TLR7 Expression Aggravates Invasive Pulmonary Aspergillosis by Suppressing Anti-*Aspergillus* Immunity of Macrophages

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ABSTRACT Toll-like receptors (TLRs) play a critical role in early immune recognition of *Aspergillus*, which can regulate host defense during invasive pulmonary aspergillosis (IPA). However, the role of TLR7 in the pathogenesis of IPA remains unknown. In this study, an *in vivo* model of IPA was established to investigate the contribution of TLR7 to host anti-*Aspergillus* immunity upon invasive pulmonary *Aspergillus fumigatus* infection. The effects of TLR7 on phagocytosis and killing capacities of *A. fumigatus* by macrophages and neutrophils were investigated *in vitro*. We found that TLR7 knockout mice exhibited lower lung inflammatory response and tissue injury, higher fungal clearance, and greater survival in an *in vivo* model of IPA compared with wild-type mice. TLR7 activation by R837 ligand led to wild-type mice being more susceptible to invasive pulmonary *Aspergillus fumigatus* infection. Macrophages, but not neutrophils, were required for the protection against IPA observed in TLR7 knockout mice. Mechanistically, TLR7 impaired phagocytosis and killing of *A. fumigatus* by macrophages but not neutrophils. Together, these data identify TLR7 as an important negative regulator of anti-*Aspergillus* innate immunity in IPA, and we propose that targeting TLR7 will be beneficial in the treatment of IPA.

KEYWORDS TLR7, infection, invasive pulmonary aspergillosis, macrophages

Invasive pulmonary aspergillosis (IPA) remains a major health problem with a rapidly evolving epidemiology and new groups of at-risk patients (1, 2). Emerging in-host adaptation and acquired triazole resistance in *Aspergillus fumigatus* highlights the urgent need for the development of new therapies (3, 4). The interplay of *A. fumigatus* and host immunity is critical to the pathogenesis of IPA; thus, a better understanding of the immune mechanisms involved in IPA may facilitate the identification of novel targets to improve host defense for future therapeutic intervention (5).

Toll-like receptors (TLRs), as a class of pattern recognition receptors, play a pivotal role in bridging innate immunity and adaptive immunity (6, 7). TLR7, primarily identified as the sensor for single-stranded RNA virus during viral infections, can also be triggered by immune modifiers that share a structure similar to that of nucleosides during bacterial or fungal infections (8, 9). A previous study demonstrated that the recognition of fungal RNA by TLR7 has a nonredundant role in host defense against systemic *Candida albicans* infection (10). A more recent study showed that TLR7 promoter flanking gene polymorphisms might be associated with invasive aspergillosis in Polish patients, suggesting that TLR7 participates in the recognition of *Aspergillus* (11). However, little is known of the functional role of TLR7 in the immunopathology of IPA.

IPA affects mostly immunocompromised patients and those undergoing

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pharmacological immunosuppression in particular. This is also reflected in infection models, and the treatment of mice with immunosuppressive agents leads to a strongly increased susceptibility to invasive *Aspergillosis* (12, 13). In the present study, we have investigated the role of TLR7 in host immune response, fungal clearance, lung injury, and mortality in a murine model of IPA. The data presented here linked TLR7 activation to the impaired anti-*Aspergillus* activity of macrophages and provided evidence for a previously unrecognized TLR7-mediated detrimental role in IPA.

RESULTS

Expression of TLR7 was upregulated during invasive pulmonary aspergillosis.

We began by measuring the gene expression of TLR7 in the lungs and spleens of wild-type (WT) mice that had been challenged intratracheally with *A. fumigatus* conidia. We found a significant induction of TLR7 mRNA levels in the lungs and spleens in response to *A. fumigatus* infection at 12 h in immunocompromised mice (see Fig. S1 in the supplemental material).

TLR7 deficiency increased the survival of mice after invasive pulmonary *A. fumigatus* infection. To investigate the role of TLR7 in response to *A. fumigatus* infection, the impact of full TLR7 deficiency on susceptibility to *Aspergillosis* was studied in an experimental model of IPA. WT C57BL/6 and TLR7^{-/-} C57BL/6 mice were compromised and subsequently subjected to lethal *Aspergillus* infection. TLR7^{-/-} mice showed a significantly increased survival rate compared to that of WT mice (Fig. 1A). In line with a decreased survival rate, WT mice presented with lethargy and piloerection at day 1 and day 3 after *Aspergillus* infection (Fig. 1B). To further elucidate the mechanism underlying increased survival of TLR7^{-/-} mice, we studied fungal loads in the lung at day 1 and day 3 after *Aspergillus* infection, showing significantly reduced fungal numbers in TLR7^{-/-} mice after *Aspergillus* inoculation (Fig. 1C and D).

TLR7 deficiency reduced pulmonary histopathological damage after invasive pulmonary *A. fumigatus* infection. Histological examination of the lungs of WT mice infected with *A. fumigatus* revealed more advanced signs of alveolar hemorrhage, fibrinous thrombi, and destruction of blood vessels compared with those of TLR7^{-/-} mice at 1 and 3 days after *A. fumigatus* infection (Fig. 2A). Lung pathology scores were also significantly lower in TLR7^{-/-} mice at 1 and 3 after *A. fumigatus* infection (Fig. 2B). Furthermore, the wet-to-dry lung tissue ratio, a marker of lung edema, was significantly lower in TLR7^{-/-} mice at day 1, and it was still lower at day 3 after *A. fumigatus* infection (Fig. 2C).

