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ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Inhibition of uric acid or IL-1β ameliorates respiratory syncytial virus immunopathology and development of asthma

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Abstract

Background: Respiratory syncytial virus (RSV) affects most infants early in life and is associated with increased asthma risk. The specific mechanism remains unknown.

Objective: To investigate the role of uric acid (UA) and IL-1β in RSV immunopathology and asthma predisposition.

Methods: Tracheal aspirates from human infants with and without RSV were collected and analyzed for pro-IL-1β mRNA and protein to establish a correlation in human disease. Neonatal mouse models of RSV were employed, wherein mice infected at 6-7 days of life were analyzed at 8 days postinfection, 5 weeks postinfection, or after a chronic cockroach allergen asthma model. A xanthine oxidase inhibitor or IL-1 receptor antagonist was administered during RSV infection.

Results: Human tracheal aspirates from RSV-infected infants showed elevated pro-IL-1β mRNA and protein. Inhibition of UA or IL-1β during neonatal murine RSV infection decreased mucus production, reduced cellular infiltrates to the lung (especially ILC2s), and decreased type 2 immune responses. Inhibition of either UA or IL-1β during RSV infection led to chronic reductions in pulmonary immune cell composition and reduced type 2 immune responses and reduced similar responses after challenge with cockroach antigen.

Conclusions: Inhibiting UA and IL-1β during RSV infection ameliorates RSV immunopathology, reduces the consequences of allergen-induced asthma, and presents new therapeutic targets to reduce early-life viral-induced asthma development.

KEYWORDS

allopurinol, asthma, interleukin-1 beta, respiratory syncytial virus, uric acid

Abbreviations: BAL, Bronchoalveolar lavage; CRA, Cockroach antigen; H/E, Hematoxylin and eosin; IACUC, Institutional Animal Care & Use Committee; IFN, Interferon; IL, Interleukin; IL-1RA, IL-1 receptor antagonist; ILC2(s), Type 2 innate lymphoid cells; LDLN, Lung draining lymph nodes; MOI, Multiplicity of infection; PAS, Periodic acid-Schiff; PFU, Plaque-forming units; PNU, Protein nitrogen units; RSV, Respiratory syncytial virus; SEM, Standard error of the mean; Th, T helper cell type; UA, Uric acid; XOI, Xanthine oxidase inhibitor.

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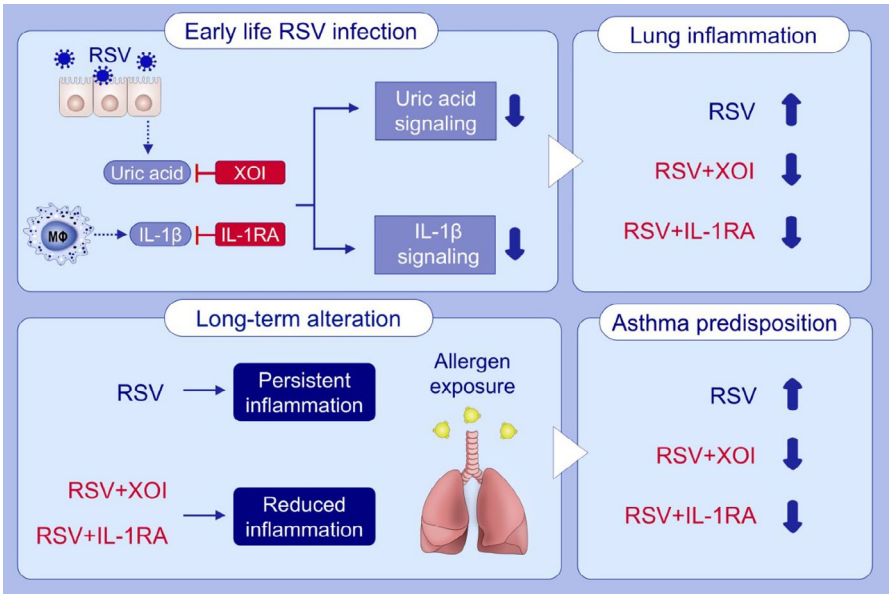

GRAPHICAL ABSTRACT

Neonatal RSV infection is associated with increases in pulmonary uric acid and IL-1β and lung immunopathology. XOI or IL-1RA administration during neonatal RSV infection leads to reduced RSV immunopathology. XOI or IL-1RA administration during neonatal RSV infection leads to reduced type 2 immune responses during a subsequent model of asthma.
Abbreviations: IL-1RA, IL-1 receptor antagonist; RSV: Respiratory syncytial virus; XOI, xanthine oxidase inhibitor.

1 | INTRODUCTION

Respiratory syncytial virus (RSV) can cause bronchiolitis and affects most infants before age two.[1,2] The global health burden of RSV includes over three million hospitalizations and ~100 000 deaths yearly among children under age five.[3] Severe RSV with bronchiolitis requiring hospitalization in infants is associated with an increased risk of childhood asthma.[4-6] RSV bronchiolitis involves airway epithelial loss, mucus overproduction, pulmonary inflammatory infiltrates, and pulmonary obstruction.[7,8] Severe RSV infection involves excessive Th2 and Th17 immune responses.[9-13] These responses persist even after viral clearance and are associated with enhanced type 2 immune responses in models of asthma induced later in life.[14] Type 2 innate lymphoid cells (ILC2s) are an important source of IL-13, which is associated with mucus production and goblet cell hyperplasia in the lung.[15-17] RSV induces IL-13-producing ILC2 accumulation, which is associated with disease severity.[7] IL-1β, a regulator of ILC2s,[18] has been described to be increased during RSV infection and may be directly involved in the pathogenesis.[19,20]

The precise connections between RSV induction of IL-1β, ILC2 activation, and RSV immunopathology remain unexplored. Uric acid production can induce reactive oxygen species production and thus activate the NLRP3 inflammasome, leading to IL-1β liberation.[21-23] Inflammasome-activating metabolic products including uric acid (UA) are associated with IL-1β production and subsequent childhood wheezing or bronchitis.[24] In this work, we investigate the roles of UA and IL-1β during neonatal RSV infection using human samples and mouse models. We demonstrate that interrupting the uric acid pathway using a xanthine oxidase inhibitor (XOI) or blocking the downstream inflammation with an IL-1 receptor antagonist (IL-1RA) can ameliorate RSV immunopathology. The protective effects of the XOI or IL-1RA during RSV persist and are each protective from subsequent cockroach allergen (CRA) induction of asthma exacerbation.

2 | MATERIALS AND METHODS

2.1 | Animals

The Institutional Animal Care & Use Committee (IACUC), University of Michigan, Ann Arbor, approved all animal use protocols, and all experiments proceeded according to IACUC guidelines. BALB/c mice 6-8 weeks old were purchased from Jackson Laboratory. These were bred 1:1 male:female to produce neonates. Each individual litter underwent a single, uniform treatment condition, and the multiple litters undergoing different treatment conditions were treated on the same dates. Treatment conditions were replicated across multiple mouse cohorts. Standard pathogen-free conditions were maintained in the Unit for Laboratory Animal Medicine at the University of Michigan.

2.2 | Patient samples

All human studies were performed in accordance with an approved University of Michigan Institutional Review Board protocol. Tracheal aspirate samples were obtained from RSV-infected infants hospitalized and mechanically ventilated in a pediatric intensive care unit; baseline clinical characteristics were collected from the medical

record and are summarized in Table S1. Infants' parents or legal guardians provided informed consent. The samples were directly aspirated from the endotracheal tube. RSV infection was detected by clinical sputum PCR for initial diagnosis; this was confirmed subsequently by PCR in the laboratory (see below). Infants intubated for other non-infectious reasons provided control samples. Samples were divided for protein and cDNA analysis. Protein samples were diluted 1:1 with PBS-containing complete anti-protease cocktail (Sigma-Aldrich) and 0.5% Triton X-100 nonionic detergent to dissociate mucus. TRIzol reagent was used for RNA extraction (Invitrogen). cDNA was synthesized using a murine leukemia virus reverse transcriptase (Applied Biosystems). Pro-IL-1β mRNA was analyzed via commercial TaqMan primers (Thermo Fisher Scientific). IL-1β and CCL5 protein were measured using a Bio-Plex 200 System (Bio-Rad Laboratories).

