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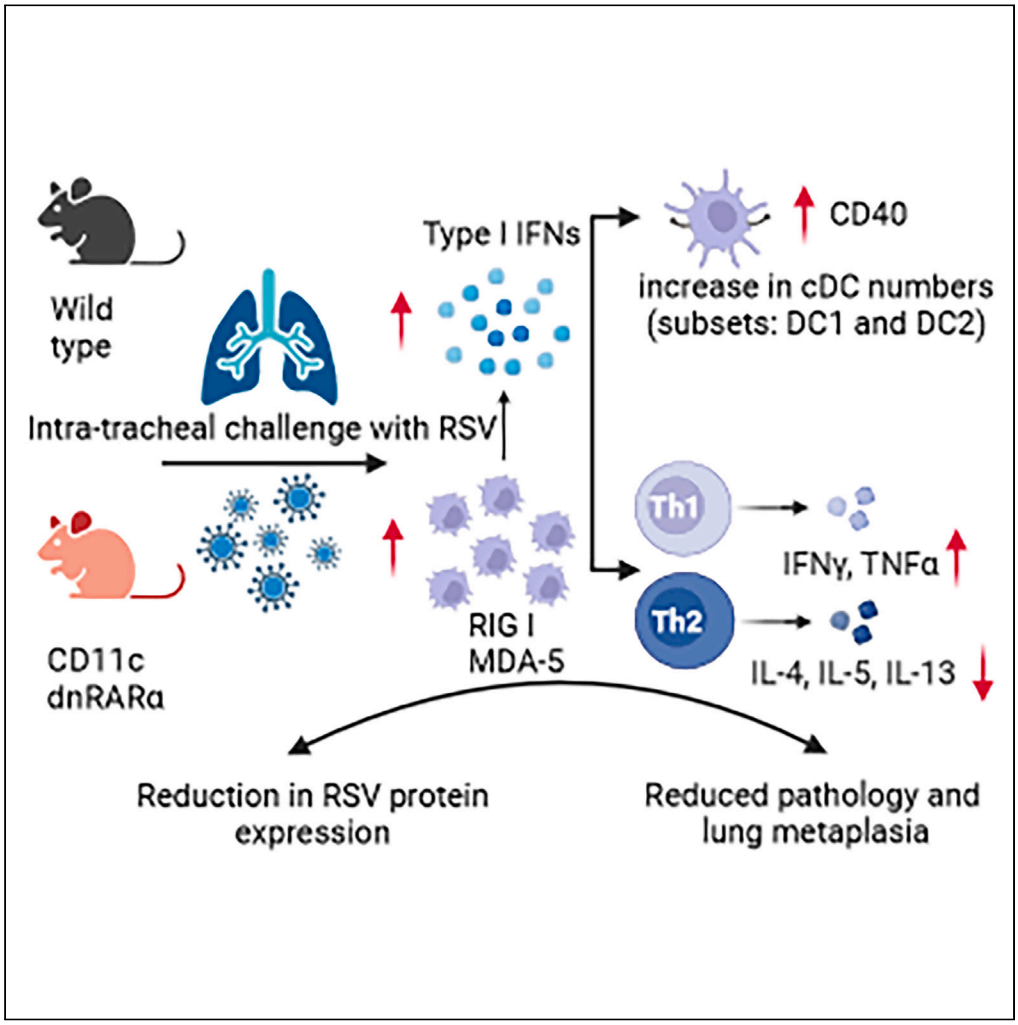

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Highlights

RA signaling in DCs regulates their innate immunity against RSV

Loss of RA signaling in DCs suppresses RSV infection and reduces lung metaplasia

Blocking RA signaling in pDCs but not AMs increase their type I and III IFNs

Increased IFNs by pDCs and CD40 on cDCs enhances Th1 and suppresses Th2 to RSV infection

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Inhibiting retinoic acid signaling in dendritic cells suppresses respiratory syncytial virus infection through enhanced antiviral immunity

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SUMMARY

Retinoic acid (RA), controls the immunoregulatory functions of many immune cells, including dendritic cells (DCs), and is important for mucosal immunity. In DCs, RA regulates the expression of pattern recognition receptors and stimulates interferon production. Here, we investigated the role of RA in DCs in mounting immunity to respiratory syncytial virus (RSV). To abolish RA signaling in DCs, we used mice expressing a dominant negative form of retinoic acid receptor-α (RARα) under the CD11c promoter (CD11c-dnRARα). Paradoxically, upon RSV challenge, these animals had lower viral burden, reduced pathology, and greater Th1 polarized immunity than wild-type (WT) mice. Moreover, CD11c-dnRARα DCs infected with RSV showed enhancement in innate and adaptive immunity genes, while genes associated with viral replication were downregulated. These findings suggest that the absence of RA signaling in DCs enhances innate immunity against RSV infection leading to decreased viral load and reduced pathogenicity.

INTRODUCTION

Respiratory syncytial virus (RSV) is one of the leading causes of infant mortality and has been associated with the development of asthma in young children as it causes bronchitis and airway inflammation.[1–3] It can also cause significant respiratory disease in the elderly or even death in immunocompromised subjects due to weakened immunity. RSV infections appear to be increasing and this is better documented due to the development of sensitive PCR-based diagnostic tests. Given there are limited treatment options it is important to better understand the pathogenesis of RSV infection.[4]

Antigen-presenting cells (APCs), particularly dendritic cells (DCs), play an important role in initiating immune responses to RSV. The virus infects the airway by binding to ciliated epithelial cells where it interacts with macrophages, natural killer cells (NK), plasmacytoid, and conventional DCs (pDC and cDC).[5] The virus activates pattern recognition receptors (PRRs) including retinoic inducible gene-I (RIG-I) like receptors (RLRs), melanoma differentiation-associated gene 5 (MDA-5), and Toll-like receptors (TLRs) to initiate the production of interferons to suppress viral replication.[6,7] Type I interferons (IFN-α and β) amplify the inflammatory response during RSV infection and signal monocytes, macrophages, and neutrophils to enhance antiviral immunity.[7,8] RSV triggers activation of TLR-2/6, 3, and 4 leading to pro-inflammatory cytokine and interferon production.[6,9] pDCs produce significant amounts of interferons that limit RSV replication and enhance its clearance.[10] Thus, DCs appear central to the innate responses that limit RSV infection.

Studies have sought to investigate the specific activities of different DC subsets in initiating specific RSV immunity during infection. Following initial RSV infection, $CD11c^{+}$ $CD11b^{high}$ $CD103^{-}$ and $CD11c^{+}$ $CD11b^{-}$ $CD103^{+}$ cDC populations significantly increase in the lung and draining lymph nodes.[11–13] These activated DCs efficiently sample viral antigens and then interact with the naive T cells in the draining lymph nodes to drive Th1, Th2, and Th17 immunity.[14–16] Thus, both innate and specific immunity to RSV is the result of DC activation.

Retinoic acid (RA) produced by DCs plays a very important role in the regulation of immune cells. Reports suggest that RA-dependent molecular pathways in DCs mediate type I interferon production to control viral infection.[17] $CD103^{+}$ DCs are crucial in maintaining mucosal homeostasis and are very efficient in converting vitamin A into RA.[18–21] RA imprints DCs, B and T cells for homing to the intestine as an immune response under different pathogenic conditions.[22–25] All-trans-retinoic acid (ATRA) is the most abundant form of RA found in the body and has been shown to regulate RIG-I pathway activation in conjunction with activated TLR ligands resulting in interferon stimulating gene (ISG) expression and interferon production.[17] Since interferons are central to initial protection from RSV and RA appears to be crucial for the generation and transcriptional programming of DCs, we endeavored to study the role of RA signaling in DCs in RSV immunity. We used transgenic

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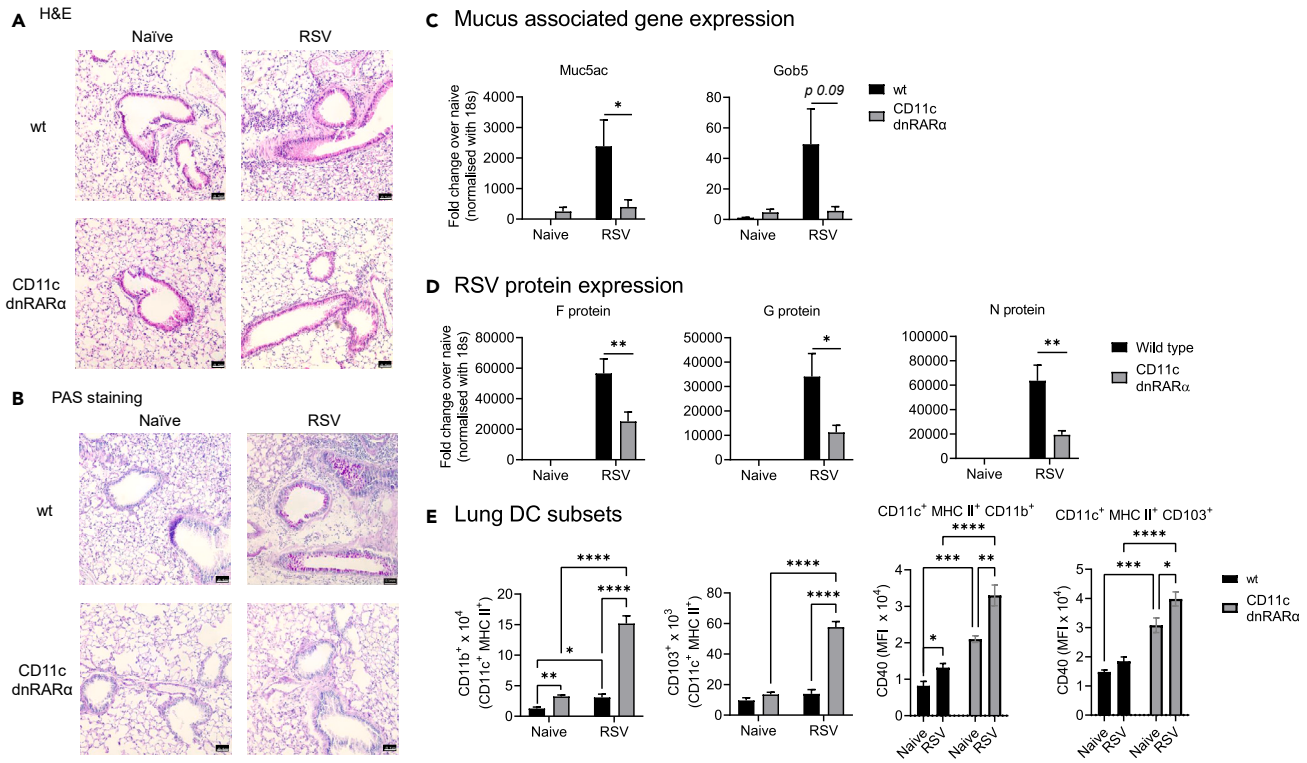

Figure 1. CD11c-dnRARα animals had reduced inflammation and mucus secretion

Lungs were isolated from naive CD11c-dnRARα and wt littermates as well as RSV (3×10^5 pfu) infected animals on day 8.

