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Journal
American Journal Of Pathology, 186(8)

ISSN
0002-9440

Authors
Birjandi, Shirin Z
Palchevskiy, Vyacheslav
Xue, Ying Ying
et al.

Publication Date
2016-08-01

DOI
10.1016/j.ajpath.2016.03.020

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The American Journal of Pathology, Vol. 186, No. 8, August 2016

The American Journal of
PATHOLOGY
ajp.amjpathol.org

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CARDIOVASCULAR, PULMONARY, AND RENAL PATHOLOGY

$CD4^+CD25^{hi}Foxp3^+$ Cells Exacerbate Bleomycin-Induced Pulmonary Fibrosis

Shirin Z. Birjandi,* Vyacheslav Palchevskiy,* Ying Ying Xue,* Stefanie Nunez,* Rita Kern,* S. Sam Weigt,* Joseph P. Lynch, III,* Talal A. Chatila,† and John A. Belperio*

From the Department of Medicine, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, California; and the Department of Pediatrics,† Harvard Medical School, Boston, Massachusetts*

Accepted for publication March 23, 2016.

Address correspondence to Shirin Z. Birjandi, Ph.D., Box 951690, 37-131 CHS, Los Angeles, CA 90095-1690. E-mail: shirin.birjandi@cshs.org.

Idiopathic pulmonary fibrosis is a fatal lung disease with a median survival of 2 to 5 years. A decade of studies has downplayed inflammation contributing to its pathogenesis. However, these studies preceded the discovery of regulatory T cells (Tregs) and all of their functions. On the basis of human studies demonstrating Tregs can decrease graft-*versus*-host disease and vasculitides, there is consideration of their use to treat idiopathic pulmonary fibrosis. We hypothesized that Treg therapy would attenuate the fibroplasia involved in a preclinical murine model of pulmonary fibrosis. IL-2 complex was used *in vivo* to expand $CD4^+CD25^{hi}Foxp3^+$ cells in the lung during intratracheal bleomycin challenge; however, this unexpectedly led to an increase in lung fibrosis. More important, this increase in fibrosis was a lymphocyte-dependent process. We corroborated these results using a $CD4^+CD25^{hi}Foxp3^+$ cellular-based therapy. Mechanistically, we demonstrated that $CD4^+CD25^{hi}Foxp3^+$ cells undergo alterations during bleomycin challenge and the IL-2 complex had no effect on profibrotic (eg, transforming growth factor-β) or type 17 immune response cytokines; however, there was a marked down-regulation of the type 1 and augmentation of the type 2 immune response cytokines from the lungs. Collectively, our animal studies show that a specific lung injury can induce Treg alterations, which can augment pulmonary fibrosis. *(Am J Pathol 2016, 186: 2008–2020; http://dx.doi.org/10.1016/j.ajpath.2016.03.020)*

Idiopathic pulmonary fibrosis (IPF) is a progressive and lethal disease of the lung.[1,2] Greater than a decade of studies has led to the conclusion that inflammation plays a minor, if any, role in the pathogenesis of IPF.[3,4] In corroboration, several studies have suggested that lymphocytes are not involved in the development of pulmonary fibrosis in humans and in animal models.[4,5] More specifically, mice with genetic defects manifesting in dysfunctional or no lymphocytes or normal mice depleted of T cells had no effect on pulmonary fibrosis.[5–7] These studies reinforced the notion that there is no role for inflammation driving fibrosis in IPF.

Many of the above observations preceded the discovery of regulatory T cells (Tregs) and their functional capabilities. Tregs are a subpopulation of $CD4^+$ T cells that are identified as $CD25^{hi}$ and expressing the transcription factor Foxp3 known to be essential for dominant self-tolerance.[8,9] Studies involving Tregs have demonstrated that they are capable of suppressing many biologically hostile inflammatory processes and prevent immunopathology by maintaining immune homeostasis.[8] These key findings, in combination with preclinical animal models, led to the human clinical trials involving therapies that augment Tregs, which has made definitive impacts on human autoimmunity and graft-*versus*-host disease (GVHD).[10,11]

Interestingly, there are studies suggesting that activated $CD4^+$ T cells from the peripheral blood of IPF patients are associated with worst clinical outcomes.[12] Furthermore, phenotypically distinct Tregs, via their expression of Semaphorin 7a+ and platelet-derived growth factor (PDGF),

Supported by NIH Pulmonary Training grant T32HL07252 to the University of California, Los Angeles (UCLA; S.Z.B.); National Center for Advancing Translational Sciences through UCLA CTSI grant UL1 TR000124 (J.A.B.); NIH grants R01 HL112990, HL131407, and CTOT 11532596 to UCLA (J.A.B.); and NIH grant 2R01AI065617 to Harvard (T.A.C.).

Disclosures: None declared.

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http://dx.doi.org/10.1016/j.ajpath.2016.03.020

have been associated with worsening pulmonary fibrosis in humans and in rodent models of lung fibrosis.[13,14] Moreover, a recent human study demonstrated that azathioprine up-regulates Treg,[15] whereas a multicenter clinical trial involving azathioprine for the treatment of IPF resulted in an increase in hospitalizations and mortality,[16] suggesting Treg may have a negative impact in patients with pulmonary fibrosis. Conversely, several studies have shown an association between decreased Treg frequencies and IPF patients.[17,18] Similarly, several animal models of acute lung injury and pulmonary fibrosis have suggested that the depletion of Tregs increases lung fibrosis, whereas Treg cell-based therapy results in the resolution of lung injury.[19−21] These desperate results underline the need for a preclinical animal study to guide further clinical trials involving Treg cells in patients with pulmonary fibrosis.

Ex vivo expansion of Tregs has multiple regulatory and production difficulties for therapeutic use in humans, suggesting that *in vivo* expansion of Tregs is a more practical approach.[22] *In vivo* expansion of Tregs using IL-2 therapies in humans has led to successful treatment of both vasculitis and GVHD.[10,11] Thus, our aim was to expand $CD4^+CD25^{hi}Foxp3^+$ cells during bleomycin-induced pulmonary fibrosis in a preclinical animal study to determine the role of $CD4^+CD25^{hi}Foxp3^+$ cells on lung fibroplasia.

We expanded $CD4^+CD25^{hi}Foxp3^+$ cells *in vivo* via the injection of IL-2 complexed to a specific IL-2 monoclonal antibody (mAb) clone (JES6-1) as well as confirmatory $CD4^+CD25^{hi}Foxp3^+$ cellular based therapies.[23−25] We show, mechanistically, that $CD4^+CD25^{hi}Foxp3^+$ cells exacerbate bleomycin-induced pulmonary fibrosis by undergoing plasticity, which leads to immune deviation of the lungs away from a type 1 and toward a type 2 immune response without significant changes in profibrotic mediators [transforming growth factor-β (TGF-β)] or the type 17 immune responses. Importantly, our work has shown the effect of increasing $CD4^+CD25^{hi}Foxp3^+$ cells in pulmonary fibrosis and has resolved the longstanding debate over the roles of both Treg cells and overall T-cell involvement in lung fibroplasia. Our research will advance the field's understanding of the role of inflammatory cells that exacerbate lung fibrosis.

Materials and Methods

Animal Model of Pulmonary Fibrosis

Female C57BL/6 (10 weeks) and *Rag1*$^{-/-}$ mice on a C57BL/6 background (10 weeks) were purchased from Charles River (San Diego, CA) or The Jackson Laboratory (Bar Harbor, ME). Foxp3 enhanced green fluorescent protein (EGFP; 10 weeks) on a C57BL/6 background mice were kindly provided by Dr. Talal A. Chatila (Harvard Medical Center, Boston, MA). On receipt all animals were housed at the animal research facility (at UCLA) under specific pathogen-free conditions in accordance with UCLA's Animal Care and Use Committee. We choose the C57BL/6 strain because they are well characterized and are susceptible to bleomycin-induced pulmonary fibrosis. Mice were challenged via intratracheal (i.t.) bleomycin (APP, Schaumburg, IL) at 1 U/mL with 40 μL delivered on day 0, as previously described.[26,27] Control animals received sterile saline (0.9% NaCl). Briefly, mice were anesthetized with 130 μL of 2 mg/mL ketamine plus 0.08 mg/mL xylazine injected i.p., followed by i.t. administration of bleomycin. At 14 days after bleomycin treatment, animals were sacrificed and lungs were perfused with 0.9% NaCl followed by 4% paraformaldehyde. Lungs were then embedded in paraffin for histological analysis.

Ashcroft Scoring

Paraffin sectioned lungs stained with H&E were examined for the severity of interstitial fibrosis using a scale of severity ranging from 0 to 8 (0 = normal lung; 8 = very severe fibrosis) via Ashcroft method.[28] Briefly, sections obtained from midsection of the lungs were evaluated and blindly scored by two independent investigators. Entire lung sections were scored under a 10× objective via light microscopy using a Zeiss Axioskop 2 Plus. Each 10× field was given a score and the average of all fields was obtained and reported for each individual animal.

Trichrome Staining and Analysis

Differences in collagen content between treatment groups were evaluated using Trichrome Stain (Abcam, Cambridge, MA) as detailed in manufacturer's protocol. Briefly, paraffin-embedded lungs were cut into sections (4 μm thick) and Trichrome staining was performed. Collagen fibers were stained blue; muscle fiber, cytoplasm, erythrocytes were stained red; and nuclei were stained black/blue. For quantitative analysis, images were taken blindly at 10× objective and analysis was performed using Fiji software version 2.0.0-rc-30/1.49v (ImageJ; NIH, Bethesda, MD; *http://fiji.sc*). Five random regions were imaged per animal, and the average was obtained and reported for each individual animal.