2.3 | RSV

RSV line 19, subgroup A, isolated from an infected infant at the University of Michigan Children's Hospital, was used for all experiments as previously described.[25] We have previously demonstrated animal models with this virus mimic human RSV with mucus hypersecretion and cytokine dysregulation.[26] Neonatal animals were infected with 1.8×10^5 plaque-forming units (PFU) via intranasal instillation.

2.4 | Primary RSV infection time course

Neonatal BALB/c mice were infected with RSV at 6-7 days old. Mice were sacrificed at days 2, 4, 6, 8, and 14 postinfection. Control mice were sham-infected with carrier fluid. Separate age-matched control groups were used at each time point. Lungs were flash-frozen for RNA evaluation. BAL was collected as below.

2.5 | Primary RSV infection

BALB/c mice were infected with RSV at 6-7 days old. Mice were treated daily with the relevant inhibitor starting on the day of infection for 7 days. At 4, 6, or 8 days postinfection, the mice were killed. Whole lungs and lung draining lymph nodes (LDLN) were isolated for flow cytometry, histology, PCR, and/or cytokine analysis (Figure S1). Control RSV-infected animals were treated with carrier fluid in these experiments. For experiments where the XOI and IL-1RA groups were both included, the IP carrier was fluid-treated, and the RSV-infected control group was used.

2.6 | Xanthine oxidase inhibitor treatment

The XOI, allopurinol, (Sigma-Aldrich) was administered at 25 μg per mouse (approximately 10 mg/kg) via intraperitoneal injection (volume 50 μL) daily starting on the day of RSV infection for seven doses. The dose was chosen based on prior dose-finding experiments (internal data) and known human dosing for allopurinol in children.[27-29]

2.7 | Interleukin-1 receptor antagonist

The IL-1RA (Cayman Chemical) was administered daily at 0.2 μg per mouse (approximately 0.08 mg/kg) via intranasal instillation (volume 5 μL) starting on the day of RSV infection for seven doses. This dose was chosen based on prior dose-finding experiments that suggested this dose was safe and potentially effective.

2.8 | Chronic RSV model

BALB/c mice were infected with RSV at days 6-7 of life. The mice were treated daily with either XOI or IL-1RA as above. After seven daily treatments, the mice rested 4 weeks. RSV-infected controls were treated with carrier fluid. The mice were euthanized, and whole lungs and LDLN were isolated for flow cytometry, histology, PCR, and/or cytokine analysis (Figure S1A).

2.9 | Cockroach antigen (CRA) model

BALB/c mice were infected with RSV as above at 6-7 days old. The mice were treated daily with either inhibitor as above. After seven treatments, the mice rested four additional weeks. The mice then began CRA sensitization and challenge as previously described.[14,30,31] Briefly, mice were sensitized with 500 protein nitrogen units (PNU) of CRA on days 0, 1, and 2 and challenged with 500 PNU on days 14, 20, 22, and 23. Clinical-grade CRA (as used in skin testing) was used for these experiments. On day 24, the mice were killed, and whole lungs and LDLN were isolated for flow cytometry, histology, PCR, and/or cytokine analysis (Figure S1B).

2.10 | Flow cytometry

Lungs were enzymatically dispersed with 1 mg/mL collagenase A (Roche) and 20 U/mL DNase I (Sigma) in RPMI with 10% FCS and further dispersed via 18-gauge needle (10-mL syringe). RBCs were lysed and samples filtered through 100-micron nylon mesh. Cells were re-suspended in PBS. LIVE/DEAD Stain Kit identified live cells (Thermo Fisher Scientific). Cells were washed and re-suspended in PBS with 1% FCS. Fc receptors were blocked with anti-CD16/32 (BioLegend). Surface markers were identified using the following clonal antibodies, all from BioLegend: anti-Gr-1 (RB6-8C5), B220 (RA3-6B2), CD3 (145-2C11), Ter119 (Ter-119), CD11b (M1/70), CD25 (PC61), CD45 (30-F11), ST2 (DIH9), c-Kit (2B8), CD90 (53-2.1), CD4 (RM4-5), CD3 (17A2), CD8 (53-5.8), CD69 (H1.2F3), CD11c (N418),

MHCII (M5/114.15.2), and CD103 (2E7). SiglecF was from BD Biosciences. For innate lymphoid staining, anti-CD3, CD11b, B220, Gr-1, TER119, and GATA3 were used (eBioscience/Thermo Fisher) in accordance with a previously published protocol.[32] For ILC2: Lin-(CD3, CD11b, B220, GR-1, TER119) CD45+ CD25+CD90+ ST2+c-Kit+ +GATA3+. For eosinophils: SSC^{high}CD11b+ SiglecF+. For neutrophils: SSC^{high}CD11b+ SiglecF-GR-1+. For conventional DC: CD11b+ CD11c+MHCII+, CD103-. For DC 103+: CD11c+ CD11b- MHCII+ CD103+. For interstitial macrophages: CD11b+ CD11c-F4/80+. For CD4+ T cells: CD3+CD4+, and CD8+ T cells: CD3+ CD8+. Data were collected using a NovoCyte Flow Cytometer (ACEA Bioscience), and analysis utilized FlowJo software (Tree Star, OR). Gating strategies are available in the Supplementary Data (Figure S4).

2.11 | Histology

The middle and inferior lobes of the right lung were perfused with formaldehyde and embedded in paraffin. Five-micron lung sections were stained with periodic acid-Schiff (PAS) or hematoxylin/eosin (H/E). A Zeiss Axio Imager Z1 with AxioVision 4.8 software (Zeiss, Munich, Germany) collected photomicrographs.

2.12 | Mucus scoring analysis

Slides from PAS-stained lungs were coded and scored by a blinded observer. Mucus was quantified on a score of 1-4, with 1 = minimal/no mucus; 2 = slight: multiple airways with goblet cell hyperplasia and mucus; 3 = moderate: multiple airways with significant mucus and some plugging; and 4 = severe: significant mucus plugging.[14]

2.13 | Quantitative RT-PCR

TRIzol reagent was used for lung tissue homogenization and RNA extraction (Invitrogen). cDNA was synthesized using murine leukemia virus reverse transcriptase (Applied Biosystems) incubated at 37°C followed by 95°C to stop the reaction. Real-time quantitative PCR (qPCR) using TaqMan (Thermo Fisher Scientific) primers with a FAM-conjugated probe measured pro-IL-1β (Mm00434228 and Hs01555410), IL-4 (Mm00445259), IL-5 (Mm00439646), IL-13 (Mm00434204), CCL5 (Mm01302428), xanthine oxidase (Mm00442110), interferon-γ (Mm00801778), and 18S (Hs99999901 and Mm03928990). A previously described primer system was used to measure Gob5.[33] Custom primers were used for RSV-G (forward: CCA AGC AAA CCC AAT AAT GAT TT; reverse: GCC CAG CAG GTT GGA TTG T) (Sigma-Aldrich). Gene expression was normalized to 18S expression with fold change values calculated using $2^{-\Delta\Delta}$ cycle threshold method relative to uninfected wild-type controls. For the human pro-IL-1β expression, human 18S expression was used for normalization. A 7500 Real-Time PCR System (Applied Biosystems) was used.