(A and B) Lungs were embedded in paraffin; PAS was performed to stain mucus secreting cells and H&E was used to examine inflammatory cell infiltration. Inflammation and PAS staining were more severe in WT than CD11c-dnRARα animals.

(C) Lungs were homogenized in Trizol and RNA was extracted from RSV infected and naive control animals. Gene expression for mucus-associated genes (muc5AC and Gob5) was determined using qPCR. Mucus production was greater after infection in the WT animals.

(D) RSV viral load was determined by gene expression of RSV F, G, and N proteins. qPCR data shown in the figure are normalized against rRNA and fold changes are calculated over naive WT littermate controls. RSV protein expression was greater in the WT animals.

(E) RSV infection induced greater numbers of activated DCs in the lungs of CD11c-dnRARα animals. Lungs were processed into single cell suspension. Cell staining was done as described in the STAR methods section. Data shown here are the absolute total number of CD11c+ MHC II + CD11b+ and CD11c+ MHC II + CD103+ DCs and CD40-MFI on these two populations. The gating strategy is presented in Figure S1. Data shown represents $n = 5$ (naive CD11c-dnRARα and wt littermate) and $n = 6$ for RSV infected mice (CD11c-dnRARα and WT littermate). Data were analyzed by paired t test or Tukey's multiple comparison test using GraphPad Prism. Significance is shown as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

animals expressing the dominant negative form of RARα driven by the CD11c promoter, which inhibits RA signaling specifically in $CD11c^+$ cells.[26] Surprisingly, we found that upon challenge with RSV inhibiting RAR signaling in $CD11c^+$ cells is associated with enhanced anti-viral innate immunity, augmented Th1 bias, and significantly reduced pathological findings and viral burden. We then sought to understand the mechanisms underlying this paradoxical finding.

RESULTS

CD11c-dnRARα animals had reduced inflammation and mucus secretion

CD11c-dnRARα mice and age-matched controls were challenged with RSV intranasally and lung pathology and RSV mRNA expression was examined. To study the regulation of inflammation and mucus secretion in RSV infected CD11c-dnRARα animals, we stained the lung tissue sections harvested on day 8 with H&E and periodic acid-Schiff stain (PAS). WT littermates infected with RSV showed the presence of cellular infiltrates and increased mucus-secreting goblet cells compared to naive controls, while CD11c-dnRARα animals had significantly reduced inflammation as well as mucus secretion (Figures 1A and 1B). We also examined the polymorphonuclear cells (PMNs) infiltrating the lung with flow cytometry in the infected animals and did not observe any difference between the WT and CD11c-dnRARα RSV-infected mice (Figure S1). We also measured the transcripts of mucus associated genes muc5AC and Gob5 from the lungs of infected animals, and both findings agreed with the PAS staining data. CD11c-dnRARα animals had significantly lower expression of muc5AC and Gob5 as compared to the WT littermates (Figure 1C). Next, we looked at RSV gene expression (protein F, G, and N) from the lungs of infected animals. In concurrence with

PAS staining and mucus associated gene expression data, we observed significantly lower expression of these viral proteins in the lungs of CD11c-dnRARα animals as compared to the WT controls (Figure 1D).

Because cDCs play a critical role in shaping adaptive immune responses and priming T cells for pathogen-specific responses, we characterized the different cDC populations in the lungs after RSV infection. We stained single cell suspensions from the lungs of RSV infected CD11c-dnRARα and WT control mice for CD11c and MHC II high population in combination with CD11b, CD103, and CD40. We found that CD11c-dnRARα animals had significantly higher numbers of $CD11c^{+}MHCII^{+}CD11b^{+}$ cDC2 in the lung basally as compared to the WT mice (Figure 1E). Both WT and CD11c-dnRARα animals showed a significant increase in this population in the lung upon RSV infection, but CD11c-dnRARα animals had a greater increase as compared to the infected WT animals. When we looked at changes to the mucosal $CD11c^{+}MHCII^{+}CD103^{+}$ cDC1 population following RSV infection, we saw a significant increase in CD11c-dnRARα compared to infected WT animals. We analyzed MHC II expression as well on different DC subsets from naive and RSV infected WT and CD11c-dnRARα mice but did not observe any difference (Figure S3). Furthermore, RSV-infected WT animals had a significant increase in the CD40 activation marker (CD40) on $CD11b^{+}$ DCs but not on $CD103^{+}$ subset as compared to naive WT controls. Interestingly, CD11c-dnRARα animals had an increase in CD40 in naive animals in both DC subsets which further increased significantly following RSV infection (Figure 1E). Together, these data suggest that the CD11c-dnRARα animals were able to clear the virus faster than WT controls. RA signaling in DCs thus regulates adaptive immunity resulting in reduced lung pathogenicity and virus gene expression.

RA signaling in DCs regulates T cell immunity to RSV

To determine the effects of RA signaling in DCs on cytokine production following RSV challenge (8 days post-infection), we examined cytokine secretion from the lung draining lymph nodes (LDLN) cells restimulated with RSV *ex vivo* for 48 h and transcripts from lungs of infected CD11c-dnRARα mice and age-matched controls. In comparison to RSV infected WT mice, CD11c-dnRARα animals showed a significantly higher increase in the production of Th1 cytokines IFN-γ and TNF-α in LDLN (Figure 2A). In contrast, Th2 cytokine production (IL-4 and IL-5) from challenged CD11c-dnRARα animals was significantly suppressed compared to the WT animals (Figure 2A). Consistently, a slight reduction in IL-13 was observed in CD11c-dnRARα animals (Figure 2A). We did not find any difference in IL-17a production between the CD11c-dnRARα and WT animals. We also analyzed the cytokine response data segregated by gender in infected WT and CD11c-dnRARα mice and there were no differences in cytokine production during the primary RSV infection based on sex (data not shown). To further evaluate the basis of the differences in the CD11c-dnRARα mice, we examined the cytokine response of naive WT and CD11c-dnRARα LDLN cells during *ex vivo* RSV infection. Contrary to the DC results there were no differences in cytokines production in LNDN of these animals after RSV was added to the culture (Figure S4). We also examined T cell cytokine transcripts from the lungs of RSV-infected animals (Figure 2B). We observed a similar pattern in the transcripts of cytokines in the lungs as we did in the LDLN. There was an increase in Th1 cytokine and suppression of Th2 cytokines. Taken together, these data suggest that RA signaling in DCs may modulate T cell immunity against RSV to enhance Th1 immunity while suppressing Th2 immunity.

RA signaling in DCs enhances RSV gene expression

Our data showed that CD11c-dnRARα animals had significantly decreased expression of RSV proteins and had reduced pathogenicity, suggesting a possible involvement of RA signaling in DCs in the process. Therefore, we aimed to study the role of RA in viral gene transcription and retinaldehyde dehydrogenase (RALDH) gene expression in BMDCs. We infected WT BMDCs with RSV (MOI of 1) in the presence or absence of RA. To create a low RA condition, we used media containing charcoal stripped FBS (without detectable RA),[27] and a complete FBS medium for a normal RA condition (25 nM).[28] We extracted RNA from these BMDCs 24 h post incubation and examined RSV mRNA expression (F, G, and N proteins) as well as *aldh1a2* for RALDH. We observed BMDCs infected in the low RA condition, had significantly reduced expression of *aldh1a2*, and RSV F, G, and N proteins as compared to the group that was infected with RSV in the presence of RA (Figures 3A and 3B). This suggested that RA signaling in DCs enhanced RSV gene transcription and that the RALDH pathway was involved. To confirm these findings, we subsequently infected WT BMDCs in the presence of RA, with or without the specific inhibitor of RARα (Ro 41–5253). The addition of the RARα inhibitor to BMDCs stimulated with RSV reduced *aldh1a2* expression and gene expression compared to the group that did not get the inhibitor (Figure 3C). These findings are consistent with the previous experiment that the presence of RA increases *aldh1a2* as well as RSV proteins expression and using RARα inhibitor blocks the RA pathway as well as infection (Figure 3D). Taken together these findings suggest that blocking RA signaling in DCs suppresses RSV mRNA expression and possibly contributes to the protective phenotype observed in CD11c-dnRARα animals.