Flow Cytometry

Lungs and spleens were dispersed by initial mincing with scissors into fine slurry and subsequently passed through wire screens for removal of tissue debris. The dispersed cells were collected in RPMI 1640 with 10% fetal bovine serum, Penstrap, L-glutamine, and HEPES. Erythrocytes were lysed with ACK and incubated on ice for 2 minutes. After multiple washes, cells were filtered through a 20-μm screen for removal of any excess tissue debris. Four-color analysis of cells from $Foxp3^+$ EGFP lungs was performed on single cell suspensions involving 1×10^6 cells per sample that were incubated with rat anti-mouse CD45-Alexa Fluor 700, rat anti-mouse CD4-PerCP-Cy5.5/APC, and

CD25-PE-Cy7/PE for 30 minutes on ice. Unless otherwise noted, all antibodies were purchased from BD Biosciences (San Jose, CA). Cells were then washed, fixed in 2% paraformaldehyde, and analyzed using HT-LSR-II (BD Biosciences, San Jose, CA) with Tree Star FlowJo software version 9.6.4 (BD, Mountain View, CA). Four-color analysis of cells from wild-type lungs treated with or without IL-2/JES6-1 antibody complex involved 1×10^6 cells per sample that were incubated with rat anti-mouse CD45-Alexa Fluor 700, rat anti-mouse CD4-APC, and rat anti-mouse CD25-fluorescein isothiocyanate on ice for 30 minutes. Intracellular staining of Foxp3, using rat anti-mouse Foxp3-PE (eBioscience, San Diego, CA), was then performed according to manufacturer's instructions.

Adoptive Transfer of $CD4^+CD25^{hi}Foxp3^+$ Cells

Spleen cells were dispersed from $Foxp3^+$ EGFP mice, as described above. $CD4^+$ T cells were then isolated from single-cell suspensions by negative selection using a mouse $CD4^+$ T-cell isolation kit (Stem Cell Technologies, Vancouver, BC, Canada), according to manufacturer's instructions. Magnetic bead isolated $CD4^+$ T cells were then cell sorted on FACSAria II (BD, San Jose, CA) for $CD25^{hi}$ GFP^+ cells with >98.0% recovery of $CD4^+CD25^{hi}Foxp3^+$ cells. Cells were collected in chilled tubes containing complete RPMI media with 20% fetal bovine serum, then washed and resuspended in sterile 0.9% NaCl and injected at 0.6×10^6 cells/200 μL vol. i.p. into recipient mice 24 hours prior i.t. bleomycin administration. Vehicle controls received 0.9% NaCl that carried the cells.

Sircol Assay

Total lung collagen was determined using the Sircol assay, as detailed in the manufacturer's protocol (Biocolor, Carrickfergus, UK). Briefly, lungs were harvested, placed on ice, and cut into 4 mm cubes, then washed with cold phosphate-buffered saline followed by overnight incubation in acid-pepsin extraction media at 4°C. Acid neutralizing reagent, cold isolation, and concentration reagent subsequently were added and allowed to incubate for 24 hours half immersed in an ice-water mix at 4°C. Tubes were pelleted and the collagen released into solution was removed by gentle pipetting. Sircol dye reagent was added to each sample as per manufacturer's instructions. Bound Sircol dye was assessed using a microplate reader (Biotek Synergy HT) set to 555 nm. Collagen standards supplied with the kit were used for assay controls.

Quantitative PCR for Cytokine Gene Expression

Whole lung homogenates were placed in Trizol reagent (Life Technologies, Grand Island, NY). Total RNA (2 μg) was DNase treated and reverse transcribed into cDNA. Gene expression assays for tumor necrosis factor-α (TNF-α), IL-1α, fibroblast growth factor (FGF)-9, PDGF-β, connective tissue growth factor (CTGF), IL-12p35, IL-12p40, IL-18, IL-4, IL-5, IL-13, TGF-β, and IL-17f were used for amplification on a StepOnePlus Q-PCR System (Life Technologies) and analyzed using StepOnePlus software version 2.3. 18S was used as endogenous control where appropriate. Quantitative analysis of gene expression was performed using the $2^{\Delta\Delta C_T}$ method as previously described.[26,27] Data are presented as fold difference compared to treatment controls.

IL-2/JES6-1 Antibody Complex Preparation

IL-2 complex consists of anti−IL-2 antibody (JES6-1) and mouse recombinant IL-2 protein incubated for 30 minutes at 37°C. Similar to work done by Webster et al,[25] we administered a short-term IL-2 complex for 3 consecutive day's i.p. at a total dose of 6 μg per injection (5 μg mAb JES6-1 + 1 μg IL-2) before i.t. bleomycin administration. Long-term IL-2 complex (6 μg per injection) consisted of every 3 days i.p. dosing starting at day -3 before i.t. bleomycin administration and continued until the lungs were harvested. The control group received i.p. vehicle 0.9% NaCl.

Statistical Analysis

Data were analyzed using Prism software version 5.0d (GraphPad Software, Inc., La Jolla, CA). Comparisons were made using the unpaired *t*-test, Mann-Whitney, and Kaplan-Meier survival analysis log-rank where appropriate. Data were considered statistically significant if $P < 0.05$.

Results

Increased Pulmonary $CD4^+CD25^{hi}Foxp3^+$ Cells Are Associated with Bleomycin-Induced Pulmonary Fibrosis

Some but not all studies involving peripheral blood, bronchoalveolar lavage fluid, and lung tissue have suggested that human patients with IPF may have a reduction in Tregs in all three compartments[17,18,29]; thus, we characterized the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells in the murine bleomycin-induced pulmonary fibrosis model. Using Foxp3 EGFP transgenic mice that coexpress Foxp3 and the enhanced green fluorescent protein (EGFP), which is under the control of the endogenous Foxp3 promoter and enhancer elements on a C57BL/6 background, we evaluated the frequency of this $CD4^+$ T-cell subpopulation in the lungs from animals receiving 40 μL of i.t. bleomycin (1 U/mL) as compared to 40 μL of i.t. control (0.9% NaCl) delivered at day 0. Whole lungs were harvested, minced, and pneumatically strained into single-cell suspensions without digests to preserve intact T-cell surface markers. Multiparameter flow cytometry using lymphocytes as the parent population by

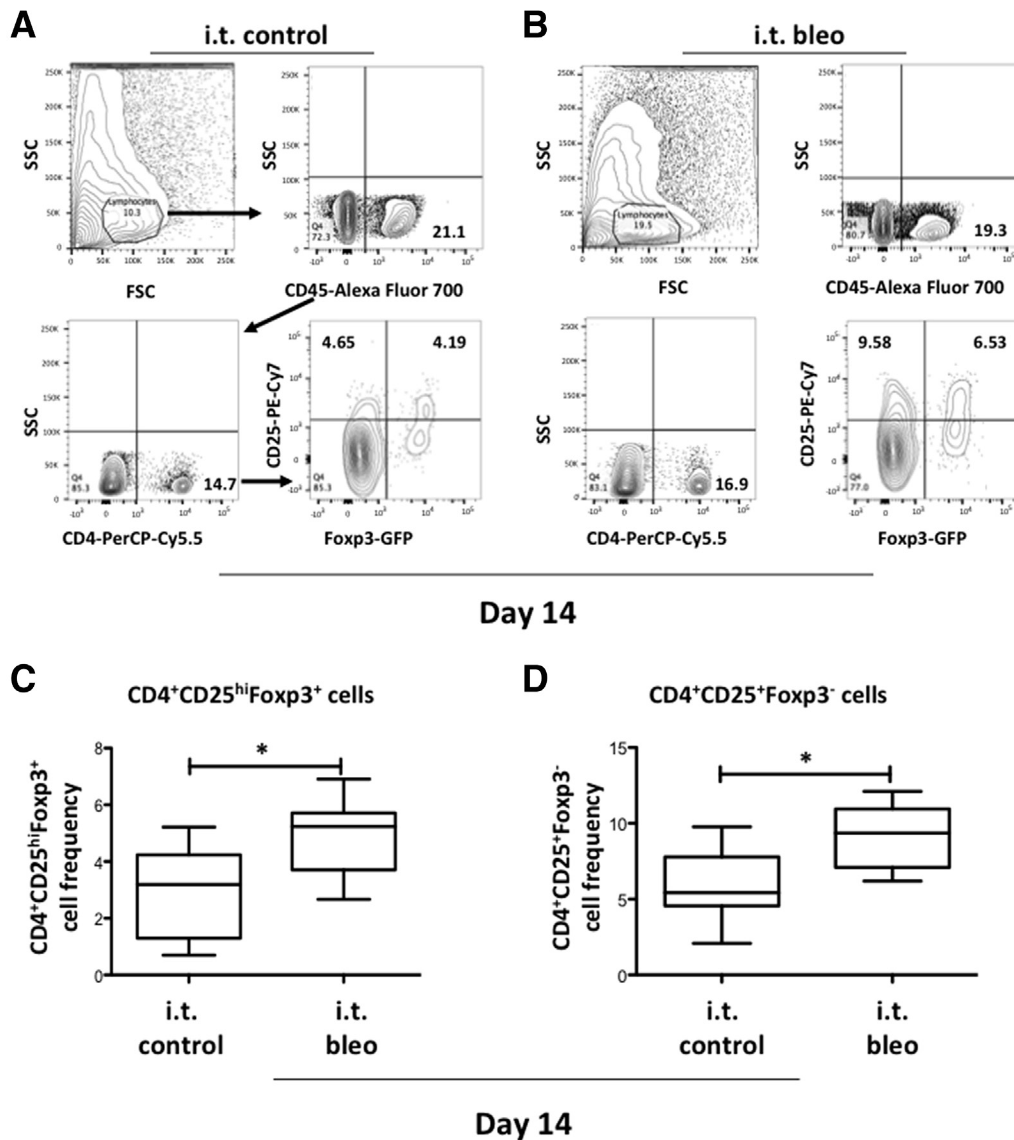

Figure 1 Increased frequency of CD4$^+$CD25^{hi}Foxp3$^+$ cells from the lungs of Foxp3 enhanced green fluorescent protein (EGFP) transgenic mice challenged with intratracheal (i.t.) bleomycin. Representative flow cytometry contour plots of whole lung single-cell suspension demonstrating the gating strategy used to evaluate the frequency of CD4$^+$CD25^{hi}Foxp3$^+$ cells 14 days after i.t. control (**A**) and i.t bleomycin (**B**). Box and whisker plots of the whole lung frequencies of CD4$^+$CD25^{hi}Foxp3$^+$ (**C**) and CD4$^+$CD25$^+$Foxp3$^-$ (**D**) cells. $n = 8$ in each group. *$P < 0.05$. bleo, bleomycin; FSC, forward scatter; SSC, side scatter.

