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MyD88-Mediated Instructive Signals in Dendritic Cells Regulate Pulmonary Immune Responses during Respiratory Virus Infection

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Respiratory syncytial virus (RSV) is the leading cause of respiratory disease in infants worldwide. The induction of innate immunity and the establishment of adaptive immune responses are influenced by the recognition of pathogen-associated molecular patterns by TLRs. One of the primary pathways for TLR activation is by MyD88 adapter protein signaling. The present studies indicate that MyD88 deficiency profoundly impacts the pulmonary environment in RSV-infected mice characterized by the accumulation of eosinophils and augmented mucus production. Although there was little difference in CD4 T cell accumulation, there was also a significant decrease in conventional dendritic cell recruitment to the lungs of MyD88−/− mice. The exacerbation of RSV pathophysiology in MyD88−/− mice was associated with an enhanced Th2 cytokine profile that contributed to an inappropriate immune response. Furthermore, bone marrow-derived dendritic cells (BMDC) isolated from MyD88−/− mice were incapable of producing two important Th1 instructive signals, IL-12 and delta-like4, upon RSV infection. Although MyD88−/− BMDCs infected with RSV did up-regulate costimulatory molecules, they did not up-regulate class II as efficiently and stimulated less IFN-γ from CD4+ T cells in vitro compared with wild-type BMDCs. Finally, adoptive transfer of C57BL/6 BMDCs into MyD88−/− mice reconstituted Th1 immune responses in vivo, whereas transfer of MyD88−/− BMDCs into wild-type mice skewed the RSV responses toward a Th2 phenotype. Taken together, our data indicate that MyD88-mediated pathways are essential for the least pathogenic responses to this viral pathogen through the regulation of important Th1-associated instructive signals. The Journal of Immunology, 2007, 178: 5820–5827.

Respiratory syncytial virus (RSV) is the leading cause of lower respiratory tract infection in infants. Despite the fact that nearly all infants are infected by 2 years of age, only 1% of infants suffer severe enough disease to require hospitalization (1–4). However, both infants and adults are susceptible to recurrent infections, and accumulating evidence suggests a strong link between RSV bronchiolitis and later development of asthma-like syndromes and episodes of wheezing (5, 6). It is likely that the age of infection can influence the production of IL-13 (12). Conversely, IL-12 in RSV-infected C57BL/6 mice creates a more protective immune environment by limiting mucus overexpression, airway hyperreactivity, and eosinophilia (13). IL-12 is primarily produced by activated APCs in the lung and promotes the development of Th1 immune cells and cell-mediated immunity (14).

The development of an appropriate immune response during viral infections relies on early events in innate immune cells to shape the immediate environment and dictate the course of infection. TLRs are essential for innate immune cells to recognize pathogen-associated molecular patterns on invading pathogens and products of their infection (15). On recognition, TLR activation through the common adapter protein MyD88 activates NF-κB, MAPK, and other signaling pathways for all TLRs except TLR3 (16, 17). TLR signaling in dendritic cells (DC) promotes the up-regulation of costimulatory, MHC molecules and cytokines that collectively regulate the priming of adaptive immune responses. A Th1 response is initiated in MyD88-activated DCs by the up-regulation of IL-12 which signals naive CD4+ cells to differentiate into Th1 cells and produce primarily IFN-γ (14, 18). Th1 immunity is the preferred pathway for protection against intracellular bacteria and viruses. It has been suggested that in the absence of MyD88, TLR-stimulated DCs do not produce IL-12 and default to Th2 responses (18). Recent evidence suggest that IL-12 is not the only instructive signal provided by DCs but that also the expression of notch ligands (delta-like4 and jagged) may account for further regulation of Th cell differentiation (19–21). The up-regulation of delta-like4 in DCs also requires MyD88 and stimulates Th1 responses (21). We demonstrate in this report that Th1 immune...
were purified using positive selection for CD11c with anti-CD11c coupled to magnetic beads (Miltenyi Biotec). BMDCs were washed and resuspended in fresh medium for an additional 2 h, washed, and resuspended in fresh medium for the indicated times.