RA signaling in DCs regulates innate immunity to RSV infection

To examine the role of RA in DCs in protective immunity, we examined differences in innate activation upon direct stimulation of BMDCs derived from WT and CD11c-dnRARα mice. DCs are the primary producer of interferons and some of the proinflammatory factors that help in virus clearance and shaping adaptive immunity. The MDA-5 and RIG-I pathways trigger interferon regulatory factor (IRF) and nuclear factor-kappa B (NF-κB) activation leading to interferon and inflammatory cytokine production.[29] Hence, to examine the activation of these pathways, we examined the cytokine profiles resulting from RSV stimulation of BMDCs differentiated from CD11c-dnRARα and WT mice. Gene expression profiles of RALDH, MDA-5, and RIG-I upon RSV stimulation were measured over time. We found in WT BMDCs that *aldh1a2* expression increases significantly over time with RSV stimulation, whereas in CD11c-dnRARα BMDCs, the expression did not change upon infection and was markedly lower at all time points (Figure 4A). In contrast to what was observed for *aldh1a2* expression,

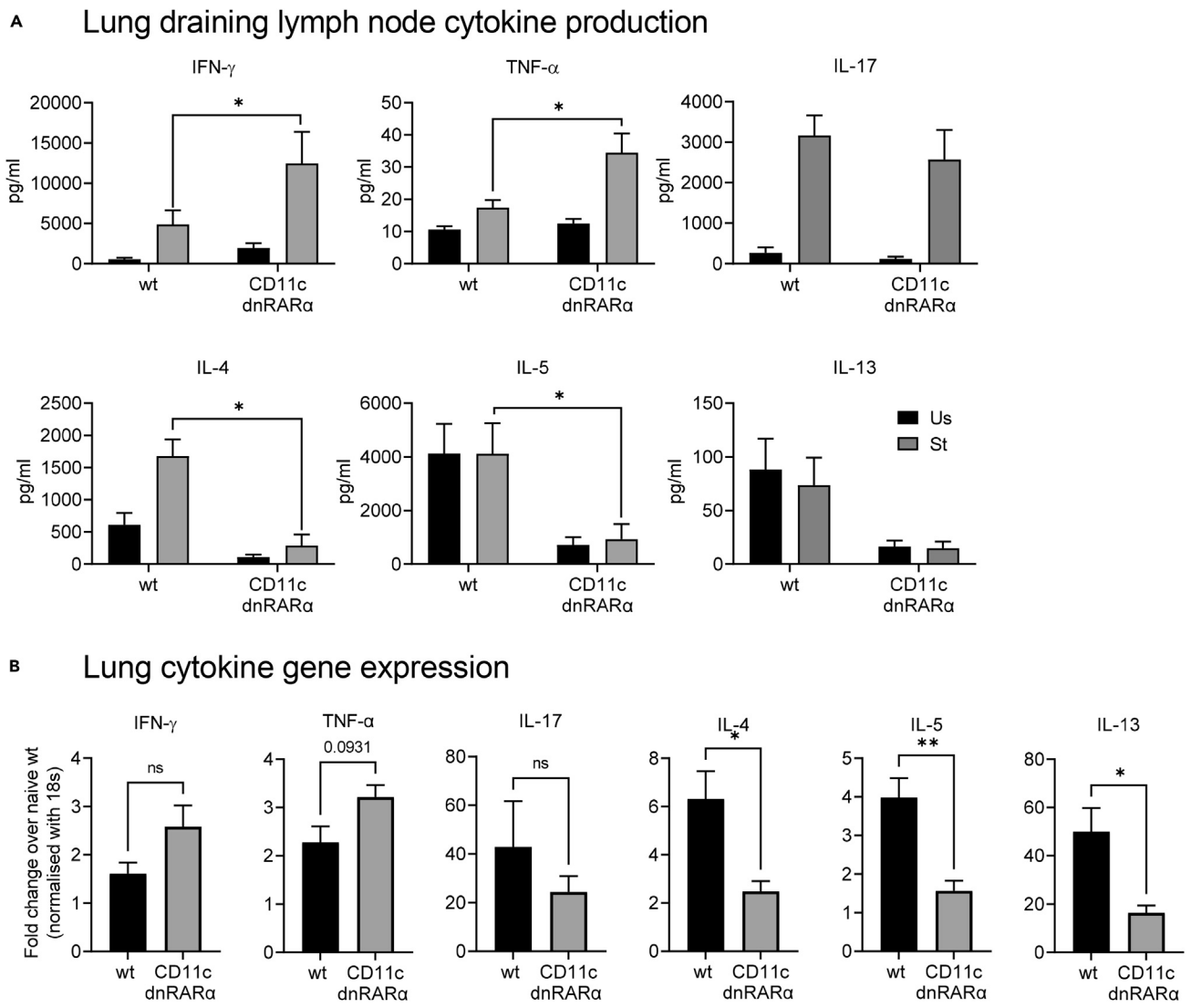

Figure 2. RA signaling in DCs regulates T cell immunity to RSV

(A) CD11c-dnRARα and WT littermate controls were infected with RSV (3×10^5 pfu), and 8 days post challenge, LDLN were isolated and processed into single cells suspension. Cells were re-stimulated with RSV *ex vivo* for 48 h. Cells were centrifuged and supernatants were collected to determine secreted cytokine.

(B) Lungs were isolated from RSV infected animals and homogenized in Trizol. RNA was extracted and the expression level of cytokines was determined using qPCR. Expression data are normalized against 18s rRNA and fold changes are calculated over naive WT littermate controls with $n = 5$ (for naive CD11c-dnRARα and WT littermates) and $n = 6$ for RSV infected mice (CD11c-dnRARα and WT littermate). Data were analyzed with paired t test and Tukey's multiple comparison test using GraphPad Prism and significance is shown as $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

in CD11c-dnRARα BMDCs, there was a significant enhancement of MDA-5 and RIG-I expression upon RSV infection. Further, CD11c-dnRARα and WT BMDC were stimulated with RSV for 24 h to measure secreted cytokines. A significant enhancement of IFN-α, IFN-β, IFN-λ2/3, and IL12-p40 secretion was observed, while production of IL-6 and TNF- α was significantly reduced (Figure 4B). RSV gene expression after 24 h of infection was also measured, demonstrating significantly reduced levels of viral gene expression in CD11c-dnRARα BMDCs, consistent with our *in vivo* data.

Further to confirm that reduction in RA signaling in DCs leads to enhanced interferon production we infected WT BMDCs with RSV in media containing physiological levels of RA. As controls, BMDCs were infected in the presence of increasing concentrations of RA alone or in combination with a RARα inhibitor. RA or its inhibitor alone did not alter IFN-β production by WT BMDCs (data not shown). In contrast, there was a significant increase in IFN-β production by WT BMDCs after RSV infection under physiological RA conditions, which appeared to be RA concentration dependent. The addition of a RARα inhibitor to infected BMDCs cultured in the presence of exogenous added RA (10, 50, or 100 nM) showed a significant enhancement in IFN-β production (Figure 4D). These datasets suggest that inhibiting RA signaling in DCs leads to enhanced production of IFN-β in response to RSV infection.

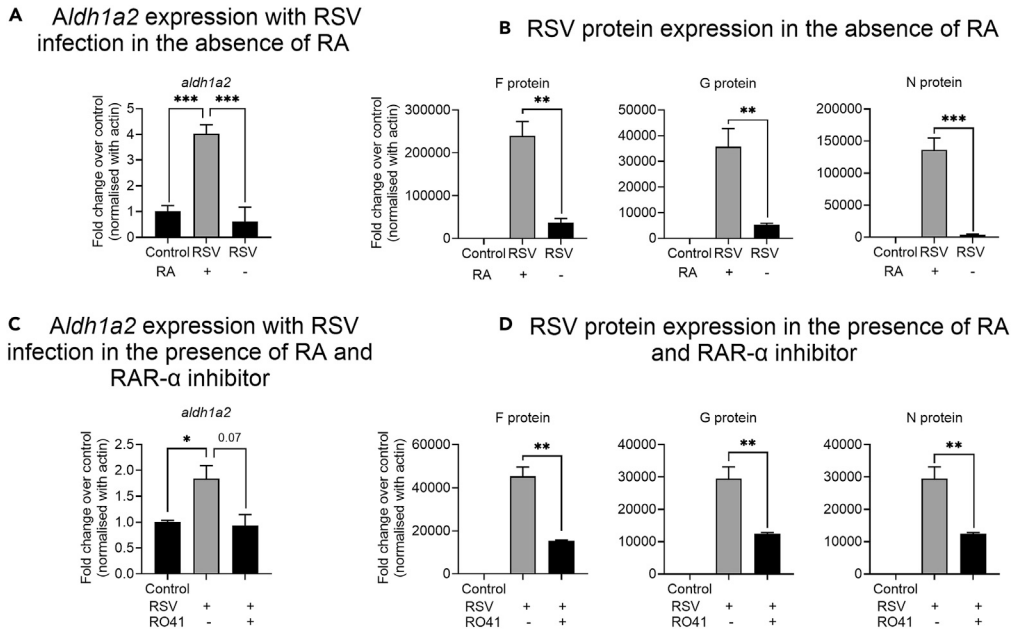

Figure 3. RA signaling in DCs enhances RSV gene expression

DCs from WT animals were infected with RSV in the presence or absence of RA.

(A and B) RA activity was measured by *aldh1a2* expression in DCs. RSV F, G, and N proteins expression was quantified using qPCR after 24 h.

(C and D) In another set of experiments, DCs were infected with RSV in the presence of RA, either with or without the RARα inhibitor (Ro41-5253). The addition of the inhibitor reduced the expression of *aldh1a2* and reduced viral protein production. *Aldh1a2* as well as RSV F, G, and N proteins expression were analyzed with qPCR. Data presented here are from experimental technical replicates, $n = 3$, mean ± SD. Statistical analysis was performed with Prism GraphPad paired t test and significance is shown as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$.

