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IL-21 Promotes Pulmonary Fibrosis through the Induction of Pro-fibrotic CD8+ T Cells

Tia Y. Brodeur[*], **Tara E. Robidoux**[*], **Jason S. Weinstein**[†], **Joseph Craft**[†], **Susan L. Swain**[‡], and **Ann Marshak-Rothstein**[*,1]

[*]Department of Medicine/Rheumatology, University of Massachusetts Medical School, Worcester, MA 01605, USA.

[†] Department of Internal Medicine/Rheumatology, Yale University School of Medicine, New Haven, CT 06520, USA

[‡]Department of Pathology, University of Massachusetts Medical School, Worcester, MA 01605, USA

Abstract

Type 2 effector production of IL-13, a demonstrated requirement in models of fibrosis, is routinely ascribed to $CD4^+$ Th2 cells. We now demonstrate a major role for $CD8^+$ T cells in a murine model of sterile lung injury. These pulmonary $CD8^+$ T cells differentiate into IL-13-producing Tc2 and play a major role in a bleomycin-induced model of fibrosis. Differentiation of these Tc2 cells in the lung requires IL-21, and bleomycin treated IL-21- and IL-21R-deficient mice develop inflammation but not fibrosis. Moreover, IL-21R-expressing $CD8^+$ cells are sufficient to reconstitute the fibrotic response in the IL-21R-deficient mice. We further show that the combination of IL-4 and IL-21 skews naïve $CD8^+$ T cells to produce IL-21, which in turn acts in an autocrine manner to support robust IL-13 production. Our data reveal a novel pathway involved in the onset and regulation of pulmonary fibrosis, and identify Tc2 cells as key mediators of fibrogenesis.

INTRODUCTION

Fibrosis, defined as the excess production and accumulation of extracellular matrix components, is the final common pathway of many chronic inflammatory conditions (1, 2). Despite the prevalence of fibrosis as both a primary disease and a consequence of common chronic diseases such as asthma, sarcoidosis, and heart failure, there are currently extremely limited FDA-approved treatments that specifically target fibrogenesis (3). Idiopathic pulmonary fibrosis (IPF)[3] can be a rapidly progressive disease with a poor prognosis and minimal therapeutic options. Fibrotic interstitial lung disease also occurs in autoimmune diseases such as systemic sclerosis (SSc) and rheumatoid arthritis (1-5). In these diseases, cells that are beneficial during normal tissue repair, including macrophages, T cells, and

[1]Address Correspondence: Ann Marshak-Rothstein, ann.rothstein@umassmed.edu, Phone: 508-856-8089, FAX: 508-856-7883.

[3]Abbreviations used: α-smAc, α-smooth muscle actin; BALF, bronchoalveolar lavage fluid; Bleo, bleomycin; IPF, Idiopathic pulmonary fibrosis; i.t., intratracheally; LDLN, lung draining lymph nodes; SSc, systemic sclerosis; Tc2, $CD8^+$ type 2

fibroblasts, drive an excessive tissue repair response, leading to fibrosis and eventual organ dysfunction (1, 2, 4).

Activation of fibroblasts by TGF-β and consequent collagen production is crucial for both wound healing and fibrosis. Additionally, Th2 cell-derived IL-4 and IL-13 are integral components of the wound healing response due to the ability of these cytokines to activate fibroblasts (1, 4). IL-13 is also a potent stimulus for macrophage production of TGF-β and can drive lung fibrosis in the absence of other lung injury (6). Th2-associated cytokines become aberrantly upregulated in fibrosis, and CD4+ T cells have been implicated in murine models of SSc (7, 8) and pulmonary fibrosis (9, 10).

Development of murine pulmonary fibrosis following bleomycin treatment, a sterile lung injury model, requires IL-13 (11-13) especially late in the response (10). Asthma, which is often accompanied by subepithelial fibrosis, has largely been considered a Th2-driven IL-13-dependent disease, (14, 15), however, a role for CD8+ T cells has been described (14, 16, 17). Fibrosis associated with schistosomiasis also requires IL-13 production, and $CD4^+$ T cells have been identified as the main producers of IL-13 in these systems (1). It has therefore been presumed that $CD4^+$ T cells are the major source of IL-13 in sterile fibrosis.

Elevated numbers of IL-17 producing Th17 cells have also been implicated in models of fibrosis (10, 18). Th17 cells secrete IL-21, a highly pleiotropic cytokine, which, in combination with other cytokines, amplifies the function of both $CD4^+$, and $CD8^+$ T cells (19, 20). Interestingly, IL-21 has been implicated in the induction of both Th17 (21) and Th2 cells (9, 22), and as such, could provide a bridge for the progression from a Th17 to a Th2 response in the fibrosing lung. Furthermore, in human fibrotic lung samples, expression of IL-21 receptor (IL-21R) has been detected in lymphocytic infiltrates, suggesting that IL-21 responsive lymphocytes may be involved in lung fibrosis (23, 24). In mice, IL-21R deficiency attenuated collagen deposition in the liver caused by parasitic infection (9). Taken together, these data implicate IL-21 as potentially deleterious in pulmonary fibrosis. We now show that IL-21 plays a critical role in the fibrotic response associated with a bleomycin model of lung injury. Unexpectedly, we also found that IL-21 drives the activation and expansion of a novel population of IL-13 producing Tc2 cells, which then serve as a critical link between inflammation and fibrosis.

MATERIALS AND METHODS

Mouse Strains

BALB/c and C57BL/6 mice were purchased from the Jackson Laboratory. IL-21R knockout mice originally made by Warren Leonard (25) were provided by Oliver Dienz (University of Vermont) and IL-21 knockout mice were obtained from Lexicon-Mutant Mouse Regional Resource Centers. Both IL-21R knockout and IL-21 knockout mice were backcrossed to C57BL/6J for >10 generations. The IL-21 reporter line IL-21-mKat has also been described (26). All mouse experiments were approved by the Institutional Animal Care and Use Committee at UMMS.

Lung treatments

12-16 week old mice were anesthetized by isoflurane inhalation. 50μl of sterile bleomycin sulfate (Sigma), 0.05U/mouse (low dose) or 0.15U/mouse (high dose), recombinant mouse IL-21 (R&D Systems) 1μg/mouse, or PBS were instilled into the lungs of mice by oropharyngeal aspiration (27). For experiments where mice of different genotypes were compared, the mice were age-matched within 2 wk difference in date of birth.

Lung and lymph node tissue harvesting

Mice were sacrificed by cervical dislocation and immediate removal of the diaphragm. Lungs were perfused with PBS through the right ventricle to remove blood. Bronchoalveolar lavage fluid (BALF) was collected with three flushes of 0.5ml PBS. LDLNs were dissected and dissociated in Hank's buffered saline solution (HBSS) using frosted glass slides. The right bronchus was sutured and the right and postcaval lobes were removed and placed in HBSS, minced, and forced through a 100μm nylon mesh filter to generate a single-cell suspension. The left lobe was inflated with 10% neutral buffered formalin, the left bronchus was sutured, and the left lobe was then removed and submerged in 10% buffered formalin at room temperature until processed for paraffin embedding (>24 hours).

Collagen Measurement

The entire left lobe was minced into fine pieces and placed in 2 ml of a solution of acetic acid (Sigma, 0.5M) and pepsin. After 24-48 hours, the solution was spun at 4C for 20 minutes, and these lung extracts were frozen until collagen concentration assay. Sircol (Biocolor, Belfast, UK) or Sirius Red Total Collagen Detection kit (Chondrex) were used according to the manufacturers instructions with similar results.

Histopathology and immunohistochemistry

Formalin fixed, paraffin embedded lung tissue was sectioned into 5 μm sections and stained by hematoxylin and eosin or Masson's trichrome stain. Immunohistochemistry for α-smooth muscle actin (Santa Cruz Biotechnology, clone 1A4, 1:500 dilution) was performed without antigen retrieval, goat anti-mouse IgG2a-HRP (Santa Cruz Biotechnology, 1:500 dilution) was used as a secondary antibody, with diaminobenzene (DAB; Vector Laboratories) as a substrate. Mayer's hematoxylin was used as a counterstain for immunohistochemistry. Before sectioning and staining, tissue blocks were coded, and the pictures were taken blinded. For H&E and trichrome staining, images were acquired randomly. For α-smooth muscle actin staining, areas of focal consolidation were chosen for image acquisition (also blinded) to identify areas of inflammation and/or fibrosis.

Flow cytometry

Single cell suspensions were surface or intracellular stained with combinations the following antibodies: IL-21R-biotin, CD3-PE-Cy7, -biotin (clone 145-2C11), CD4-efluor450, -APC (clone RM4-5), CD8-FITC, -APC, -PE-Cy7 (clone 53-6.7), CD25-PerCP-Cy5.5 (clone PC61.5), CD69-efluor450, - PerCP-Cy5.5, IL-21-APC (cloneFFA21), IL-13-PE (clone eBio13A), IL-17APerCP-Cy5.5 (clone eBio17B7), Foxp3-PE (clone FJK-16s) all from eBioscience. Strepavidin-PerCP was from BD Biosciences. For Foxp3 staining, cells were

fixed and permeabilized using the Foxp3 Transcription Factor staining buffer kit (eBioscience) according to the manufacturers instructions. For intracellular cytokine staining, single-cell suspensions were stimulated ex-vivo with plate-bound anti-CD3 (BioLegend, 5 μg/ml) or PMA (Sigma, 100ng/ml) plus ionomycin (Sigma, 1μg/ml) in complete RPMI for 2 hours at 37°C. GolgiStop (BD Biosciences, 1:100) was added and cultures were incubated for an additional 2-3 hours at 37°C. Cells were stained with Fixable Viability Dye efluor 780 (eBioscience) per manufacturer instructions. Cells were then surface stained and intracellular cytokine staining was performed using CytoFix/CytoPerm and PermWash (BD Biosciences) per manufacturer protocol.

