

# MAVS and MyD88 are essential for innate immunity but not cytotoxic T lymphocyte response against respiratory syncytial virus

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Infection by RNA viruses is detected by the host through Toll-like receptors or RIG-I-like receptors. Toll-like receptors and RIG-I-like receptors signal through the adaptors MyD88 and MAVS, respectively, to induce type I IFNs (IFN-I) and other antiviral molecules, which are thought to be essential for activating the adaptive immune system. We investigated the role of these adaptors in innate and adaptive immune responses against respiratory syncytial virus (RSV), a common human pathogen. Deletion of *Mavs* abolished the induction of IFN-I and other proinflammatory cytokines by RSV. Genome-wide expression profiling in the lung showed that the vast majority of RSV-induced genes depended on MAVS. Although *Myd88* deficiency did not affect most RSV-induced genes, mice lacking both adaptors harbored a higher and more prolonged viral load and exhibited more severe pulmonary disease than those lacking either adaptor alone. Surprisingly, *Myd88*<sup>-/-</sup>*Mavs*<sup>-/-</sup> mice were able to activate a subset of pulmonary dendritic cells that traffic to the draining lymph node in response to RSV. These mice subsequently mounted a normal cytotoxic T-lymphocyte response and demonstrated delayed but effective viral clearance. These results provide an example of a normal and effective adaptive immune response in the absence of innate immunity mediated by MAVS and MyD88.

adaptive immunity | RSV | IFN

Immune detection of pathogens is mediated by pathogen recognition receptors (PRRs), which, in the case of RNA viruses, involve a subset of Toll-like receptors (TLRs 3, 7, and 8) and the cytoplasmic RIG-I-like receptors (RLRs) (1–3). TLR 7/8 binds viral single-stranded RNA in endosomes and engages the cytosolic adaptor MyD88 to activate downstream signaling pathways leading to the activation of NF- $\kappa$ B and interferon regulatory factors (i.e., IRF3, IRF7). The RLRs, including RIG-I and MDA5, bind viral double-stranded RNA or 5'-triphosphorylated uncapped viral RNA in the cytosol (4, 5). Through their common adaptor, MAVS (also known as IPS-1, VISA, or CARDIF), RLRs also activate NF- $\kappa$ B and IRFs (6–9). Together with activated AP-1, NF- $\kappa$ B and IRFs subsequently induce transcription of antiviral genes, including type I IFNs (e.g., IFN- $\alpha$  and IFN- $\beta$ , herein referred to as IFN-I). In addition to IFN-I, proinflammatory cytokines and chemokines are induced downstream of TLR and RLR signaling. It is thought that these innate responses serve to limit the extent of infection until the adaptive immune response is able to clear the virus.

Activation of adaptive B and T cell responses is driven by dendritic cells (DCs) (10). Upon detection of viral infection through PRRs, DCs become activated and traffic to lymph nodes, where they present viral antigens and provide additional signals to activate CD4<sup>+</sup> and CD8<sup>+</sup> T cells. Activated CD4<sup>+</sup> T cells can, in turn, activate virus-specific B cells, resulting in antibody production.

Respiratory syncytial virus (RSV) belongs to the Paramyxovirus family of negative-sense single-stranded RNA viruses. RSV

infection is currently recognized as the leading cause of respiratory disease in infants and young children, and it can also be life threatening in elderly and immune-compromised adults (11–13). There is currently no vaccine available against RSV. A previous failed vaccine attempt underscores the importance of a better understanding of the host response to the virus for the design of safe and effective treatment and prophylaxis (14, 15). In this report, we generated mice lacking *Mavs*, *Myd88*, or both (double knockout, DKO) to investigate the roles of RLR and TLR signaling in the innate and adaptive immune responses to RSV.

## Results

**Innate Cytokine Induction Depends on MAVS-Mediated Signaling.** In the first series of experiments, we studied the cell-specific IFN-I response to RSV. Lung fibroblasts, bone marrow-derived macrophages, and conventional DCs (cDCs) were isolated and infected with RSV. Measurement of IFN- $\alpha$  and - $\beta$  production by ELISA, shown in Fig. 1A–E, revealed that IFN-I induction by RSV in all these cell types completely depended on MAVS. Previous studies have suggested that plasmacytoid DCs (pDCs) rely on TLR7 and MyD88 to induce IFN-I in response to RNA viruses, whereas other cell types use the RLR-MAVS pathway (16, 17). Although Sendai virus (SeV) infection triggered IFN-I production in purified pDCs, we were unable to detect any IFN- $\alpha$  or - $\beta$  in these cells after RSV infection [supporting information (SI) Fig. S1A]. Consistent with this result, the induction of IFN- $\alpha$  and - $\beta$  by RSV depended on MAVS in bone marrow cells treated with the Flt3 ligand, which leads to the generation of pDCs and cDCs (Fig. S1B and C). In contrast, infection of Flt3L-DCs with SeV triggered IFN-I production in a manner that was independent of MAVS. This is consistent with our previous finding that MAVS is dispensable for IFN-I induction by SeV in pDC, which is the major source of IFN-I in SeV-infected Flt3L-DCs (16).

