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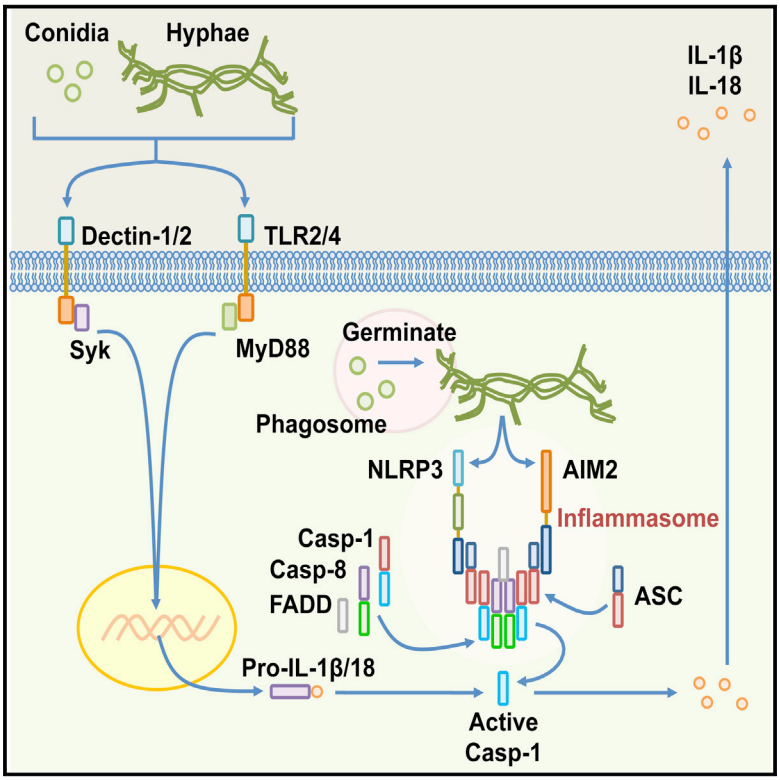
Concerted Activation of the AIM2 and NLRP3 Inflammasomes Orchestrates Host Protection against *Aspergillus* Infection

Graphical Abstract



Highlights

- Mice lacking both AIM2 and NLRP3 are highly susceptible to aspergillosis
- AIM2 and NLRP3 are required to confine *Aspergillus* to inflammatory foci in the lung
- Activation of AIM2 and NLRP3 induces a single cytoplasmic inflammasome platform
- AIM2- and NLRP3-mediated release of IL-1β and IL-18 is crucial for survival

Authors

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In Brief

Aspergillosis is a major health concern for immunocompromised individuals. Karki et al. show that two cytosolic inflammasome receptors, AIM2 and NLRP3, protect against *A. fumigatus* infection. AIM2 and NLRP3 activation induces a single cytoplasmic inflammasome platform containing ASC, caspase-1, and caspase-8 that mediates pro-inflammatory cytokine release and *A. fumigatus* clearance.

Karki et al., 2015, Cell Host & Microbe *17*, 357–368
March 11, 2015
http://dx.doi.org/10.1016/j.chom.2015.01.006

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Concerted Activation of the AIM2 and NLRP3 Inflammasomes Orchestrates Host Protection against *Aspergillus* Infection

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http://dx.doi.org/10.1016/j.chom.2015.01.006

SUMMARY

Invasive pulmonary aspergillosis is a leading cause of infection-associated mortality in immunocompromised individuals. *Aspergillus fumigatus* infection produces ligands that could activate inflammasomes, but the contribution of these host defenses remains unclear. We show that two inflammasome receptors, AIM2 and NLRP3, recognize intracellular *A. fumigatus* and collectively induce protective immune responses. Mice lacking both AIM2 and NLRP3 fail to confine *Aspergillus* hyphae to inflammatory foci, leading to widespread hyphal dissemination to lung blood vessels. These mice succumb to infection more rapidly than WT mice or mice lacking a single inflammasome receptor. AIM2 and NLRP3 activation initiates assembly of a single cytoplasmic inflammasome platform, composed of the adaptor protein ASC along with caspase-1 and caspase-8. Combined actions of caspase-1 and caspase-8 lead to processing of pro-inflammatory cytokines IL-1β and IL-18 that critically control the infection. Thus, AIM2 and NLRP3 form a dual cytoplasmic surveillance system that orchestrates responses against *A. fumigatus* infection.

INTRODUCTION

Invasive pulmonary aspergillosis is a leading cause of deaths in immunocompromised individuals (Segal, 2009). *Aspergillus fumigatus* is the major cause of invasive pulmonary aspergillosis. *Aspergillus* is a ubiquitous saprophytic mold that produces a large number of airborne spores known as conidia. These spores are inhaled on a daily basis and do not cause disease in healthy individuals owing to pulmonary defense mechanisms. In immunocompromised individuals, however, inhaled conidia metamorphosize into hyphal form to cause disease. Individuals who are highly susceptible to aspergillosis include transplant patients, patients with leukemia, those undergoing prolonged corticosteroid therapy or chemotherapy, individuals with chronic granulomatous disease, and those infected with HIV (Dagenais and Keller, 2009). Despite the advent of newer anti-fungal agents, mortality rates in transplant recipients with invasive aspergillosis ranged from 60% to over 90% (Singh and Paterson, 2005).

Innate immune defenses are critical in conferring host resistance to aspergillosis (Gresnigt and van de Veerdonk, 2014). Alveolar macrophages are able to clear conidia by phagocytosis to prevent spore germination (Philippe et al., 2003). Conidia that escape effector functions of alveolar macrophages develop into germtubes and hyphae, whereby effective killing and control require neutrophils (Prüfer et al., 2014). A number of fungal components within *A. fumigatus* serve as ligands for a family of innate immune receptors known as pattern-recognition receptors. Recognition of *Aspergillus* by host cells requires Toll-like receptors (TLRs) TLR2 (Mambula et al., 2002; Meier et al., 2003), TLR4 (Netea et al., 2003; Wang et al., 2001), and TLR9 (Ramaprakash et al., 2009; Ramirez-Ortiz et al., 2008) and C-type lectin receptors (CLRs) dectin-1 (Hohl et al., 2005; Steele et al., 2005) and dectin-2 (Barrett et al., 2009). Activation of these surface-associated TLRs or CLRs by *Aspergillus* conidia or hyphae mediates pro-inflammatory cytokine production and the synthesis of biologically inactive pro-IL-1β, which requires further processing (Gresnigt and van de Veerdonk, 2014).

Host cells are also equipped with cytoplasmic sensors, including AIM2-like receptors (ALRs) and nucleotide-binding domain and leucine-rich repeat-containing family receptors (NLRs). Activation of these sensors triggers assembly of the inflammasome, a multi-meric protein complex containing caspase-1 that converts pro-IL-1β and pro-IL-18 into their biologically active forms. Inflammasome assembly requires the adaptor protein ASC, which bridges the inflammasome receptors with caspase-1. Inflammasome receptors include AIM2, NLRP1, NLRP3, and NLRC4, which recognize diverse pathogen-associated or danger-associated molecular patterns. For example, AIM2 recognizes dsDNA (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Rathinam et al., 2010; Roberts et al., 2009), whereas NLRP3 responds to a range of activators, including ATP, silica, bacterial toxin, RNA, and DNA (Cassel et al., 2008; Dostert et al., 2008; Hornung et al., 2008; Kailasan

