a total of 30 cycles for each cytokine. For β-actin–specific primers, each cycle consisted of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C; for IL-4, 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C; for IL-10, 10 s at 91°C, 25 s at 59°C, and 25 s at 72°C; and for IL-12p40, 1 min at 94°C, 1 min at 67°C, and 1 min at 72°C. IL-4, IL-10, and IL-12p40 primers and the positive controls were as described elsewhere (15, 16, 19, 29). The β-actin primers were used as a control for both RT 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 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 at least three different experiments.

Statistical Analysis. Statistical analysis was done using the Student's *t* test; $P < 0.05$ was considered significant.

Results

IL-6−/− Are More Susceptible than IL-6+/+ Mice to C. albicans Infections. To evaluate the susceptibility of IL-6−/− and IL-6+/+ mice to *C. albicans* infection, mice were injected intravenously with different doses (10^6 or 10^5) either of highly virulent CA-6 yeast cells or of the live vaccine strain PCA-2. Groups of mice were instead infected intragastrically with 10^8 CA-6 cells. The animals were monitored for mortality, fungal load in the organs, and resistance to secondary challenge. Although the median survival times of mice injected intravenously with the higher CA-6 inoculum did not differ between the two types of infected host, IL-6–deficient mice succumbed to infection with 10^5 cells, in contrast to the long-term survival of IL-6+/+ mice (Table 1). However, both IL-6+/+ and −/− mice resisted challenge with 10^4 CA-6 cells (data not shown). IL-6−/− mice were also more susceptible to the higher (10^6 cells) inoculum of PCA-2, with a greater than 65% death rate within 3 wk of infection. With the lower (10^5 cells) inoculum, both types of mice survived primary challenge; however, they exhibited disparate susceptibilities to rechallenge with virulent CA-6, in that only the IL-6+/+ mice resisted the secondary infection (Table 1).

As the genetic background has been found to contribute to the resistance of an IL-4–deficient 129 mouse strain to murine acquired immunodeficiency syndrome (MAIDS) infection (32), we assessed the relative susceptibility to CA-6 infection of 129/SV/EV mice (24), the strain from which the embryonic stem cells (CCE) used for homologous recombination had been derived (23). The results (see note to Table 1) showed these mice to be as resistant to infection as the IL-6+/+ mice, thus ruling out that genetic background, rather than IL-6 deficiency, might be primarily responsible for the susceptibility of IL-6−/− mice to *C. albicans* infection.

Table 1. *Susceptibility of IL-6−/− and IL-6+/+ Mice to C. albicans infection*

Infection			IL-6 −/−		IL-6 +/+	
Yeast	Dose	Route	MST	D/T	MST	D/T
			d			
CA-6	10^6	i.v.	5	8/8*	8	8/8
	10^5	i.v.	8	8/8	>60	0/8
PCA-2	10^6	i.v.	18	6/8	>60	0/8
	10^5	i.v.	>60	0/8‡	>60	0/8‡
CA-6	10^8	i.g.	>60	0/6	>60	0/8

*Dead mice over total mice injected. The MST of control 129/SV/EV mice injected intravenously with 10^6 or 10^5 CA-6 cells were 14 and >60 d, respectively.

‡When parallel groups of IL-6−/− and +/+ mice surviving infection with 10^5 PCA-2 cells were rechallenged with CA-6 (10^6 cells, 2 wk after primary infection), only the former animals succumbed to virulent challenge with an MST of 3 d.

i.g., intragastric; *MST*, median survival time.

Quantification of yeast cells recovered from the systemically infected mice revealed the presence of higher numbers of *C. albicans* viable units in the kidneys, livers and, in particular, brains, of IL-6−/− mice (Fig. 1 *A*).

Based on the finding that IL-6 promotes IgA secretion by Peyer's patches (33) and that IL-6−/− mice fail to develop strong mucosal antibody responses to viral infection (34), we examined the course of GI candidiasis in IL-6−/− and IL-6+/+ mice. Course and outcome of GI infection are influenced by the host mucosal immune status (25). No differences in survival could be found after intragastric challenge (Table 1). However, analogous to what was found in the systemic infection, the number of yeast cells recovered from the stomachs of IL-6−/− mice was higher than that observed in IL-6+/+ mice, at all time points examined (Fig. 1 *B*), even though both types of host would eventually clear their mucosal infections. It is interesting to note that extensive fungal growth was detected in the esophagus of IL-6−/− mice, an occurrence typical of severely immunodeficient animals (35). Also, increased yeast dissemination occurred to the kidneys in IL-6−/− mice.

Histological examination of the kidneys from mice infected intravenously with CA-6 cells (Fig. 2, *A* and *B*) revealed the presence of microabscesses with abundant neutrophil infiltration within tubules, particularly in IL-6−/− mice. In addition, lesions tended to be larger, showed considerable necrosis, and contained hyphal aggregates and yeast cells in IL-6−/− mice, whereas signs of progressive resolution with no visible yeasts were observed in the wild-type controls. Of interest, inflammatory lesions in the kidney cortex were almost exclusively found in the IL-6+/+ mice (Fig. 2, *A* and *B*), raising the possibility that IL-6 might have an ambivalent role in the pathogenesis of renal candidiasis, as it is required for development of cortex lesions.