RA signaling in pDCs but not alveolar macrophages regulates innate immunity to RSV

In the CD11c-dnRARα transgenic mice, all CD11c expressing cells have inhibited RARα signaling. Since this includes alveolar macrophages (AMs) as well as pDCs and both have been implicated as important to the immune response to RSV, we wanted to identify which cell population(s) were important in this model. First, we investigated the total numbers of bronchoalveolar lavage (BAL) AMs and splenic pDCs to characterize two cell populations from CD11c-dnRARα mice. In comparison to WT, CD11c-dnRARα mice had a significantly reduced number of AMs while pDCs population numbers were comparable (Figure S5). Further, we isolated AMs, pDCs as well as cDCs from WT and CD11c-dnRARα animals and infected them with the RSV for 24 h. BAL AMs, stained for $CD45^+$ $CD11c^+$ Siglec F^{high} were documented to be >95% pure (Figure S6A). pDCs were identified as live $CD11c^+$MHC $II^+B220^+CD317^+$, while total cDCs were identified as live $CD11c^+$ MHC II^+ cells (Figure S6B). We examined the production of interferons and other pro-inflammatory cytokines as well as RSV protein expression following RSV infection. Though AMs isolated from CD11c-dnRARα mice showed comparative production of IFN-α, IFN-β, IFN-λ2/3, and IL12p40 with RSV stimulation, there was a decrease in TNF-α and IL-6 (Figure 5A). We did not observe any difference in RSV protein expression between the WT and CD11c-dnRARα AMs (Figure 5B). In contrast, pDCs showed significant increases in IFN-β, IFN-λ2/3, and IL12p40 while TNF-α and IL-6 were significantly reduced (Figure 5C). We did not observe detectable IFN-α in RSV stimulated pDCs. CD11c-dnRARα pDCs also showed significantly reduced expression of RSV proteins compared to WT pDCs (Figure 5D). These data were in concurrence with our BMDC data from CD11c-dnRARα mice. Although cDCs produced lower levels of IFN-β, IL12p40, TNF-α, and IL-6, they followed the same trend as CD11c-dnRARα pDCs. cDCs also showed reduced expression of viral proteins (Figure S7). Therefore, pDCs from CD11c-dnRARα animals accounted for the increase in type I interferon production.

DCs with deficient RA signaling have enhanced anti-viral response to RSV

To further characterize the role of RA signaling in inducing antiviral immunity, the total transcriptomes of *in vitro* RSV-infected BMDC cultures derived from WT and CD11c-dnRARα animals were examined 24 h after *in vitro* infection. We found a total of 996 significantly differentially expressed genes in CD11c-dnRARα BMDCs compared to WT (Figure 6A). We performed gene set enrichment analysis to examine the major antiviral pathways. We found upregulation of genes associated with innate and adaptive immunity pathways in CD11c-dnRARα BMDCs (Figures 6B and 6C). Among differentially expressed innate pathway associated genes, an increase in Il12b, STAT4, ifitm, and Clec4a (DCIR-a type lectin receptor) expression was found (Figure 6B). These genes have been shown to regulate the production of innate cytokines and the resolution of inflammation in RSV infection.[30–32] Among differentially expressed genes belonging to the adaptive pathway, significant increases in the expression of genes responsible for MHC class II antigen processing (H2-Eb2, H2Oa, H2Dmb2, H2-Eb1, H2-DMb1, H2Ab1, H2Aa, and Serpinb9) were observed (Figure 6C). MHC class II presentation of viral peptides is crucial for effective adaptive immunity and

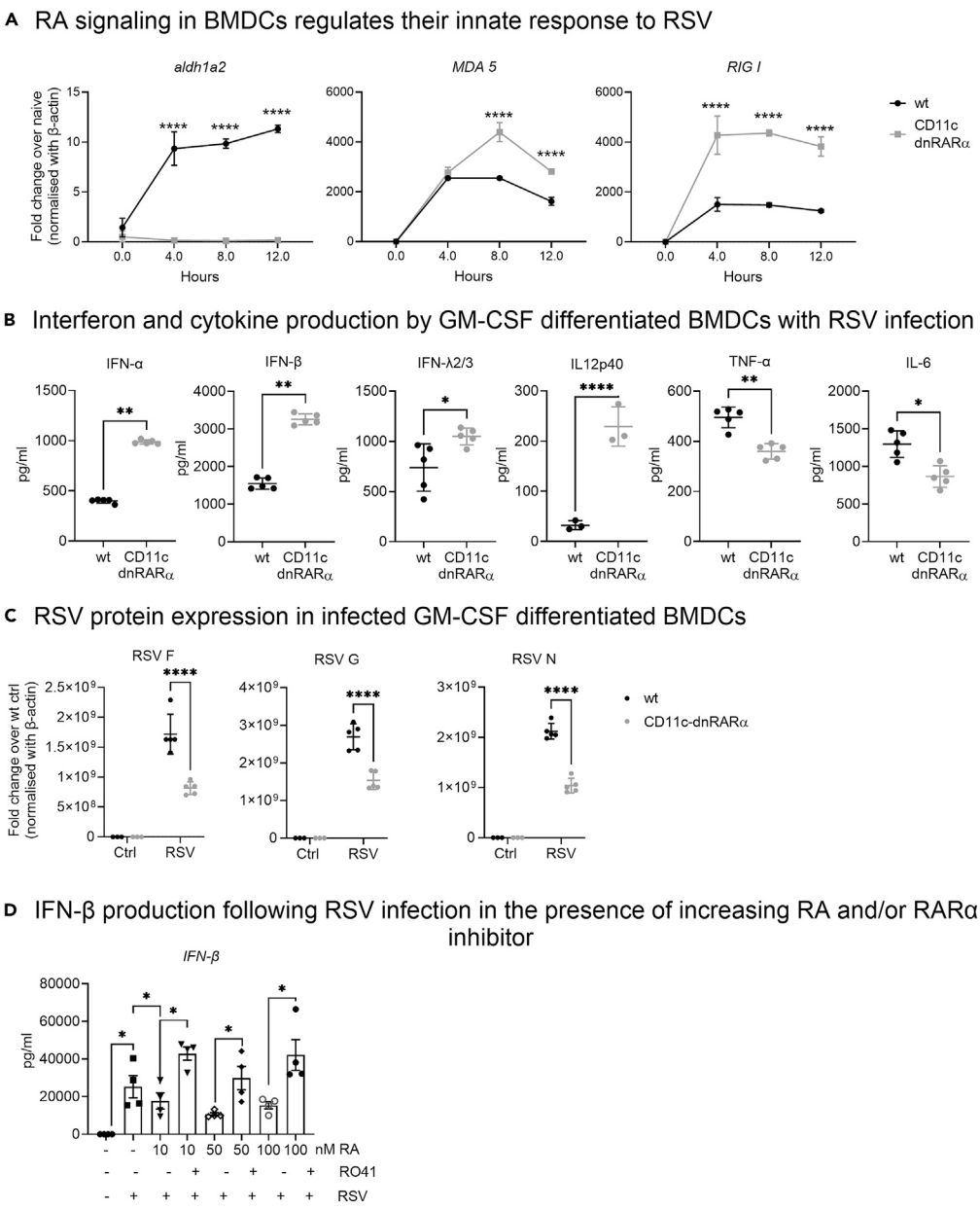

Figure 4. RA signaling in DCs regulates innate immunity to RSV infection

(A) CD11c-dnRARα BMDCs show greater expression of the innate immune-associated genes *aldh1a2*, MDA 5 ad RIG-I after RSV infection. Genes expression was quantified with qPCR at 0, 4, 8, and 12 h time points.

(B) Production of interferons and other cytokines after RSV infection (1:1 MOI) for 24 h.

(C) Decreased RSV protein expression in RSV infected CD11c-dnRARα BMDCs 24 h post stimulation.

(D) Dose dependent effect in IFN-β production from RSV infected wt BMDCs cultured with increasing concentration of RA (10, 50, and 100 nM) in combination with RARα inhibitor (RO41-5253-500 nM). Representative data of two independent experiments performed in triplicates, mean ± SD. Statistical analysis was performed using GraphPad Prism and significance is shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

clearance of pathogens.[33,34] Other genes that were enhanced (TRIM27, IL18rap, and Batf) are important in epithelial repair, reducing inflammation, and dictating DC migration following viral infections.[19,35–37] Downregulation was also observed for several genes associated with inflammation, virus entry, and replication pathways in CD11c-dnRARα BMDCs upon RSV stimulation. Among downregulated genes related to inflammation pathways were chemokines (Cxcl1, Cxcl2, and Cxcl3) and chemokine receptors (Ccr1, Ccr2, and Ccr5). Cxcl1, Cxcl2, and Cxcl3 induce chemotaxis of neutrophils, monocytes, and lymphocytes after viral infection and cause pulmonary inflammation[38–40] while monocytic expression of Ccr1, Ccr2, and Ccr5 has been shown to correlate with cellular inflammation and disease severity.[41,42] Lastly, viral entry and

replication pathways were examined, and data indicated downregulation in several genes that have been associated with the entry and replication of many viruses (Figure 6E) (TRIM25, cav1, oas2, oasl1, dicer1, and PTX3).[43–48] We also compared the basal gene expression of these pathways in untreated WT and CD11c-dnRARα BMDCs (Figure S8). We found, in comparison to WT, CD11c-dnRARα DCs have higher expression of genes related to innate and adaptive pathways while inflammatory pathway was overall down. These transcriptome datasets suggest that loss of RA signaling in DCs enhances their innate and adaptive immunity while suppressing inflammation and RSV entry and replication.

