ORIGINAL RESEARCH

Impact of Regulatory T Cells on Type 2 Alveolar Epithelial Cell Transcriptomes during Resolution of Acute Lung Injury and Contributions of IFN- γ

Jason R. Mock^{1,2}, Catherine F. Dial^{1,2}, Miriya K. Tune^{1,2}, Rodney C. Gilmore², Wanda K. O'Neal^{1,2}, Hong Dang², and Claire M. Doerschuk^{1,2,3}

¹Division of Pulmonary Diseases and Critical Care Medicine, Department of Medicine, ²Marsico Lung Institute, and ³Center for Airways Disease, University of North Carolina, Chapel Hill, North Carolina

ORCID IDs: 0000-0002-7022-0629 (J.R.M.); 0000-0003-2638-3321 (C.M.D.).

Abstract

By enhancing tissue repair and modulating immune responses, Foxp3⁺ regulatory T cells (Tregs) play essential roles in resolution from lung injury. The current study investigated the effects that Tregs exert directly or indirectly on the transcriptional profiles of type 2 alveolar epithelial (AT2) cells during resolution in an experimental model of acute lung injury. Purified AT2 cells were isolated from uninjured mice or mice recovering from LPS-induced lung injury, either in the presence of Tregs or in Treg-depleted mice, and transcriptome profiling identified differentially expressed genes. Depletion of Tregs resulted in altered expression of 49 genes within AT2 cells during resolution, suggesting that Tregs present in this microenvironment influence AT2-cell function. Biological processes from Gene Ontology enriched in the absence of Tregs included those describing responses to IFN. Neutralizing IFN- γ in Treg-depleted mice reversed the effect of Treg depletion on inflammatory macrophages and B cells by preventing the increase in inflammatory macrophages and the decrease in B cells. Our results provide insight into the effects of Tregs on AT2 cells. Tregs directly or

indirectly impact many AT2-cell functions, including IFN type I and II-mediated signaling pathways. Inhibition of IFN- γ expression and/or function may be one mechanism through which Tregs accelerate resolution after acute lung injury.

Keywords: regulatory T cells; Foxp3; acute lung injury; type 2 alveolar epithelial cells; IFN- γ

Clinical Relevance

Our results provide insight into the effects of Foxp3⁺ regulatory T cells (Tregs) on type 2 alveolar epithelial cells during acute lung injury. Tregs directly or indirectly impact many type 2 alveolar epithelial-cell functions, including IFN type I and II-mediated signaling pathways. Inhibition of IFN- γ expression and/or function may be one mechanism through which Tregs accelerate resolution following acute lung injury.

Repair of the lung epithelium is an essential component of resolution after injury, and contemporary views have proposed that the immune system plays an important role in

enhancing barrier function and promoting resolution (1–3). In acute or chronic injury, failure to regenerate damaged lung epithelium contributes to acute respiratory

distress syndrome (ARDS), pneumonia, pulmonary fibrosis, chronic obstructive pulmonary disease, and aging (2). After epithelial-cell injury, local epithelial

(Received in original form November 10, 2019; accepted in final form June 16, 2020)

Supported by the Parker B. Francis Fellowship Program (J.R.M.), the Cystic Fibrosis Foundation grant BOUCHE15R0 (W.K.O'N.), and the National Heart, Lung, and Blood Institute of the U.S. National Institutes of Health: K12HL119998 (C.M.D.), R01HL114388 (C.M.D.), R01HL145396 (C.M.D.), P30DK065988 (W.K.O'N.), K08HL129075 (J.R.M.), and R03HL145255 (J.R.M.). The University of North Carolina Flow Cytometry Core Facility is supported in part by a Cancer Center Core Support grant (P30 CA016086) to the University of North Carolina Lineberger Comprehensive Cancer Center.

Author Contributions: J.R.M., C.F.D., M.K.T., and C.M.D. conceived and designed experiments. J.R.M., C.F.D., M.K.T., R.C.G., and H.D. performed experiments and analysis. J.R.M., W.K.O'N., and C.M.D. wrote the manuscript and provided creative input. All authors have read and approved the final manuscript.

Correspondence and requests for reprints should be addressed to Jason R. Mock, M.D., Ph.D., Division of Pulmonary Diseases and Critical Care Medicine, Department of Medicine, University of North Carolina School of Medicine, Marsico Hall 7203, 125 Mason Farm Road, Chapel Hill, NC 27599. E-mail: jason_mock@med.unc.edu.

This article has a related editorial.

This article has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

Am J Respir Cell Mol Biol Vol 63, Iss 4, pp 464–477, Oct 2020

Copyright © 2020 by the American Thoracic Society

Originally Published in Press as DOI: 10.1165/rcmb.2019-0399OC on June 16, 2020

Internet address: www.atsjournals.org

progenitor cells migrate to injury sites, where they can proliferate and differentiate to facilitate repair (4–9). Type 2 alveolar epithelial (AT2) cells are a progenitor population that enhances their proliferation in response to injury (10–12).

Recently, we and others have demonstrated that a population of CD4⁺ lymphocytes, Foxp3⁺ regulatory T cells (Tregs), play essential roles in suppressing immune responses and facilitating tissue repair, particularly epithelial repair during acute lung injury (ALI) (13–17). Our prior studies demonstrated the effects of Tregs on proliferation of parenchymal cells and illustrated the important functional roles in both tissue repair and immunosuppression of Tregs during resolution of ALI (13, 16, 17).

In this study, we sought to determine the changes that Tregs exert on AT2 transcription during resolution of ALI. An established lung-injury model of intratracheal LPS-induced injury was employed to determine specific Treg-directed effects. This model mimics many aspects of ARDS, and it provides a high degree of reproducibility and uniformity during inflammatory and resolution processes (13–15). We employed cell sorting to obtain purified AT2 cell populations from uninjured and LPS-injured lungs of mice with and without Tregs. These studies identified both changes in AT2 cell transcription during resolution from lung injury and the contribution of Tregs to this transcription.

Interestingly, when Tregs were depleted during injury and resolution (16–19), 49 genes were differentially expressed by AT2 cells, indicating that Tregs present in this microenvironment are critical to AT2 cell function. Gene Ontology (GO) terms from biological processes that were enriched in the absence of Tregs included “response to IFN- γ ” and “regulation of leukocyte migration.” To determine the contributions of IFN- γ to the Treg-depleted phenotype, we neutralized IFN- γ in Treg-depleted mice. Neutralizing IFN- γ partially reversed some effects of Treg depletion, particularly the loss of inflammatory macrophages and the increase in CD19⁺ B cells induced by Treg depletion, suggesting that the impact of Tregs on these populations may be through the expression or function of IFN- γ . Our results underline the complexity of alveolar epithelial function in resolution of lung

injury and provide insight into the specific effects Tregs exert on AT2 cells during resolution of lung injury.

