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Dendritic cell NLRC4 regulates influenza A virus–specific CD4+ T cell responses through FasL expression

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Influenza A virus–specific (IAV-specific) T cell responses are important correlates of protection during primary and subsequent infections. The generation and maintenance of robust IAV-specific T cell responses relies on T cell interactions with dendritic cells (DCs). In this study, we explore the role of the nucleotide-binding domain leucine-rich repeat–containing receptor family member NLRC4 in modulating the DC phenotype during IAV infection. Nlrc4−/− mice had worsened survival and increased viral titers during infection, normal innate immune cell recruitment, and IAV-specific CD8+ T cell responses, but severely blunted IAV-specific CD4+ T cell responses compared with WT mice. The defect in the pulmonary IAV–specific CD4+ T cell response was not a result of defective priming or migration of these cells in Nlrc4−/− mice but was instead due to an increase in FasL+ DCs, resulting in IAV-specific CD4+ T cell death. Together, our data support a role for NLRC4 in regulating the phenotype of lung DCs during a respiratory viral infection and thereby influencing the magnitude of protective T cell responses.

Introduction

Influenza A virus (IAV) is a respiratory pathogen responsible for seasonal epidemics that can cause severe disease and death, most commonly in the very young, very old, and immunocompromised individuals in the population (1). Vaccination strategies have traditionally prioritized antibody responses, but there is increasing evidence that IAV-specific T cells confer heterosubtypic protection that correlates with reduced symptom severity during infection (2, 3). During a primary IAV infection, CD8+ T cells are critical for viral clearance, which they accomplish by directly killing infected cells through death receptor interactions and perforin and granzyme (4, 5). IAV-infected cells (7). IAV-specific Tregs are also present and help to restrain tissue damage caused by exaggerated nonspecific innate immune responses and targeted cytotoxic CD8+ T cell–mediated killing of infected cells (8–11).

Antigen-presenting cells (APCs) at the site of infection are indispensable for regulating the magnitude and character of T cell responses in diverse inflammatory contexts (12). Depletion of phagocytic cells in the lungs following IAV infection results in premature apoptosis of IAV-specific T cells, due to the loss of critical survival signals normally provided by dendritic cells (DCs) (13, 14). DC interactions with T cells can also result in T cell death, which is exemplified by enhanced killing of IAV-specific CD8+ T cells by plasmacytoid DCs in the lung draining lymph nodes (dLNs) during lethal IAV infection (15–17). Induction of the appropriate phenotype in DCs depends on receipt of proinflammatory signals through cytokine and chemokine receptors and pattern recognition receptors (PRRs).

NLRC4 is an intracellular PRR belonging to the nucleotide oligomerization and binding domain and leucine-rich repeat-containing (NLR) family. NLRC4 is best described for its role as part of the multiprotein inflammasome complex that mediates processing and secretion of IL-1β and IL-18 and induces a pyroptotic cell death (18). The NLRC4 inflammasome is activated in response to infection with Gram-negative bacteria, and the resultant IL-18 production in CD8+ DCs during infection is important for memory CD8+ T cell activation in this context (19). In a syngeneic subcutaneous melanoma model, NLRC4-deficient mice were reported to have defective intratumoral CD4+ and CD8+ T cell responses and increased tumor burdens (20). Considering the importance of T cell responses in viral infection and the evidence supporting a role for NLRC4 in direct or indirect modulation of T cell responses in these different settings, we assessed the role of NLRC4 in the host response to IAV infection. We report...
that Nlrc4–/– mice had decreased survival following IAV infection with an associated defective IAV-specific CD4+ T cell response. The reduction in the CD4+ T cell response in Nlrc4–/– mice was due to T cell-extrinsic signals that resulted in increased death of IAV-specific CD4+ T cells. We further showed that there was an increase in FasL+ DCs in the lungs of IAV-infected Nlrc4–/– mice and that blocking Fas-FasL interactions in vitro prevented CD4+ T cell killing by NLRC4-deficient DCs. Finally, transfer of NLRC4-deficient DCs into WT mice resulted in both the increased mortality and loss of CD4+ T cells following IAV infection seen in Nlrc4–/– mice. Together, our findings demonstrate an essential role for NLRC4 in regulating IAV-specific CD4+ T cell responses through FasL expression on DCs.