Cytokine measurements

LDLN or BAL single-cell suspensions (200,000-400,000 cells/well) were seeded onto anti-CD3 (BioLegend; 5μg/ml) coated plates in RPMI. Supernatants were collected at 24 hours and analyzed by ELISA for IL-13 (BD Biosciences), IL-21 (eBioscience), IFN-γ (BD Biosciences), and IL-17A (eBioscience). Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA Kit (R&D Systems) was used to perform ELISA on cell-free BAL supernatant without the acid treatment step to activate latent TGF-β, but otherwise performed using manufacturers instructions.

In vitro T cell differentiation

$CD8^+$ T cells were purified from spleen cell suspensions by positive selection (BD iMag CD8α beads). Cells were stimulated with plate bound anti-CD3 (2 μg/ml) and soluble anti-CD28 (BioLegend, 2 μg/ml) with or without recombinant mouse IL-4 (BD Biosciences, 10 ng/ml), recombinant human IL-2 (BD Biosciences, 50 ng/ml), recombinant mouse IL-21 (R&D Systems, 50 ng/ml), or recombinant human TGF-β (Peprotech, 5 ng/ml). Anti-CD25 antibody was purified from PC61 hybridoma supernatant (a gift from Dr. M. Shlomchik, University of Pittsburgh) and used at a concentration of 20 μg/ml. Cells were split on day 2 and then as needed. On day 4 or 5, T cells were re-stimulated with anti-CD3 for intracellular cytokine staining or re-stimulated to harvest supernatants for ELISA after 24 hours.

Statistical methods

Data analysis was performed using Prism 6 (GraphPad) software. *p* values were calculated using unpaired two-tailed Student's *t* test. p values < 0.05 were considered not significant.

RESULTS

IL-21R signaling drives fibrosis but is dispensable for inflammation after sterile lung injury

To determine whether IL-21 plays a role in a sterile lung injury model of fibrosis, we compared the effects of intratracheal (i.t.) instillation of bleomycin in wild type C57BL/6 (B6) mice and IL-21 receptor deficient (*Il21r−/−*) B6 mice. We found a striking attenuation in peribronchiolar fibrosis in the *Il21r−/−* mice at day 14 after bleomycin injury, as detected by Masson's trichrome staining (Figure 1A). Quantification of collagen concentration in lung extracts at 14 days by a colorimetric collagen binding assay further confirmed that both *Il21r−/−* and IL-21-deficient (*Il21−/−)* mice had significantly decreased collagen deposition in the lung compared to bleomycin-treated B6 mice (Figure 1B). Moreover, α-

smooth muscle actin (α-smAc), a protein expressed by collagen-producing myofibroblasts, was readily detected in the bleomycin injected B6 mice but almost completely absent from the *Il21r−/−* mice (Figure 1C). In accordance with a previous report using IL-21 reporter mice(28), we found that a small population of $CD4^+$ T cells harvested from B6 mice constitutively expressed IL-21. IL-21 expressing $CD4^+$ T cells had a roughly 7-fold higher $CD62L^+CD44^+$ surface phenotype relative to the total $CD4^+$ population. To determine whether airway T cells produced increased levels of IL-21 following bleomycin treatment, we cultured BAL cells with anti-CD3 and assayed supernatants for IL-21 by ELISA. We found that BAL from bleomycin treated mice produced more IL-21 than PBS-treated controls (Supplemental Figure 1). TGF-β has been shown to be an integral part of fibrogenesis (6, 11), although IL-13-dependent, TGF-β-independent fibrosis has been described (29). We found that TGF-β increased in both B6 and *Il21r−/−* mice in response to bleomycin (Supplemental Figure 2).

Since IL-21R deficiency led to such dramatic effects on bleomycin induced fibrosis, we next asked whether IL-21 by itself could elicit fibrosis. Strikingly, IL-21-treated mice showed evidence of inflammation and fibrosis 10 days later as detected histologically by H&E and trichrome stains (Figure 1D) and by the quantitative collagen assay (Figure 1E). Collagen deposition elicited by rIL-21 instillation was comparable to bleomycin instillation and rIL-17A, which has been previously shown to drive fibrosis (10). Thus, IL-21 is indispensible for bleomycin-induced lung fibrosis, and transient local increases in IL-21 can drive lung fibrosis.

To determine whether IL-21 deficiency led to decreased inflammation and therefore attenuated fibrosis, we assessed the extent of inflammation in the bleomycin treated mice, both by histology and by enumerating lung-infiltrating cells. Unexpectedly hematoxylin and eosin (H&E) staining indicated a similar extent of mononuclear cell infiltration in the B6 and *Il21r*$^{-/-}$ mice (Figure 2A). In addition, comparably increased numbers of mononuclear cells were recovered from the bronchoalveolar lavage fluid (BALF) of the bleomycin treated B6 and *Il21r−/−* mice compared to PBS-treated control mice, and the total number of lung draining (mediastinal) lymph nodes (LDLN) was tended to be slightly lower in the bleomycin treated *Il21r−/−* mice compared to bleomycin treated B6 mice (Figure 2B). Together, these results identify IL-21 signaling as a critical event in the transition between but was not statistically different inflammation and fibrosis.

$CD8^+$ T cell recruitment is impaired in *Il21r−/−* mice after sterile lung injury

The pathogenicity of T cells in bleomycin-induced fibrosis has been disputed (30), however, T cell depletion has been shown to attenuate fibrosis (31), and T cells produce pathogenic cytokines in this system (10, 18). Thus, we wanted to determine whether decreased T cell recruitment or activation was responsible for attenuated pulmonary fibrosis in *Il21r−/−* mice. At day 14 post-bleomycin injury, we stained single-cell suspensions of lymphocytes harvested from LDLN for T cell surface markers and markers of activation. *Il21r−/−* mice had roughly half (an average of 48% less than B6) the number of $CD8^+$ T cells in the LDLN compared to B6 mice after bleomycin injury (Figure 3A). The total average number of $CD4^+$ T cells was decreased by only 30% in *Il21r−/−* mice compared to B6 controls after

bleomycin treatment. Furthermore, surface staining of the activation marker CD69 on both $CD4^+$ and $CD8^+$ T cells was comparable between bleomycin-treated B6 and *Il21r−/−* mice (Figure 3B). $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (Tregs) can dampen inflammation, including T cell responses (32, 33). However, the frequency of Tregs in LDLN of *Il21r−/−* mice was not increased compared to B6 mice, and actually tended to be lower than B6 mice, suggesting that Tregs are not likely to be responsible for decreased $CD8^+$ proliferation or recruitment (Figure 3C). These data show that IL-21 is a key factor in maximizing the $CD8^+$ T cell response during lung fibrogenesis, and has a lesser effect on the $CD4^+$ T cell response.

Protection from fibrosis in *Il21r−/−* mice is associated with a decrease in IL-13, but not IL-17A, production by T cells

IL-21 is a reported amplifier of both Th17 and Th2 responses (21, 22). To investigate the role of IL-21 within a microenvironment that favors the differentiation of both in pro-fibrotic Th2 and Th17 responses, we analyzed LDLN T cells for cytokine production. At day 14 following bleomycin injury, LDLN cells obtained from PBS and bleomycin treated mice were stimulated *in vitro* with plate-bound anti-CD3 for 24-48 hours and supernatants were then analyzed by ELISA for IL-17A, as a readout of Th17 cells, and IL-13, as a readout for Th2 cells. T cells from the bleomycin treated B6 and *Il21r−/−* mice made comparable amounts of IL-17A (Figure 4A). However, T cells from bleomycin treated *Il21r −/−* mice secreted three-fold less IL-13 than those from bleomycin treated B6 mice (Figure 4B). To determine whether IL-13was being produced by airway lymphocytes, we treated B6 and *Il21−/−* (cytokine deficient, receptor sufficient) mice with PBS or bleomycin and harvested BAL cells at day 14. ELISA of supernatants from BAL cells cultured with plate bound anti-CD3 showed that IL-13 production by T cells was increased in mice bleomycin-treated B6 mice compared to controls, albeit at lower levels than LDLN cultures (Figure 4C). Cells that were cultured without anti-CD3 did not produce IL-13, indicating that T cells were producing the IL-13 in these cultures (data not shown). Similar to IL-21R-deficient LDLN, IL-21-deficient BAL cells produced less IL-13 in response to bleomycin (Figure 4C).

In other models of fibrosis, $CD4^+$ Th2 cells were found to be the major source of IL-13 (1, 4, 9), but since we had found $CD8^+$ T cell numbers to be most affected in the IL-21R-deficient mice, we decided to assess the subset distribution of the IL-13-producing cells in the bleomycin mice. Surprisingly, as enumerated by intracellular cytokine staining (ICS) of $CD4^+$ and $CD8^+$ T cells from LDLN, many of the IL-13 producing cells were $CD8^+$ cells. In addition, the *Il21r−/−* mice had a significantly lower frequency of these cells (Figure 4D-E). The decreased frequency of IL-13-producing $CD8^+$ T cells in the *Il21−/−* mice, taken together with the decreased total number of $CD8^+$ T cells (Figure 3A) resulted in strikingly fewer IL-13-producing $CD8^+$ T cells overall. These data indicate that bleomycin-induced $CD8^+$ T cell differentiation into pro-fibrotic, type 2 effectors is highly dependent on IL-21.

