Cutting Edge: Nlrp10 Is Essential for Protective Antifungal Adaptive Immunity against Candida albicans

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Nucleotide-binding domain leucine-rich repeat containing receptors (NLRs) are cytosolic receptors that initiate immune responses to sterile and infectious insults to the host. Studies demonstrated that Nlrp3 is critical for the control of Candida albicans infections and in the generation of antifungal Th17 responses. In this article, we show that the NLR family member Nlrp10 also plays a unique role in the control of disseminated C. albicans infection in vivo. Nlrp10-deficient mice had increased susceptibility to disseminated candidiasis, as indicated by decreased survival and increased fungal burdens. In contrast to Nlrp3, Nlrp10 deficiency did not affect innate proinflammatory cytokine production from macrophages and dendritic cells challenged with C. albicans. However, Nlrp10-deficient mice displayed a profound defect in Candida-specific Th1 and Th17 responses. These results demonstrate a novel role for Nlrp10 in the generation of adaptive immune responses to fungal infection. The Journal of Immunology, 2012, 189: 000–000.

Members of the NLRP subfamily contain a central nucleotide-binding domain (NACHT), an N-terminal pyrin domain, and C-terminal leucine-rich repeats thought to function in ligand sensing (1). Recently, we and other investigators showed that the nucleotide-binding domain leucine-rich repeat containing receptor (NLR) family member Nlrp3 plays an important role in host defense against C. albicans through triggering the assembly and activation of the Nlrp3 inflammasome (2–4). Nlrc4 also functions within the mucosal stroma to control oral C. albicans infections (5). However, other than Nlrp3 and Nlrc4, the role of NLR family members in fungal pathogenesis remains unknown.

Of interest, one NLR family member, Nlrp10, lacks the C-terminal leucine-rich repeat domain and, therefore, was hypothesized to function as a negative regulator of inflammasome activation (6, 7).

In this study, we demonstrate that Nlrp10, unlike Nlrp6, Nlrp12, and Nlrc4, is required for control of a disseminated C. albicans infection in vivo. We also show that, in contrast to Nlrp3, the absence of Nlrp10 in macrophages (MΦs) and dendritic cells (DCs) does not affect inflammasome activation in response to C. albicans or other inflammasome activators. A recent study also demonstrated that Nlrp10-deficient DCs have defective migration (8); in this study, we demonstrate that, despite normal inflammasome activation, Nlrp10-deficient mice display a profound defect in the generation of Candida-specific Th1 and Th17 responses. Thus, our results implicate Nlrp10 as a novel NLR involved in the generation of antifungal adaptive immune responses against C. albicans through a mechanism that is independent of the Nlrp3 inflammasome and the production of IL-1β.

Materials and Methods
Mice and bone marrow chimeras
The generation of Nlrp10−/−, Nlrp6−/−, Nlrp12−/−, Nlrc4−/−, and ASC−/− mice was described previously (8–12). Sex- and age-matched C57BL/6 (National Cancer Institute) mice were used as controls. All protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Iowa. Bone marrow chimeras were generated, as described (8). Reconstitution was >97% in Nlrp10−/− mice reconstituted with wild-type (WT) bone marrow and >82% in WT mice reconstituted with Nlrp10−/− bone marrow.

In vivo infection with C. albicans
The C. albicans clinical isolate FC20 was used in this study (2). Culture conditions for C. albicans yeast and hyphae were described previously (2). Mice were infected i.v. with 5 × 10^4 CFU C. albicans, and survival was assessed; mice found in a moribund state for >4 h were considered terminal and euthanized. Kidneys were harvested at the indicated time postinfection (p.i.), and dilutions...
of homogenized organs were plated and counted to determine CFU. Serum blood urea nitrogen (BUN) and creatinine levels were quantified at the Animal Fluid Analysis Core at the University of Iowa. To assay renal cytokine levels, kidneys were homogenized and resuspended in lysis buffer (50 mM Tris- HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and a protease inhibitor mixture [Roche]), and cytokine levels were measured by ELISA.

