Requirement of Interleukin-17A for Systemic Anti–Candida albicans Host Defense in Mice

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T cells are required for normal host defense against fungal infection, and individuals with T cell–deficiency syndromes are highly susceptible to fungal pathogens. Interleukin (IL)–17A is a proinflammatory cytokine that interconnects myeloid and lymphoid host defense. The role of murine (m) IL-17A/mIL-17A receptor (R) interactions was evaluated in a murine model of systemic candidiasis. In response to systemic challenge with Candida albicans, expression of mIL-17A was induced, and IL-17AR knockout (IL-17AR−/−) mice had dose-dependent, substantially reduced survival. Fungal burden in the kidneys of IL-17AR−/− mice was dramatically increased (25-fold at 96 h). In IL-17AR−/− mice, both mobilization of peripheral neutrophils and their influx to infected organs were significantly impaired and delayed. In vivo expression of mIL-17A protected normal mice from a lethal dose of C. albicans (100% at day 7 and 65% at day 42). The data suggest that the mIL-17A/mIL-17AR system is required for normal fungal host defense in vivo. IL-17A could have potential as a therapeutic cytokine for systemic C. albicans infections in immunocompromised patients with cancer or advanced acquired immunodeficiency syndrome.

Fungal infections remain a major cause of morbidity and mortality in immunocompromised hosts. Patients who are especially at risk include those with cancer who are undergoing myelotoxic or myeloablative immunosuppressive cancer chemotherapy, especially if this treatment is followed by prolonged periods of neutropenia, such as seen in bone marrow transplantation [1, 2]. In these patients, mucosal colonization with Candida albicans has been found to be associated with increased incidence of systemic candidiasis [3, 4]. Adequate numbers of functional neutrophils are required for normal fungal host defense in the circulation and are directly related to clinical outcomes [5, 6]. Despite the initiation of antifungal therapy, culture-proven fungal septicemia still has excessive mortality in immunocompromised individuals [1, 5].

Both human and murine (m) interleukin (IL)–17A are expressed almost exclusively in activated T cells (specifically, memory CD4+ T cells). mIL-17A is a T cell–derived cytokine and has been found to interconnect lymphoid and myeloid host defense, because it profoundly stimulates granulopoiesis and migration of neutrophils to tissues infected with gram-negative organisms [7–9]. T cells were previously found to be important modulators of host defense and outcomes in systemic murine candidiasis. Specifically, in C. albicans–infected mice, shifting of the immune response toward the Th1 type resulted in improved survival, whereas a shift toward the Th2 type was found to be associated with a deleterious outcome. Similarly, blockade of Th2–associated cytokines (IL-10 and IL-4) significantly enhanced survival in C. albicans sepsis [10–13]. Although many immune-modulating cytokines are defined by categorization into the Th1/Th2 host-response classification model, IL-17A could not be fitted into this model system, because of its unique effects [14]. Therefore, we decided to refine and expand this working model.

Although we had previously reported that mIL-17A
is required for bacterial host defense of the lungs in mice [7–9, 15], it was unclear what role mIL-17A might play in the setting of experimental systemic candidiasis. Given the ambiguous role of T cells in this condition, expression of mIL-17A could possibly have a deleterious effect, and, conversely, its blockade could also have a beneficial effect. Moreover, in experimental bacterial infections, the protective effect of mIL-17A did not appear to be uniform but was mostly restricted to gram-negative organisms and was of less relevance for infections with other bacterial microorganisms (authors’ unpublished data). To investigate its role and whether mIL-17A could be of relevance in systemic candidiasis, we used a murine experimental model of mIL-17A receptor (R) knockout (IL-17AR−/−) mice, as well as mIL-17A overexpression with recombinant adenovirus technology.