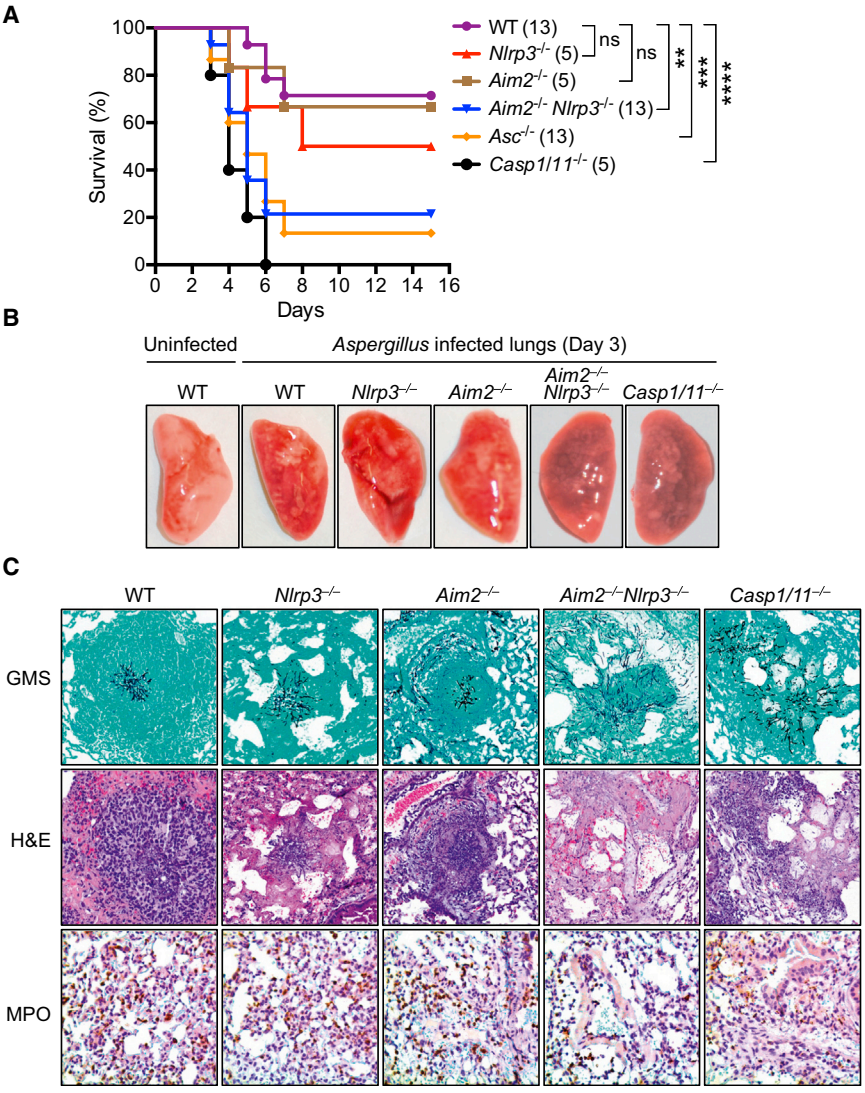

Figure 1. NLRP3 and AIM2 Inflammasomes Provide Resistance to *Aspergillus*-Induced Mortality

(A) Survival of mice infected with 1 × 10^5 *A. fumigatus* conidia after immunosuppression with cyclophosphamide and cortisone acetate.

(B) Gross pathology of *A. fumigatus*-infected lungs collected on day 3.

(C) Gomori methenamine silver (GMS), H&E, and myeloperoxidase (MPO) staining of *A. fumigatus*-infected lungs collected on day 3. Data represent two independent experiments. (A) Log-rank test, **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, not statistically significant. See also Figures S1 and S6.

Vanaja et al., 2014; Kanneganti et al., 2006; Mariathasan et al., 2006; Sander et al., 2011).

The role of inflammasomes in the host response to fungal pathogens is well established for *Candida albicans*. In human primary monocytes, *N*-mannan-linked residues, chitin, and β-glucan from *C. albicans* efficiently induce IL-1β secretion owing to the presence of constitutively active caspase-1 (Lamkanfi et al., 2009; van de Veerdonk et al., 2009). In mouse bone marrow-derived dendritic cells (BMDCs) or macrophages, TLR2, dectin-1, and Syk provide a priming signal, while NLRP3 assembles the inflammasome in response to *C. candida* infection (Gross et al., 2009; Hise et al., 2009; Joly et al., 2009). Little is known regarding the role of inflammasomes in the recognition and control of *A. fumigatus* infection. A previous study suggests that hyphal fragments derived from *A. fumigatus* induce reactive oxygen species (ROS) that provide a potential signal to trigger the NLRP3-caspase-1 inflammasome in the human THP-1 cell line (Saïd-Sadier et al., 2010). In contrast, another report found that caspase-1 is dispensable for IL-1β production in human dendritic cells in response to *A. fumigatus* conidia (Gringhuis et al., 2012). The intracellular receptor that engages inflammasome activation and the physiological function of the inflammasomes in response to *Aspergillus* infection remains to be elucidated.

In this study, we found that both AIM2 and NLRP3 engage the inflammasome to trigger innate immune responses against *A. fumigatus* infection. Mice lacking both AIM2 and NLRP3, but not mice lacking a single inflammasome receptor, are hypersusceptible to invasive aspergillosis. Bone marrow transplantation experiments revealed that both the hematopoietic and stromal compartments mediate protection against fungal dissemination in the lungs. Mechanistically, both receptors activate caspase-1 and induce processing of IL-1β and IL-18 by recruiting caspase-8 to the same inflammasome complex. IL-1β, IL-18, caspase-8, and FADD are crucial for preventing *Aspergillus*-induced mortality. Together, these results unveiled a dual cytoplasmic sensing mechanism governed by AIM2 and NLRP3 to orchestrate robust host responses against *A. fumigatus*.

RESULTS

AIM2 and NLRP3 Provide Dual Surveillance and Protection against *Aspergillus* Infection

Inflammasome receptors, including NLRP3 and AIM2, activate caspase-1 and proteolytic cleavage of pro-inflammatory cytokines IL-1β and IL-18. However, the physiological role of inflammasomes in the host defense against the fungal pathogen *A. fumigatus* is unclear. To investigate this, we infected WT, *Aim2*$^{-/-}$, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, and *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice with *A. fumigatus* conidia intranasally and monitored for their survival over time. Mice were first immunocompromised with cyclophosphamide and cortisone acetate to mimic the immunocompromised status of susceptible individuals. Following intranasal infection with 10^5 conidia, we observed that mice lacking either AIM2 or NLRP3 showed similar susceptibility to *Aspergillus*-induced mortality compared to WT mice (Figure 1A). The lungs

of mice lacking either AIM2 or NLRP3 showed similar gross damage and hemorrhage (Figure 1B). Interestingly, 100% of the mice lacking caspase-1 and -11 and 90% of the mice lacking ASC, the common inflammasome adaptor protein, succumbed to infection after 6 and 7 days, respectively (Figure 1A).

We hypothesized that inflammasome receptors are playing redundant roles in response to *A. fumigatus* infection. To address this, we crossed mice lacking AIM2 and NLRP3 to generate mice deficient in both inflammasome receptors and infected these with *Aspergillus*. In line with our hypothesis, *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice were significantly more susceptible to aspergillosis, with 80% of the *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice succumbing to *Aspergillus* infection after 6 days compared to 20% of the WT mice (Figure 1A). The lungs from mice lacking both AIM2 and NLRP3 or caspase-1 and -11 showed increased gross damage and hemorrhage (Figure 1B). Mice that were not given cyclophosphamide and cortisone acetate failed to succumb to infection; however, *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice lost more body weight after 2–4 days post-infection (Figures S1A and S1B).

We performed histological analysis on lung tissues from mice infected with *A. fumigatus* for 3 days. Gomori methenamine silver (GMS) staining was used to visualize fungal distribution and revealed that *Aspergillus* hyphae localized in small foci within the lung tissues of WT mice (Figure 1C). In contrast, *Aspergillus* hyphae were widespread and failed to confine to foci in the lung tissues of *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice (Figure 1C). The majority of blood vessels in the lungs of *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice were colonized with *Aspergillus* hyphae, with hyphae penetrating blood vessel walls and surrounding connective tissues and alveoli (Figure 1C). We quantified our histologic observations and measured the area covered by hyphae in lung tissue sections. These analyses showed that the lung area colonized by *Aspergillus* hyphae in *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice was greater than the areas colonized in WT mice (Figure S1C). Mice lacking either AIM2 or NLRP3 showed a similar susceptibility to hyphal dissemination compared to WT mice, whereas mice lacking caspase-1 and -11 phenocopied mice lacking both AIM2 and NLRP3 (Figure S1C).

H&E staining of lung tissues from WT mice revealed *Aspergillus* hyphae surrounded by a large number of immune cells composed of granulocytes, lymphocytes, histiocytes, macrophages, and plasma cells (Figure 1C). In addition, we observed a composite of immune cells encompassing most large blood vessels and associated foci of hemorrhage extending from perivascular connective tissues into the surrounding alveoli (Figure 1C). In lung tissues of *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice, we observed minimal inflammatory reaction to *Aspergillus* hyphae; however, edema and hemorrhage were present where intravascular thrombi were frequently identified in affected blood vessels (Figure 1C). Quantification of the number of myeloperoxidase (MPO)-positive cells following immunohistochemistry staining revealed a significantly greater number of MPO-positive cells in the lungs of *Aspergillus*-infected WT mice compared to *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ or *Casp1*$^{-/-}$*Casp11*$^{-/-}$ mice (Figures 1C and S1D). There was a modest reduction in the number of MPO-positive cells in mice lacking either AIM2 or NLRP3 compared to WT mice ($p > 0.05$; Figures 1C and S1D). These results collectively indicate that AIM2 and NLRP3 control *Aspergillus* dissemination within the lung tissues, which confers resistance to *Aspergillus*-induced mortality.