Results

Nlrc4–/– mice have increased morbidity and mortality during IAV infection. To determine the effect of NLRC4 deficiency on outcome during IAV infection, we compared the morbidity and mortality of WT and Nlrc4–/– mice following infection with IAV. We observed significantly increased morbidity and mortality among the Nlrc4–/– animals compared with WT animals (Figure 1, A and B), accompanied by increased viral titers in the lungs of Nlrc4–/– mice on days 1, 3, and 7 after infection (Figure 1C). The susceptibility of Nlrc4–/– mice to IAV was dose dependent, and infection with a 0.25 median lethal dose (LD50) inoculum of IAV resulted in similar mortality rates between WT and Nlrc4–/– mice (Figure 1D). Consistent with previous studies, Asc–/– mice had increased mortality compared with WT mice (Figure 1D) (21, 22).

NLRC4 is best known for its role as part of the NLRC4 inflammasome, which is formed upon recognition of bacterial flagellin and components of the type III secretion system by NAIP proteins (23, 24). Activation of the NLRC4 inflammasome results in cleavage of pro–caspase-1 into its active form, which in turn cleaves pro–IL-1β and pro–IL-18 into their mature secreted forms. Formation of the NLRC4 inflammasome within the lungs seemed unlikely in the context of a viral infection, and, indeed, we detected no defect in cleavage of pro–caspase-1 in lung homogenates from Nlrc4–/– mice 24 hours after infection with IAV (Figure 1E). These data suggest an inflammasome-independent role for NLRC4 in the control of IAV infection in vivo.
Nlrc4–/– mice have intact production of inflammatory mediators and innate immune cells in the lungs. Excessive inflammation is a well-documented cause of pathology during IAV infection (25, 26). Given the increased IAV-induced mortality seen among Nlrc4–/– mice, we compared the production of inflammatory mediators in the lungs of WT and Nlrc4–/– mice following IAV infection and found no significant differences (Supplemental Figure 1; supplemental material available online with this article; https://doi.org/10.1172/JCI124937DS1). Consistent with the similarity in the levels of innate cell chemoattractants in WT and Nlrc4–/– mice, the number of lung-infiltrating and lung-resident inflammatory cells was similar in WT and Nlrc4–/– mice on days 3 and 5 after infection (Figure 1, F–J).

Defective pulmonary IAV-specific T cell responses in Nlrc4–/– mice. The IAV-specific CD4+ T cell response occurs primarily within the lungs and comprises a wide variety of specificities, with a relatively small proportion of the cells being specific for each antigen (27–29). While useful for the immune response, this breadth of specificities complicates measurement of the total response as the sum of all the individual antigen-specific responses. Hence, we quantified the total IAV-specific CD4+ T cell response using the surrogate markers CD49d and CD11a (30), which are highly expressed on antigen-experienced cells and, in the context of IAV infection, reflect the cells that have been exposed to IAV-specific antigen (31). We detected a significant decrease in the total and IAV-specific CD4+ T cell response in the lungs of Nlrc4–/– mice compared with responses in WT mice on day 7 after infection (Figure 2, A and B). Interestingly, the decrease in IAV-specific CD4+ T cells in Nlrc4–/– mice was not present on day 3 or day 5 after infection in the lungs, dLNs, or spleen (Figure 2, A and B, and Supplemental Figure 2, A–D).

Nlrc4+/– and Nlrc4+/+ littermates had similar numbers of pulmonary IAV–specific CD4+ T cells, suggesting that the Nlrc4 gene is haplosufficient (Supplemental Figure 2, E and F).