In the GI infection (Fig. 2, *C* and *D*), sections of the stomachs from either type of mice revealed the presence of abscesses in the region of the cardiac-atrial fold, the principal site of gastric infection in mice (36). Abscesses, characterized by yeast cells surrounded by a keratinized upper layer and a basal epithelium layer, were more numerous in IL-6−/− than +/+ mice. Also, extensive mononuclear cell and neutrophil responses were evident in the submucosa and the lamina propria of only IL-6−/− mice.

All together, these results indicated that IL-6 deficiency in mice is associated with increased susceptibility and more severe pathology caused by systemic and mucosal *C. albicans* infections.

Susceptibility of IL-6−/− Mice to Candidiasis Is Associated with Activation of $CD4^+$ Th2 Cells. As protective and non-

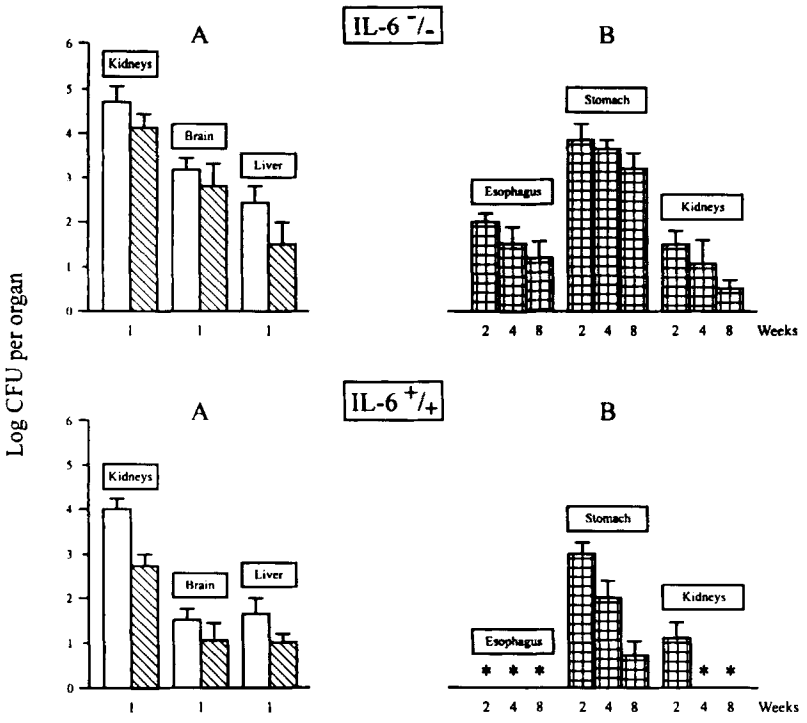

Figure 1. Fungal growth in the organs of IL-6−/− and IL-6+/+ mice. Enumeration of yeast cells recovered from different organs of IL-6−/− and +/+ mice at 1 wk after i.v. injection of 10^5 (□) or 10^4 (▨) virulent CA-6 cells (*A*) or at 2, 4, and 8 wk after intragastric challenge with 10^8 CA-6 cells (*B*). Cumulative data from two experiments (mean ± SE, six animals per group). (*Vertical bars*) Upper limit of the standard error. (*) No viable yeast units found.

protective forms of specific immunity to *C. albicans* correlate in mice with the respective expansion of Th1 and Th2 cells (13), parameters of $CD4^+$ Th cell immunity were examined in IL-6−/− and control mice with systemic or GI candidiasis. Ig levels as well as patterns of Th1/Th2 cytokine production were evaluated in mice injected intravenously with CA-6 or PCA-2 cells, or infected intragastrically with CA-6 cells. In uninfected mice, no differences were found in the production of IgG2a or IgE, although a significant decrease in total IgA fecal content was present in IL-6−/− mice (Fig. 3). However, after i.v. infection with PCA-2, *Candida*-specific IgE levels were higher in IL-6+/+ mice, whereas in contrast, specific IgG2a levels were higher in IL-6−/− mice. In GI-infected mice, the *Candida*-specific IgA content was significantly lower in fecal samples from IL-6−/− mice (Fig. 3).

For studying cytokine production, we assessed cytokine levels in sera and frequencies of cytokine-producing cells in spleens, together with the qualitative pattern of cytokine gene expression. Because circulating levels of IL-12, rather than IFN-γ, correlate with Th1 development in candidiasis (29), we quantitated the presence of bioactive IL-12 in the sera of IL-6−/− and control mice infected with PCA-2. We found (Fig. 3) these levels to be higher in resistant IL-6+/+ than in susceptible IL-6−/− mice, although IL-12 could be detected in both cases. In contrast, IL-12 levels were below the detection limits of the assay in both types of uninfected host. Fig. 3 also shows that, whereas serum levels of IL-4 did not apparently increase in response to infection in either type of mice, the levels of circulating IL-10, although increased in both types of mice, were higher in IL-6−/− mice with systemic or GI infection.