### Materials and Methods

#### RSV propagation and titer determination

RSV was derived from a clinical isolate at the University of Michigan (Ann Arbor, MI). The virus was propagated in Hep2 cells (American Type Culture Collection; ATCC) and titered using Vero cells (ATCC), as previously described (22). Plaque assays were performed on RSV-infected lungs, as previously described (23).

#### Mice

Female MyD88−/− mice on a C57BL/6 background were grown under specific pathogen-free conditions at the University of Michigan. Age- and sex-matched C57BL/6 wild-type mice were purchased from The Jackson Laboratory. All mice were maintained in specific pathogen-free facilities in the Unit for Laboratory Animal Medicine at the University of Michigan. Mice were anesthetized and infected intratracheally with RSV as previously described (9, 10, 24).

#### Bronchioalveolar lavage (BAL) analysis

PBS BAL of 1 ml were used to assess the leukocyte influx into the infected mice. The BAL samples were centrifuged, and the cellular pellets were resuspended in PBS, cytospun onto glass slides, and differentially stained (Diff-Quick); percentages of leukocytes were determined. Total cells were counted and the cell counts for each population were back-calculated.

#### Quantification of cytokines

RNA was isolated from the upper right lobes of lung, lymph nodes, and bone marrow DC (BMDC) using Trizol (Invitrogen). Levels of mRNA were assessed using quantitative PCR (qPCR) analysis (TaqMan) with pre-developed primers and probe sets from PE Biosystems. The primer/probe set used to detect gob5 have been previously described (22). Quantification of the genes of interest were normalized to GAPDH and expressed as fold change determined by ANOVA, and significance was determined as values of p < 0.05.

#### Generation of BMDC

Bone marrow was harvested from C57BL/6 or MyD88−/− mice, filtered through nylon mesh, and depleted of erythrocytes with lysis buffer. Bone marrow cells were seeded in T-150 tissue culture flasks at 10⁶ cells/ml in RPMI 1640-based complete medium with 20 ng of GM-CSF/ml (R&D Systems). After 5 days, loosely adherent cells were collected and incubated with anti-CD11c coupled to magnetic beads (Miltenyi Biotec). BMDCs were purified using positive selection for CD11c⁺ cells by running the cell suspension through a magnetic column. CD11c⁺ cells were plated overnight. The next day, BMDCs were infected with RSV (multiplicity of infection, 1.0) for 2 h, washed, and resuspended in fresh medium for the indicated times.

#### In vitro CD4⁺ T cell treatments

Mediastinal lymph node cells were isolated on day 9 after RSV infection. Lymph node cells were filtered through nylon mesh and depleted of erythrocytes with lysis buffer. CD4⁺ T cells were isolated using anti-CD4 magnetic beads (Miltenyi Biotec) and stimulated with 10 µg/ml plate-bound anti-CD3 and 1 µg/ml soluble anti-CD28 for 6 h. In experiments in which CD4⁺ T cells were stimulated with BMDCs, BMDCs were first plated in round-bottom 96-well plates and infected with RSV for 2 h. BMDCs were washed and resuspended in fresh medium for an additional 6 h. CD4⁺ T cells from RSV-infected mice were added to wells at a DC:CD4⁺ cell ratio of 1:5 and RNA was isolated 6 h later.

#### Adoptive transfer of BMDCs

BMDCs (1 × 10⁶) were resuspended in 50 µl of saline along with RSV (1 × 10⁶ PFU/mouse), and mice were infected intratracheally as described above. After 12 days of RSV infection, the mice were euthanized, and the lungs were removed for either mRNA expression data or for histology. In addition, lung-draining lymph nodes were also removed, and the T cells were isolated for restimulation responses with anti-CD3/anti-CD28 activation.