AIM2 and NLRP3 Function in the Hematopoietic and Stromal Compartments to Confer Resistance to *Aspergillus* Dissemination

Since AIM2 and NLRP3 provide protection against invasive pulmonary aspergillosis, we asked whether the bone marrow or the stromal compartments facilitate host defense against infection. We generated four groups of mice: (1) irradiated WT mice receiving WT bone marrow (control), (2) irradiated WT mice receiving DKO bone marrow (*Aim2*- and *Nlrp3*-deficient hematopoietic compartment), (3) irradiated DKO mice receiving WT bone marrow (*Aim2*- and *Nlrp3*-deficient stromal compartment), and (4) irradiated DKO mice receiving DKO bone marrow (global *Aim2* and *Nlrp3* deficiency). We infected these mice intranasally with 10^5 conidia following immunosuppression procedures and monitored for their survival. We observed that mice lacking AIM2 and NLRP3 in both the bone marrow and stromal compartments were significantly more susceptible to *Aspergillus*-induced mortality compared to WT mice receiving WT bone marrow (Figure 2A). Substantial gross damage was observed in the lungs of these mice compared to other groups (Figure 2B).

Mice lacking AIM2 and NLRP3 in either the bone marrow or stromal compartments were slightly more susceptible than WT controls; however, this was not statistically significant (Figure 2A). Indeed, GMS staining revealed that mice lacking AIM2 and NLRP3 in either the hematopoietic or stromal compartment or both were more susceptible to hyphal dissemination in the lung tissues (Figures 2C and S2A). H&E staining showed that *Aspergillus* hyphae were confined to granulomas in WT mice, whereas mice lacking AIM2 and NLRP3 in either or both the hematopoietic or stromal compartment had an impaired ability to recruit immune cells in the presence of *Aspergillus* hyphae, an observation which is further confirmed by immunohistochemistry staining and quantification of MPO-positive cells (Figures 2D, 2E, and S2B). It is, therefore, likely that AIM2 and NLRP3 operate in both the hematopoietic and stromal compartments to contribute to the host defense against aspergillosis.

AIM2 and NLRP3 Provide Redundant Roles in Mediating IL-1β and IL-18 Release in Dendritic Cells

We have shown that both AIM2 and NLRP3 provide host protection against *A. fumigatus*. To investigate the mechanism governing AIM2- and NLRP3-mediated host protection, we infected mouse BMDCs with germinating conidia and examined the level of caspase-1 activation and IL-1β or IL-18 release. *A. fumigatus* infection induced similar levels of caspase-1 and IL-1β release in unprimed BMDCs compared to *C. albicans* (Figures S3A and S3B). Indeed, priming of BMDCs with LPS was not necessary, but did enhance caspase-1 activation and IL-1β release (Figures S3C and S3D). We found that unprimed BMDCs lacking either NLRP3 or AIM2 retained the ability to induce proteolytic processing of caspase-1 following *A. fumigatus* infection (Figure 3A). In line with this observation, *Aim2*$^{-/-}$ or *Nlrp3*$^{-/-}$ BMDCs were able to release IL-1β and IL-18 upon *Aspergillus* infection (Figures 3B and 3C). However, we noted that *Aim2*$^{-/-}$ or *Nlrp3*$^{-/-}$ BMDCs consistently produced slightly less IL-1β and IL-18 than WT BMDCs ($p > 0.05$), despite their ability to produce similar levels of another pro-inflammatory cytokine, IL-6 (Figure 3D). BMDCs lacking the inflammasome receptor for bacterial flagellin or components of the type III secretion system, NLRC4,

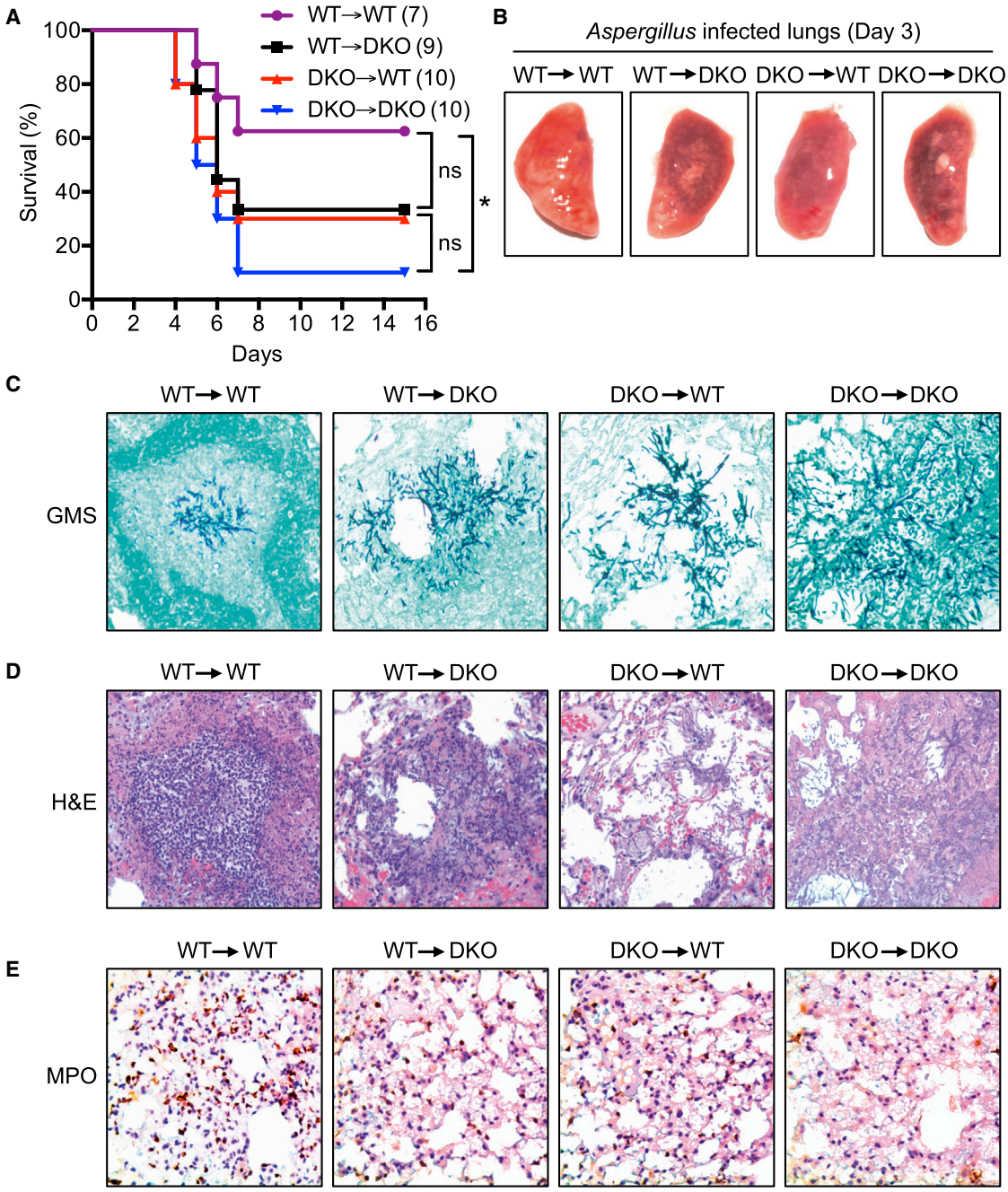

Figure 2. Bone Marrow and Stromal Compartments Contribute to the Host Defense against *Aspergillus* Infection

(A) Survival of bone marrow chimeras infected with 1×10^5 *A. fumigatus* conidia after immunosuppression with cyclophosphamide and cortisone acetate.

(B) Gross pathology of *A. fumigatus*-infected lungs collected on day 3.

(C–E) GMS, H&E, and MPO staining of *A. fumigatus*-infected lungs collected on day 3. (A) Log-rank test, *$p < 0.05$. ns, not statistically significant. See also Figure S2.

or the non-inflammasome receptor, NOD1, also exhibited normal levels of caspase-1 activation and IL-1β secretion (Figures S3E and S3F).