The combination of IL-4 and IL-21 efficiently promotes the *in vitro* differentiation of CD8$^+$ T cells into Tc2 cells

To better understand how IL-21 promotes IL-13 production by CD8$^+$ T cells in the injured lung, we decided to evaluate the importance of IL-21 and other cytokines in the *in vitro* generation of Tc2 cells. It has previously been reported that Tc2 cells require IL-2 and IL-4 for differentiation (34, 35). However, IL-2 is not strongly upregulated in the lungs in response to bleomycin injury (36). Therefore, we sought to determine whether IL-21 was sufficient for Tc2 differentiation *in vitro* as determined by IL-13 production. We activated purified CD8$^+$ T cells and assessed the ability of IL-21 to support their capacity to produce IL-13 in conjunction with IL-4, with or without IL-2. We found that IL-21 could supplant IL-2 in Tc2 differentiation (Figure 5A and 5B). Remarkably, blockade of the high-affinity IL-2 receptor, CD25, enhanced IL-13 production almost two-fold at the expense of IFN-γ production (Figure 5B and C), suggesting that upon activation, high-affinity IL-2 signaling does not drive CD8$^+$ T cells into Tc1 and Tc2 differentiation pathways evenhandedly, but rather inhibits Tc2 differentiation.

TGF-β has both potent pro-fibrotic and anti-inflammatory functions. To understand how these roles affected Tc2 differentiation, we also skewed Tc2 cells in the presence of TGF-β. As expected, TGF-β limited proliferation of CD8$^+$ T cell cultures (data not shown). However, the TGF-β treated CD8$^+$ T cells did produce IFN-γ, but not IL-13 (Figure 5B and C). Additionally, we found that TGF-β in conjunction with IL-4 decreased the level of IL-21R expression (Figure 5D), thereby downregulating CD8$^+$ T cell responsiveness to IL-21. This effect was not induced by either TGF-β or IL-4 alone. This finding suggests that while TGF-β has indispensable profibrotic effects on stromal cells such as fibroblasts, it negatively regulates pro-fibrotic CD8$^+$ T cells. Taken together, these data indicate that IL-21 can supplant IL-2 to promote Tc2 differentiation during fibrogenesis, and that high affinity IL-2R signaling and TGF-β counter-regulate the effects of IL-21 signaling.

Tc2 cells self-prime with IL-21

Since Th17 cells can be amplified by autocrine and/or paracrine IL-21 during differentiation (37), we hypothesized that autocrine IL-21 would amplify Tc2 differentiation. Using an *in vitro* activation system, we skewed wild type and IL-21-deficient purified CD8$^+$ T cells using IL-2 or IL-21 with or without IL-4, and after the cells had reverted to a resting phenotype and been washed free of any residual cytokine, they were re-stimulated with plate-bound anti-CD3. Cytokine production was initially quantified by ELISA. Intriguingly, only IL-21/IL-4, but not IL-2/IL-4 or IL-21 alone, generated CD8$^+$ effectors capable of copious IL-21 secretion upon re-stimulation (without the addition of exogenous cytokines) as measured by ELISA. The IL-21 detected is highly unlikely to have been “carried over” from the primary stimulation, since IL-21 was detected in only IL-21+IL-14 and not cultures that had been supplemented with IL-21 alone (Figure 6A). Activation of CD8+ T cells obtained from an IL-21 reporter mouse line confirmed the results of the ELISA (Figure 6B). Moreover, in accordance with our hypothesis, there was a striking difference in IL-13 production by IL-21-deficient and IL-21 sufficient Tc2 cultures. IL-21-deficient CD8$^+$ T cells did not secrete IL-13 when treated with IL-21 and IL-4, but wild type B6 CD8$^+$ T cells

secreted high levels of IL-13 (Figure 6C). Therefore, paracrine IL-21 drives $CD8^+$ T cell secretion of IL-21, which in turn promotes IL-13 production through an autocrine pathway.

IL-21R-sufficient $CD8^+$ T cells restore fibrotic phenotype of IL-21R-deficient mice

The impact of IL-21-deficiency on $CD8^+$ T cell number and function, pointed to the possibility that IL-21-responsive $CD8^+$ T cells are required for bleomycin-induced lung fibrosis. To test this hypothesis, purified splenic B6 $CD4^+$ and $CD8^+$ T cells from untreated mice were adoptively transferred into both B6 and *Il21r−/−* mice and the recipients were subsequently treated with bleomycin. Remarkably, injection of B6 $CD8^+$ but not CD4+ T cells "rescued" IL-13 production by LDLN T cells from bleomycin treated *Il21r−/−* recipients, but had little effect on the IL-13 response of B6 recipients (Figure 7A). Moreover, adoptively transferred IL-21R-sufficient $CD8^+$ T cells into *Il21r−/−* mice, but not B6 mice, led to a significant increase in collagen concentration in lung extracts after bleomycin treatment, while adoptive transfer of IL-21R-sufficient $CD4^+$ T cells had a negligible effect (Figure 7B). Restoration of fibrosis and fibroblast activation by IL-21R-sufficient $CD8^+$ T cells was also confirmed histologically by immunohistochemical staining for collagen and α-smAc Figure 7C). Thus, IL-21 responsive $CD8^+$ T cells are necessary and sufficient for reconstituting the fibrotic phenotype following lung injury in *Il21r−/−* mice.

DISCUSSION

Fibrosis is a frequent outcome of chronic inflammation, but despite extensive investigation, the pathways responsible for the transition from inflammation to fibrosis remain incompletely understood. Over-exuberant type 2 responses characterized by high levels of IL-4 and IL-13 are often linked to fibrogenesis, however, the initiating signals for such responses are not clear. Through the analysis of IL-21R-deficient mice, we have now shown that lung fibrosis and optimal IL-13 production in response to bleomycin lung injury is dependent on IL-21R signaling, thus identifying IL-21 as a potential therapeutic target for fibrotic diseases. Previous work showed that bleomycin induced lung fibrosis is ameliorated by neutralization of IL-13 or depletion of IL-13 responsive cells (12, 13, 38). In accordance with these studies, we found that IL-21R deficiency and protection from fibrosis was associated with a striking reduction in IL-13 production by lung T cells *ex vivo*. Intriguingly, protection from fibrosis in *Il21r−/−* mice was not associated with a significant reduction of inflammation or ex vivo IL-17 production. *Il21r−/−* mice have also been shown to develop less severe fibrosis in a schistosomiasis-associated liver fibrosis model, however, this effect was attributed to IL-21 induction of Th2 cells and the promotion of increased macrophage sensitivity to Th2 cytokines such as IL-4 and IL-13 (9).

We further showed that in the bleomycin model, IL-13 secreting $CD8^+$ T cells, or Tc2 cells, and not Th2 cells, were most profoundly impacted by IL-21R deficiency. This finding was unexpected since $CD4^+$ T cells have been charged with driving fibrosis through the production of both IL-17A (10, 18) and IL-13 (11, 12).

The physiological significance of Tc2 cells in vivo had been controversial since most functional studies of Tc2 cells used *in vitro*-skewed cells for adoptive transfer. *In vitro*-

differentiated Tc2 cells are less potent effectors in pulmonary viral clearance and tumor rejection when compared to Tc1 cells (39, 40). However, in murine asthma, Tc2 cells develop and increase both airway inflammation and hyper-responsiveness (14). We now show that IL-21-dependent Tc2 cells are required for lung fibrosis after sterile lung injury, as both IL-13 production and fibrosis were restored by transferring IL-21R+/+ $CD8^+$ T cells (not activated *in vitro*) into *Il21r−/−* mice treated with bleomycin. Adoptive transfer of IL-21R+/+ $CD4^+$ T cells did not "rescue" fibrosis in *Il21r−/−* mice, suggesting that $CD8^+$ T cell cytotoxicity, in addition to IL-13 production, could be crucial for fibrosis. These data are consistent with previous reports where depletion of $CD8^+$ T cells or perforin deficiency almost completely prevented bleomycin-induced pulmonary fibrosis (31, 41).

In addition to identifying a novel role for Tc2 cells during fibrosis, our results points to a unique role for IL-21 in Tc2 differentiation. Studies by other investigators have shown that Tc2 differentiation can be achieved in vitro using a combination of IL-2 and IL-4 (34, 35). Here we have shown that IL-21, in conjunction with IL-4, can replace the need for exogenous IL-2 in Tc2 differentiation. Counterintuitively, CD25 blockade enhanced Tc2 differentiation in response to IL-21/IL-4, which again pointed to a specific role for IL-21 during fibrogenesis that cannot be replaced by IL-2. Most importantly, we also found that only IL-4 + IL-21 differentiated cells acquired the capacity to produce high levels of their own self-sustaining IL-21.

We further found that $CD8^+$ T cells produced IL-21 in vivo in bleomycin treated mice. To the best of our knowledge, this is the first report of IL-21 production by $CD8^+$ T cells with relevance to a disease model. IL-21 message was detected in T-bet and Eomesodermin double-knockout Tc17 cells skewed *in vitro* by a combination of IL-6 and TGF-β, however, IL-21 was not detected in Tc17 cultures by ELISA (42). The finding that $CD8^+$ T cells produce IL-21 in vivo could reflect a phenotype that is specific to a sterile type II environment and not mounted during viral or parasitic infections.