Ex vivo lymphocyte restimulation

Mice were infected i.v. with a sublethal dose (5 × 10⁴ CFU) of C. albicans. Fourteen days p.i., spleens were collected and splenocytes were cultured in the presence or absence of 1 × 10⁵ ml⁻¹ heat-killed C. albicans for 72 h. Supernatants were collected, and IL-17 and IFN-γ levels were assessed by ELISA (eBiosciences).

CD4⁺ T cell adoptive transfer

WT mice were infected i.v. with 5 × 10⁴ CFU C. albicans; 10 d p.i., mice were rechallenged i.v. with 1 × 10⁶ heat-killed C. albicans. Five days later, splenic CD4⁺ T cells were isolated using MACS MicroBeads (Miltenyi Biotec). CD4⁺ T cells isolated from uninfected WT mice were used as naive controls. A total of 5 × 10⁵ naïve or immune CD4⁺ T cells was transferred i.v. into Nlrp10⁻/⁻ mice; 24 h following the adoptive transfer mice were infected i.v. with 5 × 10⁴ CFU C. albicans, and survival was monitored.

In vitro stimulation of MΦs and DCs

Bone marrow–derived MΦs (BM-MΦs) and bone marrow–derived DCs (BMDCs) (13, 14) were either left unprimed or were primed with 50 ng/ml LPS (InvivoGen) for 3–4 h and then infected with C. albicans (multiplicity of infection [MOI] 10:1), Francisella tularensis LPS (InvivoGen), or C. albicans LPS (InvivoGen) for 6 h. For ATP- and nigericin–treated cells, media were replaced with fresh media 30 min after stimulation. Ab pairs for ELISA were from R&D Systems.

Results and Discussion

Nlrp10-deficient mice are highly susceptible to disseminated C. albicans infection

Phylogenetic analysis of the NLR family demonstrates that Nlrp6, Nlrp10, and Nlrp12 NACHT sequences are closely related to that of Nlrp3 (1). In addition, Nlrc4, which activates caspase-1 in response to cytosolic flagellin and bacterial type III secretion systems (1), was also shown to play a role in controlling mucosal C. albicans infections (5). We hypothesized that, similarly to Nlrp3, these receptors might contribute to the in vivo immune response against a systemic C. albicans infection. To assess this, we tested the susceptibility of Nlrp6-, Nlrp10-, Nlrp12-, and Nlrc4-deficient mice to a systemic infection with C. albicans. Nlrp6-, Nlrp12-, and Nlrc4-deficient mice did not show increased susceptibility to i.v. infection with C. albicans compared to WT mice (Fig. 1A). Surprisingly, Nlrp10⁻/⁻ mice were highly susceptible to C. albicans infection, with 100% mortality by day 16 p.i. (Fig. 1B).

Renal dysfunction in Nlrp10-deficient mice reflects increased fungal invasion of kidneys at the late stage of infection

Sepsis is the main cause of death in hematogenously disseminated candidiasis; in this model, renal dysfunction strongly correlates with increased kidney fungal burdens, as well as increased mortality (15). Therefore, we evaluated kidneys of WT and Nlrp10⁻/⁻ mice 9 d p.i., with C. albicans. Histologic sections of kidneys revealed more severe early fibroplasia and parenchymal loss in kidneys of Nlrp10⁻/⁻ mice compared with WT mice (Fig. 1C). Kidneys from Nlrp10⁻/⁻ mice had significantly more collagen deposition, as shown by Masson’s trichrome stain, than did WT mice, indicating greater damage (Fig. 1C, Supplemental Fig. 1A). Very few yeast were detected histologically in kidney sections from WT mice in contrast to Nlrp10⁻/⁻ mice, in which C. albicans yeast and hyphae were readily observed in the renal cortex and medulla (Fig. 1C, Supplemental Fig. 1B). Surprisingly, despite increased C. albicans within the renal parenchyma of Nlrp10⁻/⁻ mice, there was no significant difference in the percentage of parenchymal MΦs and neutrophil staining between WT and Nlrp10⁻/⁻ mice (Supplemental Fig. 1C), suggesting a possible functional defect in the inflammatory response observed in the absence of Nlrp10.