2.14 | Re-stimulation of lung-draining lymph node (LDLN) cells with RSV or CRA

LDLN were digested via 1 mg/mL collagenase A (Roche) and 25 U/mL DNase I (Sigma-Aldrich) in RPMI with 10% FCS for 45-60 minutes at 37°C and further dispersed via 18-gauge needle (10-mL syringe). RBCs were lysed and samples filtered through 100-micron nylon mesh. Single-cell suspensions of lymph nodes at a concentration of 2.5×10^6 cells/mL (0.2 mL plated per well) in a 96-well plate were re-stimulated with RSV 5×10^5 PFU or CRA 300 PNU as appropriate. The supernatants were collected at 48 hours and analyzed for the following cytokines: IFN-γ, IL-4, IL-5, and IL-13 using a Bio-Plex bead-based cytokine assay (Bio-Rad Laboratories).

2.15 | Bronchoalveolar lavage

Bronchoalveolar lavage (BAL) was performed on neonatal mice after euthanasia. The trachea and lungs in the neck and chest were directly exposed. A 26-gauge needle was inserted into the trachea, 200 μL of PBS was instilled gently, and the lungs were directly visualized to inflate. Approximately 100 μL was returned upon application of suction. This was frozen at −20°C until analysis.

2.16 | Lung extracts

The left lung was taken for protein measurement. Each lung was placed in 1 mL Tissue Protein Extraction Reagent (Thermo Fisher), and total protein was extracted according to the manufacturer's protocol. This was frozen at −20°C until analysis.

2.17 | Uric acid measurements

BAL samples taken from neonatal mice at 6 days postinfection after euthanasia were analyzed for uric acid content. The Uric Acid Assay Kit (Cayman Chemicals) using the manufacturer's instructions was used.

2.18 | Enzyme-linked immunosorbent assays

Murine IL-1β protein was quantified from lung extract samples taken from naïve or infected mice at 6 days postinfection after euthanasia. We used the R&D DuoSet ELISA Kit (R&D Systems) and followed the manufacturer's instruction.

2.19 | Statistical analysis

Prism 7 (GraphPad Software) was used for data analysis. Data are presented as mean values ± SEM. Unpaired, two-tailed *t* test was used to

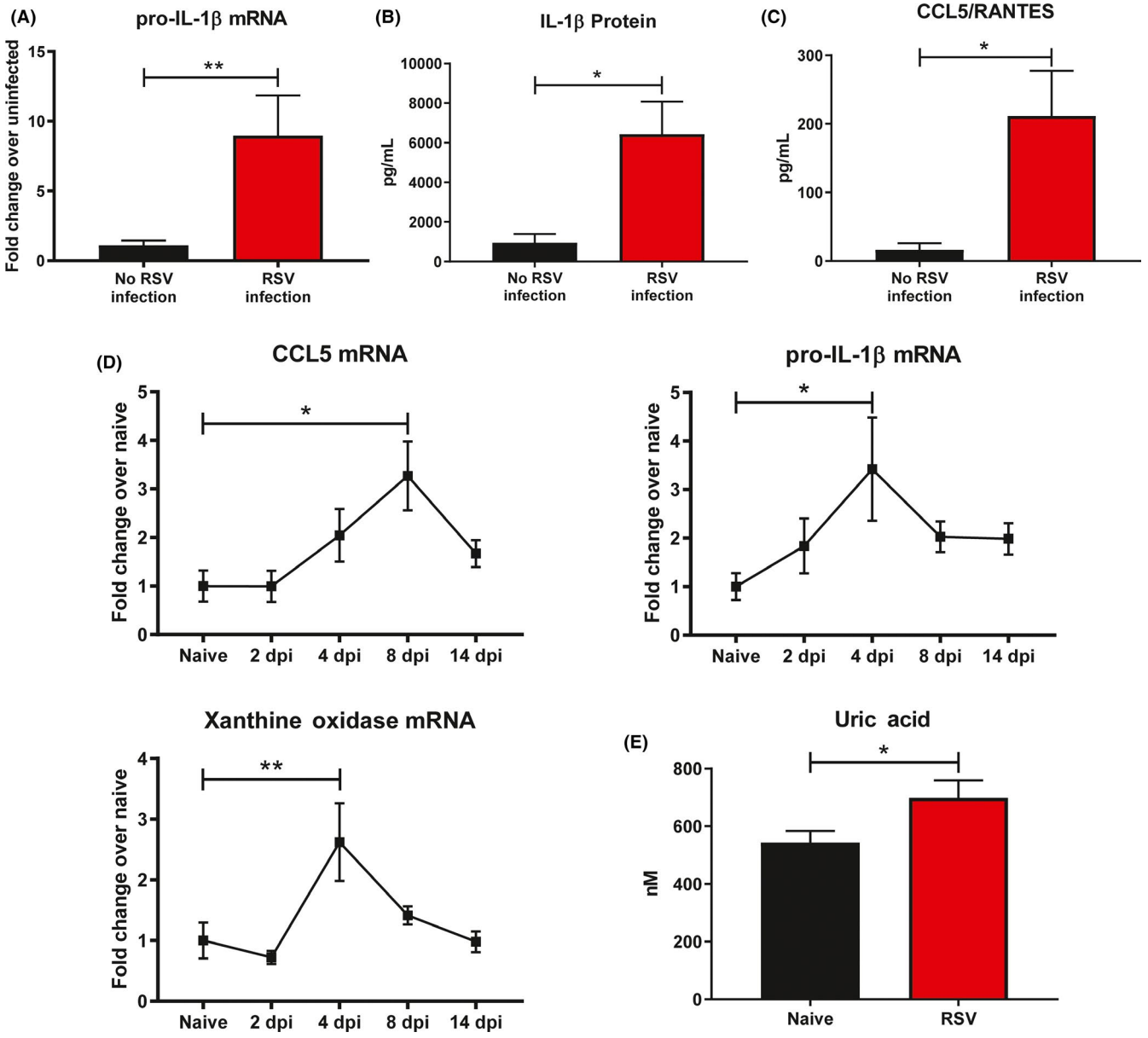

FIGURE 1 RSV induces pulmonary IL-1β expression in humans and mice neonates. (A) cDNA from tracheal aspirates from human infants with RSV and from control patients intubated for noninfectious reasons underwent qPCR to determine pro-IL-1β mRNA (N ≥ 9). (B) Tracheal aspirates from human infants underwent Bio-Plex to determine IL-1β protein concentration (N ≥ 6). (C) Tracheal aspirates from human infants were measured via Bio-Plex to determine CCL5 protein concentration (N ≥ 7). (D) Mice infected with RSV were killed at 2, 4, 8, and 14 days postinfection (dpi) and compared with age-matched controls. Lungs were homogenized and mRNA extracted to determine CCL5, pro-IL-1β, and xanthine oxidase mRNA expression (N ≥ 5). (E) BAL fluid from mice with RSV and age-matched controls was taken 8 days postinfection and assayed for uric acid (N ≥ 6). Data represent mean ± SEM. $^{*}P < .05$, $^{**}P < .01$

compare data with two groups. ANOVA was used to compare three or more groups. A P-value < .05 was considered statistically significant.

3 | RESULTS

3.1 | RSV induces pulmonary IL-1β expression in infants and neonatal mice

In this work, we initially analyzed tracheal aspirates from human infants with severe RSV for the presence of IL-1β. We detected significantly increased mRNA expression of pro-IL-1β and protein production of IL-1β in the samples of RSV+ infants compared with controls (Figure 1A,B). Protein production of CCL5, a chemokine known to be correlated with severe RSV disease,[34] was also significantly elevated in samples from RSV-infected infants (Figure 1C). IL-1β is known to be a potent cytokine that amplifies the immune response through the activation of cytokine cascades as well as activation of critical innate immune cells, such as ILC2s.[18,19,35,36] Therefore, we decided to investigate the impact of IL-1β-induced immune activation on type 2 immunity-associated RSV immunopathology.