The IAV-specific CD8+ T cell response is crucial for viral clearance during a primary IAV infection, thus we quantified this response in WT and Nlrc4–/– mice (34). We detected small decreases in the frequency and number of total lung CD8+ T cell responses in Nlrc4–/– mice compared with WT mice on day 7 after infection (Figure 2, C and D). The IAV-specific CD8+ T cell response is also Th1-polarized, with a smaller number of IAV-specific Tregs, which are protective during a primary infection (7, 32, 33). Consistent with the significantly decreased number of total IAV-specific CD4+ T cells in Nlrc4–/– mice, we found significant decreases in IAV-specific Th1 (Tbet+) cells and Tregs (Foxp3+) (Figure 2, E and F).

As a result of the defective pulmonary IAV-specific T cell response, Nlrc4–/– mice have increased lung pathology and increased IAV-induced mortality (25, 26). Given the similarity in the levels of innate cell chemoattractants, we compared the production of inflammatory mediators in the lungs of WT and Nlrc4–/– mice following IAV infection and found no significant differences (Supplemental Figure 1). Consistent with the similarity in the levels of innate cell chemoattractants in WT and Nlrc4–/– mice, the number of lung-infiltrating and lung-resident inflammatory cells was similar in WT and Nlrc4–/– mice on days 3 and 5 after infection (Figure 1, F–J).
blunted CD4+ T cell response in the lungs of Nlrc4–/– mice. Together, these data reveal a defect in the pulmonary IAV-specific CD4+ T cell response in Nlrc4–/– mice. IAV-specific CD4+ T cell responses are important for successful viral clearance and recovery from IAV infection (32, 35), thus these defects probably contribute to the enhanced mortality evident in Nlrc4–/– mice.

DC and T cell accumulation in lung dLNs is intact in Nlrc4–/– mice. To identify the cause of the blunted IAV-specific CD4+ T cell response, we evaluated key steps in the generation of this response. Initiation of robust IAV-specific T cell responses relies on successful interactions in the secondary lymphoid organs with antigen-bearing APCs, many of which have migrated from the lungs (6). Accelerated respiratory DC migration to the lung dLNs is intact in Nlrc4–/– mice (Supplemental Figure 3, A and B), indicating that defective DC migration was not likely to be contributing to the blunted IAV-specific CD4+ T cell response in Nlrc4–/– mice. Further characterization of the DC phenotype in lung dLNs revealed no differences in the abundance or expression of costimulatory molecules on DCs in WT or Nlrc4–/– mice (Supplemental Figure 3, C–H). These findings are consistent with the normal accumulation of total and antigen-experienced CD4+ T cells in the lung dLNs 3 and 5 days after infection (Supplemental Figure 2, C and D). Consid ered together, these data argue that a defect in activation and expansion of T cells in the dLNs or spleen was not the cause of the blunted CD4+ T cell response in the lungs of Nlrc4–/– mice.

Increased T cell death in the lungs of Nlrc4–/– mice following IAV infection. Given that early DC-dependent events in the development of the IAV-specific CD4+ T cell response appeared to proceed normally in Nlrc4–/– mice, we assessed whether the decrease in CD4+ T cells was due to increased death among these cells. Annexin V and viability staining showed increased dead or dying IAV-specific CD4+ T cells in the lungs of Nlrc4–/– mice compared with that seen in WT mice (Figure 3A). Consistently, IAV-specific CD4+ T cells from the lungs of Nlrc4–/– mice had more active caspase-3/-7 and active caspase-8 as measured with a fluorescent inhibitor probe 7 days after infection compared with expression in CD4+ T cells from WT mice (Figure 3, B and C). These data suggest that increased death among IAV-specific CD4+ T cells may be driving their diminished presence in Nlrc4–/– lungs.

Increased IAV-specific CD4+ T cell death in Nlrc4–/– mice is T cell extrinsic. To determine whether the cause of death in Nlrc4–/– T cells was a defect intrinsic or extrinsic to the Nlrc4–/– T cells themselves, we set up a side-by-side comparison of WT and Nlrc4–/– T cells in WT and Nlrc4–/– hosts. We adoptively transferred both WT (CD90.1/2+) and Nlrc4–/– (CD90.1+) OT-II CD4+ T cells, specific for the amino acids 323–339 of chicken OVA (OVA323–339), intrave nously into WT and Nlrc4–/– recipients (CD90.2+). One day later, we infected mice with IAV expressing the OVA323–339 epitope and quantified the IAV-specific CD4+ T cell response in the lungs 7 days after infection. We observed significantly more OT-II cells in WT and Nlrc4–/– hosts with an antigen-experienced phenotype in the lungs of WT hosts than in Nlrc4–/– hosts, regardless of the OT-II donor genotype (Figure 3D), indicating that the cause of T cell death in the Nlrc4–/– mice was T cell extrinsic.