Consistent with the increased renal damage observed by histology, Nlrp10⁻/⁻ mice had diminished renal function at 9 d p.i., as reflected by significantly higher serum BUN and
creatinine levels (Fig. 1D). Increased damage in Nlrp10-deficient kidneys correlated with elevated IL-1α and IL-6 levels within the kidney at day 9 p.i., although IL-1β, IL-18, IL-12p40, and IL-23 levels were unaffected by Nlrp10 deficiency (Supplemental Fig. 1D). Nlrp10−/− mice also had significantly higher fungal burdens in the kidney at 9 d p.i., indicating a role for Nlrp10 in controlling the replication of C. albicans in vivo (Fig. 1E). Surprisingly, during the early stages of infection, examined at days 3 and 6, there was no difference between WT and Nlrp10−/− mice with regard to renal function (Fig. 1D), fungal burdens (Fig. 1E), and kidney cytokines (data not shown). These data suggest that early innate mechanisms required to control C. albicans replication in vivo remain intact in Nlrp10-deficient mice.

Nlrp10 functions within the hematopoietic compartment to control disseminated infection with C. albicans

To better understand the biological function of Nlrp10, we examined the tissue distribution of Nlrp10 in WT mice. Consistent with previous reports, we found high expression of *Nlrp10* mRNA in the heart (7); in addition, Nlrp10 was highly expressed in the tongue, testis, and spleen (Supplemental Fig. 1E). Within the hematopoietic compartment, Nlrp10 was expressed in Mφs, DCs, CD4+ T cells, CD19+ B cells, and neutrophils but minimally in CD8+ T cells (Supplemental Fig. 1F). Stimulation of Mφs and DCs with live *C. albicans* in vitro resulted in a reduction in Nlrp10 expression in these cells (Supplemental Fig. 1G). In contrast, LPS and heat-killed *C. albicans* did not significantly alter *Nlrp10* mRNA expression (Supplemental Fig. 1G).

Given that *Nlrp10* is expressed in both hematopoietic cells and stromal cells, we wanted to determine whether the increased susceptibility of *Nlrp10*−/− mice to disseminated candidiasis was the result of a loss of Nlrp10 in the hematopoietic compartment. To do this, we generated bone marrow chimeric mice in which WT 129S6/SvEv mice were reconstituted with BM cells from *Nlrp10*−/− mice (Fig. 2A). Conversely, *Nlrp10*−/− mice that received WT bone marrow did not have significantly increased mortality compared with WT mice that received WT bone marrow (Fig. 2A). These results suggest that the increased susceptibility of *Nlrp10*−/− mice to *C. albicans* infection was primarily due to a deficiency in Nlrp10 within the hematopoietic compartment. A recent study by Lautz et al. (16) demonstrated that Nlrp10 contributes to proinflammatory cytokine release by epithelial cells and dermal fibroblasts in response to infection with *Shigella flexneri*, which may explain the reduction, although not significant, in the survival of *Nlrp10*−/− mice that received WT bone marrow.

Nlrp10 deficiency does not affect inflammasome activation

Recent studies using in vitro overexpression of Nlrp10, as well as Nlrp10 transgenic mice, suggested that Nlrp10 could inhibit the activation of Nlrp3 and Nlr4 inflammasomes and suppress NLR-kB activation (6, 7). We examined the ability of Nlrp10-deficient Mφs to secrete IL-1β in response to specific inflammasome agonists. LPS-primed BMMφs from WT and *Nlrp10*−/− mice secreted comparable levels of IL-1β when challenged with *C. albicans* yeast (Fig. 2B, 2C). Similarly, the

