

IL-2 is a critical regulator of group 2 innate lymphoid cell function during pulmonary inflammation

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Background: Group 2 innate lymphoid cells (ILC2) have been implicated in the pathogenesis of allergic lung diseases. However, the upstream signals that regulate ILC2 function during pulmonary inflammation remain poorly understood. ILC2s have been shown to respond to exogenous IL-2, but the importance of endogenous IL-2 in ILC2 function *in vivo* remains unclear.

Objective: We sought to understand the role of IL-2 in the regulation of ILC2 function in the lung.

Methods: We used histology, flow cytometry, immunohistochemistry, ELISA, and quantitative PCR with knockout and reporter mice to dissect pulmonary ILC2 function *in vivo*. We examined the role of ILC2s in eosinophilic crystalline pneumonia, an idiopathic type 2 inflammatory lung condition of mice, and the effect of IL-2 deficiency on this disease. We determined the effect of IL-2 administration on pulmonary ILC2 numbers and function in mice in the steady state and after challenge with IL-33.

Results: We discovered an unexpected role for innate cell-derived IL-2 as a major cofactor of ILC2 function during

pulmonary inflammation. Specifically, we found that IL-2 was essential for the development of eosinophilic crystalline pneumonia, a type 2 disease characterized by increased numbers of activated ILC2s. We show that IL-2 signaling serves 2 distinct functions in lung ILC2s, namely promoting cell survival/proliferation and serving as a cofactor for the production of type 2 cytokines. We further demonstrate that group 3 innate lymphoid cells are an innate immune source of IL-2 in the lung.

Conclusion: Innate cell-derived IL-2 is a critical cofactor in regulating ILC2 function in pulmonary type 2 pathology. (J Allergy Clin Immunol 2015;■■■■:■■■■-■■■■.)

Key words: Group 2 innate lymphoid cells, IL-2, eosinophilic crystalline pneumonia, IL-13, pulmonary inflammation

Type 2 cytokine-dependent immune responses are essential for protection against many helminth infections but also drive the pathology of numerous allergic diseases, such as eczema and

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Abbreviations used

DAPI:	4'-6-Diamidino-2-phenylindole dihydrochloride
ECP:	Eosinophilic crystalline pneumonia
Foxp3:	Forkhead box protein 3
FUCCI:	Fluorescent, ubiquitination-based cell cycle indicator
GFP:	Green fluorescent protein
ILC:	Innate lymphoid cell
ILC2:	Group 2 innate lymphoid cell
ILC3:	Group 3 innate lymphoid cell
JES6-1:	Anti-IL-2 antibody
MHCII:	MHC class II
NF-κB:	Nuclear factor κB
qPCR:	Quantitative (real-time) PCR
RAG:	Recombination-activating gene
STAT5:	Signal transducer and activator of transcription 5
TCR:	T-cell receptor
TLR2:	Toll-like receptor 2
YFP:	Yellow fluorescent protein

bronchial asthma. Dysregulated type 2 immune responses further underpin the life-threatening complications of viral bronchiolitis, an inflammatory response to respiratory tract infection manifesting as bronchial hyperresponsiveness. Although it is traditionally believed that CD4⁺ T_H2 cells are the principal drivers of type 2 inflammatory conditions, there is increasing evidence that such reactions can develop independently of adaptive immunity. Thus the use of T cell-independent models of type 2 inflammation has provided new insights into the innate cellular and molecular pathways that contribute to allergic pulmonary inflammation.^{1,2} Of particular note, these studies have demonstrated the capacity of innate group 2 lymphoid cells (ILC2), which are prime producers of the type 2 cytokines IL-5 and IL-13, to contribute to the pathogenesis of allergic lung diseases.^{1,3} Indeed, the importance of understanding ILC2 function *in vivo* is underscored by growing evidence that these cells likely play a role in human airway diseases, including bronchial asthma.^{4,5} Nevertheless, the upstream signals regulating ILC2 numbers and activation in the respiratory tract remain incompletely understood.

In addition to mounting innate type 2 responses to pulmonary infection,¹ immunodeficient mice spontaneously develop an idiopathic inflammatory lung condition termed eosinophilic crystalline pneumonia (ECP), also known as acidophilic macrophage pneumonia.⁶⁻⁸ ECP shares many of the pathologic hallmarks of human pulmonary diseases, such as asthma and pulmonary eosinophilic pneumonia, including increased levels of type 2 cytokines and alternatively activated macrophages. Understanding the pathogenesis of ECP might help identify candidate pathways of relevance to human diseases in which excessive type 2 inflammation drives pathology.

Our investigations have revealed a central role for ILC2s in the pathogenesis of ECP. We have further uncovered a hitherto unrecognized role for innate cell-derived IL-2 in ILC2 function in the lung, particularly in mice with ECP. Mechanistically, we show that IL-2 acts in a similar manner in ILC2s and T cells, namely by promoting cellular proliferation and augmenting nuclear factor κB (NF-κB)-dependent transcription of type 2 cytokine genes. We further identify the major cellular sources of IL-2 in the lung, which, in addition to conventional T cells, include group 3 innate lymphoid cells (ILC3s).

METHODS**ECP in immunodeficient mice**

Recombination-activating gene 1 (*Rag1*^{-/-} and *Rag2*^{-/-} and *Il2*^{-/-} mice were maintained on C57BL/6 and B10.BR backgrounds in specific pathogen-free conditions at the Centenary Institute. 5C.C7 T-cell receptor (TCR) and MHC class II (MHCII) transgenic mice have been described previously.⁹ Necropsies were performed on all mice killed during rederivation of the immunodeficient mouse colony from 2010-2014. Gross pathology was noted, and findings such as ECP were documented by using a customized database. The extent of ECP in each lung lobe was estimated visually as follows: 0, no affected tissue; 1, less than 5% of total; 2, 5% to 10%; 3, 10% to 50%; and 4, greater than 50%.

Experimental mice

All experimental *Rag1*^{-/-} mice were bred in house or purchased from the Animal Research Centre, Perth, Australia. *Rag1*^{-/-} *Cxcr6*^{+/gfp} mice,¹⁰ *Il2* fate reporter mice,¹¹ and fluorescent, ubiquitination-based cell cycle indicator (FUCCI) transgenic mice¹² were maintained in specific pathogen-free conditions at the Centenary Institute. 4C13R mice¹⁰ were bred and maintained at the Malaghan Institute, Wellington, New Zealand. Experimental investigations of ECP-affected mice and normal control mice were performed by using greater than 170-day-old mice. Eight- to 12-week-old mice were used for all other experiments. All experiments were done with the approval of the Animal Ethics Committee at the University of Sydney (Sydney, Australia) or the Victoria University Animal Ethics Committee (Wellington, New Zealand).

In vivo treatment with IL-2 and IL-33

For expansion of ILC2 populations, *Rag1*^{-/-} mice were treated with IL-2-anti-IL-2 antibody (JES6-1) complexes, as previously described.¹⁰ For activation of lung ILC2s, 50 μL of IL-33 (10 μg/mL; R&D Systems, Minneapolis, Minn) was administered intranasally.

Lung histopathology

Lung tissues were harvested from normal, ECP-affected, and IL-2-treated mice and fixed in 10% neutral buffered formalin and embedded in paraffin. Five-micrometer sections were stained with hematoxylin and eosin.

Tissue processing and flow cytometry

Lungs and spleens were filtered through an 80-μm stainless-steel mesh in running buffer (5% FCS, 2 mmol/L EDTA, and 0.02% sodium azide in PBS) to obtain single-cell suspensions. Cells were counted with a Sysmex XS-1000i Automated Hematology Analyzer (Toa Medical Electronics, Kobe, Japan). The antibodies used in this study are listed in the [Methods](#) section in this article's Online Repository at www.jacionline.org. Where appropriate, expression of markers of interest was compared with that of fluorescence minus one controls. Cells were analyzed on a FACSCanto (BD Biosciences, San Jose, Calif) or a 5-laser or custom 10-laser LSR II (BD Biosciences). Flow cytometric data were analyzed with FlowJo software (TreeStar, Ashland, Ore).

Measurement of gene expression

Cells were sorted for gene expression analysis by using quantitative RT-PCR, as previously described.¹⁰ Lungs from *Rag1*^{-/-} and *Rag2*^{-/-} mice were prepared and stained for flow cytometry, as described above. One to 2 × 10³ innate lymphoid cells (ILCs) or 5 × 10⁵ macrophages were sorted with a BD FACSAria II or custom 10-laser Influx sorter (BD Biosciences) into Buffer RLT Plus lysis buffer (Qiagen, Hilden, Germany). The primers used for quantitative PCR are listed in the [Methods](#) section in this article's Online Repository.