Increased FasL+ DCs in IAV-infected Nlrc4–/– lungs. Blunting of T cell responses by Fasl+ DCs occurs in diverse inflammatory contexts (15, 16, 37, 38), thus we explored DC expression of Fasl in WT and Nlrc4–/– mice (Supplemental Figure 4, A and B) specifically within the CD11b+ and CD11b– populations of DCs. The increase in Fasl+ DCs matched the timing and location of the decrease in IAV-specific CD4+ T cells, as there were no differences in Fasl+ DCs on day 5 after
infection in the lungs of WT and Nlrc4−/− mice (Figure 4A and Supplemental Figure 4B). The geometric mean fluorescence intensity (GMFI) for FasL was not different between DCs from WT and Nlrc4−/− mice (Supplemental Figure 4C), consistent with more cells expressing FasL rather than a higher expression level per cell.

Consistent with the normal activation of caspase-1 in lungs from Nlrc4−/− mice following IAV infection, we observed no increase in FasL+CD11b+ or FasL+CD11b− DCs in the lungs of Casp1/11−/− mice 7 days after infection (Figure 4B and Supplemental Figure 5A). We also found no difference in the proportion of Fas+ IAV-specific CD4+ T cells in the lungs of WT or Nlrc4−/− mice, in agreement with the data indicating a T cell–extrinsic cause of death (Supplemental Figure 5B).

To determine whether DCs from IAV-infected Nlrc4−/− lungs were killing CD4+ or CD8+ T cells, we modified a previously described DC–T cell coculture assay (15). Seven days after infection, we isolated lung DCs from WT or Nlrc4−/− mice, and FasL+ DCs were enumerated by flow cytometry for the indicated organ and time point after infection (A) or in the lung on day 7 after infection (B). Data are from 1 experiment (A, day 5, and B, n = 3–5 per group) or 2 separate experiments (A, day 7, n = 9–10 per group). Error bars show the SEM. **P < 0.01 and ***P < 0.001, by 2-tailed Student’s t test.

DCs isolated from IAV-infected WT and Nlrc4−/− mice, which have similar FasL expression levels (Supplemental Figure 5C). To test whether the increased killing of CD4+ T cells by Nlrc4−/− DCs was dependent on Fas-FasL interactions, we blocked this interaction by adding Fas-Fc to the cocultures. Addition of Fas-Fc to cocultures increased the survival of IAV-specific lung CD4+ T cells cultured with WT and Nlrc4−/− lung DCs to a similar degree (Figure 5A) but had no effect on CD4+ T cells cultured alone (Supplemental Figure 5D), indicating that DC FasL is responsible for killing CD4+ T cells. Consistent with the finding that CD8+ T cell numbers in the lungs of IAV-infected mice were not significantly diminished in the absence of NLRC4 (Figure 2, E and F), we observed similar survival rates of CD8+ T cells following coculture with Nlrc4−/− and WT CD11bhi, CD11b+ or CD11b− lung DCs (Figure 5B).

To confirm that the enhanced IAV-induced mortality seen in Nlrc4−/− mice was due to killing of CD4+ T cells by FasL+ DCs, 5 days after infection, WT mice received an intranasal transfer of WT or Nlrc4−/− bone marrow–derived DCs (BMDCs). Like CD11bhiCD11c+ and CD11b−CD11c+ lung DCs (Figure 4A), we observed a marked loss of total and IAV-specific CD4+ T cells in the WT hosts that received Nlrc4−/− BMDCs in comparison with the WT hosts treated with WT BMDCs (Figure 5, D and E, and Supplemental Figure 6, B and C). In contrast, no difference in IAV-specific CD8+ T cells was observed in mice that received WT or Nlrc4−/− BMDCs (Figure 5, F and G, and Supplemental Figure 6, D and E).