Depending on the yeast/host combination and outcome of infection, IL-4 (15, 37), IL-10 (16), and IL-12 (19, 29) are key contributors to the cytokine balance affecting Th cell differentiation. Therefore, we evaluated the simultaneous expression of these cytokines in the spleens of IL-6−/− and control mice injected intravenously with CA-6 or PCA-2 cells. 6 d after infection, spleens were harvested and message levels of IL-4, IL-10, and IL-12p40 were assayed in purified $CD4^+$ cells or macrophages by RT-PCR analysis. The results (Fig. 4) showed that the IL-4–specific message was not (PCA-2) or barely (CA-6) detectable in $CD4^+$ cells from *Candida*-infected IL-6−/− mice but was clearly expressed by IL-6+/+ and 129 mice. In contrast, IL-10 messages were present in IL-6−/− and +/+ mice, as well as in 129 mice. Moreover, the IL-12p40 message was present in all types of mice injected with PCA-2 but not with CA-6.

To correlate these findings with cytokine production by $CD4^+$ cells from infected mice, the frequencies of $CD4^+$ cells producing IFN-γ, IL-2, IL-4, and IL-10 cytokines were determined in IL-6−/− and +/+ mice after primary PCA-2 infection or after reinfection at 2 wk with CA-6 cells. At 3 d after primary or secondary infection, the number of IFN-γ–, IL-2–, IL-4–, and IL-10–producing $CD4^+$ cells (freshly isolated from the spleens of infected mice) was determined by means of an ELISPOT assay (Fig. 5). A fourfold increase in the number of IL-10–producing cells and an almost twofold increase for those producing IL-4 were found in the spleens of IL-6−/− mice after PCA-2 infection as compared to uninfected mice. In contrast, the frequencies of cytokine-producing cells in infected IL-6+/+ mice were not significantly different from those in unin-

