Nucleotide Oligomerization and Binding Domain 2-Dependent Dendritic Cell Activation Is Necessary for Innate Immunity and Optimal CD8⁺ T Cell Responses to Influenza A Virus Infection

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ABSTRACT

Nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) are important in the innate immune response to viral infection. Recent findings have implicated NLRP3, NOD2, and NLRX1 as important players in the innate antiviral response, but their roles in the generation of adaptive immunity to viruses are less clear. We demonstrate here that NOD2 is critical for both innate and adaptive immune responses necessary for controlling viral replication and survival during influenza A virus (IAV) infection. *Nod2*^{-/-} mice have reduced beta interferon (IFN- β) levels and fewer activated dendritic cells (DCs), and the DCs are more prone to cell death in the lungs of *Nod2*^{-/-} mice during IAV infection. In agreement with the role for DCs in priming adaptive immunity, the generation of virus-specific CD8⁺ T cells and their activation and production of IFN- γ were lower in *Nod2*^{-/-} mice. Furthermore, *Nod2*^{-/-} DCs, when cocultured with T cells *in vitro*, have a lower costimulatory capacity. Thus, *Nod2*^{-/-} DCs are unable to efficiently prime CD8⁺ T cells. These findings demonstrate that Nod2 is critical for the generation of both innate and adaptive immune responses necessary for controlling IAV infection.

IMPORTANCE

The innate immune system is the host's first line of defense against invading pathogens and is also necessary for alerting and activating T and B cells to initiate the adaptive immune response. We demonstrate here that the innate immune receptor NOD2 is required for the production of antiviral type I interferons and the activation and survival of dendritic cells that, in turn, alert T cells to the presence of influenza A virus infection. In mice that are missing NOD2, interferon levels are lower, and the CD8⁺ T cell response is impaired. As a result, the animals cannot control virus replication in their lungs as efficiently. This discovery helps us understand how the body naturally responds to virus infection and may help in the development of vaccines that use NOD2 to stimulate the CD8⁺ T cell response, thus providing better protection against influenza A virus infection.

Influenza A virus (IAV) infects hundreds of millions of people annually, with 250,000 to 500,000 deaths worldwide (1) and billions of dollars in economic loss (2–4). Although the innate immune system is able to trigger an inflammatory response to IAV, efficient clearance requires the combined efforts of both innate and adaptive immunity. During IAV infection, Toll-like receptor 7 (TLR7) and the downstream adaptor Myd88 are known to play important roles in bridging the gap between innate and humoral immunity. Specifically, TLR7 signaling is critical for efficient antibody responses to IAV and plays a critical role in antibody isotype class switching (5–7). Furthermore, TLR signaling is important for the generation of CD4⁺ T cell responses to IAV infection (6–8). Although TLR and RIG-I-like receptor signaling have been examined in the context of CD8⁺ T cell responses, neither of these pathways is important for the innate immune triggering of CD8⁺ T cell responses during IAV infection (7, 8).

The NOD-like receptor NOD2 is a cytosolic innate pattern recognition receptor that recognizes the muramyl dipeptide fragment of peptidoglycan present in bacterial cell walls. Stimulation of NOD2 results in signaling through the adaptor protein receptor interacting protein kinase 2 (RIP2) to activate NF- κ B and mitogen-activated protein kinases (9–12). In addition to its role in innate immunity to bacterial infection, NOD2 is also required for the efficient induction of both B cell and T cell responses (13–15).

More recently, NOD2 was discovered to play a role during viral infection, where it was found that NOD2 signaling through RIP2 is critical for modulating inflammation during IAV infection (16). Furthermore, NOD2 was shown to regulate innate antiviral responses through an interaction with the adaptor protein MAVS (17).

Despite obvious roles in innate immunity to virus infection, little is known about the role of NOD2 in adaptive immunity to virus infection. We report here additional roles for NOD2 in the activation of dendritic cells (DCs) and demonstrate that NOD2 is necessary for the optimal priming and generation of adaptive CD8⁺ T cell responses to IAV.