forward scatter and side scatter, then CD45$^+$ cells from which we evaluated CD4$^+$ lymphocytes for CD25 and Foxp3 (Figure 1), demonstrated a marked increase in the frequency of CD4$^+$CD25^{hi}Foxp3$^+$ cells from the bleomycin-challenged lungs as compared to control at day 14, the time period of maximal fibrosis (Figure 1, A–C).[26] There was also an increase in the frequencies of CD4$^+$CD25$^+$Foxp3$^-$ cells between the bleomycin- and control-challenged lungs at day 14 (Figure 1D).

IL-2 Complex Expands CD4$^+$CD25^{hi}Foxp3$^+$ Cells in the Lungs of Naïve C57BL/6 Mice

Because Tregs have been shown to be protective in certain forms of acute lung injury,[19–21] we hypothesized that a further expansion of CD4$^+$CD25^{hi}Foxp3$^+$ cells during the bleomycin challenge could be protective against lung fibrosis. Using a practical and clinically relevant approach, we first determined if we could expand CD4$^+$CD25^{hi}Foxp3$^+$ cells in naïve C57BL/6 mouse lungs by means of the IL-2 complex (5 μg mAb JES6-1 + mouse recombinant 1 μg IL-2) delivered i.p. 3 consecutive days via the same strategy used by Webster and associates[24,25] to expand splenic Treg[23] (Figure 2A). The control group consisted of C57BL/6 mice receiving the equivalent volume of i.p. 0.9% NaCl (200 μL) (Figure 2A). Harvested lungs were minced and pneumatically strained into single-cell suspensions and stained for flow cytometry. We found a 12.5-fold increase in the frequency of CD4$^+$CD25^{hi}Foxp3$^+$ cells from the mice receiving i.p. IL-2 complex, as

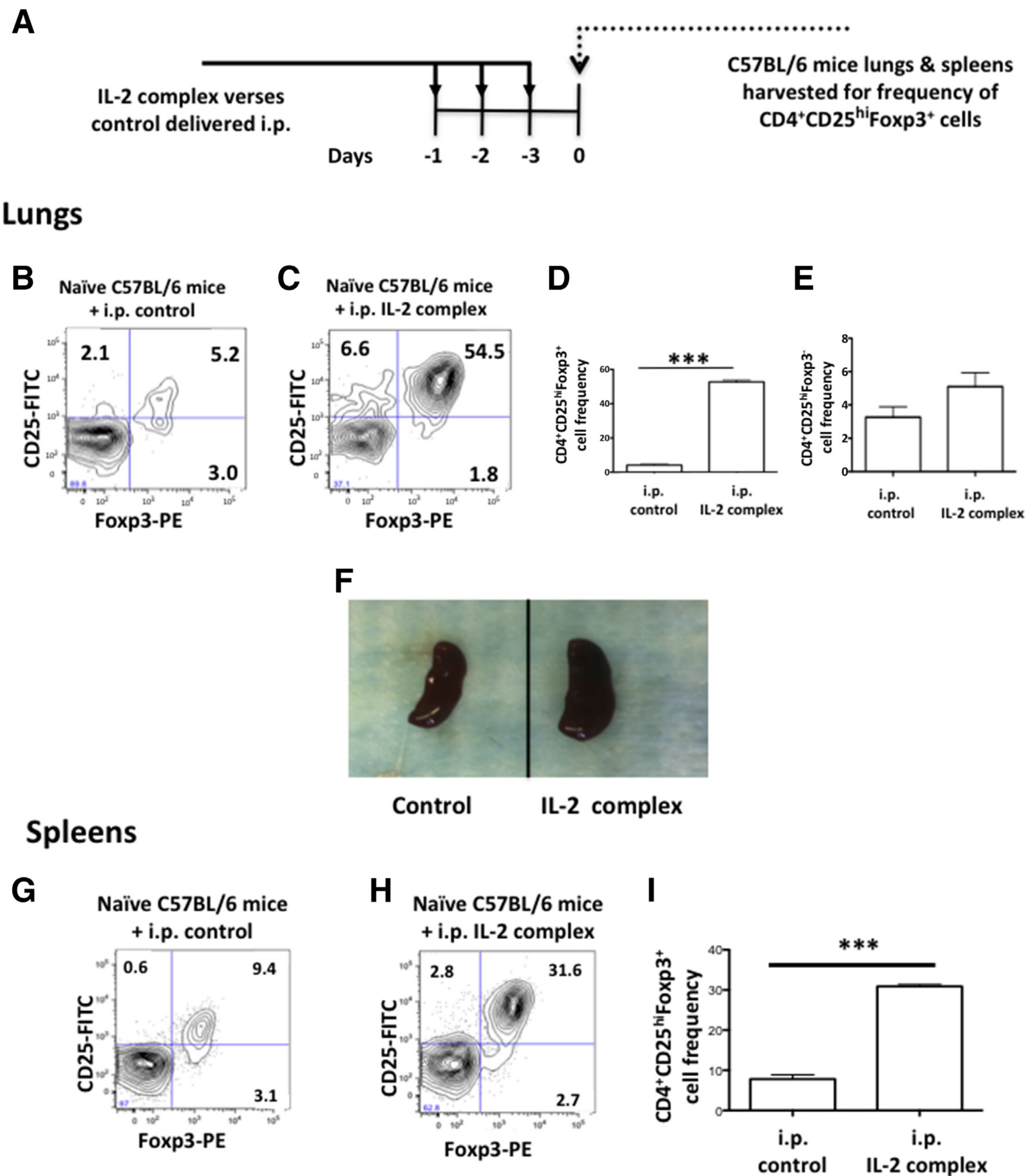

Figure 2 IL-2 complex delivered i.p. for 3 consecutive days leads to *in vivo* expansion of $CD4^+CD25^{hi}Foxp3^+$ cells in the lungs and spleens of unchallenged C57BL/6 naïve mice. **A:** Diagram of the experimental design showing 3 consecutive days of IL-2 complex as compared to control given i.p. with the lung and spleen harvested for the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells. Representative contour plots from the lungs of mice that received 3 consecutive days of i.p. control (**B**) and i.p. IL-2 complex (**C**). **D:** Column bar graph of the whole lung frequencies of $CD4^+CD25^{hi}Foxp3^+$ cells from animals treated with i.p. control as compared to IL-2 complex. **E:** Column bar graph of the whole lung frequencies of $CD4^+CD25^{hi}$ $Foxp3^-$ cells from animals treated with i.p. control as compared to IL-2 complex. **F:** Representative spleen sizes from i.p. control as compared to the IL-2 complex. Representative contour plots from the spleens of mice that received 3 consecutive days of i.p. control (**G**) and i.p. IL-2 complex (**H**). **I:** Column bar graph of the whole spleen frequencies of $CD4^+CD25^{hi}Foxp3^+$ cells from animals treated with i.p. control as compared to IL-2 complex. $n = 3$ in each group. $***P < 0.001$.

compared to i.p. control (Figure 2, B–D). Conversely, we did not find a significant increase in the frequency of $CD4^+CD25^+Foxp3^-$ cells (Figure 2E). In addition, when we harvested the spleens from mice receiving the IL-2 complex they were notably larger as compared to the normal size spleens from mice receiving the appropriate control (Figure 2F). Furthermore, when these spleens were processed in a similar manner as the lungs we found that there was a 3.9-fold increase in the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells from the mice receiving IL-2 complex, as compared to mice receiving the appropriate control (Figure 2, H and I).