We observed that BMDCs from *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice infected with *A. fumigatus* failed to activate caspase-1 and release the caspase-1 p20 subunit into the supernatant (Figures 3E and S4A). We confirmed these results and found that BMDCs lacking ASC also failed to induce caspase-1 activation (Figure 3E). Both *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ or *Asc*$^{-/-}$ BMDCs had an impaired ability to secrete IL-1β and IL-18 (Figures 3F and S4B). We observed similar levels of TNF-α, KC, and IL-6 released by WT and *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ BMDCs (Figure S4C), suggesting that there is no global defect in pro-inflammatory cytokine production by these cells infected with *A. fumigatus*. In addition, the rate of

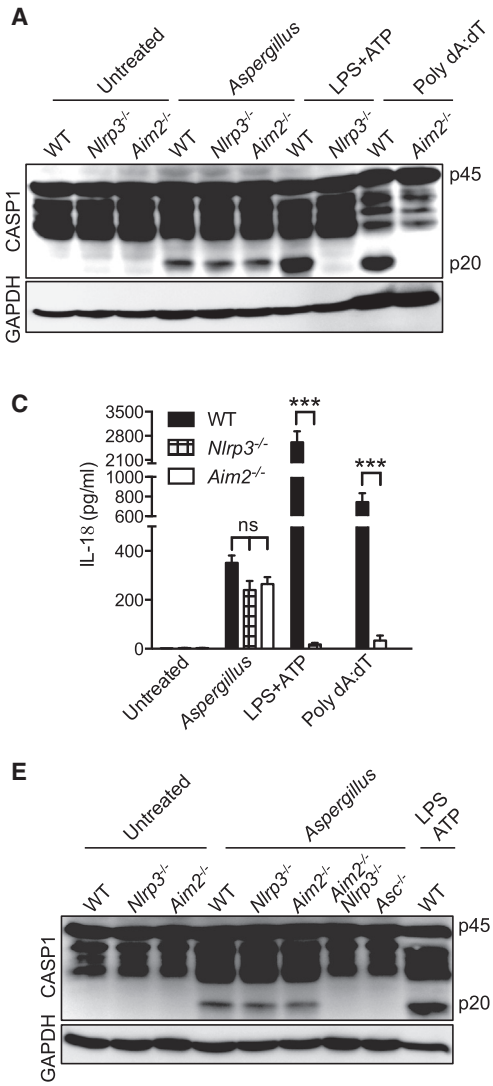

Figure 3. Both NLRP3 and AIM2 Are Required for Inflammasome Activation in Response to *Aspergillus* Infection

(A–F) BMDCs from WT, *Nlrp3*$^{-/-}$, *Aim2*$^{-/-}$, *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$, and *Asc*$^{-/-}$ mice were infected with *A. fumigatus* (MOI 20) for 20 hr. Caspase-1 activation was analyzed from the cell lysate, and levels of IL-1β, IL-18, or IL-6 released were analyzed from the supernatant. Data represent means ± SEM of triplicate wells and are representative of three or more independent experiments. (B)–(D) and (F) Tukey's multiple comparison test, $^{**}p < 0.01$; $^{***}p < 0.001$; ns, not statistically significant. See also Figures S3–S5.

phagocytosis, phagocytic index, and conidiocidal activity were comparable between WT cells and cells deficient in AIM2 and/or NLRP3 (Figure S4D). These results demonstrate that AIM2 and NLRP3 play redundant roles in driving robust inflammasome activation to mediate IL-1β and IL-18 release in response to *Aspergillus* infection.

AIM2 binds dsDNA to initiate the assembly of the inflammasome. Indeed, transfection of *Aspergillus* DNA into BMDCs activated the AIM2 inflammasome (Figures S5A and S5B). The precise activator for NLRP3 during *Aspergillus* infection is unknown. A number of mechanisms have been proposed to drive the activation of the canonical NLRP3 inflammasome, including ROS and K^+ efflux (Muñoz-Planillo et al., 2013; Perregaux and Gabel, 1994; Pétrilli et al., 2007; Zhou et al., 2011). To investigate whether these signals are required for NLRP3 inflammasome activation during *Aspergillus* infection, we infected WT and *Aim2*$^{-/-}$ BMDCs with *A. fumigatus* in the presence of a ROS inhibitor *N*-acetyl-L-cysteine (NAC) or high extracellular K^+. Inhibition of ROS or addition of KCl had minimal effects on caspase-1 activation and only partially reduced IL-1β release in WT BMDCs (Figures 4A–4D). This was not surprising given that WT cells had intact AIM2 responses. Indeed, inhibition of ROS or addition of KCl substantially impaired caspase-1 activation and IL-1β production in *Aim2*$^{-/-}$ BMDCs (Figures 4A–4D). These results indicate that both ROS and K^+ efflux contribute to NLRP3 inflammasome activation in response to *Aspergillus* infection.

To investigate whether viable *A. fumigatus* is necessary to activate the inflammasome, we stimulated BMDCs with heat-killed or paraformaldehyde-fixed *A. fumigatus* and examined the levels of caspase-1 activation and IL-1β production. Heat-killed or paraformaldehyde-fixed *A. fumigatus* failed to activate caspase-1 and induce IL-1β secretion in BMDCs (Figures 4E and 4F). In addition, inhibition of phagocytosis using cytochalasin B or cytochalasin D reduced caspase-1 activation and significantly inhibited IL-1β release (Figures S5C and S5D). Collectively, our findings indicate that phagocytosis of viable *Aspergillus* activates the NLRP3 inflammasome pathway in a manner that depends on ROS and K^+ efflux.

AIM2 and NLRP3 Assemble a Single Dynamic Inflammasome that Comprises ASC, Caspase-1, and Caspase-8

The inflammasome is a multi-meric protein complex that has the capacity to recruit distinct effector proteins to tailor the immune response (Man et al., 2013). To visualize components of the *Aspergillus*-induced inflammasome, we infected WT, *Aim2*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ BMDCs and stained for ASC, caspase-1, and caspase-8—components which assemble into an inflammasome complex to mediate IL-1β processing (Gringhuis et al., 2012; Man et al., 2013, 2014). Our confocal immunofluorescence analysis revealed that ASC, caspase-1, and caspase-8 formed a single and distinct cytoplasmic "speck" of 1 μm in diameter in WT cells infected with *A. fumigatus* (Figure 5A). We quantified the percentage of BMDCs forming the ASC speck and found that 20% of the WT cells contained the

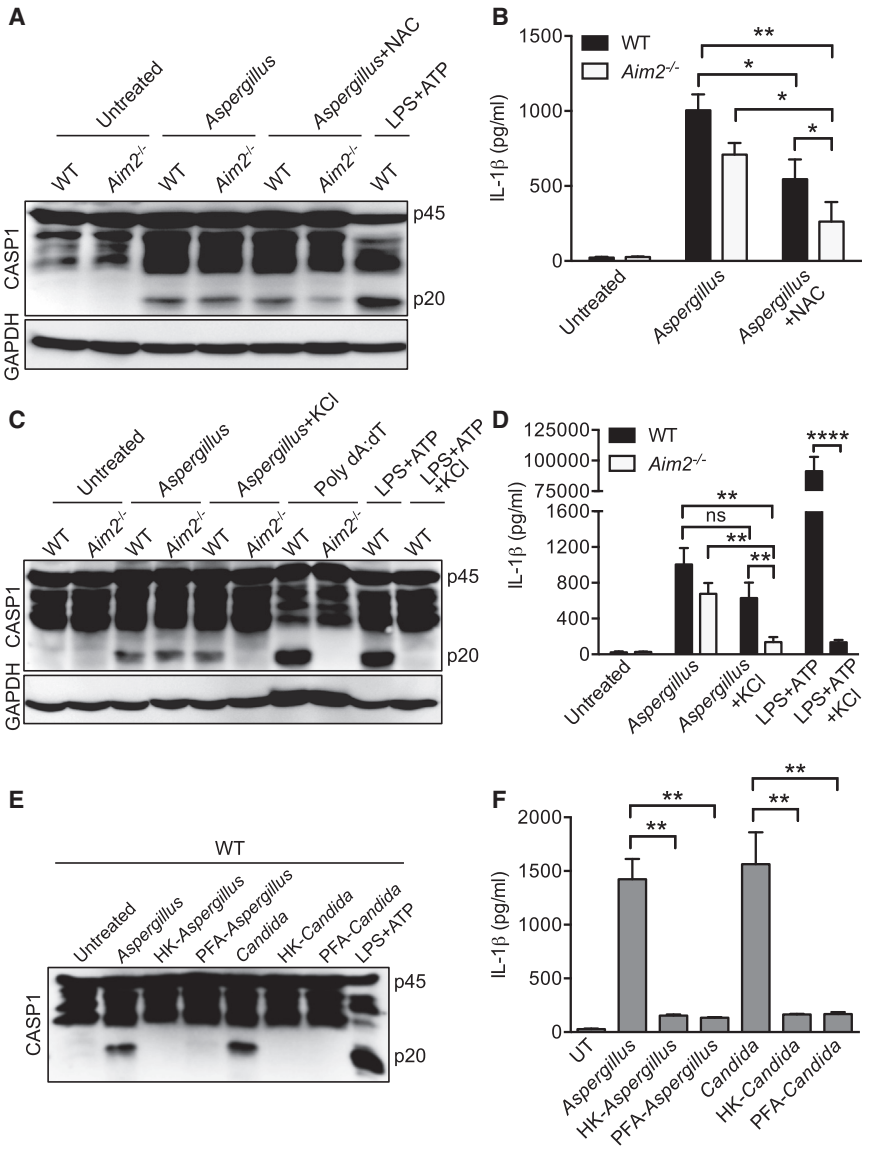

Figure 4. ROS and Potassium Efflux Contribute to NLRP3 Inflammasome Activation in Response to *Aspergillus* Infection

(A–D) BMDCs from WT and *Aim2*$^{-/-}$ mice were infected with *A. fumigatus* (MOI 20) for 20 hr in the absence or presence of 10 mM *N*-acetyl-L-cysteine (NAC) or 50 mM KCl. Caspase-1 activation was analyzed from the cell lysate, and levels of IL-1β released were analyzed from the supernatant.