TLR7-deficient mice developed a milder inflammatory response after invasive pulmonary *A. fumigatus* infection. In line with the observation of reduced fungal numbers in the lungs, TLR7-deficient mice displayed significantly lower levels of interleukin-6 (IL-6) (on day 1 and 3), IL-1 β (on day 3), IL-17A (on day 3), or CXCL1 (on day 1) in their lungs relative to WT mice after invasive pulmonary *A. fumigatus* infection (Fig. 3A). However, no significant difference of tumor necrosis factor alpha (TNF- α), IL-4, IL-10, CCL2, gamma interferon (IFN- γ), IL-27, and IFN- α/β was observed between TLR7-deficient mice and WT mice after invasive pulmonary *A. fumigatus* infection. Furthermore, there were significantly lower numbers of leukocytes in the bronchoalveolar lavage fluid (BALF) from TLR7-deficient mice than with WT mice that comprised mainly neutrophils, monocytes/macrophages, and lymphocytes (Fig. 3B and Fig. S2). Therefore, the lack of TLR7 led to a milder inflammatory response in mice with invasive pulmonary aspergillosis.

TLR7 agonist predisposed WT mice to invasive pulmonary aspergillosis. To directly determine the influence of TLR7 signaling on the progression of invasive pulmonary aspergillosis, we tested the effects of the TLR7 ligand imiquimod (R837) in WT mice with concomitant invasive pulmonary *A. fumigatus* infection. Unlike R848, R837 activates TLR7 but not TLR8. R837-treated WT mice had significantly increased mortality compared with control medium-treated WT mice after invasive pulmonary *A. fumigatus* infection (Fig. 4A). R837-mediated stimulation of TLR7 also led to a significant decrease in controlling *A. fumigatus* germination on days 1 and 3 postinfection (Fig. 4B

FIG 1 TLR7-deficient mice were resistant to invasive pulmonary aspergillosis. (A) Age- and sex-matched C57BL/6 wild-type (WT) or TLR7-deficient (TLR7^{-/-}) mice were infected intratracheally with 6×10^6 *A. fumigatus* conidia. (A) Survival of WT and TLR7-deficient mice ($n = 15$ per group) over 14 days after *A. fumigatus* infection. Kaplan-Meier survival curves are shown, and significance was determined using log-rank test. P value was determined by log-rank survival test. Results are representative of three independent experiments. (B) Representative clinical appearance of WT and TLR7-deficient mice ($n = 5$ per group) on days 1 and 3 after invasive *A. fumigatus* infection. Results are representative of three independent experiments. (C) Number of CFU in the lungs of WT and TLR7-deficient mice ($n = 5$ per group) on days 1 and 3 after invasive *A. fumigatus* infection were analyzed. (D) Fungal burden as determined by amplification of *Aspergillus* ITS2 regions by quantitative PCR from lung homogenates ($n = 5$ per group). P value was determined by Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments.

and C). However, R837 treatment had no effects on survival (Fig. 4D) and fungal clearance (Fig. 4E and F) in TLR7-deficient mice upon invasive pulmonary *A. fumigatus* infection.

Protection against invasive aspergillosis elicited by TLR7 deficiency was mediated by macrophages. To further investigate the cellular mechanism of TLR7 on immune cells in invasive pulmonary aspergillosis, we depleted pulmonary macrophages using clodronate liposomes (Fig. S3) and neutrophils using anti-Ly6G monoclonal antibodies (Fig. S4) before invasive pulmonary *A. fumigatus* infection. The depletion of macrophages significantly aggravated mortality (Fig. 5A) and increased fungal loads from the lung (Fig. 5B and C) in TLR7-deficient mice upon invasive pulmonary *A. fumigatus* infection. In contrast, there was no significant change in the survival (Fig. 6A) and fungal clearance (Fig. 6B and C) of TLR7-deficient mice after the depletion of neutrophils in invasive pulmonary aspergillosis.

TLR7 inhibited phagocytosis and killing of *A. fumigatus* by macrophages. The *in vitro* anti-*Aspergillus* activity was further evaluated using purified macrophages and bone marrow-derived neutrophils from naive mice. Fungal uptake (Fig. 5D) and killing capacities (Fig. 5E) were significantly higher in macrophages isolated from TLR7^{-/-} mice than those from WT mice, and TLR7-deficient macrophages generated

FIG 2 Effects of TLR7 deficiency on lung inflammation and injury after invasive pulmonary *A. fumigatus* infection. Age- and sex-matched C57BL/6 wild-type (WT) or TLR7-deficient (TLR7^{-/-}) mice were infected intratracheally with 6×10^6 *A. fumigatus* conidia. At the indicated time points, mice were killed and lungs were saved for analysis. (A) Representative pathological and histological analysis (hematoxylin-eosin staining) of the lungs of WT and TLR7-deficient mice ($n=5$ per group) after invasive *A. fumigatus* infection. Results are representative of three independent experiments. (B) Pathology scores of pulmonary tissue from WT and TLR7-deficient mice ($n=5$ per group) on days 1 and 3 after invasive *A. fumigatus* infection were analyzed. *P* values were determined by Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments. (C) Wet-to-dry lung weight ratios from WT and TLR7-deficient mice ($n=5$ per group) on days 1 and 3 after invasive *A. fumigatus* infection were analyzed. *P* values were determined by Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments.

significantly more reactive oxygen species (ROS) in response to *A. fumigatus* infection than WT macrophages (Fig. 7). However, TLR7 did not influence fungal phagocytosis (Fig. 6D) and killing (Fig. 6E) by mouse neutrophils. These data suggest that the observed increased killing of *A. fumigatus* by macrophages in the setting of TLR7 deficiency can be explained by enhanced phagocytosis and increased ROS production.