#### Assessment of leukocyte accumulation in lungs of RSV-infected mice

Whole lungs were dispersed using collagenase digestion as previously described (22). Briefly, the whole lungs from naive or RSV-infected wild-type or MyD88−/− mice were removed, cut into small pieces, and incubated with 0.2% type IV collagenase (Sigma-Aldrich) for 45 min. Subsequently, lungs were passed through a cannula and dispersed into a single-cell suspension after centrifugal separation from the undigested connective tissue. The isolated cell populations were then subjected to flow cytometry as indicated below. Total number of each cell population was calculated based on the percentage of a particular cell population multiplied by the total leukocytes in the lung.

#### Flow cytometric analysis

Cells were stained with the indicated Abs (BD Pharmingen) and analyzed using a FACSCalibur and Cell Quest software (BD Biosciences).

#### Statistics

Statistical significance was determined by ANOVA with a Student-Neuman-Kuhl posttest. Values of p < 0.05 were considered significant.

#### Results

MyD88 deficiency increases mucus and Th2-mediated lung inflammation in RSV-infected mice

We previously have demonstrated that C57BL/6 mice infected with RSV respond with a more protective immune response and exhibit only mild pathophysiology (13). To test the hypothesis that TLR signaling is critical in generating an appropriate nonpathogenic immune response, we first compared lung inflammation in MyD88−/− and C57BL/6 mice infected with RSV. Inflammatory
cell populations in the BAL from MyD88−/− and C57BL/6 mice were compared on 6, 9, and 12 days after RSV infection (Table I). Although the levels of lymphocytes were similar at most time points, the number of polymorphonuclear neutrophils and macrophages were reduced early at day 6 of infection, and eosinophils was significantly increased at 9 and 12 days of infection in MyD88−/− mice compared with RSV-infected C57BL/6 mice (Table I). The presence of eosinophils is most often associated with increased pathophysiological responses related to a Th2 immune phenotype and reflected a significant alteration in the overall immune environment. Because there were differences in macrophages at day 6, we further examined whether there were changes in DCs in the BAL and found that the MyD88−/− mice had lower numbers of CD11c+/class II MHC high cells in the airway (Table I). Because we observed significant changes in the DC population in the BAL at day 6, we next examined the lung infiltrating cell populations to examine whether there was a coordinate difference in the interstitium. To this end, we collagenase dispersed the lungs at 6 days postinfection and examined the lymphocyte and DC subsets within the control and infected lungs of wild-type and MyD88−/− mice. The data in Fig. 1A indicate that the number of conventional DC/myeloid DC (CD11c+/CD11b+/class II high) were significantly reduced in the MyD88−/− animals, whereas the plasmacytoid DC (CD11c+/B220) population was not affected. The number of CD4 and CD8 T cell populations were also examined and indicated no significant alteration of the CD4 T cells with a reduction in the CD8 T cell population, albeit not statistically
significant. Thus, there appeared to be an alteration in recruitment of the specific cDC subset of leukocytes migrating into the lung after RSV infection.

Excessive mucus production is a hallmark symptom of severe RSV disease in infants. Previously, we and others have monitored gob5 expression because its believed to be involved in RSV-induced mucus production (9, 10, 25, 26). To determine whether MyD88 was required to limit mucus production, we quantitate gob5 gene expression during RSV infection using qPCR. In addition, MUC5AC has been shown to be a primary mucin gene increased during severe airways diseases associated with mucus overproduction (27, 28). RSV-infected wild-type C57BL/6 mice showed little increase in expression of gob5 or MUC5AC mRNA after RSV infection. However, gob5 (Fig. 1B) expression was substantially up-regulated in MyD88−/− mice infected with RSV throughout the infection, whereas MUC5AC (Fig. 1C) was increased primarily at day 12 of infection.