MATERIALS AND METHODS

Ethics statement. All animal experiments were conducted under protocols approved by the St. Jude Children's Research Hospital Committee on

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FIG 1 NOD2 is required for influenza A virus clearance and survival. WT and *Nod2*^{-/-} mice were infected with 1,000 PFU of PR8 IAV intranasally and monitored for survival (A) or euthanized on day 2, 7, or 12 after infection (B), and virus titers were determined from lung homogenates. (C) WT and *Nod2*^{-/-} mice were infected with 1,000 PFU of IAV for 2 days, and lung homogenates were examined for IFN-β by ELISA. (D and E) Alveolar macrophages (D) or BMDCs (E) were infected with IAV at an MOI of 10 *in vitro*, and the IFN-β levels were examined by ELISA 24 h after infection. (A) Data are cumulative from three independent experiments. The totals (*n*) are shown. In panels B and C, the data are representative of three independent experiments (*n* = 3 to 7 mice per experiment). In panels D and E, the data are representative of two to three independent experiments (*n* = 2 to 3 wells per experiment; means ± the standard errors of the mean [SEM]). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 (two-sided unpaired Student *t* test, one-way ANOVA for multiple comparisons, or log-rank [Mantel-Cox] test for survival).

Use and Care of Animals (protocol 482) and were performed in accordance with institutional policies, AAALAC guidelines, the AVMA Guidelines on Euthanasia, NIH regulations (*Guide for the Care and Use of Laboratory Animals*), and the U.S. Animal Welfare Act of 1966.

Virus and mice. The influenza A/Puerto Rico/8/34 H1N1 virus (PR8) was used in all experiments (18). Wild-type (WT) and *Tcrα*^{-/-} mice were obtained from The Jackson Laboratory and bred in-house. *Nod2*^{-/-} mice have been previously reported (9, 14). All mice were on the C57BL/6J background and maintained at SJCRH.

***In vivo* responses.** Mice infected intranasally with 1,000 PFU of IAV were weighed and monitored daily for survival or euthanized on days 0, 2, or 7 for sampling the whole lung, the spleen, the mediastinal lymph nodes (MdLN), or thymus. For samples collected on day 12, mice were infected with 750 PFU of IAV to prolong survival. DC responses were examined by flow cytometry after staining with anti-CD11c, -MHCII, -CD8, -CD4, -CD11b, -GR-1, -CD103, -CD80, and -CD86 antibodies (N418, M5/114.15.2, 53-6.7, RM4-5, M1/70, RB6-8C5, 2E7, 16-10A1, and GL-1; Biolegend). DCs were first identified by gating on TCR-β⁺ cells and then gating on CD11c⁺ MHC-II^{int-hi} cells. T cell populations were determined by flow cytometry after staining with anti-TCR-β, -CD8, -CD4, and -CD44 antibodies (H57-597, 53-6.7, RM4-5, and IM7; Biolegend), and epitope-specific CD8⁺ T cells were stained with DbPA₂₂₄ or KbPB1₇₀₃ influenza-specific tetramers. Intracellular gamma interferon (IFN-γ; XMG1.2 [Biolegend]) was measured by flow cytometry after stimulation of whole lung cells with a cocktail of IAV peptides (PB1₇₀₃₋₇₁₁, PB1-F2₆₂₋₇₀, NP₃₆₆₋₃₇₄, PA₂₂₄₋₂₃₃, and NS2₁₁₄₋₁₂₁).