(E and F) WT BMDCs were infected with *A. fumigatus* (MOI 20), *C. albicans* (MOI 5), or stimulated with heat-killed (HK) or paraformaldehyde (PFA)-fixed *A. fumigatus* or *C. albicans* for 20 hr. Caspase-1 activation was analyzed from the cell lysate, and levels of IL-1β released were analyzed from the supernatant.

Data represent means ± SEM of triplicate wells. Data are representative of two independent experiments. (B), (D), and (F) Tukey's multiple comparison test, *p < 0.05; **p < 0.01; ****p < 0.0001; ns, not statistically significant.

ASC speck (Figure 5B). A significantly fewer number of *Nlrp3*$^{-/-}$ or *Aim2*$^{-/-}$ or *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ BMDCs contained an ASC speck (Figure 5B). We further analyzed the composition of the ASC speck in WT, *Aim2*$^{-/-}$, or *Nlrp3*$^{-/-}$ BMDCs and found that the majority of the ASC specks in WT cells contained both caspase-1 and caspase-8, whereas BMDCs lacking either receptor primarily harbored caspase-1 (Figure 5C). Indeed, BMDCs lacking either AIM2 or NLRP3 consistently produced less IL-1β and IL-18, and this suggests that robust secretion of these cytokines may require the recruitment of both caspases into the inflammasome.

Caspase-1 and Caspase-8 Contribute to Inflammasome Processing of IL-1β and IL-18

The role for caspase-1 and caspase-8 in the processing of IL-1β and IL-18 during *Aspergillus* infection is unclear. Here we used genetic knockout mice lacking either of these proteins to investigate the relative contribution of caspase-1 and caspase-8 in the processing of IL-1β and IL-18 during *A. fumigatus* infection. BMDCs lacking caspase-1 failed to produce appreciable levels of IL-1β and IL-18 (Figures 6A–6C). However, caspase-1-deficient mice backcrossed to the C57BL/6 background also lack caspase-11 owing to a mutation in the *caspase-11* locus, which originated from the 129 strain used to generate the caspase-1-deficient mouse strain (Kayagaki et al., 2011). However, we did not observe a role for caspase-11 in inflammasome activation because *casp11*$^{-/-}$ BMDCs infected with *A. fumigatus* induced similar levels of caspase-1 activation and IL-1β and IL-18 release compared to WT cells (Figures 6A–6C). We further confirmed these results using BMDCs lacking caspase-1 alone (*Casp1/11*$^{-/-}$ mice injected with a caspase-11 bacterial artificial chromosome transgene; *Casp1*$^{-/-}$*Casp11*^{Tg}) (Kayagaki et al., 2011) and found that caspase-1, rather than caspase-11, was required to induce processing of IL-1β and IL-18 during *A. fumigatus* infection (Figures 6A–6C).

Although caspase-1 is essential for mediating IL-1β and IL-18 processing in response to *A. fumigatus* infection, it is possible that caspase-8 might also be playing a role in this process given that this protein is recruited to the *Aspergillus*-induced inflammasome (Figure 5). Genetic deletion of caspase-8 in mice results in embryonic lethality, which can be rescued by ablation of receptor interacting protein kinase-3 (RIPK3) (Kaiser et al., 2011; Oberst et al., 2011). To unravel the role of caspase-8, we infected WT, *Rip3*$^{-/-}$, and *Casp-8*$^{-/-}$*Rip3*$^{-/-}$ BMDCs with *A. fumigatus* and examined for caspase-1 activation and IL-1β and IL-18 release. Interestingly, *A. fumigatus*-induced IL-1β and IL-18 production were impaired in *Casp-8*$^{-/-}$*Rip3*$^{-/-}$ BMDCs, but not in WT cells or *Rip3*$^{-/-}$ cells (Figures 6D and 6E). Consistently,

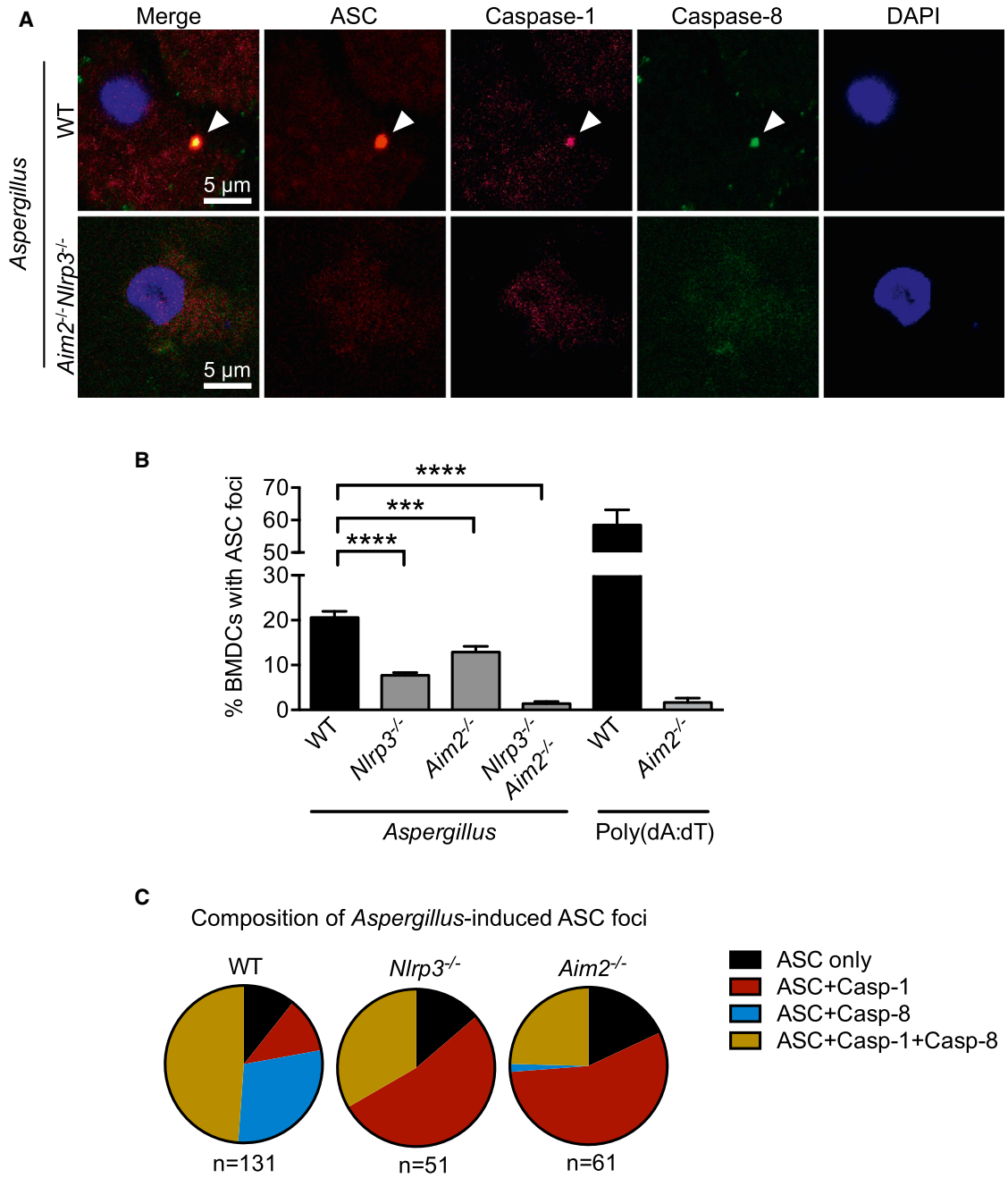

Figure 5. Activation of AIM2 and NLRP3 by *Aspergillus* Infection Induces the Assembly of a Single Inflammasome Composed of ASC, Caspase-1, and Caspase-8

(A) WT, *Aim2*$^{-/-}$, *Nlrp3*$^{-/-}$, and *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ BMDCs were infected with *A. fumigatus* for 20 hr and stained for ASC (red), Caspase-1 (magenta), Caspase-8 (green), and DNA (blue). Arrowheads indicate an inflammasome speck.