The histopathology of the lungs demonstrated increased mucus overexpression and goblet cell presence in the MyD88−/− mice compared with staining in wild-type mice (Fig. 2). As early as day 6 postinfection periodic acid-Schiff (PAS)-positive cells can be seen within the airways of RSV-infected MyD88−/− mice, and intense mucus overproduction is observed by day 12 with many airways obstructed by mucus only in the MyD88−/− mice. In contrast, very few PAS-positive cells can be found in the wild-type C57BL6 mice at any time after RSV infection, corresponding to gob5 and MUC5AC expression mentioned previously. Together, our data indicate that MyD88 plays an important role in limiting eosinophil influx and mucus production. Given these increases in disease parameters, we next compared viral titers in the lungs of RSV-infected C57BL/6 and MyD88−/− mice at 6 dpi. Using a plaque assay, we found no significant differences in viral replication in MyD88−/− mice compared with C57BL/6 controls at 2, 4, or 6 days postchallenge (data not shown).

The altered pathophysiology observed in RSV-infected MyD88−/− mice may be due to a dysregulated Th1 response. To determine whether MyD88 deficiency was associated with defects in immune regulation, CD4+ T cells were purified from the draining lymph nodes of RSV-infected C57BL/6 and MyD88−/− mice and stimulated with plate-bound anti-CD3 and soluble anti-CD28. Quantitative PCR was performed on isolated RNA to determine the cytokine profile. Our data indicate that CD4+ T cells from C57BL/6 mice predominantly up-regulate the Th1 cytokine IFN-γ upon stimulation compared with unstimulated cultures (Fig. 3). However, CD4+ T cells from MyD88−/− mice were impaired in the production of IFN-γ compared with C57BL/6 CD4+ T cells but produced significantly higher levels of IL-4, IL-5 and IL-13 overproduction (27, 28). RSV-infected wild-type C57BL/6 and MyD88−/− mice infected with RSV throughout the infection, whereas MUC5AC (Fig. 1C) was increased primarily at day 12 of infection.

The altered pathophysiology observed in RSV-infected MyD88−/− mice may be due to a dysregulated Th1 response. To determine whether MyD88 deficiency was associated with defects in immune regulation, CD4+ T cells were purified from the draining lymph nodes of RSV-infected C57BL/6 and MyD88−/− mice and stimulated with plate-bound anti-CD3 and soluble anti-CD28. Quantitative PCR was performed on isolated RNA to determine the cytokine profile. Our data indicate that CD4+ T cells from C57BL/6 mice predominantly up-regulate the Th1 cytokine IFN-γ upon stimulation compared with unstimulated cultures (Fig. 3). However, CD4+ T cells from MyD88−/− mice were impaired in the production of IFN-γ compared with C57BL/6 CD4+ T cells but produced significantly higher levels of IL-4, IL-5 and IL-13 compared with wild-type mice (Fig. 3). It has been previously shown that T cells from naive MyD88−/− mice have no intrinsic defects in cytokine production or proliferation indicating these defects are specific to RSV responses (29), and our data using T cells from uninfected mice showed no significant differences in cytokine levels in wild-type compared with MyD88−/− mice (data not shown). Although C57BL/6 mice responded to RSV with a Th1 immune bias, MyD88−/− mice exhibited a Th2-associated immune response only after RSV infection.

**MyD88 regulate Th1 polarizing signals in RSV-infected BMDCs**

Accumulating evidence suggests that T cell differentiation into Th1 and Th2 cells is regulated by the interaction between naive Th cells and activated DCs (30). To further examine the impaired Th1 response in MyD88−/− mice, we next sought to determine whether

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**Table II.** Mean fluorescent intensity of RSV-induced costimulatory molecules on BMDC