Measurement of cytokine levels in tissues

Levels of cytokines in tissue homogenates and serum were measured by using the Cytometric Bead Array assay (BD Biosciences) or ELISA (IL-33

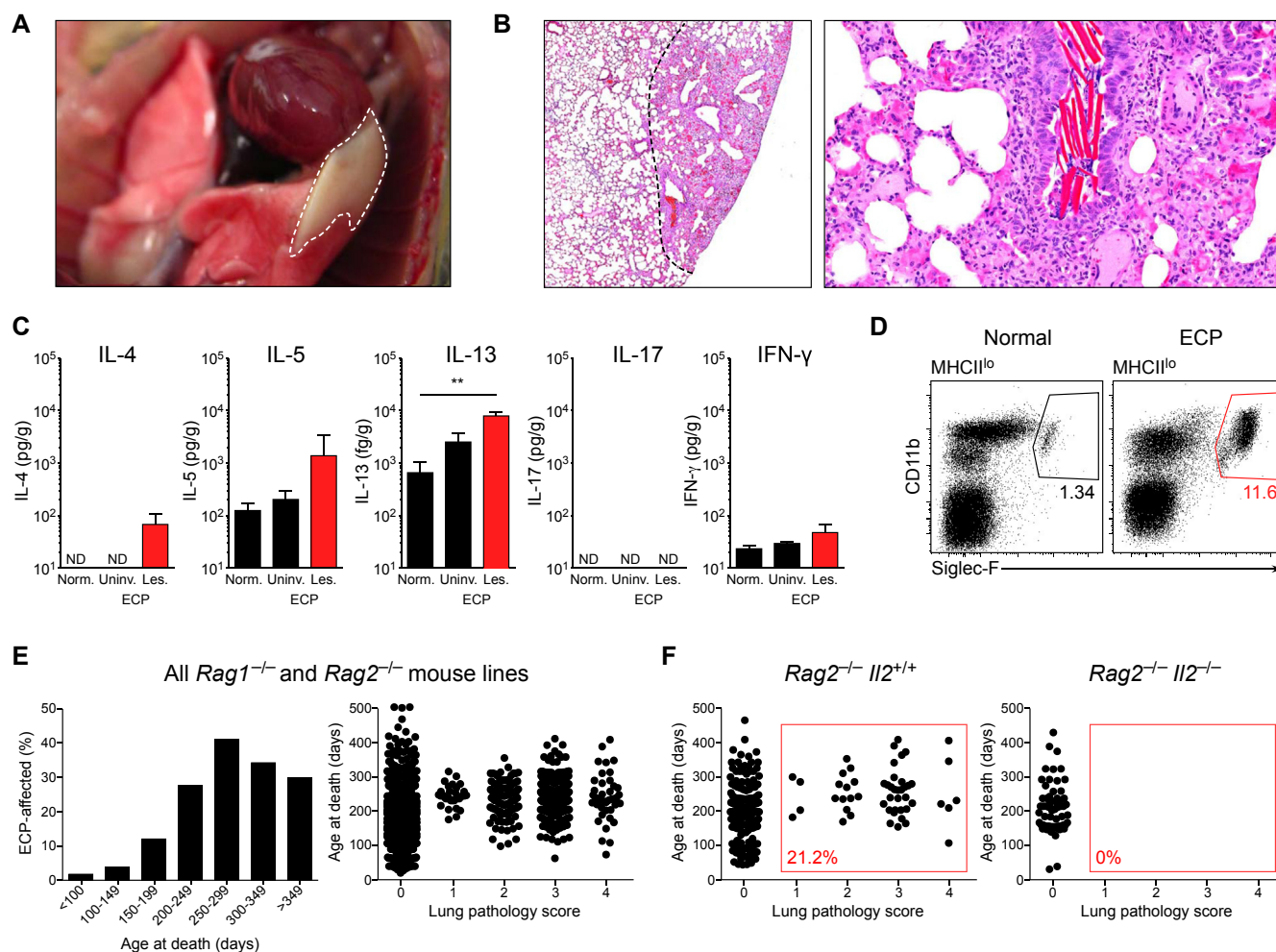


FIG 1. ECP in *Rag1*^{-/-} and *Rag2*^{-/-} mice. **A**, Lungs of mice with ECP. **B**, Lung section from an ECP-affected mouse stained with hematoxylin and eosin ($\times 100$ [left] and $\times 1000$ [right] magnification). **C**, Cytokines in lungs from mice with (n = 4) and without (n = 3) ECP. ND, Not detected. **D**, Lung eosinophils in normal and ECP-affected mice. **E**, ECP incidence in *Rag1*^{-/-} and *Rag2*^{-/-} mice stratified according to age and severity (n = 2104). **F**, ECP incidence in *Rag2*^{-/-} Il2^{+/+} (n = 250) and *Rag2*^{-/-} Il2^{-/-} (n = 64) mice.

DuoSet, R&D Systems). Tissues were mechanically homogenized with a rotor stator homogenizer (Polytron) in PBS containing protease inhibitors (Halt Protease Inhibitor; Thermo Scientific, Waltham, Mass) and 5 mmol/L EDTA. The homogenate was then centrifuged at 15,000g for 10 minutes at 4°C. The resulting supernatant was used for cytokine quantification. The amount of cytokine per gram of tissue was calculated, as previously described.¹⁰ Cytokine analysis of ECP-affected lesions presented in this article came from larger grade 3 and grade 4 lesions to minimize contamination by neighboring unaffected tissue.

Statistical analysis

For animal experiments, littermates were randomly distributed to the treatment groups so that all groups were age and sex matched. Statistical analyses, including linear regression and Pearson correlation, were performed by using Prism 6 software (GraphPad Software, La Jolla, Calif). For data presented on a log scale, log transformation was applied before statistical analysis. Data in bar graphs are means $\pm$ SEMs. *P* values of differences between 2 treatment groups were determined by using a 2-tailed unpaired *t* test (parametric data). Alternatively, a Mann-Whitney *U* test was used for nonparametric data. A statistical difference was assumed if the *P* value was less than .05.

RESULTS

IL-2 is required for ECP development

In our animal facility ECP is a nonlethal condition that presents as an incidental finding at necropsy of immunodeficient mice. Macroscopically, ECP appears as 1 or more clearly defined areas of pulmonary consolidation (Fig 1, A). Microscopically, the most prominent feature of ECP lesions is alveolar filling by large acidophilic macrophages with intracellular and extracellular crystalloid inclusions (Fig 1, B). These crystals are composed predominantly of Ym1 protein,⁶ an eosinophil chemoattractant produced by alternatively activated macrophages.¹³ ECP-affected lungs also exhibit widespread tissue infiltration with foamy macrophages, granulocytes, and lymphoid cells (see Fig E1 in this article's Online Repository at www.jacionline.org).

Cytokine analysis of lesional tissue from mice with spontaneous ECP revealed increased levels of IL-4, IL-5, and IL-13 protein but not IFN- γ or IL-17 (Fig 1, A), confirming that ECP is a type 2 cytokine-associated disease. Consistent with this, eosinophil numbers were increased in both the bronchoalveolar lavage fluid and lesional tissue of ECP-affected lungs, where

