## 8.3 The Roles of TNFα and IL-17 in the Development of Aortitis

8.3.1 Development of Aortitis in IL-1Ra$^{-/-}$ Mice

IL-1Ra$^{-/-}$ mice on the BALB/c background spontaneously developed arterial inflammation at 4 weeks of age. Approximately 50% of the mice were affected by 12 weeks (Matsuki et al. 2005). A similar observation was reported in IL-1Ra$^{-/-}$ mice on the 129/Ola x MF1 background (Nicklin et al. 2000). On the C57BL/6 background, however, there were no signs of arterial inflammation, suggesting the significant involvement of background genes in the development of aortitis, a similar observation that has been made for arthritis (Horai et al. 2000). In F2 hybrids of BALB/c- and C57BL/6-IL-1Ra$^{-/-}$ mice, arthritis was rare but aortic inflammation was common, indicating that the sets of background modifier genes that cause susceptibility to each disease are not fully overlapping (Shepherd et al. 2004).

Inflammation of the cardiovascular system was observed preferentially at the aortic root of IL-1Ra$^{-/-}$ mice (Fig. 4) (Matsuki et al. 2005). The infiltration of monocytes and occasionally neutrophils was observed in the aorta and aortic valve. A loss of elastic lamellae in the aortic media could be observed by histological examinations. Monocytes/macrophages and some neutrophils had infiltrated the inflammatory sites within the aortic sinus. Thus, the aortic inflammation in these animals may have characteristics of both acute and chronic phases of disease. We identified numerous examples of neovascularization within severe lesions. Chondrocyte-like cells were observed in the majority of IL-1Ra$^{-/-}$ mouse aortas; no such cells could be observed in the aortas of WT mice. Calcification of the media of the aorta was observed in a subset of IL-1Ra$^{-/-}$ mice. As calcification of the media, involving the degradation of smooth muscle cells, is a sign of degenerative processes (Tanimura et al. 1986a, 1986b), this result suggests the involvement of an immune response in this pathology. These mice suffered from mild aortic stenosis and hyperplasia of the interventricular septum and left ventricular posterior walls. In agreement with previous reports, these pathological findings resemble aspects of Takayasu arteritis or polyarteritis nodosa in humans (Nicklin et al. 2000).

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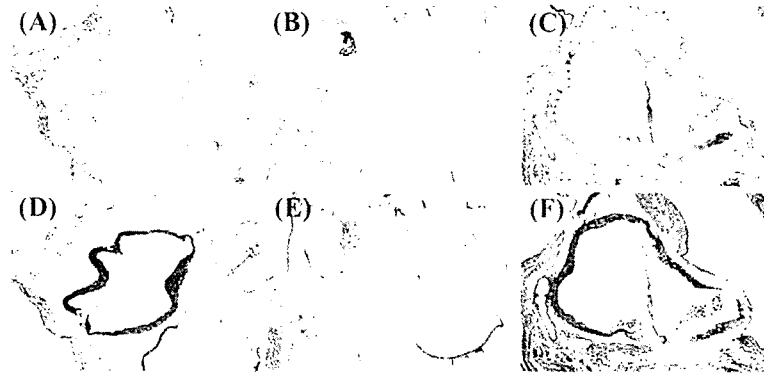
Fig. 4. Attenuation of the development of aortitis in IL-1Ra$^{-/-}$ mice by IL-17 deficiency. Sections of the aorta were examined by staining with (**A–C**) hematoxylin and eosin and (**D–F**) Masson's trichrome (Isoda et al. 2002). **A, D** Sections of the aortic valve (score 0) from a 20-week-old unaffected IL-17$^{+/-}$-IL-1Ra$^{-/-}$ mouse. **B, E** Sections from a 20-week-old affected IL-17$^{+/-}$-IL-1Ra$^{-/-}$ mouse. Severe inflammatory cell infiltration and loss of elastic lamellae over greater than two-thirds of the media of the aortic sinus are observed (score 3). **C, F** Sections from an 20-week-old IL-17$^{-/-}$-IL-1Ra$^{-/-}$ mice. Mild inflammatory cell infiltration and loss of elastic lamellae are observed (score 2)

Using peripheral T cell transplantation, we also examined the role of T cells in the development of aortitis (Matsuki et al. 2005). Purified T cells from the spleens and lymph nodes of 6- to 8-week-old IL-1Ra$^{-/-}$ mice were transplanted into BALB/c-nu/nu mice, and the development of aortitis in the recipient mice was analyzed 10 weeks after transplantation. Twelve of the 13 recipient mice developed aortitis, indicating that T cells are crucial in the pathogenesis of aortitis. As arthritis is also induced by IL-1Ra$^{-/-}$ T cell transplantation, the pathogenesis of aortitis likely utilizes a similar mechanism as that seen in arthritis, in which T cell-mediated autoimmunity caused by excess IL-1 signaling is involved.

8.3.2 The Roles of TNFα and IL-17 in the Development of Aortitis

We examined the roles of TNFα and IL-17 in the development of aortitis by intercrossing these cytokine-deficient mice to IL-1Ra$^{-/-}$ mice. The aortic valves of these cytokine-deficient IL-1Ra$^{-/-}$ mice were analyzed histologically. Interestingly, TNFα$^{-/-}$-IL-1Ra$^{-/-}$ mice showed no signs of arterial inflammation at 8 and 14 weeks of age, while approximately 50% of the TNFα$^{+/+}$-IL-1Ra$^{-/-}$ mice developed aortitis at these ages (Matsuki et al. 2005). The incidence of aortitis in IL-17$^{-/-}$-IL-1Ra$^{-/-}$ mice was similar to IL-17$^{+/-}$-IL-1Ra$^{-/-}$ mice at 20–28 weeks of age (Table 2). The disease severity score, however, was significantly reduced in these IL17$^{-/-}$-IL-1Ra$^{-/-}$ mice (Fig. 4). Thus, in IL-1Ra$^{-/-}$ mice, TNFα is crucial for the development of aortitis. While IL-17 is not essential for aortitis development, it aggravates the disease, appearing to function at both the elicitation of inflammation and the sensitization of T cells.

As mentioned already, we have demonstrated that T cell-derived TNFα plays an important role for the sensitization of T cells in the development of autoimmunity in IL-1Ra$^{-/-}$ mice (Horai et al. 2004). Other investigators have also reported the production of TNFα in T cells (Ramshaw et al. 1994; Sakaguchi et al. 1995) and the presence of TNF receptors in aortic smooth muscle and endothelial cells (Field et al. 1997). Thus, upon T cell activation, T cells produce TNFα, and this T-cell derived TNFα may activate endothelial cells to produce various

Table 2. Suppression of the development of aortitis in IL-17$^{-/-}$-IL-1Ra$^{-/-}$ mice

Genotype	Incidence (%) Severity score 20, 28 weeks
IL-17$^{+/-}$-IL-1Ra$^{-/-}$	5/6 (83%) 2.8
IL-17$^{-/-}$-IL-1Ra$^{-/-}$	6/13 (46%) 1.8*

* $p < 0.05$ vs IL-17$^{+/-}$-IL-1Ra$^{-/-}$ mice by Mann-Whitney U test

inflammatory cytokines and chemokines, resulting in the development
of inflammation (Kollias and Kontoyiannis 2002). It is also known that
TNFα induces the expression of vascular cell adhesion molecule-1 in en-
dothelial cells; this promotes the early adhesion of mononuclear leuko-
cytes to the arterial endothelium at sites of inflammation (Feldmann
2002).