T cell adoptive transfer. WT or *Nod2*^{-/-} T cells were isolated from the spleens of naive mice and purified by positive selection by using the AutoMACS Pro system and anti-CD90.2 (Thy1.2) beads (Miltenyi, catalog no. 120-000-295). A total of 7 × 10⁶ WT or *Nod2*^{-/-} T cells were then injected retro-orbitally into *Tcrα*^{-/-} mice, and T cells were allowed to propagate in the mice for 4 weeks. Mice were subsequently infected with

750 PFU of IAV, and the lungs taken on day 12 for examination of virus titer and T cell numbers, as described above.

***In vitro* T cell proliferation assay.** WT and *Nod2*^{-/-} bone marrow-derived DCs (BMDCs) were differentiated as previously described (16). BMDCs were plated at 5 × 10⁴ cells/well in round-bottom 96-well plates and infected with IAV at a multiplicity of infection (MOI) of 10. WT or *Nod2*^{-/-} T cells were purified by positive selection from spleens using the AutoMACS Pro system and anti-CD90.2 (Thy1.2) beads. T cells were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and 5 × 10⁵ cells were added to the previously infected BMDCs to generate four combinations (WT DCs + WT T cells, WT DCs + *Nod2*^{-/-} T cells, *Nod2*^{-/-} DCs + WT T cells, and *Nod2*^{-/-} DCs + *Nod2*^{-/-} T cells). Then, 10 ng of anti-CD3ε functional-grade antibody (catalog no. 553058; BD Bioscience)/ml was added to each well. On day 3, cells were stained with annexin V, anti-CD4, and anti-CD8 antibodies and examined by flow cytometry. As a positive control, T cells were stimulated with 1 μl of anti-CD3- plus anti-CD28-coated Dynabeads (Life Technologies, catalog no. 11456D)/well.

***In vitro* BMDC and alveolar macrophage stimulation.** Day 7 BMDCs were infected with IAV at an MOI of 10, and samples were collected for Western blotting at the indicated time points. Western blot analysis was performed with rabbit anti-NOD2 antibody (Santa Cruz, catalog no. H-300) and mouse anti-β-actin antibody (Sigma). Alternatively, alveolar macrophages and BMDCs were infected with IAV at an MOI of 10 for 24 h, and supernatants were collected for IFN-β enzyme-linked immunosorbent assay (ELISA; PBL Interferon Source). The BMDCs were also scraped and centrifuged and stained with an anti-CD86 antibody (GL-1; Biolegend) or with annexin V and examined by flow cytometry for activation or cell death, respectively.

Data analysis. All statistics were performed using GraphPad Prism 6.0 software using an unpaired two-tailed Student *t* test for single comparisons or one-way analysis of variance (ANOVA) with Sidak's post hoc test

FIG 2 Cytokine and chemokine levels do not correlate with reduced DC numbers in *Nod2*^{-/-} mice. WT and *Nod2*^{-/-} mice were infected with 1,000 PFU of PR8 IAV, and lungs collected on day 2 or 7 after IAV infection. Whole lungs were examined for total DC numbers by flow cytometry (A) or cytokine and chemokine responses by multiplex ELISA (B to D). For panel A, the data are representative of three independent experiments. For panels B to D, the data are cumulative from three independent experiments ($n = 3$ to 7 mice per experiment; means $\pm$ the SEM). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (two-sided unpaired Student t test).

for multiple comparisons or the log-rank (Mantel-Cox) test for survival. A P value of <0.05 was considered significant.

RESULTS

NOD2 is an important upstream pattern recognition receptor regulating survival and IAV replication. NOD2 plays key roles in innate and adaptive immunity to bacterial infections (12–15). However, its role in the immune responses to virus infection is not well characterized. To this end, we infected *Nod2*^{-/-} mice with influenza A/PR/8/34 H1N1 virus (IAV) and examined survival and virus load on days 2, 7, and 12 after infection. We observed that *Nod2*^{-/-} mice were significantly more susceptible to IAV infection than were WT mice (Fig. 1A). In addition, IAV grew to

higher titers throughout the course of infection in *Nod2*^{-/-} mice (Fig. 1B). To determine the role of NOD2 in the innate immune regulation of IAV replication on day 2, we examined lung homogenates for IFN-β levels and found that they were significantly lower in *Nod2*^{-/-} mice than WT mice (Fig. 1C). To confirm the role of NOD2 in innate immune responses, we also harvested alveolar macrophages or generated BMDCs from uninfected mice, infected them *in vitro* with IAV, and tested supernatants after 24 h for IFN-β levels. Similar to our *in vivo* results, both *Nod2*^{-/-} alveolar macrophages and *Nod2*^{-/-} BMDCs produced less IFN-β than did WT cells (Fig. 1D and E).