DISCUSSION

We have shown here a novel function of TLR7 in exerting harmful effects during invasive pulmonary aspergillosis. We found that TLR7-deficient mice were more resistant to invasive pulmonary *A. fumigatus* infection than WT mice and that macrophages were important for this phenotype. TLR7 deficiency in murine macrophages augmented phagocytosis and killing of *A. fumigatus* as well as production of ROS in response to the pathogen. Clearly, all these results point to a detrimental role for TLR7 in the host response against invasive pulmonary *A. fumigatus* infection. In invasive pulmonary aspergillosis, recognition of fungal RNA by TLR7 on the release of nucleic acids from the fungal cell envelope could serve to facilitate primary *A. fumigatus* infection in the lung. In the absence of TLR7, such as in TLR7-deficient mice, *A. fumigatus* is more limited in multiplication, which consequently may alleviate the course of infection, resulting in increased survival.

FIG 3 Effects of TLR7 deficiency on cytokine production and cell infiltration in the lung after invasive pulmonary *A. fumigatus* infection. Age- and sex-matched C57BL/6 wild-type (WT) or TLR7-deficient (TLR7^{-/-}) (Continued on next page)

Various studies have demonstrated that TLR1-deficient, TLR2-deficient, TLR3-deficient, and TLR4-deficient mice and TLR6-deficient mice were more susceptible to invasive pulmonary aspergillosis than WT mice, and these TLR-deficient mice had significantly reduced fungal clearance and increased inflammation in the lungs upon invasive pulmonary *A. fumigatus* infection compared to WT mice (12, 14–16). However, the protective effects of TLR7 deficiency were in sharp contrast with TLR1, TLR2, TLR3, TLR4, and TLR6 deficiency during invasive pulmonary aspergillosis. A previous study has also shown that mice lacking TLR7 were highly susceptible to *C. albicans* infection, and *C. albicans* burden was significantly increased in mice lacking TLR7 (10). Collectively, these results suggest that signaling through the TLR7 pathway has a different role in host defense upon different fungal infections.

During respiratory syncytial virus (RSV) infection, TLR7 activation by imiquimod treatment could improve the course of acute disease, evidenced by decreased weight loss, RSV lung titers, and attenuated airway inflammation, in a murine model of RSV infection (17). A recent study has also shown that TLR7 agonist could induce transient viremia and reduce the viral reservoir in simian immunodeficiency virus (SIV)-infected rhesus macaques on antiretroviral therapy (18). In a proof-of-concept study, TLR7 could reduce plasma virus concentration in chronic hepatitis C infection (19). TLR7 activation also could enhance IL-22-mediated colonization resistance against vancomycin-resistant enterococcus (20). However, the TLR7 agonist in the present study predisposed naive mice to invasive pulmonary aspergillosis, as evidenced by increased mortality rate and decreased fungal clearance in WT mice, which is consistent with what was observed in TLR7-deficient mice on invasive pulmonary *A. fumigatus* infection. Furthermore, we have clarified that TLR7 loss could augment the intracellular killing capacity of *Aspergillus* conidia by macrophages, which was associated with enhanced phagocytosis and increased ROS production in the setting of TLR7 deficiency. In fact, several studies have shown that macrophages play an important role in orchestrating innate antifungal immunity in the lung upon *A. fumigatus* infection (13, 21, 22). Therefore, TLR7-mediated dysfunction of macrophages upon *A. fumigatus* infection contributes to the immunopathology of IPA.

Our results are also consistent with a previous study reporting that influenza A virus infection impaired the capacity of alveolar macrophages to phagocytose *S. pneumoniae* in a subsequent bacterial infection, this impairment being alleviated in TLR7-deficient mice (23). Type I and III interferons (IFNs) could modulate host immunity in various ways, and distinct aspects of this modulation are achieved through selective TLR7 triggering (23, 24). Therefore, the contribution of type I and III IFNs to TLR7-mediated susceptibility to invasive pulmonary aspergillosis requires further studies.

There are a few important limitations to this study. First, loss of TLR7 may be compensated by increased expression of other pattern recognition receptors in the macrophages, thereby leading to enhanced detection and control of the conidia. Although we have found that TLR7 deficiency did not influence the gene expression of TLR1, -2, -3, -4, -5, -6, and -9 in the macrophages (data not shown), we are unable to exclude an immunomodulatory role of other pattern recognition receptors in mediating the effects of TLR7 *in vivo* during IPA. Second, we do not know whether the increased phagocytosis and ROS generation by TLR7-deficient macrophages is specific for *Aspergillus* conidia or is a broader consequence of the

FIG 3 Legend (Continued)

mice were infected intratracheally with 6×10^6 *A. fumigatus* conidia, and at indicated time points mice were killed and bronchoalveolar lavage fluid (BALF) and lungs were saved for analysis. (A) Cytokine and chemokine concentrations in the lungs of WT and TLR7-deficient mice ($n=5$ per group) on days 1 and 3 after invasive *A. fumigatus* infection. *P* values were determined by Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments. (B) Cytospin centrifugation and Diff-Quik staining for BALF from WT and TLR7-deficient mice ($n=5$ per group) on days 1 and 3 after invasive *A. fumigatus* infection. The total numbers of leukocytes in the BALF were counted, and the total numbers of macrophages, neutrophils, and lymphocytes were also determined. *P* values were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons *post hoc* test. Results are representative of three independent experiments.