Decreased Akt1 and FoxO3a phosphorylation in NLRC4-deficient BMDCs. To determine how NLRC4 deficiency regulated FasL expression, we examined mRNA expression of FasL in WT and Nlrc4−/− BMDCs by quantitative real-time PCR. We found that Nlrc4−/− BMDCs expressed higher levels of FasL than did WT BMDCs (Figure 6A). To confirm these findings, we assessed FasL on BMDCs from an independently generated Nlrc4−/− mouse line (39). We again observed increased FasL mRNA and protein expression levels in Nlrc4−/− BMDCs compared with levels in WT BMDCs (Supplemental Figure 6, F and G). Consistent with inflammasome-independent regulation of FasL expression, we did not observe any significant difference in FasL expression in Asc−/− or Casp11−/− BMDCs compared with WT BMDCs (Supplemental Figure 6, H and I).

FoxO3a, a member of the Forkhead family of transcription factors, has been implicated in the regulation of FasL expression. Furthermore, phosphorylation of FoxO3a by the serine/threonine kinase Akt1 prevents FoxO3a-dependent transcription by inhibiting
its translocation to the nucleus and preventing it from activating its target genes (40). We observed that BMDCs from \textit{Nlrc4}–/– mice had diminished Akt1 and FoxO3a phosphorylation compared with WT BMDCs (Figure 6, B and C), suggesting that NLRC4 may regulate FasL expression through Akt1 and FoxO3a phosphorylation.

### Discussion

The data presented here show for the first time to our knowledge that \textit{Nlrc4}–/– mice have a defective immune response during IAV infection. \textit{Nlrc4}–/– mice show increased morbidity and mortality and impaired viral clearance, but these do not appear to be the result of a defect in cytokine/chemokine production or the innate immune response. Instead, we report a blunted IAV-specific CD4+ T cell response in the lungs of \textit{Nlrc4}–/– mice. The number of pulmonary IAV-specific CD4+ T cells was dramatically decreased, but CD8+ T cells were largely unaffected. Ultimately, the loss of IAV-specific CD4+ T cells in \textit{Nlrc4}–/– mice was a result of increased CD4+ T cell death due to Fas-FasL-mediated killing by CD11b+ and CD11b- DCs in the lungs.

The increased susceptibility of \textit{Nlrc4}–/– mice to IAV infection appears to be NLRC4 inflammasome independent, as we observed no changes in the inflammasome-dependent cytokine IL-1β or caspase-1 cleavage in \textit{Nlrc4}–/– lungs during infection. The NLRP3 and AIM2 inflammasomes are activated during IAV infection (21, 22, 41), thus the caspase-1 activation and IL-1β secretion we observed are expected, given the activity of those inflammasomes. Interestingly, the level of IL-1β was slightly, but not significantly, increased in \textit{Nlrc4}–/– lungs, as determined by ELISA. This result may indicate increased activation of the NLRP3 or AIM2 inflammasomes, which could be due to the elevated viral titers and therefore more abundant activating signals in \textit{Nlrc4}–/– mice.

Previous studies focused on the NLRP3 inflammasome showed no effect of NLRC4 deficiency on morbidity or mortality following IAV infection (21, 22). We find these data intriguing in light of the very clear survival defect in \textit{Nlrc4}–/– mice we report here. The reason for the differences in these studies is probably multifactorial. When we infected mice with a lower inoculum of IAV, we no longer observed an increase in mortality among the \textit{Nlrc4}–/– mice compared with WT mice, however the \textit{Asc}–/– mice were more susceptible, even at this lower inoculum, consistent with previously published findings (Figure 1D) (21, 22). Additionally, since those studies were published, we have developed an increased appreciation for the impact that genetic background has on knockout mice. We postulate that differences in the WT sub-

