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Nlrp10 is essential for protective anti-fungal adaptive immunity against *Candida albicans* [††]

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Abstract

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 have demonstrated that Nlrp3 is critical for the control of *Candida albicans* infections and in the generation of anti-fungal Th17 responses. Here 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 (PYD) and C-terminal leucine rich repeats (LRR) thought to function in ligand sensing (1). Recently we, and others, have shown that the 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

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interest, one NLR family member, Nlrp10, lacks the C-terminal LRR domain and has therefore been 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 (Mϕ) and dendritic cells (DC) does not affect inflammasome activation in response to *C. albicans* or other inflammasome activators. A recent study has also demonstrated that Nlrp10-deficient DCs have defective migration (8); here 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 anti-fungal 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 has been described previously (8-12). Sex and age matched C57BL/6 (NCI) 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 greater than 97% in *Nlrp10*$^{-/-}$ mice reconstituted with 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 have been previously described (2). Mice were infected i.v. with 5 × 10^5 CFU of *C. albicans* and survival assessed; mice found in a moribund state for more than 4 h were considered terminal and euthanized. Kidneys were harvested at the indicated time post-infection (p.i.) and dilutions of homogenized organs were plated and counted to determine CFUs. 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, resuspended in lysis buffer (50 mM Tris·HCl, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, and a protease inhibitor cocktail (Roche)) and cytokine levels measured by ELISA.

Ex vivo lymphocyte restimulation

Mice were infected i.v. with a sublethal dose (5 × 10^4 CFU) of *C. albicans*. 14 d p.i. spleens were collected and splenocytes 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 IFNγ levels assessed by ELISA (eBiosciences).

CD4$^+$ T cell adoptive transfer

WT mice were infected i.v. with 5 × 10^4 CFU of *C. albicans*; 10 d p.i. mice were rechallenged i.v. with 1 × 10^6 heat-killed *C. albicans*. 5 d later splenic CD4$^+$ T cells were isolated using MACS microbeads (Miltenyi Biotec). CD4$^+$ T cells isolated from uninfected WT mice were used as naïve controls. 5 × 10^6 naïve or immune CD4$^+$ T cells were transferred i.v. into *Nlrp10*$^{-/-}$ mice; 24 h following the adoptive transfer mice were infected i.v. with 5 × 10^5 CFU of *C. albicans* and survival monitored.

In vitro stimulation of macrophages and dendritic cells

Bone marrow-derived Mϕ (BMMϕ) and bone marrow-derived DC (BMDC) (13, 14) were either left unprimed or primed with 50 ng/ml LPS (Invivogen) for 3-4 h and then infected with *C. albicans* (MOI 10:1), *F. tularensis* LVS (MOI 50:1) or *P. aeruginosa* PAK strain (MOI 10:1) for 6 h or as indicated. LPS-primed BMMϕ were challenged with 50 μg/cm^2 silica (Min-U-Sil-5; Pennsylvania Glass Sand Corporation), 5 mM ATP (Sigma) or 20 μM nigericin (Sigma) for 6 h. For ATP and nigericin treated cells media was replaced with fresh media 30 min after stimulation. Antibody pairs for ELISA were from eBiosciences except for IL-1β (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), has also been shown to play a role in controlling mucosal *C. albicans* infections (5). We hypothesized, that similar to Nlrp3, these receptors might contribute to the *in vivo* immune response against a systemic *C. albicans* infection. In order 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). We therefore 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 to WT (Fig. 1C). Kidneys from *Nlrp10*$^{-/-}$ mice had significantly more collagen deposition as shown by Masson's trichrome stain than WT, indicating greater damage (Fig. 1C and Suppl. Fig. 1A). Very few yeast were detected histologically in kidney sections from WT mice in contrast to *Nlrp10*$^{-/-}$ mice where *C. albicans* yeast and hyphae were readily observed in the renal cortex and medulla (Fig. 1C and Suppl. Fig. 1B). Surprisingly, despite increased *C. albicans* within the renal parenchyma of *Nlrp10*$^{-/-}$ mice there was no significant difference in the percent of parenchymal Mϕ and neutrophil staining between WT and *Nlrp10*$^{-/-}$ mice (Suppl. 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, 9 d p.i. *Nlrp10*$^{-/-}$ mice had diminished renal function as reflected by significantly higher serum blood urea nitrogen (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 (Suppl. 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 day 3 and 6, there was no difference between WT and *Nlrp10*$^{-/-}$ mice in renal function (Fig. 1D), fungal burdens (Fig.