<table>
<thead>
<tr>
<th>Costimulatory Molecules</th>
<th>Wild type</th>
<th>MyD88−/−</th>
<th>RSV Wild type</th>
<th>MyD88−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD80</td>
<td>9.85 ± 0.05</td>
<td>12.7 ± 0.12</td>
<td>14.5 ± 0.3</td>
<td>16.7 ± 0.6</td>
</tr>
<tr>
<td>CD40</td>
<td>31.8 ± 1.2</td>
<td>36.3 ± 1.4</td>
<td>45.6 ± 0.81</td>
<td>43 ± 0.61</td>
</tr>
<tr>
<td>MHC class II</td>
<td>235 ± 33.0</td>
<td>169 ± 33.5</td>
<td>443 ± 58.0a</td>
<td>223 ± 11.5</td>
</tr>
</tbody>
</table>

*Mean fluorescent intensities were statistically different between the DCs from wild-type and MyD88−/− mice.
this was linked to deficient Th1 signals from MyD88−/− DCs. To this end, we infected BMDCs from wild-type C57BL/6 and MyD88−/− mice with RSV. BMDCs were monitored for the expression of notch ligands (including delta-like4, jagged1, and jagged2) and IL-12, given that these molecules have been described as primary T cell-instructive signals produced in DCs that can regulate Th1 type responses (14, 31). RSV significantly up-regulated delta-like4 and IL-12 in C57BL/6 BMDC; however, expression of delta-like4 and IL-12 mRNA was completely abrogated in MyD88−/− BMDCs infected with RSV (Fig. 4A) as well as production of IL-12p70 protein levels (Fig. 4B). When we investigated the expression of jagged 1 and jagged 2, which have previously been shown to participate in skewing toward Th2 responses, we observed no increased expression in wild-type, MyD88−/−, or TLR3−/− BMDC (Fig. 4A). We also examined BMDC from TLR3−/− mice (which depends on Toll/IL-1 receptor domain-containing adaptor inducing IFN-β and not MyD88 for signaling), we found no defect in either IL-12 or delta-like4 expression after RSV infection. Given the altered cytokine production in MyD88−/− BMDCs, we next examined the role of MyD88 in RSV-induced costimulatory molecule expression of BMDCs. Using flow cytometric analysis, we observed a similar increase in CD40 and CD80 expression in C57BL/6 wild-type and MyD88−/− BMDCs infected with RSV as indicated by mean fluorescent intensity analysis in three repeated cell assays (Table II). Together, our data indicate that the expression of IL-12 and delta-like4 in RSV-infected DCs is MyD88 dependent. However, MyD88 is not required for RSV-induced costimulatory molecule expression in BMDC. We also examined the expression of MHC class II molecules on DCs in these same studies. The expression of class II was up-regulated on DCs from wild-type but not on DC from MyD88−/− mice (Table II), further demonstrating a difference in the ability of MyD88−/− DC to respond to the RSV stimulus.

MyD88−/− DCs are unable to stimulate IFNγ production in CD4+ T cells

To further test the hypothesis that MyD88−/− mice respond to RSV with a Th2 phenotype because of missing instructive Th1 signals in MyD88−/− DCs, we stimulated CD4+ T cells from RSV-infected C57BL/6 wild-type mice with BMDCs from either C57BL/6 or MyD88−/− mice infected with RSV. BMDCs derived from C57BL/6 and MyD88−/− mice were infected with RSV for 2 h, washed, and placed in fresh medium for an additional 6 h. CD4+ T cells isolated from draining lymph nodes of RSV-infected C57BL/6 mice were added to BMDCs for an additional 6 h, and IFN-γ mRNA expression was quantitated using real-time qPCR. C57BL/6 BMDCs infected with RSV stimulated significant levels of IFN-γ from CD4+ T cells compared with CD4+ T cells cultured with uninfected C57BL/6 BMDCs (Fig. 5A). However, we observed significantly less IFN-γ from wild-type CD4+ T cells cultured with RSV-infected MyD88 BMDCs, whereas BMDCs from TLR3−/− animals had no effect on IFN-γ expression (Fig. 5A). IFN-γ was not up-regulated in RSV-infected DCs or T cells cultured alone (data not shown). To further examine the relative role of notch ligand and IL-12, we used CD4 T cells isolated from lymph nodes of RSV-infected wild-type mice along with BMDCs infected with RSV, as above, and treated isolated cultures with anti-IL-12 or γ-secretase inhibitor (10 μM), a common blocking agent of notch ligand activation. After 6 h of RSV infection in the cultures, we examined IFN-γ expression by qPCR. The data indicate that by blocking either IL-12 with specific Ab or notch signaling with γ-secretase inhibitor, a significant decrease in IFN-γ production could be observed (Fig. 5B). These data further suggest that both of these signals are required for optimal IFN-γ production in CD4+ T cells.