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**Figure 5. Increased FasL-mediated killing of CD4+, but not CD8+, T cells by \textit{Nlrc4}–/– DCs during IAV infection.** Mice were infected with a 0.5 LD$_{50}$ inoculum of IAV or left uninfected (naive). (A and B) Pulmonary DCs and T cells were purified on day 7 after infection. Pooled WT plus \textit{Nlrc4}–/– T cells were incubated with the indicated populations of DCs for 12 hours with (+ Fas-Fc) or without (No trx) 2.5 μg/ml Fas-Fc. Live CD4+ and CD8+ T cells (annexin V– viability dye–) were enumerated by flow cytometry. The proportion of live CD4+ and CD8+ T cells cocultured with DCs was normalized to CD4+ and CD8+ T cells cultured alone. (C-G) Five days after infection, WT mice received 5 × 10$^6$ WT or \textit{Nlrc4}–/– BMDCs intranasally, and survival was assessed. (C) Pulmonary CD4+ and CD8+ T cells were quantified on day 7 after infection (C-G). Data are from 1 experiment (B, n = 4 per group) or 2 separate experiments (A, n = 8 per group, and C-G, n = 10 per group). Error bars represent the SEM. *P < 0.05 and **P < 0.01, by 2-tailed Student’s t test (A, D, and E) and Mantel-Cox test (C).
strains used as controls and for backcrossing with Nlrc4−/− mice may further contribute to the discrepancy between the results presented here and previous reports. Of particular note, we and others have recently shown differences between C57BL/6J and C57BL/6N substrains in survival and inflammatory responses to IAV (31, 42). We and others have also reported striking differences in immune responses to additional inflammatory challenges among C57BL/6 substrains (42–44). In the present study, we rigorously tested the specificity of our phenotype to a loss of NLRC4 through the use of littermate controls and by confirmation in an independently generated Nlrc4−/− mouse (Supplemental Figure 2, E and F, and Supplemental Figure 6, F and G).

NLRC4 was recently reported by our laboratory to have an inflammasome-independent role in limiting melanoma progression (20). Although IAV infection and melanoma are considerably different challenges for the host immune system, in both cases, a robust host T cell response is a correlate of protection. During both IAV infection and melanoma challenge, Nlrc4−/− mice exhibit defective T cell responses, and in both models, myeloid cell dysfunction seems to be driving the defect (20). Intriguingly, it was recently shown that FASLG is highly expressed in a large number of human cancers and that engineering tumor-specific T cells to resist Fas-mediated death is a promising strategy for enhancing immunotherapy during cancer (45). It would be of interest to determine whether there is NLRC4-mediated modulation of myeloid cell FasL during melanoma progression and whether the ineffective melanoma-specific T cell responses observed in Nlrc4−/− mice could be rescued by disrupting Fas-FasL interactions or downstream signaling.

An important remaining question to answer is how NLRC4 is acting in these myeloid cells to influence FasL expression. Offering a single explanation that accounts for all of the observations is challenging because of the dynamic nature of immune responses and their regulatory mechanisms, but we speculate that NLRC4 may be involved in differentiation or activation of myeloid cells during an inflammatory insult. That unstimulated Nlrc4−/− BMDCs, but not Casp1/11−/− or Asc−/− BMDCs, have increased FasL mRNA and protein levels is suggestive of an inflammasome-independent function of NLRC4 in either the development and/or differentiation of DCs. Fasl gene expression is controlled by a number of distinct transcription factor interactions at the Fasl promoter (46). FoxO3a can be phosphorylated by Akt1, resulting in its inactivation as a transcription factor. Conversely, dephosphorylation of FoxO3a results in upregulation of FasL and triggers increased apoptosis (40). In the absence of NLRC4, we observed diminished Akt1 and FoxO3a phosphorylation, which may have been driving increased FasL expression. As many signaling pathways involved in metabolism, cytokine sensing, and pathogen-associated molecular pattern (PAMP) and damage-associated molecular pattern (DAMP) sensing converge on Akt (47), it is possible that dysregulated activating signals in the absence of NLRC4 result in increased FasL expression.