MATERIALS AND METHODS

Mice. The generation of the IL-17AR−/− mouse has been described elsewhere [9]. C57Bl/6 mice were obtained from Jackson Laboratories. All mice were maintained under specific pathogen–free conditions in accordance with Institutional Animal Care and Use Committee approved protocols. Construction, generation, expansion, and quality control of adenovirus-expressing mIL-7A (Ad-mIL-17A) and the control virus (AdEGFP or AdCMVLuc) have been described elsewhere [7, 16]. The mice were injected in the internal jugular vein with 5 × 10⁶ pfu of Ad-mIL-17A or control virus and were killed at the time points indicated.

Tissue processing. Histologic sections of paraffin-embedded murine kidneys were generated by use of standard techniques in a clinical pathology laboratory at the Louisiana State University Health Sciences Center Morphology Core. The tissue was stained by use of standard hematoxylin-eosin (HE) techniques (Shandon) and was analyzed by use of transmission light microscopy. Complete blood counts with differential counts were performed either by use of an automated Coulter counter system (Hemavet 850) or by manual assessment of blood smears.

C. albicans culturing. Frozen glycerol stocks of C. albicans (ATCC 14053) were used to establish cultures as hydrophilic, stationary-phase yeast cells in glucose-yeast extract–peptone broth at 37°C. Cells were visually characterized and were enumerated by use of a Hemacytometer (Beckman Coulter). Before intravenous (iv) injection, cell numbers were verified by quantitative culture. C. albicans yeasts were suspended in 400 µL of PBS at predetermined numbers and were injected iv. C. albicans colonies derived from organs were determined from minced and collagenized tissue and were normalized for protein concentration. Before the colonies were scored, the diluted samples were plated on 1.0% peptone agar overnight at 37°C. Quantitative culture analysis was performed with serial 10-fold dilutions on samples that were obtained at different time points; concurrent quantification of the inocula stock solution was also performed.

Cytokine assays. Kidney tissue was homogenized and centrifuged. The supernatant was used for analysis. The mIL-17A ELISAs were purchased from R&D Systems and were conducted according to the specifications of the manufacturer. The ELISAs were adapted by establishing standard curves by use of normal kidney tissue pulsed with recombinant mIL-17A. All data points were normalized for the protein content of each individual sample.

Myeloperoxidase (MPO) activity. The assay was conducted in a fashion similar to that of a previously published method and was conducted at 4°C, unless specified otherwise [17]. In brief, kidney tissue was homogenized in homogenization buffer (20 mmol/L potassium phosphate and 0.1 mmol/L EDTA [pH 7.4]). The specimens were centrifuged for 15 min at 20 g (Beckman GS6 tabletop centrifuge), and tissue pellets were resuspended in suspension buffer (50 mmol/L potassium phosphate and 0.5% hexa-decyltrimethylammoniumbromide [pH 6.0]). Before centrifugation, the specimens were sonicated and freeze-thawed 3 times by use of the same conditions as above. Two hundred microliters of prewarmed (37°C) tetramethylbenzidine (Sigma) was added to 10 µL of supernatant in prewarmed (37°C) 96-well plates and were incubated for 3 min. The plates were then analyzed in a Uquant plate reader (Bio-Tek Instruments) at 650 nm. Separate samples were analyzed for protein content, and MPO activity was adjusted according to the protein content of each individual sample. Each sample was analyzed in triplicate, and the mean value was used for further calculations.

Statistical analysis. Means of data were calculated by use of analysis of variance with Fisher’s follow-up testing. All calculations were performed by use of mathematical functions embedded into the statistical software program Statview (Statview Software). Survival was calculated using Kaplan-Meier calculations, and statistical significance was subsequently calculated for each preset time point of analysis. P < .05 was considered to be statistically significant.