Transfer of wild-type or MyD88−/− DCs skew ongoing Th1/Th2 responses in RSV-infected mice

To examine the in vivo relevance of impaired T cell priming by MyD88−/− DCs, we adoptively transferred C57BL/6 BMDCs into RSV-infected MyD88−/− mice to see whether we could restore a Th1 response. MyD88−/− mice received either RSV and 1 × 10⁶ C57BL/6 BMDCs, or RSV and 1 × 10⁶ MyD88−/− BMDCs intratracheally. At 9 days postinfection, CD4+ T cells were isolated from draining lymph nodes of each group and stimulated with anti-CD3 and anti-CD28. MyD88−/− mice reconstituted with C57BL/6 BMDCs produced higher levels of IFN-γ and significantly lower levels of IL-13 compared with MyD88−/− mice infected with RSV alone (Fig. 6A). These data demonstrate that MyD88 signaling in DCs is required to maintain Th1 responses in RSV-infected mice.

Because our in vitro data suggested that MyD88−/− BMDC also had an effect on the IFN-γ response in T cells from wild-type mice, we transferred BMDCs from MyD88−/− mice into C57BL/6...

FIGURE 5. BMDCs induce IFN-γ and depend on MyD88 and multiple instructive signals. A, DCs grown from wild-type, MyD88−/−, or TLR3−/− mice were challenged with RSV for 2 h, washed, and combined with isolated CD4+ T cells from draining pulmonary lymph nodes of RSV-infected mice for an additional 6 h. Isolated mRNA was examined for IFN-γ mRNA expression by quantitative PCR and compared with UV-RSV incubated DC cultures. Data represent the mean ± SE from three repeat experiments. B, Isolated pulmonary lymph node CD4 cells from 12-day-infected wild-type mice were rechallenged in vitro with RSV (multiplicity of infection, 1.0)-infected BMDCs in the presence of anti-IL-12 or γ-secretase inhibitor (Inh) to examine the relative contribution of the instructive signals. Six hours post-RSV challenge, mRNA was isolated and subjected to qPCR for IFN-γ. Data represent the mean ± SE of three to five repeats. *, p < 0.05.
FIGURE 6. Transfer of DC alters the cytokine profile in MyD88−/− and wild-type mice. A, BMDC were grown and isolated from wild-type or MyD88−/− mice and injected into the airways of MyD88−/− mice (1×10^6/mouse) coincident to RSV infection (1×10^5 PFU). After 9 days, draining lymph nodes were harvested and restimulated with anti-CD3/anti-CD28 and mRNA isolated from 6-h cultures. qPCR was used to assess cytokine production. Fold increases of cytokine mRNA was assessed by comparison with unchallenged cultures. B, BMDC were grown and isolated from wild-type or MyD88−/− mice and injected into the airways of wild-type mice (1×10^6/mouse) coincident to RSV infection (1×10^5 PFU). After 9 days, draining lymph nodes were harvested and restimulated with anti-CD3/anti-CD28, and mRNA was isolated from 6-h cultures. qPCR was used to assess cytokine production. Fold increases of cytokine mRNA were assessed by comparison with unchallenged cultures.