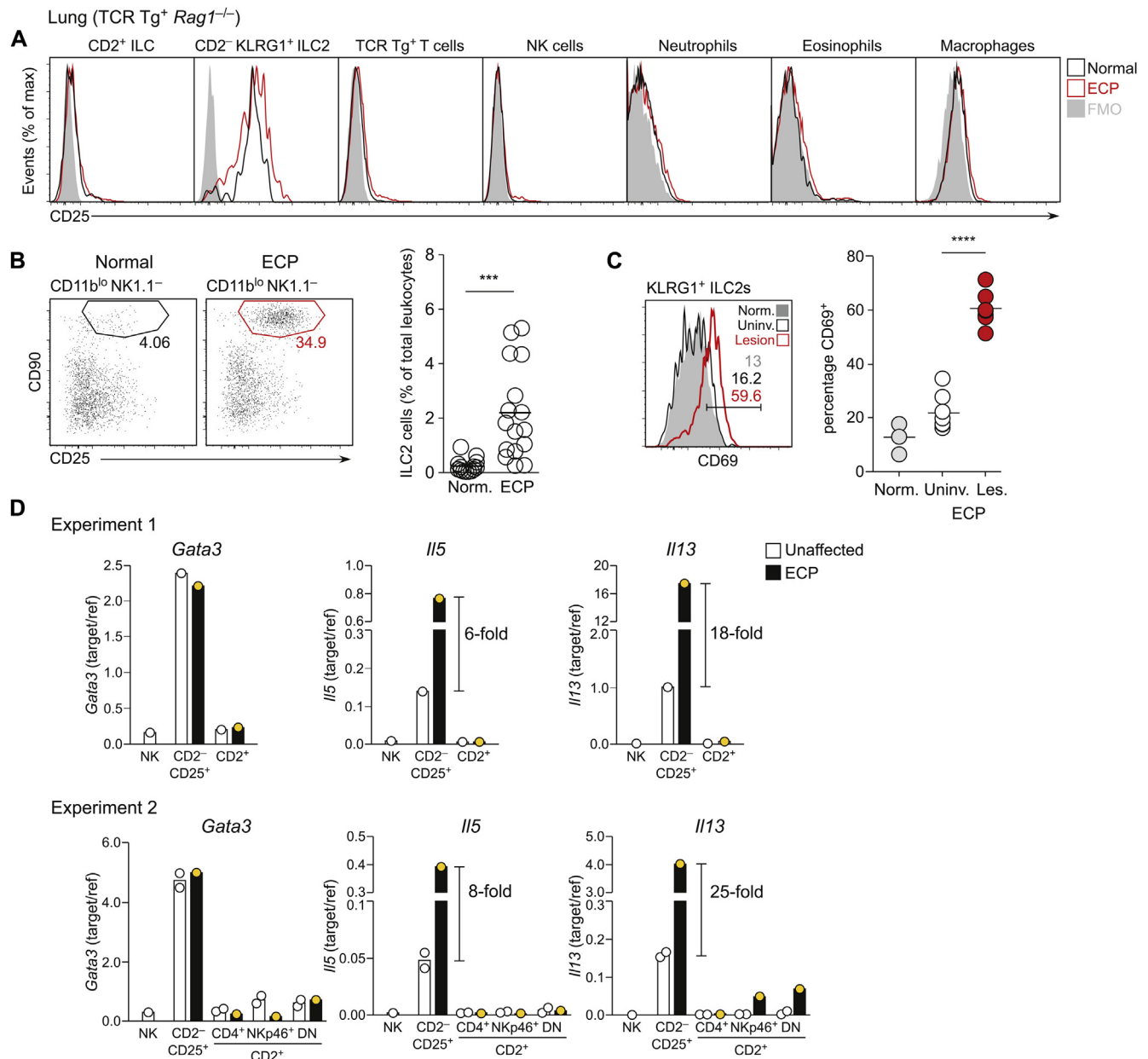


FIG 2. ILC2s in ECP-affected lungs. **A**, CD25 expression by lung leukocytes in normal and ECP-affected TCR transgenic (Tg) Rag1^{-/-} mice.²¹ **B**, ILC2s in normal (n = 14) and ECP-affected (n = 16) lungs. ***P = .0002. **C**, CD69 expression by lung ILC2s in normal (gray, n = 3), ECP-affected uninvolved (black, n = 6), and ECP-affected lesional (red) tissue. ****P < .0001. **D**, *Gata3*, *Il13*, and *Il5* expression in splenic natural killer (NK) cells and ILC2s sorted from normal and ECP-affected lung tissue.

they displayed an activated phenotype, namely increased cell-surface expression of Siglec-F and CD11c (Fig 1, D, and data not shown).

To explore possible genetic influences on ECP incidence, we performed a retrospective analysis of 2104 reports of necropsies conducted to track incidental disease during rederivation of an immunodeficient colony in our animal facility. This colony included IL-2-deficient (*Il2*^{-/-}) mice that had been bred with various TCR and MHCII transgenic mice on Rag1^{-/-} or Rag2^{-/-} backgrounds. The incidence of ECP increased with age (Fig 1, E), irrespective of the presence or absence of a TCR or MHCII transgene (see Fig E2 in this article's Online Repository

at www.jacionline.org and data not shown). In individual mouse genotypes the incidence of ECP ranged from 8% to 40%, with the exception of the *Il2*^{-/-} Rag2^{-/-} line, which never had the disease (Fig 1, F; P < .005), indicating that IL-2 was highly likely to be essential for the development of ECP in RAG-deficient mice.

ILC2s are increased and activated in mice with ECP

IL-2 promotes the survival and proliferation of T cells by means of signaling through the high-affinity IL-2 receptor (CD25). CD25 is also constitutively expressed by ILC2s, which are well-recognized producers of type 2 cytokines, particularly

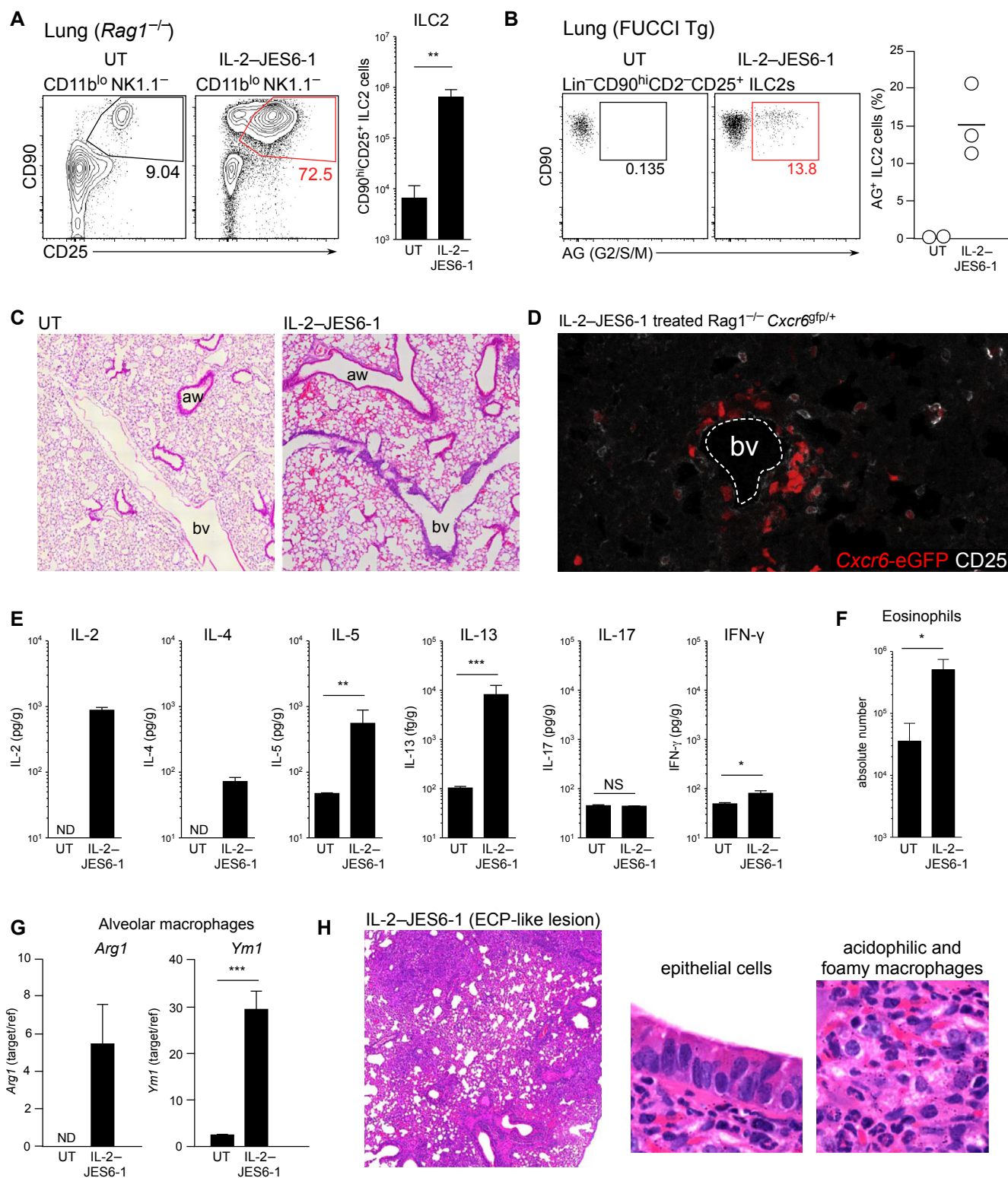


FIG 3. Lung ILC2s after IL-2 treatment. **A**, Flow cytometry of ILC2s from untreated and IL-2-treated *Rag1*^{-/-} lungs. $P = .0018$. **B**, Flow cytometry of lung ILC2s from FUCCI transgenic mice¹² left untreated or treated with IL-2 for 3 days. **C**, Lung sections of untreated and IL-2-treated *Rag1*^{-/-} mice stained with hematoxylin and eosin ($\times 100$ magnification). *aw*, Airway; *bv*, blood vessel. **D**, Lung section from a *Rag1*^{-/-} *Cxcr6*^{gfp/+} mouse treated with IL-2 and stained for CD25 (white). GFP⁺ cells are shown in red ($\times 400$ magnification). *bv*, Blood vessel. **E**, Cytokines in lungs from untreated ($n = 3$) and IL-2-treated ($n = 7$) mice. **F**, Eosinophils in untreated and IL-2-treated lungs. **G**, *Arg1* and *Ym1* expression by pulmonary macrophages sorted from untreated ($n = 4$) and IL-2-treated ($n = 4$) mice. **H**, Sections of ECP-like lung lesions from mice treated with IL-2 and stained with hematoxylin and eosin. *Left*, $\times 100$ magnification; *middle* and *right*, $\times 1000$ magnification. Data are representative of (Fig 3, A and C-H) or pooled from (Fig 3, B) 2 independent experiments. UT, Untreated. * $P < .05$, ** $P < .01$, and *** $P < .001$.