In conclusion, the work described here demonstrates a protective role for NLRC4 during IAV infection. Protection is probably NLRC4 inflammasome independent and involved support of the IAV-specific CD4+ T cell response in IAV-infected lungs. In the absence of NLRC4, expression of FasL on CD11b+ and CD11b− lung DCs was increased, triggering more CD4+ T cell death in the lungs of IAV-infected animals and the associated increased mortality. These findings are of importance, as they expand our understanding of how the IAV-specific CD4+ T cell response in the lungs is regulated and implicate DC NLRC4 in the regulation of CD4+ T cell responses.

**Methods**

**Mice.** The generation of Nlrc4−/−, Asc−/−, and Casp1/11−/− mice has been described elsewhere (48–50). Mice were backcrossed with C57BL/6N mice for at least 10 generations and maintained in a specific pathogen-free (SPF) facility. C57BL/6N mice were purchased from Charles River Laboratories and used as WT controls unless otherwise stated; B6.Cg-Tg(TcraTcrb)425Cbn/J (OT-II CD90.2+) mice were purchased from The Jackson Laboratory. Femurs from Nlrc4−/− mice (39) were a gift from Matam Vijay-Kumar (University of Toledo, Toledo, Ohio, USA). Both male and female mice (6–12 weeks of age) were used, however, mice were sex, age, and weight matched for individual experiments.

**Virus and in vivo infection.** The mouse-adapted IAV strain A/PR/8/34 (PR/8) was propagated as previously described (16). Recombinant IAV-OT-II was created using standard reverse genetics as previously described (51) and grown in 10-day-old embryonated chicken eggs (Charles River Laboratories). The OT-II epitope (Ova323–339) was inserted into the virus genome by reverse genetics.

**Implementation of death.** rescued from the lungs of IAV-infected animals and the associated increased mortality. These findings are of importance, as they expand our understanding of how the IAV-specific CD4+ T cell response in the lungs is regulated and implicate DC NLRC4 in the regulation of CD4+ T cell responses.

**Figure 6. NLRC4 regulates FasL expression at the transcriptional level in BMDCs. (A) Fasl mRNA expression was assayed in WT and Nlrc4−/− BMDCs by quantitative real-time PCR and normalized to β-actin. (B and C) p-Akt1 and p-FoxO3a protein levels in whole-cell lysates of BMDCs from WT and Nlrc4−/− mice were analyzed by immunoblotting. Densitometric analysis was performed with Bio-Rad Image Lab software (version 5.2.1). Total FoxO3a, Akt1, and β-actin were used as loading controls. Data were pooled from 3 independent experiments. *P < 0.05 and **P < 0.01, by 2-tailed Student’s t test.
inserted into the mRNA nucleotide position 186 encoding the neuraminidase stalk region, which is known to tolerate such insertions (52). Mice were anesthetized with ketamine and xylazine and infected intranasally with 0.5 LD₅₀ virus diluted in 50 μl sterile DMEM. Weight was monitored daily, and the mice were euthanized upon loss of 30% of their starting weight. CFSE labeling of lung cells followed by IAV infection were performed as described previously (36), but CFSE was administered 15 minutes prior to infection.

**Lung titers.** To measure virus titers, lungs were homogenized using a Tissue-Tearor (BioSpec), and homogenates were then centrifuged, snap-frozen in liquid nitrogen, and stored at −80°C. A standard plaque assay on Madin-Darby canine kidney (MDCK) cells was subsequently used to quantify infectious virus (53).

**ELISA.** Cytokines and chemokines were quantified in cell culture supernatants and lung homogenate supernatants using DuoSet Mouse ELISA kits from R&D Systems (for CXCL1, CXCL2, CXCL5, CXC19, CXCL10, IL-1β, CCL2, and CCL5) or ReadySetGo! Mouse ELISA kits from eBioscience (for IL-1α, IL-6, IL-10, and TNF-α) following the manufacturers’ instructions.

**Flow cytometry.** Single-cell suspensions were prepared by pressing tissues through wire mesh screens (lungs) or dissociating between frosted ends of glass slides (spleens and LNs). For some experiments, cells were minced and digested in Iscove’s DMEM (Gibco, Thermo Fisher Scientific) containing 1 mg/ml collagenase XI (Millipore Sigma) following the manufacturers’ instructions. Cells (1 × 10⁶ cells/well) on a 96-well plate (Corning) were infected with 1 μl of virus and incubated at 37°C for 24 hours. Cells were washed with PBS and stained with fluorochrome-conjugated antibodies as described in the figure legends. Live cells were sorted and analyzed using a BD FACSAria II.