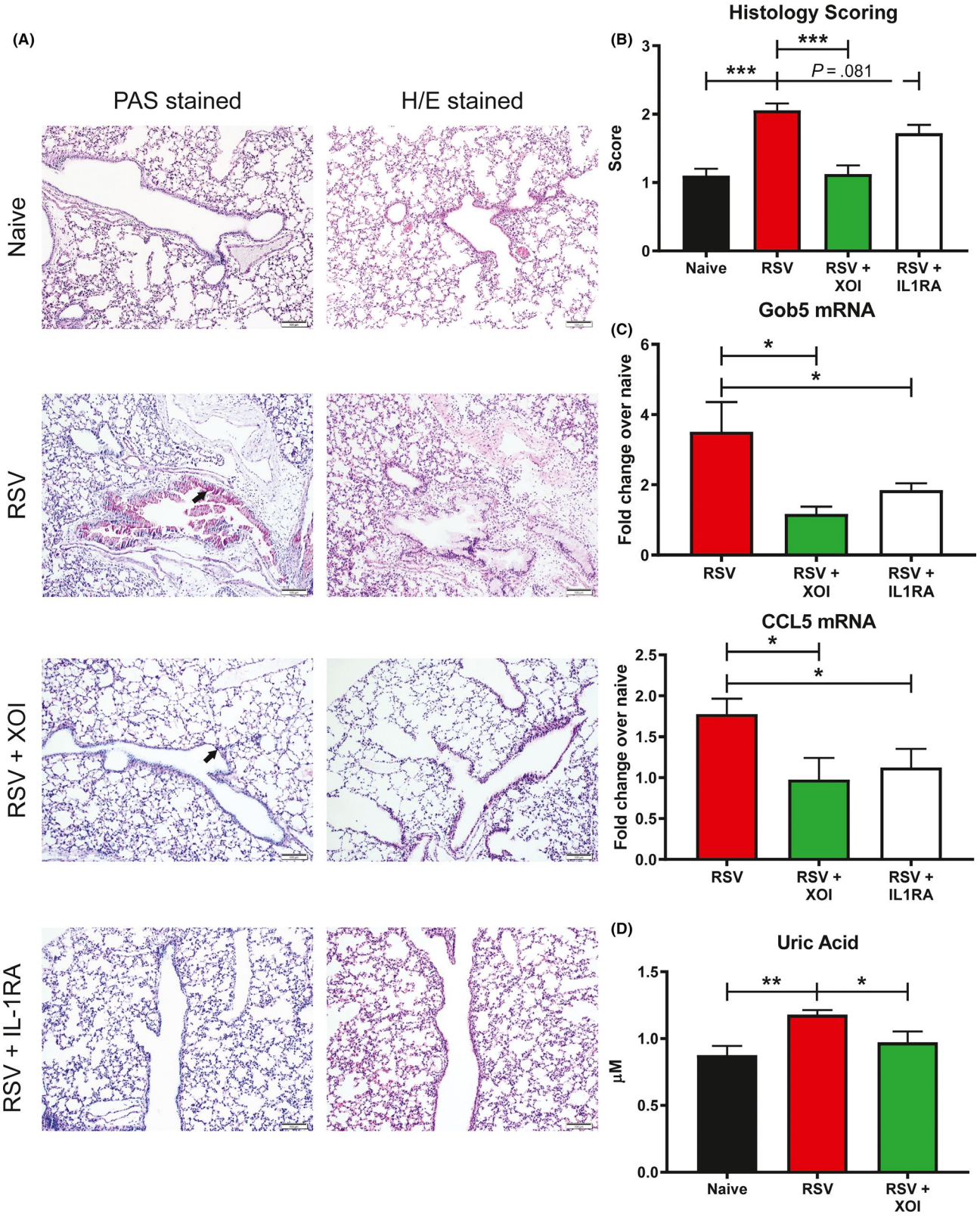

FIGURE 2 Administration of the XOI or IL-1RA ameliorates RSV infection. Mice were infected with RSV and treated with the XOI or IL-1RA and compared with infected mice at 6 or 8 days postinfection. (A) Lungs were embedded in paraffin and periodic acid-Schiff (PAS)-stained to visualize mucous (pink/purple staining) or hematoxylin/eosin (H/E)-stained to evaluate inflammatory cell infiltrates. Representative photographs are shown (N ≥ 5). (B) Mucus scoring was performed on blinded histological slides on a scale of 1-4 for mucus production (N ≥ 4). (C) Lungs were homogenized and mRNA extracted to determine Gob5 mRNA expression (N ≥ 5) and CCL5 mRNA expression (N ≥ 6). (D) BAL fluid at 6 days postinfection was collected as described and assayed for uric acid content (N ≥ 4). Data represent mean ± SEM. *$P < .05$. **$P < .01$, ***$P < .001$

