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ICOS protects against mortality from acute lung injury through activation of IL-5+ ILC2s

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a progressive lung disease causing irreversible lung scarring and loss of pulmonary function. IPF Patients suffer from a high rate of pulmonary infections and acute exacerbations of disease that further contribute to pulmonary decline. Low expression of the inducible T-cell costimulatory molecule (ICOS) in peripheral blood mononuclear cells predicts decreased survival of IPF patients, but the mechanisms by which ICOS protects are unclear. Using a model of bleomycin-induced lung injury and fibrosis, we now demonstrate that ICOS expression enhances survival from lung injury rather than regulating fibrogenesis. Of ICOS expressing cells, type 2 innate lymphocytes (ILC2s) are the first to respond to bleomycin-induced injury, and this expansion is ICOS-dependent. Interestingly, a similar decrease in ICOS+ ILCs was found in lung tissue from IPF patients. IL-5, produced primarily by ILC2s, was significantly reduced after lung injury in $ICOS^{-/-}$ mice, and strikingly, treatment with IL-5 protected both $ICOS^{-/-}$ and wild type mice from mortality. These results imply that low ICOS expression and decreased lung ILC2s in IPF patients may contribute to poor recovery from infections and acute exacerbation, and that IL-5 treatment may be a novel therapeutic strategy to overcome these defects and protect against lung injury.

Introduction

Nearly 50,000 Americans are diagnosed each year with idiopathic pulmonary fibrosis (IPF), a progressive disease with a median survival of 3 to 5 years. The etiology of IPF is thought to be due to repeated pulmonary epithelial injury, aberrant tissue repair, and excessive

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accumulation of extracellular matrix in the lung parenchyma.[1-3] While two recent FDA-approved treatments slow the loss of pulmonary function by targeting fibrogenesis, no treatments exist to reverse disease course, and lung transplant is often indicated as a final treatment option. The role of the immune system in IPF remains controversial. Recent clinical studies found that suppressing immune function in IPF patients was associated with increased mortality, suggesting a beneficial role for the immune system.[4] Further, during acute exacerbations of IPF, steroid treatment can adversely affect IPF patients and lead to decreased survival.[5] We recently reported that T cells are increased in the lungs of IPF patients, and that subsets expressing specific chemokine receptors are associated with improved lung function.[6] Gene expression profiles in peripheral blood mononuclear cells from IPF patients also have implicated a role for T cells in protection.[7] In particular, low expression of the inducible T-cell costimulatory molecule (ICOS) in peripheral blood mononuclear cells predicted decreased long-term transplant-free survival. Thus, a role for the immune system in IPF etiology or pathology is emerging.

ICOS was first identified as a Th2 cell specific costimulatory receptor[8], and $ICOS^{-/-}$ mice have defects in type 2 inflammation.[9-11] $ICOS^{+}$ cells resident in the lung include ILC2s, invariant natural killer T cells (iNKTs), and CD4 T cells.[12-14] These cells produce the cytokines IL-4, IL-5, and IL-13 associated with atopic diseases. Studies utilizing bleomycin to induce lung fibrosis in mice found that both type 17 and type 2 responses are associated with TGF-β production and subsequent fibrosis.[15] The type 17 immune response is characterized by neutrophilia that leads to lung damage from reactive oxygen species, proteinases, and MMPs. The type 2 immune response in pulmonary fibrosis is associated with M2 macrophage polarization, fibroblast activation, and collagen deposition. Since ICOS+ lymphocytes are associated with type 2 responses and fibrogenesis, why low ICOS expression in IPF patients is associated with worse outcomes is unclear.

Using bleomycin to model acute lung injury-induced fibrosis, we now demonstrate that ICOS expression is required for protecting against lung injury-induced mortality, but does not regulate fibrogenesis. Further, ICOS expression is required for IL-5 production in the lungs post-injury, and this IL-5 is produced primarily by ILC2s. Exogenous IL-5 delivered to the airways of $ICOS^{-/-}$ mice was sufficient to protect from lung injury-induced death, and importantly, IL-5 treatment was also effective in reducing mortality in wild type (WT) mice that received two-fold higher doses of bleomycin. Notably, lung injury-induced edema was greatly reduced when $ICOS^{-/-}$ mice were treated with exogenous IL-5. These findings suggest a mechanism whereby IL-5 production from ILC2s protects mice from lung injury. As acute exacerbations of IPF are associated with lung injury, IL-5 may be a novel therapeutic target for preventing exacerbation-induced decline in IPF patients.

Results

ICOS-deficient mice have an impaired ability to survive the early lung injury phase induced by bleomycin challenge

To address the role of ICOS in pulmonary fibrosis, we compared $ICOS^{-/-}$ mice to WT mice using a model of bleomycin-induced lung injury. Strikingly, compared to WT mice, $ICOS^{-/-}$ mice exhibited dramatically reduced survival and increased loss of body weight (Fig. 1A,B).

When only the ICOS$^{-/-}$ mice that survived until day 18 after challenge were analyzed, they also showed increased weight loss compared to WT mice (Fig. 1C), yet had no difference in lung fibrosis or collagen deposition (Fig. 1D,E). Mice heterozygous for ICOS were protected from weight loss despite their intermediate ICOS expression on lung lymphocyte populations (Supplemental Fig. 1).

We suspected that early mortality in ICOS$^{-/-}$ mice was caused by increased acute lung injury, as these mice lost weight within 5 days of challenge whereas WT mice exhibited little weight loss. We observed that ICOS$^{-/-}$ mice had increased RBCs in the airways on day 5 (Fig. 1F,G), indicating greater lung edema, a hallmark of lung injury. Interestingly, no difference was observed in lung inflammation in histology sections or in BAL protein levels (Fig 1H,I). Consistent with our finding of increased edema in ICOS$^{-/-}$ mice, increased pulmonary vascular permeability was also observed (Fig. 1J). Finally, lung neutrophil numbers were significantly increased when measured by flow cytometry (Fig. 1K). Together, these changes indicate a specific defect in ICOS$^{-/-}$ mice that leads to enhanced vascular leakage and neutrophilia after bleomycin challenge.

To determine whether ICOS played an essential role in other lung injury models, PR8 influenza infection and LPS airway challenge were examined in the ICOS$^{-/-}$ mice. Similar to the bleomycin model, ICOS$^{-/-}$ mice had increased weight loss and mortality in response to lung injury induced by influenza infection, with the majority of mice succumbing by day 10 post-infection (Supplemental Fig. 2).

Similarly, LPS instillation in the airways led to increased weight loss in ICOS$^{-/-}$ mice compared to WT at 48 hr (Supplemental Fig. 3). Together, these data suggest an important role for ICOS in protection of mice in three separate lung injury models, but not a direct role for ICOS in alleviating collagen deposition and fibrosis.