Interestingly, fibroblasts in the gut express IL-21R and upregulate matrix metalloproteinases in response to IL-21 (43), raising the possibility that $CD8^+$ T cell-derived IL-21 could act directly on fibroblasts. Moreover, IL-21 production by Tc2 cells could further reinforce a type II immune response due to the robust effects of IL-21 on $CD4^+$ T cells and innate immune effectors (44, 45). Strikingly, IL-21 instillation into the lungs of mice in the absence of other injurious stimuli led to fibrosis in addition to the development of both IL-13 and IL-21 producing $CD8^+$ T cells. Thus, the foregoing data show that IL-21 is both necessary and sufficient for pulmonary fibrosis.

In studying the differentiation of Tc2 cells, we found that TGF-β, a cytokine abundantly expressed in fibrotic tissues, specifically inhibited Tc2 differentiation. The finding that TGF-β decreased IL-21R expression on $CD8^+$ T cells when combined with IL-4 was surprising due to the pro-fibrotic roles of both cytokines. However, this could reflect a "brake" on fibrogenesis by decreasing positive feedback through the IL-21R. It should be noted that bleomycin-induced lung fibrosis is a self-limiting disease after single intratracheal instillations, and a TGF-β-dependent feedback mechanism could contribute for the spontaneous resolution of fibrosis. TGF-β is also produced by macrophages (46), including

alternatively activated macrophages, which are known to accumulate in the fibrotic lung (47, 48). The capacity of macrophage-derived TGF-β to suppress Tc2 differentiation might explain why macrophage depletion after fibrosis is established was found to exacerbate, and not ameliorate, collagen deposition (49). Additionally, the decrease in the expression of the IL-21R in response to TGF-β could be a mechanism by which Treg suppression of Tc2 cells is promoted. Since IL-21 impairs Treg function (50-52), Treg-derived TGF-β could thereby limit an IL-21R/IL-21 positive feedback loop, thus ensuring the potency of nearby Tregs by dampening local IL-21 production.

In addition to a role for IL-21 in Tc2 skewing, we found that IL-21R signaling was required for optimal $CD8^+$ T cell proliferation/recruitment. Both the frequency and total number of $CD8^+$ T cells were reduced in *Il21r−/−* mice during fibrogenesis compared to wild type controls. This data is consistent with a significant body of work characterizing mitogenic effects of IL-21 on $CD8^+$ T cells. In the NOD model of diabetes, lack of IL-21R signaling also decreases $CD8^+$ T cells and attenuates disease, however, in the NOD system, it has been reported that $IL\text{-}21R^+$ $CD4^+$ T cells and dendritic cells recruit cytotoxic $CD8^+$ T cells into pancreatic islets (53). By contrast, we found that direct engagement of IL-21R on $CD8^+$ T cells was required for fibrosis and optimal IL-13 production during lung fibrogenesis. Since fibrotic disease is a Th2/Tc2 driven response, rather than the Th1/Tc1-driven NOD disease, our results point to a requirement for IL-21R for a robust $CD8^+$ T cell effector activity that is specific to the type 2 environment caused by sterile fibrotic lung injury. Whether our findings could be extended to other sterile injury models of fibrosis such as silica, or an infection model such as hypersensitivity pneumonitis remains to be determined.

$CD8^+$ T cells have been positively correlated with disease severity in patients with lung fibrosis (54) and $CD8^+$ T cell activation is increased in early, diffuse SSc (55). We now establish a role for $CD8^+$ T cells among the repair/fibrosis class of effectors during sterile inflammation. Importantly, a number of studies have linked Tc2 cells to fibrosis in human patients. $CD8^+$ T cells from systemic sclerosis patients secrete copious IL-13 and can activate fibroblasts in an IL-13 and STAT-6-dependent manner (56). Additionally, in the peripheral blood of systemic sclerosis patients, a higher frequency of $CD8^+$ T cells produces IL-13 than $CD4^+$ T cells (56, 57). It has also been reported that the frequency of Tc2 cells in the lungs of IPF patients is correlated with disease severity and shortness of breath (54), and $CD8^+$ T cells are as, if not more, abundant than $CD4^+$ T cells in the lungs of IPF patients (58, 59). These studies, taken together with the current report, challenge the predominant $CD4^+$ T cell-centric view of fibrosis. Our data clearly point to an IL-21/IL-13 axis during fibrogenesis, requiring $CD8^+$ T cells as effectors on both sides of this axis. Overall, these results have important implications for the rational design of IL-21 targeted therapies that could be used to treat fibrosis resulting from conditions such as SSc, IPF, or chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Wynn TA. Fibrotic disease and the T(H)1/T(H)2 paradigm. Nat Rev Immunol. 2004; 4:583–594. [PubMed: 15286725]
2. Wick G, Grundtman C, Mayerl C, Wimpissinger TF, Feichtinger J, Zelger B, Sgonc R, Wolfram D. The immunology of fibrosis. Annu Rev Immunol. 2013; 31:107–135. [PubMed: 23516981]
3. Wynn TA, Ramalingam TR. Mechanisms of fibrosis: therapeutic translation for fibrotic disease. Nat Med. 2012; 18:1028–1040. [PubMed: 22772564]
4. Wynn TA. Integrating mechanisms of pulmonary fibrosis. J Expt Med. 2011; 208:1339–1350.
5. Luzina IG, Todd NW, Iacono AT, Atamas SP. Roles of T lymphocytes in pulmonary fibrosis. J Leuk Biol. 2008; 83:237–244.
6. Lee CG, Homer RJ, Zhu Z, Lanone S, Wang X, Koteliansky V, Shipley JM, Gotwals P, Noble P, Chen Q, Senior RM, Elias JA. Interleukin-13 induces tissue fibrosis by selectively stimulating and activating transforming growth factor beta(1). J Expt Med. 2001; 194:809–821.
7. Wallace VA, Kondo S, Kono T, Xing Z, Timms E, Furlonger C, Keystone E, Gauldie J, Sauder DN, Mak TW, et al. A role for CD4+ T cells in the pathogenesis of skin fibrosis in tight skin mice. Eur J Immunol. 1994; 24:1463–1466. [PubMed: 7911425]
8. Radojcic V, Pletneva MA, Yen HR, Ivcevic S, Panoskaltsis-Mortari A, Gilliam AC, Drake CG, Blazar BR, Luznik L. STAT3 signaling in CD4+ T cells is critical for the pathogenesis of chronic sclerodermatous graft-versus-host disease in a murine model. J Immunol. 2010; 184:764–774. [PubMed: 19995899]
9. Pesce J, Kaviratne M, Ramalingam TR, Thompson RW, Urban JF Jr. Cheever AW, Young DA, Collins M, Grusby MJ, Wynn TA. The IL-21 receptor augments Th2 effector function and alternative macrophage activation. J Clin Invest. 2006; 116:2044–2055. [PubMed: 16778988]
10. Wilson MS, Madala SK, Ramalingam TR, Gochuico BR, Rosas IO, Cheever AW, Wynn TA. Bleomycin and IL-1beta-mediated pulmonary fibrosis is IL-17A dependent. J Expt Med. 2010; 207:535–552.
11. Fichtner-Feigl S, Strober W, Kawakami K, Puri RK, Kitani A. IL-13 signaling through the IL-13alpha2 receptor is involved in induction of TGF-beta1 production and fibrosis. Nat Med. 2006; 12:99–106. [PubMed: 16327802]
12. Belperio JA, Dy M, Burdick MD, Xue YY, Li K, Elias JA, Keane MP. Interaction of IL-13 and C10 in the pathogenesis of bleomycin-induced pulmonary fibrosis. Am J Respir Cell Mol Biol. 2002; 27:419–427. [PubMed: 12356575]
13. Jakubzick C, Choi ES, Joshi BH, Keane MP, Kunkel SL, Puri RK, Hogaboam CM. Therapeutic attenuation of pulmonary fibrosis via targeting of IL-4- and IL-13-responsive cells. J Immunol. 2003; 171:2684–2693. [PubMed: 12928422]
14. Miyahara N, Swanson BJ, Takeda K, Taube C, Miyahara S, Kodama T, Dakhama A, Ott VL, Gelfand EW. Effector CD8+ T cells mediate inflammation and airway hyper-responsiveness. Nat Med. 2004; 10:865–869. [PubMed: 15258576]
15. Das J, Eynott P, Jupp R, Bothwell A, Van Kaer L, Shi Y, Das G. Natural killer T cells and CD8+ T cells are dispensable for T cell-dependent allergic airway inflammation. Nat Med. 2006; 12:1345–1346. author reply 1347. [PubMed: 17151684]
16. Miyahara N, Takeda K, Kodama T, Joetham A, Taube C, Park JW, Miyahara S, Balhorn A, Dakhama A, Gelfand EW. Contribution of antigen-primed CD8+ T cells to the development of airway hyperresponsiveness and inflammation is associated with IL-13. J Immunol. 2004; 172:2549–2558. [PubMed: 14764728]