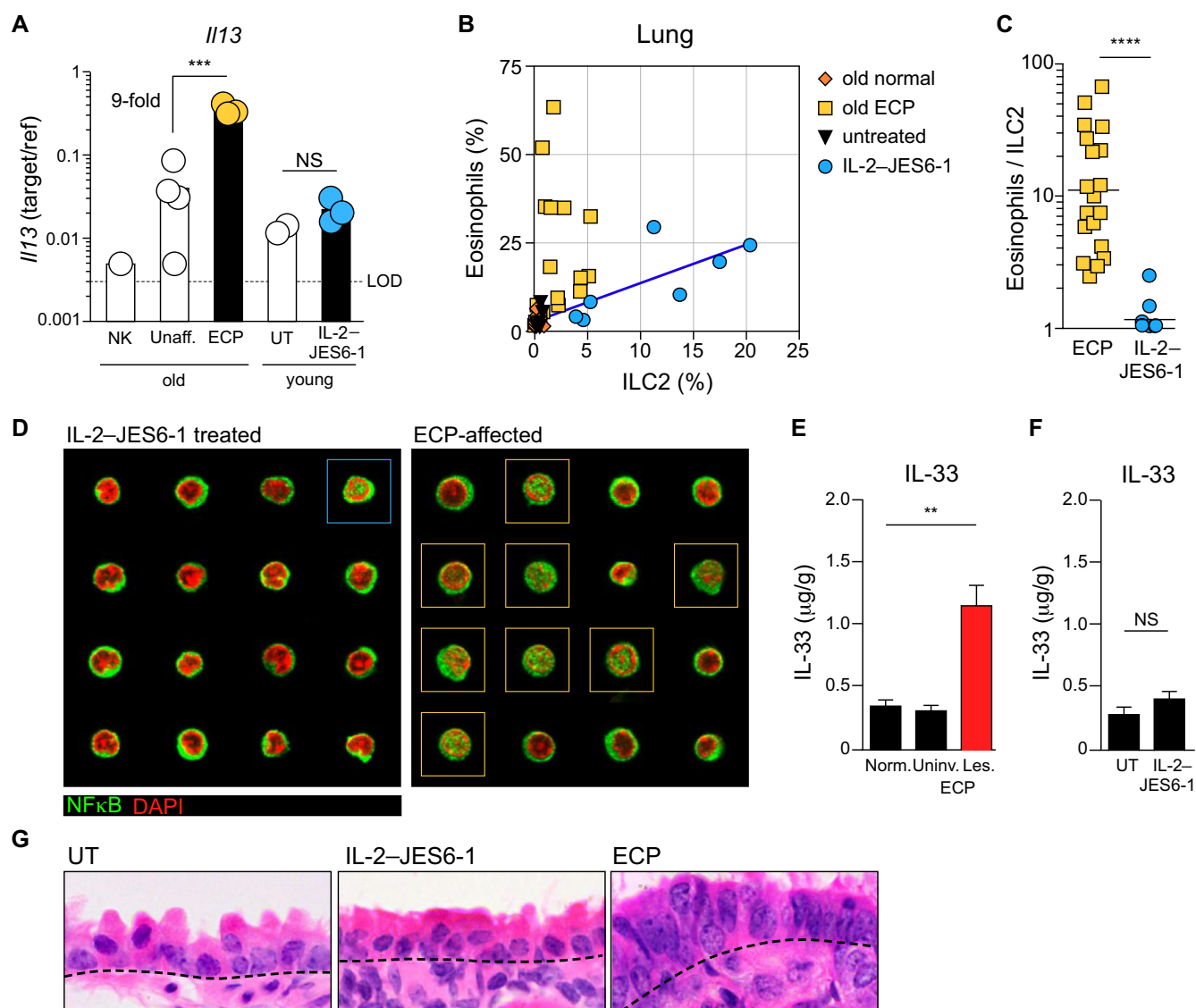


FIG 4. Comparison between ECP-affected and IL-2-treated lungs. **A**, Expression of *Il13* in ILC2s sorted from normal and ECP-affected lungs, as well as untreated and IL-2-treated mice. *** $P = .0002$. LOD, Limit of detection; NS, not significant. **B**, Scatterplot of ILC2s versus eosinophils in normal and ECP-affected lungs, as well as untreated and IL-2-treated mice. The blue line indicates linear regression for the untreated and IL-2-treated mice ($Y = 1.085X + 2.536$). **C**, Ratio of eosinophils to ILC2s in ECP-affected lesions ($n = 19$) and mice treated with IL-2 ($n = 11$). **** $P < .0001$. **D**, NF-κB staining (green) of ILC2s sorted from IL-2-treated and ECP-affected lungs. Nuclei were counterstained with DAPI (red). Boxes indicate cells with nuclear translocation of NF-κB. **E** and **F**, Concentration of IL-33 in *Rag1*^{-/-} mice with ($n = 4$) and without ($n = 4$) ECP, as well as untreated ($n = 3$) and IL-2-treated ($n = 7$) mice. ** $P = .0036$. NS, Not significant. **G**, Sections from lungs of untreated, IL-2-treated, and ECP-affected mice stained with hematoxylin and eosin. The dashed line demarks lung epithelial cells from lung parenchyma (×1000 magnification). Data are representative of 2 independent experiments (Fig 4, A and D-G) or pooled from 4 experiments (Fig 4, B and C). UT, Untreated.

IL-5 and IL-13 but also IL-4 and IL-9.¹⁴⁻¹⁸ ILC2 responsiveness to IL-2 *in vitro*, as well as exogenous IL-2 *in vivo*, has been previously established.^{10,19,20} Nevertheless, the importance of endogenous IL-2 in ILC2 function *in vivo* remains unclear. On the basis of their IL-2 responsiveness and demonstrated capacity for type 2 cytokine production, we hypothesized that ILC2s might play a role in ECP pathogenesis.

We observed that regardless of whether TCR transgenic T cells were present, Lin⁻CD90^{hi}CD2⁻KLRG1⁺ ILC2s were the

predominant CD25-expressing population in both normal and ECP-affected lungs (Fig 2, A, and data not shown).²¹ Furthermore, ILC2 numbers were increased in ECP-affected lesions (Fig 2, B and C), where they displayed an activated phenotype (Fig 2, D). To test whether type 2 cytokine production by lung ILC2s was increased in mice with ECP, we sorted ILC2s from ECP-affected tissue and normal lungs and measured *Il5* and *Il13* expression together with the ILC2-specific transcription factor *Gata3* using quantitative (real-time) PCR (qPCR). Sorted

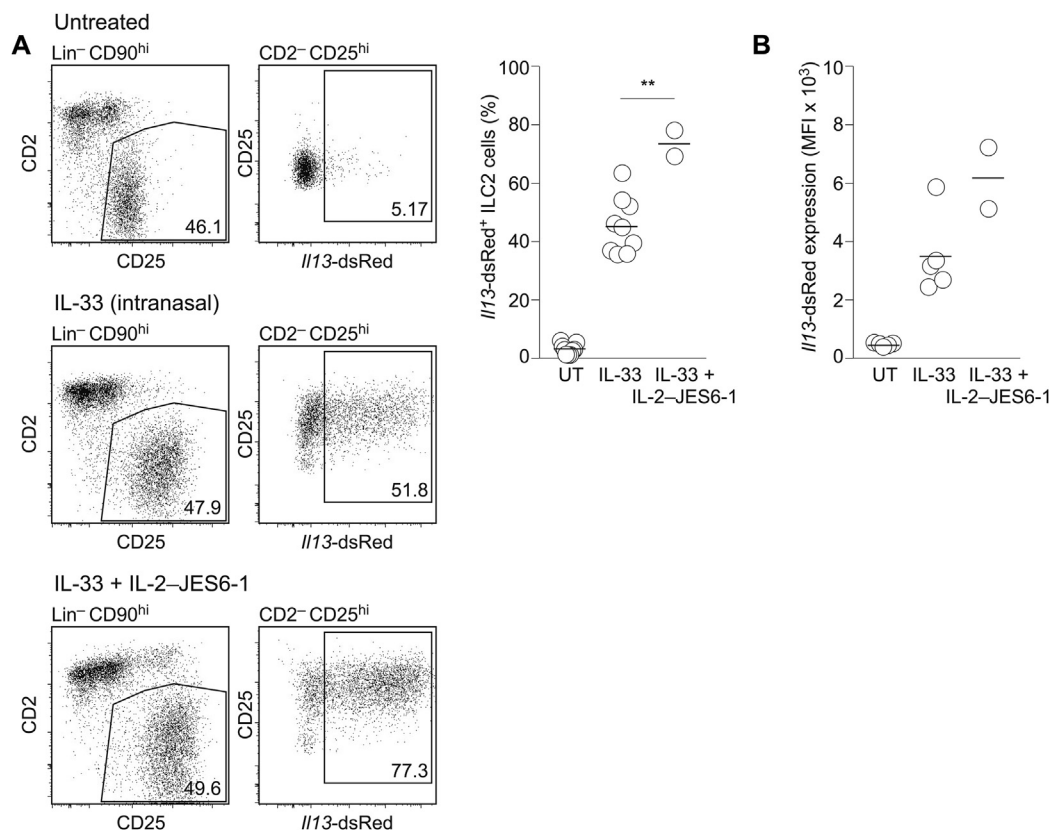


FIG 5. Effect of IL-2 on *I13* expression by ILC2s in response to IL-33. **A**, Lung ILC2s in 4C13R mice¹⁰ left untreated or challenged intranasally with IL-33 alone or in combination with treatment with IL-2. ***P* = .0038. **B**, Median fluorescence intensity of *I13*-dsRed by dsRed⁺ ILC2s isolated from the lungs as in Fig 5, A. UT, Untreated.

ILC2s from normal lungs constitutively expressed low levels of *I15* and *I13*, but expression of these genes was markedly increased in ILC2s sorted from ECP-affected tissue (Fig 2, E). In contrast, natural killer cells and Lin⁻ CD90^{hi} CD2⁺ cells, which include CD4⁺ and natural killer p46⁺ ILC3s (see Fig E3 in this article's Online Repository at www.jacionline.org), did not express *I15* or *I13* under any conditions (Fig 2, E). Collectively, these results are consistent with a central role for ILC2s in mice with ECP.