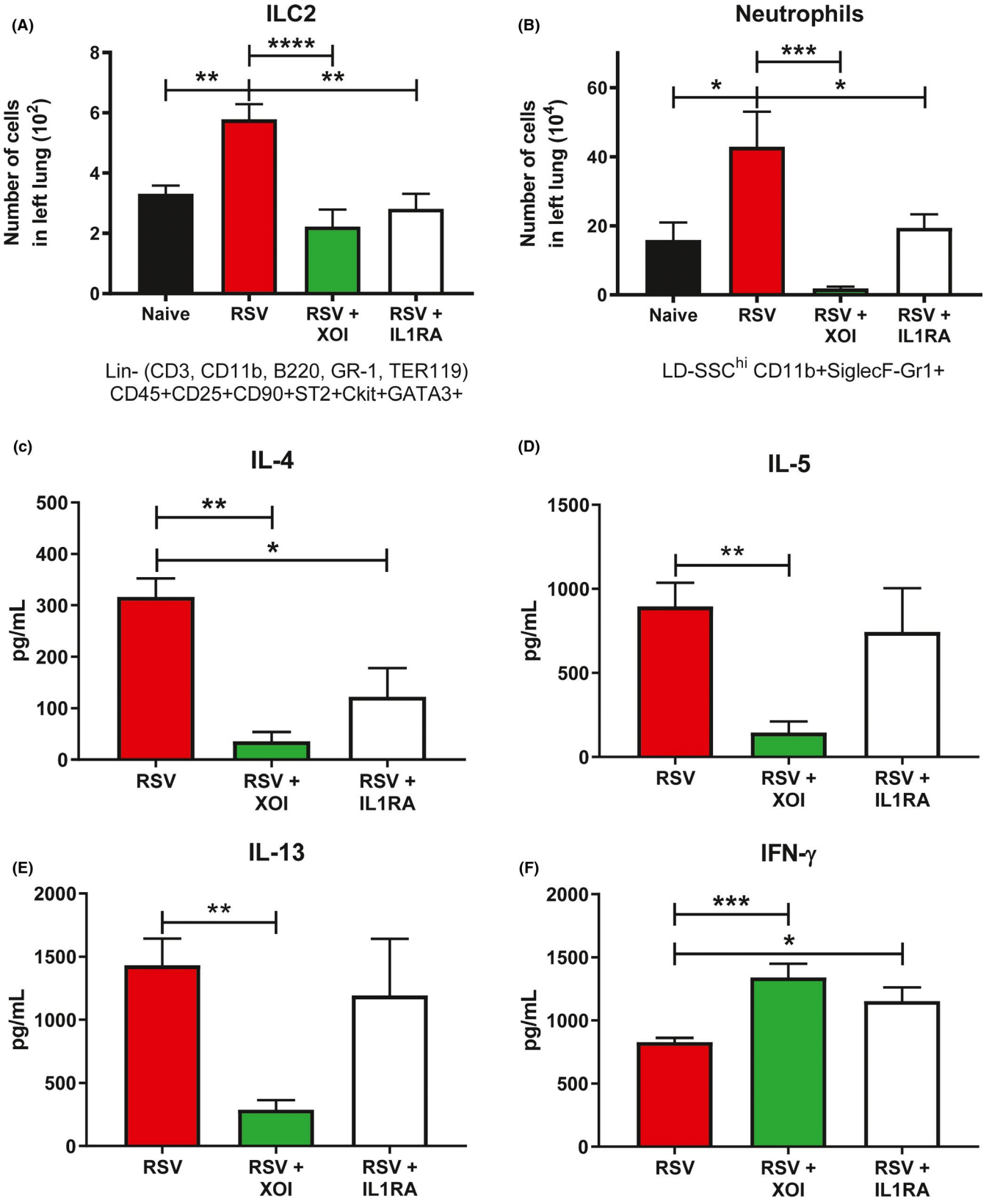

FIGURE 3 Administration of the XOI or IL-1RA reduces pulmonary immune infiltrates and lymphocyte responsiveness with RSV infection. Mice were infected with RSV and treated with the XOI or IL-1RA and compared with infected mice at 8 days postinfection. (A and B) Lungs were processed into single-cell suspension, then stained and analyzed via flow cytometry for type 2 innate lymphoid cells (N ≥ 6) and neutrophils (N ≥ 5). (C, D, E, and F) Lung draining lymph nodes were processed into single-cell suspension and re-stimulated with RSV in vitro for 48 h to determine cytokine protein levels in the supernatant including IL-4, IL-5, IL-13, and IFN-γ (N ≥ 3). Data represent mean ± SEM. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$

To study the role of IL-1β during RSV infection, a neonatal murine model was utilized to recapitulate responses in clinical disease in infants (Figure S1A).[14,37] To compare this model to the above human samples, neonatal BALB/c mice were infected with RSV line 19 and we measured mRNA expression of pro-IL-1β and CCL5 in the lung. We observed significant increases in mRNA expression of both cytokines (Figure 1D), resembling the RSV infection in infants. Various metabolic mediators have been identified to drive IL-1 pathway activation,[23,36] and RSV has been shown to alter metabolic profiles in mice as a key step driving immunopathology.[38] Examination of a key metabolic activator of IL-1β, uric acid (UA), was performed by analyzing the expression of xanthine oxidase that converts xanthine to UA. A time course of xanthine oxidase (XO) in the lungs of neonatal mice revealed high expression that peaked at 4 days postinfection (dpi) (Figure 1D). RSV infection was confirmed in the model via PCR, with RSV-G peaking on day 4 postinfection (Figure S1C). Bronchoalveolar lavage (BAL) fluid taken from neonatal mice at 8 days postinfection demonstrated elevated levels of UA in RSV-infected mice compared to controls (Figure 1E). Thus, we observed contemporaneous increased expression of XO, increased production of UA, and increased production of pro-IL-1β during neonatal RSV infection.

3.2 | Inhibition of uric acid or IL-1β pathway ameliorates RSV immunopathology

To examine the role of the XOI or IL-1RA on RSV immunopathology, neonatal BALB/c mice infected with RSV were treated daily with either the xanthine oxidase inhibitor (XOI) or interleukin 1 receptor antagonist (IL-1RA) (Figure S1A). Examination of the histopathology demonstrated that the XOI treatment in particular reduced mucus production and goblet cell metaplasia compared with infected controls as well as an overall decrease in cellular infiltrates in the treated groups (Figure 2A,B). We also observed downregulation of the mucous-associated gene Gob5 and the chemokine CCL5 in both treated groups (Figure 2C). We evaluated viral clearance rates associated with the treatments using RSV-G RNA expression. No difference between control mice and XOI-treated mice was observed at day 4 of infection (Figure S1D). However, uric acid levels in BAL at day 6 postinfection were lower in XOI-treated animals compared to control mice (Figure 2D). Local lung IL-1β levels were not significantly changed in XOI- or IL-1RA-treated animals at 4 days postinfection (dpi) (Figure S1E). These data suggest that UA and IL-1β have important roles during RSV pathogenesis.

To characterize the immune response in neonatal mice infected with RSV that were treated with either XOI or IL-1RA, we analyzed pulmonary leukocyte populations by flow cytometry. During RSV infection, increased numbers of activated ILC2s have been reported.[39] In the present studies, ILC2 (Figure 3A) and neutrophils (Figure 3B) were significantly increased in RSV infection and reduced in both XOI- or IL-1RA-treated mice compared with infected controls. Other leukocytes including macrophages, CD4+ T cells, eosinophils, and dendritic cell populations were not significantly altered by RSV infection at this time point (Figure S1F,G,H,I,J). To further evaluate the immune response, we measured cytokine levels from isolated lung draining lymph node (LDLN) cells after in vitro re-stimulation with RSV and observed that the XOI-treated neonatal group had reduced IL-4, IL-5, and IL-13 production (Figure 3C-E) and increased IFN-γ and IL-17A production (Figure 3F, Figure S3A) compared to RSV-infected control animals. The IL-1RA-treated group also demonstrated reduced IL-4 production (Figure 3C), but no change in IL-5 or IL-13 (Figure 3D,E), and an increase in IFN-γ and IL-17A levels compared to the control group (Figure 3F, Figure S3A). Altogether, both XOI and IL-1RA treatment reduce type 2 immune responses during RSV infection and promote a type 1 immune response.

3.3 | Control of RSV disease severity by XOI or IL-1RA treatments establishes long-lasting immune changes

To evaluate long-term consequences of XOI or IL-1RA treatment during RSV infection, neonatal mice were infected at days 6-7 of life and treated with the XOI or IL-1RA daily for 7 days. At 5 weeks after infection, the lungs were evaluated for their mucus expression and immune phenotype. No significant differences were observed between naïve animals, RSV-infected mice, or either treated group in visible lung pathology by PAS staining or in mucus gene expression by qPCR (Figure S2A,B,C). However, there was a persistent increase in ILC2s in the lungs of RSV-infected mice, which was significantly reduced in the groups of mice that were treated with XOI or IL-1RA (Figure 4A). In addition, increased macrophage, CD103+ dendritic cell, and eosinophil numbers seen in the RSV group were decreased in the IL-1RA-treated group, while only a nonsignificant trend toward reduction in these cell types was observed in the XOI-treated group (Figure 4B-D). CD4+ T cell and neutrophil numbers were unchanged in the treated groups (Figure S2D). To evaluate the lymphocyte responses to RSV re-stimulation, LDLN were harvested from animals infected with RSV with XOI or IL-1RA treatment and compared to infected controls. The XOI-treated mice showed an increase in IL-4 production (Figure 4E), no change in IL-5 (Figure 4F), and a significant decrease in IL-13 production (Figure 4G) compared to RSV-infected mice. The IL-1RA-treated group showed significant reduced production of IL-4, IL-5, and IL-13 (Figure 4E-G). Both treated groups showed a decrease in IFN-γ and no change in IL-17A production (Figure 4H, Figure S3B). Thus, there appear to be persistent innate immune cells in the lung and altered immune responses long after RSV infection has cleared that are attenuated in the XOI- or IL-RA-treated animals.