RESULTS

Defective fungal host defense and impaired survival in IL-17AR−/− mice after systemic C. albicans infection. IL-17AR−/− mice and age- and sex-matched normal control mice (C57Bl/6 mice) were injected iv with different doses of C. albicans. Groups of mice were injected with 2 × 10⁵, 5 × 10⁴, and 1 × 10³ viable C. albicans yeasts, with survival as the end point (figure 1A–C). IL-17AR−/− mice had dose-dependent, significantly accelerated and enhanced mortality, compared with normal littermate controls (P < .01, P < .005, and P < .01, respec-
Figure 1. Survival of interleukin (IL)-17A receptor knockout (IL-17AR<sup>−/−</sup>) mice after systemic challenge at different doses of Candida albicans. IL-17AR<sup>−/−</sup> mice and their age- and sex-matched normal littermate controls (C57Bl/6 mice) were intravenously injected with (A), 5.52 × 10<sup>5</sup>, (B), and (C) viable C. albicans yeasts (n = 10 mice/group). Survival for each group is shown. P < .01, P < .005, and P < .01, for panels A, B, and C, respectively.

Failure of IL-17AR<sup>−/−</sup> mice to clear systemic C. albicans despite a peripheral neutrophil response similar to that in C57Bl/6 mice. To determine whether death was causally linked response in murine kidney tissue. To determine whether mIL-17A is induced as a response cytokine after systemic challenge with C. albicans, levels of mIL-17A were measured in kidneys of IL-17AR<sup>−/−</sup> and C57Bl/6 mice. In C57Bl/6 mice, mIL-17A was barely detectable at baseline; however, challenge with C. albicans induced protein expression up to ~2500 pg/mL at all time points (24, 48, and 96 h). Although challenge with C. albicans led to a significant increase in levels of mIL-17A in the kidneys of IL-17AR<sup>−/−</sup> mice, given the elevated baseline levels, the relative increase was substantially less than that in C57Bl/6 mice. (Figure 2A and 2B).

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to progressive *C. albicans* infection in IL-17AR−/− mice, *C. albicans* colonies of the kidney were measured after iv injection. Mice were injected iv with 2 × 10⁵ and 5 × 10⁵ viable *C. albicans* yeasts. Kidney tissue was analyzed at different time points for *C. albicans* concentration by quantitative cultures. The fungal burden was dependent on the initial dose of the iv injection. A rapid and escalating increase of *C. albicans* colonies was observed over time in both IL-17A−/− groups. In the low-dose injection cohort (2 × 10⁵ yeasts), *C. albicans* colonies were increased by 2.5-fold (P < .06) at 96 h, after which statistical significance was reached, and *C. albicans* colonies continued to exponentially increase, compared with those in C57Bl/6 mice (data not shown). A slightly higher injection of 5 × 10⁵ yeasts showed a 25-fold increase in *C. albicans* colonies already at 96 h in IL-17AR−/− mice surviving to that time point (P < .001) (figure 3A and 3B). Analysis at the highest injection dose (1 × 10⁶ viable *C. albicans* yeasts) resulted in a dramatic increase of *C. albicans* burden in kidneys and peripheral blood at 24 and 48 h (data not shown). However, as shown in figure 1, at this injection dose, most mice had already died by 48 h. To correlate whether the enhanced outgrowth of *C. albicans* in the kidneys of IL-17AR−/− mice was caused by reduced mobilization of peripheral neutrophils, kinetics of the absolute neutrophil counts (ANCs) were established in both IL-17AR−/− mice and C57Bl/6 mice after challenge with 2 × 10⁵ viable *C. albicans* yeasts. The emergency neutrophil response in IL-17AR−/− mice was delayed up to 48 h, with their ANCss reaching levels similar to those in infected C57Bl/6 mice at 96 h (figure 4 and data not shown).