Exogenous IL-2 treatment promotes pulmonary ILC2 expansion, increased type 2 cytokine levels, and eosinophilic infiltrates in the lungs

To dissect the effects of IL-2 on lung ILC2 function *in vivo*, we treated *Rag1*^{-/-} mice with IL-2-JES6-1 antibody complexes every second day for 2 weeks, as described previously.^{10,22} In *Rag1*^{-/-} mice this protocol selectively expands ILC2s.¹⁰ After 2 weeks of IL-2 treatment, ILC2s in the lung were expanded approximately 100-fold (Fig 3, A).¹² To test whether this increase was due to local proliferation or enhanced tissue homing or retention, we used mice expressing FUCCI, in which cells in the S/G2/M phase express mAzami-Green.¹² RAG-sufficient FUCCI mice were treated with IL-2 every second day for 3 days before examination by using flow cytometry. Strikingly, 15% of ILC2s were mAzami-Green positive in the lung after IL-2 treatment (Fig 3, B), indicating that IL-2 drove lung ILC2 proliferation *in situ*.

Histologic examination of lungs from RAG-deficient mice treated with IL-2 revealed a significant leukocyte infiltrate in the lung parenchyma after 2 weeks of treatment that was predominantly comprised of lymphoid cells (Fig 3, C, and data not shown). Most of these cells adopted a perivascular distribution, with a lesser fraction associated with airway epithelial cells (Fig 3, C).

To visualize the microanatomic distribution of ILC2s *in vivo*, we treated *Rag1*^{-/-} *Cxcr6*^{gfp/+} mice with IL-2 and examined the lungs using confocal microscopy, as described in the Methods section in this article's Online Repository. ILC2s were identified as green fluorescent protein (GFP)-positive CD25⁺ cells based on their unique coexpression of these 2 markers *in vivo*.¹⁰ Consistent with the histologic results, lung ILC2s maintained a predominantly perivascular distribution after IL-2 treatment (Fig 3, D, and see Fig E4, A, in this article's Online Repository at www.jacionline.org). In addition, the GFP⁺ population in the lungs of *Rag1*^{-/-} *Cxcr6*^{gfp/+} mice included CD25⁻ CD2⁺ ILCs, numbers of which were also increased by IL-2 treatment (see Fig E4, B). Flow sorting followed by qPCR revealed that these CD2⁺ ILCs expressed both *Rorc* and *Tbx1* transcripts (see Fig E4, C), indicating that they included ILC3s, with a possible contribution of group 1 ILCs. Although we detected transcripts for the group 1 ILC-associated cytokine IFN-γ in CD2⁺ ILCs (*Ifng*; see Fig E4, C), IFN-γ protein levels were only marginally increased (1.6-fold) in IL-2-treated lungs (Fig 3, E), whereas the ILC3-associated cytokine IL-17 was unaffected (Fig 3, E). In contrast, levels of the type 2 cytokines

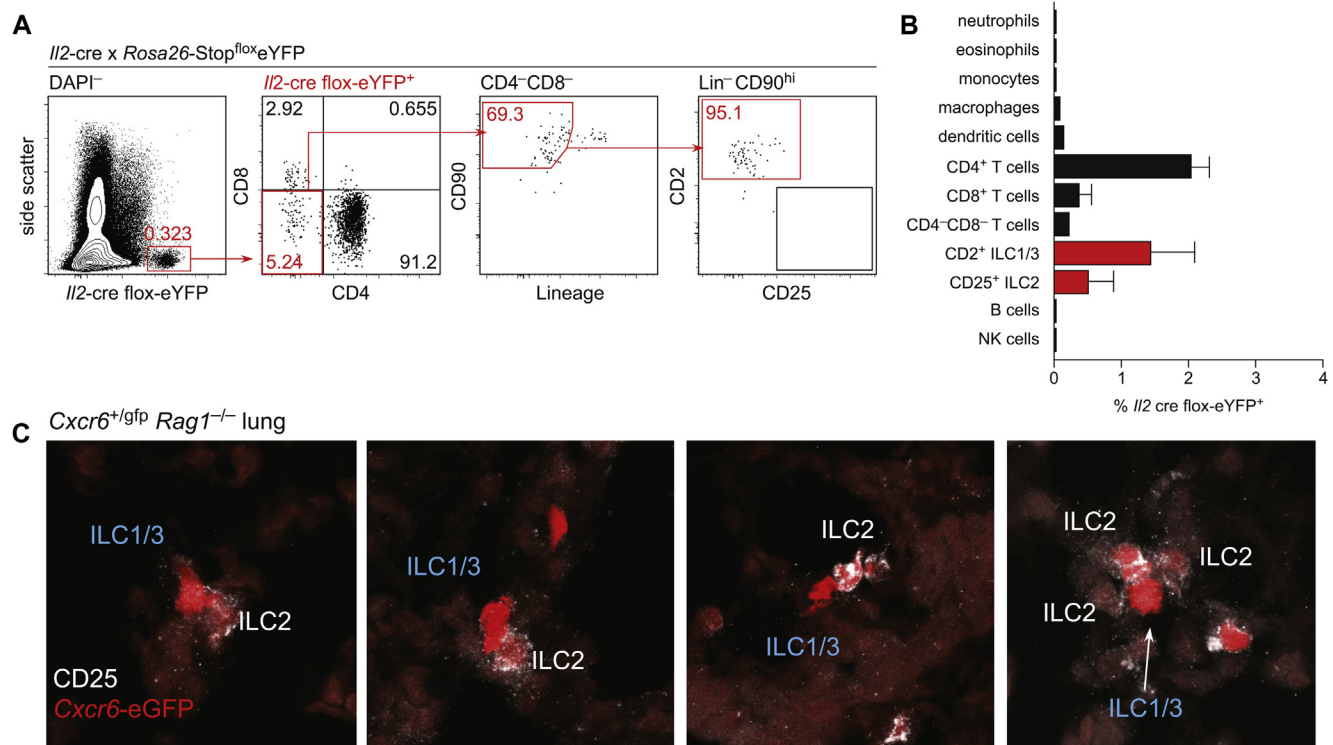


FIG 6. Source of innate IL-2. **A**, Flow cytometry of lung leukocytes from *I/2-cre* reporter mice.¹¹ **B**, Frequency of YFP⁺ cells in cells isolated from the lungs of *I/2-cre* reporter mice. **C**, Lung section from an untreated *Rag1^{-/-} Cxcr6^{+/gfp}* mouse stained for CD25 (white). GFP⁺ cells are shown in red. Data are representative of (Fig 6, A and C) or pooled from (Fig 6, B) 2 independent experiments.

IL-5 and IL-13 were increased approximately 100-fold in the lungs after IL-2 treatment (Fig 3, E).

Consistent with the increased levels of type 2 cytokines, increased numbers of eosinophils were observed in the bronchoalveolar lavage fluid and lungs of IL-2-treated mice (Fig 3, F, and data not shown), whereas alveolar macrophages expressed increased levels of *Arg1* and *Ym1* (Fig 3, G), which encode for the alternatively activated macrophage-associated proteins Arginase 1 and Ym1, respectively. Of note, some IL-2-treated mice had macroscopically visible lung consolidation, which shared many of the histopathologic features of ECP, including granulocyte infiltration, acidophilic and foamy macrophages, reactive epithelial cells with high nucleus/cytoplasm ratios, and crystals (Fig 3, H, and data not shown). Thus IL-2 treatment was sufficient to promote an inflammatory type 2 cytokine-rich milieu in the lung that was associated with increased ILC2, eosinophil, and alternatively activated macrophage numbers and a predisposition to the development of ECP-like pathology.

ILC2s do not express increased *I/13* message after IL-2 treatment

The increase in IL-13 and IL-5 protein levels in the lungs after IL-2 treatment paralleled the numeric increase in ILC2s. In contrast to ILC2s sorted from ECP-affected lungs, ILC2s sorted from the lungs of IL-2-treated mice did not express increased *I/13* relative to reference housekeeping genes (Fig 4, A). Relative *I/5* expression in ILC2s was similarly unaffected by IL-2 treatment (data not shown). These results suggest that the increased cytokine levels in IL-2-treated mice reflect the numeric

expansion of ILC2s constitutively producing a basal level of IL-13 and IL-5 rather than intrinsic changes in per-cell expression of cytokines. Consistent with this idea, lung eosinophil numbers in IL-2-treated mice correlated with ILC2 numbers (Fig 4, B; $r = 0.8270$, $P < .0001$), whereas in mice with ECP, the ratio of eosinophils to ILC2s was much higher (Fig 4, C).

