MDA5 is an essential vita-PAMP sensor necessary for host resistance against *Aspergillus fumigatus*[1]

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ABSTRACT

RIG-I like receptors (RLR) are cytosolic RNA sensors that signal through the MAVS adaptor to activate interferon responses against viruses. Whether the RLR family has broader effects on host immunity against other pathogen families remains to be fully explored. Herein we demonstrate that MDA5/MAVS signaling was essential for host resistance against pulmonary *Aspergillus fumigatus* challenge through the regulation of antifungal leukocyte responses. Activation of MDA5/MAVS signaling was driven by dsRNA from live *A. fumigatus* serving as a key vitality-sensing pattern-recognition receptor. Interestingly, induction of type I interferons after *A. fumigatus* challenge was only partially dependent on MDA5/MAVS signaling, whereas type III interferon expression was entirely dependent on MDA5/MAVS signaling. Ultimately, type I and III interferon signaling drove the expression of CXCL10, which is critical in resistance against invasive aspergillosis in transplant patients. Furthermore, the MDA5/MAVS-dependent interferon response was critical for the induction of optimal antifungal neutrophil killing of *A. fumigatus* spores. In conclusion, our data broaden the role of the RLR family to include a role in regulating antifungal immunity against *A. fumigatus*.

INTRODUCTION

Aspergillus fumigatus is a ubiquitous environmental mold that humans inhale on a daily basis. Multiple environmental surveys have demonstrated a range of several hundred to thousands of *A. fumigatus* conidia can be inhaled daily (1-3). Individuals with normal immune systems readily clear *A. fumigatus* conidia from their airways without problems. However, immunocompromised individuals are at significantly greater risk of developing invasive pulmonary aspergillosis (IPA)[4]. IPA is a life-threatening disease with over 300,000 cases globally and a high mortality rate of 30% - 50% (4). Patients at increased risk of developing IPA include those receiving chemotherapy treatments for cancer and patients receiving immunosuppressive regimens to prevent graft-versus-host disease (GVHD) following hematopoietic stem cell transplants or solid organ transplants (5-9). Furthermore, epidemiology studies predict a steady increase of IPA cases in the future due to the projected continual increase of immune compromised patients (10). Additionally, individuals with single nucleotide polymorphisms (SNPs) or primary immunodeficiencies in key antifungal signaling or effector pathways, such as CARD9 (11), pentraxin 3 (12, 13), or NADPH oxidase (14, 15) are highly susceptible to IPA. Thus, these clinical observations demonstrate that host innate immune responses are imperative in deactivating viable fungal conidia to avoid fungal establishment in the lung, and failure of such processes result in fungal germination and fungal growth and dissemination, ultimately culminating in IPA.

If the physical barriers of the lungs are bypassed by the inhaled *A. fumigatus* conidia, airway epithelial cells and lung-resident macrophages comprise the first line of defense against inhaled conidia. It is well established that lung resident macrophages, including alveolar macrophages (16, 17) and $CCR2^{+}$ monocytes (18, 19) are critical for initiating the early

inflammatory milieu necessary for the recruitment of innate immune cells to the respiratory tract to mediate host resistance against invasive disease. These innate immune effector cells include neutrophils (20, 21), inflammatory monocytes (18, 19), NKT cells (22), and plasmacytoid dendritic cells (23-25). The cytokine/chemokine signaling networks necessary to regulate the recruitment of inflammatory cells to the respiratory tract are rapidly emerging, but still much remains to be learnt.

Recently, it has become well appreciated that the host inflammatory response induced by *A. fumigatus* is tightly tuned to the virulence of the individual strain under study (26-30). Being able to recognize pathogenic traits of microbes is key to appropriately responding to infection (31), one such trait is being able to resist killing and remain viable (32). Two crucial inflammatory pathways in signaling bacterial vitality are inflammasome-dependent secretion of IL-1β and induction of the interferon response, which both can be driven by bacterial RNA (33, 34). We and others have previously demonstrated that inflammasomes are essential for IL-1β production after *A. fumigatus* challenge (35-38). Interestingly, Kanneganti and colleagues have shown to the NLRP3 and AIM2 inflammasomes could be activated by fungal RNA and DNA, respectively (35). Moreover, both type I and type III interferons have been shown to be essential for host resistance against pulmonary *A. fumigatus* challenge (39), but the fungal PAMP and host pattern-recognition pathways leading to type I and type III interferon expression following *A. fumigatus* challenge have not been well elucidated.

Type I and type III interferon responses are best understood in the context of virus infections. Following virus infections, Toll-like receptors (including TLR3, TLR7/8, TLR9), cGAS/STING, and RIG-I-like receptors (RIG-I and MDA5) are known to be critical in the induction of type I and type III interferons (review by (40-42)). However, during fungal

infections much less is currently known. Both TLR3 and TLR9 are known to be involved in the antifungal defenses against *A. fumigatus* (43-46), but only their role in the induction of type I interferons has been explored (45, 47). Following *Candida albicans* infection TLR7 is known to be involved in the induction of type I interferons (48, 49). Moreover, a Dectin-1/Syk/Irf5 signaling network has been shown to be critical in the induction of type I interferons following *C. albicans* challenge (50). Finally, Dectin-1 has been shown to be partially involved in the induction of type I and III interferons following respiratory *A. fumigatus* challenge (Dutta *et al*, in press). However, the role of cytosolic sensing pattern-recognition receptors systems in the antifungal interferon response has not been well studied.

Our data demonstrate that MDA5/MAVS-dependent signaling is necessary for host resistance against *A. fumigatus* infection through the accumulation and activation of antifungal effector functions of neutrophils in the fungal challenged airways. MAVS signaling drove expression of both type I and type III interferon, which ultimately drove the expression of the interferon inducible chemokines CXCL9 and CXCL10. Overall, our study reveals a critical role for MDA5 in host anti-fungal immunity, which supports a broader role of MDA5 in monitoring the health of the host cytosol.

MATERIALS AND METHODS

Mice. C57BL/6J (Jackson Laboratory, Stock #000664), *Mavs*$^{-/-}$ (Jackson Laboratory, Stock #008634), and *Ifih1*$^{-/-}$ (Jackson Laboratory, Stock # 015812) were all originally purchased from Jackson Laboratories and sequentially bred in-house at Geisel School of Medicine at Dartmouth. *Ifnar*$^{-/-}$, *Ifnlr*$^{-/-}$, and *Ifnar*$^{-/-}$ *Ifnlr1*$^{-/-}$ DKO were kindly provided by Dr. Sergei Kotenko and Dr. Joan Durbin (51) and bred in-house at Rutgers – New Jersey Medical School. Wild-type B6.129F2 (Jackson Laboratory, Stock #101045) mice, used as controls for the *Mavs*$^{-/-}$ mice, were purchased directly from Jackson Laboratories. All mice were 8-16 weeks of age at the time of challenge. All animal experiments were approved by either the Rutgers – New Jersey Medical School Institutional Animal Care and Use Committee or Dartmouth College Institutional Animal Care and Use Committee.

Preparation of Aspergillus fumigatus conidia. *A. fumigatus* CEA10 and Af293 strains were used for this study. Each strain was grown on glucose minimal media (GMM) agar plates for 3 days at 37°C. Conidia were harvested by adding 0.01% Tween 80 to plates and gently scraping conidia from the plates using a cell scraper. Conidia were then filtered through sterile Miracloth, were washed, and resuspended in phosphate buffered saline (PBS), and counted on a hemocytometer.

Preparation of swollen conidia and heat-killing. *A. fumigatus* CEA10 conidia were collected, as described above. To generate a homogenous population of swollen conidia, conidia were resuspended at $5x10^6$ conidia per ml in RPMI containing 0.5 μg/ml of voriconazole (Sigma-Aldrich) and incubated at 37°C for 15 h, as previously done (52). Conidia were then washed twice and adjusted to $4x10^8$ conidia per ml in PBS-Tween 80. To heat-kill the swollen conidia,

the conidial suspension was incubated in a 100°C water bath for 30 minutes. The conidial concentration and efficiency of heat-killing was verified by plating on GMM agar.