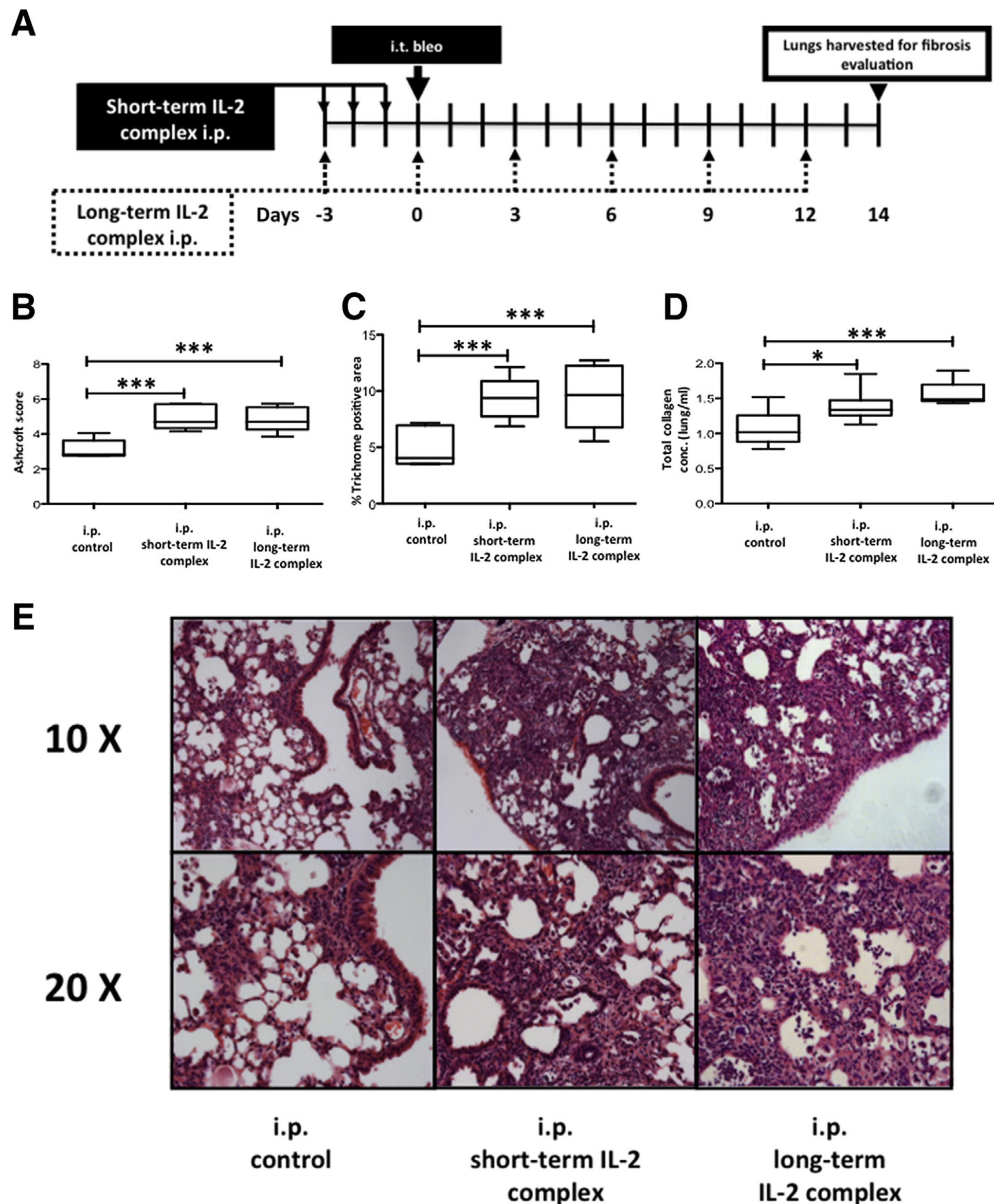

Figure 3 IL-2 complex augments bleomycin-induced pulmonary fibrosis. **A:** Strategy used to deliver i.p. short- and long-term IL-2 complex during intratracheal (i.t.) bleomycin challenge. Ashcroft score (**B**) and percentage trichrome positive area (**C**). **D:** Sircol assay of total lung collagen from mice that received i.t. bleomycin with i.p. short- and long-term IL-2 complex, as compared to i.p. controls. **E:** Representative hematoxylin and eosin stain of lungs from mice receiving i.t. bleomycin with i.p. short- and long-term IL-2 complex as compared to i.p. control. $n = 8$ in each group. $^{*}P < 0.05$, $^{***}P < 0.001$. bleo, bleomycin.

IL-2 Complex Augments Collagen Deposition during Bleomycin-Induced Pulmonary Fibrosis

Using the IL-2 complex we determined its effect on bleomycin-induced pulmonary fibrosis. Three groups of mice were examined: group 1, controls consisted of i.p. control given on days -3, -2, -1 and i.t. bleomycin administered on day 0; group 2, short-term IL-2 complex consisted of i.p. IL-2 complex given on days -3, -2, -1, and i.t. bleomycin administered on day 0; group 3, long-term IL-2 complex consisted of i.p. IL-2 complex given every 3 days starting at day -3 till harvesting of the lungs and i.t. bleomycin administered on day 0 (Figure 3A). Long-term dosing was based on a recent report demonstrating that expanded Treg may begin to decline after day 5 after short-term IL-2 complex therapy and giving the IL-2 complex every 3 days allows for persistent expansion of Treg.[23] Lungs from each group were harvested for paraffin processing, sectioning, and H&E staining for pathological assessment using the Ashcroft pulmonary fibrosis lung scoring system and Trichrome staining for collagen quantification via image analysis at day 14. The control group

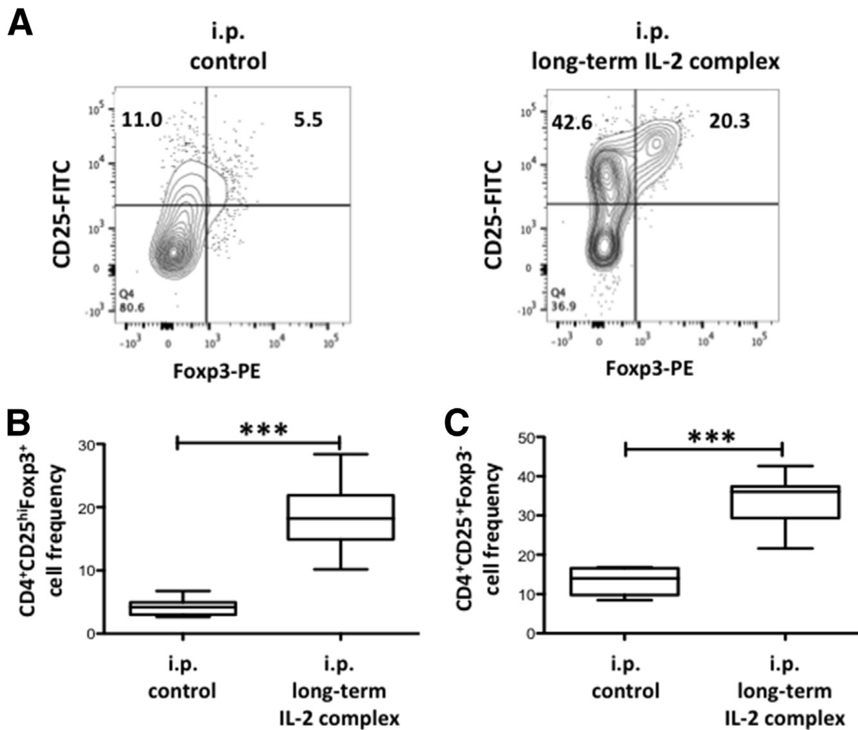

Figure 4 IL-2 complex increases $CD4^+CD25^{hi}Foxp3^+$ cells during bleomycin-induced pulmonary fibrosis. Representative contour plots of whole lung single-cell suspensions for the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells from intratracheal (i.t.) bleomycin challenge in combination with either i.p. control or i.p. long-term IL-2 complex at day 14. Box (**A**) and whisker plots of the frequency of $CD4^+CD25^{hi}Foxp3^+$ (**B**) and $CD4^+CD25^+Foxp3^-$ (**C**) cells in whole lung single-cell suspensions from i.t. bleomycin challenge with i.p. long-term IL-2 complex as compared to i.p. controls at day 14. $n = 8$ in each group. $^{***}P < 0.001$.

(i.p. control and i.t. bleomycin) was found to have a mean Ashcroft score of 3.2 (Figure 3, B and E). Unexpectedly, the IL-2 complex groups treated either for short- or long-term had markedly elevated Ashcroft scores (Figure 3, B and E). However, there was no statistically significant difference in Ashcroft scores between the IL-2 complex given short- or long-term (Figure 3, B and E). Similar results were observed in trichrome-stained tissue (Figure 3C). Confirmatory studies using the Sircol assay (Figure 3D) that evaluates total collagen content of harvested whole lungs were performed using the experimental design as shown in Figure 3A. The results of this independent experiment and assay were consistent with the Ashcroft and trichrome scores demonstrating that the i.p. IL-2 complex delivered to mice short- or long-term significantly increased total collagen in the lung, as compared to i.p. control treated mice administered i.t. bleomycin (Figure 3D). In addition, we did not find any statistically significant difference between the total collagen in the lungs from the short- and long-term IL-2 complex treated mice (Figure 3D). On the basis of these unanticipated results, we evaluated the ability of the IL-2 complex to augment $CD4^+CD25^{hi}Foxp3^+$ cell frequency during a bleomycin challenge. We chose the long-term delivery of IL-2 based on it having the greatest effect on lung fibrosis (Figure 3D). Flow cytometry performed on whole lung single cell suspensions demonstrated a 4.3-fold increase in the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells in the i.p. IL-2 complex group, as compared to the i.p. control group at day 14 after bleomycin challenge (Figure 4, A and B). In addition, there was a 2.6-fold increase in the frequency of $CD4^+CD25^+Foxp3^-$ cells in the i.p. IL-2 complex group, as compared to the i.p. control group, at day 14 after challenge with bleomycin (Figure 4, A and C).

IL-2 Complex Is Dependent on Lymphocytes to Increase Pulmonary Fibrosis

Separate experiments were performed using *Rag1*$^{-/-}$ mice on a C57BL/6 background as they are devoid of lymphocytes, allowing for an evaluation of the role of the IL-2 complex on bleomycin-induced lung fibrosis that is independent of lymphocytes. *Rag1*$^{-/-}$ mice were treated with i.p. long-term IL-2 complex as compared to i.p. control starting at day -3 and administered bleomycin i.t. on day 0. Lungs were harvested for histopathological assessment using the Ashcroft scoring system and Trichrome staining to evaluate collagen content. We found no difference in Ashcroft scores between the *Rag1*$^{-/-}$ mice receiving i.t. bleomycin with or without the administration of IL-2 complex (Figure 5, A and C). This was further supported from the analysis of trichrome-stained sections (Figure 5B). Furthermore, the percentage positive trichrome area was on average slightly higher in sections from IL-2 complex treated mice (Figure 3C and Figure 5B).