17. Dakhama A, Collins ML, Ohnishi H, Goleva E, Leung DY, Alam R, Sutherland ER, Martin RJ, Gelfand EW. IL-13-producing BLT1-positive CD8 cells are increased in asthma and are associated with airway obstruction. Allergy. 2013; 68:666–673. [PubMed: 23573812]
18. Oh K, Park HB, Byoun OJ, Shin DM, Jeong EM, Kim YW, Kim YS, Melino G, Kim IG, Lee DS. Epithelial transglutaminase 2 is needed for T cell interleukin-17 production and subsequent pulmonary inflammation and fibrosis in bleomycin-treated mice. J Expt Med. 2011; 208:1707–1719.
19. Spolski R, Leonard WJ. The Yin and Yang of interleukin-21 in allergy, autoimmunity and cancer. Curr Opin Immunol. 2008; 20:295–301. [PubMed: 18554883]
20. Liu SM, King C. IL-21-producing Th cells in immunity and autoimmunity. J Immunol. 2013; 191:3501–3506. [PubMed: 24058193]
21. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007; 448:484–487. [PubMed: 17581588]
22. Frohlich A, Marsland BJ, Sonderegger I, Kurrer M, Hodge MR, Harris NL, Kopf M. IL-21 receptor signaling is integral to the development of Th2 effector responses in vivo. Blood. 2007; 109:2023–2031. [PubMed: 17077330]
23. Parrish-Novak J, Foster DC, Holly RD, Clegg CH. Interleukin-21 and the IL-21 receptor: novel effectors of NK and T cell responses. J Leuk Biol. 2002; 72:856–863.
24. Parrish-Novak J, Dillon SR, Nelson A, Hammond A, Sprecher C, Gross JA, Johnston J, Madden K, Xu W, West J, Schrader S, Burkhead S, Heipel M, Brandt C, Kuijper JL, Kramer J, Conklin D, Presnell SR, Berry J, Shiota F, Bort S, Hambly K, Mudri S, Clegg C, Moore M, Grant FJ, Lofton-Day C, Gilbert T, Rayond F, Ching A, Yao L, Smith D, Webster P, Whitmore T, Maurer M, Kaushansky K, Holly RD, Foster D. Interleukin 21 and its receptor are involved in NK cell expansion and regulation of lymphocyte function. Nature. 2000; 408:57–63. [PubMed: 11081504]
25. Ozaki K, Spolski R, Feng CG, Qi CF, Cheng J, Sher A, Morse HC 3rd, Liu C, Schwartzberg PL, Leonard WJ. A critical role for IL-21 in regulating immunoglobulin production. Science. 2002; 298:1630–1634. [PubMed: 12446913]
26. Shulman Z, Gitlin AD, Weinstein JS, Lainez B, Esplugues E, Flavell RA, Craft JE, Nussenzweig MC. Dynamic signaling by T follicular helper cells during germinal center B cell selection. Science. 2014; 345:1058–1062. [PubMed: 25170154]
27. Walters DM, Kleeberger SR. Mouse models of bleomycin-induced pulmonary fibrosis. Curr Protoc Pharmacol. 2008 Chapter 5: Unit 5 46.
28. Spolski R, Wang L, Wan CK, Bonville CA, Domachowske JB, Kim HP, Yu Z, Leonard WJ. IL-21 promotes the pathologic immune response to pneumovirus infection. J Immunol. 2012; 188:1924–1932. [PubMed: 22238461]
29. Kaviratne M, Hesse M, Leusink M, Cheever AW, Davies SJ, McKerrow JH, Wakefield LM, Letterio JJ, Wynn TA. IL-13 activates a mechanism of tissue fibrosis that is completely TGF-beta independent. J Immunol. 2004; 173:4020–4029. [PubMed: 15356151]
30. Helene M, Lake-Bullock V, Zhu J, Hao H, Cohen DA, Kaplan AM. T cell independence of bleomycin-induced pulmonary fibrosis. J Leuk Biol. 1999; 65:187–195.
31. Piguet PF, Collart MA, Grau GE, Kapanci Y, Vassalli P. Tumor necrosis factor/cachectin plays a key role in bleomycin-induced pneumopathy and fibrosis. J Expt Med. 1989; 170:655–663.
32. Shevach EM. Mechanisms of foxp3+ T regulatory cell-mediated suppression. Immunity. 2009; 30:636–645. [PubMed: 19464986]
33. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol. 2012; 30:531–564. [PubMed: 22224781]
34. Sad S, Marcotte R, Mosmann TR. Cytokine-induced differentiation of precursor mouse CD8+ T cells into cytotoxic CD8+ T cells secreting Th1 or Th2 cytokines. Immunity. 1995; 2:271–279. [PubMed: 7697544]
35. Croft M, Carter L, Swain SL, Dutton RW. Generation of polarized antigen-specific CD8 effector populations: reciprocal action of interleukin (IL)-4 and IL-12 in promoting type 2 versus type 1 cytokine profiles. J Expt Med. 1994; 180:1715–1728.

36. Gur I, Or R, Segel MJ, Shriki M, Izbicki G, Breuer R. Lymphokines in bleomycin-induced lung injury in bleomycin-sensitive C57BL/6 and -resistant BALB/c mice. Exp Lung Res. 2000; 26:521–534. [PubMed: 11076310]
37. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. J Biol Chem. 2007; 282:34605–34610. [PubMed: 17884812]
38. Rosada RS, Moreira AP, Frantz FG, Puri RK, Rahman A, Standiford TJ, Zarate-Blades CR, Silva CL, Hogaboam CM. Therapeutic efficacy of Cintredekin Besudotox (IL13-PE38QQR) in murine lung fibrosis is unaffected by immunity to Pseudomonas aeruginosa exotoxin A. PLoS One. 2010; 5:e8721. [PubMed: 20090941]
39. Cerwenka A, Morgan TM, Harmsen AG, Dutton RW. Migration kinetics and final destination of type 1 and type 2 CD8 effector cells predict protection against pulmonary virus infection. J Expt Med. 1999; 189:423–434.
40. Helmich BK, Dutton RW. The role of adoptively transferred CD8 T cells and host cells in the control of the growth of the EG7 thymoma: factors that determine the relative effectiveness and homing properties of Tc1 and Tc2 effectors. J Immunol. 2001; 166:6500–6508. [PubMed: 11359800]
41. Miyazaki H, Kuwano K, Yoshida K, Maeyama T, Yoshimi M, Fujita M, Hagimoto N, Yoshida R, Nakanishi Y. The perforin mediated apoptotic pathway in lung injury and fibrosis. J Clin Pathol. 2004; 57:1292–1298. [PubMed: 15563670]
42. Ciric B, El-behi M, Cabrera R, Zhang GX, Rostami A. IL-23 drives pathogenic IL-17-producing CD8+ T cells. J Immunol. 2009; 182:5296–5305. [PubMed: 19380776]
43. Monteleone G, Caruso R, Fina D, Peluso I, Gioia V, Stolfi C, Fantini MC, Caprioli F, Tersigni R, Alessandroni L, MacDonald TT, Pallone F. Control of matrix metalloproteinase production in human intestinal fibroblasts by interleukin 21. Gut. 2006; 55:1774–1780. [PubMed: 16682426]
44. Rochman Y, Spolski R, Leonard WJ. New insights into the regulation of T cells by gamma(c) family cytokines. Nat Rev Immunol. 2009; 9:480–490. [PubMed: 19543225]
45. Wan CK, Oh J, Li P, West EE, Wong EA, Andraski AB, Spolski R, Yu ZX, He J, Kelsall BL, Leonard WJ. The cytokines IL-21 and GM-CSF have opposing regulatory roles in the apoptosis of conventional dendritic cells. Immunity. 2013; 38:514–527. [PubMed: 23453633]
46. Song E, Ouyang N, Horbelt M, Antus B, Wang M, Exton MS. Influence of alternatively and classically activated macrophages on fibrogenic activities of human fibroblasts. Cell Immunol. 2000; 204:19–28. [PubMed: 11006014]
47. Gibbons MA, MacKinnon AC, Ramachandran P, Dhaliwal K, Duffin R, Phythian-Adams AT, van Rooijen N, Haslett C, Howie SE, Simpson AJ, Hirani N, Gauldie J, Iredale JP, Sethi T, Forbes SJ. Ly6Chi monocytes direct alternatively activated profibrotic macrophage regulation of lung fibrosis. Am J Respir Crit Care Med. 2011; 184:569–581. [PubMed: 21680953]
48. Pechkovsky DV, Prasse A, Kollert F, Engel KM, Dentler J, Luttmann W, Friedrich K, Muller-Quernheim J, Zissel G. Alternatively activated alveolar macrophages in pulmonary fibrosis-mediator production and intracellular signal transduction. Clin Immunol. 2010; 137:89–101. [PubMed: 20674506]
49. Duffield JS, Forbes SJ, Constandinou CM, Clay S, Partolina M, Vuthoori S, Wu S, Lang R, Iredale JP. Selective depletion of macrophages reveals distinct, opposing roles during liver injury and repair. J Clin Invest. 2005; 115:56–65. [PubMed: 15630444]
50. Petrelli A, Carvello M, Vergani A, Lee KM, Tezza S, Du M, Kleffel S, Chengwen L, Mfarrej BG, Hwu P, Secchi A, Leonard WJ, Young D, Sayegh MH, Markmann JF, Zajac AJ, Fiorina P. IL-21 is an antitolerogenic cytokine of the late-phase alloimmune response. Diabetes. 2011; 60:3223–3234. [PubMed: 22013017]
51. Attridge K, Wang CJ, Wardzinski L, Kenefeck R, Chamberlain JL, Manzotti C, Kopf M, Walker LS. IL-21 inhibits T cell IL-2 production and impairs Treg homeostasis. Blood. 2012; 119:4656–4664. [PubMed: 22442347]
52. Schmitz I, Schneider C, Frohlich A, Frebel H, Christ D, Leonard WJ, Sparwasser T, Oxenius A, Freigang S, Kopf M. IL-21 restricts virus-driven Treg cell expansion in chronic LCMV infection. PLoS pathogens. 2013; 9:e1003362. [PubMed: 23696736]

53. Van Belle TL, Nierkens S, Arens R, von Herrath MG. Interleukin-21 receptor-mediated signals control autoreactive T cell infiltration in pancreatic islets. Immunity. 2012; 36:1060–1072. [PubMed: 22579473]