RA signaling differentially regulates TLR-3 and RIG-I responses in DCs

During RSV infection, a protective antiviral immune response is dependent on DCs through interferon production and their interaction with naive T cells. Double-stranded RNA (dsRNA) is a primary component of RSV that stimulates DCs by TLR3 as well as activates RIG-I and MDA-5 pathways. Previously, we found that suppression of RA signaling in DCs leads to significant enhancement of IFN-β and IL12p40, while proinflammatory cytokines (IL-6 and TNF-α) were suppressed. Viruses utilize different TLRs and the RIG-I/MDA-5 pathway in DCs to initiate innate immunity through the production of type I interferon and other cytokines. We thus aimed to study the role of RA signaling in regulating the TLR-3 and RIG-I pathways. We used polyIC to activate TLR-3 mediated signaling, while for the RIG-I pathway, we used 3php-RNA, a selective agonist of RIG-I, and measured the secreted cytokines from BMDC culture. polyIC mimics the RNA of double-stranded RNA viruses and is commonly used as a TLR-3 agonist, while 3p-hpRNA is an *in vitro* transcribed synthetic analog based-off of the differential interfering RNA of influenza virus's negative stranded RNA and was applied to study RIG-I pathway. Interestingly, both polyIC, as well as 3php-RNA stimulation, induced higher levels of IFN-β and TNF-α in CD11c-dnRARα DCs as compared to WT (Figures 7A and 7B). IL-1β and IL-6 were both significantly lower with 3php-RNA stimulation in the CD11c-dnRARα DCs as compared to WT, while these were reduced non-significantly with polyIC stimulation (Figures 7A and 7B). While IL12p40 production was significantly lower with 3php-RNA stimulation, it showed a significant increase with polyIC treatment in CD11c-dnRARα DCs as compared to WT. Taken together these data suggest that the RA signaling in DCs regulates the TLR-3 and RIG-I pathways resulting in differential type I interferon and cytokine production.

DISCUSSION

RSV is a single-stranded RNA virus that causes lower respiratory tract infection and serious morbidity and mortality in infants, immunocompromised individuals, and the elderly.[49] Many attempts have been made to develop an RSV vaccine, but until recently, candidates have failed to induce protective immunity, and some have enhanced the disease.[50–52] While recent vaccines have shown efficacy in adults,[53] future studies will examine whether additional immune modulation and adjuvant capability will be required in infants and young children where previous RSV vaccines have failed. Therefore, a better understanding of the mechanisms underlying immunity to RSV is important for the development of both treatments and vaccines. These studies have examined the potential role of retinoic acid (RA) signaling in DCs, critical innate immune cells central to regulating immune responses and pathogenic outcomes in RSV infection.

DCs are professional antigen-presenting cells that respond to infectious agents and activate innate immunity through cytokine and chemokine production. During RSV infection DCs respond to infection and process viral peptides, migrate to lymph nodes, and present antigens to naive T cells.[13,54,55] RA is a critical component of the mucosal environment as it controls local immune cells homeostasis.[26] RA has been shown to regulate DCs development and its systemic deficiency leads to altered DC subsets development in both a vitamin A diet deficient rodent model as well as in CD11c-dnRARα mice.[26,56] Among different immune cells, $CD103^+$ DCs are the main producers of RA from vitamin A.[21] Since RA plays a central role in innate and adaptive immunity on mucosal surfaces, we studied its specific role in DCs in RSV immunity.

Most prior work has suggested that RA plays a positive role in driving antiviral immunity, but some reports had conflicting findings. Besides retinoic acid receptors, IRF-1 binding to IRG-I receptor as well as IFN-γ have been shown to upregulate its expression and increase IFN-β production.[57–59] Other support for immune enhancement by RA included the finding that RA signaling stabilizes Th1 cells and blocks the conversion of T cells into Th17 cells.[60] A recent study showed that systemic deficiency of vitamin A led to enhanced production of IFN-γ by $CD4^+$ T cells while another reported dietary supplementation of vitamin A downregulates IFN-γ by $CD4^+$ T cells.[61,62] Thus, the overall role that RA plays in mucosal viral infection remains unclear.

Our previous studies on mucosal immunization of CD11c-dnRARα animals showed RA signaling is required for TLR-2 and 4 activations of DCs and effector T cell induction. However, these animals still produced antigen-specific antibody responses. Although CD11c-dnRARα mice had reduced basal numbers of splenic CD4 and CD8 subsets, the number of activated cells (CD4 CD44, CD4 CD 69, CD8 CD44, and CD8 CD69) was comparable. Additionally, we observed a reduced number of naive T cells in these animals (CD4 CD62L and CD8 CD62L) (Figure S9). To further functionally characterize T cells in these animals, we directly stimulated the T cells and found no difference in their activation or cytokine production compared to the T cells isolated from WT animals.[63] To study the response to mucosal viral pathogen, we infected CD11c-dnRARα animals with RSV. The surprising finding from these studies was that CD11c-dnRARα animals that are deficient in RA signaling had a less severe infection after RSV infection than control WT littermates with intact RA signaling. Also, RSV-infected CD11c-dnRARα animals had minimal immune cell infiltration and a reduction in mucus-producing goblet cells in their lungs, despite no observed difference in PMNs infiltration in these animals. In RSV infection, an imbalance between Th1 and Th2 immunity has been well studied and a predominant Th2 immune response has been associated with severe infection, pathogenicity, and poor clinical outcomes.[64–66] Studies on monocyte and macrophage-derived RA have suggested that RA induces cytokine secretion that favors Th2 cell development and inhibits those which promote Th1 responses.[67] Similar observations were made by Iwata et al. while elucidating the direct effect of RA on T cell polarization.[68] In previous reports, different retinoids (retinol, 9-cis, and all-trans-RA) have been shown to regulate DC maturation, but there are no data on DC-derived RA and its effect on T cell polarization.[69] In our work, we found that animals deficient in RA signaling in their DCs had increased

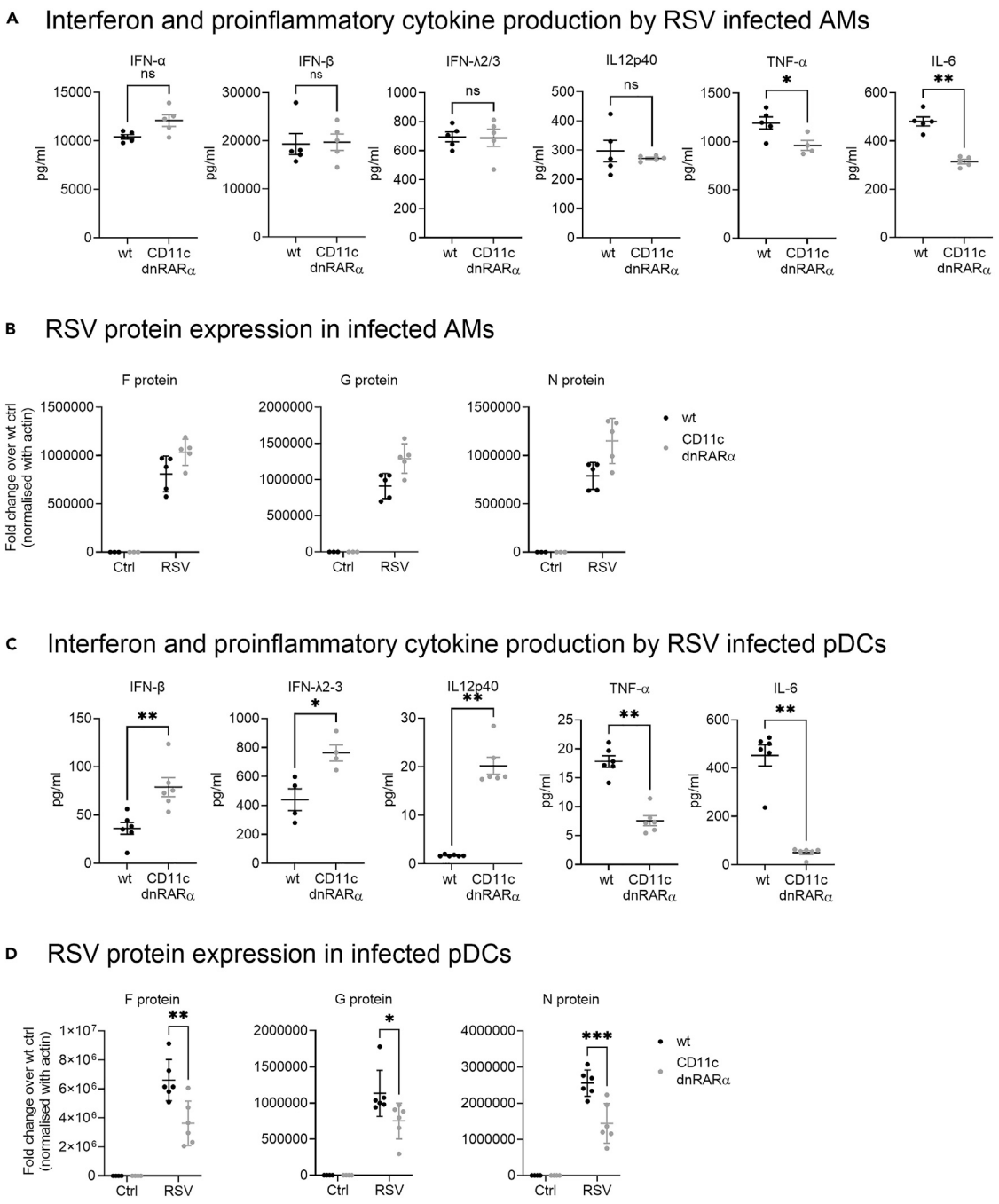

Figure 5. RA signaling in pDCs but not alveolar macrophages regulates innate immunity to RSV

BAL AMs and splenic pDCs were sorted as described in the STAR Methods. 10^5-2 × 10^5 cells were infected/stimulated with RSV. Interferons and other cytokines were measured in the supernatants of the cells 24 h post infection. Interferons (IFN-α, IFN-β, and IFN-λ2-3) and proinflammatory cytokines (TNF-α and IL-6) production by RSV infected AMs (A) and pDCs (C). RSV gene expression levels were determined by lysing the cells with TriZol and extracting the RNA from infected cells and analyzed with qPCR. Expression data are normalized with β-actin and fold changes were calculated over the respective uninfected controls. RSV proteins expressed from infected AMs (B) and pDCs (D) 24 h post infection. Ctrl represents untreated samples. Representative data of two independent experiments with the cells pooled from 5 to 6 mice each time, mean ± SD. Statistical analysis was performed using GraphPad Prism and significance is shown as *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