**Impaired and delayed kidney parenchymal neutrophil infiltration in IL-17AR−/− mice after systemic challenge with *C. albicans*.** Expression of MPO has been commonly used for quantification of neutrophils in tissue [17]. To determine neutrophil kinetics in kidney tissue after systemic challenge with *C. albicans*, a time course of MPO was established in IL-17AR−/− and C57Bl/6 mice at 0, 24, 48, and 96 h after injection with *C. albicans*. MPO activity was not different at baseline in either mouse strain (figure 5A). However, at 48 h, MPO activity had increased by 115% in C57Bl/6 mice and by only 23% in IL-17AR−/− mice (P < .001). Although MPO activity decreased in both mouse strains, it was still significantly increased (30% over baseline in C57Bl/6 mice vs. 13% over baseline in IL-17AR−/− mice; figure 5B). The data were consistent with quantitative scoring of neutrophils on representative kidney sections (data not shown). Figure 6 depicts typical histologic analysis of HE-stained IL-17AR−/− mouse kidneys 24 and 96 h after systemic challenge with *C. albicans*. Massive infiltration of neutrophils in tissue was seen in kidneys from C57Bl/6 mice at 24 and 96 h in IL-17A−/− mice, which represent the mean ± SE of 5–6 mice and are representative of 3 independent experiments/group. *P ≤ .05.

**Figure 3.** Kidney *Candida albicans* burden in normal and interleukin (IL)-17A receptor knockout (IL-17AR−/−) mice. IL-17AR−/− mice and their age- and sex-matched normal littermate controls (C57Bl/6 mice) were intravenously injected with 2 × 10⁵ (A) or 5 × 10⁵ (B) viable *C. albicans* yeasts (n = 6 mice/group and time point). Total kidney tissue of individual mice was analyzed in triplicate for outgrowth of *C. albicans* at different time points. Data points represent the mean ± SE of 5–6 mice and are representative of 3 independent experiments/group. *P ≤ .05.

**Figure 4.** Peripheral neutrophil response in interleukin (IL)-17A receptor knockout (IL-17AR−/−) mice after systemic challenge with *Candida albicans* (CA). IL-17AR−/− mice and their age- and sex-matched normal littermate controls (C57Bl/6 mice) were intravenously injected with 2 × 10⁵ viable CA yeasts, and peripheral blood counts were determined (n = 5 mice/group and time point). Data points indicate the absolute neutrophil count (ANC), which represent the mean ± SE of 5 mice. *P ≤ .05, CA-challenged IL-17AR−/− mice vs. IL-17AR−/− mice injected with PBS; **P ≤ .05, CA-challenged IL-17AR−/− mice vs. CA-challenged mice.
In contrast, neutrophil counts in IL-17AR\textsuperscript{−/−} mice were substantially lower, especially at the 24-h time point (figure 6). Although evidence for interstitial nephritis was already obvious at 24 h at higher magnification, more-serious tissue damage consistent with nephropathy (i.e., tubular regeneration and presence of proteinaceous casts) could be observed, which was limited to kidneys of IL-17AR\textsuperscript{−/−} mice at 96 h (data not shown).

**Enhancement of survival in C57Bl/6 mice through in vivo expression of mIL-17A after C. albicans infection.** To examine whether expression of mIL-17A could render mice more resistant to *C. albicans* infections, mice were injected with Ad-mIL-17A or a control virus before they were injected with a lethal dose of *C. albicans* (5 × 10\textsuperscript{9} yeasts). Although organ-specific quantitative mIL-17A measurements were not conducted during these experiments, plasma mIL-17A pharmacokinetics using this delivery system have been established and published elsewhere [7]. As previously demonstrated, expression of mIL-17A resulted in profound neutrophilia. The degree of neutrophilia was comparable to that in our previous reports in mice not challenged with *C. albicans* (9.5 × 10\textsuperscript{8} yeasts/mL in Ad-mIL-17A-treated mice vs. 1.3 × 10\textsuperscript{9} yeasts/mL in control virus–treated mice). At day 7, all control virus–treated mice had died, whereas, at day 14, 95% of Ad-mIL-17A–treated mice were still alive. At day 42, 65% of mIL-17A–treated mice were still alive (figure 7A and 7B). In a separate experiment, mice were treated with Ad-mIL-17A or control virus and were killed at different time points for quantification of kidney *C. albicans* colonies (figure 7C).