(B) Percentage of BMDCs that contained an ASC speck after *A. fumigatus* infection or transfected with 2.5 μg poly(dA:dT) for 5 hr. At least 300 BMDCs from each genotype were counted.

(C) Composition of the ASC specks.

Data represent means ± SEM of one experiment representative of two independent experiments. (B) Dunnett's multiple comparison test, ***$p < 0.001$; ****$p < 0.0001$.

Casp-8$^{-/-}$*Rip3*$^{-/-}$ BMDCs had an impaired ability to induce caspase-1 activation in response to *A. fumigatus* infection (Figure 6F). FADD is an adaptor protein for caspase-8 and has been implicated in inflammasome activation (Gurung et al., 2014). Indeed, we observed impaired caspase-1 activation and IL-1β and IL-18 production in *Fadd*$^{-/-}$*Rip3*$^{-/-}$ BMDCs compared to WT or *Rip3*$^{-/-}$ BMDCs (Figures 6D–6F). These results suggest that caspase-8 and FADD license caspase-1

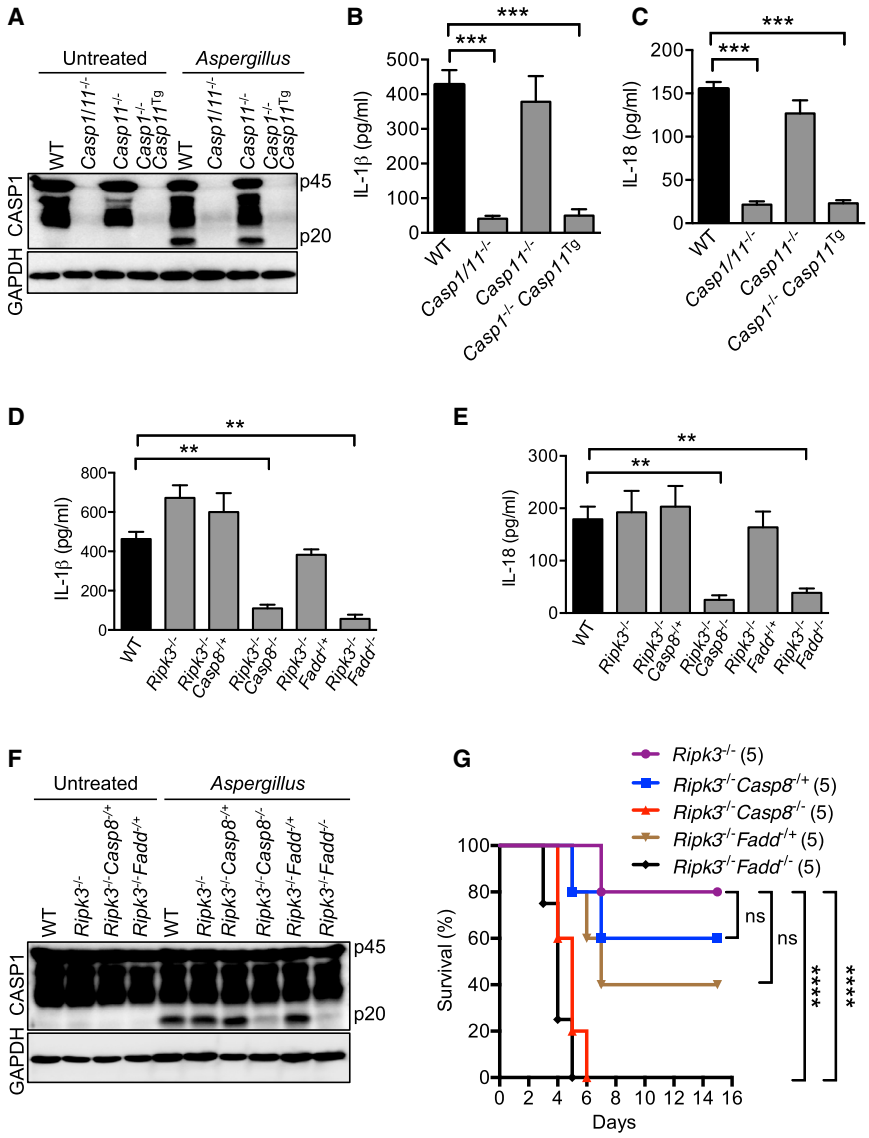

Figure 6. *Aspergillus*-Induced IL-1β and IL-18 Production Is Dependent on Both Caspase-1 and Caspase-8

(A–F) BMDCs from WT, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp11*$^{-/-}$ and *Casp1*$^{-/-}$*Casp11*^{Tg}, *Ripk3*$^{-/-}$, *Ripk3*$^{-/-}$*Casp8*$^{-/+}$, *Ripk3*$^{-/-}$*Casp8*$^{-/-}$, *Ripk3*$^{-/-}$*Fadd*$^{-/+}$, and *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ mice were infected with *A. fumigatus* (MOI 20) for 20 hr. Caspase-1 activation was analyzed from the cell lysate, and levels of IL-1β and IL-18 released were analyzed from the supernatant.

(G) Survival of mice infected with 1 × 10^5 *A. fumigatus* conidia after immunosuppression with cyclophosphamide and cortisone acetate.

(B)–(E) Data represent means ± SEM of triplicate wells. Data are representative of three or more independent experiments. Tukey's multiple comparison test, **p < 0.01; ***p < 0.001. (G) Log-rank test, ****p < 0.0001. ns, not statistically significant.

activation in the *Aspergillus*-induced inflammasome. To extend our findings to the physiological setting, we investigated the susceptibility of mice lacking caspase-8 or FADD in the absence of RIP3 to *Aspergillus* infection. Mice lacking caspase-8 and RIP3 or FADD and RIP3 succumbed to infection within 5–6 days and were significantly more susceptible than their corresponding controls (Figure 6G).

Inflammasome-Dependent Cytokines Contribute to the Host Protection against *A. fumigatus* Infection

Aspergillus-induced activation of the inflammasome leads to the production of IL-1β and IL-18 in vitro. Consistently, we observed decreased levels of both cytokines in the lungs of mice lacking AIM2 and NLRP3 compared with WT mice infected with *A. fumigatus* (Figures 7A and 7B). Caspase-1 activation in the lung tissues was substantially reduced in the absence of AIM2 and NLRP3 (Figure 7C). The levels of IFN-γ, which is produced in response to IL-18 stimulation (Ghayur et al., 1997), were markedly reduced in *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice (Figure 7D). However, there were no significant differences in the levels of IL-6, KC, and TNF-α in the lungs of *A. fumigatus*-infected WT and *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice (Figures S6A–S6C). These results confirmed that there was no global defect in pro-inflammatory cytokine production in mice lacking AIM2 and NLRP3 infected with *A. fumigatus*. Although both IL-1β and IL-18 are produced, the relative contribution of these in the host resistance to *A. fumigatus* infection is unknown. To investigate the physiological relevance of inflammasome-mediated IL-1β and IL-18 secretion in *Aspergillus* infection, we infected mice lacking IL-1β or IL-18 with *A. fumigatus* and monitored for their survival. We found significantly increased mortality in *Il-1β*$^{-/-}$ mice compared to WT mice (Figure 7E). *Il-18*$^{-/-}$ mice exhibited partial susceptibility to aspergillosis. These results, together, reveal that inflammasome-dependent cytokine release governed by AIM2 and NLRP3 contributes in the host protection against aspergillosis.