3.4 | Control of RSV infection severity by XOI or IL-1RA treatments ameliorates exacerbated development of asthma

In order to evaluate whether the changes with XOI or IL-1RA treatment during RSV infection would affect subsequent asthma development,

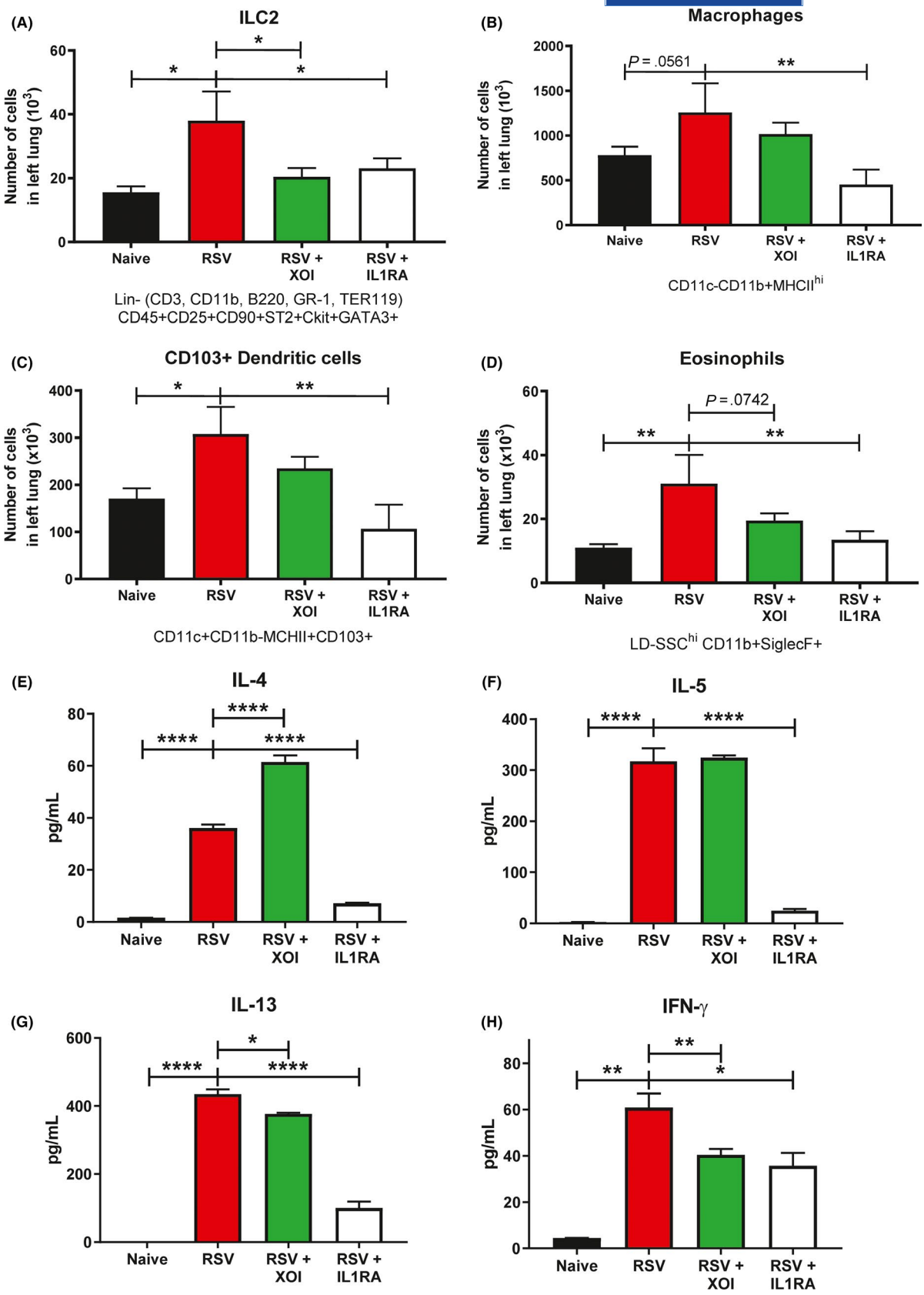

FIGURE 4 Administration of the XOI or IL-1RA during RSV infection leads to durable immune changes. Mice were infected with RSV and treated with the XOI or IL-1RA and compared with infected mice at 5 wk postinfection. (A, B, C, D) Lungs were processed into single-cell suspensions, then stained and analyzed via flow cytometry for type 2 innate lymphoid cells, macrophages, CD103+ dendritic cells, and eosinophils (N ≥ 4). (E, F, G, and H) Lung draining lymph nodes were processed into single-cell suspension and re-stimulated with RSV in vitro for 48 h to determine cytokine protein levels in the supernatant including IL-4, IL-5, IL-13, and IFN-γ (N ≥ 6). Data represent mean ± SEM. $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

RSV-infected mice were exposed to an allergen challenge model of asthma. Five-week-old mice previously infected with RSV and treated with XOI or IL-1RA were sensitized and challenged with cockroach antigen (CRA) (Figure S1B). Lung histology demonstrated a decrease in mucus deposition (Figure 5A) and less prominent inflammatory leukocyte infiltrates in the group of mice treated with XOI or IL-1RA compared to untreated animals during the neonatal RSV infection (Figure 5B). Mucus scoring was lower in the treated animal groups as well (Figure 5C). Gob5 mucus-related gene expression in the lung was reduced in XOI- and IL-1RA-treated animals (Figure 5D). CRA treatment increased IL-13 gene expression in the lung, and the enhanced IL-13 was decreased in the IL-1RA-treated group (Figure 5E).

To investigate the effect of blocking the XO and IL-1β pathways during RSV infection on the lung immune environment, flow cytometry was performed in the asthma model. Interestingly, the XOI- or IL-1RA-treated mice demonstrated no change in ILC2 numbers during CRA challenge when compared to RSV-infected/CRA-treated controls (RSV/CRA) (Figure 6A). However, reduced numbers of interstitial macrophages in the lung of the XOI- or IL-1RA-treated mice were observed (Figure 6B). Although there were no significant differences in the CD4+ T cells or CD103+ dendritic cells in either of the treated groups (Figure 6C,F), there was a significant reduction in eosinophils and CD11b+ dendritic cells in IL-1RA-treated mice (Figure 6D,E). Thus, differences in the cellular infiltrate changes in the XOI- or IL-1RA-treated groups lead to reductions in pulmonary immune infiltrates during subsequent induction of asthmatic disease.

Finally, we evaluated the acquired immune response by in vitro re-stimulation of LDLN from asthmatic mice with allergen. The groups treated with XOI and IL-1RA during neonatal RSV infection both demonstrated reduced IL-4, IL-5, and IL-13 production upon CRA re-stimulation (Figure 6G-I), with no change in IL-17A production (Figure S3C), and the IL-1RA group also showed reduced IFN-γ (Figure 6J). These data suggest that type 2 immunity induced in the RSV/CRA model is reduced by XOI or IL-1RA treatment during neonatal RSV infection. Together, these data indicate that control of neonatal RSV immunopathology, by XOI or IL-1RA treatment, translates into long-term control of type 2 immune responses in the lung upon later allergen sensitization and attenuates the RSV-associated asthma predisposition.

4 | DISCUSSION

In this work, we demonstrated that inhibition of the uric acid or IL-1β pathways by the XOI (allopurinol) or IL-1RA (Anakinra) treatments in RSV-infected neonatal mice decreased RSV immunopathology and ameliorated the long-term type 2 immunity-associated asthma induction. Given the high global health burden of RSV, with over three million hospitalizations and ~100 000 deaths worldwide yearly among children under age five, disrupting the immunopathology of early-life RSV infection with allopurinol or Anakinra could directly have a significant impact on health. In addition, because early-life viral infections, especially RSV, are associated with development of asthma, these treatments might lead to long-term reductions in childhood asthma. Prior work has shown the important role of metabolic alterations in RSV immunopathology.[38] Thus, these studies provide novel and striking evidence that interrupting this metabolic and inflammatory process could have significant clinical impact.