$CD4^+CD25^{hi}Foxp3^+$ Cellular Delivery to Rag1$^{-/-}$ Mice Receiving Bleomycin Causes Increased Mortality and Pulmonary Fibrosis with Alterations of the $CD4^+CD25^{hi}Foxp3^+$ Phenotype

The above experiments demonstrate that during bleomycin challenge the IL-2 complex therapy can augment pulmonary fibrosis, and this is dependent on lymphocytes. However, to

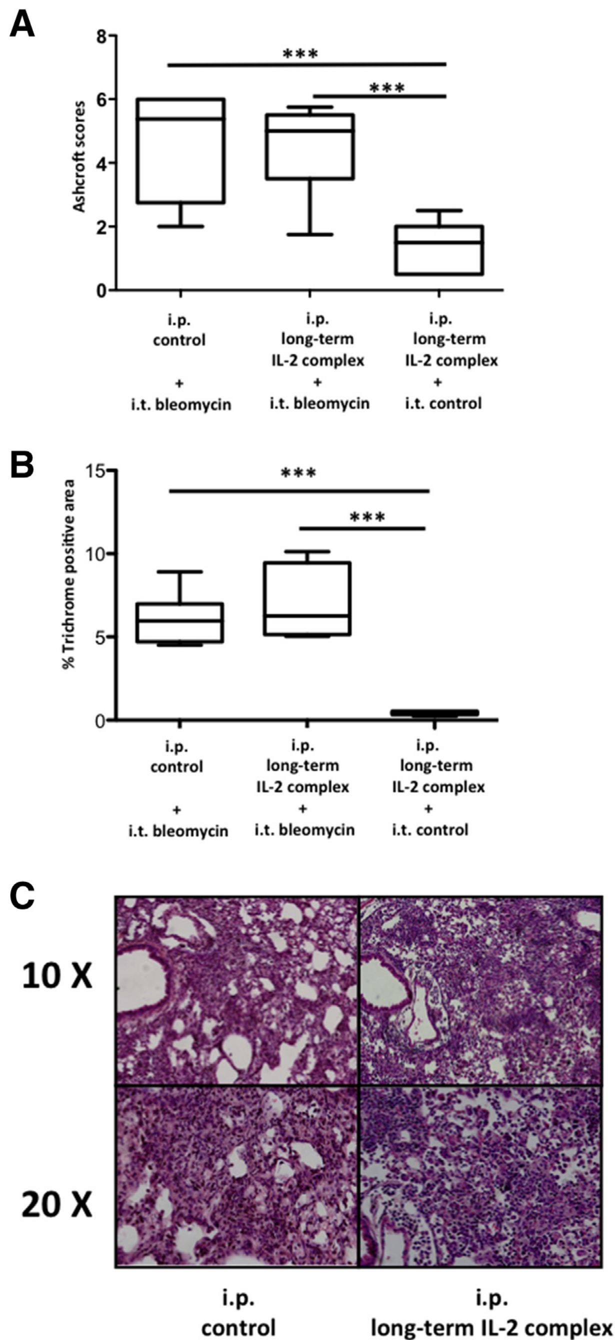

Figure 5 IL-2 complex does not alter bleomycin-induced pulmonary fibrosis in *Rag1*$^{-/-}$ mice. Ashcroft scores (**A**) and percentage positive trichrome area from intratracheal (i.t.) bleomycin challenge (**B**) in *Rag1*$^{-/-}$ mice with i.p. long-term IL-2 complex, as compared to i.p. control. **C:** Representative hematoxylin and eosin stain of lungs from *Rag1*$^{-/-}$ mice challenged with i.t. bleomycin that received i.p. long-term IL-2 complex, as compared to i.p. control. $n = 8$ in each group. $***P < 0.001$.

specifically segregate the role of CD4$^+$CD25^{hi}Foxp3$^+$ cells during bleomycin-induced fibrosis, we isolated CD4$^+$ T cells with magnetic beads from the spleens of Foxp3 EGFP transgenic mice and then sorted on CD25^{hi}GFP$^+$ cells and delivered them i.p. (0.6×10^6 cells), as compared to i.p. control (0.9% NaCl) to *Rag1*$^{-/-}$ mice 24 hours before the administration of i.t. bleomycin. There was a higher mortality rate in the mice that received CD4$^+$CD25^{hi}Foxp3$^+$ cells as compared to control mice as shown by the Kaplan-Meier survival curve ($P < 0.05$) (Figure 6A). Furthermore, when we harvested lungs from mice in both groups and performed Ashcroft scoring the mice that received CD4$^+$CD25^{hi}Foxp3$^+$ cells had significantly higher scores (Figure 6, B and C).

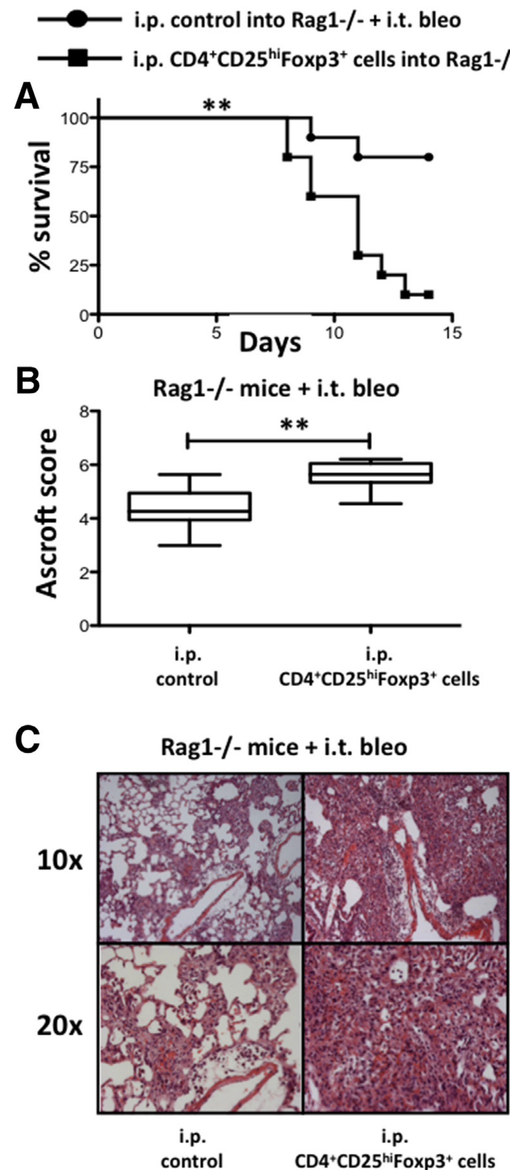

Figure 6 Adoptive transfer of CD4$^+$CD25^{hi}Foxp3$^+$ cells to *Rag1*$^{-/-}$ on a C57BL/6 background before intratracheal (i.t.) bleomycin exacerbates pulmonary fibrosis and increases mortality. **A:** Kaplan-Meier survival curves for *Rag1*$^{-/-}$ mice receiving i.t. bleomycin with i.p. control versus i.p. 0.6 $\times 10^6$ CD4$^+$CD25^{hi}Foxp3$^+$ cells sorted from naïve Foxp3 enhanced green fluorescent protein transgenic mice on a C57BL/6 background. **B:** Ashcroft scores from *Rag1*$^{-/-}$ mice receiving i.t. bleomycin with i.p. control versus i.p. 0.6×10^6 CD4$^+$CD25^{hi}Foxp3$^+$ cells at day 14. **C:** Representative hematoxylin and eosin stain of lungs from *Rag1*$^{-/-}$ mice challenged with i.t. bleomycin that received i.p. control versus i.p. 0.6×10^6 CD4$^+$CD25^{hi}Foxp3$^+$ cells at day 14. $n = 12$ in each group. $**P < 0.01$.