Aspergillus fumigatus pulmonary challenge model. Mice were challenged with *A. fumigatus* conidia by the intratracheal (i.t.) route. Mice were anesthetized by inhalation of isoflurane; subsequently, mice were challenged i.t. with ~4×10^7 *A. fumigatus* conidia in a volume of 100 µl PBS. At the indicated time after *A. fumigatus* challenge, mice were euthanized using a lethal overdose of pentobarbital. 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. After centrifugation, the cellular component of the BALF was resuspended in 200 µl of PBS and total BAL cells were determined by hemocytometer count. BALF cells were subsequently spun onto glass slides using a Cytospin4 cytocentrifuge (Thermo Scientific) and stained with Diff-Quik (Siemens) or Hema 3™ Stat Pack (Fisher Scientific) stain sets for differential counting. For histological analysis lungs were filled with and stored in 10% buffered formalin phosphate for at least 24 hours. Lungs were then embedded in paraffin and sectioned into 5-micron sections. Sections were stained with Grocott-Gomori methenamine silver (GMS) using standard histological techniques to assess lung inflammatory infiltrates and fungal germination, respectively. Representative pictures of lung sections were taken using an Olympus BX50WI microscope with a QImaging Retiga 2000R camera.

Luminex assay for cytokine and chemokine secretion from experimental murine models of invasive aspergillosis. BALF and lung homogenates from B6/129F2 mice and *Mavs*$^{-/-}$ mice challenged with *A. fumigatus* 12 or 48 h prior were analyzed for cytokines and chemokines using

Milliplex Mouse Cytokine & Chemokine 32-plex (Millipore). Plates were read using a BioPlex 200 (Bio-Rad) in the Immune Monitoring and Flow Cytometry Core Facility at Dartmouth College.

Quantitative RT-PCR analysis. Total RNA from lungs was extracted with Trizol (Invitrogen). One microgram of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). TaqMan Fast Universal Master Mix (2X) No AmpErase UNG and TaqMan probes (Applied Biosystems, Catalog #4331182) for *Ifna2* (Mm00833961_s1), *Ifnb1* (Mm00439552_s1), *Ifnl2/3* (Mm0404158_gH), *Ifng* (Mm01168134_m1), *Cxcl9* (Mm00434946_m1), and *Cxcl10* (Mm00445235_m1) were used and normalized to *Gapdh* (Mm99999915_g1). Gene expression was calculated using ΔΔCT method relative to naïve sample.

Western blot analysis. Total protein from whole lungs was extracted using RIPA buffer. Total protein was quantified using a DC™ Protein Assay (Bio-Rad). For Western Blot analysis, 40μg of total protein was loaded per well. Gels were run at 100V for ~1 hour at room temperature and then transferred to PVDF membranes at 100V for ~1 hour at 4°C. After transfer, the membranes were blocked with TBST + 5% milk for 1 hour at room temperature. Following blocking, blots were rinsed twice with TBST buffer. Staining with primary antibodies was done in TBST with 5% BSA at 4°C for overnight on a roller. Primary antibodies used for these studies were rabbit anit-STAT1 (Cell Signaling, #9172S, 1/1000), rabbit anti-pSTAT1 (Cell Signaling, #7649P, 1/1000 dilution), and rabbit anti-β-actin (Abcam, ab75186, 1/40,000). After primary antibody

staining blots were washed four times with TBST. Blots were then stained with a donkey anti-rabbit IgG conjugated to HRP (Cell Signaling, #7074P2, 1/4000 dilution) in TBST + 5% milk for 1 hour at room temperature with gentle shaking. After secondary antibody staining blots were washed four times with TBST. Blots were developed using ECL Clarity (Bio-Rad) for 5 minutes, then analyzed using an Alpha Innotech FluorChem Q imager.

Generation of a Fluorescent Aspergillus fumigatus reporter (FLARE) strain in the CEA10 background. CEA10:H2A.X$^{A.\ nidulans}$::*ptrA* was constructed in two steps. First, *gpdA*(p) was amplified from DNA isolated from strain *A. nidulans* A4 (Source: FGSC) using primers RAC2888 and RAC2799. Histone variant H2A.X (AN3468) was amplified (without stop codon) from A4 DNA using primers RAC4582 and RAC4583. mRFP fragment was amplified from plasmid pXDRFP4 (Source: FGSC) using primers RAC2600 and RAC4575 and terminator for *A. nidulans trpC* gene was amplified using primers RAC2536 and RAC2537. The four fragments were then fused together with primers RAC1981 and RAC4134 using fusion PCR resulting in H2A.X first round fragment as described earlier (53). All primers are listed in Supplemental Table 1.

Secondly, we targeted integration of the H2A.X::rfp to the intergenic locus between AFUB_023460 and AFUB_023470. For this, left homology arm was amplified from CEA10 genomic DNA using primers RAC3873 and RAC3874. Right homology arm was amplified with primers RAC3875 and RAC3876. Dominant selection marker gene *ptrA* conferring resistance to pyrithiamine hydrobromide was amplified from plasmid pSD51.1 with primers RAC2055 and

RAC2056. The four fragments were then fused together with primers RAC3877 and RAC3878 using fusion PCR as described earlier (53). After the construct generation, polyethylene glycol mediated transformation of protoplast was performed as described earlier (53). Transformants were screened by PCR (data not shown) and confirmed with southern blot analysis as described earlier (54). mRFP fluorescence was confirmed with FACS (Fluorescence activated cell sorting) analysis.

Fluorescent Aspergillus fumigatus reporter (FLARE) assay. To measure both conidial uptake and viability in distinct immune cell populations we used fluorescent *Aspergillus* reporter (FLARE). The CEA10-based FLARE was labelled with AlexaFlour 633 membrane labeling, as described elsewhere (55). Bronchoalveolar lavage (BAL) and lung cell suspensions were prepared as described elsewhere (55) and stained with anti-Ly6G (1A8), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD45 (30-F11), anti–CD206 (C068C2) and a fixable viability dye (eBioscience; Catalog #65-0865-14). Neutrophils were identified as $CD45^{+}CD11b^{+}Ly6G^{+}$, alveolar macrophages as $CD45^{+}CD11b^{+}Ly6G^{-}CD206^{dim}$, interstitial macrophages as $CD45^{+}CD11b^{+}Ly6G^{-}CD206^{-}$, and monocytes as $CD45^{+}CD11b^{dim}Ly6G^{-}CD206^{+}$ cells, as previously defined (56, 57). Flow cytometric data were collected on a CytoFLEX (Beckman Coulter). All data was analyzed using FlowJo software.

Primary murine fibroblast cultures. Ears from 6-12 week-old C57BL/6J and *Ifih1*$^{-/-}$ mice were collected, washed in 70% EtOH, then PBS, and finally minced using scissors. For each ear, 500 µl of 1000 U/ml collagenase (Gibco, REF 17101-015) in HBSS was used to digest them at 37°C

for 25 minutes. The tissues were then centrifuged at 500 *x g* for 3 minutes and then washed once with HBSS. Ears were subsequently digested with 500 μl of 0.05% trypsin in HBSS for a further 20 minutes in 37°C. The tissues were then centrifuged again at 500 *x g* for 3 minutes, and the trypsin was discarded and replaced by 0.5ml fibroblast media [10% FCS, 1% MEM Non-essential amino acids, 1% pencillin-streptomycin in DMEM]. The tissues were then pushed through a sterile 40 μm cell strainer via the plunger of a 3ml syringe to obtain single cell suspensions. For every ear, 25ml of fibroblast media was used to initiate the murine fibroblast culture in tissue culture flasks and a media change was conducted every two days. The fibroblasts were usually confluent and harvested on day 6 by 0.25% trypsin digest for 5min in 37°C and then collected with fresh fibroblast media.

Isolation of total RNA from Aspergillus fumigatus. *A. fumigatus* conidia was harvested from GMM plates and inoculated at 1 x 10^6 per ml in liquid GMM. Cultures were grown for 24h in 37°C with shaking at 250 rpm in an orbital shaker. Mycelia were then filtered and wrapped in sterile Miracloth, dried by repeated paper towel absorption, and weighed. For every 50 mg of mycelium, 1 ml of Trisure (Bioline) was added and mixed. The Trisure-mycelium mixture was frozen by liquid nitrogen to release cellular content and then homogenized using a mortar and pestle. For every 1 ml of Trisure used, 0.2 ml chloroform was added and vortexed, and the solution was centrifuged for 15 min at 12000 *x g* at 4°C. The aqueous phase was then transferred to a new tube and 0.5 ml of isopropanol was added, vortexed, and centrifuged for 10 min at 12000 *x g* at 4°C. The supernatant was then removed, and the pellet was washed by 1 ml 75% ethanol via vortexing followed with centrifuging for 5 min at 7500 *x g* at 4°C. The 75% ethanol

was then removed to allow a complete air dry of the RNA, followed by dissolving in molecular biology grade water and stored in -80°C.