54. Atamas SP, Yurovsky VV, Wise R, Wigley FM, Goter Robinson CJ, Henry P, Alms WJ, White B. Production of type 2 cytokines by CD8+ lung cells is associated with greater decline in pulmonary function in patients with systemic sclerosis. Arthritis Rheum. 1999; 42:1168–1178. [PubMed: 10366109]

55. Radstake TR, van Bon L, Broen J, Hussiani A, Hesselstrand R, Wuttge DM, Deng Y, Simms R, Lubberts E, Lafyatis R. The pronounced Th17 profile in systemic sclerosis (SSc) together with intracellular expression of TGFbeta and IFNgamma distinguishes SSc phenotypes. PloS one. 2009; 4:e5903. [PubMed: 19536281]

56. Fuschiotti P, Larregina AT, Ho J, Feghali-Bostwick C, Medsger TA Jr. Interleukin-13-producing CD8+ T cells mediate dermal fibrosis in patients with systemic sclerosis. Arthritis Rheum. 2013; 65:236–246. [PubMed: 23001877]

57. Fuschiotti P, Medsger TA Jr. Morel PA. Effector CD8+ T cells in systemic sclerosis patients produce abnormally high levels of interleukin-13 associated with increased skin fibrosis. Arthritis Rheum. 2009; 60:1119–1128. [PubMed: 19333920]

58. Kradin RL, Divertie MB, Colvin RB, Ramirez J, Ryu J, Carpenter HA, Bhan AK. Usual interstitial pneumonitis is a T-cell alveolitis. Clin Immunol Immunopathol. 1986; 40:224–235. [PubMed: 3521973]

59. Papiris SA, Kollintza A, Kitsanta P, Kapotsis G, Karatza M, Milic-Emili J, Roussos C, Daniil Z. Relationship of BAL and lung tissue CD4+ and CD8+ T lymphocytes, and their ratio in idiopathic pulmonary fibrosis. Chest. 2005; 128:2971–2977. [PubMed: 16236974]

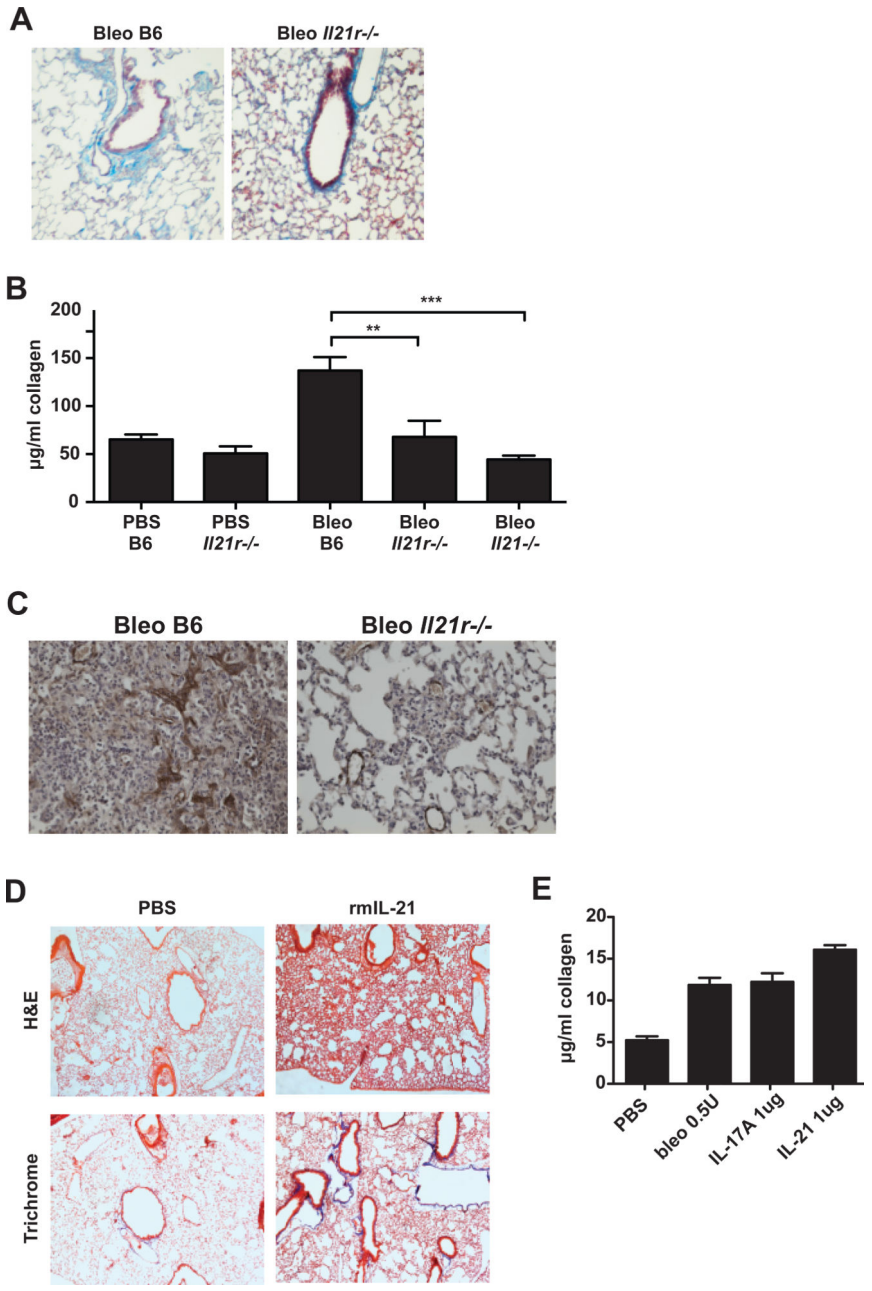

Figure 1. IL-21 promotes pulmonary fibrosis following lung injury

B6 and *Il21r−/−* mice were treated i.t with bleomycin or PBS and sacrificed at day 14. (A) Formalin-fixed, paraffin-embedded lung sections were stained with Masson's trichrome. Magnification = 20X. (B) Collagen concentrations in lung extracts were quantitated by a colorimetric assay. (C) Lung sections were stained by immunohistochemistry for α-smAc to detect activated fibroblasts Data in A-C are representative of three independent experiments (PBS→B6, n=6; PBS→*Il21r−/−*, n=4; Bleomycin→B6, n=11; Bleomycin→*Il21r−/−*, n=11; Bleomycin→*Il21−/−*, n=5). (D) BALB/c mice were treated with a single instillation of rmIL-21 i.t. or PBS and at day 10, lungs were harvested and formalin fixed, paraffin-embedded sections stained by H&E and Masson's Trichrome. Magnification = 20X. (E)

Collagen concentrations in lung extracts from PBS, bleomycin, IL-17At, and IL-21 treated mice were measured by colorimetric assay. Bars represent means ± SEM. Results shown are compiled from four independent experiments with three to four mice per group. Data shown are means ± SEM. ** denotes $p \leq 0.01$; Student's *t* test. Error bars represent standard error of the mean (SEM).

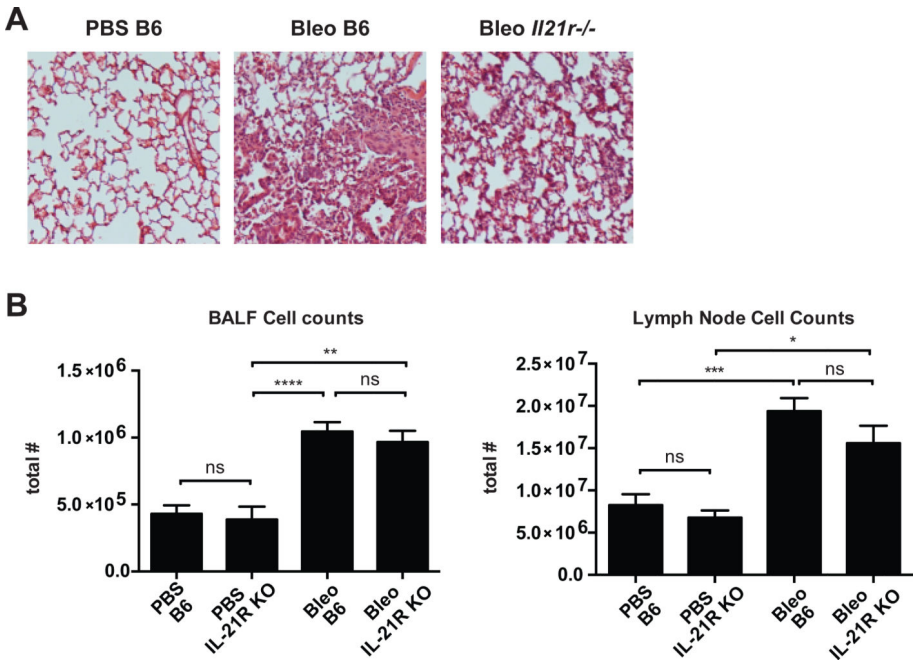

Figure 2. IL-21R deficiency does not prevent lung inflammation after bleomycin injury

B6 and *Il21r−/−* mice were treated i.t with bleomycin or PBS and sacrificed at day 14. (A) Formalin-fixed, paraffin-embedded lung sections were stained by H&E. Magnification = 20X. (B) BALF and LDLN cells were collected at day 14 and cell numbers/mouse were determined. Data are compiled from 3-4 independent experiments.