Discussion

In this study it was established that the activation of MyD88 in DCs is required for optimal antiviral and Th1 responses in RSV-infected mice. This conclusion is based on the following results: 1) RSV-infected MyD88−/− BMDCs are incapable of producing the Th1 instructive signals delta-like4 and IL-12; 2) Ag-specific CD4+ T cells from wild-type mice cultured with MyD88−/− BMDCs fail to induce optimal IFN-γ levels; and 3) adoptive transfer of C57BL/6 BMDCs into MyD88−/− mice infected with RSV reconstitutes Th1, whereas transfer of MyD88−/− BMDC skew wild-type mice toward Th2 immune responses. Although MyD88−/− DCs have the ability to respond to RSV as evidenced by the expression of costimulatory molecules, there was a decrease in numbers of DCs in the lungs and airway, an altered expression of class II MHC, as well as a general ineffectiveness to activate T cells in a similar manner as wild-type DCs. The fact that MyD88−/− DCs can express important costimulatory molecules after activation has previously been reported (29). It is likely that all of these aspects greatly impact on the outcome of the local pulmonary response. These data correspond with studies that have previously recognized the global effects that MyD88-mediated activation has on the development of the immune response, given that multiple models have now suggested that without MyD88 signaling the immune outcome is significantly skewed (29, 32–37). The changing environment from the loss of MyD88 has generally

wild-type mice infected with RSV. Similar to previous experiments, C57BL/6 mice were infected with RSV in conjunction with C57BL/6 or MyD88−/− BMDCs (1×10^6/mouse). After infection, CD4+ T cells were isolated from the draining lymph nodes and stimulated by anti-CD3/anti-CD28, and levels of IFN-γ and IL-13 were determined. Results show that transferring MyD88−/− BMDCs into C57BL/6 mice impaired IFN-γ synthesis but elevated IL-13 production (Fig. 6B). In separate studies when DCs were transferred into uninfected mice, no difference was observed in IFN or IL-13 from unstimulated lymph node CD4 T cells after anti-CD3/anti-CD28 restimulation (IFN, wild type 25.9 ± 1.5 vs MyD88 21.6 ± 1.1; IL-13, wild type 4.9 ± 2.0 vs MyD88 6.2 ± 1.7 ng/ml). Thus, adoptive transfer of MyD88−/− BMDCs into wild-type mice initiated Th2 responses in C57BL/6 mice infected with RSV only, suggesting that the DC from the MyD88−/− mice provide Th2 skewing signals after infection.

To further assess the pathogenic response in the above mouse transfer experiments we also examined the expression of the mucus-associated gene MUC5AC, which was shown to be differentially expressed at day 12 of infection in MyD88−/− mice. In separate experiments, when we transferred wild-type DC into the MyD88−/− mice, we observed a ~6-fold decrease in MUC5AC expression compared with MyD88−/− control infected animals (Fig. 7A). In contrast, when MyD88−/− DCs were transferred into wild-type mice, a ~3-fold increase was observed in comparison to the control infected wild-type mice. We also examined the mice histologically and although the MyD88−/− DC into wild-type mice did not seem to significantly up-regulate mucus expression, when wild-type DC were transferred into the MyD88−/− mice, there was a visible difference in the amount of mucus induced in the airways (Fig. 7B). Although there was still some goblet cell staining, the numbers appear to be decreased, and the airways were not filled with mucus and therefore appeared less obstructed. These data followed the above responses for the change in cytokine phenotype observed in reactivated lymph node T cells where IL-13 production regulates mucus overexpression in the airway.
results in altered immunopathology, including inflammatory, autoimmune, allergen, and parasitic responses. Although the concepts portrayed in this article will lead to a better understanding of RSV pathogenesis, they also contribute to the overall understanding of innate molecule function determining antiviral immune responses.