Stimulation of murine fibroblasts with fungal RNA. Freshly harvested murine fibroblasts were dosed at 5 x10^4/ml in fibroblast media and transferred to 24-well tissue culture treated plates with 0.5 ml per well. The plate was incubated for one day to allow the attachment of murine fibroblasts. Fibroblasts were then transfected via LyoVec™ (Invivogen)/RNA complexes. For every 200 µl of LyoVec™, 2 µg of either poly-IC (Invivogen), 5'-ppp RNA (Invivogen), or *A. fumigatus* RNA was diluted in 80 µl molecular biology grade water, mixed, and incubated in room temperature for 30 minutes to assemble the liposome. The assembled liposome complexes were then added to 5 ml of fibroblast medium and 0.5 ml of the above transfection media was transferred to each well of 24-well plates after removal of the original medium. Twenty-four hours after transfection the supernatant was collected for subsequent ELISA analysis.

ELISA analysis for cytokine and chemokine secretion. Cell culture supernatants from fibroblast stimulated with *A. fumigatus* RNA were analyzed by ELISA for CXCL10 (R&D Systems, Cat. DY466) and Interferon alpha (Invitrogen, Cat. BMS6027TWO). Plates were read using an Epoch BioTek Gen5 microplate reader at 450nm, and the background was subtracted at 570nm.

Statistical analysis. Statistical significance for *in vitro* and *ex vivo* data was determined by a Mann-Whitney U test, one-way ANOVA using a Tukey's or Dunn's post-test, or two-way ANOVA with Tukey's post-test through the GraphPad Prism 7 software as outlined in the figure

legends. Mouse survival data were analyzed with the Mantel-Cox log rank test using GraphPad Prism.

RESULTS

Induction of an optimal interferon response by *Aspergillus fumigatus* is dependent on viable conidia.

The polysaccharide-rich cell wall is a major driver of the inflammatory response against *A. fumigatus* and other fungi. Following *C. albicans* challenge the Dectin-1 (*Clec7a*) receptor is critical in the induction of type I interferons following (50). Dectin-1 is only partially responsible for induction of the type I and type III interferon response induced by *A. fumigatus* (Dutta *et al*, in press). To test whether the fungal cell wall was essential for driving the type I and type III interferon response, we stimulated C57BL/6J mice with $4x10^7$ live or heat-killed swollen conidia of the CEA10 strain of *A. fumigatus*. Forty hours post-challenge with live swollen conidia there was an induction of IL-28 (IFN-λ), CXCL10, and TNFα secretion (Figure 1). In contrast, when challenged with heat-killed swollen conidia secretion of both IL-28 (IFN-λ) and CXCL10 was markedly reduced, while TNFα was still induced (Figure 1). These finding demonstrate that while cell carbohydrates in heat-killed swollen conidia still drove robust inflammatory cytokines (such as TNFα), as has previously been demonstrated (52, 58), there is an alternative pattern-recognition receptor pathway which is largely responsible for the interferon response induced by live *A. fumigatus* conidia.

dsRNA from *Aspergillus fumigatus* drives an MDA5-dependent interferon response.

Vitality sensing during bacterial infection through recognition of bacterial RNA has been shown to be critical for the induction of protective immune responses through the secretion of both IL-1β and type I interferons (33). Thus, we next wanted to test whether *A. fumigatus* RNA could drive interferon release. To test this hypothesis, primary murine fibroblasts from C57BL/6J

mice were stimulated with naked or liposome packaged total RNA from the Af293 strain of *A. fumigatus*. As expected, untreated fibroblast or empty liposome treated did not drive significant secretion of IFNα or CXCL10, while liposome packaged polyI:C drove significant secretion of both (Figure 2A). Liposome-packaged Af293 RNA drove secretion of both IFNα and CXCL10, while naked Af293 could not drive their production (Figure 2A). Taken together, these data demonstrate that intracellular sensing of *A. fumigatus* RNA can drive an interferon-dependent inflammatory response.

Intracellular sensing of RNA is mediated by the RIG-I-like receptor family, composed of RIG-I and MDA5, for the induction of interferons. Both RIG-I and MDA5 recognize distinct structures in foreign RNA (59, 60). To test whether ssRNA or dsRNA from *A. fumigatus* was required to drive type I IFN secretion, we extended these *in vitro* fibroblast stimulation studies by pre-treating the *A. fumigatus* RNA pool with either RNase S1 or RNase III, which will selectively cleave ssRNA and dsRNA, respectively, prior to liposome packaging. We also treated the fungal RNA pool with DNase I as a control. Similar to our previous result, total *A. fumigatus* RNA packaged in liposomes was able to induce IFNα secretion from murine fibroblast (Figure 2B). Pre-treatment of the *A. fumigatus* RNA with either RNase S1 or DNase I had no impact on the secretion of IFNα (Figure 2B). In contrast, pre-treatment of the *A. fumigatus* RNA with RNase III completely ablated the secretion of IFNα from primary murine fibroblasts (Figure 2B). These data suggest that recognition of double-stranded fungal RNA in the cytosol is necessary for the interferon response induced by *A. fumigatus.*

To date the most well characterized intracellular receptor for dsRNA is MDA5 (59). To test whether *A. fumigatus* RNA initiates an interferon response in a MDA5-dependent manner we treated primary murine fibroblasts isolated from C57BL/6J or *Ifih1*$^{-/-}$ mice liposome packaged

fungal RNA. As positive controls, we utilized high molecular weight polyI:C, a known MDA5 ligand, and 5'ppp-RNA, a known RIG-I ligand. Eighteen hours are stimulation cell culture supernatants were collected for ELISA analysis to assess IFNα secretion. Unstimulated or LyoVec™ only stimulated fibroblast did not secrete any IFNα (Figure 2B). As positive controls, 5'ppp-RNA stimulated equivalent secretion of IFNα from both wild-type and *Ifih1*-deficient fibroblasts; while high molecular weight polyI:C stimulated robust IFNα secretion from wild-type fibroblast, which was significantly reduced in *Ifih1*-deficient fibroblasts (Figure 2B). The induction of IFNα secretion by *A. fumigatus* RNA was partially dependent on MDA5, like what we also observed with high molecular weight polyI:C (Figure 2B). Interestingly, RNase III treatment ablated both the MDA5-dependent and -independent IFN-α response (Figure 2B), which suggests there is another dsRNA receptor that contributions to the IFN-α response in response to encapsulated fungal RNA.

MAVS-dependent signaling is required for interferon expression after *Aspergillus fumigatus* challenge

During viral infections RLR family members are critical in initiating both the type I and type III interferon responses (61). Moreover, stimulation of *Ifih1*-deficient murine macrophages with *C. albicans* resulted in a slight decrease in *Ifnb* mRNA expression, which did not reach significance (62). However, the specific role of MDA5/MAVS in initiating the induction of a broad range of interferons to other fungal pathogens has not been explored. To test the role of MAVS signaling in the induction of interferons after respiratory challenge with *A. fumigatus*, we challenged B6.129F2 mice and *Mavs*$^{-/-}$ mice with $4x10^7$ conidia of CEA10. We collected lungs from the *A. fumigatus* challenged mice at 3 and 48 h post-inoculation for quantitative RT-PCR

analysis to assess interferon expression patterns. Similar to previous observations with C57BL/6J mice (39), wild-type B6.129F2 mice expressed high levels of type I interferons, *Ifna4* (Figure 3A) and *Ifnb* (Figure 3B), at 3 h post-inoculation that waned with time. Both type II interferon (*Ifng*) (Figure 3D) and type III interferon (*Ifnl2/3*) (Figure 3C) were expressed at higher levels at 48 h post-inoculation in wild-type B6.129F2 mice. Interestingly, in the *Mavs*-deficient mice type I interferon levels were only decreased by approximately 50% (Figure 3A-B), whereas the expression of type II and type III interferons were nearly completely ablated in the absence of MAVS (Figure 3C-D). Thus, MAVS-dependent signaling is essential for the late expression of type III interferon and only partially responsible for early expression of type I interferons.