Methods

The METHODS are presented in brief below. Please see the data supplement for details of each procedure and analysis.

Mice

C57BL/6 wild-type *Foxp3*^{EGFP} (strain coexpressing *Foxp3* and enhanced GFP [*EGFP*]), and *Foxp3*^{DTR} (strain coexpressing *Foxp3* and diphtheria-toxin receptor [*DTR*]) mice were obtained from Jackson Laboratory (16, 17, 20). Mice were housed in ventilated cages, and colonies were maintained within a pathogen-free facility at the University of North Carolina (UNC) at Chapel Hill. Animal procedures and protocols were approved by the UNC Animal Care and Use Committee.

Experimental Design

Eight to twelve-week-old male and female mice were anesthetized with tribromoethanol before tracheal intubation and administration of *Escherichia coli* LPS O55:B5 (3 mg/kg) (Sigma-Aldrich). To deplete Tregs, diphtheria toxin (DT) (List Biologicals Laboratories) was given intraperitoneally to *Foxp3*^{DTR} or *Foxp3*^{EGFP} mice before and after LPS (Figure 1) (15, 18, 21). At Day 7 after LPS, mice were killed, and lungs were either lavaged or removed for generation of single-cell suspensions for flow-cytometric analysis or cell sorting, using methods similar to those outlined in previous descriptions (15, 18, 19).

In Vivo Antibody-mediated Neutralization of IFN- γ

Foxp3^{DTR} animals were given 20 μ g/dose/mouse of intraperitoneal injections of a rat monoclonal anti-IFN- γ antibody (505847, Ultra-LEAF antimouse IFN- γ , clone XMGI.2; BioLegend) or isotype control (400457, Ultra-LEAF Rat IgG1 κ isotype, clone RTK2017; BioLegend) on Days 1, 2, and 3 after LPS administration.

Quantifying Cytokine and Chemokine Concentrations in BAL Fluid

BAL in either *Foxp3*^{EGFP} or *Foxp3*^{DTR} mice was performed in unchallenged lungs or at Day 1, 3, or 7 after LPS-induced lung

injury, as previously described (15, 21). Cytokine and chemokine concentrations were determined using multiplex ELISAs.

RNA Isolation and Analysis for Transcriptomic Analysis

RNA was isolated from frozen cells stored at -80°C using the Zymo Direct-zol RNA MiniPrep with the TRI Reagent kit (Zymo Research).

mRNA Profiling and Data Analysis and Statistics

Expression of mRNA transcripts from sorted AT2 cells was profiled using the Affymetrix MoGene ST version 2.1 microarrays (Affymetrix) performed by the UNC School of Medicine Functional Genomics Core Facility ($n = 5$ replicates in each group). The analysis was performed as previously described (19).

RNA Isolation, cDNA Preparation, and RT-PCR Conditions for Single-Gene Transcript Analysis

Isolated RNA was reverse transcribed, and qRT-PCR was performed.

Statistics

Analysis of data other than gene expression used comparisons using the Mann-Whitney rank sum test or one-way ANOVA with Tukey or Holm-Sidak methods for multiple t test comparisons. Data are expressed as the mean $\pm$ SEM. Statistical analysis was performed using GraphPad Prism 7 software. Statistical difference was accepted at $P < 0.05$.

Accession Code

The microarray data have been deposited into the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>), series GSE106081.

Results

Tregs Affect the AT2-Cell Transcriptome during ALI Resolution

To examine AT2 cells during the resolution of ALI, we employed an LPS-induced ALI model using two transgenic strains (*Foxp3*^{DTR} and *Foxp3*^{EGFP}) of the same genetic background (16, 17, 19). The *Foxp3*^{DTR} strain expresses both the human DT receptor (DTR) and the gene for GFP in the 3' untranslated region of the *Foxp3* locus (17). The *Foxp3*^{EGFP} strain has the gene

Figure 1. Regulatory T cells (Tregs) impact LPS-induced lung-injury resolution. (A) *Foxp3^{EGFP}* (gene for enhanced GFP [EGFP] inserted downstream of the *Foxp3* locus) or *Foxp3^{DTR}* (human gene for the diphtheria toxin receptor [DTR] inserted in the 3' untranslated region of the *Foxp3* locus) mice received intraperitoneal diphtheria toxin (DT) (50 µg/kg or 10 µg/kg) on Days -2, -1, 1, 3 and 5. Intratracheal LPS was administered on Day 0. Type 2 alveolar epithelial (AT2) cells were isolated and sorted from the lungs of unchallenged C56BL/6J wild-type mice (Uninjured) or the lungs from either *Foxp3^{EGFP}* mice (Injured [Tregs Present]) or *Foxp3^{DTR}* mice (Injured [Tregs Depleted]) 7 days after LPS (3 mg/kg) challenge. (B) Body weight relative to baseline measured daily before and after injury (*Foxp3^{EGFP}* mice *n* = 24; *Foxp3^{DTR}* mice *n* = 34). Data are expressed as the mean ± SEM. (C) Survival of *Foxp3^{EGFP}* and *Foxp3^{DTR}* mice following LPS injury and DT administration (*P* = 0.032 using log rank test).

for EGFP inserted downstream of the *Foxp3* locus (16). DT administration (Figure 1A) depletes endogenous Tregs in the *Foxp3^{DTR}* strain, whereas the *Foxp3^{EGFP}* strain's Tregs are resistant to DT and remain present throughout injury and resolution, as previously reported (19).

We hypothesized that Tregs impact AT2 transcription during ALI resolution. The effects that Tregs exert on AT2 cells during ALI resolution were determined by comparing the AT2-cell transcriptome in the presence (*Foxp3^{EGFP}*) or near-absence (*Foxp3^{DTR}*) of Tregs, achieved by administering intratracheal LPS and intraperitoneal DT to both strains (Figure 1A). Mice depleted of Tregs during LPS-induced ALI have similar weight loss and recovery compared with control animals after LPS (Figure 1B). However, the mortality after LPS injury is significantly greater in Treg-depleted mice (Figure 1C). We selected the time point of resolution on the basis of multiple criteria, including body weights, a maximal point of AT2 proliferation, Treg kinetics, and decreasing neutrophils and inflammatory macrophages, as previously demonstrated by our group (15). Building on published

methods of studying alveolar epithelial populations using multicolor flow cytometry of lung single-cell digests, we sorted AT2 cells from uninjured mice or mice resolving from ALI (Figures 1B and 1C; see Figure E1 in the data supplement) (7, 15, 18, 22–24). Three sets of AT2 cells were examined: 1) AT2 cells sorted from uninjured lungs (Uninjured), 2) AT2 cells sorted at 7 days after injury from *Foxp3^{EGFP}* mice (Injured [Tregs Present]), and 3) AT2 cells sorted at 7 days after injury from *Foxp3^{DTR}* mice (Injured [Tregs Depleted]). To obtain adequate numbers of AT2 cells for mRNA analysis, single-cell suspensions from multiple mice were combined before sorting (*n* = 3–8 mice/experimental replicate). This study used male mice aged 8–12 weeks, and five replicates were performed for each of the three conditions.