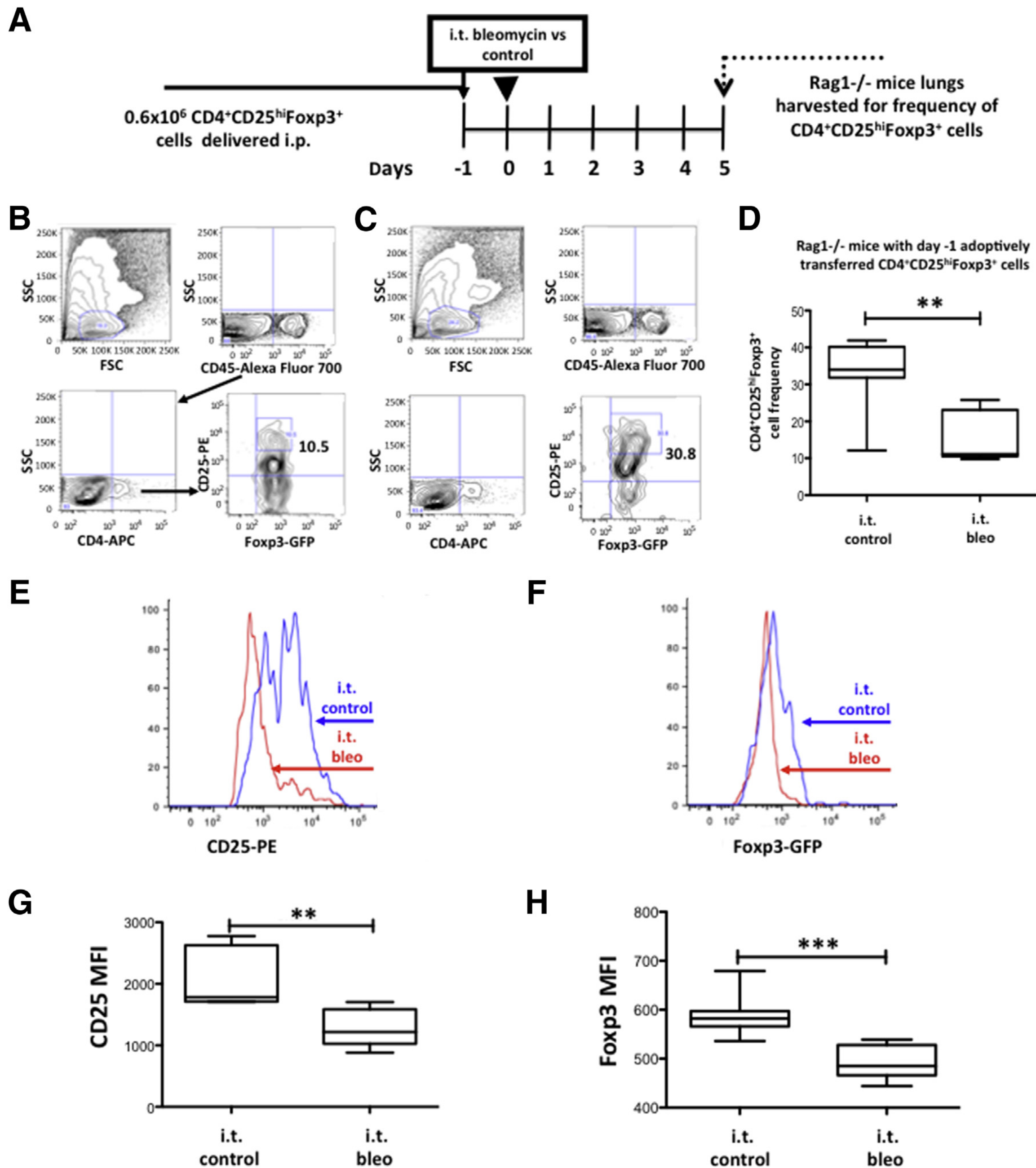

Figure 7 CD4$^+$CD25^{hi}Foxp3$^+$ cells adoptively transferred to *Rag1*$^{-/-}$ on a C57BL/6 background before intratracheal (i.t.) bleomycin causes alteration in their frequency and cellular expression of CD25 and Foxp3 at day 5. **A:** Experimental design involving the i.p. adoptive transfer of CD4$^+$CD25^{hi}Foxp3$^+$ cells at day -1 to *Rag1*$^{-/-}$ mice with a day 0 challenge of either i.t. bleomycin or i.t. control. Representative flow cytometry contour plots of whole lung single-cell suspensions demonstrating the gating strategy used to evaluate the frequency of CD4$^+$CD25^{hi}Foxp3$^+$ cells at 5 days after i.t. bleomycin (**B**) and control (**C**). **D:** Box and whisker plot of the whole lung frequencies of CD4$^+$CD25^{hi}Foxp3$^+$ from i.t. control and bleomycin. Histogram and box and whisker plots of the CD4$^+$CD25$^+$Foxp3$^+$ cells demonstrating their expression of CD25 (**E** and **G**) and Foxp3 (**F** and **H**) after i.t. bleomycin as compared to control. $n = 8$ in each group. $^{**}P < 0.01$, $^{***}P < 0.001$. bleo, bleomycin; FSC, forward scatter; SSC, side scatter.

Interestingly, some studies suggest that Tregs have marked plasticity, whereas others do not[8]; thus, we evaluated the effects of i.t. bleomycin on CD4$^+$CD25^{hi}Foxp3$^+$ cells. More specifically, to isolate an ability to alter CD4$^+$CD25^{hi}Foxp3$^+$ cells we challenged *Rag1*$^{-/-}$ mice with i.t. bleomycin as compared to i.t. control at day 0 after adoptive transfer of 0.6×10^6 sorted CD4$^+$CD25^{hi}Foxp3$^+$ cells from the spleens of Foxp3 EGFP transgenic mice delivered i.p. at day -1. On the basis of our Kaplan-Meier curves demonstrating increased mortality beginning between 5 and 10 days we choose to evaluate alterations in CD4$^+$CD25^{hi}Foxp3$^+$ cells in the lungs at day 5 after i.t. challenge (Figure 7A). Using undigested single-cell suspensions from the lungs we found a 2.2-fold reduction in the frequency of CD4$^+$CD25^{hi}Foxp3$^+$

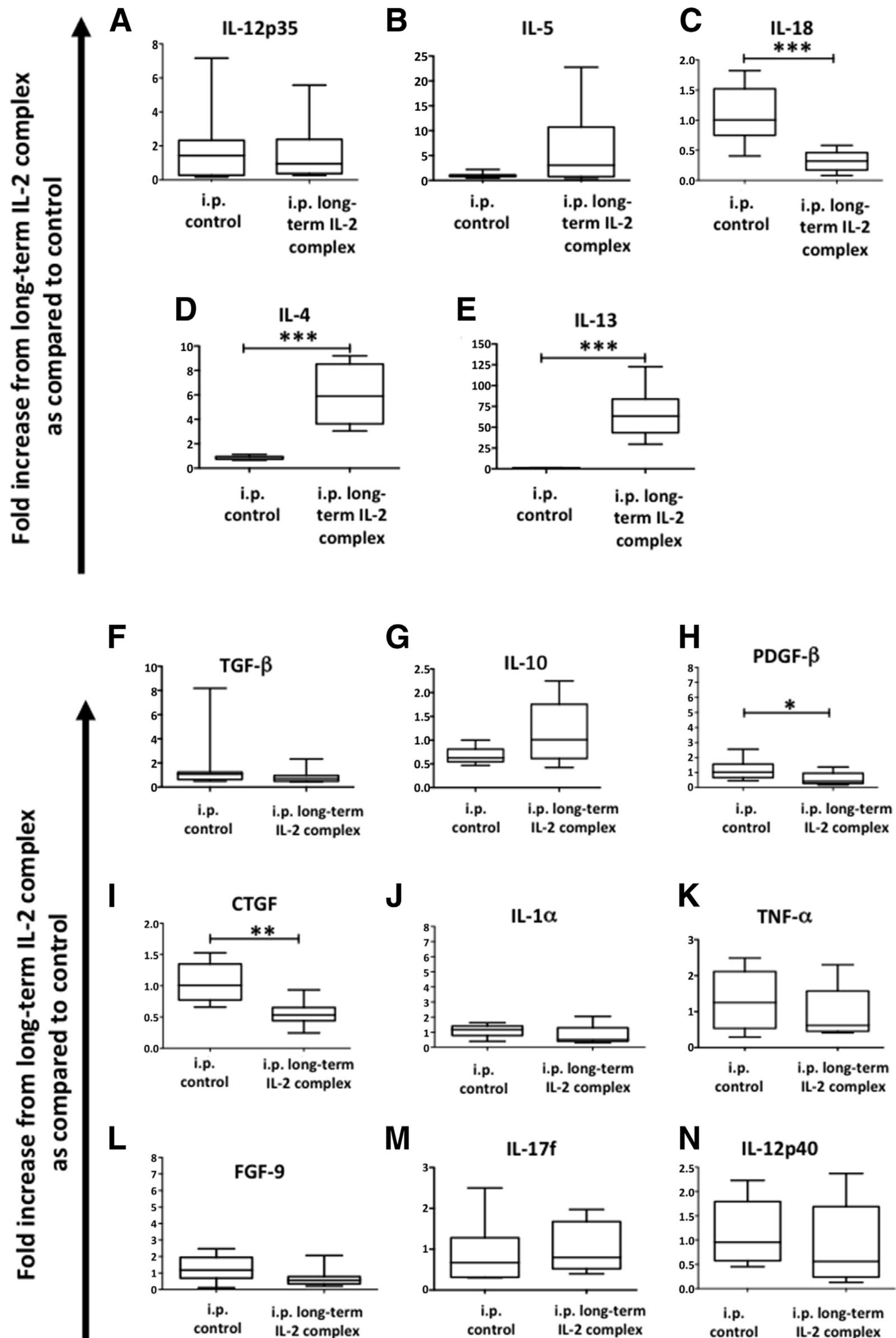

Figure 8 IL-2 complex polarizes the lung toward a type 2 immune response during bleomycin-induced pulmonary fibrosis. Quantitative PCR performed on day 14 whole lungs from C57BL/6 mice challenged with intratracheal (i.t.) bleomycin and given i.p. IL-2 complex long-term as compared to i.p. control demonstrate: no change in the type 1 immune response cytokine IL-12p35 (**A**); no change in the type 2 immune response cytokine IL-5 (**B**); marked reduction in type 1 immune response cytokine IL-18 (**C**); dramatic increase in the type 2 immune response cytokines IL-4 (**D**) and IL-13 (**E**); no change in the profibrotic cytokine transforming growth factor (TGF)-β (**F**); reduction in the profibrotic growth factor IL-10 (**G**); no change in the Treg-associated cytokine platelet-derived growth factor (PDGF)-B (**H**); no change in connective tissue growth factor (CTGF; **I**); no change in the inflammatory cytokines IL-1α (**J**) and tumor necrosis factor (TNF)-α (**K**); no change in the inflammatory/fibrotic growth factor fibroblast growth factor (FGF)-9 (**L**); no change in the type 17 immune response cytokines IL-17f (**M**) and IL-12p40 (**N**). $n = 8$ in each group. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

cells from the bleomycin, as compared to control challenge (Figure 7, B–D). More important, when we focused on the $CD4^+CD25^+Foxp3^+$ cells, there was dramatic reduction in the cells surface expression of CD25 as well as expression of Foxp3 from the i.t. bleomycin challenge group as compared to the i.t. control group (Figure 7, E–H).