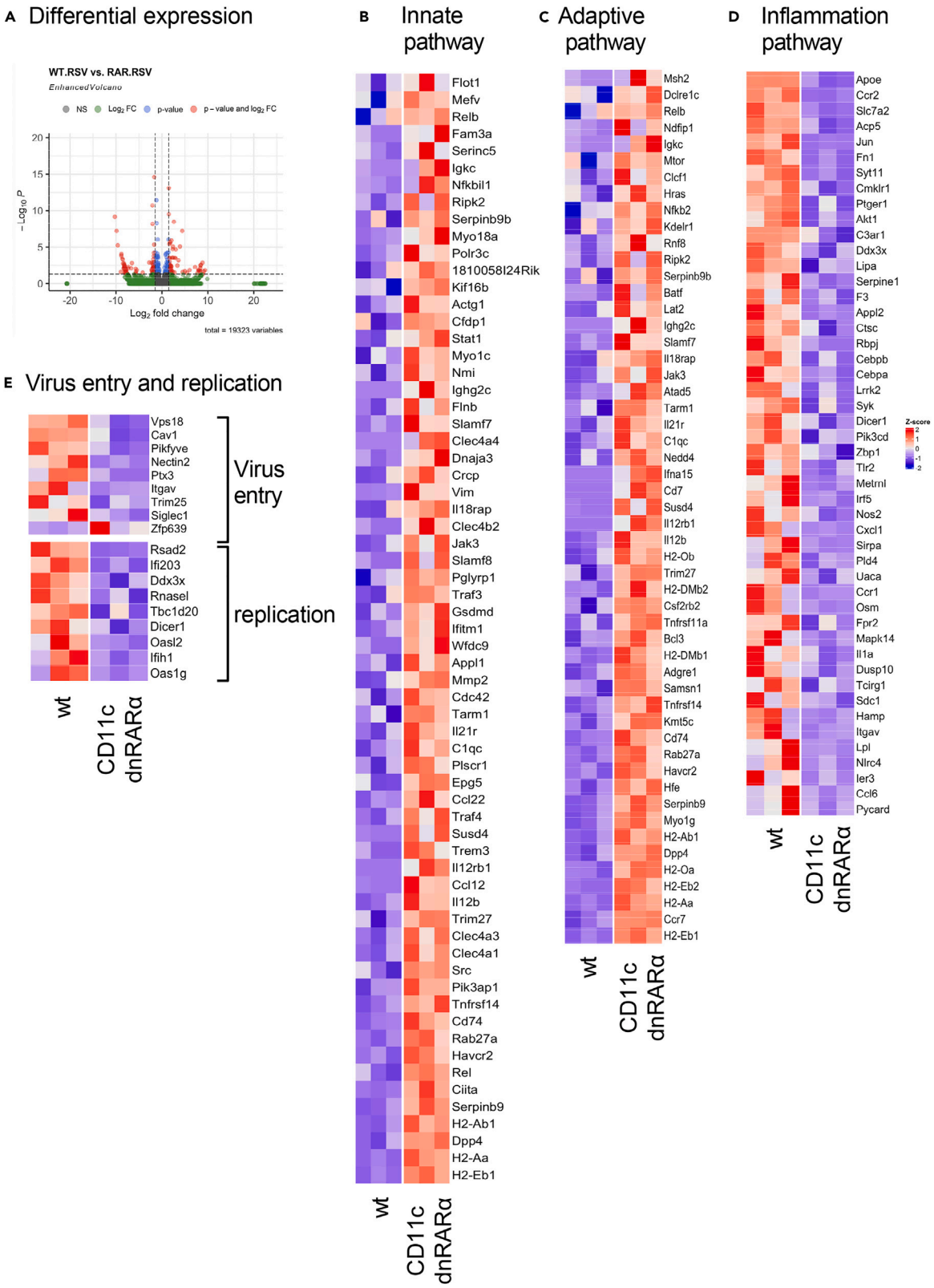
A Differential expression
WT.RSV vs. RAR.RSV
EnhancedVolcano
NS
Log2 FC
p-value
p – value and log2 FC
−Log10 P
Log2 fold change
total = 19323 variables
B Innate pathway
Flot1
Mefv
Relb
Fam3a
Serinc5
Igkc
Nfkbil1
Ripk2
Serpinb9b
Myo18a
Polr3c
1810058I24Rik
Kif16b
Actg1
Cfdp1
Stat1
Myo1c
Nmi
Ighg2c
Flnb
Slamf7
Clec4a4
Dnaja3
Crcp
Vim
Il18rap
Clec4b2
Jak3
Slamf8
Pglyrp1
Traf3
Gsdmd
Ifitm1
Wfdc9
Appl1
Mmp2
Cdc42
Tarm1
Il21r
C1qc
Plscr1
Epg5
Ccl22
Traf4
Susd4
Trem3
Il12rb1
Ccl12
Il12b
Trim27
Clec4a3
Clec4a1
Src
Pik3ap1
Tnfrsf14
Cd74
Rab27a
Havcr2
Rel
Ciita
Serpinb9
H2-Ab1
Dpp4
H2-Aa
H2-Eb1
wt
CD11c dnRARα
C Adaptive pathway
Msh2
Dclre1c
Relb
Ndfip1
Igkc
Mtor
Clcf1
Hras
Nfkb2
Kdelr1
Rnf8
Ripk2
Serpinb9b
Batf
Lat2
Ighg2c
Slamf7
Il18rap
Jak3
Atad5
Tarm1
Il21r
C1qc
Nedd4
Ifna15
Cd7
Susd4
Il12rb1
Il12b
H2-Ob
Trim27
H2-DMb2
Csf2rb2
Tnfrsf11a
Bcl3
H2-DMb1
Adgre1
Samsn1
Tnfrsf14
Kmt5c
Cd74
Rab27a
Havcr2
Hfe
Serpinb9
Myo1g
H2-Ab1
Dpp4
H2-Oa
H2-Eb2
H2-Aa
Ccr7
H2-Eb1
wt
CD11c dnRARα
D Inflammation pathway
Apoe
Ccr2
Slc7a2
Acp5
Jun
Fn1
Syt11
Cmklr1
Ptger1
Akt1
C3ar1
Ddx3x
Lipa
Serpine1
F3
Appl2
Ctsc
Rbpj
Cebpb
Cebpa
Lrrk2
Syk
Dicer1
Pik3cd
Zbp1
Tlr2
Metrnl
Irf5
Nos2
Cxcl1
Sirpa
Pld4
Uaca
Ccr1
Osm
Fpr2
Mapk14
Il1a
Dusp10
Tcirg1
Sdc1
Hamp
Itgav
Lpl
Nlrc4
Ier3
Ccl6
Pycard
Z-score
wt
CD11c dnRARα
E Virus entry and replication
Vps18
Cav1
Pikfyve
Nectin2
Ptx3
Itgav
Trim25
Siglec1
Zfp639
Virus entry
Rsad2
Ifi203
Ddx3x
Rnasel
Tbc1d20
Dicer1
Oasl2
Ifih1
Oas1g
replication
wt
CD11c dnRARα

Figure 6. DCs with deficient RA signaling have enhanced antiviral response to RSV

BMDCs cultured from WT and CD11c-dnRARα animals were infected with RSV for 24 h and RNA sequencing was performed on total RNA. (A) Differentially expressed genes between WT and CD11c-dnRARα BMDCs. Specific pathways were analyzed in CD11c-dnRARα BMDCs in comparison to BMDCs from WT animals. Heatmaps show genes associated with innate immunity (B), adaptive immunity (C), inflammatory pathway (D), and virus entry and replication (E). Data presented here are from experimental technical replicates, $n = 3$.

Th1 (TNF-α and IFN-γ) and suppressed Th2 cytokines (IL-4, IL-5, and IL-13) following RSV infection. This suggested that the improved outcome observed in these animals was due to polarization toward Th1 over Th2 immune responses. In contrast, no differences in IL-17A production between RSV-infected CD11c-dnRARα and WT animals were observed. IL-17A has been suggested to contribute to RSV-associated pathogenicity by increasing mucus secretion, reducing viral clearance, and exacerbating airway hypersensitivity.[70] Our data suggest that IL-17A is not associated with RSV pathogenicity in the absence of Th2 cytokines.

Different DC subsets express distinct TLRs and RLRs repertoires, contributing to their activation and functional specialization. DCs sense single and double strands of viral RNA and signal either through TLR-3/7/8 in the endosome or through RIG-I/MDA-5 in the cytoplasm. This activates interferon-stimulating genes (ISGs) leading to interferon secretion and NF-κB activation, resulting in pro-inflammatory cytokine production.[4,71] In the case of RSV, it triggers RLRs (RIG-I) and TLRs (TLR-2, 3, 4, and 7) leading to DC maturation and cytokine production.[6,7,72] IRF3 activation in DCs amplifies type I interferons and IL12p40 and regulates T cell priming.[73] Our findings indicated that RA signaling in WT BMDCs was associated with increased RSV gene expression, as it was suppressed in CD11c-dnRARα animals. To verify this, we used a specific inhibitor of RA signaling *in vitro* and this significantly blocked RSV gene expression in conjunction with the inhibition of RA mediated *aldh1a2* transcription. Our data showed a significant increase in *aldh1a2* over time upon RSV infection in WT BMDCs, while this signaling was inhibited in CD11c-dnRARα BMDCs. Thus, it appears that RA signaling in DCs enhances RSV infection.