Consistent with our observations, it was recently reported that In-
fliximab, an anti-TNFα antibody, improved endothelial dysfunction in
antineutrophil cytoplasmic antibody-associated systemic vasculitis in
humans (Booth et al. 2004). Although the etiopathogenesis of this vas-
culitis has not been completely elucidated, it is thought that both aortitis
in IL-1Ra$^{-/-}$ mice and antineutrophil cytoplasmic antibody-associated
systemic vasculitis in humans share a similar pathogenic process involv-
ing TNFα. These observations provide new insight into the pathogenesis
of vasculitis, and the IL-1Ra$^{-/-}$ mice should be a useful model for the
study of the pathogenic mechanisms of vasculitis (Fig. 5).
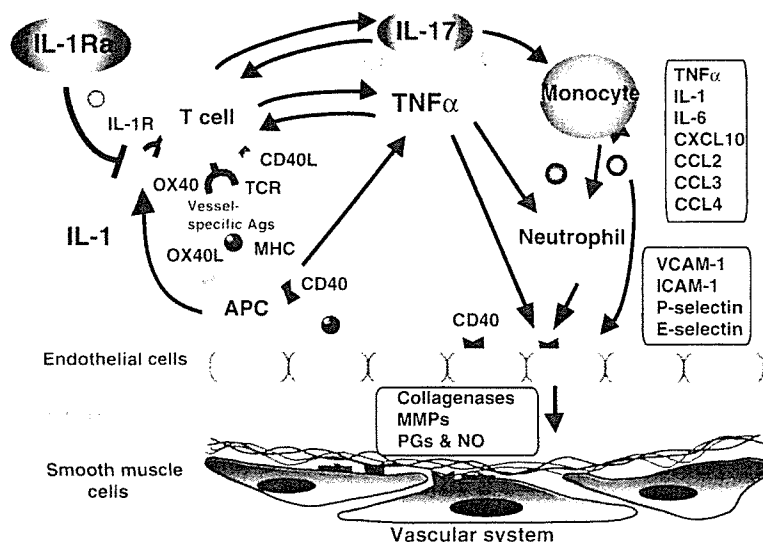
Fig. 5. Pathogenesis of aortitis: TNFα plays an important role in the development
of aortitis in IL-1Ra$^{-/-}$ mice

8.4   The Role of TNFα in the Development of Dermatitis

8.4.1   Development of Psoriasis-Like Dermatitis in IL-1Ra$^{-/-}$ Mice

Psoriasis-like skin disease, first reported by Shepherd et al. (2004), was evident in IL-1Ra$^{-/-}$ mice on the BALB/c background. However, we could not identify disease in animals on the C57BL/6 background, indicating that strain-specific background genes are also involved in the development of this disease. The mice on the BALB/c background develop redness and scaling of their ears and tail (Fig. 6), a pathology characterized by extensive thickening of the epidermis associated with hyperkeratosis of the skin. The majority of keratinocytes retained their nucleus in the cornified cellular layer. We also observed massive neutrophil infiltration into the epidermis and dermis. With increasing disease progression, the epidermal layer gradually penetrated into the dermal layer, forming ridges. Aseptic microabscesses formed under the skin. CD4^{+} T cells were occasionally observed within the dermis.

Interestingly, however, significant disease developed in scid/scid-IL-1Ra$^{-/-}$ mice. Furthermore, IL-1Ra$^{-/-}$ T cell transfer could not induce dermatitis in nu/nu mice, in contrast to cases of arthritis or aortitis in

(A)   (B)
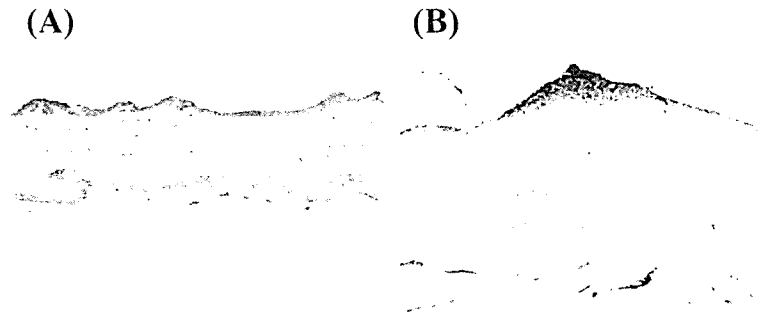
Fig. 6. Histological examination of the skin in IL-1Ra$^{-/-}$ mice. A Hematoxylin and eosin staining of the ear pinna of a normal 20-week-old WT mouse. B An affected IL-1Ra$^{-/-}$ mouse at 20 weeks of age. The epidermis becomes thickened and hypertrophic associated with hyperkeratosis of the skin. Massive infiltration of neutrophils into the epidermis and dermis are observed in IL-1Ra$^{-/-}$ mouse

which diseases could be induced by T cell transfer. Thus, in this case, an autoimmune process is not likely to be involved in disease pathogenesis; rather, excess IL-1 signaling directly induces inflammation within the skin.

In humans, the involvement of IL-1 in the development of psoriasis has not been elucidated completely. Some studies have indicated that IL-1α concentrations were reduced in psoriatic lesional skin as compared to nonlesional and normal skin, although IL-1β concentrations were increased (Cooper et al. 1990; Debets et al. 1997). Our data clearly show that excess IL-1 signaling can induce psoriasis-like lesions in mice, suggesting involvement of IL-1 in the development of psoriasis in humans. In agreement with our observations, Tg mice that express IL-1α from K14 promoter in the basal epidermis also develop scaly and erythematous inflammatory skin lesions (Groves et al. 1995).

8.4.2 The Role of TNFα in the Development of Dermatitis

The development of dermatitis in IL-1Ra$^{-/-}$ mice was completely absent in TNFα-deficient IL-1Ra$^{-/-}$ mice, indicating a crucial role for TNFα in disease pathogenesis. The importance of IL-1 and TNFα were also seen in contart hypersensitivity (CHS) reactions, in which antigen-specific CD4$^+$ T cells play a central role. 2, 4, 6-trinitrochlorobenzene (TNCB)-induced CHS was suppressed in IL-1α/β$^{-/-}$ and IL-1α$^{-/-}$, but not IL-1β$^{-/-}$, mice, and these responses were augmented in IL-1Ra$^{-/-}$ mice, suggesting an important role for IL-1α in CHS responses (Nakae et al. 2001c, 2003b). We demonstrated that the IL-1 produced by APCs of the epidermis enhances the sensitization of allergen-specific T cells and induces inflammation via TNFα production during the elicitation phase (Nakae et al. 2003b). TNFα elicits inflammatory cell infiltration in the skin through the induction of CXCLIO.