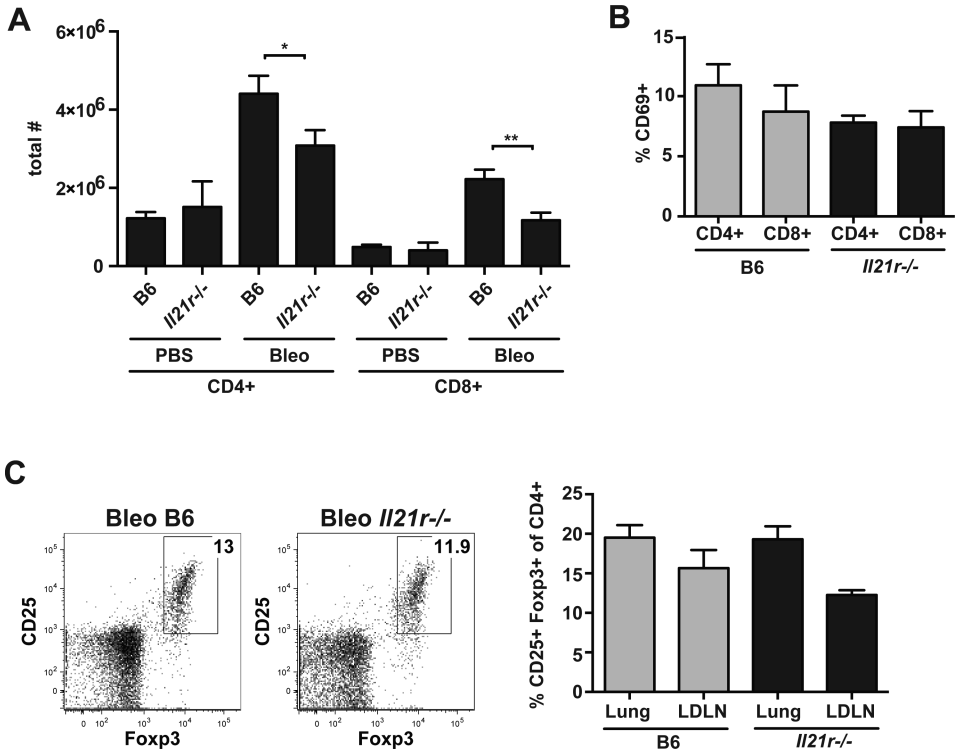

Figure 3. IL-21/IL-21R interactions are required for optimal CD8$^+$ T cell recruitment to the lung following bleomycin injury

B6 and *Il21r*−/− mice were treated with bleomycin or PBS i.t. and sacrificed at day 14. (A) Summary of the total number of CD4$^+$ and CD8$^+$ T cells in LDLN at day 14. (B) LDLN single-cell suspensions were analyzed by flow cytometry for surface expression of CD69. (C) Surface and intranuclear staining of LDLN single-cell suspensions was performed to determine Treg frequency by co-expression of CD25 and Foxp3 as determined by flow cytometry. Data are compiled from two independent experiments with similar results (PBS→B6, n=6; PBS→*Il21r*−/−, n=4; Bleomycin→B6, n=8; Bleomycin→*Il21r*−/−, n=8). Data shown are means with an error bar representing SEM. * denotes a $p<0.05$, **, $p\leq0.01$ and, ***, $p\leq0.001$; Student's *t* test. Error bars represent standard error of the mean (SEM).

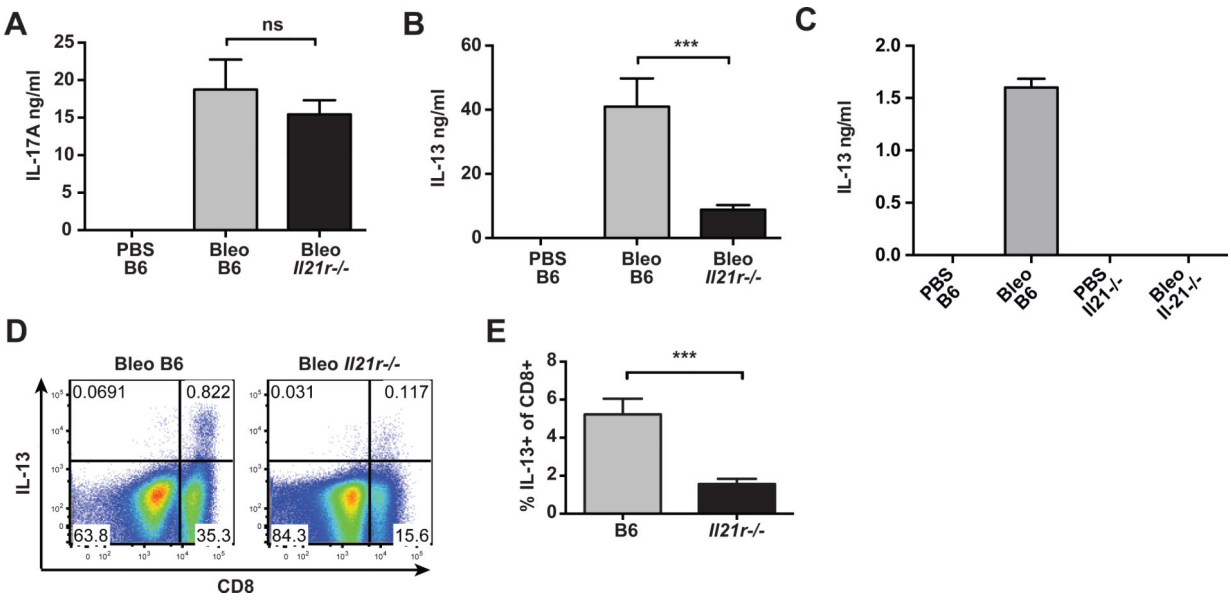

Figure 4. Lack of IL-21R significantly impairs IL-13 production in response to sterile lung injury

(A, B) B6 and *Il21r−/−* were treated i.t. with PBS or bleomycin. At day 14, the mice were sacrificed LDLN were harvested. LDLN cell suspensions collected from PBS and bleomycin-treated mice at day 14 were stimulated with anti-CD3 and culture supernatants were harvested 24 hours later and assayed for IL-17A and IL-13 by ELISA. Data are compiled from two independent experiments with similar results. (PBS→ B6, n=6, Bleomycin→B6, n=9; Bleomycin→*Il21r−/−*, n=7). (C) B6 and *Il21−/−* were treated i.t. with PBS or bleomycin. At day 14, the mice were sacrificed and BAL cells harvested. BAL cells were stimulated with anti-CD3 and culture supernatants were harvested 24 hours later and assayed for IL-13 by ELISA. Data in *C* shows PBS→B6, n=3; PBS→*Il21−/−*, n=2 Bleomycin→B6, n=6; Bleomycin→*Il21−/−*, n=4. (D, E) LDLN T cells from bleomycin-treated mice at day 14 were re-stimulated and cytoplasmic staining was performed for IL-13. Data in *D* are compiled from three independent experiments. (Bleomycin→B6, n=12; Bleomycin→*Il21r−/−*, n=10). Data in *E* are representative of three independent experiments that yielded similar results with >3 mice per group. Data shown are means with an error bar representing SEM. ns denotes $p>0.05$, ***, $p\leq0.001$; Student's *t* test.