The enrichment of the AT2 cells after sorting was determined by measuring the expression of lineage-specific genes and by flow cytometry. Epithelial-cell expression of lineage-specific genes (*Aqp5* [aquaporin 5], *Ccsp* [club cell secretory protein], *Foxj1* [forkhead box J1], and *Sftpc* [surfactant protein C]) were used to confirm

enrichment of AT2 cells (Figure E2). These data show that expression of *Sftpc*, a marker of AT2 cells, was enriched fivefold in sorted AT2 cells compared with AT1 cells. The AT2 cells expressed only 15–20% of the amount of *Aqp5* expressed in AT1 cells (Figure E2A). Neither *Ccsp* nor *Foxj1*, markers of club and cilia cells, were enriched in AT2 cells. Purity measured by flow cytometry was very high when AT2 cells were isolated from uninjured mice (>95%) and was slightly lower in AT2 cells isolated from resolving lungs (85–90%) (Figure E3).

Transcriptome analysis identified 1,974 differentially expressed genes (DEGs) between AT2 cells sorted from uninjured and injured lungs with and without Tregs, when filtering at an adjusted *P* value < 0.05 and a fold change greater than two (genes listed in Tables E1–E3). Principal component analysis of gene expression showed that the top three principal components accounted for 38% of the variance between AT2 sets (Figure 2A).

A Venn diagram demonstrates the overlap of DEGs (Figure 2B and Tables E1–E3). When Tregs were depleted, AT2 cells from lungs at 7 days after injury

Figure 2. Treg impact on the AT2-cell transcriptome during resolution of lung injury. *Foxp3^{EGFP}* or *Foxp3^{DTR}* mice were challenged with intraperitoneal DT (50 $\mu\text{g/kg}$ or 10 $\mu\text{g/kg}$) administered at Days -2 , -1 , 1 , 3 and 5 . Intratracheal LPS was administered on Day 0 . Uninjured, Injured (Tregs Present), or Injured (Tregs Depleted) samples 7 days after LPS (3 mg/kg) challenge. (A) Principal component analysis of mRNA expression demonstrates the contribution of the top three principal components to the variance in the three sets. Each symbol represents one sort of pooled AT2 cells from mice (3–8 mice/experiment; five arrays/set). (B) Venn diagram demonstrating the overlap of significantly differentially expressed transcripts between sets (adjusted P value < 0.05 , fold change > 2). Circle areas reflect the relative number of differentially expressed transcripts for each comparison. Numbers in parentheses indicate the total from the respective pairwise comparison. These genes are listed in Tables E1–E3 (see the data supplement). (C) Unsupervised hierarchical clustering with Euclidean distance metric of 1,974 differentially expressed genes (DEGs) identified in all three comparisons. The dendrogram identifies clustering across gene (top) or experimental samples (right). The heat map depicts the $\log_2$ -normalized intensity, and each row represents AT2 RNA from a pooled set of mice. As described in the METHODS, genes between sorted AT2 sets were identified by filtering at an adjusted P value < 0.05 and a fold change > 2 (bright blue for lower expression amounts and bright red for high expression amounts). (D) DEGs between Injured (Tregs Depleted) and Injured (Tregs Present) mice. Unsupervised hierarchical clustering of the unique 49 DEGs between AT2 samples. The heat map depicts the $\log_2$ -normalized intensity across samples (bright blue for lower expression amounts and bright red for high expression amounts). Each row represents AT2 RNA from pooled AT2 cells, and each column represents a DEG. The dendrogram identifies clustering across experimental samples (right) or DEGs (top). (E–G) Volcano plots showing differential gene expression between pair-wise conditions. Select genes with a greater than twofold change between comparisons are identified in gray dots and labeled with the gene name. *Clca1* = chloride channel accessory 1; *Cst8* = cystatin 8; *Entpd4* = ectonucleoside triphosphate

Table 1. The Number of DEGs for Each Condition

Comparison of AT2 Cell Condition	Number of Genes		
	Upregulated	Downregulated	Total (%)
Injured (Tregs Depleted) vs. Injured (Tregs Present)	45	4	49 (2.5)
Injured (Tregs Present) vs. Uninjured	777	679	1,456 (74)
Injured (Tregs Depleted) vs. Uninjured	925	675	1,600 (81)

Definition of abbreviations: AT2 = type 2 alveolar epithelial; DEG = differentially expressed gene; DTR = diphtheria-toxin receptor; EGFP = enhanced GFP; Injured (Tregs Depleted) = AT2 cells sorted at 7 days after injury and diphtheria toxin administration from *Foxp3^{DTR}* mice; Injured (Tregs Present) = AT2 cells sorted at 7 days after injury and diphtheria toxin administration from *Foxp3^{EGFP}* mice; Treg = regulatory T cell; Uninjured = AT2 cells sorted from uninjured lungs from C56BL/6J wild-type mice.

The number of up- or downregulated genes that were differentially expressed among the three conditions. The genes are upregulated or downregulated more than twofold and filtered at an adjusted *P* value < 0.05. The percentage of the total number of DEGs (1,974) is described.

differentially expressed 50 transcripts coding for 49 unique genes, when compared with the expression profile of injured AT2 cells with Tregs present (Injured [Treg-Depleted] vs. Injured [Tregs Present]) (Figure 2B; genes listed in Table E1). AT2 cells from lungs at 7 days after injury with Tregs present differentially expressed 1,456 genes compared with the AT2 cells from uninjured lungs (Injured [Tregs Present] vs. Uninjured) (Figure 2B; genes listed in Table E2). AT2 cells from lungs at 7 days after injury with Tregs absent differentially expressed 1,600 genes compared with the AT2 cells from uninjured lungs (Injured [Treg-Depleted] vs. Uninjured), 1,067 of which overlapped with those changed when Tregs were present (Figure 2B; genes listed in Table E3). The numbers of genes that were up- or downregulated are shown in Table 1 for each comparison.

Unsupervised hierarchical clustering of all 1,974 DEGs among the three conditions is shown in Figure 2C. Except for a single Injured (Tregs Depleted) sample that clustered within the Injured (Tregs Present) samples, all samples clustered by group. Unsupervised hierarchical clustering of the 49 DEGs between Injured (Tregs Depleted) and Injured (Tregs Present) is shown in Figure 2D. The dendrogram identifies clustering across samples (right) and DEGs (top). These data indicate that each sample clustered by condition. Importantly, most of the changed genes were upregulated by the absence of Tregs.