IL-2 Complex during a Bleomycin Challenge Leads to an Immune Deviation of the Lung toward a Type 2 Immune Response

On the basis of the $CD4^+CD25^{hi}Foxp3^+$ cells demonstrating an ability to be altered during a bleomycin challenge, we wanted to determine whether this was associated with immune response polarization (eg, type 1, type 2, type 17, or profibrotic immune responses) during the IL-2 complex delivery. Whole lung mRNA expression by quantitative PCR was evaluated after bleomycin challenge with or without the long-term IL-2 complex. Expression of type 1 (IL-12p35 and IL-18) as well as type 2 (IL4, IL-5, and IL13) immune response cytokines at day 14 demonstrated no difference with regard to IL-12p35 and IL-5 (Figure 8, A and B), but a dramatic down-regulation of IL-18 (Figure 8C) and augmentation of IL-4 and IL-13 (Figure 8, D and E).

We also explored other pathways of fibrosis in our bleomycin model with or without long-term IL-2 complex. Notably, whole lung mRNA expression by quantitative PCR did not demonstrate any difference in TGF-β or IL-10; however, there was a reduction in the expression of PDGF-β and CTGF (Figure 8, F–I). In addition, we did not find differences in lung mRNA expression of IL-1α, TNF-α, FGF-9, IL-17f, and IL-12p40 from mice that did and did not receive the IL-2 complex (Figure 8, J–N).

Discussion

Contemporary strategies used to treat diabetes, GVHD, and vasculitides with Tregs have raised the possibility that they may limit or even reverse pulmonary fibrosis.[10,11,30] We tested this hypothesis in a preclinical animal model of bleomycin-induced pulmonary fibrosis. Characterization of the model with regard to the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells in the lungs demonstrated an increase that paralleled pulmonary fibrosis (Figure 1C), which would be consistent with some, but not all, human studies involving pulmonary fibrosis.[17,18,29] On the basis of the strong evidence for the protective effects of Tregs during injurious events,[19–21,23–25] these data would insinuate that the increased frequency of $CD4^+CD25^{hi}Foxp3^+$cells in the lungs from mice that received bleomycin is a protective mechanism that may be overcome by a severe injury that ultimately results in fibrosis. However, an ability to further augment the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells in the lung may lead to an opportunity to attenuate bleomycin-induced pulmonary fibrosis.

The *in vivo* expansion of Tregs is considered to be a superior therapeutic intervention than *ex vivo* expansion.[22,24] One *in vivo* expansion approach would be stimulating the immune system with superagonistic mAb to CD28, which inhibited crescentic glomerulonephritis in a rodent model.[31] Unfortunately, its use via TGN1412 led to life-threatening systemic organ failure in human trials.[31,32] An alternative approach involves low-dose IL-2, which has recently been demonstrated to be beneficial in alleviating chronic hepatitis C–mediated vasculitis and chronic GVHD in humans.[10,11]

IL-2 acts on cells expressing the high-affinity trimeric IL-2R and to a degree on the low-affinity dimeric IL-2R.[33,34] CD25 (IL-2Rα) is the third chain of the trimeric IL-2R, which does not appear to participate in signaling, but functions to increase the affinity of the IL-2R for its ligand by 10- to 100-fold (Kd approximately 10^{-11} mol/L).[33,34] Unlike other T-cell subsets, Tregs constitutively express high levels of CD25 and are easily expanded using continuous low levels of IL-2. Problematically, studies have shown that unbound IL-2 given by i.v., s.c., or i.p. injections has a short half-life that is within minutes.[33,34] However, this problem can be overcome by the injection of IL-2 in complex with the neutralizing IL-2–specific monoclonal antibody JES6-1A12 (JES6-1).[33,34] JES6-1 binds to a specific site of IL-2 that inhibits its interaction with the dimeric IL-2R yet allows for IL-2 to interact with CD25 causing the selective expansion of peripheral Treg with a half-life of >48 hours.[33,34] Therefore, JES6-1 focuses the activity of IL-2 selectively on $CD25^{hi}$ cells and has virtually no effect on $CD25^-$ cells with multiple studies demonstrating a specific expansion of $CD4^+CD25^{hi}Foxp3^+$ cells.[25,33,34] However, we cannot entirely rule out the possibility that other cells may increase their expression of CD25 enough during a bleomycin challenge and interact with the IL-2 complex.

We found the IL-2 complex markedly augments $CD4^+CD25^{hi}Foxp3^+$ cells in the lungs and spleens of mice with or without bleomycin challenge (Figure 2, B–I). Although the lung results, to our knowledge, have never been shown, the increased frequency of $CD4^+CD25^{hi}Foxp3^+$ cells in the spleen of naïve mice is similar to that found by Webster and associates[24,25] and others.[23] Overall, this demonstrates the IL-2 complex is an effective way to expand the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells in the lung of mice that can be easily translated to humans.

Consistently, using multiple short- and long-term dosing regimens, the IL-2 complex augments collagen deposition during bleomycin challenges (Figure 3, B–E). These results were unanticipated on the basis of multiple studies demonstrating this same IL-2 complex via the *in vivo* expansion of Tregs has tremendous protection in mice with regard to adriamycin nephropathy, experimental allergic encephalitis, and islet cell transplantation rejection.[24,25] What makes these results even more surprising is the converse results found in other forms of lung injury.[21] More specifically, in a lipopolysaccharide-induced lung injury model, it was noted that the adoptive transfer of Tregs leads to improved resolution of lung disease.[21]

Exploring the mechanism involved in the increased fibrosis from the IL-2 complex, we performed the bleomycin model in $Rag1^{-/-}$ mice with our without long-term IL-2 complex and found no difference in pulmonary fibrosis (Figure 5, A and B). More important, bleomycin-challenged $Rag1^{-/-}$ mice given IL-2 complex therapy have lower percentage trichrome scores as compared to wild-type mice given IL-2 complex therapy. Collectively, this established that the IL-2 complex was predominately working via a lymphocyte-dependent pathway and supports a role for the ability of lymphocytes to exacerbate bleomycin-induced pulmonary fibrosis. The ability of lymphocytes to exacerbate lung fibrosis is a novel finding and is in contrast to other studies, suggesting there is no physiological consequence of inflammation during pulmonary fibrosis.[5–7]

Interestingly, although the IL-2 complex increased the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells during the bleomycin challenge (Figure 4C), it was not to the same magnitude as that seen in the unchallenged mice (Figure 2C). These findings suggest that a hostile environment generated by a bleomycin challenge may lead to instability of the $CD4^+CD25^{hi}Foxp3^+$ cells and this is associated with the development of worsening lung disease. More important, we found that $CD4^+CD25^{hi}Foxp3^+$ cellular delivery during a bleomycin challenge results in phenotypic changes as demonstrated by a dramatic down-regulation in the cellular expression of CD25 and Foxp3 (Figure 7, B–H). These findings demonstrate $CD4^+CD25^{hi}Foxp3^+$ cellular instability during bleomycin challenge and are supported by a study by Zhou et al[35] describing that Tregs down-regulate CD25 and Foxp3 during autoimmune-induced end organ damage.

These alterations can be involved in an increased early acute lung injury as indicated by the increased mortality that began day 5 after bleomycin challenge as well as the augmented fibrosis at day 14. We suspect the plasticity of the $CD4^+CD25^{hi}Foxp3^+$ cells associated with more injury is likely the reason we find more fibrosis, whereas previous studies using Treg cellular based therapy in less robust inflammatory/fibroproliferative models of acute lung injury demonstrated protection.[21] This is reminiscent of other studies that expanded Tregs by the same IL-2 complex and found protection in a minimally alloreactive murine heterotopic islet cell transplant model, yet was not in a highly alloreactive/fibroproliferative vascularized murine cardiac transplantation model.[25] Collectively, these and our studies suggest that hostile environments can alter $CD4^+CD25^{hi}Foxp3^+$ cells into a damaging rather than protective phenotype.

On the basis of prior studies suggesting that Tregs can alter the production of lung PDGF-β, CTGF, TGF-β,[20] IL-1α, TNF-α, FGF-9, as well as the Th17 pathways[36,37] during injury and fibrosis, we evaluated the bleomycin-challenged lungs for immune deviation because of the IL-2 complex and the alterations of the $CD4^+CD25^{hi}Foxp3^+$ cellular phenotype. Although we did not find any significant changes in TGF-β, IL-1α, TNF-α, and FGF-9 expression patterns (Figure 8, F and J–L), there was a reduction in PDGF-β and CTGF (Figure 8, H and I) with the IL-2 complex. Notably, the Treg-associated cytokine IL-10, normally elevated with respect to Treg cells, showed no differences between groups (Figure 8G). More important, the IL-2 complex led to a tremendous down-regulation of type 1 with a concomitant up-regulation of type 2 immune responses, which is consistent with ours and other investigators' previous studies demonstrating that type 2 polarization induces fibroplasia.[26,27]

In conclusion, this study reveals novel findings with regard to the pathogenesis and exacerbations of pulmonary fibrosis. IL-2 complex has profibrotic effects that are, in part, lymphocyte dependent. In addition, the IL-2 complex increased the frequency of $CD4^+CD25^{hi}Foxp3^+$ cells during bleomycin challenge. However, the environment induced by bleomycin has altering effects on the $CD4^+CD25^{hi}Foxp3^+$ cells. Ultimately, this leads to immune deviation that is dominated by type 2 immune responses within the lung, which is associated with heightened pulmonary fibroplasia. More important, bleomycin has a modifying and profibrotic effect on the $CD4^+CD25^{hi}Foxp3^+$ cells that was corroborated via adoptive transfer experiments in $Rag1^{-/-}$ mice. This suggests that a therapeutic strategy of expanding $CD4^+CD25^{hi}Foxp3^+$ in humans may be detrimental via the augmentation of type 2 immune responses in patients with IPF and other fibroproliferative lung diseases. Furthermore, our findings collectively with others[13,14] support the importance of inflammation and more specifically a subpopulation of lymphocytes contributing to the exacerbation of pulmonary fibrosis via diverse and different profibrotic mediators.