To confirm that the expression of interferons resulted in down-stream IFN signaling, we next examined the phosphorylation of STAT1. STAT1 phosphorylation is essential for the signaling of all interferon classes. Lungs from C57BL/6J mice challenged 6, 24, or 48 h prior with either the CEA10 or Af293 strains were collected, and total proteins were extracted for Western blot analysis. Both the CEA10 and Af293 strains induced a bi-phasic induction of STAT1 phosphorylation (Figure 4A). Specifically, significant STAT1 phosphorylation was observed at 6 and 48 h after challenge, but minimal phosphorylation was observed at 24 h (Figure 4A). These data fit with our earlier observation that type I interferons are produced by 3 h post-challenge, while type II and type III interferons are highly expressed at later times (Figure 3). We next examined the phosphorylation of STAT1 in the lungs of B6.129F2 and *Mavs*$^{-/-}$ mice challenged with the CEA10 stain of *A. fumigatus* at 6 and 48 h after challenge, times when we saw high levels of STAT1 phosphorylation. Like C57BL/6J mice, wild-type B6.129F2 mice induced robust phosphorylation of STAT1 at both 6 and 48 h (Figure 4B). Interestingly, at 6 h after challenge with *A. fumigatus* the phosphorylation of STAT1 in the *Mavs*$^{-/-}$ mice was not

significantly different, although there was a slight but reproducible reduction (Figure 4B-C), which fits with our earlier observation that type I interferon mRNA levels were only mildly reduced at 3 h post-challenge with *A. fumigatus* (Figure 3A-B). In contrast, phosphorylation of STAT1 in the *Mavs*$^{-/-}$ mice was significantly decreased at 48 h post-challenge with *A. fumigatus* (Figure 4B-C), which corresponds with the significant decrease in type III interferon mRNA levels we observed at this time (Figure 3C). Taken together, these results strongly support the conclusion that MAVS signaling is critical for the late expression of type III interferons in response to *A. fumigatus* instillation and initiation of the interferon response that is necessary for host resistance against *A. fumigatus* infection.

Alteration of the airway inflammatory milieu after *Aspergillus fumigatus* challenge in the absence of MAVS-dependent signaling

While our data demonstrates that all the classes of interferons are decreased to vary degrees after pulmonary *A. fumigatus* challenged, we next wanted to more broadly understand the cytokine and chemokine response in the airways of *Mavs*$^{-/-}$ mice. To achieve this, we challenged B6.129F2 and *Mavs*$^{-/-}$ mice with $4x10^7$ conidia of *A. fumigatus*. At 12 and 48 h after challenge with *A. fumigatus*, the BALF was analyzed using a 32-plex Milliplex cytokine assay. Numerous cytokines and chemokines--TNFα, IL-17A, CCL2, and CCL4--were expressed in a manner that was completely independent of MAVS signaling (Figure 5). Interestingly, IL-1α, IL-1β, CXCL1, and CXCL2 secretion were significantly greater in the absence of MAVS signaling (Figure 5). In contrast, IL-12p70, CXCL9, and CXCL10 secretion was significantly impaired in the absence of MAVS signaling, particularly at 48 h post-challenge despite the large increase in

fungal burden (Figure 5). These data suggest that MAVS-dependent signaling is critical for induction and/or maintenance of these cytokines.

Cxcl9 and _Cxcl10_ mRNA expression after pulmonary _Aspergillus fumigatus_ challenge requires both type I and type III interferons

The marked decrease in CXCL9 and CXCL10 expression in the absence of MAVS following *A. fumigatus* challenge was quite striking, because both CXCL9 and CXCL10 are interferon-inducible chemokines (63). To test the role of type I and type III interferons in the induction of CXCL9 and CXCL10 expression, we challenged C57BL/6, *Ifnar*$^{-/-}$, *Ifnlr*$^{-/-}$, and *Ifnar*$^{-/-}$ *Ifnlr*$^{-/-}$ (DKO) mice with $4x10^7$ conidia of *A. fumigatus*. Forty-eight hours later, lungs were collected for quantitative RT-PCR analysis to assess *Cxcl9* and *Cxcl10* mRNA expression patterns. Both *Ifnar*- and *Ifnlr*-deficient mice had a significant decrease in their expression of both *Cxcl9* (Figure 6A) and *Cxcl10* (Figure 6B). In general, the defect in *Cxcl9* and *Cxcl10* mRNA expression was greater in the absence of type III interferon signaling, particularly regarding *Cxcl9* expression. Importantly, the *Ifnar*$^{-/-}$ *Ifnlr*$^{-/-}$ (DKO) mice had the most severe defect in *Cxcl9* and *Cxcl10* expression (Figure 6). Overall, these data demonstrate that type I and type III interferon signaling are essential for the expression of both *Cxcl9* and *Cxcl10*.

MDA5/MAVS-dependent signaling is required for host resistance against _Aspergillus fumigatus_ infection

These previous data suggest that MDA5 can sense *A. fumigatus* RNA to drive an interferon response, which could be critical in vitality sensing following an *in vivo* challenge with *A. fumigatus*. Thus, we wanted to test the role of MDA5/MAVS signaling in host resistance

against respiratory challenge with the human fungal pathogen, *A. fumigatus*. To globally assess the role of the RLR family in antifungal immunity, we initially challenged B6.129F2 mice and *Mavs*$^{-/-}$ mice with 4x10^{7} conidia of the CEA10 strain, since MAVS is the central signaling adaptor for both RIG-I and MDA5-mediated responses (64-67). We monitored the survival of the *A. fumigatus* challenged mice over the next two weeks. *Mavs*-deficient mice were more susceptible to pulmonary challenge with *A. fumigatus* than wild-type, B6/129F2 mice (Figure 7A; Mantel-Cox log rank test, $p = 0.0001$). Additionally, susceptibility of the *Mavs*$^{-/-}$ mice to *A. fumigatus* challenge is not dependent on the strain of *A. fumigatus* used because *Mavs*$^{-/-}$ mice challenged with the Af293 strain were also highly susceptible to infection (Supplemental Figure 1; Mantel-Cox log rank test, $p < 0.0001$).

It is widely acknowledged that neutrophils and macrophages are critical antifungal effector cells for clearing *A. fumigatus* from the lungs. Thus, we assessed inflammatory cell accumulation in the airways via differential microscopic counting of cytospins stained with Diff-Quik from the BALF. The increased susceptibility of *Mavs*-deficient mice to *A. fumigatus* challenge was associated with decreased accumulation of macrophages and neutrophils in the airways compared with B6.129F2 mice at 40 h post-challenge (Figure 7B). Next, we assessed fungal growth and tissue invasion in the lung by histological analysis at 40 h after conidial instillation. Strikingly, GMS staining of lung tissue from *Mavs*-deficient mice revealed the presence of germinating *A. fumigatus* conidia at 40 h that was not observed to the same extent in B6.129F2 mice (Figure 7C). When the presence of germinating *A. fumigatus* conidia was quantified, wild-type B6.129F2 mice displayed low levels of fungal germination (17.6% ± 2.9) compared with *Mavs*$^{-/-}$ mice in which most of the fungal material had germinated (73.8% ± 7.6) by 40 h post-challenge (Figure 7C). B6.129F2 mice had very few areas of fungal material

throughout their lungs, but it was associated with robust cellular infiltration and tissue destruction (Figure 7C).

To specifically address which RLR sensor is involved in regulating the innate anti-fungal immune response against *A. fumigatus* we chose to assess the role of MDA5 because of its role in the *in vitro* sensing of *A. fumigatus* RNA (Figure 2). Specifically, we challenged C57BL/6J and *Ifih1*$^{-/-}$ mice with 4x10^{7} conidia of *A. fumigatus*. We monitored the survival of the *A. fumigatus* challenged mice over the next ten days. Like the *Mavs*-deficient mice, *Ifih1*-deficient mice were more susceptible to pulmonary challenge with *A. fumigatus* than wild-type C57BL/6J mice (Figure 7D; Mantel-Cox log rank test, $p = 0.0004$). Analogous to what we observed in the *Mavs*-deficient mice, the increased susceptibility of *Ifih1*-deficient mice to *A. fumigatus* challenge was associated with decreased accumulation of neutrophils in the airways compared with C57BL/6J mice at 40 h post-challenge (Figure 7E). GMS staining of lung tissue from *Ifih1*-deficient mice revealed the presence of germinating *A. fumigatus* conidia at 42 h that was not observed to the same extent in C57BL/6J mice (Figure 7F) similar to our observation in the *Mavs*-deficient mice.

While immune competent animal models have provided important insights into the host immune pathways necessary for antifungal control (11, 13, 55, 68-70), we wanted to assess the importance of MDA5/MAVS signaling in a clinically relevant immune compromised model of IPA. For this we utilized the triamcinolone model of IPA (71). Briefly, C57BL/6J and *Ifih1*$^{-/-}$ mice were immunosuppressed with triamcinolone and then subjected to challenge with 10^{6} conidia of the Af293 strain of *A. fumigatus*. At 72 h post-inoculation lungs were collected for histological analysis for fungal growth. Interestingly, *Ifih1*$^{-/-}$ mice had a significantly greater number of fungal lesions per lung section than C57BL/6J mice (Supplemental Figure 2A).