Furthermore, BMDCs deficient in RA signaling showed significantly increased MDA-5 and RIG-I gene expression over time with RSV infection. The cytokine profile from *in vitro* RSV-stimulated BMDCs showed significantly enhanced production of interferons and IL12p40 while IL-6 and TNF-α were suppressed. Most of the published research has shown that retinoids enhance the antiviral response by upregulating ISGs and increasing the production of type I interferons.[74] However, our data indicate that RA signaling-deficient DCs had significantly higher interferons but decreased IL-6 and TNF-α production. In RSV infection, type I interferons are mainly produced by AMs and pDCs.[7,75,76] When these studies examined interferons and proinflammatory cytokines with RSV stimulation, an increase in interferons in CD11c-dnRARα pDCs and cDCs was observed, while AMs produced comparable levels to WT. Due to the presence of high levels of TLR7 and TLR9 on lung pDCs, they account for most of the interferons produced in response to RSV infection.[77–79] This could be the possible reason why we see an increase in interferons from pDCs in CD11c-dnRARα mice. Since type I Interferon production has been associated with a reduction in RSV-induced immunopathology.[80,81] The greater production of interferon and IL12p40 by the transgenic animal DCs might be involved in the modulation of T cell immunity toward Th1 since these molecules enhance the expression of co-stimulatory molecules promoting DC maturation, antigen uptake, and their MHC II presentation resulting in Th1 polarization.[82–85] Our data showed that following RSV infection, DC subsets ($CD11b^+$ and $CD103^+$) increased significantly in CD11c-dnRARα compared to the littermates. Furthermore, DC ($CD11b^+$ and $CD103^+$) subsets from the CD11c-dnRARα animals had a significant increase in CD40. RNA sequencing data from RSV infected BMDCs from CD11c-dnRARα mice also showed significant enhancement of antigen processing and presentation pathways. Total transcriptome analysis of RSV infected CD11c-dnRARα BMDCs showed significant enhancement of innate and adaptive immunity associated genes while inflammatory as well as virus entry and replication pathways were suppressed. While it is possible that the dnRARα transgenic expression may have non-specific targets, it has not been observed in this or previous studies using the mice.[56,63,86] Altogether these findings suggest that following RSV infection RA deficiency in the DCs enhances innate immunity leading to increased type I interferon production that promoted cDC maturation and their antigen presentation resulting in Th1 polarization in these animals.

Next, we stimulated RA-signaling deficient DCs with 3php-RNA for RIG-I pathway activation and with polyIC for TLR-3. We found a similar response to both ligands in the RA signaling deficient DCs: increased IFN-β and TNF-α and downregulated IL-6 and IL-1 β. However, IL12p40 was suppressed with 3php-RNA stimulation while it was enhanced with pIC. Upon activation different TLRs couple with distinct TIR-domain signaling adapter proteins (MYD88, TRIF, TRAP, and TRIM) to activate IRF3 or NF-κB pathway. Differences in cytokine profile toward RIG-I versus TLR-3 ligands suggest that RA signaling in DCs regulates these pathways differentially and may respond variably to different viruses.[87,88] Differences in the cytokine profile between RSV and 3php-RNA/polyIC stimulated DCs are likely because RSV activates RIG-I as well as other TLR ligands, and there is significant crosstalk between these pathways.

Taken together, these studies demonstrated that inhibiting RA signaling in DCs suppresses RSV replication and results in reduced lung inflammation. In RSV infection the absence of RA signaling in DCs leads to increased production of type I interferons as well as IL12p40 while decreasing IL-6 and TNF-α, enhancing the Th1 phenotype of the immune response. This suggests that the produced type I interferons skew specific immune responses, resulting in suppressed RSV replication and improved lung pathology. Thus, inhibition of RA signaling in innate cells could be explored in vaccine and therapeutic approaches for RSV infection. This phenomenon should also be evaluated in other respiratory viruses that activate similar immune pathways during infection.

Limitations of the study

Our results indicate that RA signaling deficiency in pDC protects against severe RSV infection. This is associated with increased interferon and IL12p40 production, which appears to be the result of enhanced Th1 polarization and suppression of Th2 immunity. The major limitation of this study is the use of the transgenic mice. These mice had their immune systems develop in the absence of RA signaling in dendritic cells,

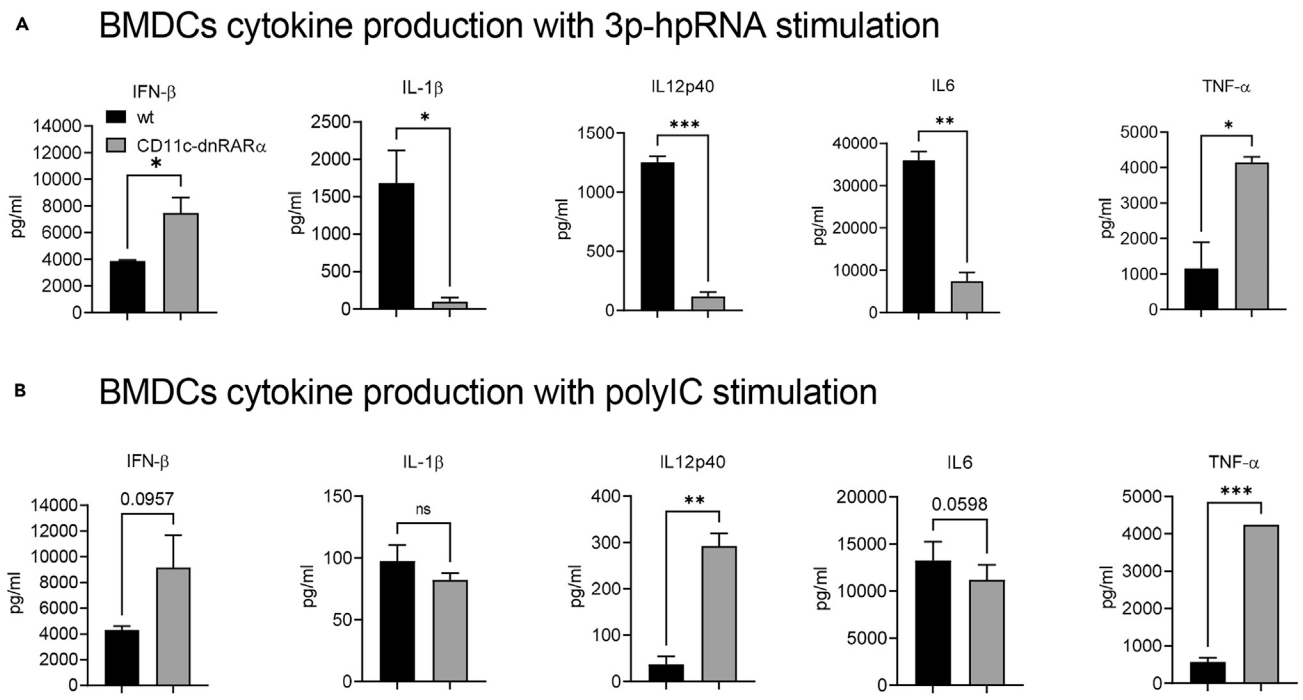

Figure 7. RA signaling differentially regulate TLR-3 and RIG-I responses in DCs

(A) 3p-hpRNA (500 ng/ml) was transfected into DCs using lipofectamine 3000 and cultured for 24 h or (B) stimulated with polyIC (1 μg/mL) for 24 h. Post incubation, cells were centrifuged, and the supernatant was collected to analyze for secreted cytokine. Representative data are from 3 independent experiments performed in technical triplicates presented here as mean ± SD (n = 3). Statistical analysis was performed using GraphPad Prism and significance is shown as *p, 0.05, **p, 0.01, ***p, 0.001, and #p, 0.0001.

which could have resulted in subtle changes not reflective of normal murine or human immunity. These studies also have not determined the exact mechanism by which this signaling abnormality creates a protective phenotype. Experiments using more specific means of RA signaling inhibition would clarify this process. Finally, studies using retinoic acid signaling inhibitors in wild-type mice could determine the therapeutic application of these findings in the prevention of severe RSV infection.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2024.110103.

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AUTHOR CONTRIBUTIONS

M.F. and J.R.B. conceptualized the studies in this manuscript. C.H.K. and N.W.L. provided reagents and gave input on experimental design. M.F., G.A., J.Z., P.T.W., J.J.O., A.J.R., and S.M. performed experiments and data analysis. M.F. and J.R.B. wrote the manuscript with input from P.T.W., J.J.O., N.W.L., and C.H.K.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PerCP anti-mouse CD45 Antibody	Biolegend	Cat:103130 RRID:B_893339
Brilliant Violet 605™ anti-mouse CD11c Antibody	Biolegend	Cat#117334 RRID:AB_11204262
PE/Cyanine7 anti-mouse I-A/I-E Antibody	Biolegend	cat:107630 RRID:AB_2290801
APC anti-mouse CD103 Antibody	Biolegend	Cat:121414 RRID:AB_1227503
APC/Cyanine7 anti-mouse/human CD11b Antibody	Biolegend	Cat:101226 RRID:AB_830642
Brilliant Violet 510™ anti-mouse CD80 Antibody	Biolegend	Cat:104741 RRID:AB_2810337
BV650 Rat Anti-Mouse Siglec-F	BDBiosciences	Cat: 740557 RRID:AB_2740258
FITC anti-mouse CD317 (BST2, PDCA-1) Antibody	Biolegend	Cat 127008 RRID:AB_2028462
PerCP/Cyanine5.5 anti-mouse/human CD45R/B220 Antibody	Biolegend	Cat:103236 RRID:AB_893354
PE anti-mouse CD4 Antibody	Biolegend	Cat: 100408 RRID:AB_312692
APC anti-mouse CD8b Monoclonal Antibody	Thermofisher	Cat:17-0083-81 RRID: AB_657760
APC/Cyanine7 anti-mouse/human CD44 Antibody	Biolegend	Cat: 103028 RRID: AB_830785
PerCP anti-mouse CD62L (L-Selectin) Monoclonal Antibody	Thermofisher	Cat:45-0621-82 RRID:AB_996667
PE-Cy7 Hamster Anti-Mouse CD69	BD Pharmingen	Cat: 552879 RRID: AB_394508
Recombinant Murine GM-CSF	Peprotech	Cat: 315-03
Chemicals, Peptides, and Recombinant Proteins		
HyClone RPMI 1640 media	Thermofisher	Cat: SH30096
MEM Non-Essential Amino Acids Solution (100X)	Thermofisher	Cat: 11140050
Penicillin-Streptomycin (10,000 U/mL)	Thermofisher	Cat: 15140148
L-Glutamine (200 mM)	Thermofisher	Cat: 25030081
2-Mercaptoethanol (50 mM)	Thermofisher	Cat: 31350010
UltraPure™ 0.5M EDTA	Thermofisher	Cat: 15575020
Critical commercial assays		
IFN-α	PBL Bioassay	Cat: 42120
IFN-β	R&D	Cat: DY8234-05
IFN-2-3	R&D	Cat: DY1789B
TNF-α	R&D	Cat: DY410

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
IL-6	R&D	Cat: DY406
Multiplex assay	EMD-Millipore	Cat: MTHMAG-47K
Direct-zol RNA Microprep kit and TRI Reagent	Zymoresearch	Cat: R2063
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems	Cat: 4368814
Power SYBR™ Green PCR Master Mix	Applied Biosystems	Cat: 4368702
Experimental model: Cell lines		
HEp2	ATCC	Cat: CCL-23

RESOURCE AVAILABILITY

Lead contact

Further information and request for resources should be directed to Dr. James R Baker Jr at jrbakerjr@med.umich.edu.