Innate ICOS-expressing cells are reduced in the lungs of ICOS$^{-/-}$ mice after bleomycin challenge

To determine the mechanisms by which ICOS protects from early mortality, we first examined cellular infiltrates in the lung tissue at 3 days post-challenge. Total isolated cell numbers were not different in the mouse strains, nor where the percent of alveolar macrophages, neutrophils, or eosinophils at this early time point (Fig. 2A), although neutrophils were increased in ICOS$^{-/-}$ mice by day 5 (Fig. 1K). Next we assessed the contributions of ICOS-expressing cell types. As previously reported[12, 13], we found that untreated ICOS$^{-/-}$ mice had fewer ILC2s and iNKTs in the lungs (Supplementary Fig. 4). In WT mice, bleomycin induced an expansion of ILC2 cells 3 days after challenge, but ILC2s from ICOS$^{-/-}$ mice failed to expand until day 7 (Fig. 2B,C). In contrast to ILC2s, the proportion of iNKTs did not increase in ICOS$^{+/+}$ mice until day 7 after challenge, an increase not observed in ICOS$^{-/-}$ mice (Fig. 2D). CD4 T cells were found at a similar percentage and number in both mouse strains (Fig. 2E), although the proportion identified as FoxP3+ regulatory T cells (Tregs) was only increased in WT mice (Supplemental Fig 5A). Surprisingly, bleomycin also induced increased ICOS expression on ILC2 cells over time, but not on iNKTs or CD4 T cells (Fig 2F, Supplemental Fig 5B). In addition, we found an increase in ICOSL on alveolar macrophages and monocyte-derived macrophages over time

in both WT and ICOS$^{-/-}$ mice, suggesting a role for macrophages in early ICOS signaling (Supplemental Fig 6). Thus, ICOS-dependent ILC2s, and not iNKTs or T cells, are the first ICOS-expressing cells to expand during the early inflammatory phase after bleomycin challenge. Further, the failure of iNKTs and Tregs to expand at day 7 after lung injury may contribute to the poor recovery of the ICOS$^{-/-}$ mice.

To determine whether ICOS-expressing innate cells might contribute to human disease, we obtained lung tissue from 9 IPF patients at the time of lung transplant. Control lungs were obtained through the Gift of Hope Organ Bank of Illinois from organ donors with no known lung disease. A group of 13 control lungs were selected to match the IPF patients based on age, sex, race, and smoking history (Table 1). As previously shown by others, we found that IPF lungs had significantly increased eosinophils (measured by histologic counts) compared to the control lungs (39.25 +/−29.93 eos/field in IPF patients vs. 2.1+/−1.7 in controls; $P<0.0001$). We compared the percent of ICOS$^+$ ILCs (lin$^-$ CD127$^+$ ICOS$^+$) or ICOS$^+$ iNKTs (CD3$^+$CD56$^+$ICOS$^+$) between IPF lungs and the non-fibrotic donor lungs, and found that explanted IPF lungs contained lower percentages of ICOS$^+$ ILCs compared to control lungs, but comparable percentages of ICOS$^+$ iNKTs (Fig. 3A-C). Based on our findings in mice, it is possible that the decrease in ICOS-expressing innate cells in the IPF lungs may play a role in the recovery of IPF patients from lung injury during acute exacerbations.

IL-5, produced primarily by ILC2s, is reduced in ICOS$^{-/-}$ mice at day 3 post-bleomycin challenge

To determine the function of ICOS$^+$ cells in protection from lung injury-associated death, we examined their cytokine profiles after bleomycin challenge. We found that day 3 IL-5 expression in the lungs of mice was dependent on ICOS, although IL-13 was unaffected (Fig. 4A,B). The majority of IL-5-producing lung cells were ILC2s, rather than iNKTs or CD4 T cells (Fig. 4C). Total IL-13+ cell percentages and numbers were not reduced in ICOS$^{-/-}$ mice (Fig. 4B,D). While the ILC2 did produce IL-13, they were not the major source of IL-13$^+$ cells in the lungs. Thus, the decrease in ILC2s did not affect the total number of lung IL-13$^+$ producing cells after bleomycin challenge. Interestingly, the loss of ICOS did not affect the cell-intrinsic ability of ILC2s to produce IL-5 or IL-13 (Fig. 4E), it only affected the absolute number of ILC2s. However, the overall decrease in ILC2 at day 3 (Fig 2C) led to reduced numbers of total IL-5+ ILC2s and IL-13+ ILC2s (Fig. 4E-F). The defect in IL-5$^+$ cells in the ICOS$^{-/-}$ mice was gone by day 7, suggesting that early, not late, ICOS-dependent IL-5 production was key to the protection from lung injury induced death in the WT mice (Supplemental Fig. 7). ICOS-dependent variation in CD4 T cell cytokines was observed at day 7, but these differences were independent of bleomycin challenge (Supplemental Fig. 8). Thus, ICOS$^{-/-}$ mice produce less IL-5 early after bleomycin challenge primarily due to loss of total ILC2s.

Treatment with exogenous IL-5 protects both ICOS$^{-/-}$ and WT mice from bleomycin-induced mortality

As IL-5-producing ILC2 expansion was associated with protection from lung injury, we speculated that IL-5 could be a protective effector cytokine during the early response. Strikingly, when ICOS$^{-/-}$ mice were treated with IL-5 at the time of bleomycin challenge,

they were protected from death despite similar weight loss (Fig. 5A). Protection against death was lost when treatment was started 24 hours after bleomycin challenge (Fig. 5B). These data indicate that IL-5 alone is sufficient to protect ICOS$^{-/-}$ mice from lung injury-induced death when treated at the time of injury. While our findings in ICOS$^{-/-}$ mice were dramatic, lung injury in humans occurs in patients that express ICOS. To determine the therapeutic potential of IL-5 to treat lung injury, we challenged WT mice with a higher dose of bleomycin that induced 80% mortality. Surprisingly, WT treated with IL-5 at the time of bleomycin challenge had only 20% mortality that occurred at a later time point (Fig. 5C). Treatment starting 24hrs post- bleomycin challenge had no effect on survival (Fig 5D). We found that IL-5 treatment caused a transient increase in total cells, including eosinophils, in the BAL at day 3 post- bleomycin, but by day 5 the BAL numbers and eosinophilia was similar in both groups (Fig. 6A,B). Importantly, mice receiving IL-5 treatment had reduced RBCs in the airways indicating a reduction in pulmonary edema (Fig. 6C-E), suggesting IL-5 acts to suppress early vascular leakage and reduce the effects of bleomycin induced-lung injury. In both ICOS$^{-/-}$ and WT mice, IL-5 treatment had no effect of the number of Tregs recruited to the lungs on day 7 (Supplementary Fig. 9). These data suggest that IL-5 has potential therapeutic effects in bleomycin-induced lung injury independent of the presence of functional ILC2s, Tregs, or endogenous IL-5 production.

Discussion

Our study demonstrates that early innate type 2 immune responses, driven by cells expressing ICOS, protect against lung injury-associated death. We found that ICOS$^{-/-}$ mice have increased mortality in two models of lung injury. Further, they had increased edema and lung permeability as early as 5 days post-challenge, suggesting that ICOS expression is important for maintaining barrier function in the lungs. IL-5+ ILC2s expanded quickly after lung injury in WT mice, but not ICOS−/− leading to an overall defect in IL-5+ cells in the lungs. We now demonstrate that IL-5 is a critical ILC2 product that protects mice from lung injury-associated death. In fact, IL-5 was sufficient to improve survival in WT mice where ICOS signaling and ILC2 responses are intact. IPF patients also had fewer ICOS^{+} ILCs in their lungs at the time of transplant suggesting that ICOS^{+} innate lung cells may impact human disease as well.

Our mouse studies revealed that the protective role of ICOS is driven not by attenuation of fibrosis, but by protection during the lung injury phase. While the type 2 response may have pathogenic effects in lung fibrosis, mostly through IL-13[16,17], activation of the type 2 pathway is also critical for effective tissue repair after lung damage. Previous studies have shown ILC2s produce tissue-reparative factors such as amphiregulin[18, 19], IL-4[20], and IL-13, but interestingly, we found ILC2s produced little of the total IL-13 measured in the bleomycin model. Our findings now argue for a novel role for IL-5, produced primarily by ILC2s, in preventing lung injury or mediating repair. In fact, our data that 1) IL-5 treatment must start concurrent with the lung injury, and 2) IL-5 reduced RBC influx into the airways, leads us to speculate that prevention of lung injury is the primary function of IL-5 in this model. The mechanisms by which IL-5 protects from lung injury remain unknown.