Nlrp3 agonists silica and nigericin induced similar levels of IL-1β secretion from Nlrp10-deficient BMMφs compared with WT BMMφs (Fig. 2D). In addition, *P. aeruginosa* and *F. tularensis* LVS, activators of the Nlrc4 and AIM2 inflammasomes, respectively (1), induced comparable levels of IL-1β secretion from *Nlrp10*−/− and WT BMMφs (Fig. 2C). As expected, IL-1β secretion in response to *C. albicans*, *P. aeruginosa*, and *F. tularensis* LVS was dependent on the presence of the inflammasome adaptor molecule ASC (Fig. 2C). Similar to our findings with BMMφs, BMDCs from WT and

![FIGURE 2. Nlrp10 in hematopoietic cells is required for control of a systemic *C. albicans* infection. (A) Kaplan–Meier survival curves of bone marrow chimeras (donor→recipient) infected i.v. with 5 × 10⁵ CFU of *C. albicans* yeast. Results are pooled from two independent experiments (n = 14–15). There was no significant (ns) difference between WT→*Nlrp10*−/− and WT→WT mice or between *Nlrp10*−/−→WT and *Nlrp10*−/−→*Nlrp10*−/− mice. *p < 0.01, WT→*Nlrp10*−/− versus *Nlrp10*−/−→WT mice, log–rank test. (B) Unprimed and LPS-primed BMMφs from WT and *Nlrp10*−/− mice were stimulated for 6 h with *C. albicans* yeast (MOI 10:1), and IL-1β secretion was quantified with ELISA. (C) LPS-primed BMMφs from WT, *Nlrp10*−/− and ASC−/− mice were stimulated for 6 h with *C. albicans* (MOI 10:1) or *P. aeruginosa* (MOI 10:1) or for 9 h with *F. tularensis* LVS (MOI 50:1); IL-1β secretion was quantified by ELISA. (D) LPS-primed BMMφs from WT, *Nlrp10*−/−, and ASC−/− mice were stimulated with silica (50 μg/cm²) or nigericin (20 μM) for 6 h, and IL-1β secretion was quantified by ELISA. (E) BMMφs from WT and *Nlrp10*−/− mice were stimulated for 6 h with 50 ng/ml LPS, and IL-12 p40, TNF-α, and IL-6 levels were assessed by ELISA. Determinations were performed in triplicate and expressed as the mean ± SEM; results are representative of three independent experiments.]
Nlrp10−/− mice secreted comparable levels of IL-1β when challenged with C. albicans yeast (Supplemental Fig. 2A). In addition, both unprimed and LPS-primed WT and Nlrp10−/− BMΦs failed to secrete IL-1β in response to C. albicans hyphae (Supplemental Fig. 2B). C. albicans hyphae were capable of inducing the secretion of IL-1β from LPS-primed WT BMDCs, although again this was similar to the levels of IL-1β secreted from LPS-primed Nlrp10−/− BMDCs (Supplemental Fig. 2C). These data indicate that a deficiency in Nlrp10 in MΦs or DCs does not affect the activation of Nlrp3, Nlrc4, and AIM2 inflammasomes. Consistent with a recent study by Eisenbarth et al. (8), in response to stimulation with the TLR4 agonist LPS, the production of IL-6, TNF-α, and IL-12 p40 was unaffected by Nlrp10 deficiency in both BMΦs and BMDCs (Fig. 2E, Supplemental Fig. 2D), suggesting that Nlrp10 also does not suppress NF-κB activation in these cells.

Internalization and killing of Candida is an indispensable function of MΦs in the control of candial infections. Nlrp10-deficient BMΦs and BMDCs did not display any defect in their ability to phagocytose C. albicans compared with WT BMΦs and BMDCs (Supplemental Fig. 2E). Growth of C. albicans within MΦs was also comparable between WT and Nlrp10−/− BMΦs (Supplemental Fig. 2F). Similarly, we did not observe defects in the phagocytosis or growth of C. albicans within Nlrp10−/− thioglycollate-elicited peritoneal neutrophils (Supplemental Fig. 2E, 2F). Taken together, these data suggest that phagocytosis, intracellular killing, and the generation of proinflammatory cytokines by MΦs and DCs remain intact in the absence of Nlrp10.