DISCUSSION

In this study, we unveiled a requirement for inflammasome receptors, AIM2 and NLRP3, in the cytoplasmic sensing of and host protection against the human pathogen *A. fumigatus*. Emerging evidence suggests that multiple inflammasome receptors can be activated upon infection by microorganisms, which ultimately determines the nature and dynamics of the innate immune response (Broz et al., 2010; Kalantari et al., 2014; Liu et al., 2012; Man et al., 2014). Indeed, we and others have previously shown that NLRC4 and NLRP3 mediate the clearance of *Salmonella* infection, and that NLRP3 and AIM2 contribute to the host defense against plasmodium infection (Broz et al., 2010;

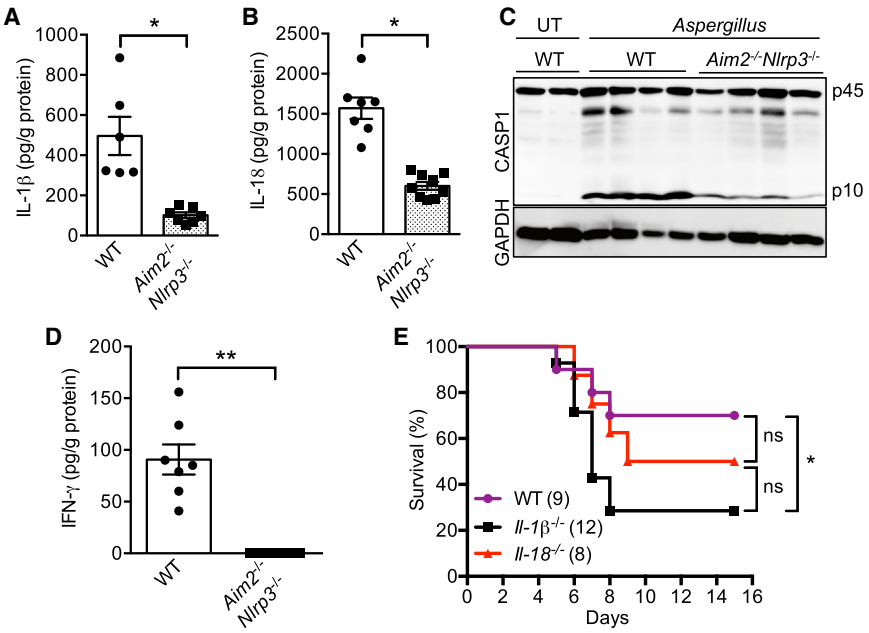

Figure 7. IL-1β and IL-18 Are Critical in Controlling *Aspergillus* Infection

WT and *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice were infected with 1 × 10^5 *A. fumigatus* conidia after immunosuppression with cyclophosphamide and cortisone acetate.

(A, B, and D) The levels of IL-1β, IL-18, and IFN-γ in lung homogenates after 3 days of *A. fumigatus* infection.

(C) Caspase-1 activation in lung homogenates after 3 days of *A. fumigatus* infection.

(E) Survival of WT, *Il-1β*$^{-/-}$, and *Il-18*$^{-/-}$ mice infected with 1 × 10^5 *A. fumigatus* conidia after immunosuppression.

(A, B, and D) Data represent means ± SEM. Unpaired t test. (E) Log-rank test. *p < 0.05; **p < 0.01, ns, not statistically significant.

Kalantari et al., 2014; Man et al., 2014). However, in response to the fungal pathogen *C. albicans*, NLRP3 is strictly required for the activation of the inflammasome in macrophages or dendritic cells (Gross et al., 2009; Hise et al., 2009; Joly et al., 2009). It is unclear why *C. albicans* activates NLRP3 exclusively, whereas *A. fumigatus* activates both NLRP3 and AIM2. It is possible that *C. albicans* fails to release its DNA into the host cytosol to activate AIM2 or that it harbors a virulence factor that mediates evasion of AIM2 detection. Furthermore, NLRP3 is required for robust IL-1β secretion in the human THP-1 cell line infected with *A. fumigatus* (Saïd-Sadier et al., 2010), whereas our study showed that both NLRP3 and AIM2 are required in mouse dendritic cells. Whether AIM2 is also required in human immune cells is unknown. Regardless, it is important to note that the composition of the human and mouse NLRP3 inflammasomes may differ. Nevertheless, our study now provides insights into the cooperative and synergistic relationship between different inflammasome receptors in the context of *Aspergillus* infection.

AIM2, a member of the HIN-200 family, is activated by dsDNA of bacterial or viral origin (Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Rathinam et al., 2010; Roberts et al., 2009). It has been shown that germination of *A. fumigatus* results in biofilm formation, which leads to the release of extracellular DNA (Rajendran et al., 2013). The release of extracellular DNA by *A. fumigatus* may activate the AIM2 inflammasome. We and others have also shown that bacterial nucleic acid molecules, including RNA and DNA:RNA hybrids, are recognized by NLRP3 (Kailasan Vanaja et al., 2014; Kanneganti et al., 2006; Sander et al., 2011). Therefore, it may be possible that NLRP3 and AIM2 recognize a common nucleic acid structure released by *Aspergillus* that ultimately triggers the formation of a single heteroduplex inflammasome.

ROS generated in the cell in response to *A. fumigatus* infection has been suggested to activate NLRP3 in the human THP-1 cell line (Saïd-Sadier et al., 2010). In addition, K^+ efflux is one of the proposed mechanisms in the activation of the canonical NLRP3 inflammasome (Muñoz-Planillo et al., 2013; Perregaux and Gabel, 1994; Pétrilli et al., 2007). We provide evidence to show that both ROS and K^+ efflux act upstream of NLRP3 and contribute to NLRP3-induced inflammasome activation in response to *Aspergillus* infection. It is possible that damage of the host cell causes both ROS production and K^+ efflux that collectively leads to activation of NLRP3. Recent studies indicate that, depending on the pathogens countered, NLRP3 inflammasome activation can be driven by a canonical caspase-11-independent or a non-canonical caspase-11-dependent pathway (Kayagaki et al., 2011). Here we identified a canonical pathway for NLRP3 activation in response to *A. fumigatus*.

We have also shown that the *Aspergillus*-induced inflammasome, composed of ASC, caspase-1, and caspase-8, localized in a single cytoplasmic location to regulate processing of pro-IL-1β and pro-IL-18. Our study clearly supports the view that the inflammasome is a dynamic complex which recruits different components to a single molecular platform depending on the contextual cue received by the cell upon infection. In this study, we have demonstrated that caspase-8 drove caspase-1 proteolytic activities during *Aspergillus* infection. A previous study has shown that inhibition of caspase-8, but not caspase-1, using chemical inhibitors abolished pro-IL-1β processing in dendritic cells exposed to fungi and mycobacteria, suggesting that caspase-8, rather than caspase-1, mediates IL-1β processing (Gringhuis et al., 2012). In contrast, another study suggests that caspase-1 is required for the processing of IL-1β (Saïd-Sadier et al., 2010). Both of these studies used chemical inhibitors, which are prone to off-target effects (Pereira and Song, 2008). Here, we provide genetic evidence and demonstrate that genetic deletion of caspase-1 or caspase-8 leads to impaired IL-1β and IL-18 release in response to *A. fumigatus* infection, suggesting that both caspases are required in this process. The requirement for caspase-8 to induce caspase-1 activation suggests that caspase-8 might be upstream of caspase-1. The involvement of caspase-8 and the NLRP3 inflammasome has also been described for β-glucan-induced IL-1β processing in Pam_2CSK_4-primed BMDCs (Ganesan et al., 2014).

In line with our in vitro data, mice lacking both AIM2 and NLRP3 are hypersusceptible to aspergillosis. These mice

phenocopied mice deficient in caspase-1 and -11 or ASC. In addition, our data suggest that mice lacking caspase-1 and -11 appear to be more susceptible than mice lacking IL-1β alone, suggesting that pyroptosis may play a role in the host defense during *Aspergillus* infection. It would be interesting to compare the susceptibility between *Casp1/11*$^{-/-}$ and *Il-1b*$^{-/-}$/*Il-18*$^{-/-}$ mice to *A. fumigatus* infection to fully elucidate the role of pyroptosis in future studies. Our observation that mice deficient in only AIM2 or NLRP3 exhibit little to no susceptibility to aspergillosis supports our view that these two inflammasome receptors play redundant roles in the host defense against *Aspergillus* infection. However, BMDCs lacking either inflammasome receptor consistently produce less IL-1β and IL-18 compared to WT BMDCs, suggesting that the relationship between NLRP3 and AIM2 is not always strictly redundant. The partnership between AIM2 and NLRP3 in the host defense against infectious diseases extends beyond that of *Aspergillus* infection, since a concerted inflammasome effector function mediated by AIM2 and NLRP3 has been observed in response to *Plasmodium* and *Listeria* infection (Kalantari et al., 2014; Kim et al., 2010; Wu et al., 2010). In conclusion, our findings reveal that AIM2 and NLRP3 form a dual surveillance system within the cytoplasm to orchestrate a robust inflammasome-mediated response against *A. fumigatus* infection.