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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 understand better 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 (Suppl. Fig. 1D). Within the hematopoietic compartment *Nlrp10* was expressed in Mϕ, DCs, CD4$^+$ T cells, CD19$^+$ B cells and neutrophils, but minimally in CD8$^+$ T cells (Suppl. Fig. 1E). Stimulation of Mϕ and DCs with live *C. albicans in vitro* resulted in a reduction of *Nlrp10* expression in these cells (Suppl. Fig. 1F). In contrast, LPS and heat-killed *C. albicans* did not significantly alter *Nlrp10* mRNA expression (Suppl. Fig. 1F).

Given that *Nlrp10* is expressed in both hematopoietic cells as well as stromal cells we wanted to determine whether the increased susceptibility of *Nlrp10*$^{-/-}$ mice to disseminated candidiasis was the result of 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 non-hematopoietic compartment. WT mice that received *Nlrp10*$^{-/-}$ bone marrow were susceptible to disseminated candidiasis and recapitulated the phenotype observed in Nlrp10-deficient mice (Fig. 2A). Conversely, *Nlrp10*$^{-/-}$ mice that received WT bone marrow did not have significantly increased mortality compared to 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 of Nlrp10 within the hematopoietic compartment. A recent study by Lautz *et al.* demonstrates that Nlrp10 contributes to proinflammatory cytokine release by epithelial cells and dermal fibroblasts in response to infection with *Shigella flexneri* (16) and may possibly explain the reduction, although not significant, in 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 Nlrc4 inflammasomes as well as suppress NF-κB activation (6, 7). We examined the ability of Nlrp10-deficient Mϕ to secrete IL-1β in response to specific inflammasome agonists. LPS-primed BMMϕ from WT and *Nlrp10*$^{-/-}$ mice secreted comparable levels of IL-1β when challenged with *C. albicans* yeast (Fig. 2B and C). Similarly, the Nlrp3 agonists silica and nigericin induced similar levels of IL-1β secretion from Nlrp10-deficient BMMϕ when compared to WT BMMϕ (Fig. 2D). In addition, *Pseudomonas aeruginosa* and *Francisella tularensis* LVS, activator of the Nlrc4 and AIM2 inflammasomes respectively (1), also induced comparable levels of IL-1β secretion from *Nlrp10*$^{-/-}$ and WT BMMϕ (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 BMMϕ, BMDC from WT and *Nlrp10*$^{-/-}$ mice secreted comparable levels of IL-1β when challenged with *C. albicans* yeast (Suppl. Fig. 2A). In addition, both unprimed and LPS-primed WT and *Nlrp10*$^{-/-}$ BMMϕ failed to secrete IL-1β in response to *C. albicans* hyphae (Suppl. Fig. 2B). *C. albicans* hyphae were capable of inducing the secretion of IL-1β from LPS-primed WT BMDC, although this was again similar to levels of IL-1β secreted from LPS-primed *Nlrp10*$^{-/-}$ BMDC (Suppl. Fig. 2C). These data indicate that a deficiency of Nlrp10 in Mϕ 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, TNFα and IL-12 p40 was unaffected by Nlrp10-deficiency in both BMMϕ and BMDC (Fig. 2E and Suppl. 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 Mϕ in the control of candidal infections. Nlrp10-deficient BMMϕ and BMDC did not display any defect in their ability to phagocytose *C. albicans* compared to WT BMMϕ and BMDC (Suppl. Fig. 2E). Growth of *C. albicans* within Mϕ was also comparable between WT and *Nlrp10*$^{-/-}$ BMMϕ (Suppl. Fig. 2F). Similarly, we did not observe defects in the phogocytosis or growth of *C. albicans* within *Nlrp10*$^{-/-}$ thioglycollate-elicited peritoneal neutrophils (Suppl. Fig. 2E and F). Taken together these data suggest that phagocytosis, intracellular killing and the generation of proinflammatory cytokines by Mϕ 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*. Th17 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-17 receptor A (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 pro-inflammatory cytokines we next examined if the generation of CD4^{+} T helper 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*; 15 d p.i. Th1 and Th17 responses were evaluated by measuring IFNγ 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 infection with *C. albicans* as evidenced by the secretion of IFNγ and IL-17 by restimulated splenocytes (Fig. 3A). Surprisingly, *Nlrp10*$^{-/-}$ mice displayed a profound defect in the generation of both *Candida*-specific Th1 and Th17 responses (Fig. 3A), which indicate that Nlrp10 is required for driving appropriate adaptive immune responses to *C. albicans in vivo*.