FIG 4 TLR7 agonist R837 reduced resistance of wild-type (WT) mice to invasive pulmonary aspergillosis. (A) Immunocompromised WT mice were pretreated with R837 (2.5 mg/kg), followed by an intratracheal challenge with 6×10^6 *A. fumigatus* conidia. Medium control served as control saline. Survival of WT mice ($n = 15$ per group) with or without R837 treatment over 14 days after *A. fumigatus* infection is shown. Kaplan-Meier survival curves are shown, and significance was determined using log-rank test. P value was determined by log-rank survival test. Results are representative of three independent experiments. (B) Fungal numbers in lungs of WT mice ($n = 5$ per group) with or without R837 pretreatment on days 1 and 3 after invasive *A. fumigatus* infection were analyzed. P values were determined by Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results were representative of three independent experiments. (C) Fungal burden as determined by amplification of *Aspergillus* ITS2 regions by quantitative PCR from lung homogenates ($n = 5$ per group). P values were determined by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments. (D) Immunocompromised TLR7-deficient mice were pretreated with R837 (2.5 mg/kg), followed by an intratracheal challenge with 6×10^6 *A. fumigatus* conidia. Medium control served as control saline. Survival of WT mice ($n = 15$ per group) with or without R837 treatment over 14 days after *A. fumigatus* infection is shown. Kaplan-Meier survival curves are shown, and no statistical significance was observed using log-rank test. Results are representative of three independent experiments. (E) Fungal numbers in lungs of TLR7-deficient mice ($n = 5$ per group) with or without R837 pretreatment on days 1 and 3 after invasive *A. fumigatus* infection were analyzed. No statistical significance was observed using Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments. (F) Fungal burden as determined by amplification of *Aspergillus* ITS2 regions by quantitative PCR from lung homogenates ($n = 5$ per group). No statistical significance was observed using Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments.

loss of TLR7, which requires further studies. Finally, we will continue to pursue the role of TLR7 in differentially regulating the expression of cytokines and chemokines after invasive pulmonary *A. fumigatus* infection.

Taken together, our present data highlight a detrimental role of TLR7 in the pathogenesis of invasive pulmonary aspergillosis, which placed TLR7 in a unique position in anti-*Aspergillus* host defense. Therefore, our findings not only reveal a previously unknown mechanism of action of TLR7 but also indicate that pharmacological TLR7 signaling inhibitors are promising targets for future therapeutic approaches to treat invasive pulmonary aspergillosis.

MATERIALS AND METHODS

Ethics statement. The study was approved by the Clinical Research Ethics Committee of The First Affiliated Hospital of Chongqing Medical University. All animal experiments were discussed with and approved by the Animal Care and Use Committee of the Chongqing Medical University and carried out according to the recommendations in the guide for the care and use of laboratory animals, conformed to animal protection laws of China and applicable guidelines.

FIG 5 Macrophages contributed to resistance to invasive pulmonary *Aspergillosis* in TLR7-deficient mice. (A) Mortality after macrophage depletion by clodronate liposomes and subsequent challenge with 6×10^6 *A. fumigatus* conidia in TLR7-deficient mice ($n = 15$ per group). Comparison between groups was done by Kaplan-Meier analysis followed by log-rank tests. P value was determined by log-rank survival test. Results are representative of three independent experiments. (B) Fungal numbers in lungs from TLR7-deficient mice ($n = 5$ per group) with or without macrophage depletion on days 1 and 3 after invasive pulmonary *A. fumigatus* infection. P values were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons *post hoc* test. Results are representative of three independent experiments. (C) Fungal burden as determined by amplification of *Aspergillus* ITS2 regions by quantitative PCR from lung homogenates ($n = 5$ per group). P values were determined by Kruskal-Wallis test followed by Dunn's multiple comparisons *post hoc* test. Results are representative of three independent experiments. (D) Macrophages were challenged with heat-inactivated fluorescein isothiocyanate (FITC)-labeled *Aspergillus* conidia for 4 h at 37°C. Arrows indicate engulfed conidia (as determined by overlay of green conidia) by macrophages, and cell nuclei were stained with DAPI (blue). Representative figures from five independent experiments were shown. Phagocytosis assay data are expressed as means $\pm$ SD from five independent experiments. P value was determined by Student's t test. (E) Macrophages isolated from WT and TLR7-deficient mice were infected with live *Aspergillus* conidia for 24 h. Percent conidicidal activity was then analyzed. Data were expressed as means $\pm$ SD from five independent experiments. P value was determined by Student's t test.

Mice. Female Toll-like receptor 7^{-/-} mice (Tlr7^{tm1Flv/J}) mice raised on the C57BL/6 background were purchased from the Jackson Laboratory. Sex- and age-matched wild-type (WT) C57BL/6 mice were used as controls. All animal experiments were done in accordance with the Institutional Animal Care and Use Committee's guidelines at the Chongqing Medical University.

Preparation of *Aspergillus fumigatus* conidia. WT *A. fumigatus* strain 293 was obtained from the Fungal Genetic Stock Center, and it was grown on Sabouraud dextrose agar plates for 5 days at 37°C. Conidia were harvested by adding 0.01% Tween 20 to plates and gently scraping conidia from the plates

FIG 6 Neutrophils played a limited role for resistance to invasive pulmonary aspergillosis in TLR7-deficient mice. (A) Mortality after neutrophil depletion using anti-Ly6G monoclonal antibodies and subsequent challenge with 6×10^6 *A. fumigatus* conidia in TLR7-deficient mice ($n=15$ per group). Comparison between groups was done by Kaplan-Meier analysis followed by log-rank tests. No statistical significance was observed by log-rank survival test. Results are representative of three independent experiments. (B) Fungal numbers in lungs from TLR7-deficient mice ($n=5$ per group) with or without neutrophil depletion on days 1 and 3 after invasive pulmonary *A. fumigatus* infection. No statistical significance was observed by Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments. (C) Fungal burden as determined by amplification of *Aspergillus* ITS2 regions by quantitative PCR from lung homogenates ($n=5$ per group). No statistical significance was observed using Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test. Results are representative of three independent experiments. (D) Murine bone marrow-derived neutrophils were challenged with heat-inactivated fluorescein isothiocyanate (FITC)-labeled *Aspergillus* conidia for 4 h at 37°C. Phagocytosis assay data are expressed as means $\pm$ SD from five independent experiments. No statistical significance was observed by Student's *t* test. (E) Murine bone marrow-derived neutrophils isolated from WT and TLR7-deficient mice were infected with live *Aspergillus* conidia for 24 h. Percent conidicidal activity was then analyzed. Data are expressed as means $\pm$ SD from five independent experiments. No statistical significance was observed by Student's *t* test.

using a cell scraper. Conidia were then filtered through a sterile 40- μ m cell strainer (BD Falcon), washed, resuspended in phosphate-buffered saline (PBS), and counted on a hemacytometer.