Uric acid is a product of purine metabolism produced by xanthine oxidase[40] and is produced during cell injury with viral infections.[41] UA has been implicated in house dust mite pulmonary injury and as an adjuvant promoting asthma; allopurinol can disrupt UA's deleterious effects.[40,42-44] Allopurinol is well-studied with a long-standing safety record[45] and has been used for many years in diseases such as gout to normalize the level of system UA to reduce flares.[46,47] In addition, dosing and safety data are available in children, particularly with treating tumor lysis syndrome.[27-29] This process of blocking UA production may be especially viable in disease such as viral infections that promote high levels of production since humans do not produce uricase to further process UA.[48] Thus, while further clinical research will be needed, safety and dosing have already been established in children and infants making this a potential clinical option to add to the clinical "toolbox."

IL-1β is a major inflammasome output, which can be activated by UA through the NLRP3 pathway.[23,36] IL-1β is elevated in nasopharyngeal aspirates of infants with RSV and in murine models plays a role in RSV immunopathology,[19,49,50] and a role for NLRP3-inflammasome activation of IL-1β has been shown in models of rhinovirus.[51] IL-1β promotes type 2 immune responses in asthma, IL-1β is upregulated in BAL fluid from Th2/Th17-polarized asthmatic patients, IL-1β elevation is associated with increased rates of hospitalization in asthmatic patients, and murine asthma models demonstrate a role for IL-1β in regulating barrier function and mucin production.[20,52-54] While we suspect that the IL-1RA affects IL-1β signaling through the IL-1 receptor, we acknowledge that these data do not definitively prove this connection; future studies are needed to understand this mechanism fully.

Like allopurinol, there are established safety and dosing profiles for IL-1RAs in children.[55-57] IL-1RAs, particularly Anakinra, are widely used in juvenile rheumatoid arthritis to reduce IL-1-related inflammation.[55-57] Furthermore, IL-1RAs are effectively and safely deployed in various autoinflammatory syndromes, including cryopyrin-associated periodic fever syndromes, tumor necrosis factor-associated periodic fever syndromes, familial Mediterranean fever, and adult-onset Still's disease.[58-61] However, IL-1RAs have not been evaluated as a therapy for RSV to potentially reduce asthma.

There are differences in the outcome of disease phenotypes between the XOI and IL-1RA treatments in this study. These differences are likely because UA can affect multiple pathways including inflammasome activation, while IL-1β may have a more targeted and specific effect on the downstream inflammation. In this case, the lack of decrease in IL-1β during RSV infection with XOI treatment suggests IL-1 independent pathways may play a role. Allopurinol appears to provide a more robust reduction in

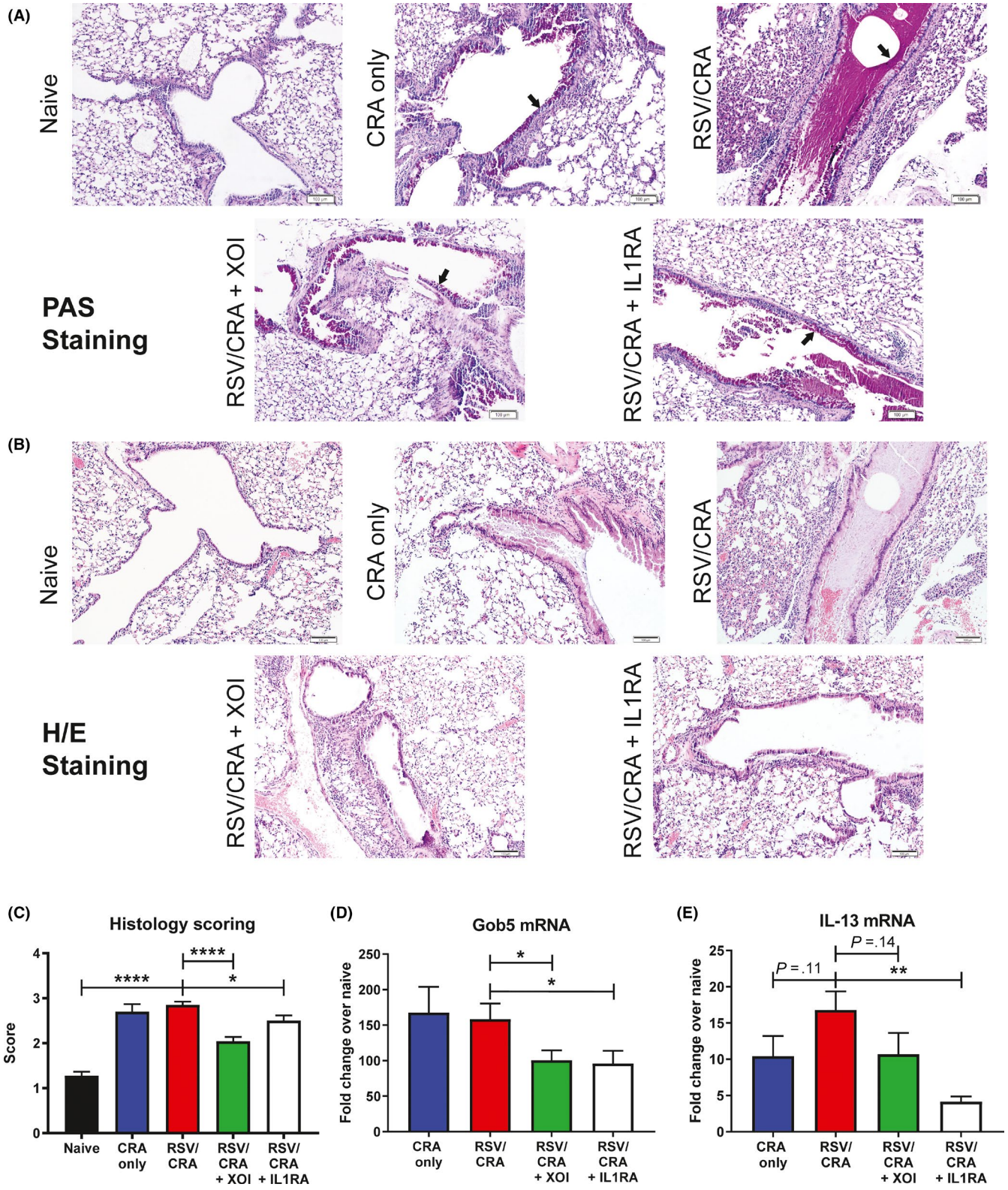

FIGURE 5 Administration of the XOI or IL-1 receptor antagonist during RSV infection ameliorates lung immunopathology during subsequent cockroach antigen sensitization. Mice were infected with RSV and treated with the XOI or IL-1RA. Five weeks after the start of infection, mice underwent CRA sensitization and challenge. Control mice were uninfected, CRA-treated mice (CRA only), and RSV-infected/CRA-treated mice (RSV/CRA). (A and B) Lungs were embedded in paraffin and periodic acid-Schiff (PAS)-stained to visualize mucous (pink/purple staining) or hematoxylin/eosin (H/E)-stained to evaluate inflammatory cell infiltrates. Representative photographs are shown (N ≥ 9). (C) Mucus scoring was performed on blinded histological slides on a scale of 1-4 for mucus production (N ≥ 9). (D and E) Lungs were homogenized and mRNA extracted to determine Gob5 (N ≥ 8) and IL-13 mRNA expression (N ≥ 3). Data represent mean ± SEM. $^{*}P < .05$, $^{**}P < .01$