It has been shown that optimal *I/13* production by T_H2 cells requires NF- κ B signaling,^{23,24} and the same might hold true for ILC2s.²⁵ Therefore we sorted lung ILC2s from ECP-affected and IL-2-treated mice and assessed them for nuclear translocation of p65, a central mediator of the canonical NF- κ B signaling pathway. In IL-2-treated mice less than 10% of ILC2s showed evidence of NF- κ B translocation (Fig 4, D, and data not shown). In contrast, nuclear translocation of NF- κ B was observed in more than 60% of ILC2s isolated from ECP-affected lungs. NF- κ B is critical for mediating signaling from IL-25 and IL-33,²⁵ epithelium-derived cytokines that have been shown to activate ILC2s *in vivo*.^{16,17} IL-33 levels were significantly increased in ECP-affected lungs (Fig 4, E) but remained unchanged in IL-2-treated mice (Fig 4, F). Similarly, epithelial cells displayed an activated phenotype in ECP-affected tissue in contrast to those in IL-2-treated lungs, which resembled cells from untreated control mice (Fig 4, G), except in cases of ECP-like lesions (Fig 3, H).

IL-2 augments IL-33-dependent *I/13* production by lung ILC2s

In addition to its role in proliferation, IL-2 has been shown to augment NF- κ B-mediated activation of type 2 cytokine genes in T_H2 cells through activation of signal transducer and activator of

transcription 5 (STAT5).²⁶ To test whether IL-2 could play a similar role in ILC2s *in vivo*, we tested whether IL-2 would augment ILC2 production of IL-13 in response to IL-33.^{16,17} To measure *in vivo* changes in *Il13* expression by ILC2s at the cellular level, we used the 4C13R dual reporter mouse in which transgenic expression of the cyan fluorescent protein AmCyan and red fluorescent protein dsRed indicates *Il4* production and *Il13* production, respectively, without affecting endogenous expression of these cytokines.¹⁰

We challenged 4C13R mice intranasally with IL-33 with or without (intraperitoneal) IL-2 treatment and examined lung ILC2s 24 hours later for changes in *Il13*-dsRed expression. In untreated mice a small percentage of ILC2s expressed *Il13*-dsRed in the steady state (Fig 5, A),¹⁰ which is consistent with our qPCR results (Fig 2, E). After challenge with IL-33, lung ILC2s dramatically upregulated their expression of CD25 and *Il13*-dsRed (Fig 5, A). In the presence of IL-2, both the percentage of *Il13*-dsRed⁺ ILC2s and the level of dsRed expression in positive cells were increased compared with IL-33 alone (Fig 5). Collectively, these results suggest that IL-2 alters the magnitude and duration of *Il13* expression by ILC2s *in vivo*, which is consistent with the predicted function of STAT5-mediated activation and stabilization of the type 2 cytokine loci.²⁵ We conclude that IL-2 alone is insufficient to induce *Il13* upregulation but is capable of enhancing the cytokine response by ILC2s to other external stimuli, such as IL-33.

Lung CD2⁺ ILCs are a major source of IL-2 *in vivo*

We and others have previously speculated that ILC2s likely respond to T cell–derived IL-2,^{10,18,19} the predominant source of IL-2 *in vivo*.^{11,27} However, because the development of ECP was IL-2 dependent but arose in RAG-deficient mice, our data suggested that ILC2s in the lungs might also be able to access a non-T cell–derived source of IL-2. A number of non-T-cell sources of IL-2 have been reported, including dendritic cells,²⁸ eosinophils,²⁹ and ILC3s.³⁰ However, *in vivo* data for these populations are mostly lacking. Although the IL-2 used by lung ILC2s could conceivably be derived from any organ, a number of lines of evidence suggested a local source. First, IL-2 protein was undetectable in the serum (data not shown). Second, most ECP-affected mice did not exhibit increased serum IL-5 or IL-13 levels, which might be expected in response to systemic IL-2 (see Fig E5 in this article's Online Repository at www.jacionline.org). Finally, we detected low levels of *Il2* mRNA (but not IL-2 protein) in both ECP-affected and unaffected lungs (data not shown).

Because *Il2* expression is usually transient,³¹ we sought to identify IL-2–producing cells *in vivo* using *Il2*-fate reporter mice, in which expression of yellow fluorescent protein (YFP) is an indicator of past or present *Il2* expression.¹¹ As previously reported,¹¹ the predominant YFP⁺ population in these mice was CD4⁺ T cells, with a lesser representation of CD8⁺ T cells (Fig 6, A).¹¹ Strikingly, the major YFP⁺ non-T-cell population was Lin[−]CD90^{hi}CD2⁺ ILCs (Fig 6, A and B), most likely ILC3s.³⁰ In contrast, all myeloid populations, including dendritic cells and eosinophils, were YFP[−] in these mice. Of note, in *Rag1*^{−/−} *Cxcr6*^{gfp/+} mice CD25⁺GFP⁺ ILC2s could be observed clustering with CD25[−]GFP⁺ (CD2⁺) ILCs in the lungs (Fig 6, C), indicating that ILC2s would likely have access to a local source of IL-2.

DISCUSSION

In this study we have uncovered an absolute requirement for IL-2 in the pathogenesis of ECP, an enigmatic type 2 lung disease. Further investigation identified a central role for ILC2s in the pathogenesis of this disease. *Il2*^{−/−} mice were completely protected from ECP, and increased numbers of activated IL-2–responsive ILC2s were observed in ECP lesions. Furthermore, expansion of lung ILC2s with exogenous IL-2 predisposed mice to the development of ECP-like lesions. Thus it appears that the IL-2–ILC2 axis represents an important pathway of type 2 cytokine–driven inflammation of the lungs.

Previous studies have implicated Ym1 protein, which is produced by macrophages in response to IL-4 and IL-13,¹³ as central to the pathogenesis of ECP.⁶ Overabundance of Ym1 leads to its crystallization, which might in turn trigger an inflammatory response by resident and recruited macrophages, possibly through activation of the inflammasome.³² Consistent with this hypothesis, ECP lesions have increased levels of IL-1β, a key downstream effector of inflammasome function (data not shown).

On the basis of our findings, we speculate that ILC2-derived IL-13 is important for the progression of ECP. Our histologic identification of reactive epithelial cells, combined with increased IL-33 expression, is consistent with activation of the now well-recognized epithelial cell–ILC2 axis³ and provides a mechanism that could underpin ECP progression, in which inflammation-driven activation of epithelial cells promotes IL-13 production by ILC2s, which in turn drives more production of Ym1 by macrophages (see Fig E6 in this article's Online Repository at www.jacionline.org). Although this model provides a basis for understanding the progression of lung consolidation in mice with ECP, we cannot yet explain the essentially Poissonian disease incidence, which suggests a relatively uncommon concurrence of factors that constitute the primary inflammatory “trigger” of ECP.

The requirement for IL-2 in ECP development in RAG-deficient mice implicated a non-T-cell source of this cytokine. Using *Il2* fate reporter mice, we identified the cellular sources of IL-2 in the lung, which, in addition to conventional T cells, include CD2⁺ ILCs, most likely corresponding to ILC3s.³⁰ How IL-2 production by ILC3s is regulated *in vivo* awaits investigation. *In vitro* human ILC3s have been shown to make IL-2 in response to Toll-like receptor 2 (TLR2) signaling.³⁰ TLR2 agonists include a wide range of exogenous and endogenous ligands, including respiratory syncytial virus,³³ the most common etiologic agent causing viral bronchiolitis in children. Whether TLR2 mediates IL-2 production by lung ILC3s during respiratory syncytial virus infection remains to be determined.

Mechanistically, we have demonstrated that IL-2 alone was insufficient to increase *in vivo* IL-13 production in ILC2s on a per-cell basis. Rather IL-2 signaling synergized with IL-33 to augment *Il13* expression by ILC2s, thereby serving as a potent cofactor for driving type 2 cytokine production in the lungs. These findings are consistent with those reported for other type 2 effector cells, including T_H2 cells and basophils, which require signaling through both NF-κB and STAT5 for optimal expression of type 2 cytokines.^{23,25} STAT5 translocation can occur in response to multiple upstream cytokine–cytokine receptor interactions, including IL-2, IL-7, IL-9, and thymic stromal lymphopoietin binding to their respective receptors (ie, CD25,

CD127, IL-9 receptor, and thymic stromal lymphopoietin receptor, respectively). Although ILC2s express receptors for all of these cytokines, our data suggest that optimal IL-13 production by lung ILC2s is nevertheless uniquely reliant on IL-2 under the conditions that predispose to ECP.

This study has highlighted the utility of studying ECP, a naturally occurring disease of immunodeficient mice, to identify and dissect the role of IL-2 in innate immune cell regulation. Because RAG-sufficient *I12^{-/-}* mice die prematurely of autoinflammatory disease, the IL-2 dependence of ILC2s *in vivo* could only be revealed through the investigation of RAG-deficient animals. Nevertheless, our findings might have important implications for understanding the pathogenesis of T cell-dependent diseases. In particular, the link between IL-2 and ILC2 function suggests that ILC2s might respond to T cell-derived IL-2 during lung inflammation, providing a novel means of cross-talk between T_H1 and T_H2 responses. They further raise the intriguing possibility that regulatory T cells, which are a major consumer of T cell-derived IL-2 *in vivo*, might control ILC2 activity in the lung by means of IL-2 availability. This might be relevant to the pathogenesis of allergic disease in human subjects and mice with deficiencies of regulatory T cells.³⁴⁻³⁶ An interplay between ILC2s and regulatory T cells might also be involved in viral bronchiolitis, in which reduced regulatory T-cell numbers are believed to contribute to the severity of the inflammatory pathology.³⁷⁻³⁹

Finally, we raise the possibility of cross-talk between ILC2s and ILC3s, both in terms of their physical proximity and the likely reliance of ILC2s on ILC3-derived IL-2 in the lungs. Spencer et al⁴⁰ have previously reported an inverse relationship between ILC2 and ILC3 numbers in the context of vitamin A deficiency, likely reflecting changes in ILC lineage choices in the bone marrow and competition for IL-7 in the periphery. However, direct communication between the 2 cells has not previously been proposed.