NOD2 deficiency results in impaired DC responses. Although the ability of NOD2 to regulate IFN-β has been demon-

FIG 3 NOD2 regulates DC activation and survival. (A to E) WT and *Nod2*^{-/-} mice were infected with 1,000 PFU of PR8 IAV, and lungs were collected on day 2 or 7 after infection. The DC numbers (A) and CD86 expression as determined from the geometric mean fluorescence intensity (B) were examined by flow cytometry on day 2 after IAV infection. DC numbers (C) and CD86 expression (D) were examined in whole lungs on day 7 after IAV infection. (E) DC cell death was measured by annexin V staining on day 2 after IAV infection in the whole lung. (F) BMDCs were infected with IAV and examined for cell death 72 h after infection. In panels A to D, the data are representative of two to three independent experiments ($n = 4$ to 7 mice per experiment; means $\pm$ the SEM). For panel E, the data are representative of two independent experiments ($n = 6$ wells per experiment; means $\pm$ the SEM). *, $P < 0.05$; **, $P < 0.01$ (two-sided unpaired Student *t* test).

strated previously (17), we discovered that the total number of DCs in the lungs of *Nod2*^{-/-} mice on days 2 and 7 were lower than in the lungs of WT mice, which likely contributes to the inability of *Nod2*^{-/-} mice to control IAV replication (Fig. 2A). To understand why there were fewer DCs in the lungs, we examined several chemokines and cytokines that would affect inflammation and trafficking but found them to be similar or elevated in most instances in *Nod2*^{-/-} mice compared to WT mice, except that IFN- γ and MIP-1 α levels were lower only on day 7 (Fig. 2B to D).

Since the defects in cytokine and chemokine production did not correlate with the early defects seen in DC responses, we next examined the effect of activation markers, such as CD80 and CD86, on DCs. The numbers of CD80⁺ and CD86⁺ DCs on day 2 after IAV infection were also lower in the lungs of *Nod2*^{-/-} mice than in the lungs of WT controls (Fig. 3A). Intriguingly, CD86 expression was significantly lower in DCs taken from the lungs of *Nod2*^{-/-} mice compared to WT mice (Fig. 3B). Furthermore, this phenotype persisted on day 7 after IAV infection, except that the

number of CD80⁺ DCs was no longer lower than for the WT controls (Fig. 3C and D). Although decreased activation might play some role in the recruitment of DCs into the lungs, we also found that there were increased numbers of dead DCs in the lungs of *Nod2*^{-/-} mice compared to WT controls (Fig. 3E). *Nod2*^{-/-} BMDCs infected *in vitro* with IAV also died more readily than WT BMDCs (Fig. 3F), indicating that lower numbers of DCs in the lungs of *Nod2*^{-/-} mice are also the result of increased cell death.