The IL-5 receptor is expressed on eosinophils, B1 B cells, monocytes, and neutrophils[21-23]. Eosinophils in particular have been shown to have immunoregulatory function and to produce anti-inflammatory cytokines including IL-10 and TGF-β, and the immunoregulatory molecule indoleamine 2,3-dioxygenase (IDO).[24-26] While eosinophils have been shown to be increased in IPF lungs, IL-5 levels were not elevated in BAL fluid.[27, 28] Eosinophils can be either tissue-resident with anti-inflammatory function or recruited during inflammation and have pro-inflammatory function[29]. We expect that IL-5 may act on eosinophils as well as other cell types shown to express the IL-5 receptor, such as neutrophils and monocytes. A role of IL-5 on monocytes was recently reported in sepsis, and IL-5 was able to protect mice from cecal-ligation-induced sepsis in an eosinophil-independent manner[22]. Therefore, monocytes are also a potential target of IL-5 that may mediate protection from lung injury-associated death. Neutrophils have been shown to be a major player in lung injury, however how IL-5 may affect these cells remains unstudied. Additionally, it is possible that IL-5 could act directly on structural cells in the lungs, such as epithelium or endothelium, to reduce permeability. Understanding the mechanisms by which IL-5 protects against lung injury-induced mortality in mice will provide essential knowledge about how the immune system safeguards lungs against irreversible damage.

The role of lung injury in IPF is not completely understood, but repeated damage to pulmonary epithelium has been implicated.[15] Acute exacerbations are a major cause of morbidity and mortality of IPF patients. Although acute exacerbations often occur without identifiable cause, lung injury has been proposed to be a factor in the decline of IPF patients after acute exacerbations.[30, 31] Interestingly, corticosteroid treatment in IPF patients during acute exacerbations contributed to increased mortality, and a clinical trial using immune suppressants in patients was terminated due to worse outcomes in the treatment group.[4, 5] These trials, in combination with our current data, suggest that IPF patients may benefit from increased immune activation through an ICOS-dependent pathway or therapeutic use of IL-5. In our model, one treatment with IL-5 at the time of injury was sufficient for protection, suggesting that IL-5 has lasting downstream effects. We expect that IL-5 treatment would be a benefit during acute exacerbations, when pulmonary function is reduced due to a lung injury event.

IL-5 treatment may broadly benefit recovery from other types of lung injury. A variety of factors, including respiratory infections and sepsis, can result in lung injury that leads to Acute Respiratory Distress Syndrome (ARDS). ARDS is characterized by lung edema and hypoxemia, and currently has no specific therapy[32]. Bleomycin is widely used as a model of ARDS, and therefore our findings may also be applicable to ARDS and related syndromes.[33] Our data that $ICOS^{-/-}$ mice die early during influenza infections supports the hypothesis that ICOS deficiency is a risk factor for lung injury-related mortality. Interestingly, ILC2s expand during early infection with influenza, and are associated with viral clearance and recovery.[18, 34] Thus, our findings may have important implications for the treatment of ARDS and other forms of acute lung injury.

Methods

Human lung procurement from donors and IPF patients

Lung tissue specimens were collected from 9 IPF patients who underwent lung transplantation at the University of Chicago from August 2013 to May 2015. IPF diagnosis was established by a multidisciplinary group of pulmonologists and dedicated chest pathologists, and radiologists in concordance with the American Thoracic Society/European Respiratory Society criteria, and in agreement with recent guidelines[30]. IPF patients were excluded at study entry if they had clinical or serological markers of an autoimmune disease including rheumatoid arthritis, malignancies, active infection, and drug exposure or occupational exposures known to cause interstitial lung disease. The study is approved by the University of Chicago Institutional Review Board and all transplant patients signed informed consent. All IPF patients had severe hypoxemia but were stable, uninfected, and called from home to receive their transplant surgery. Control lungs were obtained through the Gift of Hope Regional Organ Bank of Illinois and processed within 48hr of organ clamp time. The University of Chicago IRB has determined that use of human donor lungs that could not be transplanted does not constitute human subjects research and is exempt from IRB oversight.

Isolation of human lung leukocytes

To obtain lung leukocytes from IPF or control lungs, right lower lobes were perfused with 150-200mL sterile 2% FBS/PBS using a 50mL syringe. Tissue was minced using dissection scissors to an approximate size of $2mm^3$. Minced tissue was digested in RPMI media containing 10% FBS, 120 Units/mL Collagenase D (Sigma), and 2 μg/mL DNase I (Worthington Biochemical Corporation) at 37C for 90m. Mononuclear cells were enriched by gradient centrifugation using Histopaque-1077 (Gibco). Isolated leukocytes from IPF and control lungs were cryopreserved until analysis.

Mice

C57BL/6 mice (WT) were purchased from Harlan Laboratories. $ICOS^{-/-}$ ($B6.ICOS^{-/-}$) mice were a generous gift of Dr. Flavell[35]. $ICOS^{+/+}$, $ICOS^{+/-}$, and $ICOS^{-/-}$ littermates were generated from WT × $B6.ICOS^{-/-}$ parents. All animal procedures and housing were approved by the University of Chicago Animal Resources Center. The studies conformed to the principles set forth by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Cellular analysis and flow cytometry

Perfused mouse lungs were dissociated by mechanical mincing followed by digestion with 150 U/mL Collagenase D (Gibco) in 10 ml DMEM for 1 hr. Samples were then washed and counted. For phenotyping, 5×10^5 cells were suspended in 50 μl of buffer (PBS containing 0.1% sodium azide and 0.2% BSA) and block using 2.4G2 (anti-CD16/32) and then stained with surface antibodies. To measure intracellular cytokine production, 1×10^6 lung cells/mouse were stimulated with Phorbol myristate acetate (10ng/mL) and Ionomycin (500ng/mL) for 30 min followed by Brefeldin A (1ug/mL) for an additional 4-5 hr prior to

staining. Isotype controls or no Brefeldin A controls were used for all cytokine staining. For intracellular staining, cells were fixed with 1% paraformaldehyde and permeabilized with 0.5% saponin in PBS. Flow antibodies were purchased from Biolegend, eBioscience, and BD Biosciences, and iNKTs were identified using PE-conjugated CD1d^{PBS57} tetramers (National Institutes of Health Tetramer Facility at Emory University). Flow cytometric analysis was performed on an LSRFortessa or LSRII (BD Biosciences), and the data were analysed with FlowJo software (Tree Star). All instruments are maintained by the University of Chicago Flow Cytometry and Antibody Technology Core Facility.