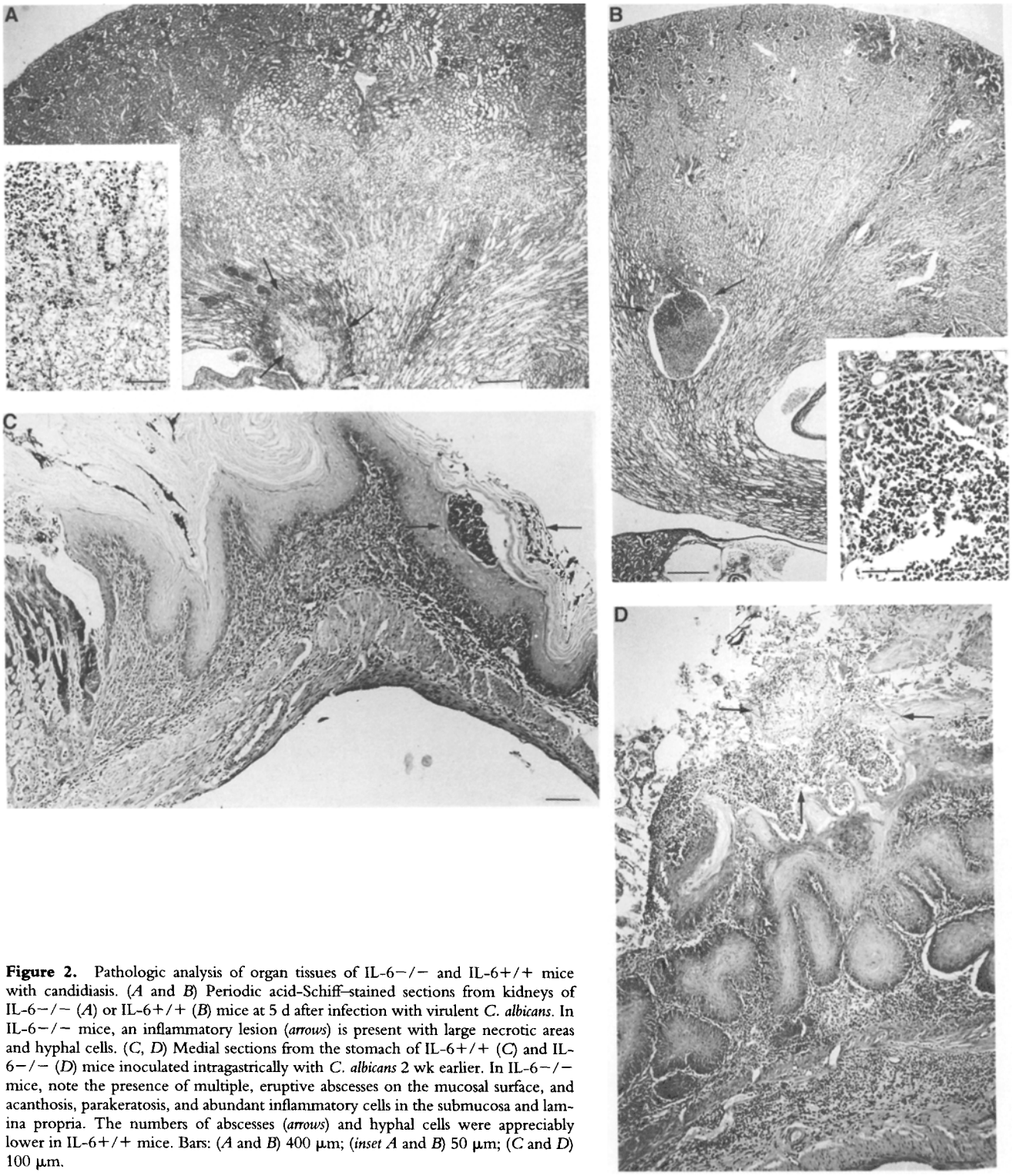

Figure 2. Pathologic analysis of organ tissues of IL-6−/− and IL-6+/+ mice with candidiasis. (*A* and *B*) Periodic acid-Schiff–stained sections from kidneys of IL-6−/− (*A*) or IL-6+/+ (*B*) mice at 5 d after infection with virulent *C. albicans*. In IL-6−/− mice, an inflammatory lesion (*arrows*) is present with large necrotic areas and hyphal cells. (*C*, *D*) Medial sections from the stomach of IL-6+/+ (*C*) and IL-6−/− (*D*) mice inoculated intragastrically with *C. albicans* 2 wk earlier. In IL-6−/− mice, note the presence of multiple, eruptive abscesses on the mucosal surface, and acanthosis, parakeratosis, and abundant inflammatory cells in the submucosa and lamina propria. The numbers of abscesses (*arrows*) and hyphal cells were appreciably lower in IL-6+/+ mice. Bars: (*A* and *B*) 400 μm; (*inset A* and *B*) 50 μm; (*C* and *D*) 100 μm.

fected controls. After reinfection, a significant increase in the number of IL-4–producing cells was observed in the IL-6–deficient mice, yet the numbers of IFN-γ– and IL-2–producing cells did not increase, as opposed to what was observed in IL-6+/+ mice.

Taken together, these results indicated that susceptibility of IL-6−/− mice to candidiasis might be associated with the activation of $CD4^+$ Th2 cells and a preponderant production of IL-10.

IL-10 Neutralization Enhances Th1-mediated Resistance of IL-6−/− Mice to Candidiasis. As IL-10 impairs development of protective anticandidal Th1 responses by inhibiting

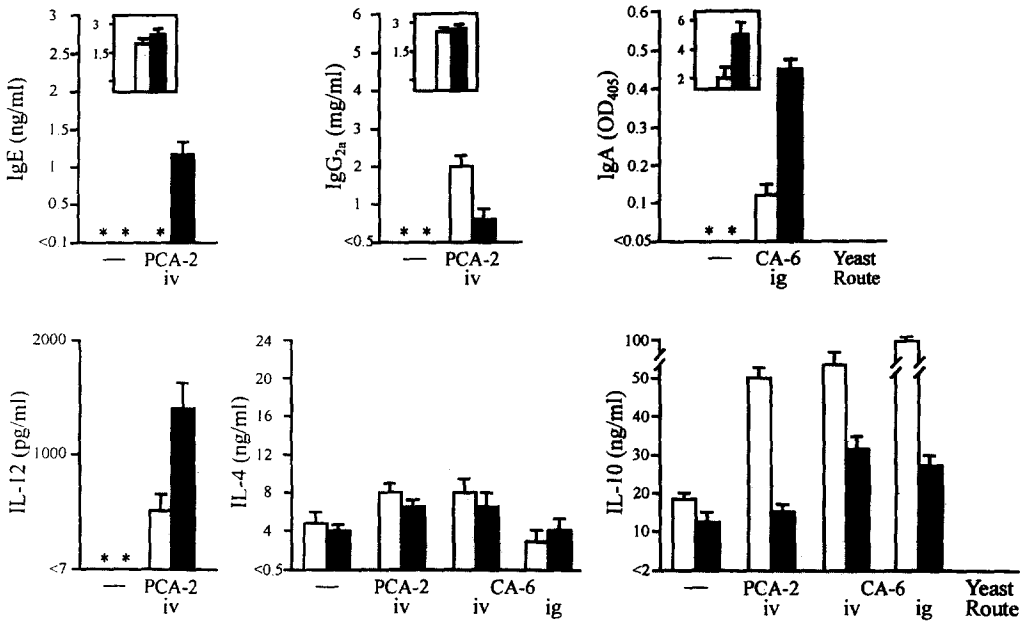

Figure 3. Ig production and serum cytokine levels in *C. albicans*–infected IL-6−/− and IL-6+/+ mice. Mice were injected intravenously with 10^5 CA-6 or 10^6 PCA-2 cells or intragastrically with 10^8 CA-6 cells. Cytokines and *Candida*-specific IgG2a and IgE in serum and IgA in fecal samples were assessed 6 d after infection. (——) Uninfected control mice; (□) IL-6−/− mice; and (■) IL-6+/+ mice. (*Insets*) Levels of total Ig production by uninfected mice. (*) Below the detection limit of the assay, as indicated on the y-axis.