TNFα production is increased in psoriatic lesional skin as compared to nonlesional and healthy skin (Ettehadi et al. 1994). Moreover, direct correlation between TNFα concentration either at the lesional skin or serum levels and the psoriasis area severity index scores has been reported (Bonifati et al. 1994). Thus, TNFα may also be involved in the development of psoriasis in humans (Fig. 7).
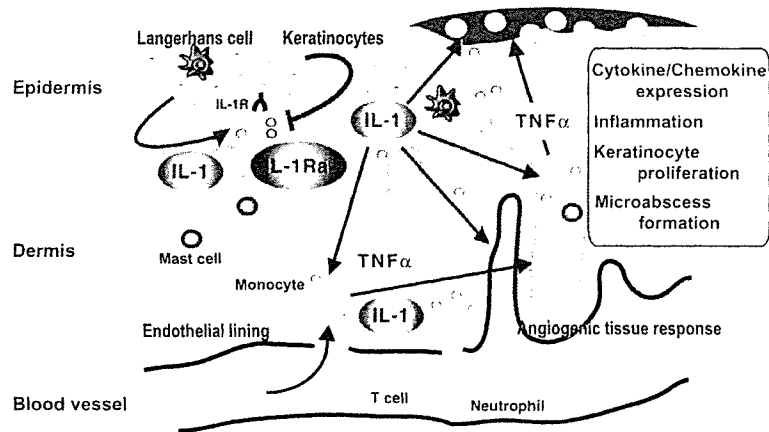
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Fig. 7. Pathogenesis of dermatitis

In human psoriasis, the importance of an acquired immune response is suggested, because linkage association with MHC class I is observed (Elder et al. 1994), the dermis and the epidermis are heavily infiltrated by $CD4^+$ T cells (Baker and Fry 1992), cyclosporin A efficiently suppresses psoriasis (Griffiths et al. 1989), and depletion of $CD25^+$ T cells ameliorates the disease (Gottlieb et al. 1995). Furthermore, it was shown that injection of prepsoriatic skin engrafted onto *scid/scid* mice with $CD4^+$ T cells induces psoriasis (Nickoloff and Wrone-Smith 1999). However, so far, no conclusive evidence has been presented for the involvement of autoimmunity in this disease. On the other hand, it has been argued that keratinocytes of psoriatics suffer from an intrinsic abnormality in the regulation of their activation by cytokines, which trigger proliferation and migration, and stimulated keratinocytes may act as initiators of an inflammatory process by means of the secretion of various cytokines able to induce the expression of cell adhesion molecules and the recruitment of inflammatory cells (Bonifati and Ameglio 1999; Shepherd et al. 2004). Our observations suggest that keratinocyte-derived pathogenesis rather than an autoimmune mechanism is involved in the development of dermatitis in $IL-1Ra^{-/-}$ mice. Since the infiltration of inflammatory cells is not prominent at the beginning of the disease and gradually increases, immune mechanisms may be involved at the later phase.

8.5 Conclusion

We have demonstrated that a variety of inflammatory diseases, including arthritis, aortitis, and psoriatic dermatitis, develop spontaneously in IL-1Ra$^{-/-}$ mice. Although excess IL-1 signaling is responsible for the development of these diseases, the pathogenic mechanisms differ significantly; both arthritis and aortitis result from the development of autoimmunity, while such autoimmune processes are not involved in the development of dermatitis. Both TNFα and IL-17 play important roles in the activation of T cells downstream of IL-1 signaling, in addition to the roles in the elicitation of inflammation. TNFα activates T cells by inducing OX40 expression, leading to increased IL-17 production. Although IL-17 was also shown to be involved in the sensitization of T cells, the mechanism underlying this activation remains to be elucidated. Thus, both cytokines play crucial roles in the development of the autoimmunity that can cause arthritis and aortitis in this knockout mouse model. In contrast, in the case of psoriatic dermatitis, excess IL-1 signaling and TNFα signaling directly induce inflammation of the skin without the involvement of autoimmunity. Thus, IL-1 and TNFα have dual functions, the activation of T cells and the direct induction of inflammation. It is interesting that excess IL-1 signaling can induce several different diseases in an animal via different mechanisms. In any case, these observations suggest that the suppression of IL-1, TNFα, and IL-17 is important in the control of inflammatory diseases; suppression of cytokine expression or action should be beneficial for the treatment of these diseases.

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Endogenous Interleukin (IL)–1α and IL-1β Are Crucial for Host Defense against Disseminated Candidiasis

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Background. Interleukin (IL)–1α and IL-1β are protective proinflammatory cytokines involved in host defense against *Candida albicans*. It is, however, unknown whether they provide protection through similar mechanisms. We investigated the effect of endogenous IL-1α and IL-1β on disseminated *C. albicans* infection.

Methods. Mice deficient in the genes encoding IL-1α (IL-1α$^{-/-}$), IL-1β (IL-1β$^{-/-}$), or both molecules (IL-1α$^{-/-}$β$^{-/-}$) were used. Survival and *C. albicans* outgrowth in the kidneys was assessed after intravenous injection of *C. albicans*.

Results. Both mortality and *C. albicans* outgrowth in the kidneys were significantly increased in IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice, compared with those in control mice, with the IL-1α$^{-/-}$β$^{-/-}$ mice being most susceptible to disseminated candidiasis. The host defense mechanisms triggered by IL-1α and IL-1β differed from one another. IL-1β$^{-/-}$ mice showed decreased recruitment of granulocytes in response to an intraperitoneal *C. albicans* challenge, and generation of superoxide production was diminished in IL-1β$^{-/-}$ granulocytes. IL-1α$^{-/-}$ mice had a reduced capacity to damage *C. albicans* pseudohyphae. Protective type 1 responses were deficient in both IL-1α$^{-/-}$ and IL-1β$^{-/-}$ mice, as assessed by production of interferon-γ by splenocytes in response to heat-killed *C. albicans*.

Conclusion. Although IL-1α and IL-1β have differential effects on the various arms of host defense, both cytokines are essential for mounting a protective host response against invasive *C. albicans* infection.

Despite the availability of potent antifungal agents, acute disseminated candidiasis remains a life-threatening disease that occurs mainly in immunocompromised patients [1]. Immunotherapies with cytokines have great potential to augment host resistance and as adjunctive treatment for invasive candidiasis. For further development of these strategies, a better understanding of the protective immune mechanisms against invasive candidiasis is needed.