Bleomycin model

Bleomycin for Injection USP (15 Units/Vial from Teva Pharmaceuticals USA, Sellersville PA) was reconstituted at 3 Units/mL in PBS and stored at -80°C until use. Stock solution was diluted in additional PBS for intratracheal instillations (i.t.). Mice were weighed one day prior to i.t. to determine appropriate unit/kg dose. Recombinant murine IL-5 (50ng/mouse, Biolegend) was added to calculated bleomycin doses prior to i.t. instillation. Lung collagen content was quantified by hydroxyproline assay and trichrome staining of formalin-fixed tissue sections. Lung inflammation was determined by H&E staining of lung tissue sections or flow cytometry. Lung edema was scored as follows for one lung lobe per mouse: 0, no RBCs present; 1, small patch of airway with RBCs infiltrating; 2, 1/8 of lung lobe contains RBCs; 3, ¼ of lung lobe contains RBCs; 4, ½ of lobe contains RBCs; 5, Entire lobe shows RBCs in airways. For all histology scoring, the scorer was blinded to the mouse genotype and/or treatment, and slides were presented in a random order. The same lung lobe was scored for each mouse. Lung permeability was determined by administering FITC-dextran 70 (10mg/kg, Sigma) intratracheally and measuring FITC in the serum 1hr later[36]. Total protein in BAL was determined by BCA assay (Sigma).

Statistical Analysis

Unless specified in figure legends, statistical analyses were performed with GraphPad Prism software, and a P-value less than 0.05 was considered significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns = not significant). Experiments with two groups were analyzed using an unpaired Student's two-tailed t test. Experiments with greater than two groups were analyzed with a one-way ANOVA and post-hoc Tukey test. Error bars represent the SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Borensztajn K, Crestani B, Kolb M. Idiopathic pulmonary fibrosis: from epithelial injury to biomarkers--insights from the bench side. Respiration; international review of thoracic diseases. 2013; 86(6):441–452. [PubMed: 24356558]
2. Ahluwalia N, Shea BS, Tager AM. New therapeutic targets in idiopathic pulmonary fibrosis. Aiming to rein in runaway wound-healing responses. American journal of respiratory and critical care medicine. 2014; 190(8):867–878. [PubMed: 25090037]
3. Zoz DF, Lawson WE, Blackwell TS. Idiopathic pulmonary fibrosis: a disorder of epithelial cell dysfunction. The American journal of the medical sciences. 2011; 341(6):435–438. [PubMed: 21613930]
4. Wells AU, Behr J, Costabel U, Cottin V, Poletti V. Triple therapy in idiopathic pulmonary fibrosis: an alarming press release. The European respiratory journal. 2012; 39(4):805–806. [PubMed: 22467722]
5. Papiris SA, Kagouridis K, Kolilekas L, Papaioannou AI, Roussou A, Triantafillidou C, et al. Survival in Idiopathic pulmonary fibrosis acute exacerbations: the non-steroid approach. BMC pulmonary medicine. 2015; 15(1):162. [PubMed: 26666385]
6. Adengunsoye A, H CL, Bonham CA, Jaffery MR, Blaine KM, Sullivan M, Churpek MM, Strek ME, Noth I, Sperling AI. Skewed Lung CCR4 to CCR6 CD4+ T Cell Ratio in Idiopathic Pulmonary Fibrosis Is Associated with Pulmonary Function. Frontiers in Immunology. 2016 Nov 23.
7. Herazo-Maya JD, Noth I, Duncan SR, Kim S, Ma SF, Tseng GC, et al. Peripheral blood mononuclear cell gene expression profiles predict poor outcome in idiopathic pulmonary fibrosis. Science translational medicine. 2013; 5(205):205ra136.
8. Rudd CE, Schneider H. Unifying concepts in CD28, ICOS and CTLA4 co-receptor signalling. Nature reviews Immunology. 2003; 3(7):544–556.
9. Moore TV, Clay BS, Cannon JL, Histed A, Shilling RA, Sperling AI. Inducible costimulator controls migration of T cells to the lungs via down-regulation of CCR7 and CD62L. Am J Respir Cell Mol Biol. 2011; 45(4):843–850. [PubMed: 21421907]
10. Tesciuba AG, Shilling RA, Agarwal MD, Bandukwala HS, Clay BS, Moore TV, et al. ICOS costimulation expands Th2 immunity by augmenting migration of lymphocytes to draining lymph nodes. Journal of immunology (Baltimore, Md: 1950). 2008; 181(2):1019–1024.
11. Shilling RA, Pinto JM, Decker DC, Schneider DH, Bandukwala HS, Schneider JR, et al. Cutting edge: polymorphisms in the ICOS promoter region are associated with allergic sensitization and Th2 cytokine production. Journal of immunology (Baltimore, Md: 1950). 2005; 175(4):2061–2065.
12. Maazi H, Patel N, Sankaranarayanan I, Suzuki Y, Rigas D, Soroosh P, et al. ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. Immunity. 2015; 42(3):538–551. [PubMed: 25769613]
13. Akbari O, Stock P, Meyer EH, Freeman GJ, Sharpe AH, Umetsu DT, et al. ICOS/ICOSL interaction is required for CD4+ invariant NKT cell function and homeostatic survival. Journal of immunology (Baltimore, Md: 1950). 2008; 180(8):5448–5456.
14. Simpson TR, Quezada SA, Allison JP. Regulation of CD4 T cell activation and effector function by inducible costimulator (ICOS). Current opinion in immunology. 2010; 22(3):326–332. [PubMed: 20116985]
15. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nature medicine. 2012; 18(7):1028–1040.
16. Gause WC, Wynn TA, Allen JE. Type 2 immunity and wound healing: evolutionary refinement of adaptive immunity by helminths. Nature reviews Immunology. 2013; 13(8):607–614.
17. Firszt R, Francisco D, Church TD, Thomas JM, Ingram JL, Kraft M. Interleukin-13 induces collagen type-1 expression through matrix metalloproteinase-2 and transforming growth factor-

beta1 in airway fibroblasts in asthma. The European respiratory journal. 2014; 43(2):464–473. [PubMed: 23682108]