NOD2 is important for CD8⁺ T cell responses. From these data, it is clear that NOD2 is important for the generation of appropriate innate immunity to IAV infection. However, innate immune responses are also necessary for the appropriate initiation of adaptive immunity. Therefore, we examined T cell responses on day 7 after IAV infection and found that CD8⁺ T cell numbers were lower in the lungs of *Nod2*^{-/-} mice than in the lungs of WT control mice (Fig. 4A). We determined that IAV-specific responses were lower in *Nod2*^{-/-} mice by staining CD8⁺ T cells for two different IAV-specific tetramers (K^bPB1₇₀₃ and D^bPA₂₂₄)

FIG 4 NOD2 is necessary for optimal T cell responses. Adaptive immunity was examined on day 7 or 12 postinfection with PR8 IAV in WT and *Nod2*^{-/-} mice. (A) Frequency of CD4⁺ and CD8⁺ T cells in whole lungs and whole-lung CD8⁺ T cell numbers. (B) Whole lung CD8⁺ T cells from WT and *Nod2*^{-/-} mice were stained with D^bPA or K^bPB1 influenza tetramers. (C) Percentage of CD44⁺ and IFN-γ⁺ CD8⁺ T cells from whole lungs of WT and *Nod2*^{-/-} mice after stimulation with an influenza peptide pool (see Materials and Methods). (D) Whole-lung CD4⁺ T cells and percentage of CD44⁺ CD4⁺ T cells from WT and *Nod2*^{-/-} mice. (E) Whole-lung T cell numbers on day 12 after infection. In panels A to D, the data are representative of three independent experiments with *n* = 5 to 7 mice per experiment. For panel E, the data are representative of two independent experiments with *n* = 4 to 7 mice per experiment (means ± the SEM). *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 (two-sided unpaired Student *t* test).

(Fig. 4B). Finally, *Nod2*^{-/-} mice had lower percentages of CD8⁺ T cells that expressed IFN-γ or the activation marker CD44 after stimulation with epitope-specific IAV peptides (Fig. 4C). However, the total CD4⁺ T cells, as well as the CD44⁺ activated CD4⁺ T cells, in *Nod2*^{-/-} mice were comparable to those in WT mice (Fig. 4A and D). Furthermore, the lower numbers of CD8⁺ T cells in the lungs of *Nod2*^{-/-} mice were also seen on day 12 after IAV infection (Fig. 4E). Overall, these data demonstrate that NOD2 is essential for optimal IAV-specific CD8⁺ T cell responses. Furthermore, the increased viral loads on days 7 and 12 after infection that we observed in Fig. 1B were also observed in mice defective in CD8⁺ T cell responses (19–21), suggesting that the increased viral load in *Nod2*^{-/-} mice is likely the result of impaired innate and adaptive immunity.

NOD2 is not required for the development or maintenance of T cell numbers in naive mice. To determine the role of NOD2 in CD8⁺ T cell generation, we examined thymic development of T cells. In the thymus, nascent T cells are initially devoid of the coreceptors CD4 and CD8 (double negative). As the T cell matures, it expresses both CD4 and CD8 (double positive). As T cell development proceeds, the cell is selected for either the CD4 or the CD8 lineages (CD4 single positive and CD8 single positive). However, we found no differences between naive WT and *Nod2*^{-/-} mice at any of these stages of T cell development (Fig. 5A). Similarly, the numbers of T cells in uninfected *Nod2*^{-/-} mice were

normal in the spleens and MdLN compared to WT mice (Fig. 5B and C). These data demonstrate that NOD2 is not required for development or maintenance of T cells in naive mice.

NOD2 regulates specific DC subsets. To further characterize the defective DC responses in *Nod2*^{-/-} mice and their effects on CD8⁺ T cell responses, we examined specific DC populations in the lungs and draining lymph nodes. Several populations of DCs are known to be important for antigen presentation to CD8⁺ T cells in the MdLN during IAV infection. These include CD11b⁻ CD103⁺ DCs and CD8⁺ DCs (22–24). CD11b⁻ CD103⁺ DCs were present in similar numbers in the MdLN of both WT and *Nod2*^{-/-} mice on day 2 but were slightly lower on day 7 (Fig. 6A). On the other hand, CD8⁺ DCs were significantly lower in the MdLN on both days 2 and 7 after IAV infection (Fig. 6B). As a control, we also examined CD4⁺ DCs, which have been associated with antigen presentation to CD4⁺ T cells (25, 26), and found similar numbers in WT and *Nod2*^{-/-} mice (Fig. 6C). To determine why there are fewer CD8⁺ DCs in the MdLN, we examined cell death by annexin V staining and found that cell death is increased in the CD8⁺ DCs population in *Nod2*^{-/-} mice specifically (Fig. 6D). Although these data suggest that antigen presentation to CD8⁺ T cells is impaired in *Nod2*^{-/-} mice, reactivation is also critical for the expansion and survival of CD8⁺ T cells once they migrate to the lungs. In this regard, TipDCs (i.e., CD11b^{hi}, Ly6c/GR-1^{hi} DCs) and CD8⁺ DCs in the lungs are known to regulate