To determine if 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 naïve 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 naïve 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 to *Nlrp10*$^{-/-}$ control mice (Fig. 3B). Hence, taken together these data suggest that Nlrp10 is required for the generation of protective anti-fungal adaptive immune responses *in vivo*.

The defect in generation of specific T helper cell responses in *Nlrp10*$^{-/-}$ mice was not restricted to *C. albicans*; immunization of *Nlrp10*$^{-/-}$ mice with antigen in the presence of a number of adjuvants, including LPS, aluminum hydroxide and complete Freund's adjuvant has also been 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 antigen transport to the draining lymph node and explaining the lack of priming of naïve CD4^{+} T cells in *Nlrp10*$^{-/-}$ mice (8). Hence Nlrp3 and Nlrp10 play distinct roles in shaping adaptive immune responses against fungal pathogens. Whereas Nlrp3 inflammasome-driven IL-1β production drives Th17 differentiation during a *C. albicans* infection (19), the role of Nlrp10 in the generation of specific T helper cell responses is likely to be at the level of appropriate DC migration and antigen presentation to naïve CD4$^+$ T cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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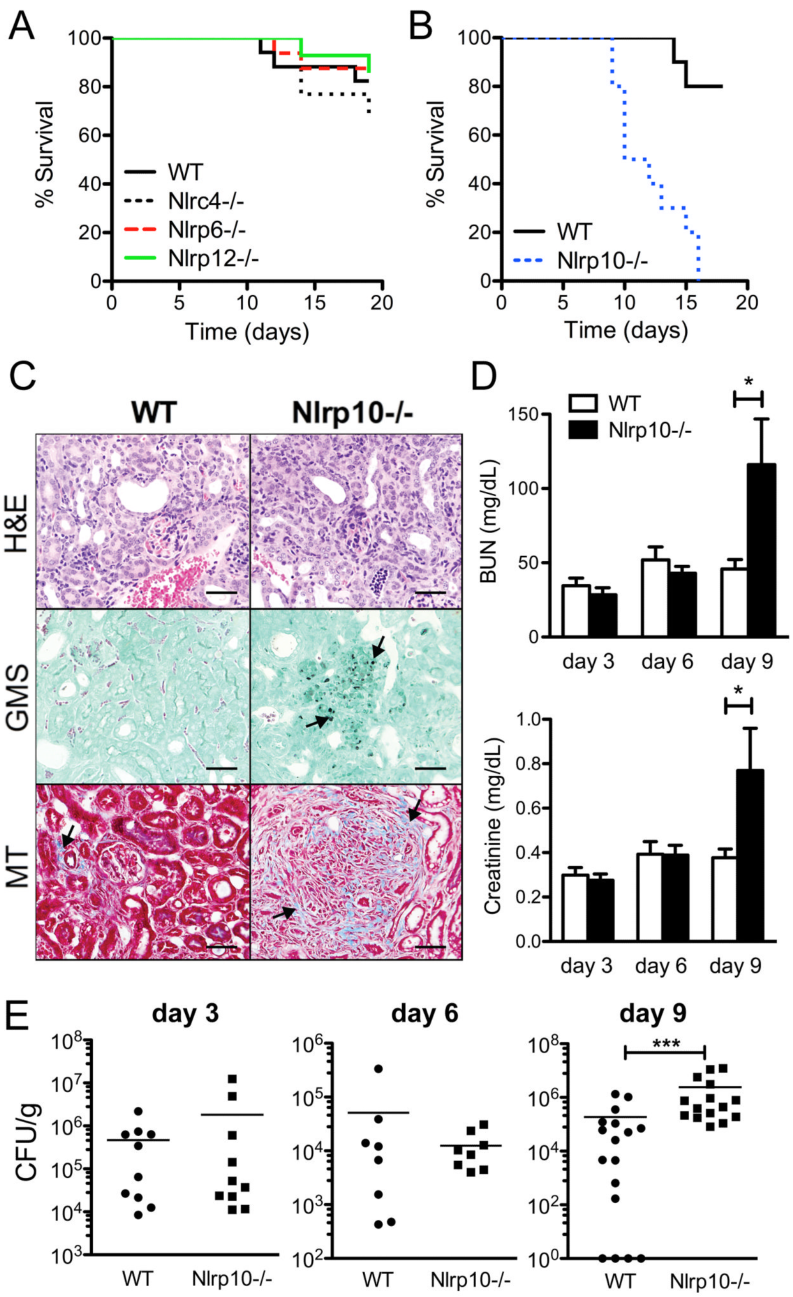