18. Monticelli LA, Sonnenberg GF, Abt MC, Alenghat T, Ziegler CG, Doering TA, et al. Innate lymphoid cells promote lung-tissue homeostasis after infection with influenza virus. Nature immunology. 2011; 12(11):1045–1054. [PubMed: 21946417]
19. Manzo ND, Foster WM, Stripp BR. Amphiregulin-dependent mucous cell metaplasia in a model of nonallergic lung injury. American journal of respiratory cell and molecular biology. 2012; 47(3): 349–357. [PubMed: 22493011]
20. Pelly VS, Kannan Y, Coomes SM, Entwistle LJ, Ruckerl D, Seddon B, et al. IL-4-producing ILC2s are required for the differentiation of TH2 cells following Heligmosomoides polygyrus infection. Mucosal immunology. 2016
21. Gregory B, Kirchem A, Phipps S, Gevaert P, Pridgeon C, Rankin SM, et al. Differential regulation of human eosinophil IL-3, IL-5, and GM-CSF receptor alpha-chain expression by cytokines: IL-3, IL-5, and GM-CSF down-regulate IL-5 receptor alpha expression with loss of IL-5 responsiveness, but up-regulate IL-3 receptor alpha expression. Journal of immunology (Baltimore, Md: 1950). 2003; 170(11):5359–5366.
22. Linch SN, Danielson ET, Kelly AM, Tamakawa RA, Lee JJ, Gold JA. Interleukin 5 is protective during sepsis in an eosinophil-independent manner. American journal of respiratory and critical care medicine. 2012; 186(3):246–254. [PubMed: 22652030]
23. Mesnil C, Raulier S, Paulissen G, Xiao X, Birrell MA, Pirottin D, et al. Lung-resident eosinophils represent a distinct regulatory eosinophil subset. The Journal of clinical investigation. 2016
24. Spencer LA, Szela CT, Perez SA, Kirchhoffer CL, Neves JS, Radke AL, et al. Human eosinophils constitutively express multiple Th1, Th2, and immunoregulatory cytokines that are secreted rapidly and differentially. J Leukoc Biol. 2009; 85(1):117–123. [PubMed: 18840671]
25. Odemuyiwa SO, Ghahary A, Li Y, Puttagunta L, Lee JE, Musat-Marcu S, et al. Cutting edge: human eosinophils regulate T cell subset selection through indoleamine 2,3-dioxygenase. Journal of immunology (Baltimore, Md: 1950). 2004; 173(10):5909–5913.
26. Lee JJ, Jacobsen EA, McGarry MP, Schleimer RP, Lee NA. Eosinophils in health and disease: the LIAR hypothesis. Clinical and experimental allergy: journal of the British Society for Allergy and Clinical Immunology. 2010; 40(4):563–575. [PubMed: 20447076]
27. Taniguchi H, Katoh S, Kadota J, Matsubara Y, Fukushima K, Mukae H, et al. Interleukin 5 and granulocyte-macrophage colony-stimulating factor levels in bronchoalveolar lavage fluid in interstitial lung disease. The European respiratory journal. 2000; 16(5):959–964. [PubMed: 11153599]
28. Boomars KA, Schweizer RC, Zanen P, van den Bosch JM, Lammers JW, Koenderman L. Eosinophil chemotactic activity in bronchoalveolar lavage from idiopathic pulmonary fibrosis is dependent on cytokine priming of eosinophils. The European respiratory journal. 1998; 11(5): 1009–1014. [PubMed: 9648948]
29. Mesnil C, Raulier S, Paulissen G, Xiao X, Birrell MA, Pirottin D, et al. Lung-resident eosinophils represent a distinct regulatory eosinophil subset. The Journal of clinical investigation. 2016; 126(9):3279–3295. [PubMed: 27548519]
30. Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med. 2011; 183(6):788–824. [PubMed: 21471066]
31. Ghatol A, Ruhl AP, Danoff SK. Exacerbations in idiopathic pulmonary fibrosis triggered by pulmonary and nonpulmonary surgery: a case series and comprehensive review of the literature. Lung. 2012; 190(4):373–380. [PubMed: 22543997]
32. Ferguson ND, Fan E, Camporota L, Antonelli M, Anzueto A, Beale R, et al. The Berlin definition of ARDS: an expanded rationale, justification, and supplementary material. Intensive care medicine. 2012; 38(10):1573–1582. [PubMed: 22926653]
33. Aeffner F, Bolon B, Davis IC. Mouse Models of Acute Respiratory Distress Syndrome: A Review of Analytical Approaches, Pathologic Features, and Common Measurements. Toxicologic pathology. 2015; 43(8):1074–1092. [PubMed: 26296628]

34. Gorski SA, Hahn YS, Braciale TJ. Group 2 innate lymphoid cell production of IL-5 is regulated by NKT cells during influenza virus infection. PLoS pathogens. 2013; 9(9):e1003615. [PubMed: 24068930]

35. Dong C, Juedes AE, Temann UA, Shresta S, Allison JP, Ruddle NH, et al. ICOS co-stimulatory receptor is essential for T-cell activation and function. Nature. 2001; 409(6816):97–101. [PubMed: 11343121]

36. Chen H, Wu S, Lu R, Zhang YG, Zheng Y, Sun J. Pulmonary permeability assessed by fluorescent-labeled dextran instilled intranasally into mice with LPS-induced acute lung injury. PloS one. 2014; 9(7):e101925. [PubMed: 25007191]

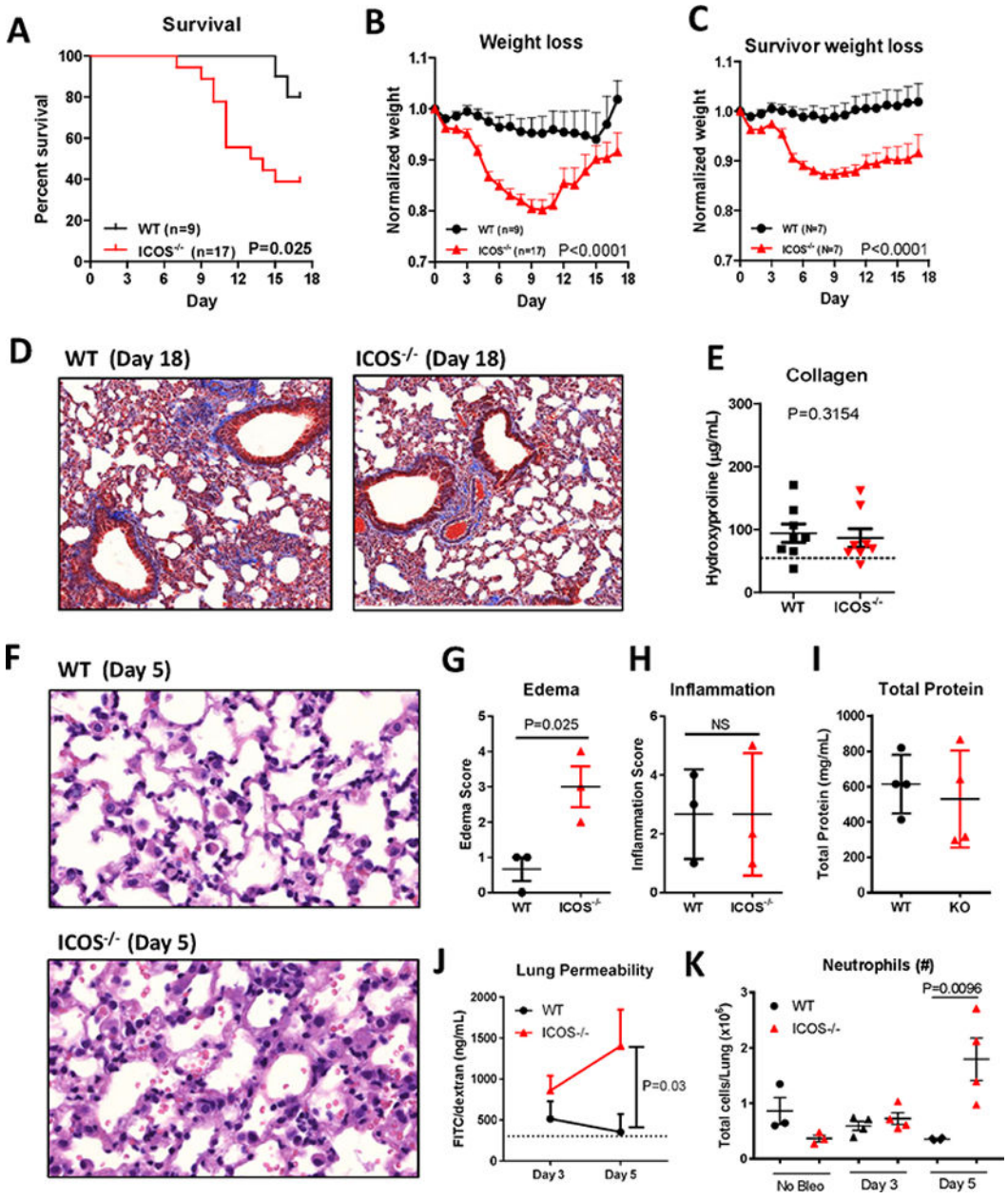

Figure 1. Bleomycin-challenged ICOS$^{-/-}$ mice exhibit similar lung collagen deposition compared to WT mice, but lose more weight and have increased mortality