Acknowledgments

We thank Dr. Steven Dubinett (Department of Medicine Pulmonary Division, UCLA) for providing the Pulmonary Training Grant, the UCLA FACS Core Facility for assistance with flow cytometry, and Dr. Talal A. Chatila (Harvard Medical Center, Boston, MA) for providing Foxp3 EGFP mice.

References

1. Lynch JP 3rd, Fishbein MC, Saggar R, Zisman DA, Belperio JA: Idiopathic pulmonary fibrosis. Expert Rev Respir Med 2007, 1:377–389
2. Ryu JH, Moua T, Daniels CE, Hartman TE, Yi ES, Utz JP, Limper AH: Idiopathic pulmonary fibrosis: evolving concepts. Mayo Clin Proc 2014, 89:1130–1142
3. Munger JS, Huang X, Kawakatsu H, Griffiths MJ, Dalton SL, Wu J, Pittet JF, Kaminski N, Garat C, Matthay MA, Rifkin DB, Sheppard D: The integrin alpha v beta 6 binds and activates latent TGF beta 1: a mechanism for regulating pulmonary inflammation and fibrosis. Cell 1999, 96:319–328

4. Selman M, King TE, Pardo A: Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med 2001, 134:136−151
5. Helene M, Lake-Bullock V, Zhu J, Hao H, Cohen DA, Kaplan AM: T cell independence of bleomycin-induced pulmonary fibrosis. J Leukoc Biol 1999, 65:187−195
6. Christensen PJ, Goodman RE, Pastoriza L, Moore B, Toews GB: Induction of lung fibrosis in the mouse by intratracheal instillation of fluorescein isothiocyanate is not T-cell-dependent. Am J Pathol 1999, 155:1773−1779
7. Szapiel SV, Elson NA, Fulmer JD, Hunninghake GW, Crystal RG: Bleomycin-induced interstitial pulmonary disease in the nude, athymic mouse. Am Rev Respir Dis 1979, 120:893−899
8. Hori S: Stability of regulatory T-cell lineage. Adv Immunol 2011, 112:1−24
9. Chatila TA, Blaeser F, Ho N, Lederman HM, Voulgaropoulos C, Helms C, Bowcock AM: JM2, encoding a fork head-related protein, is mutated in X-linked autoimmunity-allergic disregulation syndrome. J Clin Invest 2000, 106:R75−R81
10. Saadoun D, Rosenzwajg M, Joly F, Six A, Carrat F, Thibault V, Sene D, Cacoub P, Klatzmann D: Regulatory T-cell responses to low-dose interleukin-2 in HCV-induced vasculitis. N Engl J Med 2011, 365:2067−2077
11. Koreth J, Matsuoka K, Kim HT, McDonough SM, Bindra B, Alyea EP 3rd, Armand P, Cutler C, Ho VT, Treister NS, Bienfang DC, Prasad S, Tzachanis D, Joyce RM, Avigan DE, Antin JH, Ritz J, Soiffer RJ: Interleukin-2 and regulatory T cells in graft-versus-host disease. N Engl J Med 2011, 365:2055−2066
12. Herazo-Maya JD, Noth I, Duncan SR, Kim S, Ma SF, Tseng GC, Feingold E, Juan-Guardela BM, Richards TJ, Lussier Y, Huang Y, Vij R, Lindell KO, Xue J, Gibson KF, Shapiro SD, Garcia JG, Kaminski N: Peripheral blood mononuclear cell gene expression profiles predict poor outcome in idiopathic pulmonary fibrosis. Sci Transl Med 2013, 5:205ra136
13. Reilkoff RA, Peng H, Murray LA, Peng X, Russell T, Montgomery R, Feghali-Bostwick C, Shaw A, Homer RJ, Gulati M, Mathur A, Elias JA, Herzog EL: Semaphorin 7a+ regulatory T cells are associated with progressive idiopathic pulmonary fibrosis and are implicated in transforming growth factor-beta1-induced pulmonary fibrosis. Am J Respir Crit Care Med 2013, 187:180−188
14. Lo Re S, Lecocq M, Uwambayinema F, Yakoub Y, Delos M, Demoulin JB, Lucas S, Sparwasser T, Renauld JC, Lison D, Huaux F: Platelet-derived growth factor-producing CD4+ Foxp3+ regulatory T lymphocytes promote lung fibrosis. Am J Respir Crit Care Med 2011, 184:1270−1281
15. Tselios K, Sarantopoulos A, Gkougkourelas I, Boura P: The influence of therapy on CD4+CD25(high)FOXP3+ regulatory T cells in systemic lupus erythematosus patients: a prospective study. Scand J Rheumatol 2015, 44:29−35
16. Idiopathic Pulmonary Fibrosis Clinical Research Network, Raghu G, Anstrom KJ, King TE Jr, Lasky JA, Martinez FJ: Prednisone, azathioprine, and N-acetylcysteine for pulmonary fibrosis. N Engl J Med 2012, 366:1968−1977
17. Kotsianidis I, Nakou E, Bouchliou I, Tzouvelekis A, Spanoudakis E, Steiropoulos P, Sotiriou I, Aidinis V, Margaritis D, Tsatalas C, Bouros D: Global impairment of CD4+CD25+FOXP3+ regulatory T cells in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 2009, 179:1121−1130
18. Shimizu Y, Dobashi K, Endou K, Ono A, Yanagitani N, Utsugi M, Sano T, Ishizuka T, Shimizu K, Tanaka S, Mori M: Decreased interstitial FOXP3(+) lymphocytes in usual interstitial pneumonia with discrepancy of CXCL12/CXCR4 axis. Int J Immunopathol Pharmacol 2010, 23:449−461
19. Garibaldi BT, D'Alessio FR, Mock JR, Files DC, Chau E, Eto Y, Drummond MB, Aggarwal NR, Sidhaye V, King LS: Regulatory T cells reduce acute lung injury fibroproliferation by decreasing fibrocyte recruitment. Am J Respir Cell Mol Biol 2013, 48:35−43
20. Song L, Weng D, Liu F, Chen Y, Li C, Dong L, Tang W, Chen J: Tregs promote the differentiation of Th17 cells in silica-induced lung fibrosis in mice. PLoS One 2012, 7:e37286
21. D'Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, Pipeling MR, Brower RG, Tuder RM, McDyer JF, King LS: CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. J Clin Invest 2009, 119:2898−2913
22. Boyman O, Sprent J: The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol 2012, 12:180−190
23. Liu R, Zhou Q, La Cava A, Campagnolo DI, Van Kaer L, Shi FD: Expansion of regulatory T cells via IL-2/anti-IL-2 mAb complexes suppresses experimental myasthenia. Eur J Immunol 2010, 40:1577−1589
24. Polhill T, Zhang GY, Hu M, Sawyer A, Zhou JJ, Saito M, Webster KE, Wang Y, Grey ST, Sprent J, Harris DC, Alexander SI, Wang YM: IL-2/IL-2Ab complexes induce regulatory T cell expansion and protect against proteinuric CKD. J Am Soc Nephrol 2012, 23:1303−1308
25. Webster KE, Walters S, Kohler RE, Mrkvan T, Boyman O, Surh CD, Grey ST, Sprent J: In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. J Exp Med 2009, 206:751−760
26. 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
27. Belperio JA, Dy M, Murray L, Burdick MD, Xue YY, Strieter RM, Keane MP: The role of the Th2 CC chemokine ligand CCL17 in pulmonary fibrosis. J Immunol 2004, 173:4692−4698
28. Ashcroft T, Simpson JM, Timbrell V: Simple method of severity of pulmonary fibrosis on a numerical scale. J Clin Pathol 1988, 41:467−470
29. Galati D, De Martino M, Trotta A, Rea G, Bruzzese D, Cicchitto G, Stanziola AA, Napolitano M, Sanduzzi A, Bocchino M: Peripheral depletion of NK cells and imbalance of the Treg/Th17 axis in idiopathic pulmonary fibrosis patients. Cytokine 2014, 66:119−126
30. Rosenzwajg M, Churlaud G, Hartemann A, Klatzmann D: Interleukin 2 in the pathogenesis and therapy of type 1 diabetes. Curr Diab Rep 2014, 14:553
31. Takabatake Y, Li XK, Mizui M, Miyasato K, Matsui I, Kawada N, Imai E, Hunig T, Takahara S, Wada T, Furuichi K, Rakugi H, Isaka Y: A superagonistic monoclonal antibody for CD28 ameliorates crescentic glomerulonephritis in wistar-kyoto rats. Mol Med 2011, 17:686−696
32. Schraven B, Kalinke U: CD28 superagonists: what makes the difference in humans? Immunity 2008, 28:591−595
33. Boyman O, Kovar M, Rubinstein MP, Surh CD, Sprent J: Selective stimulation of T cell subsets with antibody-cytokine immune complexes. Science 2006, 311:1924−1927
34. Boyman O, Surh CD, Sprent J: Potential use of IL-2/anti-IL-2 antibody immune complexes for the treatment of cancer and autoimmune disease. Expert Opin Biol Ther 2006, 6:1323−1331
35. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA: Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol 2009, 10:1000−1007
36. Peng X, Moore MW, Peng H, Sun H, Gan Y, Homer RJ, Herzog EL: CD4+CD25+FoxP3+ regulatory Tregs inhibit fibrocyte recruitment and fibrosis via suppression of FGF-9 production in the TGF-beta1 exposed murine lung. Front Pharmacol 2014, 5:80
37. 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 Exp Med 2010, 207:535−552