The pairwise differences among the three conditions are shown in volcano plots (Figures 2E–2G), and particular genes are labeled. As noted in the Venn diagram, only 49 genes were significantly different when Tregs were depleted during injury (Injured [Tregs Depleted] vs. Injured [Tregs Present]; Figure 2G and Table E1). The genes upregulated in AT2 cells from Treg-depleted mice with resolving ALI included *Ccl20*, *CD59a*, *Stat2* (signal transducer and activator of transcription 2), and *Wdly1*. Only four genes were downregulated, *Entpd4* (ectonucleoside triphosphate diphosphohydrolase 4), *Entpd4b*, *Ide* (insulin-degrading enzyme), and *Lrmda* (leucine-rich melanocyte differentiation-associated) (Figure 2G).

To identify biological pathways that were impacted by Treg depletion, we performed gene-set enrichment analysis using gene sets developed from the public GO Biological Process annotations and the ROAST (rotation gene set tests for complex microarray experiments) statistical gene-set test (25, 26). When the resolving AT2 cells with Tregs depleted were compared with resolving AT2 cells with Tregs present, eight significantly upregulated gene sets were identified (false discovery rate < 0.05), three of which contained identical genes (Table 2). Of these eight gene sets, five described the cellular response to type I IFN, and three described multiorganism intracellular transport and cellular localization.

AT2 cells with Tregs present had 332 significant gene sets compared with AT2

from uninjured lungs (Injured [Tregs Present] vs. Uninjured). For example, upregulated gene sets (Table 3) include a response to oxygen radical (GO:0000305). Dampened gene sets included positive regulation of the Wnt signaling pathway (GO:0030177) and regulation of the LPS-mediated signaling pathway (GO:0031664).

AT2 cells with Tregs depleted had 298 significantly regulated gene sets compared with AT2 cells from uninjured lungs (Injured [Tregs Depleted] vs. Uninjured). Selected interesting examples of upregulated enriched gene sets (Table 4) include response to IFN- γ (GO:0034341), negative regulation of response to wounding (GO:1903035), negative regulation of cytokine production (GO:0001816), regulation of leukocyte migration (GO:0002685), α - β T-cell activation involved in immune response (GO:0002287), and regulation of fibroblast migration (GO:0010762).

Overall, the transcriptomic analysis revealed that during ALI resolution, many transcriptional changes are occurring in AT2 cells. Moreover, the depletion of Tregs elicits a substantial effect on the AT2 transcriptome.

Chemokine and Cytokine Expression in the Bronchoalveolar Compartment during LPS-induced ALI in the Presence or Absence of Tregs

On the basis of the AT2 transcriptome analysis, we examined whether depletion of Tregs during ALI affects concentrations of type I IFN and type II IFN and other

Figure 2. (Continued). *diphosphohydrolase 4*; *Gbp* = guanylate binding protein; *Gm4951* = predicted gene 4951; *Ide* = insulin-degrading enzyme; *Lrmda* = leucine-rich melanocyte differentiation-associated; *Ly6i* = lymphocyte antigen 6i; *Malt1* = mucosa-associated lymphoid tissue lymphoma translocation protein 1; *Mgl1* = monoacylglycerol lipase; *Mki67* = marker of proliferation Ki-67; PC = principal component; PCA = principal component analysis; *Pon1* = paraoxonase 1; *Saa3* = serum amyloid A; *Scgb1c1* = secretoglobin 1C member 1; *Stat2* = signal transducer and activator of transcription 2; *Tnfrsf9* = tumor necrosis factor receptor superfamily member 9; *Wdly1* = WD repeat and FYVE domain-containing protein 1.

Table 2. Gene-Set Enrichment Analysis Identified GO Biological Pathways Showing Highly Significant Enrichment in Injured (Tregs Depleted) versus Injured (Tregs Present)

	Ontology ID	Functional Term	Number of Genes	Genes
Upregulated in Injured (Tregs Depleted)				
	GO:0035458	Cellular response to IFN- β	32 (13)	<i>Ube2g2</i> , <i>Irf1</i> , <i>Cdc34</i> , <i>Pnpt1</i> , <i>Ddx41</i> , <i>Acod1</i> , <i>Tmem173</i> , <i>Stat1</i> , <i>Gbp3</i> , <i>Gbp2</i> , <i>Ube2k</i> , <i>Iffit1</i> , <i>Ndufa13</i> , <i>Iff203</i> , <i>Iff209</i> , <i>Irgm1</i> , <i>Gm12185</i> , <i>Trex1</i> , <i>Ilgp1</i> , <i>Iff205</i> , <i>Gm5431</i> , <i>Htra2</i> , <i>Gm4841</i> , <i>Irgm2</i> , <i>9930111J21Rik1</i> , <i>Iff204</i> , <i>Gm4951</i> , <i>Iffit3</i> , <i>Igtp</i> , <i>Tgtp2</i> , <i>Tgtp1</i> , <i>F830016B08Rik</i>
	GO:0034340	Response to type I IFN	25 (5)	<i>Irf3</i> , <i>Cdc37</i> , <i>Iffar1</i> , <i>Iffar2</i> , <i>Mx2</i> , <i>Ptpn2</i> , <i>Shmt2</i> , <i>Iffitm3</i> , <i>Irf7</i> , <i>Stat1</i> , <i>Zbp1</i> , <i>Adar</i> , <i>Ube2k</i> , <i>Fadd</i> , <i>Iffit1</i> , <i>Cactin</i> , <i>Isg15</i> , <i>Mavs</i> , <i>A230050P20Rik</i> , <i>Stat2</i> , <i>Mul1</i> , <i>Ikake</i> , <i>Trim56</i> , <i>Lsm14a</i> , <i>Nlrc5</i>
	GO:0071357	Cellular response to type I IFN	19 (5)	<i>Irf3</i> , <i>Cdc37</i> , <i>Iffar1</i> , <i>Iffar2</i> , <i>Ptpn2</i> , <i>Iffitm3</i> , <i>Irf7</i> , <i>Stat1</i> , <i>Zbp1</i> , <i>Adar</i> , <i>Ube2k</i> , <i>Fadd</i> , <i>Iffit1</i> , <i>Cactin</i> , <i>Mavs</i> , <i>Stat2</i> , <i>Mul1</i> , <i>Lsm14a</i> , <i>Nlrc5</i>
	GO:0060337	Type I IFN signaling pathway	18 (5)	<i>Irf3</i> , <i>Cdc37</i> , <i>Iffar1</i> , <i>Iffar2</i> , <i>Ptpn2</i> , <i>Iffitm3</i> , <i>Irf7</i> , <i>Stat1</i> , <i>Zbp1</i> , <i>Adar</i> , <i>Ube2k</i> , <i>Fadd</i> , <i>Cactin</i> , <i>Mavs</i> , <i>Stat2</i> , <i>Mul1</i> , <i>Lsm14a</i> , <i>Nlrc5</i>
	GO:0060338	Regulation of type I IFN-mediated signaling pathway	13 (3)	<i>Irf3</i> , <i>Cdc37</i> , <i>Ptpn2</i> , <i>Irf7</i> , <i>Zbp1</i> , <i>Adar</i> , <i>Ube2k</i> , <i>Fadd</i> , <i>Cactin</i> , <i>Mavs</i> , <i>Mul1</i> , <i>Lsm14a</i> , <i>Nlrc5</i>
	GO:1902583	Multiorganism intracellular transport	11 (2)	<i>Fmr1</i> , <i>Ddx39b</i> , <i>Thoc1</i> , <i>Tap2</i> , <i>Thoc3</i> , <i>Thoc5</i> , <i>Tap1</i> , <i>Thoc2</i> , <i>Thoc6</i> , <i>Bst2</i> , <i>Thoc7</i>
	GO:1902581	Multiorganism cellular localization	11 (2)	<i>Fmr1</i> , <i>Ddx39b</i> , <i>Thoc1</i> , <i>Tap2</i> , <i>Thoc3</i> , <i>Thoc5</i> , <i>Tap1</i> , <i>Thoc2</i> , <i>Thoc6</i> , <i>Bst2</i> , <i>Thoc7</i>
	GO:0075733	Intracellular transport of virus	11 (2)	<i>Fmr1</i> , <i>Ddx39b</i> , <i>Thoc1</i> , <i>Tap2</i> , <i>Thoc3</i> , <i>Thoc5</i> , <i>Tap1</i> , <i>Thoc2</i> , <i>Thoc6</i> , <i>Bst2</i> , <i>Thoc7</i>
Downregulated in Injured (Tregs Depleted)	None*	—	—	—