WT or ICOS$^{-/-}$ were challenged intratracheally with 1.0U/kg bleomycin. (A) ICOS$^{-/-}$ mice had increased mortality and (B) lost more weight. (C) Weight loss in ICOS$^{-/-}$ and WT mice that survived until day 18 after challenge. Survival P value shown from Log-rank test. Weight loss was normalized to each mouse's starting weight on day 0 and P values were determined using a mixed-effects linear regression model to account for missing values from mortality. Shown is indicated N per group from two independent experiments combined. (D) Representative histologic sections from the left lung were stained with trichrome to show similar collagen deposits and cell infiltration in surviving WT or ICOS$^{-/-}$ mice. (E) On day 18 of the experiment surviving mice were sacrificed and right lung was removed for

hydroxyproline assay as a surrogate for collagen deposition. Dotted line indicates mean hydroxyproline level detected in untreated WT mice. Unpaired Student's *t* test was used to determine significance. (F) Representative H&E stained tissue sections from WT or ICOS−/− mice on day 5 post-bleomycin challenge. Left lungs were scored for (G) edema by the presence of RBCs in airways or (H) inflammation by the presence of leukocytic infiltration. Sections were blindly scored based on the overall area of the lung exhibiting edema or inflammation and the degree to which cell influx was observed. (I) Total protein measured in BAL day 5. (J) Lung permeability was determined by administering FITC-dextran intratracheally and measuring FITC in the serum 1 hr later. P value is 2-way ANOVA comparing the genotypes with 4-5 mice per group per time point. Dotted line indicates the average FITC measurement in serum from PBS-treated control mice. (K) Lung neutrophils were identified by flow cytometry as live $CD11b^{+}Ly6G^{+}$ cells in total digested lung tissue.

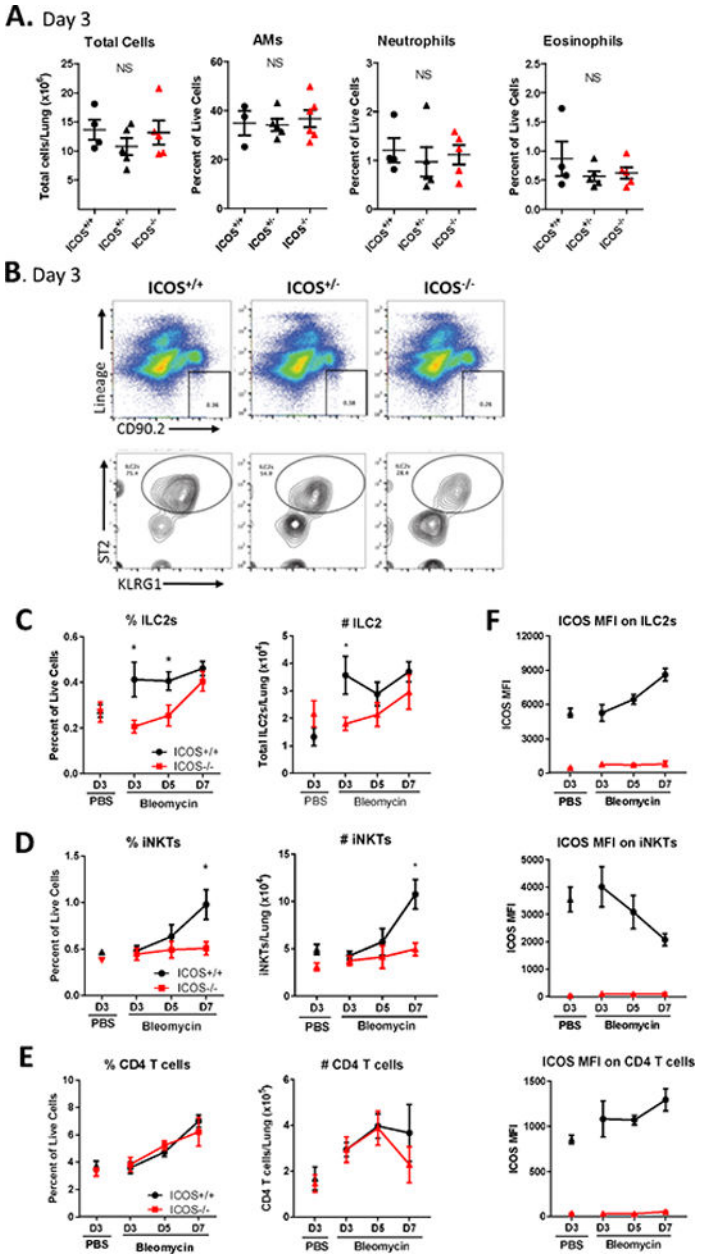

Figure 2. Increased early inflammation in ICOS$^{-/-}$ mice is associated with failed expansion of ILC2s and iNKTs, but not CD4 T cells

(A) Total cells recovered from the lungs on day 3, and the proportion identified as AMs (CD11c^{+}Siglec-F^{+}), neutrophils (CD11b^{+}Ly6G^{+}), and eosinophils (Siglec-F^{+}CD11c^{lo}). No significant differences (NS) were found by ANOVA with Tukey post-test. (B) ILC2s were identified as Lineage^{-} Thy1.2^{+}ST2^{+}KLRG1$^{+/-}$. Representative flow plots of ILC2 proportions in lungs are shown at 3 days post-bleomycin challenge. Proportion and total number of (C) ILC2s, (D) iNKTs (CD3^{+} CD1d Tetramer^{+}), and (E) CD4 T cells (CD3^{+}CD4^{+}) are shown for ICOS$^{-/-}$ and WT mice over days 3-7. Shown is mean and SEM with N=6-15 mice per time point per group. P value determined by unpaired Student's *t* test comparing ICOS$^{+/+}$ to ICOS$^{-/-}$ mice at each time point and significance <0.05 is indicated

by *. (F) Expression of ICOS was measured by mean fluorescent intensity (MFI) using flow cytometry on the indicated cell types.