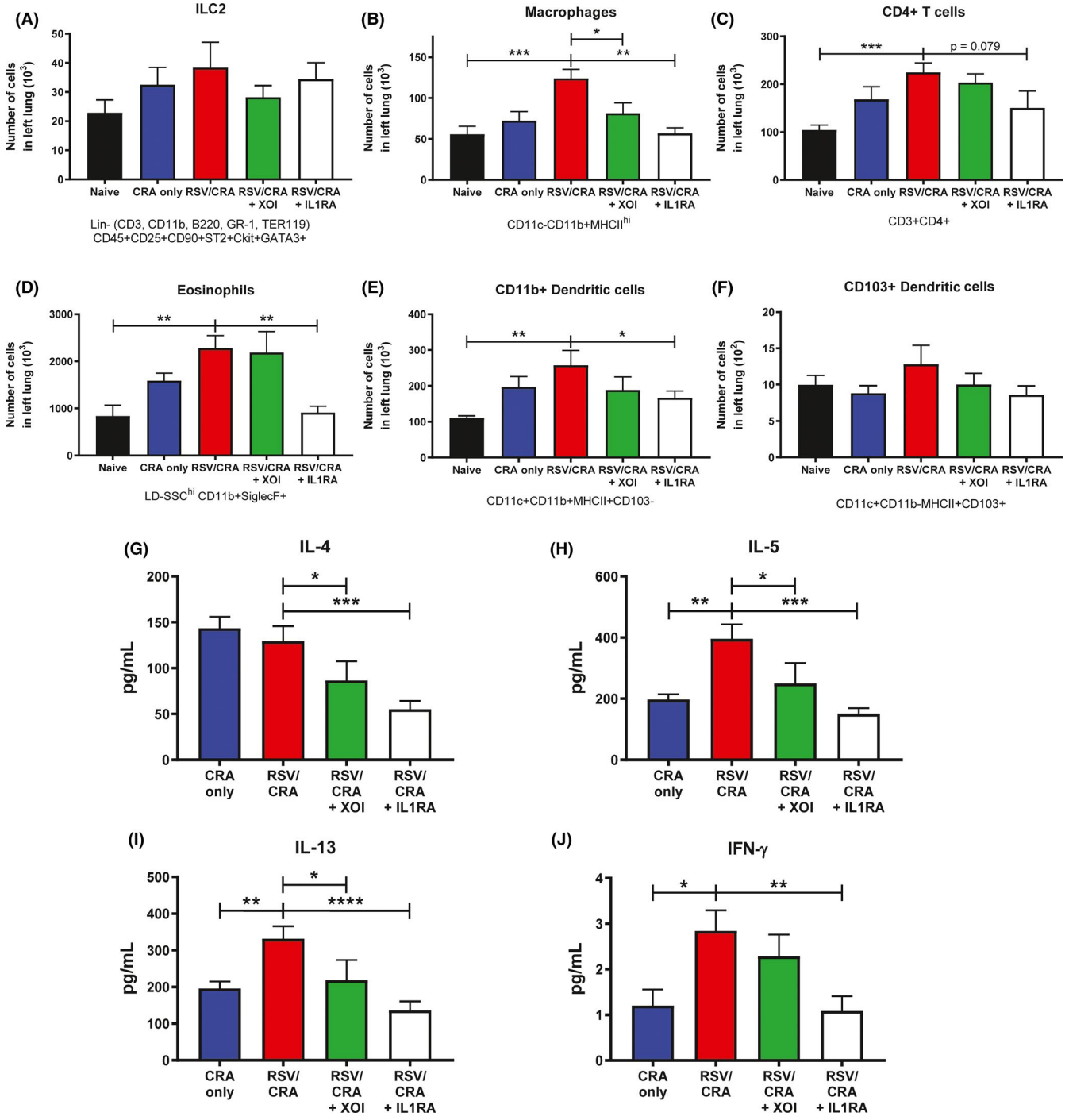

FIGURE 6 Administration of the XOI or IL-1 receptor antagonist during RSV infection ameliorates type 2 immune responses during subsequent cockroach antigen sensitization. Mice were infected with RSV and treated with the XOI or IL-1RA. Five weeks after the start of infection, mice underwent CRA sensitization and challenge. Control mice were uninfected, CRA-treated mice (CRA only) as well as RSV-infected/CRA-treated mice (RSV/CRA). (A, B, C, D, E, F) Lungs were processed into single-cell suspensions, then stained and analyzed via flow cytometry for type 2 innate lymphoid cells (N ≥ 7), macrophages (N ≥ 8), CD4+ T cells (N ≥ 4), eosinophils (N ≥ 8), CD11b+ dendritic cells (N ≥ 8), and CD103+ dendritic cells (N ≥ 8). (G, H, I, and J) Lung draining lymph nodes were processed into single-cell suspension and re-stimulated with RSV in vitro for 48 h to determine cytokine protein levels in the supernatant including IL-4, IL-5, IL-13, and IFN-γ (N ≥ 8). Data represent mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$

RSV immunopathology acutely than the IL-1RA, whereas the IL-1RA effects are more persistent when examining pulmonary infiltrates and LDLN at later time points, despite the slightly higher viral levels seen in the acute infection. Further, CD103+ dendritic cells were reduced in the IL-1RA-treated mice in the later allergen model in the IL-1RA treatment, which corresponded with a reduction in IFN-γ on LDLN re-stimulation consistent with prior work.[62,63] Both inhibitors lead to marked reductions in ILC2s,

which can play a role in RSV immunopathology and type 2 immune responses.[39] Given the perceived ability of ILC2s to establish an allergic immune environment in the lung,[64] this modified phenotype may be desirable and play a key role. The differences extend to the later development of allergic disease, where the IL-1RA-treated mice demonstrate greater reductions in pulmonary IL-13 expression, pulmonary cellular infiltrates, and type 2 immune responses in LDLN lymphocytes than XOI-treated animals. However, since both treatments reduce the overall disease, the use of them individually or together may be a clinical decision based upon the viral-induced phenotype and severity presented in each infant. These differences may depend upon, for example, the level of UA vs IL-1β in airway samples or the metabolic state of the infected patient.

This novel work verifies and connects the metabolic consequences and immunopathology of RSV infection. We propose a conceptual model (see graphical abstract) to explain how these two processes might be linked. We show that UA production is induced by RSV infection and that this activates the inflammasome, leading to IL-1β production. IL-1β, known to induce ILC2 activation and proliferation, likely promotes a type 2 immune environment coupled with persistent changes in the airway, such as goblet cell metaplasia. By inhibiting either xanthine oxidase or the IL-1 receptor, we can interrupt this process, with beneficial immediate and long-term consequences. This neonatal murine model reflects the time in life that this infection has the most consequences in humans.[2] However, since the clinical burden of RSV is not limited to infants, these treatments could have impacts on RSV infection in the elderly as well as those with chronic lung disease, such as COPD.[65-67] This work extends to development of asthma early in life, which may be a clinical consequence of RSV infection, and these inhibitors may attenuate subsequent asthma. Further research, preclinical and clinical, could provide better definitions of how and when to use specific inhibitors during disease.

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CONFLICTS OF INTEREST

Dr Schuler, Dr Malinczak, Ms Best, Mr Rasky, Ms Morris, Dr Ptaschinski, and Dr Fonseca have nothing to disclose. Dr Lukacs reports grants from the NIH during the conduct of the study.

AUTHOR CONTRIBUTIONS

CFS, NWL, and WF designed the experiments. CFS, CAM, SKB, SBM, AJR, CP, and WF performed the experiments. CFS, NWL, and WF wrote the manuscript. CFS, NWL, and WF performed the data analysis. All authors participated in editing the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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