To examine the role of MAVS and MyD88 in RSV infection *in vivo*, we infected *Mavs*<sup>-/-</sup>, *Myd88*<sup>-/-</sup>, and DKO mice with RSV via the intranasal route (Fig. 1F). Consistent with the *in vitro* results, 1 day after infection, WT mice secreted a large amount of IFN-I, which was detected in bronchoalveolar lavage fluid (BALF). This response was normal in MyD88-null mice but abolished in mice lacking MAVS. Consistently, MAVS-deficient

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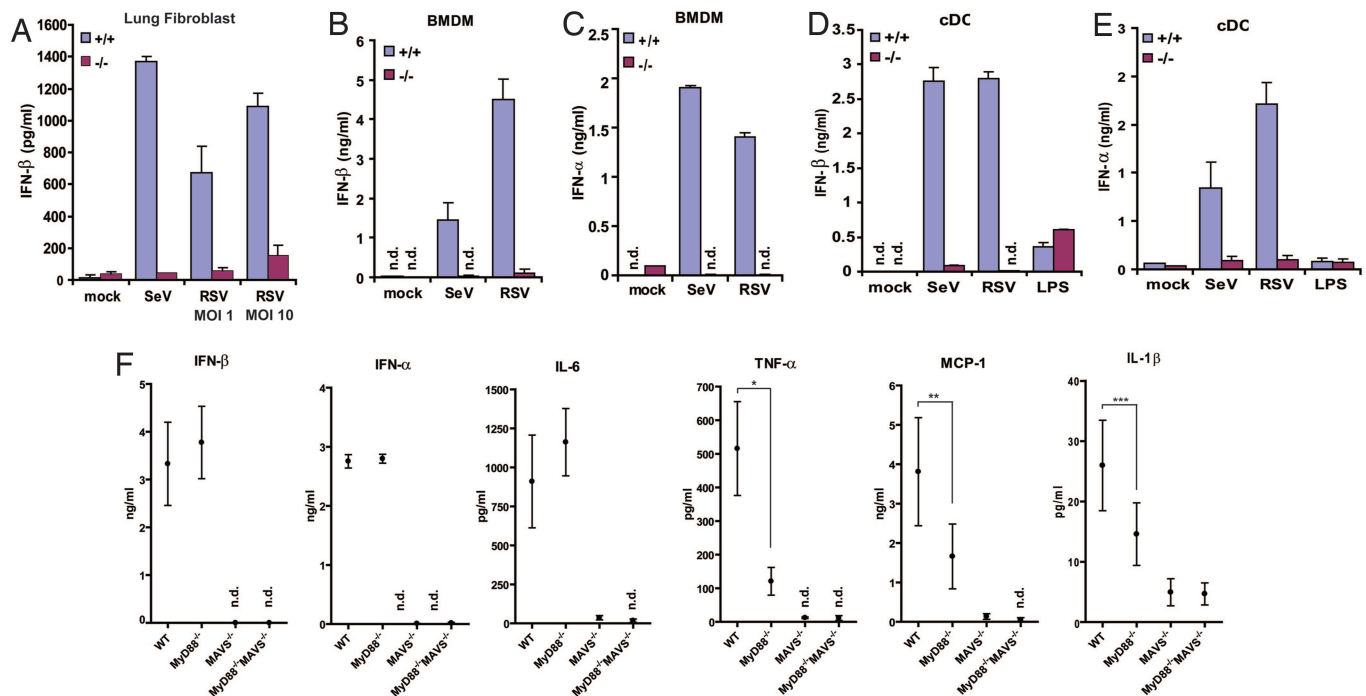
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**Fig. 1.** *In vitro* and *in vivo* cytokine responses to RSV infection are lost in the absence of MAVS. Adult lung fibroblasts (A), bone marrow-derived macrophages (BMDM; B and C), and cDCs (D and E) from WT<sup>+/+</sup> and *Mavs*<sup>-/-</sup> mice were infected with SeV or RSV for 24 h before culture supernatants were analyzed for IFN-β or -α by ELISA. Data are represented as mean ± SD. (F) Mice of the indicated genotypes were intranasally infected with RSV (10<sup>7</sup> pfu) for 24 h, and then BALF was harvested for cytokine measurements by ELISA (for IFN-α, IFN-β, and IL-1β) or Cytometric Bead Array (for IL-6, TNF-α, and MCP-1) (\*, *P* < 0.001; \*\*, *P* < 0.01; \*\*\*, *P* < 0.05; ANOVA, Tukey's test) (*n* = 4 per group). n.d., not detected. Data are represented as mean ± SEM.