FIG 5 NOD2 does not regulate T cell development in naive mice. Uninfected naive WT and *Nod2*^{-/-} mice were examined for T cell responses. (A) Percentages of thymocytes in WT and *Nod2*^{-/-} mice for the indicated subgroups. DN, double negative; DP, double positive; CD4sp, CD4 single positive; CD8sp, CD8 single positive. (B) Splenic T cell numbers in WT and *Nod2*^{-/-} mice. (C) Mediastinal lymph node (MdLN) T cell numbers in WT and *Nod2*^{-/-} mice. The data are representative of three independent experiments with *n* = 3 to 5 mice per experiment (means $\pm$ the SEM).

the reactivation and expansion of CD8⁺ T cells once they traffic to the lung (27–29). The CD8⁺ DC numbers are lower in the lungs of *Nod2*^{-/-} mice on day 7 after IAV infection (Fig. 6E); however, the numbers of TipDCs were similar for WT and *Nod2*^{-/-} mice (Fig. 6E). It should be noted that all DC populations were similar between WT and *Nod2*^{-/-} mice prior to infection (Fig. 7). These results demonstrate that specific DC subsets known to be important for the activation of CD8⁺ T cells are lower during IAV infection in *Nod2*^{-/-} mice.

Nod2-deficient DCs are poor primers of adaptive immunity. Based on the results presented above, we hypothesized that the defects seen in DC activation and survival in *Nod2*^{-/-} mice were resulting in subsequent defects in CD8⁺ T cell responses. Therefore, we examined whether NOD2 plays a role in antigen presentation in response to IAV infection. To do this, we developed an *in vitro* assay. We first determined that BMDCs are appropriate for

FIG 6 NOD2 regulates specific DC subsets. WT and *Nod2*^{-/-} mice were infected with 1,000 PFU of PR8 IAV, and the MdLNs (A to D) and lungs (E) were collected on day 2 or 7 after infection and examined for the indicated DC subsets or cell death by annexin V staining. The data are representative of two to three independent experiments (*n* = 4 to 7 mice per experiment; means $\pm$ the SEM). **, *P* < 0.01; ***, *P* < 0.001 (two-sided unpaired Student *t* test).

this assay by examining the expression of NOD2 during IAV infection (Fig. 8A). Next, we infected BMDCs with IAV and examined CD86 expression on the surface of either WT or *Nod2*^{-/-} BMDCs. In agreement with our *in vivo* results, *Nod2*^{-/-} BMDCs did not upregulate CD86 as efficiently as WT cells after IAV infection (Fig. 8B). Since BMDCs expressed NOD2 and phenocopied DCs taken from the lungs of *Nod2*^{-/-} mice infected with IAV, we paired WT or *Nod2*^{-/-} BMDCs with naive WT or *Nod2*^{-/-} T cells. The BMDCs were infected with IAV prior to coincubation

FIG 7 NOD2 does not regulate DC numbers in naive mice. Uninfected naive WT and *Nod2*^{-/-} mice were examined for DC populations in the lungs (A), MdLN (B), and spleen (C). The data are representative of two independent experiments with $n = 3$ to 5 mice per experiment (means $\pm$ the SEM).