Interleukin (IL)–1α and IL-1β are proinflammatory cytokines that exert similar biological activities after

interaction with the IL-1 type I receptor (IL-1RI) and the IL-1R accessory protein [2]. Exogenous recombinant human IL-1α or IL-1β has been administered in studies of disseminated murine candidiasis, and these studies have clearly indicated a protective role for IL-1 in this infection model [3, 4]. The mechanisms of this beneficial effect have been only partly elucidated. IL-1 has no direct antifungal effect, and the protective effect of IL-1 in host defense against *Candida albicans* does not depend on the presence of granulocytes or humoral factors, such as acute-phase proteins [3, 4]. To characterize the role of endogenous IL-1α and IL-1β in disseminated candidiasis and to gain further insight into the mechanisms through which both IL-1 molecules confer protection against disseminated candidiasis, mice in which the genes encoding IL-1α (IL-1α$^{-/-}$), IL-1β (IL-1β$^{-/-}$), or both (IL-1α$^{-/-}$β$^{-/-}$) had been disrupted were used in the present study. The IL-1–deficient mice and their immunocompetent littermates were subjected to experimental disseminated *C. albicans* infection.

MATERIALS AND METHODS

Mice. IL-1$\alpha^{-/-}$, IL-1$\beta^{-/-}$, and IL-1$\alpha^{-/-}\beta^{-/-}$ mice were produced as described elsewhere [5]. As control mice for the IL-1–deficient mice, age-matched C57Bl/6 (IL-1$\alpha^{+/+}\beta^{+/+}$) mice were used. The mice were allowed to become accustomed to laboratory conditions for 1 week before experimental use as described below.

Infection model. Mice were injected intravenously with 1×10^5 *C. albicans* (ATCC MYA-3573; UC820) blastoconidia, and survival was assessed. For measurement of circulating granulocytes and cytokines, mice were bled from the retroorbital plexus on day 1, 3, or 7 of infection. To quantify fungal outgrowth, the kidneys were removed aseptically, weighed, and homogenized, and serial dilutions were plated on Sabouraud agar, as described elsewhere [3]. Colony-forming units were counted, and results were expressed as log colony-forming units per kidney. For histologic analysis, kidneys of subgroups of mice (5 mice/group) were fixed in buffered formaldehyde (4%). Paraffin-embedded sections were stained with periodic acid–Schiff or hematoxylin-eosin.

To investigate whether the role of endogenous IL-1 in candidiasis is mediated by polymorphonuclear neutrophils (PMNs), mice were rendered granulocytopenic by use of cyclophosphamide (Bristol-Myers Squibb), administered subcutaneously at a dose of 150 mg/kg on day −4 and at a dose of 100 mg/kg on days −1 and 2 of infection with 1×10^4 *C. albicans* blastoconidia [3]. Daily differential counts in peripheral blood smears confirmed granulocytopenia ($<100 \times 10^6$ cells/L; data not shown). Hyperuricemia-induced tumor necrosis factor (TNF) production was prevented by gastric instillation of sodium bicarbonate at a dose of 100 mg/kg twice per day, starting on day −4 and continuing until the end of the experiment [6]. *C. albicans* outgrowth in the kidneys on day 1 or 3 of infection was determined as described elsewhere [3].

PMN recruitment. PMN recruitment to an infection site was determined after intraperitoneal injection of 1×10^7 cfu of heat-killed *C. albicans* in uninfected mice. After 4 h, mice were killed by CO_2 asphyxiation, and peritoneal exudates were obtained by washing with ice-cold PBS. The numbers of PMNs were assessed in Giemsa-stained cytocentrifuge preparations.

Superoxide production. Superoxide production was studied in a luminol-enhanced peroxidase-catalyzed chemiluminescence assay [7]. Briefly, peritoneal exudate PMNs were suspended at a concentration of 2×10^6 cells/mL of Hanks' balanced salt solution without phenol red (Gibco), supplemented with 0.25% human serum albumin. Cells (2×10^5/well) were incubated in 96-well microtiter plates (Costar Corning) with 50 μmol/L luminol and 4.5 U/mL horseradish peroxidase (Sigma) and were stimulated with either medium (as a negative control) or 2×10^8 heat-killed blastoconidia and PMA (50 ng/mL). Chemiluminescence was measured on a Victor^2 1420 counter and ex-

pressed as the total amount of superoxide produced in 35 min, by integrating the area under the curve per PMN.

Phagocytosis and intracellular killing of C. albicans. Exudate peritoneal phagocytes were collected 4 h (PMN) or 72 h (macrophages) after intraperitoneal injection of 10% proteose peptone. Cells were centrifuged at 550 g, counted, and resuspended in RPMI 1640 Dutch modification (with 20 mmol/L HEPES, without glutamine; RPMI-dm; ICN Biomedicals) supplemented with 5% heat-inactivated fetal calf serum.

Phagocytosis and intracellular killing were studied in an adherent monolayer of phagocytes, as described elsewhere [8]. Briefly, monolayers were incubated with opsonized blastoconidia (ATCC 10261) in modified Eagle's medium (Gibco) (effector-to-target cell [E:T] ratio, 40:1). After 15 min, supernatants containing noningested blastoconidia were plated on Sabouraud agar. The percentage of phagocytosed blastoconidia was calculated as [1 − (number of uningested cfu/cfu at the start of incubation)] × 100. To assess the percentages of *C. albicans* blastoconidia internalized versus those only attached to the membrane, fluorescein isothiocyanate–labeled *C. albicans* was opsonized and incubated for 15 min on a macrophage monolayer (E:T ratio, 40:1, as described above). After washing with sterile medium to remove the extracellular nonadherent yeasts, the total fluorescence was measured in the monolayer to enumerate the total of internalized and adherent *C. albicans* cells. The fluorescence of the extracellular, membrane-adhered *C. albicans* was quenched by adding methylene blue, the cells were washed, and the number of fluorescent internalized blastoconidia was assessed. In 3 separate experiments, 90%–97% of the total number of internalized and adherent blastoconidia were shown to be intracellular, and no differences in the ratios that were adherent to phagocytosed *C. albicans* cells between wild-type and IL-1–deficient mice were apparent.

After removal of the nonphagocytosed blastoconidia, killing of blastoconidia by PMNs was assessed in the same monolayers in fresh medium. After 3 h of incubation at 37°C and 5% CO_2, the wells were gently scraped with a plastic paddle and washed with 200 μL of distilled H_2O to achieve lysis of macrophages. This procedure was repeated 3 times, after which the pooled washes were adjusted to a final volume of 1 mL with distilled water. Microscopic examination of the culture plates showed that there was a complete removal of phagocytes. Viable intracellular blastoconidia were quantified as described elsewhere [8]. The percentage of phagocyte-killed yeasts was determined as [1−(cfu after incubation/number of phagocytized cfu)] × 100. Phagocyte-free incubations of blastoconidia were included as controls for yeast viability.