In the absence of MyD88, not only are Th1 immune responses reduced but also Th2 responses are up-regulated. It has been widely accepted that IL-12 is critical in the Th1/Th2 differentiation process. This concept was originally established by studies with Leishmania major that showed that a Th1 response in C57BL/6 could be skewed to a Th2 response in IL-12−/− mice (38, 39). The importance of IL-12 during RSV infection has been demonstrated using rIL-12 treatment, IL-12 neutralization, or mice deficient in IL-12 (13, 40–43). However, not all pathogenic infections result in a Th2 response in the absence of IL-12 (37). These latter studies, along with the recently described role of notch ligands (19, 21, 44, 45), suggest that there may be both Th1 and Th2 instructive signals that regulate the decision process. The data from the present studies further indicate that although MyD88−/− DCs had a deficient expression of important instructive signals, they were able to provide stimulatory signals that are required for initial T cell activation. Thus, these instructive signals likely provide directional in-

formation for the T cells once activated. When MyD88−/− DCs were assessed in vitro we observed no up-regulation of jagged1 or jagged2 that have been implicated in skewing Th2 responses (21). The use of the inhibitor of γ-secretase is a convenient way to determine whether Notch is involved, and its use demonstrated a similar decrease in IFN-γ as using anti-IL-12. It will also be important in future experiments to determine whether IL-12 and/or delta-like promote Th1 immune responses, inhibit Th2 responses, or together do both in the course of viral infections. This is the first report to show the induction of delta-like4/Notch ligand by a virus and it is exciting to consider delta-like4/Notch signaling as a critical modulator of virus-induced airway disease.

Although various cell types can contribute to the initiation of Th1 responses, we found conventional BMDCs to be critical in regulating this process in C57BL/6 mice. RSV may also activate MyD88-dependent pathways in plasmacytoid DCs, and it would be interesting to transfer wild-type plasmacytoid DCs into MyD88−/− mice to determine whether different DC subsets could also restore Th1 responses. This latter aspect is quite important because our laboratory has recently published data that indicate the importance of pDC in controlling development of a more pathogenic response (24). Thus, it is likely that the appropriate immune responses can be initiated only with the participation of multiple innate immune molecules on functionally diverse cell populations. For example, TLR7 recognizes single-stranded RNA viruses via a MyD88-dependent mechanism, and TLR7 has been implicated in the response to specific respiratory viral infections (46); however, the functional role of TLR7 in other viral infections is still unclear (47) and is primarily expressed on the plasmacytoid DC subset of APC. TLR4 has also been suggested to be activated via the F protein of RSV and may contribute to the MyD88-dependent pathways leading to the activation of instructive signals (48–50). MyD88 also regulates IL-1 and IL-18 signaling (51, 52). IL-18 has been demonstrated that multiple TLR ligands synergize in the production of IL-12 and delta-like4 in DCs (31). Thus, RSV may trigger multiple TLR and non-TLR signaling pathways that program a Th1 response that is blocked in MyD88−/− mice. TLR3−/− DCs, which have no defect in IL-12 or delta-like4 expression, were not impaired in their ability to generate an IFN-γ-producing response. However, TLR3−/− mice infected with RSV do have altered immune responses centered on the up-regulation of IL-13 and mucus, with little effect on IFN-γ production (9). Altogether, these data suggest that DC provide multiple signals to T cells that regulate Th1 and Th2 type responses that determine the immune environment in the lung.

Because DCs link innate and adaptive immune responses, it is crucial to understand how the recognition pathways in DCs cooperate with the activation pathways in T cells. Impairment in delta-like4 and IL-12 in MyD88−/− BMDCs correlated with hampered IFN-γ production in CD4+ T cells but elevated levels of IL-4, IL-5, IL-13. These studies are an important step for understanding innate recognition pathways involved in favorable immune responses to RSV as well as other infectious organisms in vivo.

Disclosures
The authors have no financial conflict of interest.

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