Moreover, the fungal lesions appear to be larger and contain more fungi (Supplemental Figure 2A). Additionally, the BALF from *Ifih1*$^{-/-}$ challenged mice had higher levels of LDH activity (Supplemental Figure 2B) and albumin (Supplemental Figure 2C), which are indicators of lung damage and leakage, respectively, than C57BL/6J challenged mice. Taken together, these results strongly support the conclusion that MDA5/MAVS signaling is critical for host resistance against *A. fumigatus* in an immune competent murine model and critical for disease progression in a corticosteroid immune compromised murine model.

Neutrophil antifungal effector functions are decreased in the absence of MDA5.

To determine whether MDA5/MAVS signaling regulates antifungal effector cell functions, we used FLARE conidia challenge of C57BL/6 or *Ifih1*$^{-/-}$ mice to assess neutrophil-mediated conidial uptake and killing. FLARE conidia encode a fungal viability indicator (DsRed) and contain a tracer fluorophore (Alexa Fluor 633) (55). For this study we generated a novel FLARE strain in the CEA10 strain background (Supplemental Figure 3). FLARE conidia emit two fluorescence signals (DsRed and Alexa Fluor 633) when the conidia are alive, but only emit a single fluorescence signal (Alexa Fluor 633) when the conidia are dead. This approach allows us to determine the frequency of conidia-engaged immune cells that contain either live or dead fungal cells in the BAL and lungs. The frequency of conidia-engaged neutrophils was similar between the C57BL/6 and *Ifih1*$^{-/-}$ mice (Figure 8A). However, the frequency of conidia-engaged neutrophils that contain live conidia was increased among *Ifhi1*$^{-/-}$ mice compared with C57BL/6J mice, indicating a defect in conidial killing (Figure 8B). Specifically, the frequency of neutrophils that contain live conidia was 1.9-fold and 2.1-fold higher for BAL and lung fluid of *Ifih1*$^{-/-}$ mice compared with their C57BL/6J counterparts (Figure 8B). Thus, these data indicate

that neutrophil-mediated antifungal killing of *A. fumigatus* conidia requires MDA5/MAVS activation and signaling.

DISCUSSION

It is now well appreciated that the host inflammatory response induced by *A. fumigatus* is tightly tuned to the virulence of the individual strain under study (26-30). In filamentous fungi one key determinant for sensing the threat posed by the fungi is the changes in that occur in the fungal cell wall composition. Specifically, detection of β-1,3-glucan linked polysaccharides by Dectin-1 (*Clec7a*) occurs only upon conidial swelling and germling formation, which are the earliest steps of fungal growth (52, 58). Interestingly, our data demonstrate that while heat-killed swollen conidia of *A. fumigatus* can induce the secretion of pro-inflammatory cytokines, such as TNFα, they do not drive the secretion of IFNα and CXCL10 (Figure 1). These data suggest that fungal factors other than cell wall polysaccharides are responsible for driving the interferon response following respiratory *A. fumigatus* challenge. Consistent with this observation, individual polysaccharides commonly found in the *A. fumigatus* cell wall are not sufficient to induce an interferon response (72).

During bacterial infections one key virulence trait sensed by the host immune system to tune the inflammatory response is the ability of the bacteria to resist killing and remaining viable. Two crucial inflammatory pathways in signaling bacterial vitality are inflammasome secretion of IL-1β and interferon response through TRIF and cGAS/STING signaling (33, 34, 73). Following respiratory challenge with *A. fumigatus* both IL-1β and type I/III interferons have been shown to be critical for antifungal immunity (35, 39). While we and others have previously demonstrated that inflammasomes are essential for the IL-1β response after respiratory challenge with *A. fumigatus* (35-38), the innate pattern-recognition receptor(s) leading to type I/III interferon expression have not been elucidated.

In bacteria one critical PAMP associated with vitality (vita-PAMP) is RNA. Using an *E. coli* infection model, bacterial RNA was shown to be sufficient to drive both inflammasome-dependent IL-1β secretion and TRIF-dependent IFNβ secretion (33). Heat-killing of *E. coli* drove the loss of RNA and its subsequent inflammatory response (33). Several innate RNA sensors have been identified in mammalian hosts, including TLR3 (74), TLR7 (75), MDA5 (76), and RIG-I (77). TLR3 and TLR7 signal through TRIF (78), while MDA5 and RIG-I signal through MAVS (64-67) to drive the interferon response. Our data using RNase III treatment of total RNA isolated from *A. fumigatus* demonstrates that dsRNA from *A. fumigatus* is sufficient to drive an interferon response (Figure 2B). TLR3 and MDA5 are both receptors for dsRNA (59, 74, 77). Our data demonstrate that mice lacking *Mavs* displayed a markedly reduced type III interferon (IL-28/IFNλ), CXCL9, and CXCL10 secretion, but only a moderate defect in type I interferons (Figure 4). In contrast, the expression of *Ifna1* and *Ifnb1* are regulated by both TRIF (46) and TLR3 (45). Interestingly, the role of TRIF appears to be restricted to the non-hematopoietic compartment (46) likely epithelial cells (79). Taken together these data suggest the type I interferon response following *A. fumigatus* challenge is primarily driven by TLR3/TRIF-dependent signaling, while the type III interferon response is primarily drive by MDA5/MAVS-dependent signaling.

The purpose of vitality sensing is to drive robust inflammation that is in line with the threat posed by the invading pathogen. Neutrophils are well known to be critical antifungal effector cells needed for the prevention of fungal germination and clearance of *A. fumigatus* from the lungs (20). Our results demonstrate that MDA5/MAVS-dependent signaling is necessary for both the optimal accumulation (Figure 7) and activation of neutrophils in the *Aspergillus* challenged airways (Figure 8). Following pulmonary challenge with *A. fumigatus* it has been

established that both type I and type III interferons are essential for host resistance against through enhancing neutrophil ROS production and antifungal effector functions (39). Our FLARE data support as neutrophils in both the lungs and airways of *Ifih1*-deficient mice are significantly impaired in their antifungal killing capacity at 48 h after challenge (Figure 8), which corresponds with a time when type III interferons are nearly absent mice lacking MDA5/MAVS signaling. Decreased accumulation of neutrophils in the *Mavs*- and *Ifih1*-deficient mice did not correlate with classical neutrophil chemoattractants, such as CXCR2 ligands, that are required to maintain host resistance against *A. fumigatus* (36, 69, 80). Rather, in the absence of *Mavs* and *Ifih1* there was a marked decrease in CXCL9 and CXCL10, which are both ligands for CXCR3. Recently, CXCR3 has been suggested to be a potential chemoattractant for neutrophils in certain systems (81, 82). However, *Cxcr3*-deficient mice appear to recruit normal numbers of neutrophils following *A. fumigatus* challenge (25). Thus, why *Mavs*- and *Ifih1*-deficient mice accumulate few neutrophils in their lungs following challenge warrants further research.

While the role of RLRs in host immunity was originally associated with antiviral innate immune responses (reviewed by (42)), recent studies have begun to expand the role of RLRs in immunity to other pathogens, including *C. albicans*, *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Plasmodium falciparum* (40, 62, 83-87). Our study adds *A. fumigatus* to the list of non-viral pathogens which can activate the RLR family. Thus, cytosolic sensing of pathogens by RLRs, both RIG-I and MDA5, may have a broader role in initiating innate immune responses in several other clinically relevant non-viral pathogens. Additionally, MDA5/MAVS signaling appears to be crucial in sensing vita-PAMPs for the enhancing the inflammatory response to invading pathogens.

544

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XW, AKC, AR, RC, JJO. Performed the experiments: XW, AKC, VE, KL, WC, SD. Analyzed the data: XW, AKC, VE, KL, WC, AR, JJO. Wrote the paper: XW, JJO.

XW and AKC are co-first authors: AKC initiated these studies before graduating, while XW picked up and completed these studies and helped write the manuscript, which warranted listing XW first.

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FOOTNOTES:

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[4]Abbreviations: RLR, RIG-I like receptors; IPA, invasive pulmonary aspergillosis; GVHD, graft-versus-host disease; SNP, single nucleotide polymorphism; GMM, glucose minimal medium; PBS, phosphate buffered saline; i.t., intratracheal; BALF, bronchoalveolar lavage fluid; GMS, Grocott-Gomori methenamine silver; FLARE, fluorescent *Aspergillus* reporter; vita-PAMP, vitality pathogen-associated molecular pattern

FIGURE LEGENDS

Figure 1. Heat-killed swollen conidia have a decreased ability to induce CXCL10. C57BL/6J mice were challenged i.t. with $4x10^7$ CEA10 live or heat-killed swollen conidia. Forty hours later BALF was collected and IL-28/IFN-λ **(A)**, CXCL10 **(B)**, and TNFα **(C)** levels were determined by ELISA. Data are pooled from 2 independent experiments with 10-15 total mice per group. Statistical significance was determined using a one-way ANOVA with Dunn's post-test (*$p<0.05$; **$p < 0.01$).