Assessment of PMN-mediated pseudohyphal damage. PMN-mediated pseudohyphal damage was determined by the XTT dye assay, as described elsewhere [9]. ATCC MYA-3573 blastoconidia were suspended at 1×10^6 cfu/mL of RPMI-dm (pH
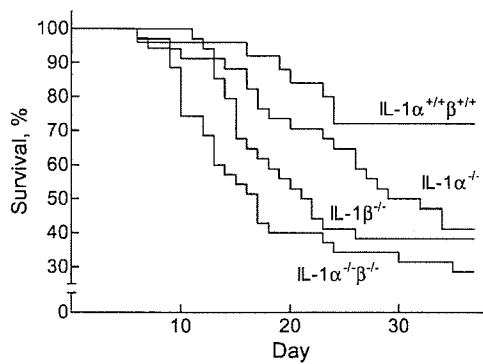
Figure 1. The survival of interleukin (IL)–1–deficient mice after an intravenous injection of 1×10^5 cfu of *Candida albicans*. Survival of IL-$1\alpha^{-/-}$ mice and IL-$1\beta^{-/-}$ mice was significantly impaired, compared with that of IL-$1\alpha^{+/+}\beta^{+/+}$ mice ($P < .05$). IL-$1\alpha^{-/-}\beta^{-/-}$ mice showed significantly impaired survival, compared with that of IL-$1\alpha^{+/+}\beta^{+/+}$ mice ($P < .001$) and IL-$1\alpha^{-/-}$ mice ($P < .05$). Data are the cumulative results of experiments performed in quadruplicate for at least 25 mice/group and were analyzed using the Kaplan Meier log rank test.

6.4). After incubation at 37°C for 24 h, pseudohyphae were obtained and resuspended in RPMI 1640 without phenol red and L-glutamine (RPMI-wp; ICN Biomedicals). Pseudohyphae (1×10^5) and PMNs in RPMI-wp were added to the wells in the presence of 10% fresh IL-$1\alpha^{+/+}\beta^{+/+}$ serum (E:T ratio, 8:1). Control wells contained pseudohyphae or PMNs only. After incubation for 2 h, PMNs were lysed with sterile H_2O. After 15 min, sterile XTT (400 μg/mL; Sigma Chemical) and coenzyme Q_0 (50 μg/mL; Sigma) were added. After 1 h of incubation at 37°C, the plate was centrifuged (770 *g*), the supernatants were transferred to a microtiter plate, and the absorbance was measured in a spectrophotometer at 450 nm. The percentage of fungal damage was calculated as $1 - [(A_{450}$ hyphae and PMNs$-A_{450}$ PMNs)/A_{450} hyphae] $\times 100$.

Production of cytokines and NO. Resident peritoneal macrophages were obtained aseptically with ice-cold PBS. Cells were resuspended in RPMI-dm in a round-bottom 96-well plate (1 $\times 10^5$ cells/well). For cytokine production, macrophages were stimulated with culture medium as a negative control, heat-killed blastoconidia (ATCC MYA-3573; 1×10^7), or pseudohyphae (1×10^6). For cell-associated IL-1α, fresh RPMI was added to the remaining macrophages, which were subsequently disrupted by 3 freeze-thaw cycles. Supernatants collected after 24 h of incubation and cell lysates were stored at -80°C until assay.

For NO production, macrophages (5×10^6 cells/mL) were stimulated with heat-killed *C. albicans* (1×10^7 cfu/mL) and interferon (IFN)–γ (100 U/mL), or lipopolysaccharide (LPS) (1 μg/mL) for 24 h at 37°C. The nitrite concentration in the supernatants was determined by the Griess reaction [10].

Splenocyte stimulation. Fourteen days after an intravenous injection of 1×10^4 *C. albicans*, spleens were removed and gent-

ly squeezed in sterile 200-μm filter chambers. Of the splenocytes, 95% were lymphocytes, 2% were monocytes, and 3% were granulocytes. Splenocytes (5×10^6 cells/mL) were stimulated with control medium or 1×10^7 heat-killed *C. albicans* (ATCC MYA-3573) blastoconidia (E:T ratio, 2:1). IFN-γ and IL-10 concentrations were measured in supernatants after 48 h of incubation at 37°C in 5% CO_2 in 24-well plates (Greiner).

Cytokine assays. TNF-α, IL-1α, and IL-1β concentrations were determined using specific radioimmunoassays, as described elsewhere [11]. The detection limit was 40 pg/mL for TNF-α and 20 pg/mL for IL-1α and IL-1β. IL-10, IFN-γ, and IL-6 concentrations were determined by ELISA (Biosource), and the detection limits were 8, 15.6, and 150 pg/mL, respectively. Murine keratinocyte-derived chemokine (KC), macrophage inhibitory protein (MIP)–2, and monocyte chemotactic protein–1 (MCP-1) were measured by ELISA (R&D Systems).

Statistical analysis. Parametric data are expressed as mean $\pm$ SD, and data that showed normal distribution after log transformation are expressed as means and 95% confidence intervals (CIs). Nonparametric values are expressed as medians. Since $\geqslant 3$ groups were compared, parametric data were analyzed using 1-way analysis of variance (ANOVA), and nonparametric data were analyzed using the Kruskal-Wallis 1-way ANOVA. For posttest comparisons of nonparametric data, the Mann-Whitney U test was applied. The Kaplan-Meier log rank test was used to analyze survival data. The data represent the pooled results of all experiments performed.