**DISCUSSION**

Although prior studies have proven mIL-17A to be of critical importance for bacterial host defense with certain gram-negative bacteria, this observation could not be generalized to all microbial pathogens [8, 9] (and authors’ unpublished observations). To determine the possible relevance of mIL-17A and mIL-17AR in *C. albicans* host defense, we have conducted experiments using a model of in vivo systemic candidiasis. Our data show that mIL-17A is induced in response to *C. albicans* sepsis within the kidney very early on and that its expression is sustained during the course of the infection, at levels previously found to be biologically active in vitro and in vivo [7]. Of interest, mIL-17A also appears to play a significant role early in infection in mice maintained under specific pathogen–free conditions. Although activated T cells are considered to be the main source for mIL-17A, the cellular sources were not isolated or determined in these experiments. The exact mechanisms of presumed T cell activation leading to expression of mIL-17A during *C. albicans* sepsis also needs to be determined. Since bacterial lipoproteins can directly induce expression of mIL-17A, it is possible that certain fungal proteins could also directly induce expression of mIL-17A, thus leading to early expression in pathogen free–maintained mice. However, since neutrophils can also express mIL-17A, their contribution as a source of mIL-17A during *C. albicans* sepsis also needs to be determined. The elevated mIL-17A baseline levels in IL-17AR\textsuperscript{−/−} mice could be explained by the possible role of the receptor in clearance of the cytokine. This mechanism of receptor-mediated clearance has been reported for other cytokines, such as granulocyte colony-stimulating factor (G-CSF) [18].

With the profound biological effects of mIL-17A on granulopoiesis and neutrophils, the reasons for its critical role during fungal infection would most likely appear to also be neutrophil related. In the present study, in IL-17AR\textsuperscript{−/−} mice, we have observed a direct association of *C. albicans* host defense reflected by increased mortality, delayed and impaired peripheral neutrophil response, and impaired neutrophil migration...
Figure 6. Impaired and delayed neutrophil influx into infected kidneys after systemic challenge with Candida albicans in interleukin (IL)–17A receptor knockout (IL-17AR−/−) mice. IL-17AR−/− mice and normal control mice (C57Bl/6 mice) were systemically infected with C. albicans, and hematoxylin-eosin–stained histologic kidney sections were obtained at predetermined time points. Image at 0 h depicts typical sections before injection with C. albicans. Images at 24 and 96 h are typical in both mouse strains after C. albicans infection. A 50-μm bar was inserted into the 0-h C57Bl/6 image for size comparison.