**Western blotting.** Lysates were prepared in RIPA buffer (Cell Signaling Technology) with 1 mM PMSF according to the manufacturer’s instructions. Lungs were homogenized in RIPA buffer using a Tissue-Tearor (BioSpec Products). A biotinylated antibody (BCA) assay (Thermo Fisher Scientific) was performed to measure total protein in lung homogenates, and then samples were diluted to the same concentration in RIPA buffer. Lysates were stored at −80°C. Proteins were separated on a NuPAGE gel (Invitrogen, Thermo Fisher Scientific) and transferred onto a PVDF membrane using the XCell II blotting system (Invitrogen, Thermo Fisher Scientific). Membranes were blocked with 5% nonfat milk or BSA and incubated with anti-caspase-1 (p20) antibody (1:1000, AG-20B-0042-C100, Adipogen), β-actin (1:2000, sc-47778, Santa Cruz Biotechnology), phosphorylated Akt1 (p-Akt1) (1:1000, CST 9018, Cell Signaling Technology), Akt1 (1:1000, CST 75692, Cell Signaling Technology), p-FoxO3a (1:1000, CST 9466), or FoxO3a (1:1000, CST 12829, Cell Signaling Technology) overnight at 4°C. Following washing, the membranes were incubated with HRP-tagged anti-mouse IgG (1706516, Bio-Rad) or anti-rabbit IgG (NA934, GE Healthcare) and developed using SuperSignal West Pico or Femto substrate (Thermo Fisher Scientific).

**Real-time PCR.** Total RNA was isolated from BMDCs using a NNeasy Mini Kit (QIAGEN), and cDNA was generated with PrimeScript RT Master Mix (Takara Bio) according to the manufacturer’s instructions. Real-time PCR was carried out using the CFX Real-Time PCR System (Bio-Rad) with a SYBR Green molecular probe (Applied Biosystems). mRNA levels were quantitatively analyzed using Bio-Rad CFX Manager 3.1 software. Relative mRNA expression levels were normalized to the housekeeping gene β-actin, forward, 5′-CGAGGTATCCTGACCTG-3′, reverse, 5′-CCACCGTGGGAGACACCA-3′, and 18S, forward, 5′-CGAGGTATCCTGACCCCTGGA-3′, reverse, 5′-GTTGTTGTGGTCCAGATCTTCT-3′.

**DC adoptive transfer.** BMDCs were differentiated as previously described (54). BMDCs were administered by flow cytometry and/or 5 × 10⁵ BMDCs in 50 μl sterile DMEM were administered intranasally to IAV-infected mice 5 days after infection.

**Statistics.** Statistical tests used to determine significance are indicated in the figure legends. Error bars represent the mean ± SEM. Statistical significance was based on the Mantel-Cox test for Figure 1A and D, and Figure 5C; 1-way ANOVA with Tukey’s post hoc analysis for Figure 1B; and a 2-tailed Student’s t test for all remaining data. P values of 0.05 or less were considered statistically significant. Data were graphed and statistical tests performed using GraphPad Prism (GraphPad Software).
Study approval. All animal studies were approved by and performed according to the guidelines of the IACUC of the University of Iowa and Cedars-Sinai Medical Center.

Author contributions
EEH, FSS, and SLC conceptualized the project. EEH, KLL, FSS, and SLC developed the methodology. EEH and JD performed the experiments with assistance and/or reagents from ZRZ, AMM, RAL, and KLL. EEH, JD, FSS, and SLC performed formal analysis. EEH, FSS, and SLC wrote the original draft of the manuscript. EEH, JD, ZRZ, AMM, KLL, GAB, PC, FSS, and SLC reviewed and edited the manuscript. FSS and SLC acquired funding. GAB, FSS, and SLC supervised the project.

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