early macrophage production of NO (16) and IL-12 (29), we assessed such productions together with the pattern of resistance in IL-6−/− and control mice after intravenous challenge with PCA-2 in the presence of IL-10–neutralizing mAb (Table 2). IL-10 neutralization did not affect resistance in IL-6+/+ mice, yet significantly increased survival in IL-6−/− mice. The protective effect of anti-IL-10 was associated with elevated serum levels of bioactive IL-12 and upregulation of NO release by activated macrophages; both functions were defective in IL-6−/− mice, infected (Table 2) or not (data not shown) with PCA-2.

Therefore, overproduction of IL-10 seemed to play an important role in the susceptibility to *Candida* infection and the development of nonhealing Th2 cell responses in IL-6−/− mice. However, the T cell response could be redirected to a Th1 pattern of reactivity by anti-IL-10 treatment.

Susceptibility and Resistance to Candidiasis Correlate with the Levels of Endogenous IL-6 in Conventional Mice. To further define the role of IL-6 in candidiasis, we measured IL-6 production in previously established models of healing or nonhealing infections in which the patterns of Th cell responses have been best characterized (19). Accordingly, IL-6 levels were measured in culture supernatants of splenocytes from $CD2F_1$ mice, injected intravenously 2 d earlier with escalating doses of either CA-6 (nonhealing infection) or PCA-2 (healing infection) after restimulation in vitro with *Candida* antigens (Fig. 6). In both infections, the produc-

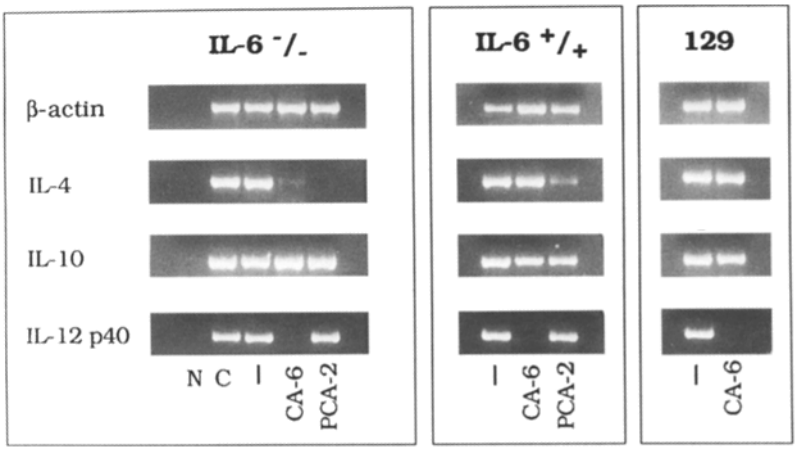

Figure 4. Analysis of cytokine gene expression in IL-6−/−, IL-6+/+, and 129 mice with systemic candidiasis. Mice were injected intravenously with 10^5 CA-6 or 10^6 PCA-2 cells, 6 d before spleen harvesting for purification of $CD4^+$ T cells and macrophages. IL-4 and IL-10 (in $CD4^+$ T cells) and IL-12p40 (in macrophages) gene expression was assessed by RT-PCR. (*N*) No DNA added to the amplification mix during PCR; (*C*) β-actin– or cytokine–specific controls; and (——) uninfected control mice.