Definition of abbreviations: 9930111J21Rik1 = RIKEN cDNA 9930111J21 gene 1; A230050P20Rik = RIKEN cDNA A230050P20 gene; Acod1 = acornite decarboxylase 1; Adar = RNA-specific adenosine deaminase 1; Bst2 = bone marrow stromal cell antigen 2; Cactin = cactin, spliceosome C complex subunit; Cdc = cell division cycle; Ddx = aspartic acid/glutamic acid/alanine/aspartic acid-box helicase; F830016B08Rik = RIKEN cDNA F830016B08 gene; Fadd = Fas associated via death domain; Fmr1 = fragile X mental retardation 1; Gbp = guanylate binding protein 1; Gm = predicted gene; GO = gene ontology; Htra2 = high temperature requirement protein A2; ID = identifier; Iff = IFN-induced protein; Iffit = Iff with tetrapeptide repeats; Iffitm3 = IFN-induced transmembrane protein 3; Iffar = IFN- α and IFN- β receptor subunit; Igtp = IFN- γ -induced GTPase; ilgp1 = IFN-inducible GTPase 1; Ikake = inhibitor of NF- κ B kinase subunit epsilon; Irf = IFN regulatory factor; Irgm = immunity-related GTPase family M; Isg15 = IFN-stimulated gene 15; Lsm14a = family with sequence similarity 61, member A; Mavs = mitochondrial antiviral signaling; Mul1 = mitochondrial E3 ubiquitin protein ligase 1; Mx2 = IFN-induced GTP-binding protein Mx2; Ndufa13 = nicotinamide adenine dinucleotide reduced:ubiquinone oxidoreductase subunit A13; Nlrc5 = nucleotide-binding oligomerization domain-like receptor family caspase recruitment domain-containing 5; Pnpt = polynucleotide nucleotidyltransferase 1; Shmt2 = serine hydroxymethyltransferase, mitochondrial; Stat = signal transducer and activator of transcription; Tap = transporter associated with antigen processing; Tgtp = T cell-specific GTPase 1; Thoc = THO complex; Tmem173 = transmembrane protein 173; Trex1 = three prime repair exonuclease 1; Trim56 = tripartite motif-containing 56; Ube = ubiquitin-protein ligase E; Zbp1 = Z-DNA binding protein 1.

All significant gene sets that were differentially regulated between comparisons are listed. Sets are listed only if the false discovery rate was less than 0.05. The number of genes in each GO is provided, and the total number of DEGs are listed in parentheses. Gene names in bold italic are DEGs from the array analysis.

*No gene sets were significantly downregulated.

Table 3. Gene-Set Enrichment Analysis Identified GO Biological Pathways Showing Highly Significant Enrichment in Injured (Tregs Present) versus Uninjured

Upregulated in Injured (Tregs Present)			Downregulated in Injured (Tregs Present)		
Ontology ID	Functional Term	Number of Genes	Ontology ID	Functional Term	Number of Genes
GO:0071450	Cellular response to oxygen radical	18	GO:2000188	Regulation of cholesterol homeostasis	11
GO:0000070	Mitotic sister chromatid segregation	119	GO:0006895	Golgi to endosome transport	16
GO:0001916	Positive regulation of T cell-mediated cytotoxicity	13	GO:0046321	Positive regulation of fatty acid oxidation	13
GO:0006953	Acute phase response	24	GO:0044320	Cellular response to leptin stimulus	11
GO:0002227	Innate immune response in mucosa	12	GO:0000187	Activation of MAPK activity	77
GO:0051301	Cell division	440	GO:0030177	Positive regulation of Wnt signaling pathway	71
GO:0060337	Type I IFN signaling pathway	18	GO:0009755	Hormone-mediated signaling pathway	130
GO:0003351	Epithelial cilium movement	11	GO:0031664	Regulation of LPS-mediated signaling pathway	17
GO:0006753	Nucleoside phosphate metabolic process	389	GO:0032374	Regulation of cholesterol transport	24
GO:0000305	Response to oxygen radical	24			
GO:0007005	Mitochondrion organization	475			
GO:0031589	Macromolecule glycosylation	162			

Definition of abbreviation: MAPK = mitogen-activated protein kinase.

