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.

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 lipid 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 Candida 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 C7BL/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 >98% 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 × 10^2 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

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; BMMΦ, bone marrow–derived macrophage; BUN, blood urea nitrogen; DC, dendritic cell; LVS, live vaccine strain; MΦ, macrophage; MOI, multiplicity of infection; NLR, nucleotide–binding domain leucine-rich repeat containing receptor; p.i., postinfection; WT, wild-type.

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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 assess 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 × 10^4 CFU) of *C. albicans*. Fourteen days p.i., spleens were collected and splenocytes were cultured in the presence or absence of 1 × 10^7 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 × 10^4 CFU *C. albicans*; 10 d p.i., mice were rechallenged i.v. with 1 × 10^5 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 × 10^5 naive 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 × 10^4 CFU *C. albicans*, and survival was monitored.

**In vitro stimulation of MΦs and DCs**

Bone marrow–derived MΦs (BMMΦs) 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 live vaccine strain (LVS) (MOI 50:1), or *Pseudomonas aeruginosa* PAK strain (MOI 10:1) for 6 h or as indicated. LPS-primed BMMΦs were challenged with 50 μg/cm^2 silica (Min-U-Sil-5; Pennsylvania Glass Sand), 5 mM ATP (Sigma), or 20 μM nigericin (Sigma) 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, with the exception of IL-1β (eBiosciences).

### 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 Nlrp10 deficiency was restricted to either the hematopoietic or nonhematopoietic compartment. WT mice in which Nlrp10 deficiency was restricted to the hematopoietic compartment. To do this, we generated bone marrow chimeric mice in which Nlrp10 deficiency was restricted to either the hematopoietic or nonhematopoietic compartment. WT mice were challenged for 6 h with 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 or not for 6 h with C. albicans yeast (MOI 10:1), and IL-1β secretion was quantified by ELISA. (C) LPS-primed BMMφs from WT, Nlrp10−/−, and ASC−/− mice were challenged 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 challenged with silica (50 mg/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 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 NF-κB 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 Nlr4 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
**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**−/− BMDCs 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, Nlr4, 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 BMMΦs 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 candidal infections. **Nlrp10**−/− deficient BMMΦs and BMDCs did not display any defect in their ability to phagocytose *C. albicans* compared with WT BMMΦs and BMDCs (Supplemental Fig. 2E). Growth of *C. albicans* within MΦs was also comparable between WT and **Nlrp10**−/− BMMΦs (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). 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. **Nlrp10**−/− mice were infected i.v. with a sublethal dose of *C. albicans*. Th1 and Th17 responses were evaluated 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.

**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 × 10⁶ CFU) of *C. albicans* yeast. Fourteen days p.i., splenocytes were restimulated with heat-killed *C. albicans* and 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 × 10⁶ CD4+ T cells from either naive or immune WT mice was adoptively transferred into **Nlrp10**−/− mice, followed by i.v. infection with 5 × 10⁶ CFU of *C. albicans* yeast; survival was monitored. Data are pooled from two independent experiments (*n* = 13–15). *p* < 0.05, Student *t* test. **Nlrp10**−/− mice that received naive 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.
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Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Data

Supplementary Figure 1. Nlrp10-deficient mice have increased fungal burdens and renal damage following systemic *C. albicans* infection. (A-C) Mice were infected i.v. with 5 x 10^5 CFU of *C. albicans*. 9 d p.i. kidneys were harvested for histology and digital images collected at 100X (DP72, Olympus) and analyzed with ImageJ software (NIH). Collagen deposition was semi-quantified from Masson’s trichrome stained images by averaging the percent collagen staining from two 100X fields per animal (A). The number of yeast and hyphae in the renal parenchyma of Grocott’s methenamine silver (GMS) stained sections were counted in ten 400X fields and averaged (B). *p < 0.05 and **p < 0.01 by the Mann-Whitney U test. (C) Parenchymal macrophage and neutrophil infiltration was semi-quantified by staining with anti-F4/80 and myeloperoxidase (MPO) respectively and assessed using ImageJ software. The majority of identifiable neutrophils stained with MPO, however there were scattered degranulated neutrophils that were not MPO positive. (D) Kidneys were harvested from WT and *Nlrp10*−/− mice at day 9 p.i. with 5 x 10^5 CFU of *C. albicans* and tissue cytokine levels assessed by ELISA. Data represent the mean ± SEM. n=6-8 mice per group. **p < 0.01 by the Mann-Whitney U test. (E, F) *Nlrp10* expression in C57BL/6 mouse organs and the indicated cell types was quantified by real-time quantitative RT-PCR. *Nlrp10* mRNA expression levels were normalized to HPRT. Primers used for RT-PCR amplification were as follows: HPRT, 5′-GTTGGATAACAGGCCAGACTTTGTT and 3′-GAGGGTAGGCTGGCCTATAGGCT; *Nlrp10*, 5′-GGAGCTTGTAGACTACCTCA and 3′-AAAGTCTCCACATCGACAGG. (G) BMMφ and BMDC from WT mice were challenged with live *C. albicans* (MOI 10:1), heat killed *C. albicans* (MOI 10:1) or LPS (50 ng/ml) for 6 h and *Nlrp10* expression quantified by real-time quantitative RT-PCR. *Nlrp10* mRNA expression levels were normalized to HPRT. Results are expressed as the mean ± SD of two independent experiments.

Supplementary Figure 2. Innate immune functions of phagocytosis, intracellular killing and the generation of proinflammatory cytokines by BMMφ and BMDCs remains intact in the absence of *Nlrp10*. (A) BMDC from WT and *Nlrp10*−/− mice were stimulated with *C. albicans* yeast (MOI 10:1); 6 h later culture supernatants were collected and IL-1β secretion quantified by ELISA. (B, C) Unprimed and LPS-primed BMMφ or BMDC from WT and *Nlrp10*−/− mice were stimulated for 6 h with *C. albicans* hyphae (MOI 10:1). Culture supernatants were collected and IL-1β secretion quantified by ELISA. (D) BMDC from WT and *Nlrp10*−/− mice were stimulated for 6 h with 50 ng/ml LPS and IL-12 p40, TNFα and IL-6 levels assessed by ELISA. (E) BMMφ, BMDC and thioglycollate-elicited peritoneal neutrophils from WT and *Nlrp10*−/− mice were challenged with *C. albicans* yeast (MOI 5:1) for 2 h and the number of internalized yeast scored microscopically. (F) BMMφ or thioglycollate-elicited peritoneal neutrophils from WT and *Nlrp10*−/− mice were infected with *C. albicans* yeast (MOI 5:1); at the indicated time p.i. BMMφ monolayers were lysed and the number of *C. albicans* per well quantified by dilution plating. Determinations were performed in triplicate and expressed as the mean ± SEM; results are representative of three (A-D) and two (E, F) independent experiments.
Supplementary Figure 1
Supplementary Figure 2