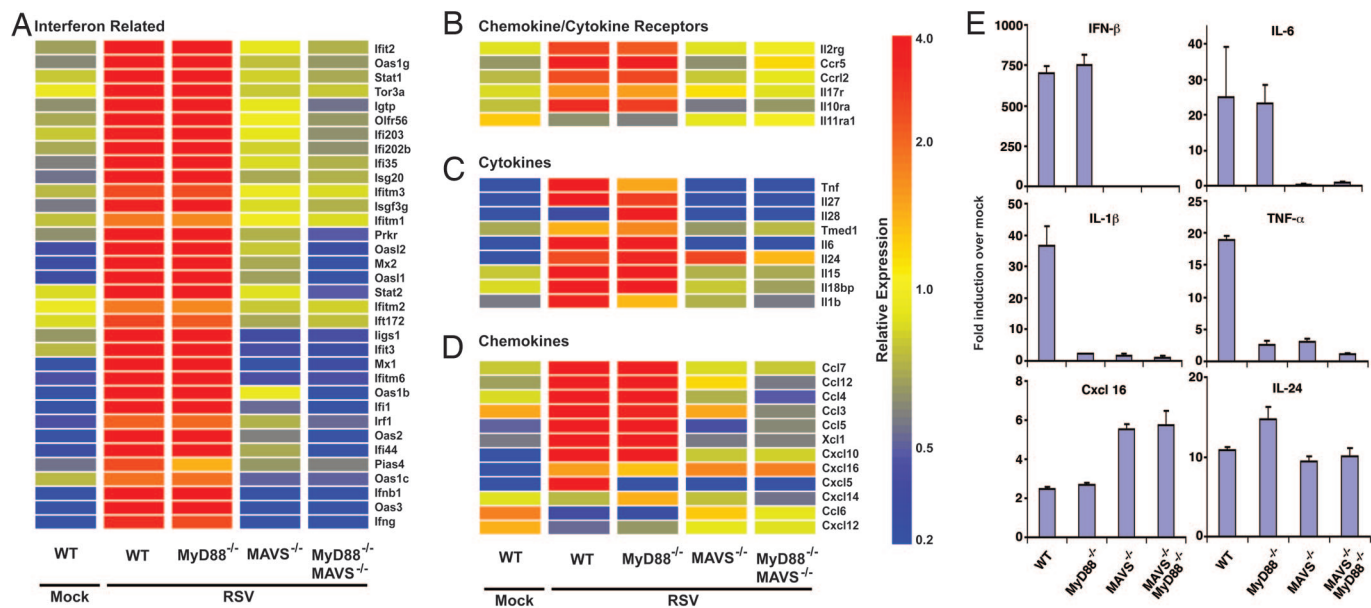
mice failed to induce and activate STAT1 in the lung after RSV infection, whereas this IFN signaling response was normal when these mice were infected with vesicular stomatitis virus, an RNA virus known to induce IFN-I independent of MAVS (Fig. S2; ref. 16). Other proinflammatory cytokines, including IL-6, TNF-α, monocyte chemoattractant protein (MCP)-1, and IL-1β, also depended on MAVS for their expression. Interestingly, maximal production of TNF-α, MCP-1, and IL-1β also required MyD88. At the same time point, day 1, we were unable to detect significant levels of IFN-γ, IL-12, or IL-10 in the BALF (data not shown). Neither IFN-α nor -β was detectable in sera and mediastinal lymph nodes by ELISA or quantitative PCR in mice of any genotype (data not shown). Recently, it has been shown that in response to intranasal Newcastle disease virus infection, IFN-α-producing alveolar macrophages and cDCs could be detected after 24 h in WT mice (18). In MAVS-deficient animals, however, this response was abolished and a compensatory IFN-α response was detected in pDCs at 48 h. However, we found no evidence of IFN-I production 2, 5, 9, or 14 days after RSV infection in *Mavs*<sup>-/-</sup> mice (Fig. S3, data not shown), consistent with our *in vitro* observation that RSV does not induce IFN-I in pDCs.

To evaluate the roles of MyD88- and MAVS-mediated signaling in the global pulmonary innate immune response to RSV, we analyzed lung RNA by microarray 24 h after infection (Fig. 2). Of the 659 genes induced by ≥2-fold in WT mice, 440 (~66.8%) depended on MAVS but not on MyD88, 12 (~1.8%) depended on MyD88 but not on MAVS, and 90 (13.7%) depended on both (Fig. S4). The induction of most IFN-related genes was MAVS-dependent and MyD88-independent (Fig. 2A). A similar pattern of dependence was seen for most of the cytokine-, chemokine-, and PRR-related genes (Fig. 2B–D, Fig. S5). In contrast, optimal expression of TNF-α and IL-1β depended on both adaptors (Fig. 2E). Interestingly, both IL-24 and

Cxcl-16 were induced in all RSV-infected groups compared with mock-treated groups. With the exception of these chemokines, innate immune responses were largely absent in *Mavs*<sup>-/-</sup> and DKO mice.

**Viral Clearance in the Absence of MAVS and MyD88.** Next, we evaluated viral clearance in these mice by measuring viral loads in the BALF at various times after infection. In WT mice, viral loads peaked on day 5 after infection and reached undetectable levels by day 9 as previously observed in this model (Fig. 3A) (19). Consistent with a previous study, MyD88-deficient mice showed no significant differences in viral clearance compared with WT mice (20). In contrast, viral loads in *Mavs*<sup>-/-</sup> mice were ≈100 times higher than those in the WT mice on days 1 and 5 after infection. Surprisingly, despite the absence of IFN-I and other antiviral molecules, these mice were able to clear the virus to undetectable levels by day 9. The DKO mice showed no significant difference in viral loads compared with the *Mavs*<sup>-/-</sup> group 1 day after infection, suggesting the importance of MAVS- but not MyD88-mediated viral clearance in the early phase of infection. This is consistent with the lack of a pDC response, which would otherwise be the major source of early TLR-dependent antiviral interferons. As the infection progressed, however, DKO mice harbored significantly higher viral loads compared with all other genotypes. Remarkably, DKO mice were also able to clear the infection by day 28 (Fig. 3B).