A murine model of invasive pulmonary aspergillosis. A murine model of invasive pulmonary aspergillosis was established using *A. fumigatus* strain Af293, as described in our previous study (25).

FIG 7 TLR7-mediated reactive oxygen species (ROS) release by macrophages upon *A. fumigatus* infection. Macrophages isolated from WT and TLR7-deficient mice were infected with *A. fumigatus* germs for 1 h, and ROS levels were then assayed. Representative examples from five independent experiments were shown for ROS release, and the data are shown as the means $\pm$ SD and compared to the control group by Student's *t* test. *P* value was determined by Student's *t* test.

Briefly, mice were immunosuppressed at day 3 and day 1 before infection and at day 1 after infection by intraperitoneal injection of 200 ml cyclophosphamide (Sigma-Aldrich) at 200 mg/kg of body weight. On the day of infection, mice were anesthetized with a cocktail of ketamine and xylazine administered intraperitoneally (i.p.), and subsequently mice were challenged with 6×10^6 live conidia per mouse using an intratracheal infection procedure.

Preparation of mouse organ samples. At designated time points, mice were sacrificed by CO₂ asphyxiation. Blood was drawn into heparinized tubes, and spleens and lungs were removed aseptically. For histologic examinations, lungs were perfused, inflated, fixed in 4% paraformaldehyde in PBS, and processed and stained with hematoxylin-eosin. For quantitative reverse transcriptase PCR for TLR7 expression, spleens and lungs were frozen in liquid nitrogen and stored at -80°C , and RNA of lungs and spleens was extracted as soon as possible. For cytokine assays, lung vasculature was perfused with 1 ml PBS containing 5 mM EDTA through the right ventricle, excised, frozen in liquid nitrogen, and stored at -20°C until the day of the assay. On the day of cytokine assay, samples were homogenized in complete protease inhibitor cocktail buffer (Roche Applied Science, Indianapolis, IN, USA) in PBS and sonicated. The resulting slurry was pelleted, and supernatants were passed through 0.45- μm filters (Gelman Sciences, Inc., Ann Arbor, MI, USA) and stored at 4°C for up to 3 days until cytokine assay.

Wet-to-dry weight ratio calculation. The wet-to-dry weight ratio for each group was calculated as the mean ratios from all 5 lung tissue samples. It was measured by weighing the lung samples immediately at the end of mouse sacrifice (wet weight). This lung was then placed in an Eppendorf tube, which was left open at room temperature for at least 14 days. Once the tissue was desiccated, it was weighed again (dry weight). A wet-to-dry lung weight ratio was then calculated.

BALF collection. Bronchoalveolar lavage fluid (BALF) was collected by washing the lungs with 2 ml of PBS containing 0.05 M EDTA. BALF was clarified by centrifugation and stored at -20°C until analysis. BALF cells were resuspended in 200 μl of PBS, and the total number of BALF cells was determined by hemacytometer count. BALF cells were subsequently spun onto glass slides using a Cytospin4 cytocentrifuge (Thermo Scientific) and stained with Diff-Quik stain set (Siemens) for differential counting. Differential cell counts were made from a minimum count of 300 cells in light microscopy. Cell counts were expressed for neutrophils, monocytes/macrophages, and lymphocytes.

Quantitative reverse transcriptase PCR for TLR7 expression. RNA was isolated according to the protocol supplied with the TRIzol reagent. Isolated mRNA (1 μg) was reverse transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa). Quantitative real-time PCR (qPCR) was performed using SYBR premix Ex Taq II (TaKaRa) and the following primers (all manufactured by Sangon Biotech [Shanghai]) for mouse samples: mTlr7 forward (Fwd), 5'-GATCGTGGACTGCACAGACA-3', and reverse (Rev) 5'-CCAGATGGTTCAGCTACGG-3'; m β -actin Fwd, 5'-GCCAGCACAGCTTCTT-3', and Rev, 5'-TGACCCATTCCACCAT-3'. PCR was performed using PCR conditions of 10 min 95°C followed by 40 cycles at 95°C for 10 s and 60°C for 1 min. Amplification efficiencies were validated and normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Each data point was examined for integrity by analysis of the amplification plot. The mRNA-normalized data were expressed as relative gene mRNA in treated compared to untreated experimental groups.

Determination of *A. fumigatus* CFU numbers. Lungs were collected aseptically and homogenized in 1 ml of sterile PBS. Three replicates of 50 μl of serial dilutions of the lung homogenate were then cultured overnight on Sabouraud dextrose agar plates at 37°C . CFU numbers were determined after 24 h.

***Aspergillus* assessment by quantitative PCR.** To further determine *Aspergillus* burden in the lungs, mice were sacrificed at the indicated times postinoculation, and lungs were harvested and immediately frozen in liquid nitrogen. Homogenized lungs were used for DNA isolation by using the automated MagNA Pure system and the MagNA Pure LC total nucleic acid isolation kit according to the manufacturer's protocol (Roche Applied Science). The fungal burden was determined by amplification of *Aspergillus* ITS2 regions. *Aspergillus* loads were assessed by quantitative PCR using the LC480 instrument and the probe master kit (Roche Applied Science) as described previously (13).