with CFSE-labeled T cells. Since the frequency of IAV specific T cells was expected to be low in naive mice, we also added low levels of anti-CD3 ϵ antibody to each well. Therefore, all cells would receive T cell receptor stimulation, and the only role of BMDCs would be in costimulation. On day 3 of coinoculation, we found that the proliferation of both WT and *Nod2*^{-/-} CD8⁺ T cells was diminished when paired with *Nod2*^{-/-} BMDCs (Fig. 8C). Furthermore, WT and *Nod2*^{-/-} CD8⁺ T cell death was increased when paired with *Nod2*^{-/-} BMDCs (Fig. 8D). Taken together, these data demonstrate that defects in *Nod2*^{-/-} DCs contribute to the decreased CD8⁺ T cell responses in *Nod2*^{-/-} mice following IAV infection. It should be noted, however, that even in the presence of WT DCs, *Nod2*^{-/-} CD8⁺ T cells still did not replicate as efficiently as WT CD8⁺ T cells (Fig. 8C). However, this appears to be context dependent, since the stimulation of *Nod2*^{-/-} CD8⁺ T cells with anti-CD3- plus anti-CD28-coated beads resulted in a proliferation similar to that seen with WT cells (Fig. 8C). Finally, to verify the role of innate immune cell priming on the function of CD8⁺ T cells *in vivo*, we purified WT or *Nod2*^{-/-} T cells and adoptively transferred them into *Tcr α* ^{-/-} mice. After 4 weeks, we infected these mice with IAV and then examined T cell numbers in the lungs and viral clearance on day 12 after infection. Importantly, the *Nod2*^{-/-} CD8⁺ T cell numbers in the lungs were similar to the number of WT CD8⁺ T cells when adoptively transferred into a WT antigen-presenting environment (Fig. 8E). Furthermore, we did not find any significant difference in viral clearance under these conditions (Fig. 8F). These data indicate

that in a WT priming environment, *Nod2*^{-/-} CD8⁺ T cells can respond normally and that the defects in CD8⁺ T cells seen in *Nod2*^{-/-} mice are due primarily to defects in priming from the innate immune system, particularly defects in DC activation and survival.

DISCUSSION

Numerous studies have sought to address the innate pattern recognition receptors (PRRs) and signaling pathways that are responsible for the generation of protective adaptive immunity to IAV infection. It was previously reported that TLR7 and MyD88 were required for humoral responses (5–7) and that Myd88 was important for CD4⁺ T cell responses (6–8). Although type I IFN (30, 31) and interleukin-1 (IL-1) (32–35) signaling have been shown to play central roles in the generation of cytotoxic CD8⁺ T cell responses, the specific upstream PRRs that sense IAV and initiate cellular immunity have remained elusive. We demonstrate here that the NOD-like receptor NOD2 plays an important role in activating the innate immune system so that it can prime the development of CD8⁺ T cell responses. Subsequently, the overall impact of diminished IFN- β , lower DC numbers, and reduced CD8⁺ T cell responses in *Nod2*^{-/-} mice results in an increased virus load throughout the course of IAV infection.

In addition to our current findings, we reported previously that NOD2 signaling through the adaptor RIP2 is critical for modulating inflammation during IAV infection (16). In particular, *Rip2*^{-/-} and *Nod2*^{-/-} mice have enhanced NLRP3 inflammasome activation and higher levels of IL-18, which contributed significantly to mortality in these mice. Furthermore, we found that *Rip2*^{-/-} mice have hyperactivated CD8⁺ T cell responses, which differs from that of *Nod2*^{-/-} mice, where we observed reduced CD8⁺ T cell responses. Therefore, NOD2 plays multiple roles in regulating the innate immune response during virus infection, and the increased susceptibility of *Nod2*^{-/-} mice to IAV infection is likely the combined effect of regulating inflammasome activation, IL-18, IFN- β , DC activation/survival, and the priming of adaptive immunity. In the future, it will be interesting to determine whether specific mutations in NOD2 can be made that affect only the RIP2-dependent or -independent pathways and to examine the individual contributions of these pathways to the susceptibility of *Nod2*^{-/-} mice to IAV infection.