Selected gene sets of interest that were differentially regulated between these comparisons are listed (false discovery rate < 0.05). The unique gene sets found only in this comparison are identified in italics.

cytokines and chemokines during ALI injury. We measured cytokine and chemokine concentrations in the BAL fluid at 1, 3, or 7 days after LPS-induced ALI and DT administration in mice with and without Tregs (Table 5). These time points were chosen on the basis of our prior work examining the kinetics of cytokines during ALI injury (19). The Holm-Sidak method for multiple comparisons was employed to determine the statistical significance of these results. Only two chemokines, CCL19 and CXCL16, were significantly different between groups, and this was only at Day 7 (Table 5). Interestingly, the mRNA coding for both CXCL16 and its receptor CXCR6 are more highly expressed by Tregs in injured lungs, compared with splenic Tregs (19). CXCL16, but not CXCR6, is expressed by uninjured and injured AT2 cells similarly in the presence or absence of Tregs (Gene Expression Omnibus, series GSE106081). The function of this CXCL16 and CXCR6 axis during resolution of inflammation has not been studied, but it may regulate immune-cell recruitment and function. CCL19 and its ligand CCR7 are

expressed by uninjured and injured AT2 cells at similar degrees with and without Tregs present, as well as by Tregs from injured and uninjured lungs (19). The observed increase in the CCL19 concentration in the BAL supernatant after Treg depletion may reflect downstream inhibitory effects of Tregs.

A second statistical analysis, which evaluated each individual cytokine or chemokine between *Foxp3^{EGFP}* and *Foxp3^{DTR}* at the three time points was also performed (Figures E4 and E5). This analysis found that at Day 3, a time point of peak inflammation (15, 21), three mediators, IFN- γ , CCL22, and CXCL16, were significantly different between the two groups (Table 5; concentrations of selected mediators and total protein are plotted in Figures E4 and E5). Only IFN- γ was higher in the Treg-depleted mice (*Foxp3^{DTR}*) compared with control mice (*Foxp3^{EGFP}*) at Day 3 (Figure E5), whereas the two chemokines CCL22 and CXCL16 had lower concentrations (Table 5 and Figure E4). At Day 7, when mice have begun to recover weight and resolve injury, twice as many cytokines were significantly different

between conditions. The concentrations of the chemokines CCL1 and CCL19 were higher in the Treg-depleted mice compared with control animals, whereas the concentrations of five cytokines or chemokines, IL-4, IL-10, CXCL11, CXCL12, and CXCL16 were lower (Figures E4 and E5). Type I IFN concentrations, for IFN- α and IFN- β , did not differ in the BAL fluid between strains (Figure E5).

To determine the cellular source of the increased IFN- γ , we employed flow cytometry with intracellular staining to assess changes in IFN- γ expression. At Day 3 after LPS in lung digests of Treg-depleted mice, IFN- γ was significantly increased in CD4⁺ and CD8⁺ lymphocytes and decreased in natural killer (NK) cells and NK T lymphocytes compared with control mice (Figure 3A) (19). No differences in IFN- γ expression were detected in neutrophils or macrophage subpopulations (Figure 3B; representative gating shown in Figure E6) (27).

These results demonstrate the effects Tregs exert on cytokine and chemokine concentrations during ALI. The near-

Table 4. Gene-Set Enrichment Analysis Identified GO Biological Pathways Showing Highly Significant Enrichment in Injured (Tregs Depleted) versus Uninjured

Upregulated in Injured (Tregs Depleted)			Downregulated in Injured (Tregs Depleted)		
Ontology ID	Functional Term	Number of Genes	Ontology ID	Functional Term	Number of Genes
GO:0035456	Response to IFN- β	39	GO:0008202	Steroid metabolic process	155
GO:0051301	Cell division	440	GO:0070076	Histone lysine demethylation	22
GO:0045824	Negative regulation of innate immune response	33	GO:1903209	Positive regulation of oxidative stress-induced cell death	13
GO:1903035	Negative regulation of response to wounding	44	GO:0006631	Fatty acid metabolic process	243
GO:0034341	Response to IFN- γ	63	GO:0010669	Epithelial structure maintenance	12
GO:0034446	Substrate adhesion-dependent cell spreading	78	GO:0019395	Fatty acid oxidation	80
GO:0001818	Negative regulation of cytokine production	144	GO:0006637	Acyl-CoA metabolic process	49
GO:0002287	α - β T-cell activation involved in immune response	36	GO:0045104	Intermediate filament cytoskeleton organization	20
GO:0002685	Regulation of leukocyte migration	114			
GO:0007004	Telomere maintenance via telomerase	49			
GO:0007162	Negative regulation of cell adhesion	173			
GO:0010762	Regulation of fibroblast migration	31			

Definition of abbreviation: acyl-CoA = acyl-coenzyme A.

Selected gene sets of interest that were differentially regulated between these comparisons are listed (false discovery rate < 0.05). The gene sets identified uniquely in this comparison are identified in italics.

depletion of Tregs may intensify the IFN- γ -driven responses in AT2 cells because 42 of the 49 DEGs in resolving AT2 cells with and without Tregs are regulated by either or both type I and type II IFN, as determined from the Interferome database (Table E1 and Figure E5D; www.interferome.org) (28). On the basis of these findings, we sought to examine which effects of Treg depletion are due to IFN- γ .

IFN- γ Has Functions Independent of Tregs but Also Modulates the Effect of Tregs on the Expansion of Inflammatory Macrophages and CD19⁺ B Cells

These studies assessed what aspects of Treg function during injury and resolution are due IFN- γ . To identify the functions of Tregs, Treg-depleted mice were compared with mice with a full complement of Tregs. To determine which of the observed effects of Treg depletion are reversed by neutralizing IFN- γ , Treg-depleted *Foxp3^{DTR}* mice were given an IFN- γ -neutralizing antibody or isotype control at Days 1, 2, and 3 after LPS-induced injury and were studied on Day 7. In addition, to examine the

effects of IFN- γ neutralization alone in the presence of Tregs, *Foxp3^{DTR}* mice were given IFN- γ -neutralizing antibody or isotype control at Days 1, 2, and 3 after LPS-induced injury and were studied on Day 7. The data showed that Treg depletion had no effect on weight changes after LPS, but mice administered IFN- γ -neutralizing antibody had less weight loss and a faster recovery of this loss compared with those given the isotype antibody, whether Tregs were depleted or not (Figure 4A). Treg depletion also did not affect protein concentration or cell numbers in the BAL fluid compared with mice with a full complement of Tregs (Figures 4B and 4C). IFN- γ -neutralizing antibody or isotype control also did not affect BAL-fluid protein concentration or cell numbers in the presence of Tregs. However, neutralizing IFN- γ in Treg-depleted mice resulted in fewer lavageable cells (Figure 4C).