Pulmonary histopathology. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: bronchitis, edema, interstitial inflammation, intra-alveolar inflammation, pleuritis, endothelialitis, and percentage of the lung surface demonstrating confluent inflammatory infiltrate. Each parameter was graded from 0 (absent) to 4 (severe). The total pathology scores for lungs were analyzed by a pathologist blinded for groups using a BH2 microscope (Olympus) and expressed as the sum of the score for all parameters.

Total protein assessment. Total proteins (bicinchoninic acid protein assay kit; Beyotime Biotechnology, Shanghai, China) for lung permeability assessment were quantified according to the manufacturer's instructions.

Cytokine/chemokine measurements. The cytokine/chemokine levels in the supernatants of lung homogenate were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the protocol supplied by the manufacturer. IL-27, CXCL1, and CCL2 assays were from R&D Systems, and IFN- α/β , IFN- γ , TNF- α , IL-1 β , IL-4, IL-6, IL-10, and IL-17A were from BioLegend.

Flow cytometry. Cells in BALF were washed in PBS, pelleted, and subsequently stained for flow cytometry. Cell counts were characterized accordingly with peridinin chlorophyll protein (PerCP)-conjugated anti-CD45, allophycocyanin (APC)-conjugated anti-CD11b, fluorescein isothiocyanate (FITC)-conjugated anti-Ly6G, and phycoerythrin (PE)-cy5-conjugated anti-F4/80 monoclonal antibodies (BD Pharmingen). The absolute numbers of CD11b⁺ Ly6G⁺ neutrophils and CD11b⁺ F4/80⁺ macrophages were then quantified by dividing the number of positive cellular events by the number of bead events and multiplying the result by the bead concentration using a FACScan flow cytometer (Becton, Dickinson). Absolute numbers were derived from the pooled sample of 5 biopsy specimens.

Murine macrophage and neutrophil harvesting and culture. Alveolar macrophages were isolated by adherence of bronchoalveolar lavage fluids for 1 h in Dulbecco's minimum essential medium at 37°C, 5% CO₂, and nonadherent cells were removed by the replacement of culture medium with antibiotic-free medium. Neutrophils from the bone marrow were purified by discontinuous Percoll gradient centrifugation followed by magnetic cell sorting using a mouse neutrophil isolation kit (Miltenyi Biotec Inc, Auburn, CA, USA) according to the manufacturer's instructions.

In vivo macrophage depletion. The clodronate-encapsulated liposomes and PBS-encapsulated liposomes were prepared as described previously (26). Clodronate-encapsulated liposomes were delivered intranasally (200 µl) to deplete macrophages. PBS-encapsulated liposomes were delivered in a similar fashion as a control. Clodronate liposome treatment resulted in a 90% decrease of macrophages in the lung by flow cytometry using PE-cy5-conjugated anti-F4/80 (eBioscience).

In vivo neutrophil depletion. Neutrophil depletion was performed as previously described (27, 28). Briefly, mice were injected intravenously (i.v.) with 0.1 mg of RB6-8C5 monoclonal antibodies (MAb) to mouse Ly6G (clone RB6-8C5; eBioscience) with rat IgG2b as a control. This treatment depleted 95% of neutrophils in the blood and 90% of neutrophils in the spleen by flow cytometry using APC-conjugated anti-CD11b (BD Pharmingen) and FITC-conjugated anti-Ly6G (BD Pharmingen).

Phagocytosis assays. For macrophages, FITC-labeled *Aspergillus* conidia were prepared by incubation with 0.5 mg/ml FITC (Sigma, Poole, UK) for 20 min at 37°C. Macrophages (1×10^5 cells) were incubated with heat-inactivated FITC-labeled conidia at a multiplicity of infection (MOI) of 10 for 4 h at 37°C. After washing steps, cell nuclei were stained with DAPI (Invitrogen), followed by visualization using confocal laser scanning microscopy (LSM 510; Zeiss). Neutrophils were plated in 24 flat-bottom plates at 5×10^5 cells/well. Cells were allowed to phagocytose 5×10^6 (MOI, 1:10) heat-inactivated FITC-labeled conidia for 4 h. Subsequently, the fluorescence signal of extracellular nonphagocytosed conidia was quenched using 0.2% trypan blue. The cells were measured on a Cytoflex flow cytometer (Becton, Dickinson), and the data were analyzed using Kaluza software (Becton, Dickinson). The neutrophils that phagocytosed one or more conidia were enumerated by their positivity for the FITC signal and could be divided into an FITC-negative (neutrophils that did not engulf conidia) and an FITC-positive (neutrophils that engulfed conidia) population. In some experiments, macrophages and neutrophils were pretreated with imiquimod (R837) (100 ng/ml; InvivoGen) before infection with *Aspergillus* conidia.

***Aspergillus* killing assays.** Freshly isolated macrophages (1×10^5) were exposed to 1×10^5 live *Aspergillus* conidia (MOI, 1:1) in 24-well plates at a final volume of 500 µl. After 24 h at 37°C, extracellular nonphagocytosed conidia were washed in PBS and new culture medium was added to the wells for killing. The additional wells of cells were lysed in water and plated in serial dilution on Sabouraud agar plates. CFU were counted after 24 h at 37°C. In another experiment, 5×10^5 neutrophils were exposed to *Aspergillus* conidia (MOI, 1:1) after 24 h at 37°C, and the cells were washed in water and plated in serial dilution on Sabouraud agar plates. CFU were counted after 24 h at 37°C. In some experiments, macrophages and neutrophils were pretreated with imiquimod (R837) (100 ng/ml; InvivoGen) before infection with *Aspergillus* conidia.