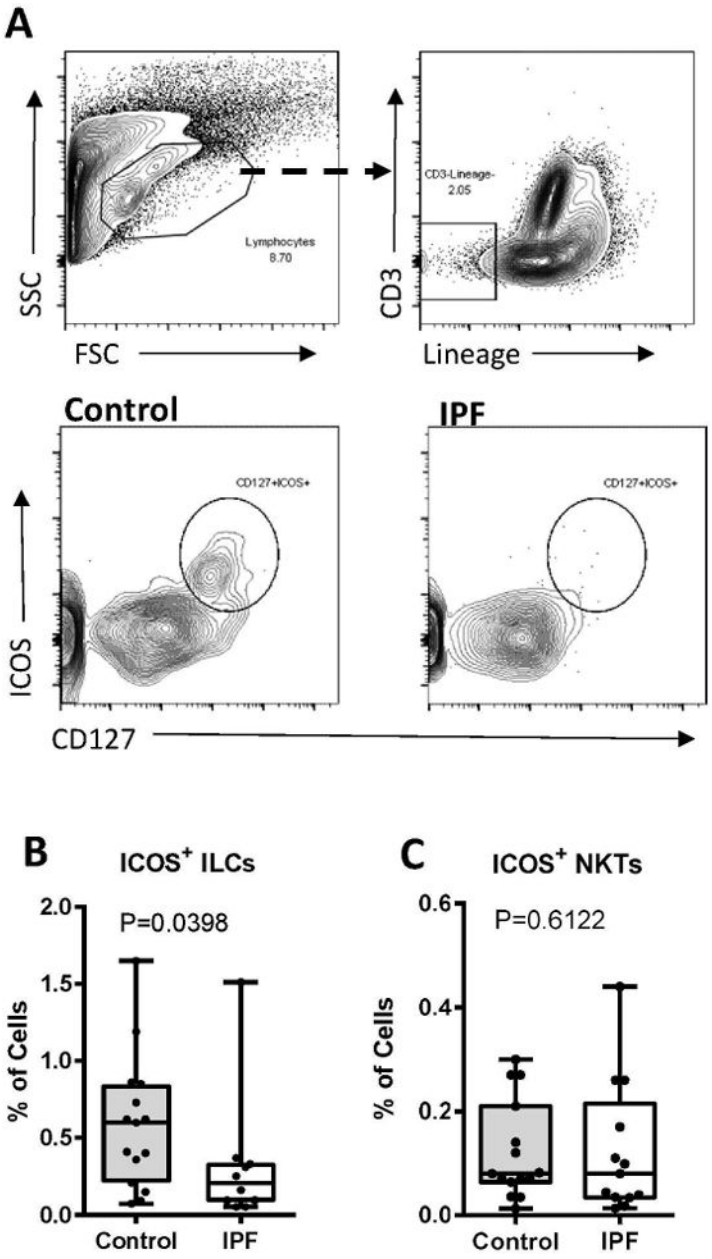

Figure 3. Lower percentage of ICOS+ ILCs are found in the lungs of IPF patients

(A) Gating strategy to identify $ICOS^+$ ILCs in human lung tissue from IPF patients or controls. For IPF patients, tissue was taken from the diseased lung at the time of lung transplant, and for controls, lung tissue was taken from deceased organ donors with no history of lung disease or fibrosis. Lymphocytes were gated on forward and side scatter for size (left top panel), followed by a lineage-negative selection (right top panel). Of these cells, $ICOS^+$ ILCs were identified as $ICOS^+CD127^+$. Shown are a representative control lung (left bottom panel) and a representative IPF lung (right bottom panel). Enumeration of (B) ILCs or (C) iNKTs, defined as $CD3^+CD56^+ICOS^+$, in IPF and control lungs. Shown is the proportion of each cell type out of total isolated lung leukocytes, with significance determined by Mann-Whitney non-parametric test.

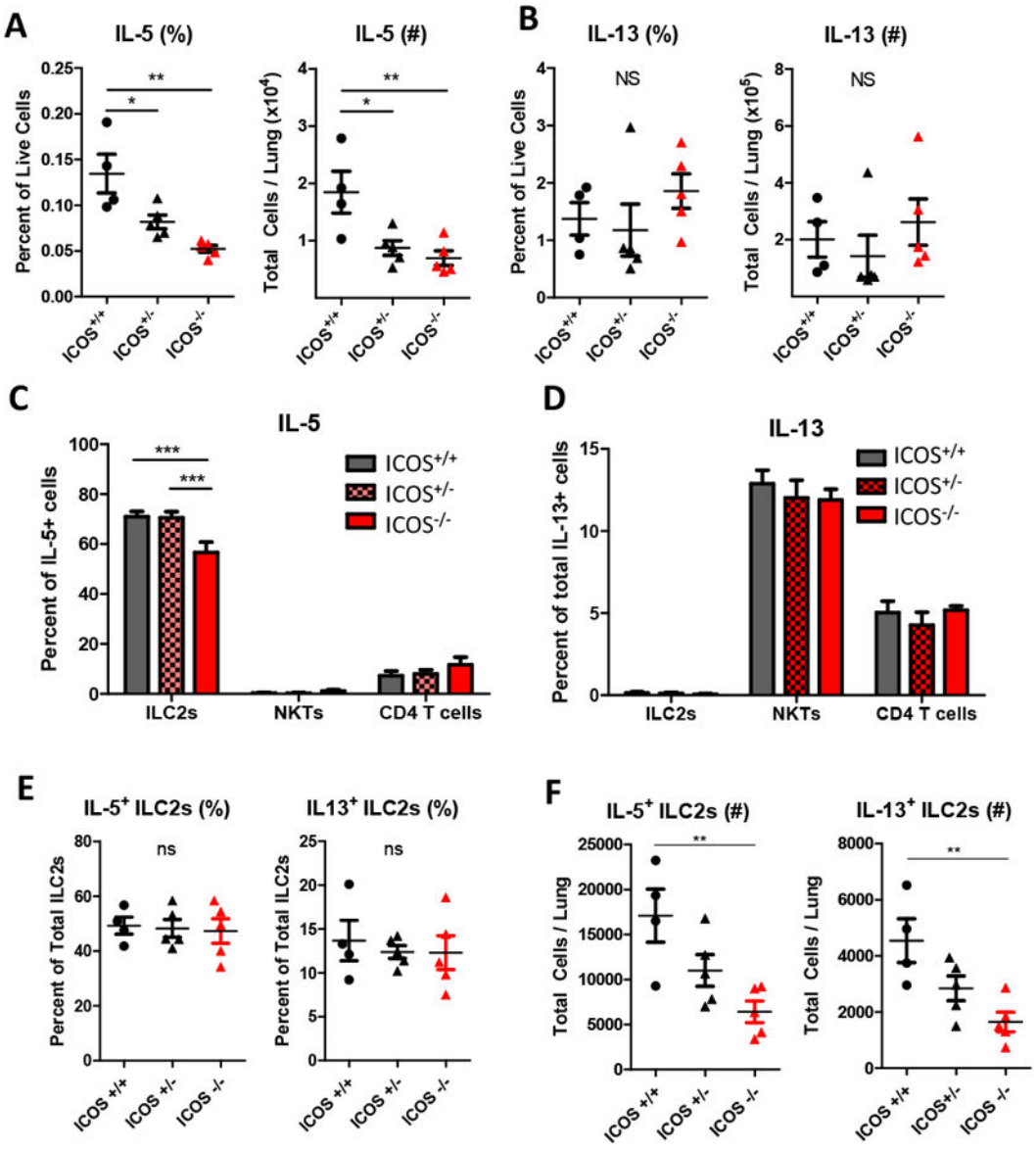

Figure 4. ILC2 cells are the major producer of IL-5 three days after bleomycin challenge

The percent and total number of IL-5-producing (A) or IL-13-producing cells (B) was determined 3 days after bleomycin challenge. Significant differences were determined by ANOVA with asterisk indicating *p<0.05 or **p<0.01 by Tukey post-test. Shown is one representative of three independent experiments. IL-5-producing (C) and IL-13-producing (D) cells were identified as ILC2s, iNKTs, or CD4 T cells and shown as the percent of each cell type out of total IL-5+ or IL-13+ lung cells. Significant differences were determined by ANOVA with asterisks indicating ***p<0.001 by Tukey post-test. The percentage of ILC2s producing IL-5+ or IL-13+ was similar between strains (E), however differences were found in total IL-5+ and IL-13+ ILC2s due to the overall decrease in ILC2 numbers (F).