Fig. 1. Nlrp10-deficient mice have increased susceptibility to systemic *C. albicans* infection
(A, B) Kaplan-Meier survival curves of WT (n=17), *Nlrp6*$^{-/-}$ (n=16), *Nlrp12*$^{-/-}$ (n=14), *Nlrc4*$^{-/-}$ (n=13) mice (A) or WT (n=10) and *Nlrp10*$^{-/-}$ (n=10) mice (B) infected i.v. with 5 × 10^5 CFU of *C. albicans* yeast. Results are pooled from two independent experiments. $p < 0.01$ by log-rank test for WT compared to *Nlrp10*$^{-/-}$. (C) Histology of kidneys 9 d p.i. from WT control and *Nlrp10−/−* mice stained with hematoxylin and eosin (H&E), Grocott's methenamine silver stain (GMS) and Masson's trichrome (MT) stain. Black arrows indicate yeast and hyphae in the GMS stain and collagen deposition (light blue) in the MT stain. (D) Serum creatinine and blood urea nitrogen (BUN) levels were measured at the indicated times p.i. with 5 × 10^5 CFU of *C. albicans* yeast. Data represent the mean ± the SEM. n=9

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for day 3; n=6 for day 6; and n=14-16 for day 9. *$p < 0.05$ by Student's t test. (E) WT and *Nlrp10*$^{-/-}$ mice (n=10 for day 3; n=8 for day 6; n=15-18 for day 9) were infected i.v. with 5×10^5 CFU of *C. albicans*; at the indicated times p.i. fungal burdens in the kidney were assessed. ***$p < 0.001$ by the Mann-Whitney *U* test.