Nlrp10 is necessary for generating Candida-specific Th1 and Th17 responses

Adaptive immune responses play a crucial role in host defense against C. albicans. Th1 responses, in particular, are important for control of C. albicans infections through the recruitment of neutrophils to the infection site. As such, mice deficient in the cytokine receptor IL-17RA have increased susceptibility to both disseminated and mucosal candidiasis (17, 18). Given our findings that Nlrp10 deficiency had little effect on MΦ and DC production of proinflammatory cytokines, we next examined whether the generation of CD4+ Th cell responses to C. albicans remained intact in Nlrp10−/− mice. WT and Nlrp10−/− mice were infected i.v. with a sublethal dose of C. albicans; Th1 and Th17 responses were evaluated 15 d p.i. by measuring IFN-γ and IL-17 release, respectively, from splenocytes restimulated with heat-killed C. albicans for 72 h. As expected, WT mice displayed a mixed Th1 and Th17 response to C. albicans as evidenced by the secretion of IFN-γ and IL-17 by restimulated splenocytes (Fig. 3A). Surprisingly, Nlrp10−/− mice displayed a profound defect in the generation of Candida-specific Th1 and Th17 responses (Fig. 3A), which indicates that Nlrp10 is required for driving appropriate adaptive immune responses to C. albicans in vivo.

To determine whether the inability to generate appropriate adaptive immune responses was the cause of the increased mortality of Nlrp10−/− mice following C. albicans infection, we adoptively transferred naive and C. albicans–immune WT CD4+ T cells into Nlrp10−/− mice. Following CD4+ T cell adoptive transfer, Nlrp10−/− mice were infected i.v. with C. albicans. Nlrp10−/− mice that received CD4+ T cells from naive WT mice succumbed to C. albicans infection at a similar rate to Nlrp10−/− control mice (Fig. 3B). However, Nlrp10−/− mice that received CD4+ T cells from WT mice that had previously been challenged with a sublethal dose of C. albicans and boosted with heat-killed C. albicans displayed significantly improved survival in response to a lethal C. albicans challenge compared with Nlrp10−/− control mice (Fig. 3B). Hence, taken together, these data suggest that Nlrp10 is required for the generation of protective antifungal adaptive immune responses in vivo.

The defect in the generation of specific Th cell responses in Nlrp10−/− mice was not restricted to C. albicans immunization of Nlrp10−/− mice with Ag in the presence of a number of adjuvants, including LPS, aluminum hydroxide, and CFA, was also shown to result in defective adaptive immune responses (8). In addition, Nlrp10-deficient DCs were shown to have an intrinsic defect in their ability to emigrate from a site of inflammation, resulting in a lack of Ag transport to the draining lymph node and explaining the lack of priming of naive CD4+ T cells in Nlrp10−/− mice (8). Hence, Nlrp3 and Nlrp10 play distinct roles in shaping adaptive immune responses against fungal pathogens. Although Nlrp3 inflammasome-driven IL-1β production drives Th17 differentiation during a C. albicans infection (19), the role of Nlrp10 in the generation of specific Th cell responses is likely to be at the level of appropriate DC migration and Ag presentation to naive CD4+ T cells.

FIGURE 3. Nlrp10-deficient mice fail to mount a protective T cell response against C. albicans. (A) WT and Nlrp10−/− mice were infected with a sublethal dose (5 × 10^6 CFU) of C. albicans yeast. Fourteen days p.i., splenocytes were restimulated with heat-killed C. albicans; 72 h later, IL-17 and IFN-γ secretion into the supernatant was assessed by ELISA. Data are pooled from three independent experiments (n = 6–8) and expressed as the mean ± SEM. (B) A total of 5 × 10^6 CD4+ T cells from either naive or immune WT mice was adoptively transferred into Nlrp10−/− mice, followed by i.v. infection with 5 × 10^6 CFU of C. albicans yeast; survival was monitored. Data are pooled from two independent experiments (n = 13–15). *p < 0.05, Student t test, **p < 0.01, Nlrp10−/− mice that received naive CD4+ T cell or PBS versus immune CD4+ T cells, log-rank test.
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Disclosures
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References