EXPERIMENTAL PROCEDURES

Mice

Aim2$^{-/-}$, *Nlrp3*$^{-/-}$, *Asc*$^{-/-}$, *Casp1*$^{-/-}$*Casp11*$^{-/-}$, *Casp11*$^{-/-}$, *Casp1*$^{-/-}$*Casp11*^{Tg}, *Nlrc4*$^{-/-}$, *Nod1*$^{-/-}$, *Il1β*$^{-/-}$, *Il18*$^{-/-}$, *Rip3*$^{-/-}$, *Rip3*$^{-/-}$*Casp8*$^{-/-}$, and *Rip3*$^{-/-}$*Fadd*$^{-/-}$ mice were described previously (Jones et al., 2010; Kanneganti et al., 2006; Kayagaki et al., 2011; Mariathasan et al., 2004, 2006; Oberst et al., 2011; Shornick et al., 1996; Zaki et al., 2010). *Aim2*$^{-/-}$*Nlrp3*$^{-/-}$ mice were generated by crossing *Aim2*$^{-/-}$ and *Nlrp3*$^{-/-}$ mice. All mice were backcrossed to the C57BL/6 background. Mice were bred at St. Jude Children's Research Hospital. Animal studies were conducted under protocols approved by St. Jude Children's Research Hospital on the use and care of animals.

In Vivo *Aspergillus* Infection

Aspergillus fumigatus strain AF293 was used in this study. This is a clinical isolate from an individual who died of invasive aspergillosis (Giles et al., 2011). Cyclophosphamide (Sigma) was dissolved in sterile water and given by intraperitoneal injection (150 mg/kg of body weight). Cortisone acetate (Sigma) was suspended in 0.02% Tween 80 in PBS and administered by subcutaneous injection (112 mg/kg of body weight). Mice were given a combination of cyclophosphamide and cortisone acetate 3 days and 1 day before *Aspergillus* infection. Mice were injected again with cyclophosphamide (150 mg/kg of body weight) 3 days after infection. On the day of infection, *Aspergillus* conidia were swollen in RPMI media at 37°C for 5 hr followed by two washes using 0.02% Tween 80 in PBS. Mice were anesthetized by isoflurane inhalation and inoculated intranasally with 1 × 10^5 conidia in 25 μl of 0.02% Tween 80 in PBS. For histopathology and cytokine levels, the mice were sacrificed on day 3 after infection, and lungs were submitted for histopathology and processed for cytokine measurement or western blotting.

In Vitro Stimulation of BMDCs

BMDCs were prepared as described previously (Lamkanfi et al., 2009). In brief, bone marrow cells were grown in RPMI supplemented with 10% FBS, 1% penicillin-streptomycin, 1% non-essential amino acid, 1% sodium pyruvate, and 20 ng/ml GM-CSF for 7 days. BMDCs (5 × 10^6) were seeded in six-well cell culture plates and infected with *Aspergillus* conidia with the indicated multiplicities of infection (MOIs) for 20 hr. In experiments which required LPS priming, 500 ng/ml ultrapure LPS from *Salmonella minnesota* R595 (InvivoGen) was added to BMDCs for 4 hr. To activate the NLRP3 inflammasome, 5 mM ATP (10519979001, Roche), 20 μM nigericin (N7143, Sigma), or 2.5 mg/ml silica (MIN-U-SIL 15, US Silica) was added for 30 min, 45 min, and 8 hr, respectively.

For DNA transfection, each reaction consisted of 2.5 μg of poly(dA:dT) (InvivoGen) resuspended in PBS and mixed with 0.6 μl of Xfect polymer in Xfect reaction buffer (Clontech Laboratories). After 10 min, DNA complexes were added to BMDCs in Opti-MEM (Gibco) and incubated for 5 hr. To transfect *Aspergillus* DNA, *A. fumigatus* conidia were grown in RPMI media supplemented with 10% FBS and 1% penicillin-streptomycin overnight at 37°C. The next day, *A. fumigatus* was collected and centrifuged to remove the culture supernatant. *A. fumigatus* DNA was extracted using the QIAamp DNA Mini Kit according to the manufacturer's instructions (51304, QIAGEN). DNA transfection was performed as described above.

The concentrations of inhibitors used in our studies were 5 and 50 μM cytochalasin B (C6762, Sigma), 5 and 50 μM cytochalasin D (C8273, Sigma), 10 mM NAC (A7250, Sigma), and 50 mM KCl (P217500, Fisher Scientific).

Bone Marrow Chimeras

Mice were subjected to a single dose of irradiation (1,000 rad). Mice were retro-orbitally injected with 10^7 bone marrow cells from the femur and tibia of donor mice 2 hr after irradiation. The reconstitution rate evaluated 6 weeks after bone marrow transfer was determined by flow staining of CD45.1 and CD45.2 in blood leukocytes, which was at least 95%. After 6 weeks, mice were infected as described above.

Western Blot

BMDCs were lysed in 1 × RIPA buffer and sample loading buffer containing SDS and 100 mM DTT. Lung tissues were homogenized in PBS containing protease inhibitors (Roche). Homogenates were then mixed 1:1 with 2 × RIPA buffer, and protein concentrations determined and diluted to 1 μg/μl using sample loading buffer. Proteins (15 μg) were separated on 10%–12% polyacrylamide gels. Following electrophoretic transfer of protein onto PVDF membranes, membranes were blocked in 5% skim milk and incubated with primary antibodies against caspase-1 (Adipogen, AG-20B-0042) followed by secondary anti-rabbit or anti-mouse HRP antibodies (Jackson ImmunoResearch Laboratories).

Immunofluorescence Staining

Following infection, BMDCs were washed three times with PBS and incubated with media containing 1 × FLICA far-red 660 active caspase-1 for 1 hr (ImmunoChemistry Technologies). Cells were then fixed in 4% paraformaldehyde for 15 min, followed by blocking with 10% normal goat serum (Dako) in 0.1% saponin (Sigma) for 1 hr. Cells were incubated with a mouse anti-Asc antibody (1:500 dilution, clone 2EI-7; Millipore) overnight followed by incubation with a rabbit anti-caspase-8 (1:500 dilution, 8592; CST) for an additional 1 hr. The secondary antibodies used were Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-mouse IgG. Cells were counterstained in DAPI mounting medium (1:250 dilution; Vector Laboratories). Cells and inflammasomes were visualized, counted, and imaged using a Nikon C1 confocal microscope at the Cell and Tissue Imaging Center Light Microscopy Facility (CTIC-LM) at St. Jude.

Phagocytosis and Conidiocidal Assays

Phagocytosis, phagocytic index, and intracellular conidiocidal activity were performed as described previously (Roilides et al., 1998). In brief, percentage phagocytosis was determined by calculating the number of BMDCs that had one or more conidia phagocytosed or attached divided by the total number of BMDCs observed × 100. The phagocytic index was generated by determining the average number of conidia that had been phagocytosed or attached per BMDC. The conidiocidal activity was calculated by the following formula: (1 − the number of CFU observed at 6 hr / the number of CFU observed at 1 hr) × 100.

Cytokine Analysis

Cell supernatants were collected for ELISA. Lung tissues were homogenized in PBS containing protease inhibitors (Roche). Cytokine levels were determined by using multiplex ELISA (Millipore) or IL-18 ELISA (MBL International) according to the manufacturers' instructions.

Immunohistochemistry

Formalin-preserved livers were processed and embedded in paraffin according to standard procedures. Sections (5 μm) were stained with H&E and examined by a pathologist blinded to the experimental groups. For immunohistochemistry, formalin-fixed paraffin-embedded livers were cut into 4-μm sections and stained with an anti-MPO antibody (1:500 dilution, A0398; Dako) for 30 min, followed by rabbit on rodent polymer-HRP (RMR622L; Biocare Medical) for 30 min.

Statistical Analysis

GraphPad Prism 6.0 software was used for data analysis. Data were represented as mean ± SEM. Statistical significance was determined by Student's t test, ANOVA with multiple comparison tests, or log-rank test as specified in the figure legends. $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2015.01.006.

AUTHOR CONTRIBUTIONS

R.K., S.M.M., R.K.S.M., and P.G. performed the experiments; P.V. performed the histological analysis; R.K., S.M.M., M.L., and T.-D.K. analyzed the data. S.M.M. and R.K. wrote the paper. T.-D.K. oversaw the project.

ACKNOWLEDGMENTS

We thank V.M. Dixit (Genentech), R.A. Flavell (Yale), D.R. Green (St. Jude), and D.D. Chaplin (University of Alabama) for the supply of mutant mice. We thank F.L. van de Veerdonk and L.A.B. Joosten (Radboud University Medical Center) for their suggestions. S.M.M. is a recipient of the Neoma Boadway Endowed Fellowship, and P.G. is a recipient of the Paul Barrett Endowed Fellowship, both funded by St. Jude Children's Research Hospital. M.L. is supported by European Union Marie-Curie grant 256432, ERC grant 281600, and grants G030212N, 1.2.201.10.N.00, and 1.5.122.11.N.00 from the Fund for Scientific Research - Flanders. This work was supported by grants from the NIH and the American Lebanese Syrian Associated Charities (ALSAC) to T.-D.K.

Received: September 20, 2014
Revised: December 8, 2014
Accepted: January 5, 2015
Published: February 19, 2015

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