RESULTS

Disseminated candidiasis in nonneutropenic mice. Whereas 72% of the IL-$1\alpha^{+/+}\beta^{+/+}$ mice survived infection with 1×10^5 cfu of *C. albicans*, only 41% of IL-$1\alpha^{-/-}$ and 38% of IL-$1\beta^{-/-}$ mice survived ($P < .05$, control mice vs. IL-$1\alpha^{-/-}$ or IL-$1\beta^{-/-}$
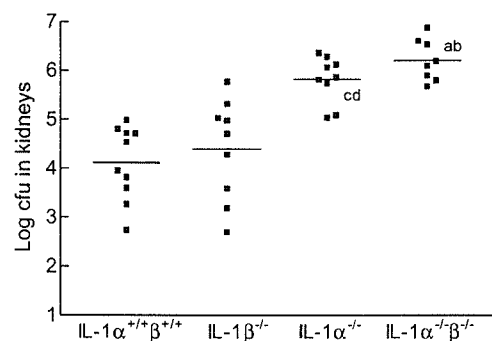
Figure 2. Outgrowth of *Candida albicans* in the kidneys 7 days after an intravenous injection of 1×10^5 cfu of *C. albicans*. The log-transformed data represent the cumulative results of experiments performed twice and were analyzed using 1-way analysis of variance and the Mann-Whitney U test for posttest comparisons. Horizontal bars indicate the means. [a]$P < .001$, vs. interleukin (IL)–$1\alpha^{+/+}\beta^{+/+}$ mice; [b]$P < .01$, vs. IL-$1\beta^{-/-}$ mice; [c]$P < .01$, vs. IL-$1\alpha^{+/+}\beta^{+/+}$mice; [d]$P < .05$, vs. IL-$1\beta^{-/-}$ mice.
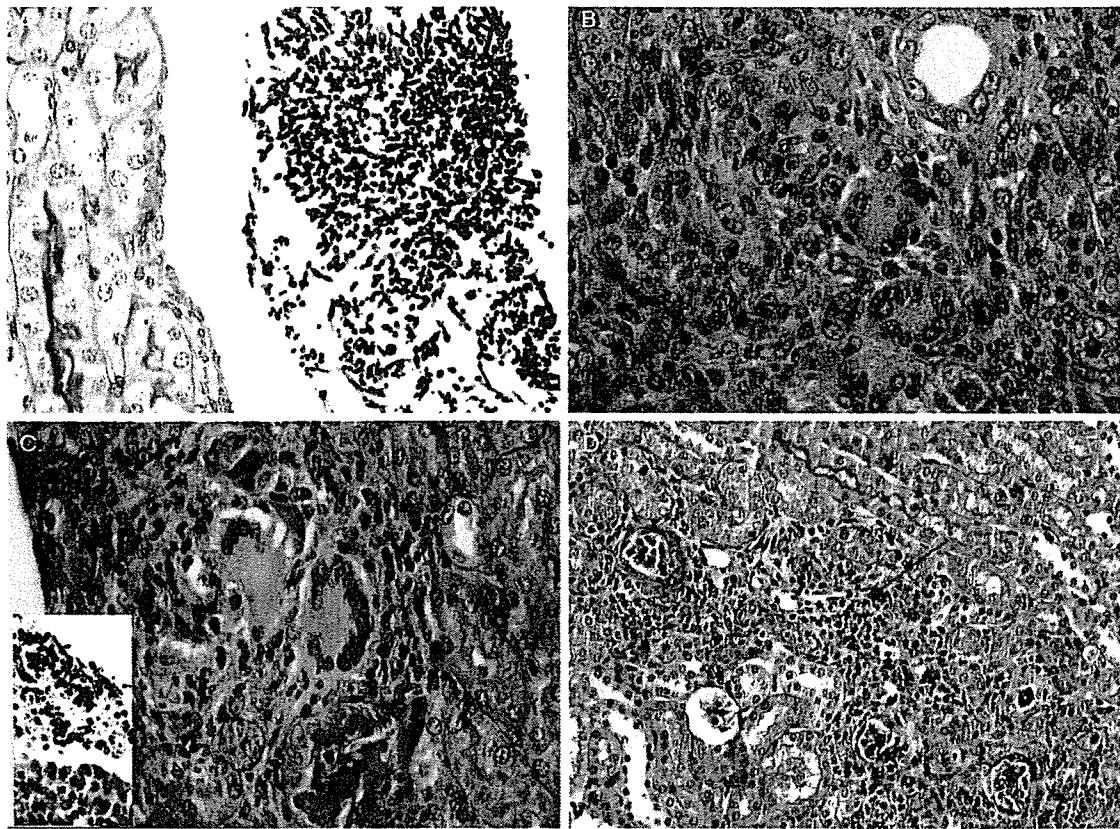
Figure 3. Histopathologic assessment of the kidneys 7 days after an intravenous injection of 1×10^5 cfu of *Candida albicans* ($\times$400; periodic acid–Schiff–stained sections). *A,* Large number of *C. albicans* blastoconidia and pseudohyphae in the collecting duct of an interleukin (IL)–$1\alpha^{-/-}$ mouse. *B,* Granulomatous response with foreign body giant cells in the kidney of an IL-$1\beta^{-/-}$ mouse. *C,* Outgrowth of *C. albicans (inset),* as well as a granulomatous response, in an IL-$1\alpha^{-/-}\beta^{-/-}$ mouse. *D,* Resolving inflammation *(arrow)* with fibroblasts and few granulocytes in the kidney of an IL-$1\alpha^{+/+}\beta^{+/+}$ mouse.

mice, Kaplan Meier log rank test) (figure 1). Moreover, survival was even further impaired (29%) in mice lacking both IL-1α and IL-1β; this difference was significant, in comparison with IL-$1\alpha^{+/+}\beta^{+/+}$ mice ($P<.001$) and with IL-$1\alpha^{-/-}$ mice and IL-$1\beta^{-/-}$ mice ($P<.05$) (figure 1).

Since the kidneys are the main target organs in disseminated candidiasis [3, 12], *C. albicans* outgrowth in the kidneys was determined. On day 7 of infection, *C. albicans* outgrowth was increased 51-fold in IL-$1\alpha^{-/-}$ mice ($P<.01$), 3-fold in IL-$1\beta^{-/-}$ mice ($P>.05$), and 125-fold in IL-$1\alpha^{-/-}\beta^{-/-}$ ($P<.001$) (figure 2), when means of outgrowth in knockout and IL-$1\alpha^{+/+}\beta^{+/+}$ mice were compared. At this point in time, the *C. albicans* burden in IL-$1\beta^{-/-}$ mouse kidneys was significantly higher than that in IL-$1\alpha^{-/-}$ mouse kidneys ($P<.05$) (figure 2). Circulating concentrations of IL-1α, IL-1β, TNF-α, IFN-γ, IL-10, and IL-6 in blood obtained from mice on day 7 of infection were under the detection limit (data not shown).

To determine the difference in outgrowth between IL-$1\alpha^{-/-}$ and IL-$1\beta^{-/-}$ mice at a later point in time during infection, subgroups of IL-$1\alpha^{-/-}$, IL-$1\beta^{-/-}$, or IL-$1\alpha^{+/+}\beta^{+/+}$ mice received an intravenous injection of 5×10^4 cfu of *C. albicans.* On day

14 of infection, outgrowth in the kidneys of IL-$1\alpha^{-/-}$ mice (mean, 6.03 log cfu [95% CI, 5.50–6.57 log cfu]) and IL-$1\beta^{-/-}$ mice (mean, 6.10 log cfu [95% CI, 5.44–6.77 log cfu]) was significantly increased, compared with that in IL-$1\alpha^{+/+}\beta^{+/+}$ mice (mean, 4.25 log cfu [95% CI, 2.54–5.96 log cfu]) ($P<.05$). However, no difference between the numbers of *C. albicans* colony-forming units recovered from the kidneys of IL-$1\alpha^{-/-}$ mice and IL-$1\beta^{-/-}$ mice was observed at this time point.

Histopathologic assessment. Seven days after an intravenous injection of 1×10^5 *C. albicans* blastoconidia, the inflammatory lesions in the kidneys of IL-$1\alpha^{+/+}\beta^{+/+}$ mice were healing, showing few PMNs and fibroblasts (figure 3D). In the kidneys of IL-$1\beta^{-/-}$ and IL-$1\alpha^{-/-}\beta^{-/-}$ mice, a granulomatous response was observed without the presence of yeast cells (figure 3B and 3C). In IL-$1\alpha^{-/-}$ mice and IL-$1\alpha^{-/-}\beta^{-/-}$ mice however, a large amount of *C. albicans* had accumulated in the collecting ducts, surrounded by PMNs and lymphocytes (figure 3A and 3C).

Disseminated candidiasis in neutropenic mice. To assess whether the protective effect of IL-1 is PMN mediated, mice were rendered granulocytopenic by use of cyclophosphamide. Three days after infection with 1×10^4 *C. albicans* blastoconid-

Table 1. No. of circulating polymorphonuclear neutrophils (PMNs) before or on day 3 of infection with *Candida albicans* blastoconidia (1 × 10⁵ cfu administered intravenously), and recruitment of PMNs 4 h after intraperitoneal injection of heat-killed *C. albicans* blastoconidia (1 × 10⁷ cfu).