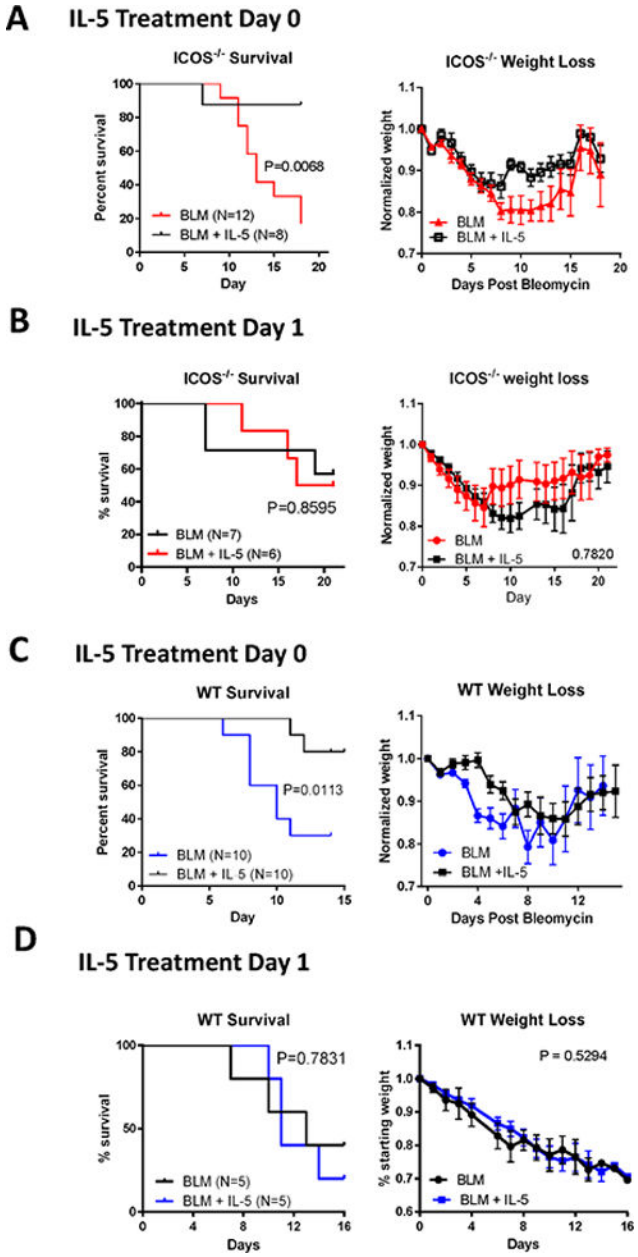

Figure 5. Treatment with recombinant IL-5 improves survival from bleomycin-induced lung injury

ICOS$^{-/-}$ mice were treated with 1.0U/kg bleomycin with or without recombinant IL-5 (50ng/mouse) concurrent with challenge (A) or one day after challenge (B). Survival and weight loss were measured. Wild-type C57Bl/6 mice were treated with a *two-fold higher* dose of bleomycin (2.0U/kg) with or without recombinant IL-5 (50ng/mouse) on day 0 (C) or day 1 after challenge (D). Survival curve P values determined by log-rank test.

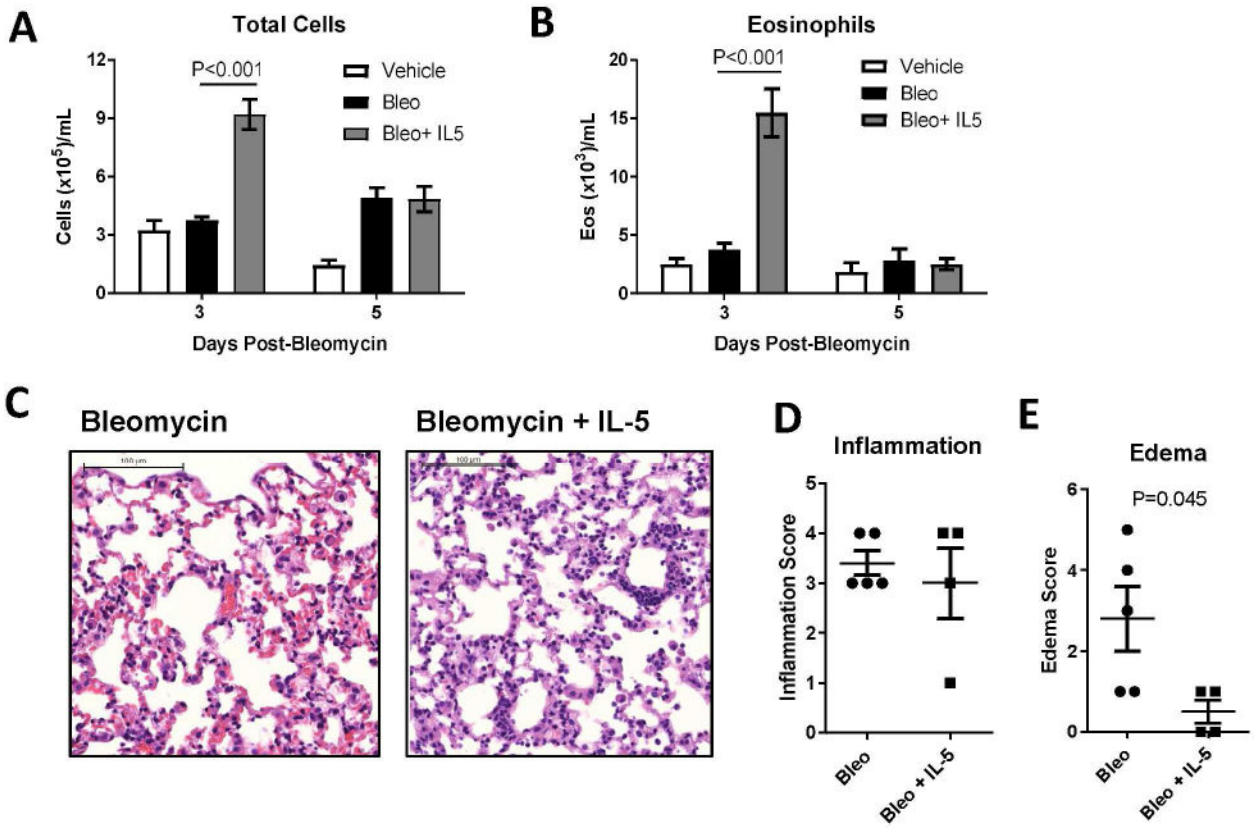

Figure 6. IL-5 treatment results in increased eosinophils in the BAL and reduced RBCs in the airways

ICOS$^{-/-}$ mice were challenged with bleomycin and treated with IL-5 or vehicle control. (A) Total cells and (B) eosinophils (Ly6G^{-} Siglec-F^{hi}CD11c^{low}) were measured in the BAL on days 3 and 5. Significance determined by ANOVA with Tukey post-test with 3-4 mice per group per time point. (C) Representative histology from ICOS$^{-/-}$ mice challenged with bleomycin (left panel) or bleomycin with IL-5 (right panel). (D) Inflammation and (E) edema was scored as described in Fig 1, and significance determine by unpaired t test.

Table 1

Characteristics of Study Subjects*

Characteristic	IPF (n=9)	Control (n=13)	p-value
Age, mean (SD)	58.7 (7.6)	58.0 (4.1)	0.782
Male, n (%)	6 (67)	4 (31)	0.192
Race/Ethnicity			
White, n (%)	6 (67)	8 (62)	1.000
Non-White, n (%)	3 (33)	5 (37)	1.000
Ever smoker, n (%)	6 (67)	7 (54)	0.674
Pack years, mean (SD)	28.2 (25.6)	16.9 (12.9)	0.325
Immunosuppressive therapy, n (%)	2 (22)	2 (15)	1.000
Diabetes, n (%)	1 (11)	4 (31)	0.360

*p-value determined by t-test for age and pack years. All other p values were determined by Pearson Chi squared or Fisher's exact test.