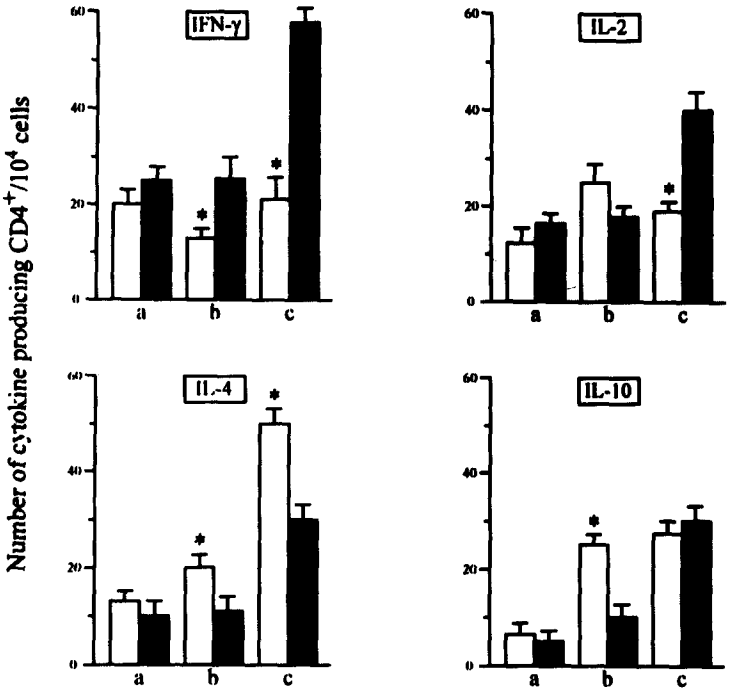

Figure 5. Frequencies of cytokine-producing cells in IL-6−/− and IL-6+/+ mice with candidiasis. The number of cytokine-producing $CD4^+$ cells was determined in IL-6−/− (□) or IL-6+/+ (■) noninfected mice (*a*), at 3 d after challenge with 10^6 PCA-2 cells (*b*), or with 10^6 CA-6 cells 14 d after PCA-2 (*c*). Values are the means ± SE of four replicates obtained with pooled cells from four mice per group. (*) Significantly different from IL-6+/+ mice.

tion of IL-6 directly correlated with the fungal dose used for the in vivo challenge, being the highest in mice given the largest inoculum of the yeast. A similar dependence of IL-6 production on size of microbial challenge has been observed in *Listeria* (38) and HIV (39) infections. In our experiments with $CD2F_1$ mice challenged with high- or low-virulence *Candida*, the highest production of IL-6 was detected in nonhealer mice (Fig. 6) and relatively high levels of IL-6 were also found in early sera from these animals (data not shown), analogous to the results of others in another system (21). However, in experiments not reported here, we found that administration of IL-6 to healer mice (with an apparently low endogenous production of the cytokine) decreased fungal load, which would instead be increased by IL-6 neutralization in nonhealer mice. Therefore, the early production of IL-6 may represent an important defense mechanism to limit candidal infection. Because IL-6 does not affect the growth of *Candida* in vitro (our unpublished observations), it is likely that the effects of IL-6 in vivo occur via activation of antifungal effector functions, such as those typically mediated by phagocytic cells.

Table 2. *IL-10 Neutralization Increases Th1-mediated Resistance in IL-6−/− Mice with Systemic Candidiasis*

Mice	Anti-IL-10 mAb‡	MST	D/T	NO_2^-	IL-12p70
		d			
IL-6 −/−*	−‡	14	6/6	9.4§	984 ± 44‖
IL-6 −/−	+	>60	2/6	18¶	1,850 ± 141
IL-6 +/+	−	>60	0/6	14	2,180 ± 194
IL-6 +/+	+	>60	0/6	16	2,240 ± 155

*Mice were injected intravenously with 10^6 PCA-2 cells.
‡Anti-IL10–neutralizing mAb (100 μg/mouse) was given intraperitoneally 4 h before challenge. Control mice received an equal amount of isotype-matched purified antibodies.
§Adherent macrophages from 3-d–infected mice were cultured overnight with IFN-γ (100 U/ml) and LPS (10 ng/ml) before determination of nitrite content (μm/10^7 cells).
‖IL-12p70 (ng/ml) was determined in sera pooled from three to five mice at 6 d of infection.
¶$P < 0.05$ (anti-IL-10–treated versus controls).

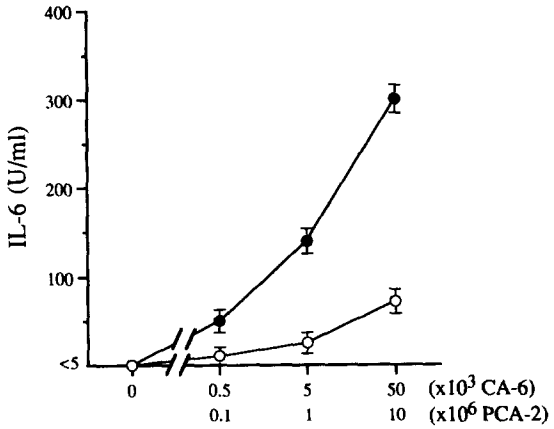

Figure 6. IL-6 production in mice with candidiasis. $CD2F_1$ mice were injected with escalating doses of CA-6 (●-●) or PCA-2 (○-○) cells. Splenocytes from 2-d–infected mice were stimulated in vitro with *Candida* antigen before IL-6 determination in culture supernatants by ELISA. IL-6 production by splenocytes from noninfected mice was below the detection limit of the assay (<5 U/ml). Values are the means ± SE of two independent experiments.

Exogenous IL-6 Restores Neutrophil Effector Function and Increases Resistance in IL-6–deficient Mice. One such effector function is mediated by neutrophils, such that their ablation in vivo increases susceptibility to candidal infection and promotes Th2 development (13). Significantly, IL-6–deficient mice are unable to mount peripheral blood neutrophilia in response to *Listeria* infection (40). Therefore, it seemed of interest to measure the number of peripheral blood neutrophils in IL-6−/− and +/+ mice before and after *C. albicans* infection. Neutrophil counts did not significantly differ in uninfected IL-6–deficient and control mice (Table 3, groups 1 and 6); however, at 2 d after infection, the number of peripheral blood neutrophils failed to increase in IL-6−/− mice in contrast to what observed in IL-6+/+ mice (groups 2 and 7).