It was reported previously that NOD2 plays an intrinsic role in the activation of CD4⁺ T cells and the production of Th1 immunity during *T. gondii* infection (36). Although NOD2 may play a T cell intrinsic role for CD4⁺ T cells during infection with other pathogens, Lin et al. demonstrated that defects seen in *Nod2*^{-/-} CD8⁺ T cells during IAV infection are not truly intrinsic but instead are derived from the environment in which the T cells develop (37). Our data indicate that, during IAV infection, defects in *Nod2*^{-/-} CD8⁺ T cell responses are the result of defective priming from lower numbers and impaired activation of *Nod2*^{-/-} DCs. Furthermore, NOD2 is important for specific DC populations in the lung and MdLN that are required for the generation of cytotoxic CD8⁺ T cell responses (22–24), as well as the reactivation and expansion of CD8⁺ T cells once they traffic to the lung (27–29). The totality of these findings indicates that during virus infection, NOD2 is required for priming of CD8⁺ T cell and does not play a CD8⁺ T cell intrinsic role.

FIG 8 NOD2 is necessary for optimal DC priming of CD8⁺ T cells. (A) WT BMDCs were infected with PR8 IAV at an MOI of 10, and samples were collected and analyzed for NOD2 protein expression by Western blotting at the indicated time points. (B) BMDCs were mock infected or infected with PR8 IAV at an MOI of 10, and samples were analyzed after 24 h for CD86 expression by flow cytometry. (C and D) *In vitro* stimulation of T cells in the presence of IAV-infected BMDCs and anti-CD3ε antibody for 3 days. (C) Proliferation of CD8⁺ T cells was measured by CFSE dilution. As a control, T cells were stimulated *in vitro* with anti-CD3- plus anti-CD28-beads for 3 days. (D) The death of CD8⁺ T cells was measured by using annexin V staining. (E and F) Virus titer and CD8⁺ T cell numbers on day 12 after IAV infection from *Tcrα*^{-/-} mice, which had WT or *Nod2*^{-/-} T cells adoptively transferred 30 days prior to infection. For panels A and B, the data are representative of at least three independent experiments: *n* = 2 wells per experiment (A) and *n* = 3 wells per experiment (B). For panels C and D, the data are representative of seven independent experiments (*n* = 3 to 4 wells per experiment). For panels E and F, *n* = 4 to 6 mice per experiment (means ± the SEM). ***, *P* < 0.001 (one-way ANOVA for multiple comparisons).

Adaptive immunity has been harnessed during the last two centuries for its public health benefits through the successful generation of vaccines, which have now effectively eradicated or drastically reduced some of the most deadly pathogens to humans (38). Although there is an influenza vaccine available, it has many shortcomings. It is generally thought that an effective vaccine should mimic the host response to the natural infection (39–42). However, the subunit vaccines that are the staple of seasonal influenza vaccine strategies do not mirror the breadth or depth of immunity that natural infection can offer. Primarily, there is little

or no CD8⁺ T cell immunity generated by these vaccines, which is critical for cross-protective immunity, and the humoral response is dominated by IgG1 instead of IgG2a/c (43–47).

The finding that NOD2 regulates CD8⁺ T cell responses during IAV infection could aid in the development of vaccines and adjuvants that are more physiologically relevant and can provide a broader spectrum and potentially longer lasting immunity to IAV. It will be important to determine whether viral RNA and the bacterial cell wall component muramyl dipeptide, both known to activate NOD2, have similar potential for DC

activation and subsequent T cell responses, or if the use of different NOD2 ligands has different adjuvant potentials in the development of vaccines.

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