ROS measurement. Macrophages (1×10^5) were incubated with *A. fumigatus* germs (multiplicity of infection, 100) for 1 h. To measure the total intracellular ROS levels, macrophages were treated with the fluorogenic probe H2DFFDA (Life Technologies) at 5 µM for 30 min at 37°C. The medium was then removed, and the cells were returned to prewarmed fresh growth medium. The emitted fluorescence was detected by a fluorescent microplate reader using 490/520-nm excitation/emission filters (Molecular Devices, Sunnyvale, CA). The ROS levels are reported as fluorescence intensity.

Statistical analysis. Mouse data were expressed as box-and-whisker plots showing the smallest observation, lower quartile, median, upper quartile, and largest observation or as medians with interquartile ranges. *Aspergillus* phagocytosis/killing assay and ROS release data were expressed as means $\pm$ standard deviations (SD). Mann-Whitney *U* test or Kruskal-Wallis test followed by Dunn's multiple-comparison *post hoc* test was performed for data of nonnormal distribution. Student's *t* test was for data of normal distribution. For murine survival studies, Kaplan-Meier analyses followed by log-rank tests were performed. All analyses were done using GraphPad Prism version 5.01 (GraphPad Software). *P* values of less than 0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

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We have no competing interests to declare.

REFERENCES

1. Camargo JF, Husain S. 2014. Immune correlates of protection in human invasive aspergillosis. *Clin Infect Dis* 59:569–577. <https://doi.org/10.1093/cid/ciu337>.
2. Kosmidis C, Denning DW. 2015. The clinical spectrum of pulmonary aspergillosis. *Thorax* 70:270–277. <https://doi.org/10.1136/thoraxjnl-2014-206291>.
3. Lestrade PPA, Meis JF, Melchers WJG, Verweij PE. 2019. Triazole resistance in *Aspergillus fumigatus*: recent insights and challenges for patient management. *Clin Microbiol Infect* 25:799–806. <https://doi.org/10.1016/j.cmi.2018.11.027>.
4. Rybak JM, Ge W, Wiederhold NP, Parker JE, Kelly SL, Rogers PD, Fortwendel JR. 2019. Mutations in *hmg1*, challenging the paradigm of clinical triazole resistance in *Aspergillus fumigatus*. *mBio* 10:e00437-19. <https://doi.org/10.1128/mBio.00437-19>.
5. Lehnbecher T, Kalkum M, Champer J, Tramsen L, Schmidt S, Klingebiel T. 2013. Immunotherapy in invasive fungal infection—focus on invasive aspergillosis. *Curr Pharm Des* 19:3689–3712. <https://doi.org/10.2174/1381612811319200010>.
6. Hennessy EJ, Parker AE, O'Neill LA. 2010. Targeting Toll-like receptors: emerging therapeutics? *Nat Rev Drug Discov* 9:293–307. <https://doi.org/10.1038/nrd3203>.
7. Liu CH, Liu H, Ge B. 2017. Innate immunity in tuberculosis: host defense vs pathogen evasion. *Cell Mol Immunol* 14:963–975. <https://doi.org/10.1038/cmi.2017.88>.
8. Jian W, Gu L, Williams B, Feng Y, Chao W, Zou L. 2019. Toll-like receptor 7 contributes to inflammation, organ injury, and mortality in murine sepsis. *Anesthesiology* 131:105–118. <https://doi.org/10.1097/ALN.0000000000002706>.
9. Maeda K, Akira S. 2016. TLR7 structure: cut in Z-loop. *Immunity* 45:705–707. <https://doi.org/10.1016/j.immuni.2016.10.003>.
10. Biondo C, Malara A, Costa A, Signorino G, Cardile F, Midiri A, Galbo R, Papasergi S, Domina M, Pugliese M, Teti G, Mancuso G, Beninati C. 2012. Recognition of fungal RNA by TLR7 has a nonredundant role in host defense against experimental candidiasis. *Eur J Immunol* 42:2632–2643. <https://doi.org/10.1002/eji.201242532>.
11. Skonieczna K, Woźniacka A, Czajkowski R, Styczyński J, Krenska A, Robak E, Gawrych M, Kaszewski S, Wysocki M, Grzybowski T. 2018. X-linked TLR7 gene polymorphisms are associated with diverse immunological conditions but not with discoid lupus erythematosus in polish patients. *Postepy Dermatol Alergol* 35:26–32. <https://doi.org/10.5114/pdia.2017.69984>.
12. Balloy V, Si-Tahar M, Takeuchi O, Philippe B, Nahori MA, Tanguy M, Huerre M, Akira S, Latgé JP, Chignard M. 2005. Involvement of Toll-Like receptor 2 in experimental invasive pulmonary aspergillosis. *Infect Immun* 73:5420–5425. <https://doi.org/10.1128/IAI.73.9.5420-5425.2005>.
13. Gresnigt MS, Cunha C, Jaeger M, Gonçalves SM, Malireddi RKS, Ammerdorffer A, Lubbers R, Oosting M, Rasid O, Jouvion G, Fitting C, Jong DJ, Lacerda JF, Campos A, Jr, Melchers WJG, Lagrou K, Maertens J, Kanneganti TD, Carvalho A, Ibrahim-Granet O, van de Veerdonk FL. 2018. Genetic deficiency of NOD2 confers resistance to invasive aspergillosis. *Nat Commun* 9:2636. <https://doi.org/10.1038/s41467-018-04912-3>.