The lungs of Treg-depleted mice (*Foxp3^{DTR}* mice) contained more cells than those of mice with unaltered Treg numbers (*Foxp3^{EGFP}* mice), and neutralizing IFN- γ did not affect the total cell number in either the presence or absence of Tregs (Figure 4D). Measurement of Treg numbers in the

lungs of *Foxp3^{DTR}* mice confirmed a nearly complete depletion of *Foxp3⁺* cells, as well as in the percentage of CD4⁺ cells that coexpress *Foxp3⁺*, compared with *Foxp3^{EGFP}* mice (Figures 4E and 4F). Interestingly, the number of lung *Foxp3⁺* cells and the percentage of *Foxp3⁺* cells of total CD4⁺ cells were significantly higher in Treg-depleted mice administered the IFN- γ -neutralizing antibody compared with the isotype antibody (Figures 4E and 4F), suggesting that IFN- γ prevents expansion of Tregs, potentially through proliferation and/or recruitment.

Neither Treg depletion nor IFN- γ blockade in Treg-depleted mice had any effect on the number of alveolar or interstitial macrophages (Figures 4G and 4H). Blockade of IFN- γ in the presence of Tregs results in more alveolar macrophages and fewer interstitial macrophages (Figures 4G and 4H), confirming our previously observations in C57Bl/6J mice (29). In contrast, mice with Treg depletion had more lung neutrophils than mice with a full complement of Tregs, and IFN- γ blockade had no impact compared with an isotype control in the presence or absence of Tregs (Figure 4I), suggesting that IFN- γ does not

Table 5. Cytokines Measured in BAL Fluid of *Foxp3^{EGFP}* or *Foxp3^{DTR}* mice at Day 1, 3, or 7 after LPS-induced Injury and DT Administration

Analyte (pg/ml)	<i>Foxp3^{EGFP}</i> Day 1 (n = 7)	<i>Foxp3^{DTR}</i> Day 1 (n = 10)	<i>Foxp3^{EGFP}</i> Day 3 (n = 6)	<i>Foxp3^{DTR}</i> Day 3 (n = 10)	<i>Foxp3^{EGFP}</i> Day 7 (n = 17)	<i>Foxp3^{DTR}</i> Day 7 (n = 19)
CXCL13	11,188.1 ± 4,197.0	13,995.0 ± 4,249.1	24,653.9 ± 10,662.9	29,163.2 ± 11,090.8	15,464.7 ± 16,049.6	6,698.4 ± 10,590.6 (n = 18)
CCL27	333.5 ± 68.5	398.5 ± 97.5	655.7 ± 50.8 (n = 5)	630.2 ± 147.0	532.9 ± 274.9 (n = 14)	3,742 ± 298.3 (n = 13)
CXCL5	229.7 ± 67.8	661.7 ± 140.3	379.9 ± 140.3	352.6 ± 215.0	215.2 ± 173.1	305.3 ± 256.0 (n = 18)
CCL11	35.4 ± 14.6	38.4 ± 19.6	139.0 ± 82.2	115.5 ± 53.9	81.0 ± 52.1	114.5 ± 146.3 (n = 18)
CCL24	1,144.2 ± 348.4 (n = 6)	1,289.3 ± 756.5	1,978.5 ± 1,492.7 (n = 5)	1,006.4 ± 514.8	4,623.9 ± 4,712.0	8,036.4 ± 8,499.3 (n = 18)
CX3CL1	25.8 ± 7.4	26.4 ± 6.7	43.2 ± 8.8 (n = 5)	48.5 ± 11.6	31.3 ± 18.2	24.3 ± 16.3 (n = 18)
GM-CSF	4.5 ± 1.3	7.4 ± 9.6	6.7 ± 3.0 (n = 4)	6.7 ± 4.3 (n = 7)	11.5 ± 5.4 (n = 8)	13.9 ± 0.0 (n = 1)
CCL1	<LLOQ	2.1 ± 0.3 (n = 3)	3.6 ± 1.1 (n = 5)	3.5 ± 0.8	6.6 ± 4.8 (n = 13)	18.2 ± 15.0 (n = 18)
IFN-γ	5.1 ± 0.8	8.8 ± 3.2	27.8 ± 19.4	173.9 ± 220.0	9.4 ± 6.6 (n = 16)	8.2 ± 10.9 (n = 16)
IL-1β	64.6 ± 9.8	86.9 ± 26.2	247.4 ± 148.0	255.1 ± 126.8	81.4 ± 49.6	71.6 ± 36.2 (n = 18)
IL-2	4.8 ± 1.8	5.8 ± 1.5	9.1 ± 2.7	10.3 ± 2.6	6.9 ± 5.0 (n = 16)	4.5 ± 2.9 (n = 18)
IL-4	0.9 ± 0.1	1.2 ± 0.3	2.6 ± 1.2	2.8 ± 1.1	2.1 ± 1.5 (n = 14)	0.9 ± 0.9 (n = 16)
IL-6	149.7 ± 74.9	205.0 ± 69.6	2,196.2 ± 2,418.1	2,422.9 ± 2,890.6	219.6 ± 368.8 (n = 14)	28.7 ± 43.7 (n = 11)
IL-10	45.8 ± 10.1 (n = 6)	58.9 ± 10.4	132.5 ± 40.9 (n = 5)	139.9 ± 43.8	95.5 ± 41.4 (n = 10)	56.3 ± 35.5 (n = 9)
IL-16	312.8 ± 108.6	562.7 ± 233.5	1,579.2 ± 865.6	2,011.2 ± 630.5	546.6 ± 390.0	269.1 ± 263.8
CXCL10	138.9 ± 39.7	296.8 ± 211.3	422.7 ± 180.6	513.1 ± 281.2	182.8 ± 101.9 (n = 16)	232.3 ± 106.5 (n = 18)
CXCL11	42.7 ± 10.8	57.4 ± 11.2	105.4 ± 47.3	122.6 ± 37.8	100.8 ± 61.0 (n = 12)	55.5 ± 43.6 (n = 9)
CXCL1	27.2 ± 16.1	123.0 ± 58.0	19.5 ± 7.3	35.6 ± 27.5	7.9 ± 2.8 (n = 6)	10.4 ± 4.0 (n = 9)
CCL2	313.2 ± 190.4	623.7 ± 211.3	304.6 ± 78.9	263.9 ± 314.4	417.6 ± 317.7 (n = 10)	129.7 ± 92.0 (n = 5)
CCL7	185.6 ± 54.8	239.2 ± 22.5	189.5 ± 59.5	185.1 ± 60.6	103.5 ± 86.6	72.0 ± 67.1 (n = 18)
CCL12	87.0 ± 48.4	159.5 ± 53.3	108.1 ± 58.1	78.8 ± 46.0	32.0 ± 19.7 (n = 14)	21.1 ± 36.3 (n = 17)
CCL22	207.2 ± 123.3	209.3 ± 50.0	399.3 ± 226.8	246.1 ± 102.4	219.8 ± 140.5	167.6 ± 87.8
CCL3	166.1 ± 34.7	230.0 ± 75.5	321.2 ± 109.7	250.8 ± 194.7	12.4 ± 7.2	8.1 ± 3.3 (n = 18)
CCL4	326.1 ± 59.4	570.7 ± 243.5	991.1 ± 748.8	1,122.1 ± 1,005.9	43.6 ± 16.1 (n = 10)	25.7 ± 10.1 (n = 14)
CXCL2	240.7 ± 80.9	310.1 ± 167.3	320.5 ± 96.2	230.3 ± 159.5	30.5 ± 17.5	26.4 ± 11.8 (n = 18)
CCL20	806.2 ± 221.4	1,257.3 ± 460.9	598.3 ± 157.5	517.8 ± 160.7	37.2 ± 23.8	140.2 ± 133.0
CCL19	40.6 ± 9.1	51.3 ± 11.4	160.4 ± 90.1	172.8 ± 64.9	58.7 ± 32.4	170.3 ± 102.8* (n = 18)
CCL5	319.7 ± 107.4	568.7 ± 125.6	454.1 ± 420.1	488.3 ± 288.7	37.2 ± 20.8	41.1 ± 34.1 (n = 18)
CXCL16	350.0 ± 104.5	408.5 ± 59.5	597.8 ± 257.6	324.6 ± 113.5	461.8 ± 115.0	265.2 ± 122.2†
CXCL12	93.1 ± 20.3	105.4 ± 33.1	334.2 ± 103.5	206.1 ± 92.8	219.5 ± 170.9	107.9 ± 87.0 (n = 17)
CCL17	4,279.5 ± 2,303.4 (n = 5)	6,191.7 ± 1,734.5 (n = 2)	271.6 ± 125.4	174.4 ± 81.7	304.4 ± 218.5	266.7 ± 164.0
CCL25	328.3 ± 60.4	457.8 ± 70.3	598.2 ± 83.5	541.7 ± 140.4	366.9 ± 127.0 (n = 9)	277.1 ± 47.0 (n = 4)
TNF-α	376.3 ± 221.4	738.7 ± 534.0	110.2 ± 73.7	141.4 ± 77.6	49.9 ± 60.7 (n = 16)	15.8 ± 15.9 (n = 17)