Consistent with unrestricted viral replication in *Mavs*<sup>-/-</sup> and DKO mice in the early phase of RSV infection, immunohistochemical staining with RSV antibodies in the lungs of these mice showed evidence of infection of ciliated respiratory epithelium lining the bronchioles and bronchi on days 1 and 5 after infection (Fig. 3C). This staining was undetectable by day 14. Histological examination of lungs of infected mice revealed no striking differences on day 1 (Fig. S6A). On day 5, the inflammation seen in DKO mice appeared in patches compared with the diffuse



**Fig. 2.** Global gene expression analysis of lung RNA after infection with RSV reveals profound defects in mice lacking MAVS. For each genotype, two mice were mock treated and three were inoculated with RSV ( $10^7$  pfu) for 24 hours before lungs were harvested for total RNA extraction, which was analyzed by microarray. (A–D) Mean relative expression of genes known to be involved in immune responses, including interferon-related (A), chemokine or cytokine receptors (B), cytokines (C), and chemokines (D). (E) Induction of selected genes was confirmed by qPCR. Data are represented as mean  $\pm$  SEM.

inflammation in the other three genotypes. At this time point, there was no gross difference in the composition of the cellular infiltrate among all groups. However, few multinucleated syncytial cells were observed in the bronchioles of DKO mice (Fig. S6B). On day 14, inflammation had largely subsided in WT and *Myd88*<sup>-/-</sup> mice but remained in the other two groups. Although *Mavs*<sup>-/-</sup> mice had some chronic inflammation at this stage, DKO mice exhibited patchy acute pneumonia, numerous syncytial cells, and reactive bronchial epithelium (Fig. S6C). By day 28, inflammation was significantly reduced and comparable in all genotypes (Fig. S6D).

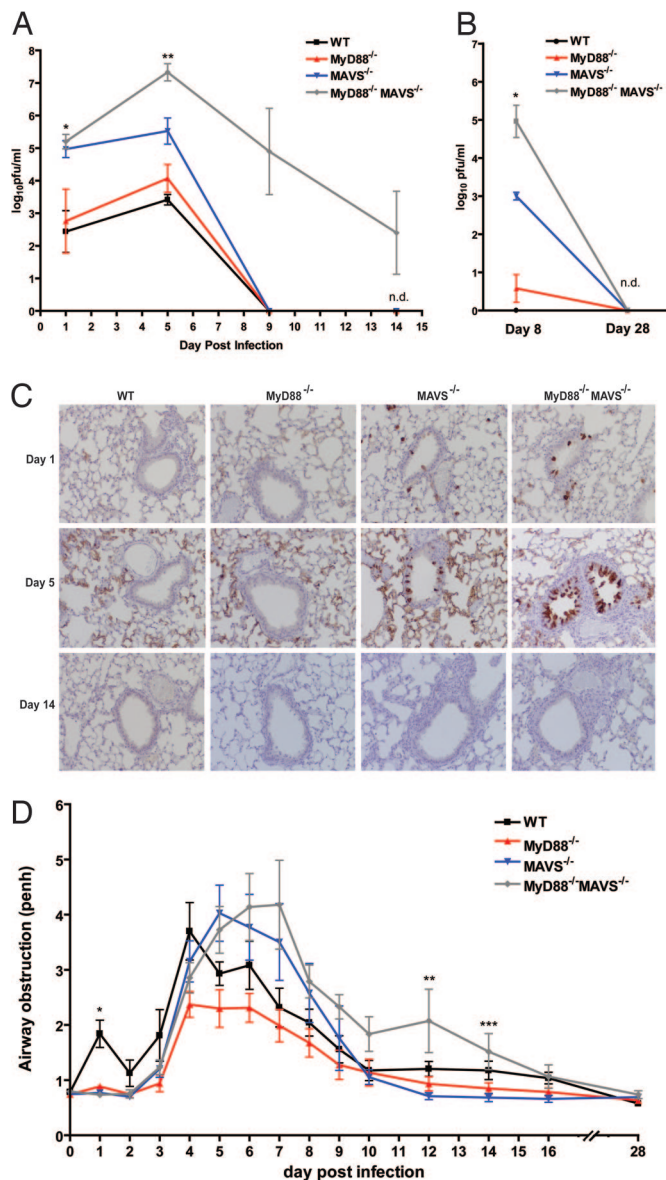
**MAVS or MyD88 Deficiency Alleviates only the Initial Phase of Pulmonary Disease, and All Mice Eventually Recover.** Next, we evaluated the roles of MAVS and MyD88 signaling in the pulmonary function of mice by plethysmography (Fig. 3D). One day after infection, only WT mice exhibited pulmonary dysfunction, possibly attributable to inflammatory cytokine responses that cause airway obstruction ( $P < 0.0001$ ). We also consistently noted that only WT mice had ruffled hair and were less active than other genotypes on day 1. This is consistent with cytokine measurements, which showed that mice lacking MAVS, MyD88, or both had markedly reduced levels of the proinflammatory cytokines TNF- $\alpha$ , IL-6, and IL-1 $\beta$  (Figs. 1 and 2). Between days 2 and 10, all groups developed increased airway obstruction compared with their baseline function. Whereas the WT, *Mavs*<sup>-/-</sup>, and *Myd88*<sup>-/-</sup> mice returned to normal function around day 10, the DKO mice experienced slightly prolonged pulmonary dysfunction but eventually also returned to normal lung function (Fig. 3D; day 12,  $P = 0.03$ ; day 14,  $P = 0.04$ ).

**Antibody Production Is Defective in the Absence of MAVS or MyD88.** The surprising ability of *Mavs*<sup>-/-</sup> and DKO mice to clear RSV led us to examine anti-RSV adaptive immune responses in these mice. Serum anti-RSV antibody responses, including the production of both IgG1 and IgG2a, were significantly attenuated in mice lacking either MAVS or MyD88 and were reduced even further in the DKO mice (Fig. 4A). These results suggest that both MAVS and MyD88 play an important role in the generation

of anti-RSV antibodies in the adaptive phase of the antiviral response. This is in contrast to antibody production in response to influenza, which is MyD88 dependent and MAVS independent (21). However, depletion of B cells, followed by RSV infection, has suggested that the antibody response is unlikely to be a major mediator of primary RSV clearance (22).