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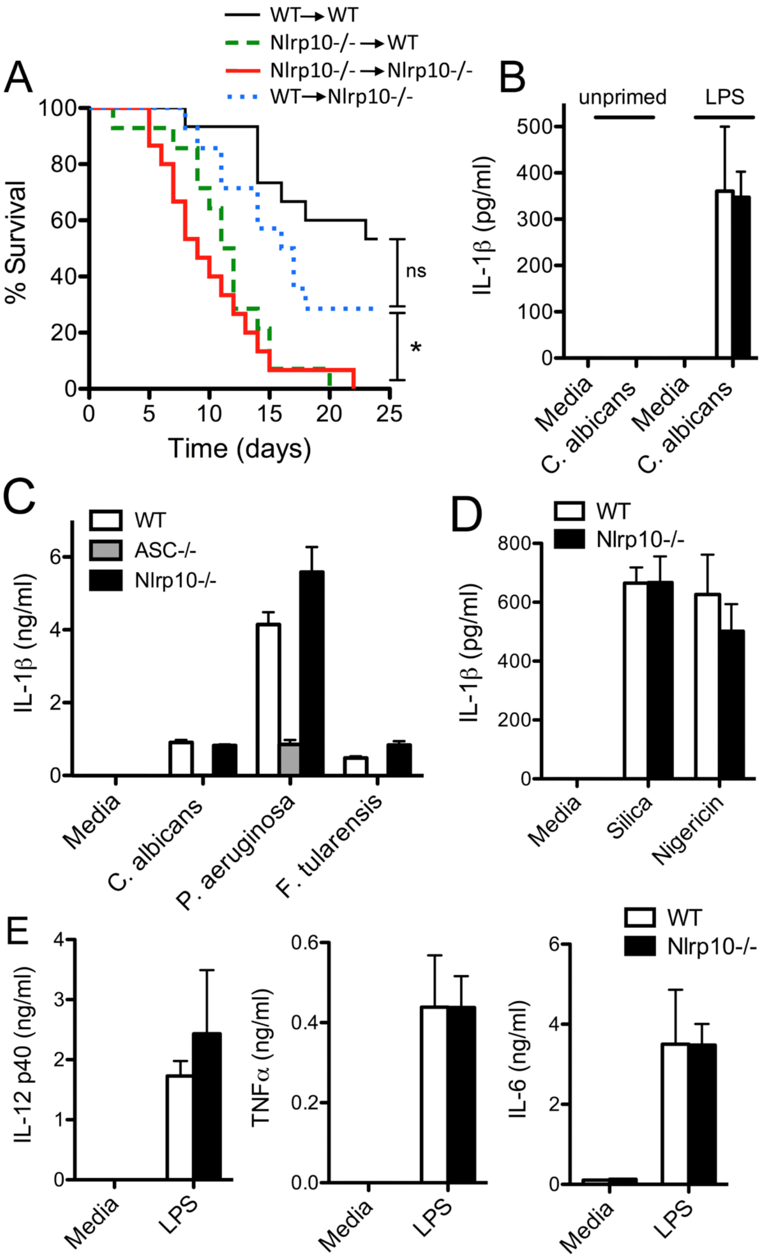

Fig. 2. Nlrp10 in hematopoietic cells is required for control of a systemic *C. albicans* infection

(A) Kaplan-Meier survival curves of bone marrow chimeras (donor→recipient) infected i.v. with 5×10^5 CFU of *C. albicans* yeast. Results are pooled from two independent experiments (n=14-15). *p < 0.01 by log-rank test comparing WT→*Nlrp10*$^{-/-}$ to *Nlrp10*$^{-/-}$→WT; but no significant (ns) difference between WT→*Nlrp10*$^{-/-}$ and WT→WT and between *Nlrp10*$^{-/-}$→WT and *Nlrp10*$^{-/-}$→*Nlrp10*$^{-/-}$. (B) Unprimed and LPS-primed BMMϕ from WT and *Nlrp10*$^{-/-}$ mice were stimulated for 6 h with or without *C. albicans* yeast (MOI 10:1) and IL-1β secretion quantified by ELISA. (C) LPS-primed BMMϕ from WT, *Nlrp10*$^{-/-}$, and *ASC*$^{-/-}$ mice were challenged for 6 h with *C. albicans* (MOI 10:1) and *P. aeruginosa* (MOI 10:1), and for 9 h with *F. tularensis* LVS (MOI 50:1); IL-1β secretion

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was quantified by ELISA. (D) LPS-primed BMMϕ from WT, *Nlrp10*$^{-/-}$, and *ASC*$^{-/-}$ mice were challenged with silica (50 μg/cm^2) or nigericin (20μM) for 6 h and IL-1β secretion quantified by ELISA. (E) BMMϕ 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. Determinations were performed in triplicate and expressed as the mean ± SEM; results are representative of 3 independent experiments.