Strain	Circulating PMNs, no. ×10⁹/L		Intraperitoneal PMN recruitment	
	Before infection	On day 3 of infection	%[a]	No. ×10⁶
IL-1α⁺/⁺β⁺/⁺	0.89 ± 0.31	0.84 ± 0.71	63 ± 13	5.81 ± 3.26
IL-1α⁻/⁻	0.42 ± 0.10	0.86 ± 0.30	54 ± 6	5.56 ± 1.62
IL-1β⁻/⁻	1.38 ± 0.47	3.20 ± 0.99	37 ± 12[b]	1.85 ± 0.84[c]
IL-1α⁻/⁻β⁻/⁻	0.67 ± 0.35	2.95 ± 0.52	39 ± 8[b]	3.21 ± 0.70

NOTE. Data are the mean ± SD of at least 3 mice/group, were obtained from 1 experiment, and were analyzed using 1-way analysis of variance and the Mann-Whitney *U* test for posttest comparisons. IL, interleukin.

[a] Percentage of the total no. of peritoneal exudate cells
[b] *P* < .005, vs. control or IL-1α⁻/⁻ mice.
[c] *P* < .01, vs. control or IL-1α⁻/⁻ mice.

ia, the largest quantity of *C. albicans* was recovered from the kidneys of IL-1α⁻/⁻β⁻/⁻ mice (mean ± SD, 5.23 ± 0.37 log cfu), and the difference was significant in comparison with IL-1α⁺/⁺β⁺/⁺ mice (mean ± SD, 3.64 ± 0.51 log cfu) (*P* < .01). Outgrowth in the kidneys of IL-1α⁻/⁻ mice (mean ± SD, 4.27 ± 1.31 log cfu) was similar to that in the kidneys of IL-1β⁻/⁻ mice (mean ± SD, 4.13 ± 0.67 log cfu. (Data are cumulative results of 2 experiments for at least 6 mice/group.)

PMN circulating numbers and recruitment. To study the role of endogenous IL-1α and IL-1β in the recruitment of PMNs to the site of a localized *C. albicans* infection, peritoneal PMNs were collected after intraperitoneal injection of heat-killed *C. albicans* (table 1). Whereas the recruitment of PMNs in IL-1α⁻/⁻ mice did not differ from that in IL-1α⁺/⁺β⁺/⁺ mice, significantly lower numbers of PMNs were recruited to the site of infection in IL-1β⁻/⁻ mice, indicating that IL-1β is most important for PMN recruitment to the site of infection. In contrast, during the early days of infection, increased numbers of circulating PMNs were observed in IL-1β⁻/⁻ mice and IL-1α⁻/⁻β⁻/⁻ mice, compared with those in IL-1α⁻/⁻ and IL-1α⁺/⁺β⁺/⁺ mice (table 1), underscoring the importance of IL-1β in the migration of PMNs from the bloodstream to the site of infection. The mean numbers of peripheral blood PMNs were similar for all groups on day 7 of infection (data not shown).

Anticandidal response of PMNs against **C. albicans** *blastoconidia and pseudohyphae.* To further characterize the influence of endogenous IL-1α and IL-1β on the activity of PMNs against *C. albicans*, the fungicidal capacity of PMNs was determined in vitro. Whereas IL-1α⁻/⁻ PMNs showed a tendency only toward reduced superoxide production (mean ± SD, 58.3 × 10³ ± 15.4 × 10³ cps), IL-β⁻/⁻ PMNs produced significantly less superoxide than did control PMNs (mean ± SD, 30.6 × 10³ ± 15.9 × 10³ vs. 71.5 × 10³ ± 10.5 × 10³ cps) (*P* <

.01). However, this observation was not accompanied by an altered capacity of IL-1–deficient PMNs to phagocytose or kill internalized *C. albicans* blastoconidia; all groups phagocytosed ~25% of the blastoconidia and killed ~75% of internalized blastoconidia.

In contrast, IL-1 modulated the capacity of PMNs to damage pseudohyphae (figure 4). Pseudohyphal damage caused by IL-1α⁻/⁻ or IL-1α⁻/⁻β⁻/⁻ PMNs was significantly less efficient than that caused by IL-1α⁺/⁺β⁺/⁺ or IL-1β⁻/⁻ PMNs (*P* < .001) (figure 4). Deficiency of both IL-1 molecules showed a tendency to further impair PMN antipseudohyphal resistance, compared with IL-1α deficiency alone. Deficiency of IL-1β alone did not diminish the capacity of PMNs to damage *C. albicans* pseudohyphae.

Macrophage function and stimulation. Macrophage recruitment was determined by harvesting peritoneal exudate cells 72 h after an intraperitoneal injection of 1 × 10⁷ cfu of heat-killed *C. albicans*. No differences in the mean ± SD numbers of macrophages that were recruited into the peritoneal cavity were observed, compared with those in control mice (IL-1α⁺/⁺β⁺/⁺, 1.01 × 10⁹ ± 0.12 × 10⁹; IL-1α⁻/⁻, 1.10 × 10⁹ ± 0.02 × 10⁹; IL-1β⁻/⁻, 0.96 × 10⁹ ± 0.19 × 10⁹; IL-1α⁻/⁻β⁻/⁻, 1.18 × 10⁹ ± 0.15 × 10⁹). IL-1 influenced neither the mean ± SD capacity of exudate peritoneal macrophages to phagocytose *C. albicans* blastoconidia (IL-1α⁺/⁺β⁺/⁺, 41% ± 5%; IL-1α⁻/⁻, 50% ± 4%; IL-1β⁻/⁻, 56% ± 3%; IL-1α⁻/⁻β⁻/⁻, 41% ± 16%) nor their capacity to damage *C. albicans* pseudohyphae (data not shown). No differences between NO production by macrophages from the mouse strains were observed in response to either heat-killed *C. albicans* (all around the detection limit) or
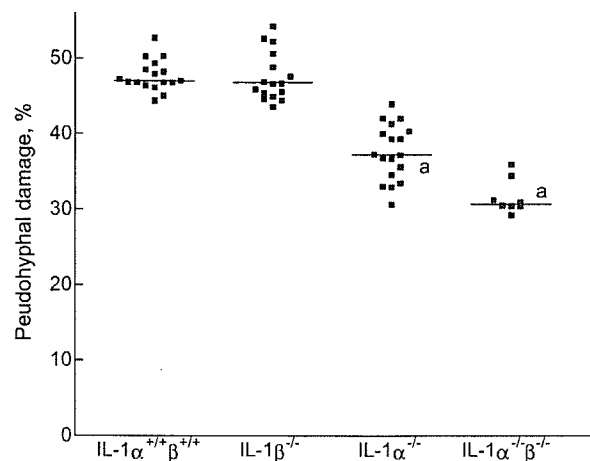
Figure 4. Percentage of exudate polymorphonuclear neutrophil (PMN)–mediated pseudohyphal damage. Pseudohyphae (1 × 10⁵) were incubated in the presence of 8 × 10⁵ PMNs for 2 h. Data are the cumulative results of 2 separate experiments and were analyzed using the Kruskal-Wallis 1-way analysis of variance. For posttest comparisons, the Mann-Whitney *U* test was applied. Horizontal bars indicate medians. ª*P* < .001, vs. interleukin (IL)–1α⁺/⁺β⁺/⁺ or IL-1β⁻/⁻ mice.