#### Normal CD8<sup>+</sup> T Cell Response in the Absence of MAVS and MyD88.

Next, we examined the activation of the CD8<sup>+</sup> cytotoxic T-lymphocyte (CTL) response in the lungs of mice after intranasal infection of RSV. Eight days after infection, lung cells were stimulated *in vitro* with an RSV-derived peptide previously identified as a CD8<sup>+</sup> T cell stimulating epitope or with a control SeV-derived peptide, both of which bind H2-Db (23). The percentage of IFN- $\gamma$ -producing CD8<sup>+</sup> pulmonary T cells was quantified by FACS (Fig. 4B and Fig. S7). Unexpectedly, RSV-specific CD8<sup>+</sup> T cell activation was similar in *Myd88*<sup>-/-</sup> and *Mavs*<sup>-/-</sup> mice compared with WT counterparts. Even the DKO mice were clearly able to activate CD8<sup>+</sup> T cells in response to RSV, although the responses were slightly but not significantly lower ( $P = 0.35$ ). In fact, measurement of IFN- $\gamma$  in the BALF of WT and DKO mice at various times after infection revealed that DKO mice produced this cytokine more robustly than WT counterparts (Fig. 4C). In WT mice, IFN- $\gamma$  peaked on day 6 after RSV infection and was undetectable by day 10. In DKO mice, however, IFN- $\gamma$  peaked on day 8 at a higher level than in WT mice, suggesting that the higher viral load in DKO mice led to a corresponding increase in IFN- $\gamma$  production. Additionally, analysis of CD8<sup>+</sup> T cell cytolytic activity showed that, compared with cells from mock-treated animals, lung cells from RSV-infected mice of all genotypes demonstrated increased ability to specifically kill target cells loaded with the MHC-I-binding RSV epitope (Fig. 4D). Furthermore, depletion of CD8<sup>+</sup> T cells in conjunction with RSV infection firmly established a role for these cells. Analysis of BALF 8 days after infection revealed that production of IFN- $\gamma$  was intact in all mice treated with control IgG but absent in mice depleted of CD8<sup>+</sup> T cells, pointing to these cells as the likely source of the cytokine (Fig. 4E). Finally, measurement of viral titers in *Mavs*<sup>-/-</sup> and DKO mice showed



**Fig. 3.** *Mavs*<sup>-/-</sup> and DKO mice are able to clear RSV and resolve pulmonary disease. (A and B) WT, *Myd88*<sup>-/-</sup>, *Mavs*<sup>-/-</sup>, and *Myd88*<sup>-/-</sup>*Mavs*<sup>-/-</sup> (DKO) mice were infected with RSV (10<sup>7</sup> pfu) and BALF was harvested at the indicated times to measure viral loads by plaque assay. (A: \*, *mavs*<sup>-/-</sup> vs. *myd88*<sup>-/-</sup>,  $P < 0.001$ ; \*\*, *mavs*<sup>-/-</sup> vs. DKO and *mavs*<sup>-/-</sup> vs. *myd88*<sup>-/-</sup>,  $P < 0.001$ ; B: \*, *mavs*<sup>-/-</sup> vs. DKO and *mavs*<sup>-/-</sup> vs. *myd88*<sup>-/-</sup>,  $P < 0.01$ ; ANOVA) ( $n = 3-5$ ). (C) Lungs from infected mice and mock controls were analyzed by immunohistochemistry using a polyclonal anti-RSV antibody. (D) Mice infected with RSV were assessed by whole-body plethysmography to measure airway obstruction (\*,  $P < 0.0001$ ; \*\*,  $P < 0.03$ ; \*\*\*,  $P < 0.04$ ; ANOVA). n.d., not detected; Penh, enhanced pause. Data are represented as mean  $\pm$  SEM.

much higher levels of virus when CD8<sup>+</sup> T cells were depleted (Fig. 4F). In contrast, depletion of CD8 T cells did not increase the viral titers in WT and *Myd88*<sup>-/-</sup> mice, presumably because these mice still had an intact MAVS pathway to clear the virus. Together, these data clearly show that CTL activation was intact and effective in DKO mice.

**Subset of Pulmonary DCs Is Activated in *Mavs*<sup>-/-</sup> and DKO Mice.** To investigate how *Mavs*<sup>-/-</sup> and DKO mice mount a largely normal CTL response in the absence of innate IFNs and cytokines known to be important for activating adaptive immunity, we

examined the activation of DCs. We first measured up-regulation of CD86 in CD11c<sup>+</sup> GM-CSF-derived bone marrow-derived DCs (BMDCs) in response to RSV and SeV infection *in vitro*. CD86 up-regulation was observed in WT and *Myd88*<sup>-/-</sup> DCs but not in *Mavs*<sup>-/-</sup> and DKO DCs (Fig. S8), suggesting a complete dependence on MAVS-mediated signaling. As a control, LPS-induced CD86 surface expression was observed in DCs of all genotypes.