In conclusion, our results shed new light on the multifaceted role of IL-2 *in vivo*, which might have important implications for the pathogenesis of bronchial asthma and other type 2 cytokine-driven immune responses in the lung. They also might explain why increased serum IL-5 levels and eosinophilia are invariably observed in patients treated with IL-2.⁴¹

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Key messages

- IL-2 is a key driver of pathogenesis in mice with ECP.
- ILC2 populations are expanded and activated in mice with ECP.
- IL-2 signaling promotes proliferation of ILC2s in the lung.
- IL-2 enhances the cytokine response by ILC2s to IL-33 *in vivo*.

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METHODS

Antibodies

Fluorochrome-conjugated antibodies to the following cell-surface molecules were used: CD2 (RM2-5), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11c (HL3), CD11b (M1/70), CD25 (PC61), CD45 (30-F11), B220 (RA3-6B2), CD69 (H1.2F3), CD90.2 (53-2.1), Ly6C/G (RB6-8C5), MHCII (2GB), natural killer (NK) 1.1 (PK136), NKp46 (29A1.4), Siglec-F (E50-2440; all from BD Biosciences), EpCAM (G8.8), F4/80 (BM8), KLRG1 (2F1), and Ly6G (1A8; all from BioLegend, San Diego, Calif). For intracellular staining of forkhead box protein 3 (Foxp3), cells were first stained with mAbs directed against the surface molecules of interest and then fixed, permeabilized, and stained with a murine Foxp3 staining kit (eBioscience, San Diego, Calif) and the anti-Foxp3 mAb FJK-16s (eBioscience), according to the manufacturer's instructions. For flow cytometry, cell suspensions were resuspended in running buffer containing 0.5 $\mu\text{g/mL}$ 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Life Technologies, Eugene, Ore) for exclusion of dead cells.

For intracellular NF- κ B staining, ILC2s were sorted from ECP-affected lesions and from IL-2-treated mice before fixation and permeabilization (Cytofix/Cytoperm kit, BD Biosciences). Cells were then stained in suspension with rabbit anti-NF- κ B (p65 subunit, N-terminus; Cell Signaling Technology, Danvers, Mass), followed by anti-rabbit Alexa Fluor 647 and DAPI counterstaining to identify nuclei.

Primers

Quantitative PCR was done with TaqMan Gene Expression Master Mix and the following TaqMan assays (Life Technologies assay identifier in parentheses): *Gapdh* (Mm99999915_g1), *Actb* (Mm00607939_s1), *Hprt* (Mm00446968_m1), *Gata3* (Mm00484683_m1), *Il5* (Mm00439646_m1), *Il13* (Mm00434204_m1), *Arg1* (Mm00475988_m1), *Chi3l3* (*Ym1*) (Mm00657889_mH), *Rorc* (Mm01261022_m1), *Tbx21* (Mm00450960_m1), *Ifng* (Mm01168134_m1), and *Il2* (Mm00434256_m1).

Confocal microscopy of the lungs

For histologic staining of CD25, killed *Rag1*^{-/-} *Cxcr6*^{gfp/+} mice were perfused with PBS before perfusion with 10% formalin. The lungs were filled *in situ* with Tissue-Tek optimal cutting temperature (OCT) compound (Sakura Finetek, Torrance, Calif) through the trachea by using a 28-gauge needle with a blunted bevel. Lungs were then embedded in OCT compound in vinyl cryomolds, snap-frozen, and stored at -70°C until sectioning. Five- to 10- μm sections were placed onto poly-L-lysine-coated slides, air-dried for 60 minutes, and fixed in cold (4°C) formalin for 20 minutes. Sections were then blocked with blocking buffer (10% FCS in PBS) for 60 minutes at room temperature before staining with purified anti-CD25 (clone PC61) for 60 minutes at room temperature. Sections were counterstained with anti-rat Alexa Fluor 647 (Life Technologies) for 60 minutes at room temperature. Images were acquired on a Leica TCS SP5 confocal microscope system (Leica, Wetzlar, Germany) and processed with ImageJ software (National Institutes of Health, Bethesda, Md).

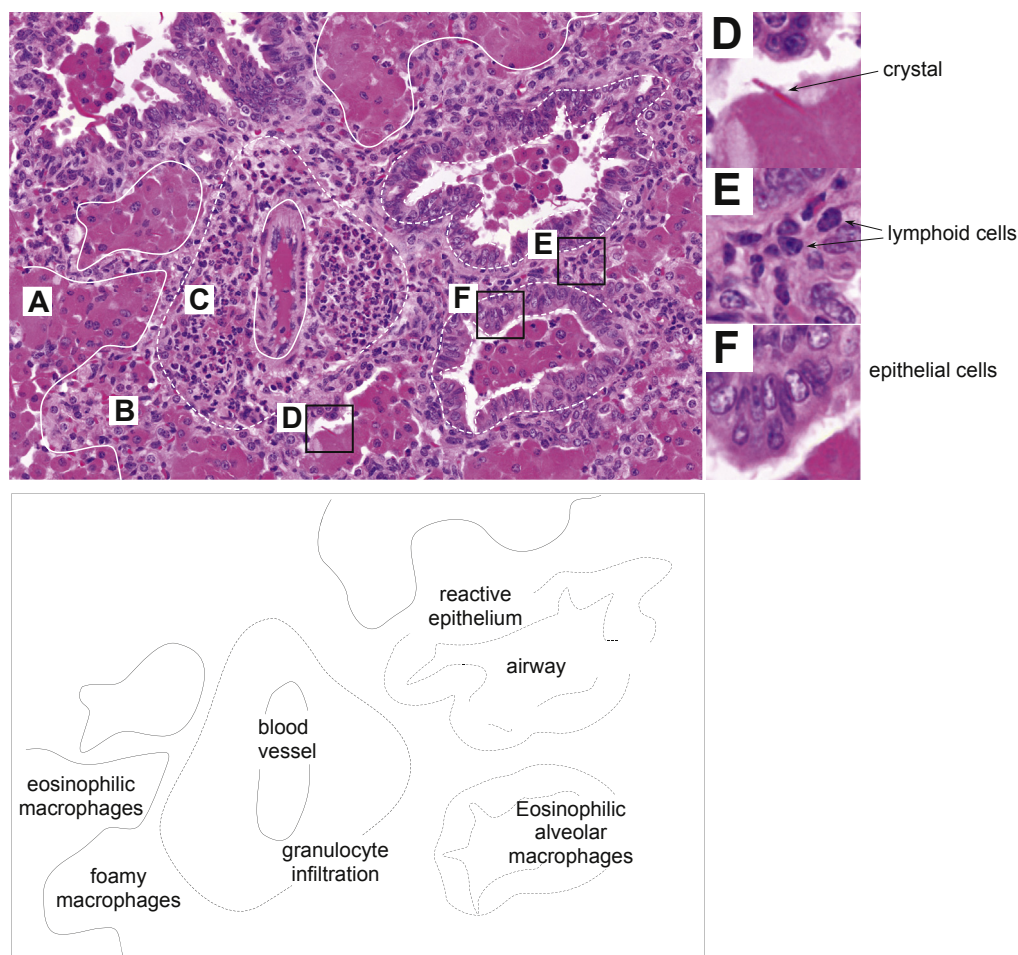


FIG E1. Pathologic characteristics of ECP. *Top*, Lung section from a 179-day-old *Rag1*^{-/-} mouse with ECP stained with hematoxylin and eosin (×400 magnification). *Bottom*, Annotated schematic of lung section. **A**, Eosinophilic macrophages. **B**, Foamy macrophages. **C**, Perivascular infiltration of granulocytes. **D**, Eosinophilic crystal. **E**, Lymphoid cells. **F**, Reactive epithelial cells.

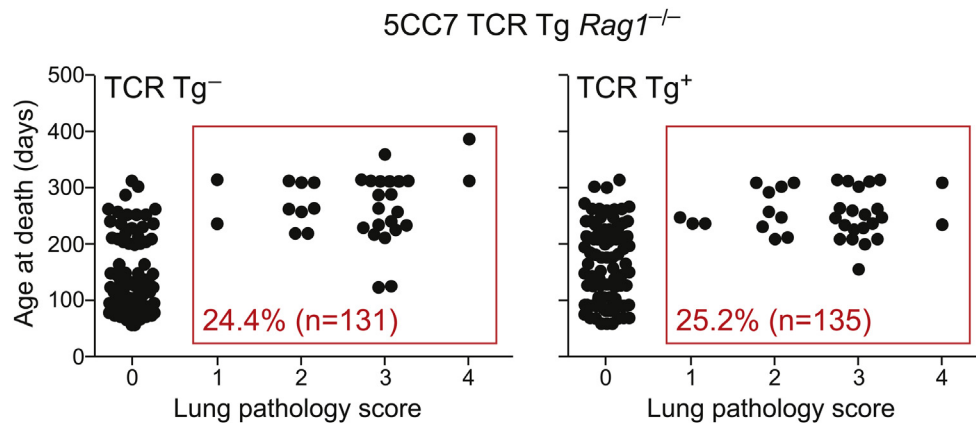


FIG E2. ECP in TCR transgenic (*Tg*) mice. Incidence of ECP in 5C.C7 TCR transgenic *Rag1*^{-/-} mice. Mice have been subgrouped into those that carry the TCR transgene (*TCR Tg*⁺) and those that do not (*TCR Tg*⁻).