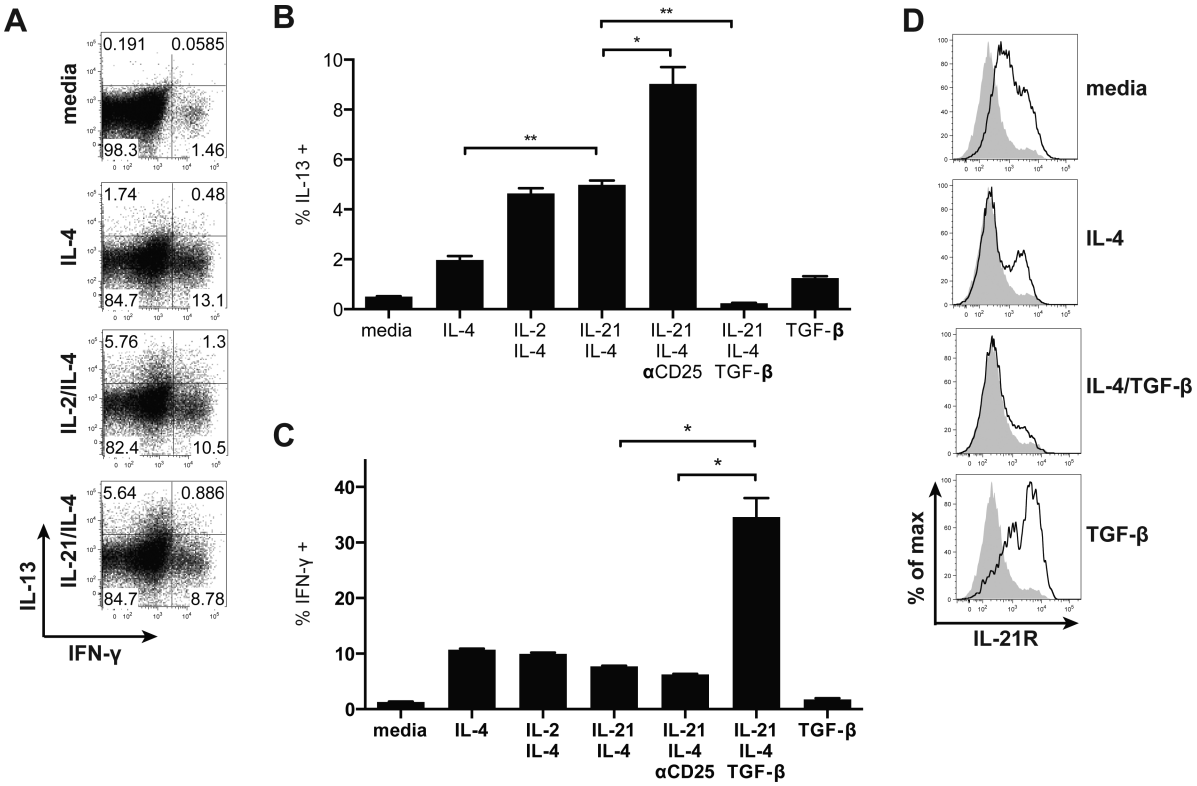

Figure 5. IL-21 drives Tc2 differentiation *in vitro*

Purified splenic CD8$^+$ T cells from naïve B6 mice were cultured in the presence of the indicated cytokines and antibodies and activated with plate-bound anti-CD3 and soluble anti-CD28 for 4-5 days. (A-C) CD8+ T cell cultures were re-stimulated using anti-CD3 in the presence of GolgiStop and cytoplasmic staining for IL-13 and IFN-γ was performed. A representative flow cytometry plot is shown in *A* and a summary is shown in *B* and *C*. Bars represent means ± SEM. Data are representative of five independent experiments. (D) Surface IL-21R expression on cultured CD8$^+$ T cells was analyzed by flow cytometry at day 5 post-primary stimulation. Grey histogram shows *Il21r*−/− control subjected to the same stain. Data are representative of three independent experiments. Bars represent are means with an error bar representing SEM. * denotes a $p<0.05$, **, $p\leq0.01$ and, ***, $p\leq0.001$; unpaired Student's *t* test.

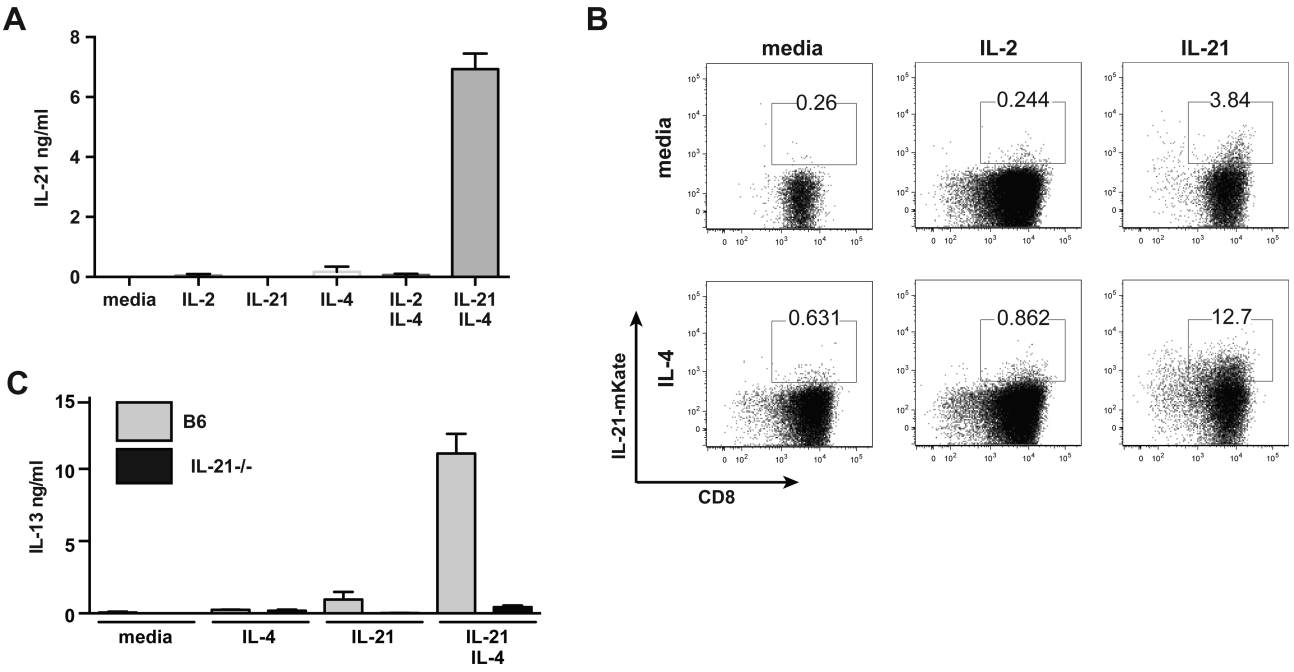

Figure 6. Autocrine IL-21 promotes Tc2 phenotype

(A) Purified splenic $CD8^+$ T cells from naïve B6 mice were cultured in the presence of the indicated cytokines and antibodies for 4 days, washed and then re-stimulated using anti-CD3. Cell supernatants were harvested at 24 hours and assayed for IL-21. Data shown are compiled three independent experiments. (B) Purified $CD8^+$ T cells from WT and IL-21-mKate reporter mice were stimulated as indicated and mKate expression was analyzed by flow cytometry at day 4 without restimulation or addition of exogenous cytokines. . Representative of two independent experiments. (C) Purified $CD8^+$ T cells from WT and *Il21−/−* mice were stimulated as indicated and the concentration of IL-13 in the culture supernatants was determined. Results in C are representative of three independent experiments. Bars show means ± SEM.

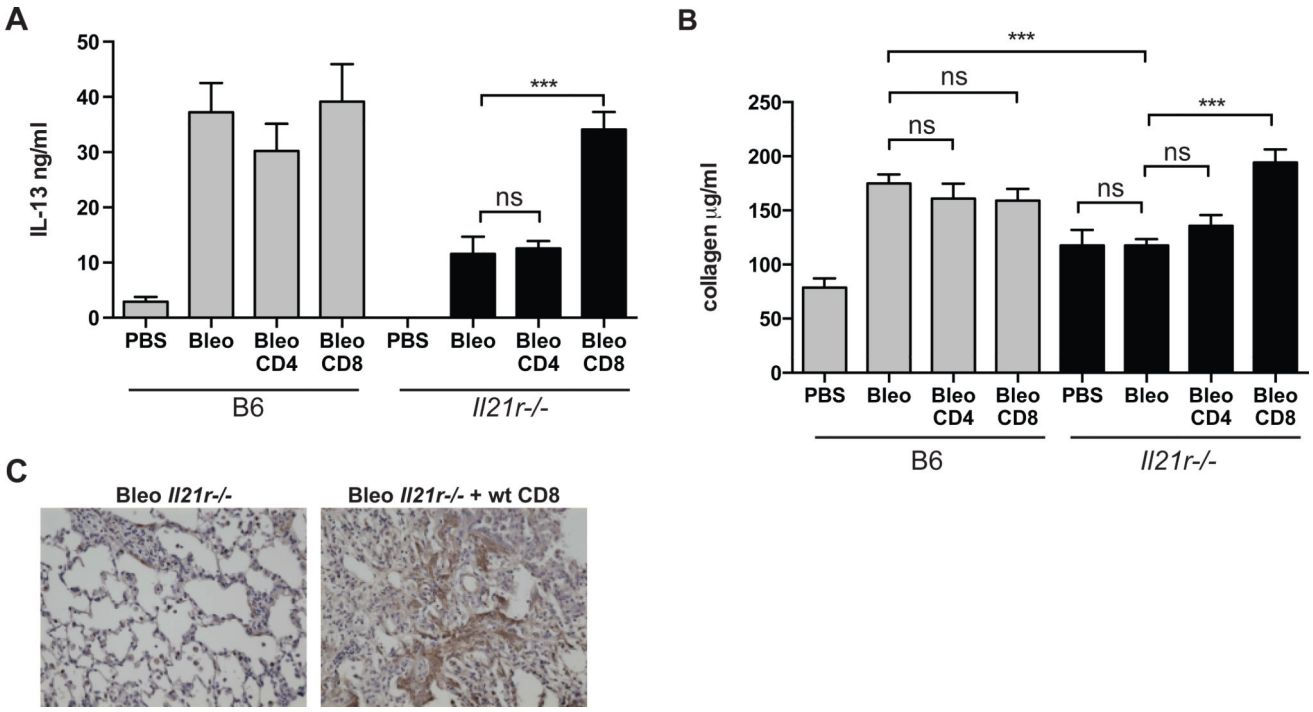

Figure 7. IL-21R on CD8$^+$ T cells is required for optimal IL-13 production and collagen deposition

(A) 10^6 purified splenic CD4$^+$ or CD8$^+$ T cells from naïve B6 mice were injected i.v. into B6 and *Il21r−/−* mice at day −1. At day 0, mice were treated i.t. with PBS or bleomycin. LDLN cell suspensions from PBS and bleomycin-treated mice were collected at day 14 and stimulated with anti-CD3. Cell supernatants were harvested at 24 hours and assayed for IL-13. (B) Lungs were harvested at day 14 and lung extracts were quantitated for collagen concentration by Sircol assay. (C) Lung sections obtained from day 14 were stained for α-smooth muscle actin to detect fibroblast activation Magnification = 20X. Data in *A-B* are compiled from two independent experiments (PBS→B6, n=5; PBS→ *Il21r−/−*, n=4; Bleomycin→B6, n=9; Bleomycin→ *Il21r−/−* , n=8; Bleomycin→B6 + wt CD4, n=5; Bleomycin→B6 + wt CD8, n=8; Bleomycin→*Il21r−/−* + wt CD4, n=5 ;Bleomycin→*Il21r −/−* + wt CD8, n=9). Bars represent means ± SEM. * denotes a $p<0.05$, **, $p\leq0.01$ and, ***, $p\leq0.001$; unpaired Student's *t* test.