To define the role of neutrophils as antifungal effectors in IL-6−/− and +/+ mice, both types of mice were depleted of neutrophils by treatment with granulocyte-depleting mAb, according to a procedure established previously in yeast-infected $CD2F_1$ mice (13). Neutrophil depletion, at the time of *C. albicans* challenge, dramatically increased susceptibility to infection in both types of mice, as shown by the occurrence of median survival times of 2 d (Table 3, groups 3 and 8). However, recombinant human IL-6 administered to yeast-infected IL-6−/− mice (group 4), resulted in a dramatic increase in the number of peripheral blood neutrophils, provided that the animals had not been treated with the mAb (group 5). Concomitantly, there was a twofold decrease in the number of yeast cells recovered from the kidneys (groups 2 and 4). The number of yeast units in the kidneys was also lower at 7 d of infection (fourfold decrease, data not shown). It is interesting to note that no such effect was observed in neutrophil-depleted mice. Indeed, similar to the effect of IL-10 neutralization (Table 2), the administration of rIL-6 to the yeast-infected IL-6−/− mice significantly increased their survival times, from 14 to >60 d (groups 2 and 4, respectively, Table 3).

Discussion

This study demonstrates that IL-6 deficiency, resulting from gene targeting, increases mouse susceptibility to *C. albicans* infection and prevents development of protective Th1-mediated immunity. This effect is reversed by neutralization of endogenous IL-10 or administration of rIL-6, suggesting that production of IL-6 may represent an important defense mechanism against candidal infection. However, the limited IL-6 production found in conventional mice with healing infection of the yeast would suggest that the production of IL-6 must be strictly regulated, in accordance with the notion that immunopathological consequences may result from its deregulation (41). This is also consistent with previous findings of a deleterious effect of IL-6 overexpression in C/EBPβ mice infected with *Candida* (22). The increase in fungal load found not only in the kidneys and livers, but also in the brains of systemically infected IL-6–deficient mice may indicate a possible unique role for IL-6 in immunosurveillance in this compartment (42). However, another observation in this study was that highly susceptible IL-6–deficient mice did not develop as severe renal pathology in the cortex as the otherwise resistant IL-6+/+ mice. Because of the known involvement of IL-6 in mesangial proliferative disorders (43), it is possible that this cytokine plays a deleterious role in renal disease by *Candida*.

The interplay between the innate and acquired immune

Table 3. *IL-6 Administration Induces Peripheral Blood Neutrophilia and Increases Resistance in C. albicans–infected IL-6−/− Mice*

Group	Mice	Treatment‡		Neutrophils/mm³ (± SE)	CFU (×10³)	MST	D/T
		Antigranulocyte mAb	Recombinant human IL-6				
						d	
1	IL-6−/−*	−	−	980 ± 120§	−‖	−	−
2	IL-6−/−	−	−	1020 ± 165	150 ± 10	14	6/6
3	IL-6−/−	+	−	21 ± 8	ND	2	10/10
4	IL-6−/−	−	+	2340 ± 204	78 ± 8	>60	1/6
5	IL-6−/−	+	+	38 ± 11	ND	2	10/10
6	IL-6+/+	−	−	1521 ± 280	−	−	−
7	IL-6+/+	−	−	2780 ± 220	124 ± 27	>60	0/6
8	IL-6+/+	+	−	52 ± 18	ND	2	10/10

*Mice were injected intravenously with 10^6 PCA-2 cells, with the exception of groups 1 and 6.
‡Antigranulocyte mAb (30 μg i.p. of affinity-purified RB6-8C5 mAb) was given 4 h before infection. A total of 30 μg/mouse of recombinant human IL-6 was given subcutaneously 4 h before and 1 d after infection.
§Absolute numbers of peripheral blood neutrophils as determined by tail bleeds at 2 d after infection.
‖CFUs (mean ± SE) were determined in kidneys 2 d after infection. The difference between groups 2 and 4 is statistically significant ($P < 0.05$).

system is considered to be fundamental in the general defense against infections (44), and IL-6, through either endocrine, paracrine, or autocrine pathways of activation, appears to be important to the successful development of both forms of immunity. However, IL-6 may be more critical in the promotion of cellular immunity (45) than in short-term events, such as shock (46) and LPS-induced systemic inflammation (47). IL-6 acts as a potent costimulatory signal in $CD4^+$ T cell activation and promotes both cytotoxic T cell differentiation and NK function (41). A deficient IL-6 production is known to impair T cell activation and to increase susceptibility to *Listeria* infection (45). IL-6 is also required for the induction of T cell–mediated resistance to *Mycobacterium avium* (48).