into infected organs. Although statistically significant, this finding was less pronounced than that observed in our previous studies of gram-negative bacteria challenge, in which IL-17AR−/− mice failed entirely to mount a peripheral neutrophil response [8, 9]. The data suggest that, during C. albicans host defense, IL-17AR−/− mice have a neutrophil defect at various levels, which includes neutrophil demargination, mobilization, and migration into infected organs. Neutrophils are considered to be the most important host defense cells in systemic fungal infections, although other effector cells or ancillary cells were found to be required for normal fungal host defense. It is possible that mIL-17A is also either directly or indirectly involved in the enhancement of the anti-C. albicans activity of neutrophils. mIL-17A was found to exert significant effects on neutrophils and its precursors, not directly, but, to a large extent, via the induction and release of secondary or downstream cytokines from stromal cells [7, 15]. Because mIL-17A is induced in response to C. albicans infection, it is likely that the proinflammatory cytokine/chemokine milieu is altered within the infected tissue microenvironment of IL-17AR−/− mice that are unresponsive to mIL-17A. Several of these mIL-17A–induced downstream cytokines, such as G-CSF and IL-8, have direct neutrophil-activating properties [19, 20]. Therefore, it is reasonable to hypothesize that IL-17AR−/− mice also have a defect of neutrophil activation within the infected tissue, although additional investigations, such as neutrophil-depletion studies, will be required to fully test this hypothesis. Other cells that have been reported to play a primary fungicidal role in fungal host defense are macrophages and NK cells. Although T cells in fungemias have long been recognized as host-defense modulators, their exact role in that complex process has not been well defined. Some researchers have established direct interactions between T cells and phagocytes and have reported that this phenomenon directly augments fungicidal properties [21–24]. The concept of a host immune-enhancing role for T cells is supported by various epidemiologic and experimental studies. For instance, the incidence of both mucosal and systemic fungal infections was found to be greatly increased in CD4+ T cell–depleted patients with AIDS [25, 26]. However, other investigators have suggested that activation of T cells during fungal tissue inflammation can lead to accelerated tissue injury, which could result in increased mortality [27]. The type of T cell–mediated immune response to C. albicans infection was also found to be of importance, because Th1-directed responses were found to favor clinical outcomes; in contrast, Th2 modulation affects survival adversely [10–13]. Because the proinflammatory molecule mIL-17A is not believed to fit into the current Th1/Th2 paradigm, it appears to be an independent T cell–derived modulator involved in the balancing of a physiologic host response in fungemias. It is unknown what the impact of the defect in the physiologic mIL-17A downstream cytokine cascade in IL-17AR−/− mice following C. albicans sepsis might be on the T helper type cytokine profile. It is possible that, in addition to the abnormal neutrophil response in these
The therapeutic protective effect of adenovirus-mediated in vivo expression of mIL-17A on a systemic C. albicans challenge in C57Bl/6 mice was associated with neutrophilia. In these experiments, as well as in preceding published studies investigating in vivo expression of mIL-17A, we did not find significant expansion of host defense–related lineages other than neutrophils and their precursors [7, 9] (data not shown). The therapeutic expression of mIL-17A resulted in substantially reduced quantitative outgrowth of C. albicans from treated mouse kidneys, implying a direct association of in vivo expression of mIL-17A with reduced fungal growth and reduction of mortality as a result of reduction of C. albicans.

Although not all possible host defense mechanisms have been investigated in the present study, the data suggest that, in fungal host defense, mIL-17A functions primarily via expansion and mobilization of neutrophils. Nevertheless, there are already several other known cytokines and chemokines available that can effectively stimulate granulopoiesis and mobilize neutrophils to an extent comparable to mIL-17A or even higher. Therefore, could mIL-17A possibly be viewed as a redundant cytokine, at least as far as its role as a therapeutic agent would be concerned? Specifically, G-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF) are already approved agents for clinical use. Moreover, mIL-17A was found to function, at least in part, via release of G-CSF from stromal cells, and administration of G-CSF has been reported to protect mice from lethal C. albicans challenges in neutropenic and nonneutropenic mice [15, 28, 29]. However, there are substantial biologic differences that distinguish mIL-17A from G-CSF and GM-CSF in systemic C. albicans infections. For instance, G-CSF and GM-CSF cytokine knockout mice respond completely different to challenge with C. albicans. Although the data on IL-17AR−/− mice indicate that mIL-17A is required for normal C. albicans host defense, Basu et al. [30] reported, in studies with G-CSF and GM-CSF knockout mice, that both cytokines were dispensable during systemic candidiasis. In contrast to IL-17AR−/− mice, in which the emergency neutrophil response to C. albicans infection was impaired, the emergency response in G-CSF– and GM-CSF–deficient mice was paradoxically greatly enhanced [30]. This suggests that compensatory mechanisms must exist in G-CSF and GM-CSF mice, leading to an overshooting neutrophil response after challenge with C. albicans that exceeds the response seen in C57Bl/6 mice. These findings suggest that significant differences in neutrophil regulation during fungal infection exist and that the mIL-17A pathway is distinct and required for normal fungal host defense.

In summary, these data demonstrate that mIL-17AR is re-
quired for normal fungal host defense, which is related to dysfunctional neutrophil regulation in vivo. Therefore, mIL-17A could be used in vivo as a therapeutic cytokine. The data are encouraging for pursuing further development of mIL-17A in fungal infections, which could benefit immunocompromised individuals, especially patients with cancer or AIDS.

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