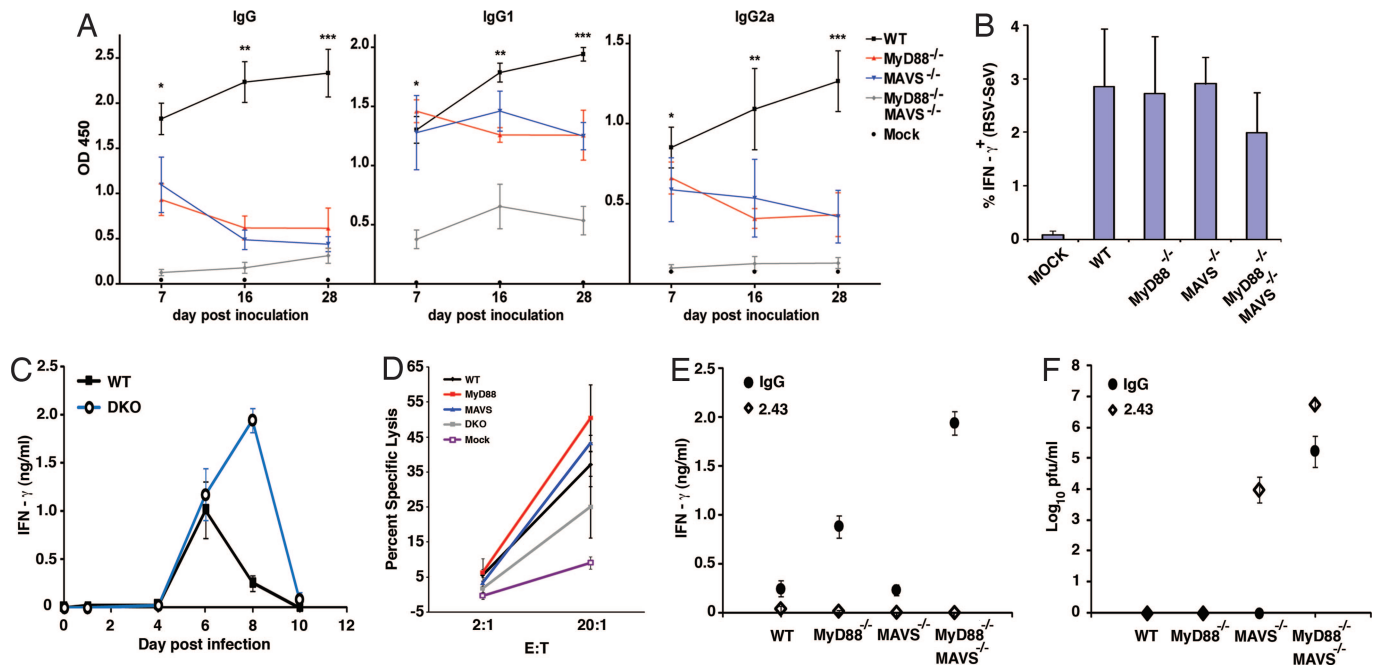
Because the RSV-induced response in these cultured DCs did not explain intact CD8<sup>+</sup> T cell activation in mice lacking MAVS, we examined *in vivo* activation of pulmonary cDCs, which were identified as CD11c<sup>+</sup>, CD2<sup>-</sup>, and F4/80<sup>-</sup> cells (18). Both WT and *Myd88*-deficient CD11c<sup>+</sup> DCs up-regulated surface levels of CD86 (Fig. 5A) and CD80 (data not shown) after RSV infection compared with mock-infected mice. In both MAVS-deficient and DKO mice, up-regulation of these molecules was drastically reduced. However, a small percentage of the pulmonary DCs in these mice consistently up-regulated these molecules, indicating their activation. In contrast, CD86 up-regulation was completely MAVS-dependent in CD11c<sup>+</sup>CD2<sup>+</sup>F4/80<sup>+</sup> alveolar macrophages of the same mice (Fig. 5A).

After activation, antigen-loaded DCs traffic from their peripheral tissue location to draining lymph nodes, where they activate T cells. Therefore, we examined the phenotype of DCs in the mediastinal lymph nodes draining the lung (Fig. 5B). Upon RSV infection, the majority of lymph node DCs in WT and *Myd88*<sup>-/-</sup> mice were CD86<sup>high</sup> compared with DCs in mock-treated mice. Consistent with the notion that the subset of pulmonary DCs activated in the absence of MAVS may subsequently traffic to the lymph node, we found a significant percentage of CD86<sup>high</sup> DCs in the lymph nodes of *Mavs*<sup>-/-</sup> and DKO mice. This subset of DCs may be responsible for the effective activation of RSV-specific CTLs in the absence of innate immunity mediated by MAVS and MyD88.

## Discussion

We have provided both *in vivo* and *in vitro* evidence that MAVS is essential for the production of IFN-I and inflammatory cytokines in response to RSV infection. Global gene expression profiling further demonstrated that MAVS is required for RSV-induced production of the vast majority of antiviral molecules. In contrast to MAVS, MyD88 is dispensable for the induction of IFN-I and the majority of antiviral cytokines, with a few exceptions such as TNF- $\alpha$ , IL-1 $\beta$ , and MCP-1. The defective production of these molecules in all of the mutant mice may reflect the complex regulation unique to these potent inflammatory mediators. Notably, *Il-24* and *cxcl-16* were induced independently of MAVS and MyD88. Whether these cytokines play any role in the immune response to RSV requires further study.