We have observed that IL-6−/− mice default to the Th2 pathway in response to a low-virulence *C. albicans* strain that, in contrast, induces a preponderance of Th1 responses in IL-6+/+ mice. IL-10, rather than IL-4, was the main cytokine produced by the IL-6−/− former mice in response to primary infection. This could explain the pattern of Ig production observed in sera as well as the low levels of bioactive IL-12p70 produced by the IL-6–deficient mice. IL-10 induces the synthesis of IgG with no direct effect on IgE production (49) and strongly inhibits IL-12 production (50). However, the number of IL-4–producing $CD4^+$ cells was greatly increased by yeast reinfection, which indicates that memory $CD4^+$ T cells will produce substantial amounts of IL-4 upon secondary challenge. The finding of a relatively low production of IL-4 in IL-6+/+ and 129 mice challenged with virulent *Candida* suggests that genetically determined factors in addition to IL-4 could mediate susceptibility to infection in these mice, a possibility that may be at work in MAIDS infection as well (32). The genetic T cell background is likewise important in determining default Th phenotype development in an in vitro system (51).

Whether IL-6 has a direct effect on the developing $CD4^+$ T cells in candidiasis is unclear. We do not favor the hypothesis of a direct role, also considering the recent finding that the protective effect of rIL-6 in murine listeriosis is independent of lymphocytes (40). The low levels of IL-12 produced by IL-6–deficient mice may well explain their failure to mount a sufficient Th1 response. As a matter of fact, the finding of upregulated IL-12 production and increased resistance to infection after IL-10 neutralization suggests that an appropriate immune response will take place in IL-6−/− mice when the effects of the opposing cytokine, IL-10, are neutralized.

That IL-10 may prevent development of protective Th1 responses in mice with candidiasis has already been shown (52). In addition, in genetically susceptible DBA/2 mice, the emergence of protective Th1 reactivity after IL-10 neutralization is concomitant with upregulation of NO release by splenic macrophages (16). We have assessed NO production by macrophages from IL-6−/− and +/+ mice infected with *C. albicans*. Although NO release by elicited peritoneal (45) or splenic macrophages in *Listeria* infection (40) is not different in independently derived IL-6−/− and control mice, we did observe defective NO production by activated splenic macrophages from *C. albicans*–infected IL-6–deficient mice. Because IL-10 neutralization was followed by upregulation of NO production, it is likely that IL-10 was primarily responsible for the impaired NO release observed in IL-6−/− mice. Whether the (relative) overproduction of IL-10 is directly related to IL-6 deficiency remains to be clarified.

It is interesting to note that increased production of IL-10 is a common finding in *C. albicans*–infected neutropenic mice (Romani L., A. Meucacci, E. Cenci, R. Spaccapelo, P. Puccetti, and F. Bistoni, manuscript in preparation). After neutrophil depletion, otherwise resistant mice exhibit high susceptibility to infection associated with Th2, rather than Th1 responses. Neutrophils may actively participate in the selection of the Th response by modifying the cytokine milieu at the time of infection, this being individually the most critical factor in Th cell differentiation to *Candida* (13). We found that the number of neutrophils was lower in IL-6−/− than in IL-6+/+ mice, and, in particular, that IL-6−/− mice were unable to mount peripheral blood neutrophilia in response to infection. A similar condition has recently been observed in IL-6–deficient mice in response to *Listeria* infection (40). However, an anticandidal effect was found to result from administration of exogenous IL-6; this correlated with increased counts of circulating neutrophils. The therapeutic activity of IL-6 was lost in neutrophil-depleted mice, which suggests a neutrophil dependence of the effect. These findings are in line with the IL-6–promoting activity on granulocyte and macrophage colony development from murine progenitor cells (53) and with the significant increase in neutrophils observed after IL-6 injection (54). These data also suggest that IL-6 participates in the early defense to *C. albicans* infection by stimulating neutrophils, either directly or indirectly. IL-6 is known to influence the oxidative burst and destructive capacity of human neutrophils (55), raising the possibility that the neutrophil effector function may be impaired in IL-6–deficient mice as well.

All together, these results indicate that a defective innate immune response, such as an inefficient neutrophil function, together with the negative effect of unopposed cytokines, such as IL-10, on macrophage activity, may contribute to the inability of IL-6–deficient mice to mount protective Th1 responses in candidiasis. In GI infection, a further negative effect could result from impaired IgA secretion, even though the precise role of IgA in mucosal candidiasis is still to be defined (25).

Th1 development in candidiasis appears to require a critical balance of cytokines acting directly or indirectly on $CD4^+$ subset differentiation. In addition to previous evidence for an obligatory role of IFN-γ (18), IL-12 (19), and TGF-β (20), the present data establish IL-6 as another cytokine that, presumably via effects on innate response mechanisms, permits optimal development of Th1 reactivity leading to lasting anticandidal protection.

The authors thank Eileen Zannetti for editorial assistance, Paolo Mosci for histological analysis, and Gennaro Ciliberto for critically reading the manuscript.

This study was supported by VIII Progetto AIDS (9305-02) Italy.

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Received for publication 2 October 1995 and in revised form 12 December 1995.

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