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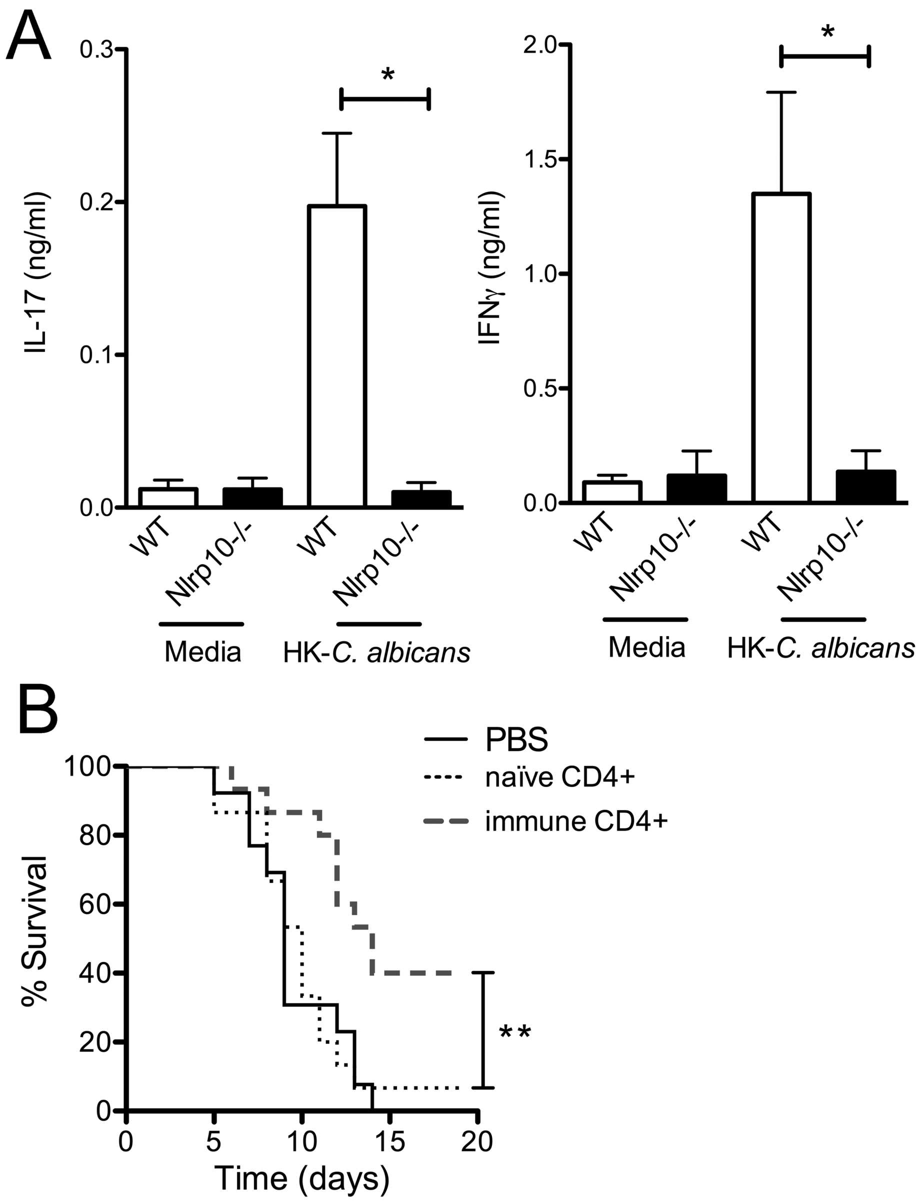

Fig. 3. Nlrp10-deficient mice fail to mount a protective T cell response against *C. albicans*

(A) WT and *Nlrp10*$^{-/-}$ mice were infected with a sublethal dose (5 × 10^4 CFU) of *C. albicans* yeast. 14 d p.i. splenocytes were restimulated with heat-killed *C. albicans*; 72 h later, IL-17 and IFNγ secretion into the supernatant was assessed by ELISA. Data are pooled from three independent experiments (n=6-8) and expressed as the mean ± SEM; *p < 0.05 by Student's t test. (B) 5 × 10^6 CD4$^+$ T cell from either naïve or immune WT mice were adoptively transferred into *Nlrp10*$^{-/-}$ mice which was then followed by i.v. infection with 5 × 10^5 CFU of *C. albicans* yeast and survival monitored. Data are pooled from two independent experiments (n=13-15). **p < 0.01 by log-rank test comparing *Nlrp10*$^{-/-}$ mice that received naïve CD4$^+$ T cell or PBS versus immune CD4$^+$ T cell.