Figure 2. *Aspergillus fumigatus* RNA induces an interferon response in a partially MDA5-dependent manner. **(A)** Primary murine fibroblasts from C57BL/6J mice were stimulated with LyoVec™ encapsulated polyI:C (pIC) or total RNA isolated from Af293 for 18 h. After stimulation cell supernatants were collected and analyzed for IFNα (left) and CXCL10 (right) by ELISA. Data from 2 independent experiments with 3 samples per group. **(B)** Primary murine fibroblasts from C57BL/6J and *Ifih1*$^{-/-}$ mice were stimulated with LyoVec™ encapsulated polyI:C (pIC) (MDA5 agonist), 5'-ppp (RIG-I agonist), or total RNA isolated from Af293 for 18 h. The total RNA pool isolated from Af293 was also treated with either RNase S1, RNase III, or DNase to degrade ssRNA, dsRNA, or DNA, respectively prior to encapsulation in LyoVec™. After stimulation cell supernatants were collected and analyzed for IFNα by ELISA. Data from 2 independent experiments with 3 samples per group. Statistical significance was determined by a two-way ANOVA with a Tukey's post-test (τττ $p<0.001$ – LyoVec™ only vs. experimental group; *** $p<0.001$ – B6 vs. *Ifih1*$^{-/-}$; ^^^ $p<0.001$ – enzyme treated vs. Af293).

<u>Figure 3.</u> *Mavs*$^{-/-}$ mice have decreased interferon mRNA levels after *Aspergillus fumigatus* challenge particularly at later times. B6.129F2 and *Mavs*$^{-/-}$ mice were challenged with $4x10^7$ conidia of CEA10**.** Lungs were collected 3 or 48h post-inoculation. Total RNA was extracted from whole lungs. Gene expression as determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using TaqMan probes for *Ifna* **(A)**, *Ifnb* **(B)**, *Ifnl2/3* **(C)**, and *Ifng* **(D)**, which were normalized to *Gapdh* expression. Bars represent data means ± SEM with each dot representing individual mice. Data are representative of results from at least 2 independent experiments with at least 3 mice per group. Data were analyzed using a Mann-Whitney U-test (** $p < 0.01$; *** $p < 0.0001$).

<u>Figure 4.</u> *Mavs*$^{-/-}$ mice have decreased STAT1 phosphorylation after *Aspergillus fumigatus* challenge at later times. (A) C57BL/6J mice were challenged with $4x10^7$ conidia of CEA10 or Af293. Lungs were collected at 6, 24, and 48 hours post-inoculation and total protein was extracted from whole lungs. Protein expression and phosphorylation was determined by Western blot analysis. **(B)** B6.129F2 and *Mavs*$^{-/-}$ mice were challenged with $4x10^7$ conidia of CEA10**.** Lungs were collected at 6- or 48-hours post-inoculation and total protein was extracted from whole lungs. Protein expression and phosphorylation was determined by Western blot analysis. **(C)** Western blots from panel B were quantified by densitometry. Bars represent data means ± SEM with each dot representing individual mice. Data are representative of results from at least 2 independent experiments with at least 3 mice per group. Data were analyzed using a Mann-Whitney U-test.

<u>Figure 5.</u> *Mavs*$^{-/-}$ mice have an altered inflammatory milieu after *Aspergillus fumigatus* challenge in the airways. B6.129F2 and *Mavs*$^{-/-}$ mice were challenged with 4x10^{7} conidia of CEA10**.** Bronchoalveolar lavage fluid (BALF) was collected 12 or 48h post-inoculation. Cytokine and chemokine levels were determined using a Milliplex Mouse Cytokine & Chemokine 32-plex (Millipore). Bars represent data means ± SEM. Data are representative of results 2 independent experiments with 4-6 mice per group. Data were analyzed using a Mann-Whitney U-test (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

<u>Figure 6.</u> Expression of *Cxcl9* and *Cxcl10* are dependent on IFN signaling. C57BL/6J, *Ifnar*$^{-/-}$, *Ifnlr*$^{-/-}$, and *Ifnar*$^{-/-}$*Ifnlr*$^{-/-}$ (DKO) mice were challenged with 4x10^{7} conidia of CEA10**.** Lungs were collected 48h post-inoculation and total RNA was extracted. Gene expression as determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) using TaqMan probes for *Cxcl9* **(A)** and *Cxcl10*, which were normalized to *Gapdh* expression. Bars represent data means ± SEM with each symbol representing individual mice. Data are representative of results from at least 2 independent experiments with at least 4 mice per infected group. Data were analyzed using a Mann-Whitney U-test (n.s. = not significant; **** $p < 0.0001$).

<u>Figure 7.</u> Both *Mavs*$^{-/-}$ and *Ifih1*$^{-/-}$ mice are highly susceptible to *Aspergillus fumigatus*. (A-C) B6.129F2 and *Mavs*$^{-/-}$ mice or **(D-F)** C57BL/6J and *Ifih1*$^{-/-}$ mice were challenged with 4x10^{7} conidia of CEA10. **(A & D)** Survival analysis in immune-competent wild-type and knock-out mice were tracked over the first 8-15 days. Statistical significance was assessed using a Mantel-Cox log rank test (**(A)** $p = 0.0001$ and **(D)** $p = 0.0004$). **(B & E)** At 36 hpi, mice were euthanized

and BALF collected for quantification of macrophage and neutrophil recruitment to the airways. Data from 2 independent experiments for a total of 7-10 mice per group. Data are presented as box-and-whisker plots with Tukey whiskers and outliers displayed as dots. Statistical significance was determined using a Mann-Whitney U test. **(C & F)** At 40-42 hpi mice were euthanized and lungs saved for histological analysis. Formalin-fixed lungs were paraffin embedded, sectioned, and stained with GMS for analysis by microscopy. Representative lung sections from are shown to the right using the 40× objective. *A. fumigatus* germination rates were determined by microscopically counting both the number of conidia and number of germlings in GMS-stained sections. Data from 2 independent experiments with 4-6 mice per group. Statistical significance was determined using a Mann-Whitney U test (*$p < 0.05$; **$p < 0.01$).

Figure 8. *Ifih1*$^{-/-}$ mice have decrease antifungal killing by neutrophils *in vivo*. C57BL/6J and *Ifih1*$^{-/-}$ mice were challenged with $3x10^7$ conidia of CEA10 fluorescent *Aspergillus* reporter (FLARE). Bronchoalveolar lavage (BAL) fluid and lungs were harvested at 48 hours after infection and fungal uptake and viability were analyzed with flow cytometry. Fungal uptake **(A)** and viability **(B)** in neutrophils were measured. Data pooled from 2 independent experiments with 7-9 mice per group. Statistical significance was determined using a Mann-Whitney U test (***$p < 0.01$).

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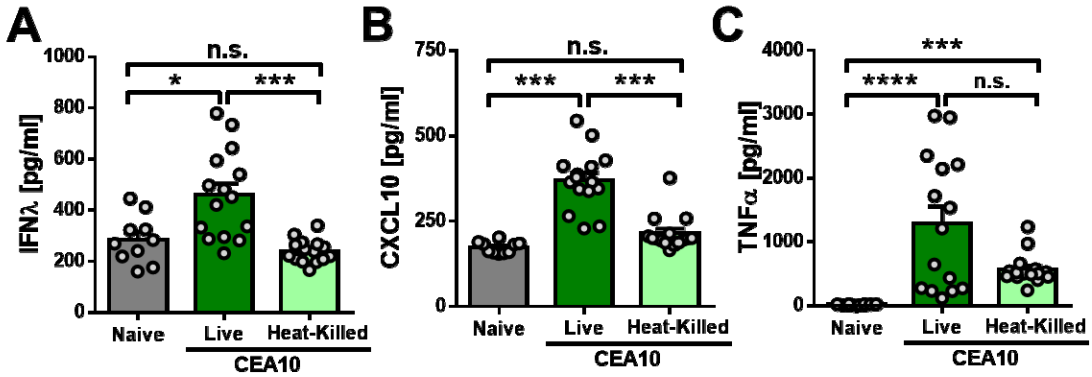
Figure 1