It has recently been shown that systemic infection with the RNA virus, LCMV, results in IFN-I, inflammatory cytokine and chemokine production in a MyD88-dependent but largely MAVS-independent manner (24). Using an intranasal mode of infection, the same group found that influenza virus-induced IFN-I is defective only in mice lacking both MAVS and MyD88 (21). Further, pulmonary NDV infection indicated that mice lacking either MAVS or MyD88 are still capable of producing IFN- $\alpha$  (18). These results indicate that the requirement of MAVS and MyD88 for innate cytokine responses depends on the pathogen and on the route of infection. Our data suggest that RSV solely relies on the RLR-MAVS pathway for IFN-I induction *in vivo*. Further, we find no evidence of compensatory MyD88-mediated IFN-I production, which is consistent with recent data on the lack of IFN production by pDCs in response to RSV infection (25) but inconsistent with other reports suggesting IFN-I induction in these cells by RSV (26, 27). Our



**Fig. 4.** Adaptive immune responses in mice lacking *Mavs* and *Myd88*. (A) RSV-specific antibody subtypes, IgG, IgG1, and IgG2a, were measured by ELISA using sera taken on the indicated day after RSV infection (IgG; \*, WT vs. *Myd88*<sup>-/-</sup>;  $P < 0.01$ , *Myd88*<sup>-/-</sup> vs. DKO,  $P < 0.05$ ; \*\* and \*\*\*, WT vs. *Myd88*<sup>-/-</sup>;  $P < 0.001$ ) (IgG1; \*, *Mavs*<sup>-/-</sup> vs. DKO,  $P < 0.01$ ; \*\* and \*\*\*, WT vs. *Myd88*<sup>-/-</sup> and *Myd88*<sup>-/-</sup> vs. DKO,  $P < 0.05$ ) (IgG2a; \*, WT vs. *Myd88*<sup>-/-</sup> and *Mavs*<sup>-/-</sup> vs. DKO,  $P < 0.01$ ; \*\*, WT vs. *Mavs*<sup>-/-</sup>,  $P < 0.001$ ; \*\*\*, WT vs. *Myd88*<sup>-/-</sup>,  $P < 0.001$ ) (ANOVA, Tukey's test) ( $n = 7$ – $10$  per group). (B) Mice were mock-infected or infected with RSV for 8 days before lung cells were harvested for stimulation with an RSV- or SeV (control)-derived peptide. Six hours after stimulation, intracellular IFN- $\gamma$  levels in CD3<sup>+</sup>CD8<sup>+</sup> cells were measured by FACS. The percentage of IFN- $\gamma$ <sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> cells in RSV-peptide treated cultures minus that from SeV-peptide cultures is plotted for each genotype ( $P = 0.35$ ; ANOVA) ( $n = 5$  per group). (C) Bronchoalveolar lavage fluid IFN- $\gamma$  was measured in WT and DKO mice at the indicated times after RSV infection ( $n = 3$  per group). (D) Lung cells (effectors) from day 8 RSV- or mock-infected mice were incubated with EL4 target cells loaded with a peptide (RSV peptide or SeV control peptide). The target cells were differentially labeled with CFSE and incubated with the effector cells at the indicated effector:target (E:T) ratios for 4 hours. Cells were then analyzed by flow-cytometry and the specific killing of RSV peptide-loaded targets was calculated ( $n = 3$  per group). (E) Mice were either depleted of CD8<sup>+</sup> T cells (with antibody 2.43) or mock depleted (IgG) and infected with RSV. BALF was extracted on day 8 and IFN- $\gamma$  was measured by ELISA ( $n = 3$  per group). (F) BALF viral titers were measured for mice described in E. Data are represented as mean  $\pm$  SEM.

observation that RSV-infected *Myd88*<sup>-/-</sup> mice induce normal levels of IFN-I suggests that in this model, pDCs do not produce these cytokines because they are expected to use TLR7, which requires MyD88 for signaling. The reason for these seemingly conflicting results is unclear but may be partially explained by differences in virus strain or cell depletion methods.

Despite such a drastically defective cytokine response and the fact that *Mavs*<sup>-/-</sup> mice harbored higher viral loads shortly after RSV infection, these mice still cleared the virus effectively by activating a normal CTL response. Antibody-mediated depletion of CD8<sup>+</sup> T cells significantly prolonged RSV replication, confirming their appropriate activation and demonstrating the important role of these cells in controlling the virus. Although deletion of *Myd88* alone had no effect on RSV loads, mice lacking both adaptors had higher and more prolonged viral loads than those lacking either one alone, suggesting that in the absence of MAVS, MyD88 signaling contributed to antiviral immunity through a mechanism independent of IFN-I induction. Remarkably, even the DKO mice were able to activate CD8<sup>+</sup> T cells and clear the virus effectively.

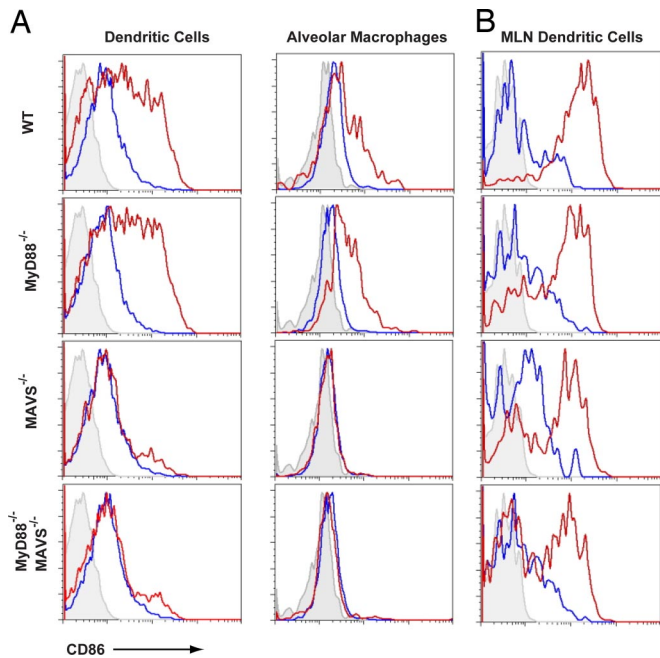
It is surprising that CD8<sup>+</sup> T cell activation is normal in the absence of MAVS, because it is widely believed that IFNs and cytokines produced during innate antiviral responses are required for activating adaptive immunity, including cross-priming (28, 29). A possible answer to this conundrum is our finding that a subset of pulmonary DCs is activated by RSV in the lung and then migrates to the mediastinal lymph nodes in the absence of MAVS and MyD88. It is possible that this subset of DCs may be responsible for the cross-priming of CD8<sup>+</sup> T cells in a manner