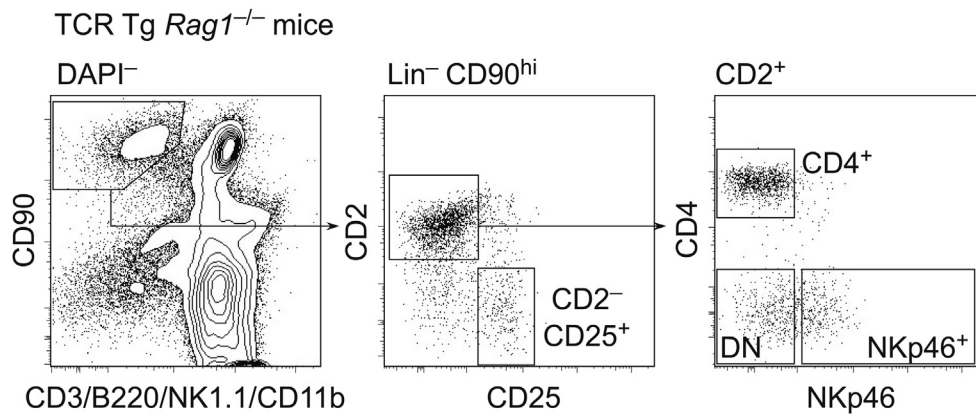
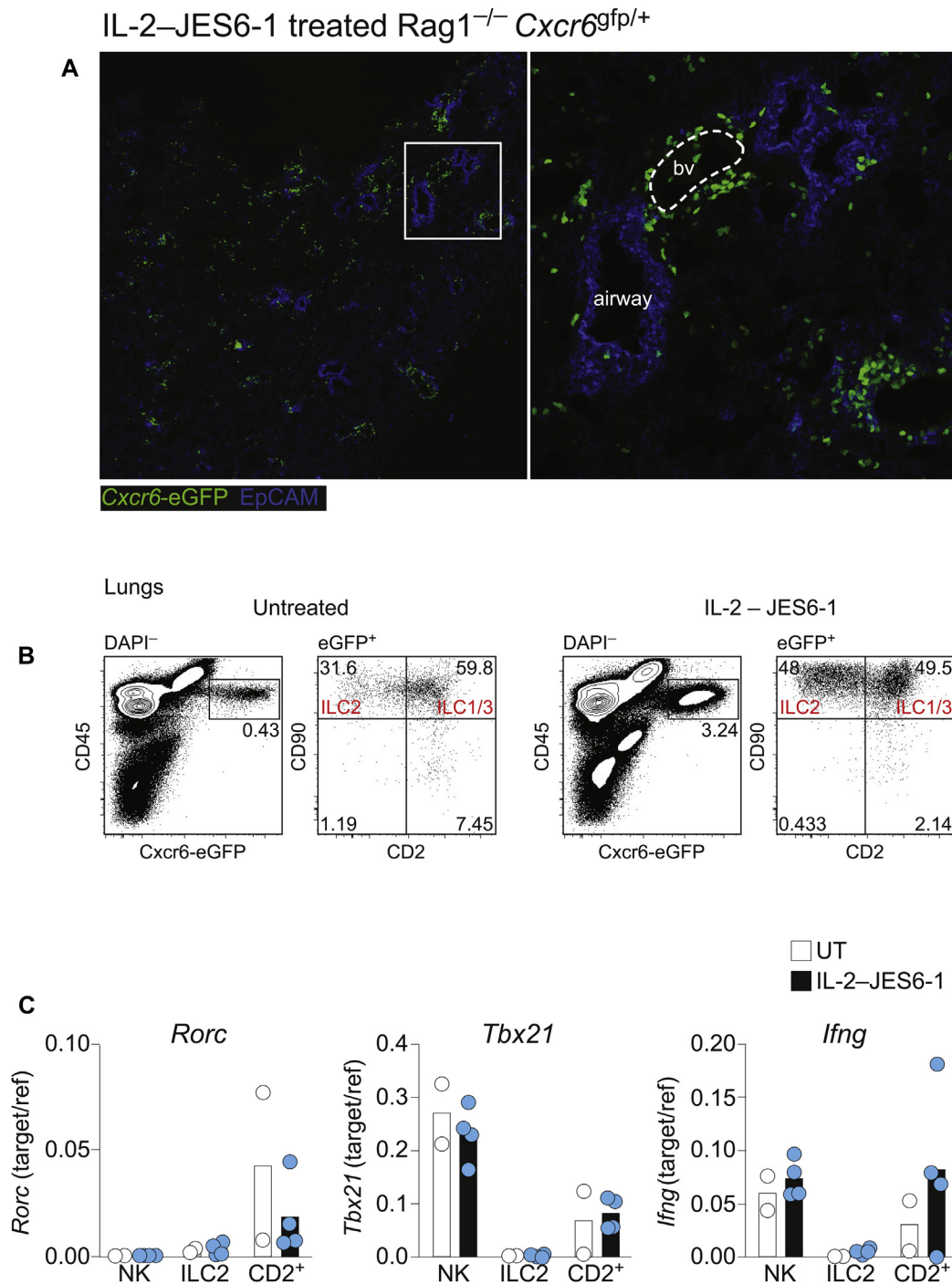


FIG E3. Cell-surface phenotype of lung ILCs. Results of flow cytometry of leukocytes isolated from lungs of 5C.C7 TCR Tg⁺ *Rag1*^{-/-} mice is shown.



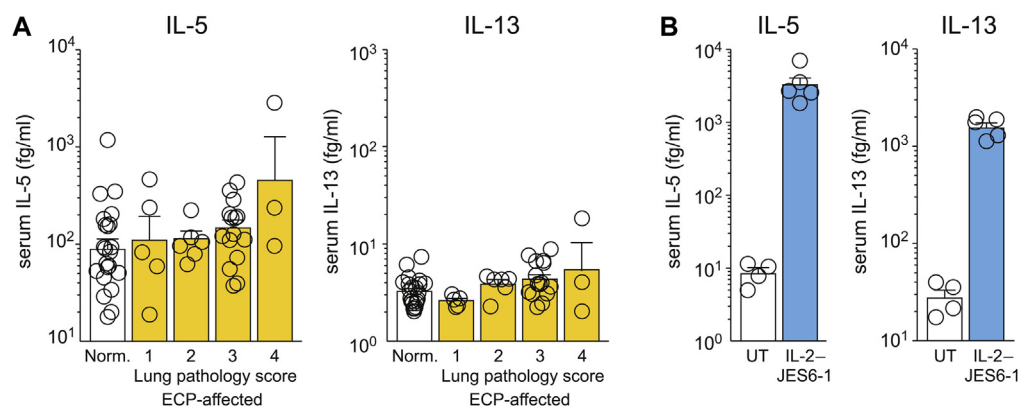


FIG E5. Serum cytokines in ECP-affected and IL-2-treated lungs. **A**, Concentration of cytokines in the sera of more than 170-day-old normal (white bars) and ECP-affected (orange bars) mice. **B**, Concentration of cytokines in the sera of young (<85 days old) *Rag1*^{-/-} mice left untreated (white bars) or treated with IL-2-JES6-1 for 2 weeks (blue bars). Each circle in Fig 5, A and B, represents an individual mouse. UT, Untreated.

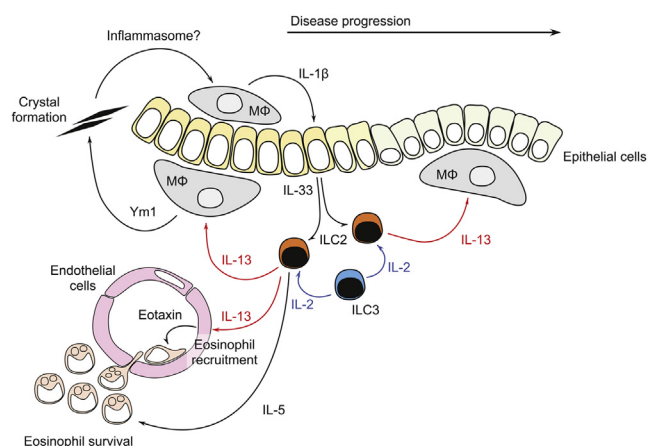


FIG E6. Proposed model of disease progression in the setting of ECP. IL-2-dependent ILC2 production of IL-13 drives both Ym1 production by macrophages and eosinophil recruitment to the lung. Crystallization of Ym1 triggers inflammasome activation and IL-1 β production by resident and recruited macrophages, which in turn activates epithelial cells to produce IL-33. Epithelial cell-derived IL-33 further drives ILC2 activation, establishing a positive feedback loop that promotes the expansion of ECP-affected lesions.