Materials availability

All materials and methodologies communicated in this study are available from the lead contact with a completed Materials Transfer Agreement.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Mice

All studies involving the use of animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Michigan and were performed by these guidelines. Chang H Kim provided the CD11c-dnRARα animals. To abolish specific RA signaling in DCs, we cross-bred mice expressing dominant negative RAR-α (RAR403) with CD11c-CRE mice to generate CD11c-dnRARα mice.[26] Littermates lacking the insertion of RAR403 were used as wild-type (wt) controls in the studies. In the studies, male and female mice were used at the age of 6–8 weeks. Animals were maintained in specific pathogen free conditions.

METHOD DETAILS

RSV infection studies

A chimeric RSVA2 strain with recombinant Line19 fusion protein was used for all experiments as previously described.[89] RSV strain A2 and line 19 differ in their clinical outcome and disease model.[89,90] RSV A2 line 19 variant clinical manifestation and symptoms (i.e., mucus production, lymphocyte activation, and inflammation) peak at day 8–10 in this model of disease.[89–91] CD11c-dnRARα and littermate control mice were infected intratracheally (50μL/animal) with 3×10^5 PFU of RSV A2/L19-F. Analysis of the viral response was performed on day 8 post-infection.

Viral stocks were grown in Hep-2 cells and concentrations were determined by plaque assay. The virus was ultracentrifuged (100,000 × g for 30 min at 4°C) and resuspended in fresh complete RPMI 1640 culture media (RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, nonessential amino acids, sodium pyruvate, and 2-mercaptoethanol).

Lung histology

Lungs were removed from naive and RSV infected animals on day 8 post infection. The middle and inferior lobes of the right lung were harvested and perfused with 10% formalin for fixation and embedded in paraffin. Five micrometer lung sections were stained with periodic acid–Schiff (PAS) to detect mucus production and H&E (hematoxylin and eosin) staining to examine inflammatory infiltrates. Photomicrographs were captured using a Leica microsystem imager and application suite v3.1.0 software was used.

Flow cytometry

Single cell suspensions were prepared by digesting lungs with 1 mg/mL collagenase A (Roche) and 20 U/mL DNase I (Roche) in RPMI 1640 with 10% FCS for 60 min at 37°C. Digested tissue was passed through an 18-gauge needle in a 5 mL syringe to dissociate the matrix. RBCs were lysed and single cell suspensions were filtered through a 70 μm filter. Filtered cells were washed with PBS and cells were suspended in PBS. Fc

receptors were blocked using an anti-CD16/32 antibody. Cells were re-suspended in FACS buffer (0.1%BSA in PBS) containing antibodies and stained on ice for 20 min. The following antibodies were used to stain for DCs: CD11c-AF647 (N418), CD11b-APC-Cy7 (M1/70), MHC II-PE-Cy7 (M5/114.15.2), CD103, CD40-PE (1C10) and for live/dead staining Fixable Viability Dye eFluor 450 was used. All antibodies were purchased from either eBiosciences, BD Pharmingen, or BioLegend. Cells were washed with FACS buffer and events were acquired on a Novocyte 3000 flow cytometer (Acea Biosciences) and analyzed using FlowJo software (v10).

Lung-draining lymph node cells *ex vivo* stimulation and cytokine production assay

Lung-draining lymph nodes (LDLN) were isolated by enzymatic digestion using 1 mg/mL collagenase A (Roche) and 20 U/ml DNase I (Sigma-Aldrich) in RPMI 1640 with 10% FBS for 45 min at 37°C. Lymph nodes were further dispersed through an 18-gauge needle (1-mL syringe). RBCs were lysed, and samples were filtered through a 70-μm nylon mesh. Cells ($5x10^5$) from LDLN were plated in 96-well plates and restimulated with RSV L19 MOI 5:1 for 48 h. IFN-γ, TNF-α IL-4, IL-5, IL-13, and IL-17a levels were quantified in the supernatants with a Milliplex multiplex immunoassay kit (EMD Millipore).

BMDC culture

Bone marrow was harvested as previously described.[24] BMDCs were cultured for 10 days in RPMI-1640 containing 10% HI-FBS, 1× non-essential amino acids, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, GM-CSF (20 ng/mL), 100 units penicillin/mL and 100 μg/mL streptomycin. Bone marrow cells were counted and 2×10^6 cells were seeded in 10 mL of complete media in bacterial dishes. 10 mL of fresh media was added on day 3 of culture and hemi-depletion was performed on days 5, 7, and 9. On Day 10, BMDCs were analyzed for maturity by analyzing $CD11c^+CD11b^+$ cell surface expression. More than 90% of cells were double-positive for the expression of CD11c and CD11b.

Bronchoalveolar lavage (BAL) AM isolation

BAL AMs were isolated as per the protocol described elsewhere.[7] Briefly, BAL cells were collected by flushing the lungs 4 times with 0.8 mL sterile PBS supplemented with 5 mM EDTA (Lonza). BAL cells were pooled from 5 to 6 mice together and centrifuged at 2000 × rpm for 5 min. Cells were washed twice with sterile PBS and suspended in 1 mL PBS. To check the purity, cell suspension was stained with live-dead dye in combination with CD11c, CD45, and Siglec F antibodies. Live $CD11c^+$ $CD45^+$ Siglec F^+ was identified as AMs. The purity of AMs was >95% assessed by flow cytometry.

pDCs and cDCs sorting

pDCs and cDCs were sorted as per the published protocol.[92] Briefly, spleens were isolated and single cell suspensions were prepared as described elsewhere.[24] Total cells pooled from 5 mice were stained for live-dead dye in combination with antibodies for CD11c, MHC II, B220, and CD317. Live MHC II^+ $CD11c^+$ B220- cells were considered as total cDCs while live MHC II^+ $B220^+$ $CD11c^+$ $CD317^+$ were identified as pDCs. The BD FACS Melody system was used to sort these two cell populations.

qPCR

BMDCs were stimulated with RSV 1:1 MOI for different time points. At the end of incubation, cells were centrifuged and lysed with Trizol to extract RNA. RNA was isolated from BMDCs using the DirectZol RNA MiniPrep Plus (Zymo Research) according to the manufacturer's protocol. Isolated RNA was quantified using NanoDrop 1000 Spectrophotometer (Thermofisher Scientific) and cDNA was synthesized with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Power SYBR green PCR master mix (Applied Biosystems) was used for qPCR. Gene expression was calculated by ΔΔCt analysis and normalized to 18S or β-actin levels. Fold change was calculated over unstimulated wt samples. Primers were purchased from IDT technologies and sequences are provided in Table S1.

Analysis of secreted chemokines and cytokines in cultures

For cytokine analysis, BMDCs, AM, pDCs, and cDC (2.5×10^5) were stimulated with RSV (MOI = 1) for 24 h in a 96-well plate. Cells were centrifuged, and supernatants were collected to study secreted cytokines. IFN-α was measured with an ELISA kit from PBL Assay Science (catalog number 42120). IFN-β, IFN-λ2/3, TNF-α, and IL-6 were measured with R&D DuoSet ELISA kits (catalogs DY8234-05, DY1789B, DY410, and DY406 respectively). A Luminex Multiplex detection system (Millipore, Billerica, Mass) was also used to measure other cytokines from the collected cell culture supernatant.

RNA sequencing and analysis

Total RNA sequencing was performed on RNA samples extracted from RSV treated BMDCs. Briefly, RNA was extracted using DirectZol miniprep (Zymoresearch), and RNA purity was confirmed with NanoDrop (ND-1000 spectrophotometer, Thermofisher Scientific). Samples were examined for RNA integrity prior to sequencing. Library preparation and sequencing were accomplished using the NovaSeq PE150 sequencing platform and strategy. 35 million reads per sample were acquired to generate the data and for analysis differential gene expression was performed using the DESeq2 method using a negative binomial generalized linear model.[93] Annotation data from ENSEMBL 102 was used, and genes were additionally annotated with Entrez Gene IDs and text descriptions. Functional analysis, including candidate

pathways activated or inhibited in comparison, was performed using iPathway Guide (iPathwayGuide, Advaita).[94] Gene ontology enrichment analysis was performed using a previously published method.[95]

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using GraphPad (Prism). Results presented here are the representatives of at least two independent experiments. Data are expressed as mean ± SD. Paired t test was used to compare the two groups. In experiments comparing multiple groups, statistical differences were calculated by using Holm-Sidak/Tukey method for two-way ANOVA. *p* values of **<0.05*, ***<0.01*, ****<0.001* and *****<0.0001* were considered as significant.