that depends on TLR3 and Trif (30). We have assessed the expression of several surface markers, including CD8 $\alpha$ , CD4, CD11b, B220, mPDCA1, and GR-1, on the pulmonary DCs and found that none are specifically enriched or lower in the activated subset in the WT mice or in those lacking *MAVS* or *MyD88*. Therefore, it is still not clear what makes this subset of DCs unique in getting activated by RSV in the absence of MAVS and MyD88. Further characterization of this DC subset should provide important insights into the regulation of T cell responses to RSV and possibly other pathogens.

Our results highlight the sophistication of the host's antiviral defense mechanisms and provide an example of an effective adaptive immune response in the absence of known innate immune mediators. Our results also have important implications for the development of RSV therapeutics and vaccines, as a significant contributor of RSV disease is an inappropriate CD8<sup>+</sup> T cell response. Indeed, antibody-mediated depletion of CD8<sup>+</sup> T cells alleviates disease progression in RSV-infected mice (22, 31). Because MAVS is required for optimal anti-RSV antibody production but not for a CD8<sup>+</sup> T cell response, agents that activate the RLR-MAVS pathway may serve as important adjuvants in future RSV vaccines and therapeutics.

## Materials and Methods

**Mice.** The generation of *Mavs*<sup>-/-</sup> mice has been described in ref. 16. Briefly, *Mavs*<sup>-/-</sup> mice were made by homologous recombination with a targeting vector in 129/Sv ES cells, which were then injected into C57BL/6 blastocysts to create chimeric mice. *Myd88*<sup>-/-</sup> mice, which had been back-crossed to the C57BL/6 background, were kindly provided by Shizuo Akira (Osaka University).



**Fig. 5.** A subset of pulmonary DCs is activated by RSV in mice lacking *Mavs*. (A) Mice of the indicated genotypes were infected with RSV or mock treated for 24 hours before CD86 levels were assessed on pulmonary DCs and alveolar macrophages by flow cytometry. Shaded histograms represent isotype-control antibody staining, blue histograms represent DCs from mock-infected mice and red histograms represent RSV infected mice. (B) Mediastinal lymph nodes (MLNs) were taken from mice described in A and surface expression of CD86 was assessed on dendritic cells by flow cytometry. Representative mice are shown for each genotype ( $n = 3$  per group).

To obtain mice lacking *mavs*, *myd88*, or both (DKO), *mavs*<sup>+/-</sup> and *myd88*<sup>+/-</sup> mice were bred with each other, and the resulting progeny with appropriate genotypes were used in the experiments. All mice described in this report were

engineered and housed in animal facilities at the University of Texas Southwestern Medical Center, and the experimental protocols were approved by the Institutional Animal Care and Use Committee.

**In Vivo Infection.** Mice were anesthetized with inhaled isoflurane before intranasal inoculation with  $10^7$  pfu of live RSV in 100  $\mu$ l of Eagle Minimal Essential Medium supplemented with L-glutamine, Hepes, penicillin, streptomycin, and 10% FBS. Control animals were sham-inoculated with 100  $\mu$ l of cell-culture media (19).

**CD8<sup>+</sup> T Cell Analyses.** For IFN- $\gamma$  production, lung cells were incubated in complete media with either an SeV-derived peptide (FAPGNYPAL) or an RSV-derived peptide (NAITNAKII) (23) (10  $\mu$ g/ml) and brefeldin A (10  $\mu$ g/ml; Sigma) for 6 h. Cells were then incubated with antibodies against CD3 and CD8, followed by fixation, permeabilization, and staining for IFN- $\gamma$  using the Becton Dickinson fix/perm reagent according to the manufacturer's instructions. Samples were run on a Becton Dickinson FACSCalibur and analyzed using FlowJo 8.3 (Tree Star, Inc.). Cytotoxicity assays were performed as described (23). EL4 targets were incubated with either RSV- or SeV-derived peptides (10  $\mu$ g/ml) overnight. Respiratory syncytial virus peptide-loaded targets were then labeled with 5  $\mu$ M carboxyfluorescein diacetate succinimidyl ester (CFSE) and control targets with 0.5  $\mu$ M CFSE before mixing them at a 1:1 ratio. Mixed targets were then incubated at the indicated effector/target (E/T) ratios with lung cells from mice infected with RSV for 8 days. Cells were incubated at 37°C for 4 h and then were analyzed by flow cytometry. Percent specific lysis was calculated as  $100 - (100 \times \text{Percent CFSE}^{\text{high}} [\text{at E/T of 2 or 20}]/\text{Percent CFSE}^{\text{high}} [\text{at 0:1}])$ .

More information on materials and methods, including statistical analyses, can be found in *SI Text*.

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