LPS (control mice, 1.37 ± 0.54 ng/mL; IL-$1\beta^{-/-}$ mice, 0.78 ± 1.35 ng/mL; IL-$\alpha^{-/-}\beta^{-/-}$ mice, 5.28 ± 0.2 ng/mL; $P > .05$).

IL-1–deficient resident peritoneal macrophages showed impaired production of IL-6 and of the chemokine KC in response to heat-killed *C. albicans* blastoconidia or pseudohyphae. The difference in IL-6 production was significant for IL-$1\alpha^{-/-}$ macrophages and IL-$1\alpha^{-/-}\beta^{-/-}$ macrophages, in comparison with IL-$1\alpha^{+/+}\beta^{+/+}$ macrophages, and the difference in KC production was significant for IL-$1\beta^{-/-}$ macrophages and IL-$1\alpha^{-/-}\beta^{-/-}$ macrophages, in comparison with IL-$1\alpha^{+/+}\beta^{+/+}$ macrophages ($P < .05$) (table 2). No differences between groups were observed in the production of TNF-α, MCP-1, or MIP-2.

Stimulation of splenic lymphocytes. To assess whether IL-1 induces a type 1 or a type 2 immune response, splenocytes were stimulated with heat-killed *C. albicans.* Whereas IL-$1\alpha^{+/+}\beta^{+/+}$ splenocytes produced IFN-γ, neither IL-$1\alpha^{-/-}$ nor IL-$1\beta^{-/-}$ splenocytes did ($P < .05$) (table 3), indicating that both IL-1α and IL-1β are important for protective IFN-γ production. Whereas IL-$1\beta^{-/-}$ and IL-$1\alpha^{-/-}\beta^{-/-}$ splenocytes did not produce any IL-10, both IL-$1\alpha^{+/+}\beta^{+/+}$ splenocytes and IL-$1\alpha^{-/-}$ splenocytes did ($P < .05$) (table 3), indicating that both IL-1α and IL-1β are important for protective IFN-γ production, whereas the residual IL-10 production in IL-$1\alpha^{-/-}$ mice further contributed to a type 2 response.

DISCUSSION

The results of the present study indicate that deficiency of endogenous IL-1α or IL-1β has deleterious effects on the outcome of disseminated candidiasis. Both IL-$1\alpha^{-/-}$ mice and IL-$1\beta^{-/-}$ mice showed increased mortality associated with an increased outgrowth of *C. albicans* in the kidneys. IL-1β proved to be important for PMN recruitment and generation of su-

peroxide production. IL-1α was essential for the capacity of PMNs to damage *C. albicans* pseudohyphae, and both IL-1α and IL-1β were required for the induction of protective Th1 responses.

One of the mechanisms through which IL-1 has been suggested to confer protection is the enhancement of granulopoiesis and influx of PMNs to the site of infection. Although IL-$1\alpha^{-/-}$ mice had 50% fewer circulating granulocytes at the time of infection than control mice, their number in the circulation on day 3 of infection and PMN recruitment to the site of infection did not differ from that of control mice. In IL-$1\beta^{-/-}$ mice, however, a trend toward an increased number of circulating granulocytes during infection was observed, and this coincided with significantly decreased PMN migration to the site of infection in a standardized model of intraperitoneal *C. albicans* challenge. This is in line with earlier evidence that exogenous administration of IL-1α or IL-1β induces peripheral blood granulocytosis [13, 14]. Our data on PMN recruitment in response to heat-killed *C. albicans* also suggest an important role for endogenous IL-1β in the early PMN influx, in line with studies showing that exogenous administration of IL-1 induces PMN accumulation [15, 16]. Moreover, PMN recruitment in IL-1–deficient mice may be further impaired as a result of decreased production of neutrophil chemoattractants. Whereas IL-$1\alpha^{-/-}$ mice showed a tendency toward reduced production of the CXC chemokine KC, KC production by IL-$1\beta^{-/-}$ macrophages was significantly reduced, thus likely contributing to impaired granulocyte recruitment in IL-$1\beta^{-/-}$ mice. Whereas the differences in chemokine production between IL-$1\alpha^{-/-}$ and IL-$1\beta^{-/-}$ mice may not be impressive, the major difference between the mouse strains was the complete absence of IL-1β in the IL-$1\beta^{-/-}$ mice. Since IL-1β has been shown to be an important mediator of

Table 2. In vitro cytokine and chemokine production by resident peritoneal macrophages (1×10^5) stimulated with either heat-killed *Candida albicans* blastoconidia (1×10^7) or heat-killed pseudohyphae (1×10^6).

Stimulation, strain	TNF-α, ng/mL	IL-6, pg/mL	MCP-1, pg/mL	MIP-2, pg/mL	KC, pg/mL
Blastoconidia					
IL-$1\alpha^{+/+}\beta^{+/+}$	0.56 ± 0.14	926 ± 547	87 ± 45	2190 ± 1633	1842 ± 880
IL-$1\alpha^{-/-}$	0.88 ± 0.41	154 ± 9^a	40 ± 1	2660 ± 1630	380 ± 197
IL-$1\beta^{-/-}$	0.46 ± 0.22	329 ± 235	50 ± 22	2166 ± 1769	308 ± 345^a
IL-$1\alpha^{-/-}\beta^{-/-}$	0.72 ± 0.41	159 ± 21^a		1674 ± 753	265 ± 254^a
Pseudohyphae					
IL-$1\alpha^{+/+}\beta^{+/+}$	1.32 ± 0.82	1378 ± 796	221 ± 135	3350 ± 2699	1920 ± 879
IL-$1\alpha^{-/-}$	0.95 ± 0.42	366 ± 203	152 ± 154	2550 ± 1656	435 ± 196
IL-$1\beta^{-/-}$	0.74 ± 0.29	417 ± 268	98 ± 56	2495 ± 1860	99 ± 87^a
IL-$1\alpha^{-/-}\beta^{-/-}$	0.98 ± 0.39	233 ± 187^a		1295 ± 510	303 ± 326^a

NOTE. Data are the mean $\pm$ SD production in 5 mice/group, obtained from 1 experiment, and were analyzed using 1-way analysis of variance and the Mann-Whitney U test for posttest comparisons. IL, interleukin; KC, keratinocyte-derived chemokine; MCP, monocyte chemotactic protein; MIP, macrophage inhibitory protein; TNF, tumor necrosis factor

^{a} $P < .05$, vs. IL-$1\alpha^{+/+}\beta^{+/+}$ mice.