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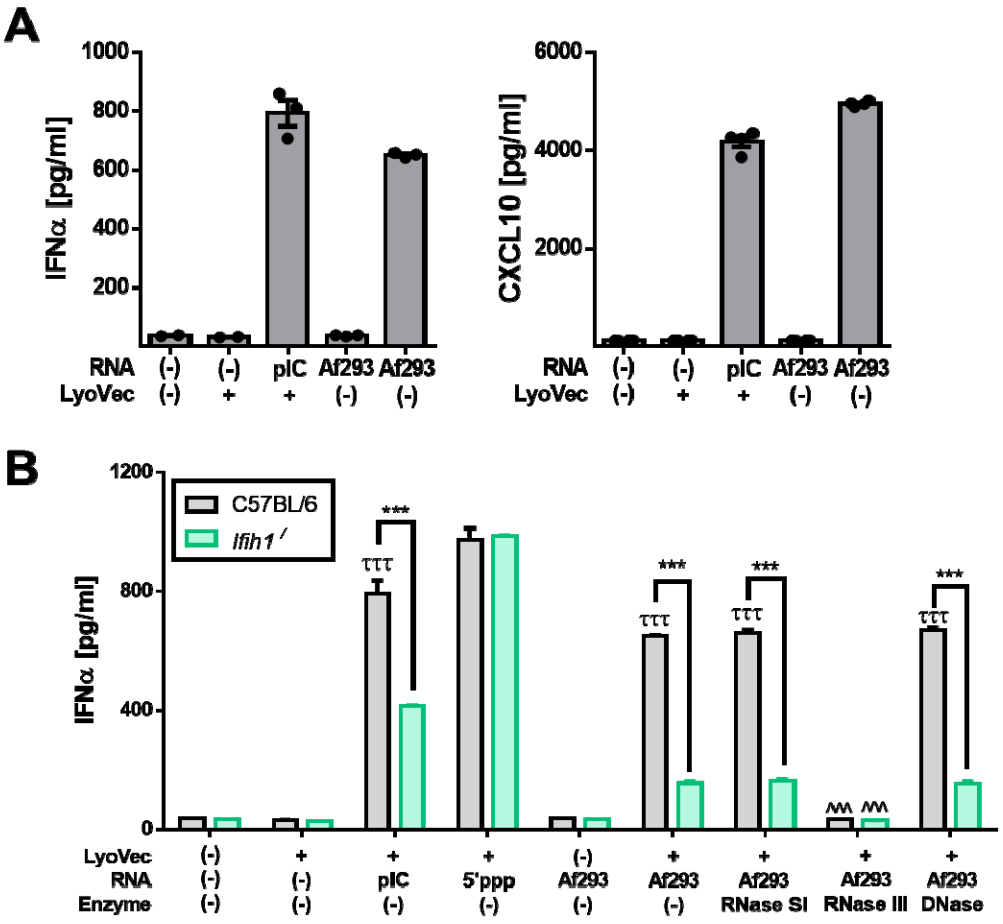

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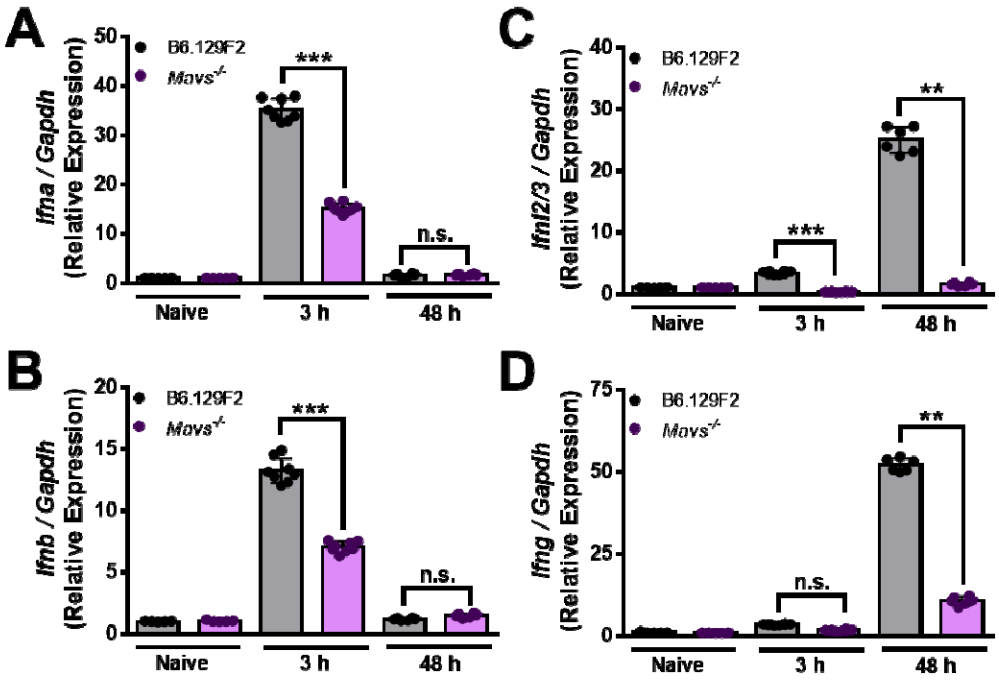

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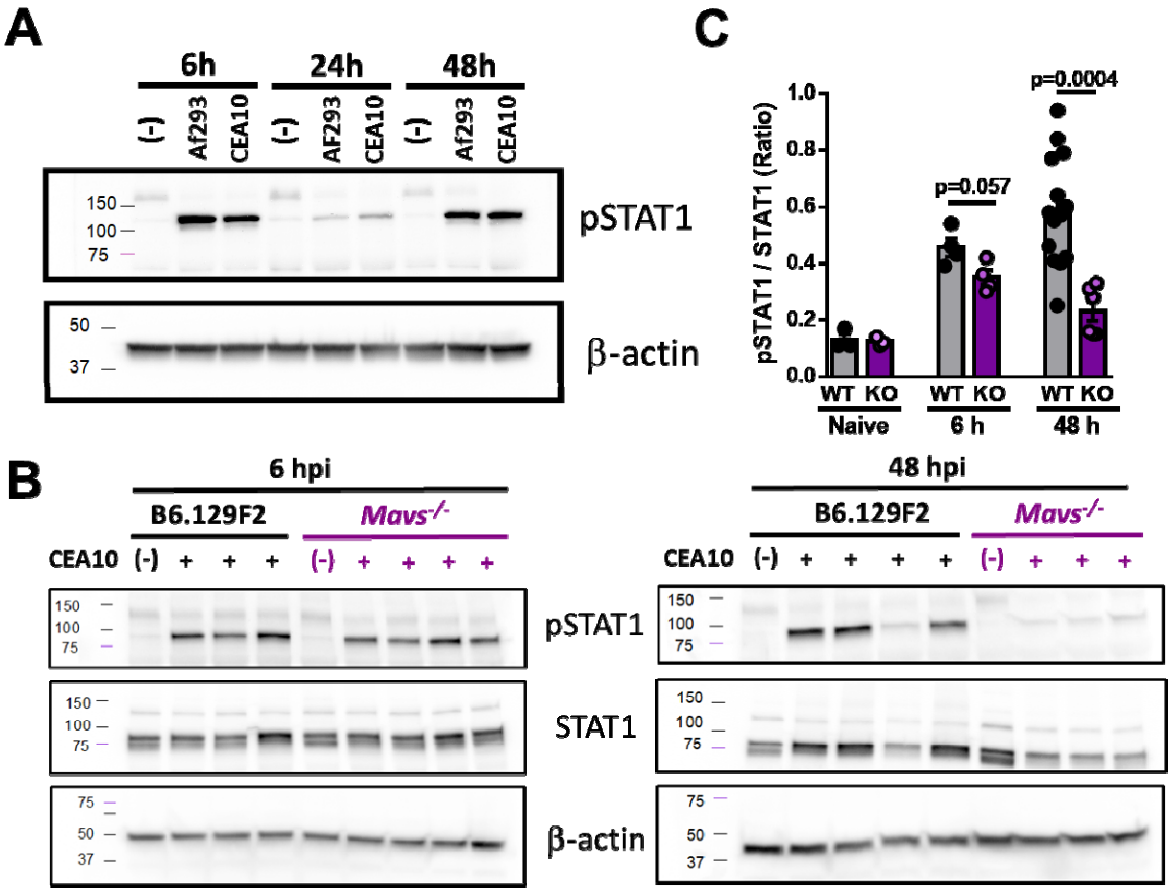