14. Bellocchio S, Moretti S, Perruccio K, Fallarino F, Bozza S, Montagnoli C, Mosci P, Lipford GB, Pitzurra L, Romani L. 2004. TLRs govern neutrophil activity in aspergillosis. *J Immunol* 173:7406–7415. <https://doi.org/10.4049/jimmunol.173.12.7406>.
15. Carvalho A, De Luca A, Bozza S, Cunha C, D'Angelo C, Moretti S, Perruccio K, Iannitti RG, Fallarino F, Pierini A, Latgé JP, Velardi A, Aversa F, Romani L. 2012. TLR3 essentially promotes protective class I-restricted memory CD8⁺ T-cell responses to *Aspergillus fumigatus* in hematopoietic transplanted patients. *Blood* 119:967–977. <https://doi.org/10.1182/blood-2011-06-362582>.
16. Rubino I, Coste A, Roy DL, Roger T, Jaton K, Boeckh M, Monod M, Latgé JP, Calandra T, Bochud PY. 2012. Species-specific recognition of *Aspergillus fumigatus* by Toll-like receptor 1 and Toll-like receptor 6. *J Infect Dis* 205:944–954. <https://doi.org/10.1093/infdis/jir882>.
17. Salinas FM, Nebreda AD, Vázquez L, Gentilini MV, Marini V, Benedetti M, Nabaes Jodar MS, Viegas M, Shayo C, Bueno CA. 2020. Imiquimod suppresses respiratory syncytial virus (RSV) replication via PKA pathway and reduces RSV induced-inflammation and viral load in mice lungs. *Antiviral Res* 179:104817. <https://doi.org/10.1016/j.antiviral.2020.104817>.
18. Lim SY, Osuna CE, Hrabert PT, Hesselgesser J, Gerold JM, Barnes TL, Sanisetty S, Seaman MS, Lewis MG, Gelezianus R, Miller MD, Cihlar T, Lee WA, Hill AL, Whitney JB. 2018. TLR7 agonists induce transient viremia and reduce the viral reservoir in SIV-infected rhesus macaques on antiretroviral therapy. *Sci Transl Med* 10:eaa04521. <https://doi.org/10.1126/scitranslmed.aao4521>.
19. Horsmans Y, Berg T, Desager JP, Mueller T, Schott E, Fletcher SP, Steffy KR, Bauman LA, Kerr BM, Averett DR. 2005. Isatoribine, an agonist of TLR7, reduces plasma virus concentration in chronic hepatitis C infection. *Hepatology* 42:724–731. <https://doi.org/10.1002/hep.20839>.
20. Abt MC, Buffie CG, Sušac B, Becattini S, Carter RA, Leiner I, Keith JW, Artis D, Osborne LC, Pamer EG. 2016. TLR-7 Activation enhances IL-22-mediated colonization resistance against vancomycin-resistant *Enterococcus*. *Sci Transl Med* 8:327ra25. <https://doi.org/10.1126/scitranslmed.aad6663>.
21. Espinosa V, Jhingran A, Dutta O, Kasahara S, Donnelly R, Du P, Rosenfeld J, Leiner I, Chen CC, Ron Y, Hohl TM, Rivera A. 2014. Inflammatory monocytes orchestrate innate antifungal immunity in the lung. *PLoS Pathog* 10:e1003940. <https://doi.org/10.1371/journal.ppat.1003940>.
22. Rosowski EE, Raffa N, Knox BP, Golenberg N, Keller NP, Huttenlocher A. 2018. Macrophages inhibit *Aspergillus fumigatus* germination and neutrophil-mediated fungal killing. *PLoS Pathog* 14:e1007229. <https://doi.org/10.1371/journal.ppat.1007229>.
23. Stegemann-Koniszewski S, Gereke M, Orrskog S, Lienenklaus S, Pasche B, Bader SR, Gruber AD, Akira S, Weiss S, Henriques-Normark B, Bruder D, Gunzer M. 2013. TLR7 contributes to the rapid progression but not to the overall fatal outcome of secondary pneumococcal disease following influenza A virus infection. *J Innate Immun* 5:84–96. <https://doi.org/10.1159/000345112>.
24. Goel RR, Wang X, O'Neil LJ, Nakabo S, Hasneen K, Gupta S, Wigerblad G, Blanco LP, Kopp JB, Morasso MI, Kotenko SV, Yu ZX, Carmona-Rivera C, Kaplan MJ. 2020. Interferon lambda promotes immune dysregulation and tissue inflammation in TLR7-induced lupus. *Proc Natl Acad Sci U S A* 117:5409–5419. <https://doi.org/10.1073/pnas.1916897117>.
25. Liu S, Chen D, Luo Q, Gong Y, Yin Y, Cao J. 2020. IL-27 negatively controls antifungal activity in a model of invasive pulmonary aspergillosis. *Am J Respir Cell Mol Biol* 62:760–766. <https://doi.org/10.1165/rcmb.2019-0391OC>.
26. Cao J, Xu F, Lin S, Song Z, Zhang L, Luo P, Xu H, Li D, Zheng K, Ren G, Yin Y. 2014. IL-27 Controls sepsis-induced impairment of lung antibacterial host defence. *Thorax* 69:926–937. <https://doi.org/10.1136/thoraxjnl-2014-205777>.
27. Lin X, Luo H, Yan X, Song Z, Gao X, Xia Y, Zhang L, Yin Y, Cao J. 2018. Interleukin-34 ameliorates survival and bacterial clearance in polymicrobial sepsis. *Crit Care Med* 46:e584–e590. <https://doi.org/10.1097/CCM.0000000000003017>.
28. Yang X, Yin Y, Yan X, Yu Z, Liu Y, Cao J. 2019. Flagellin attenuates experimental sepsis in a macrophage-dependent manner. *Crit Care* 23:106. <https://doi.org/10.1186/s13054-019-2408-7>.