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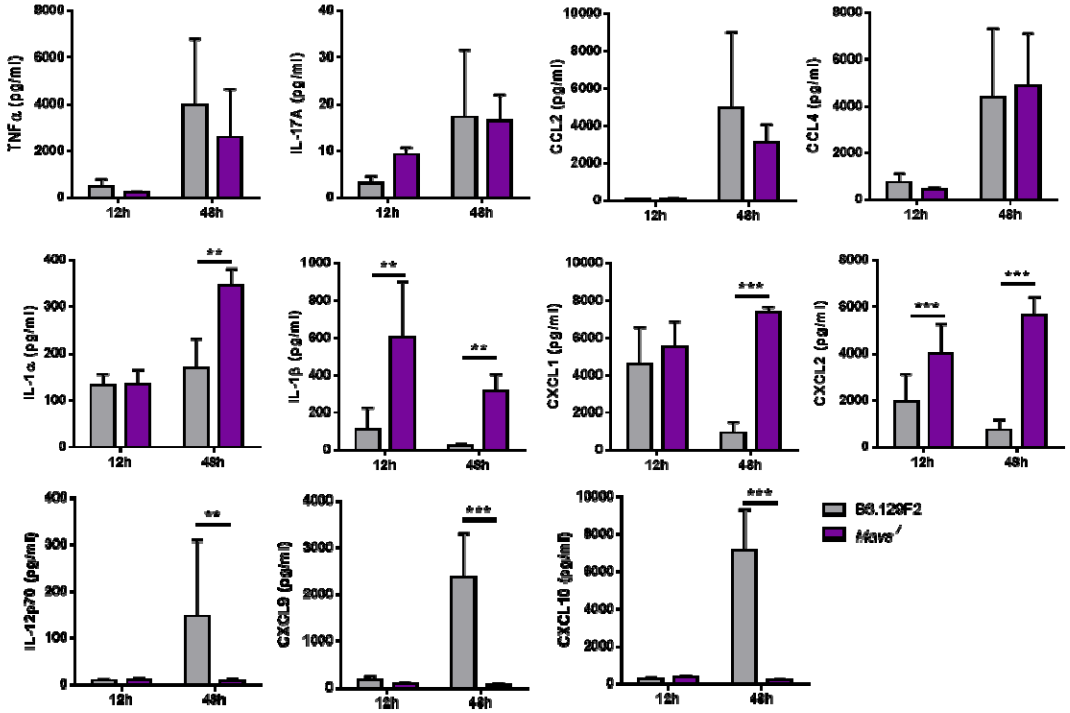

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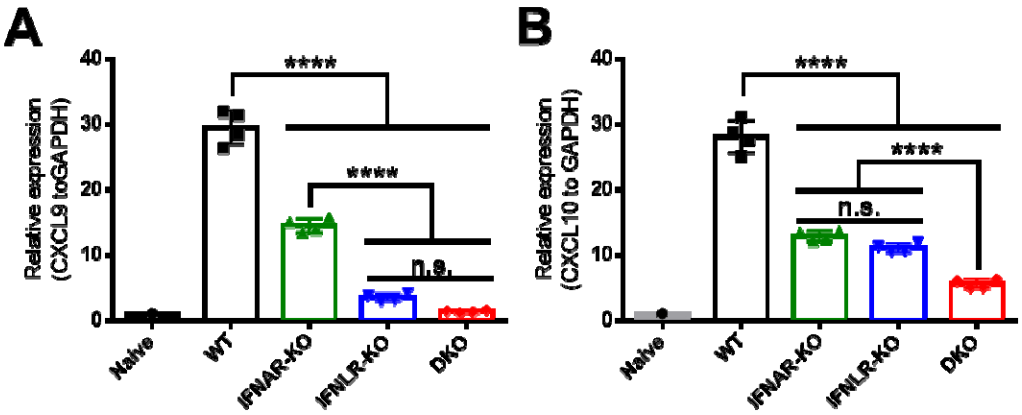

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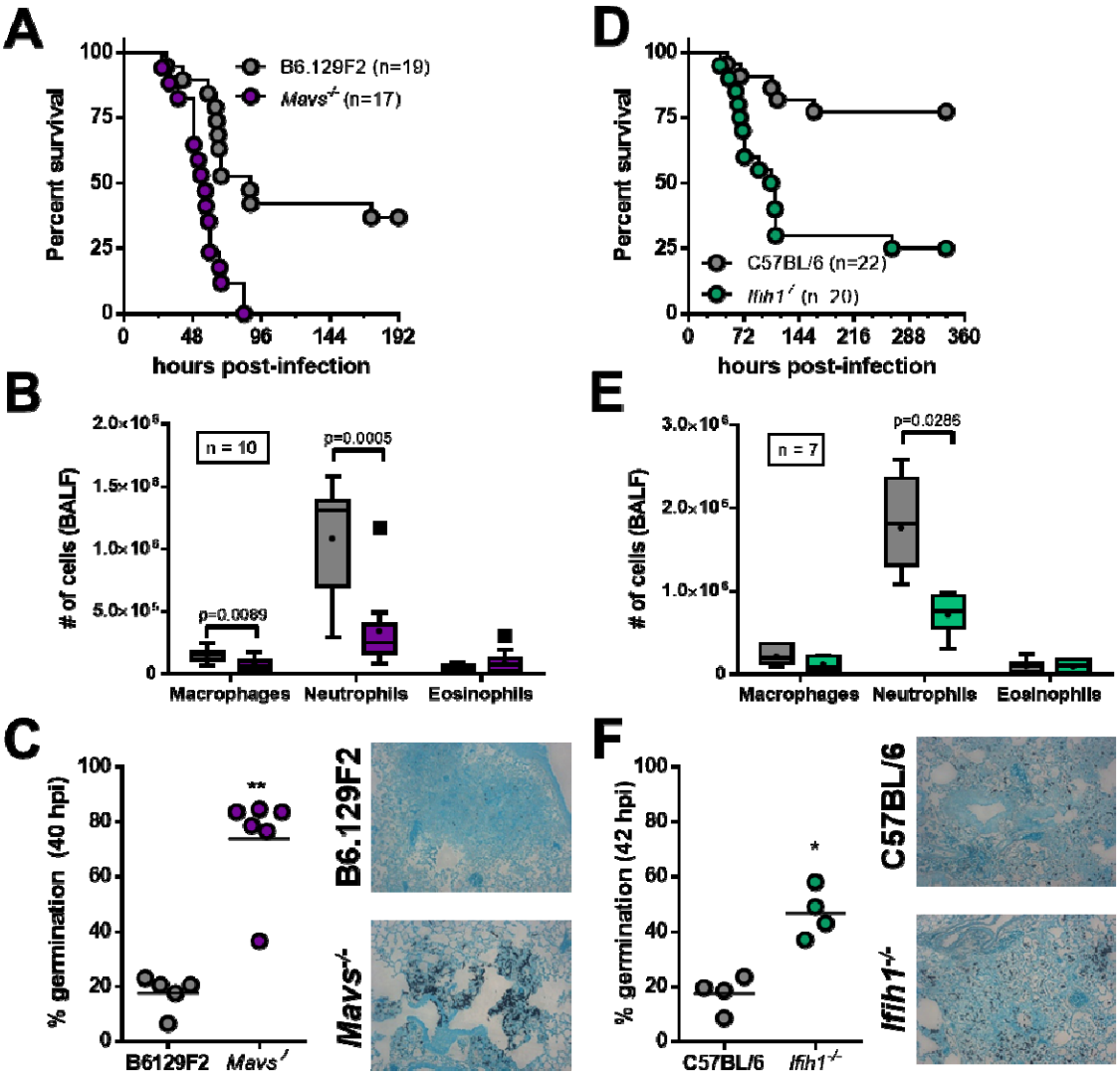

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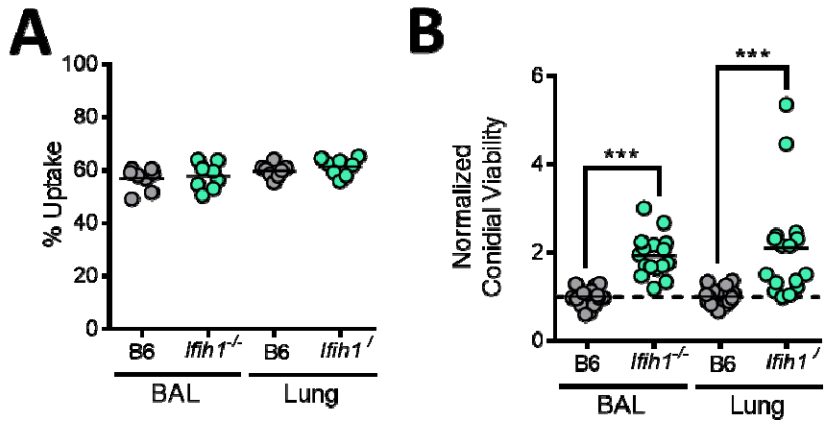

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