Definition of abbreviations: DT = diphtheria toxin; DTR = DT receptor; EGFP = enhanced GFP; *Foxp3^{DTR}* = strain has the human DTR gene inserted in the 3' untranslated region of the *Foxp3* locus; *Foxp3^{EGFP}* = strain has the EGFP gene inserted downstream of the *Foxp3* locus; GM-CSF = granulocyte-macrophage colony-stimulating factor; LLOQ = lower limit of quantitation; TNF-α = tumor necrosis factor-α.

Cytokines measured in BAL fluid at Day 1, 3, or 7 after intratracheal LPS (Day 0) and intraperitoneal DT administered at Days -2, -1, 1, 3 (for Day 3 and 7) and 5 (for Day 7) in *Foxp3^{EGFP}* (Injured [Tregs Present]) or *Foxp3^{DTR}* mice (Injured [Tregs Depleted]). Only two chemokines, CCL19 (boldface, increased in the Treg-depleted mice) and CXCL16 (boldface, decreased in Treg-depleted mice) at Day 7 reached significance. Values are the mean ± SD. Samples with values below the LLOQ for the assay were removed from the analysis. The number of samples used for calculations (n) is noted in the column subheading unless otherwise indicated in the individual cells when concentrations for the specific cytokine or chemokine sample fell below the LLOQ in the condition. Samples are combined from two independent experiments. P values were determined using a one-way ANOVA for all values (all cytokines and all time points; 66 conditions), followed by the Holm-Sidak multiple comparison test.

*P < 0.05.

†P < 0.01.

Figure 3. Treg depletion affects IFN- γ expression during lung injury. *Foxp3^{EGFP}* or *Foxp3^{DTR}* mice were challenged with intraperitoneal DT (50 μ g/kg or 10 μ g/kg) administered at Days -2, -1, and 1 together with intratracheal LPS administered at Day 0. Mice were studied on Day 3 after LPS. Uninjured, baseline *Foxp3^{DTR}* mice were also examined. (A and B) Median fluorescence intensity (MFI) of IFN- γ was determined by flow cytometry in lymphocyte subtypes, macrophage subpopulations, and neutrophils ($n=3-8$ /group, representative of two independent experiments). Data are expressed as the mean $\pm$ SEM. P values were determined using the Holm-Sidak multiple comparison test. Please note that because the lymphoid and myeloid cells were studied in different flow-cytometric panels of antibodies, the MFIs can be compared only within and not between these two panels. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. NK = natural killer.

mediate this enhanced recruitment. Treg-depleted mice had increased numbers of inflammatory macrophages compared with the *Foxp3^{EGFP}* mice (Figures 4J and E6). Strikingly, this increase in inflammatory macrophages was abrogated in the presence or absence of Tregs after IFN- γ neutralization compared with an isotype control (Figure 4J), suggesting that the Treg-induced increase in inflammatory macrophages was entirely mediated through IFN- γ .

The numbers of CD3⁺, CD4⁺, and CD8⁺ lymphocytes in Treg-depleted mice were increased compared with *Foxp3^{EGFP}* mice, and neutralization of IFN- γ did not affect these changes (Figures 4K–4M). Depletion of Tregs also led to increased numbers of effector populations of CD4⁺ and CD8⁺ lymphocytes (Figures E7A–E7C). In the presence of Tregs, blockade of IFN- γ led to less activation for CD4⁺ cells, but not when Tregs were absent. Treg-depleted mice had fewer CD19⁺ lymphocytes (40% fewer) and $\gamma\delta$ ⁺ lymphocytes (65% fewer) compared with the *Foxp3^{EGFP}* mice and no significant differences in NK cell or NK T-lymphocyte numbers (Figures 4N–4Q). Neutralization of IFN- γ in the presence of Tregs led to lower $\gamma\delta$ ⁺ lymphocyte numbers and no difference in CD19⁺ lymphocytes compared with the isotype control. Strikingly, the number of CD19⁺ B lymphocytes was higher after IFN- γ

neutralization compared with an isotype control (Figure 4O) in Treg-depleted mice (1.6-fold higher), suggesting that the Treg-induced decrease in B (CD19⁺) lymphocytes was mediated through IFN- γ .

Multicolor flow cytometry was also used to identify lung endothelial (CD31⁺) and epithelial (CD326⁺) cells in lung digests obtained 7 days after LPS (15, 18). The number of total endothelial cells was similar between genotypes and conditions; however, Treg-depleted mice administered an IFN- γ -neutralizing antibody had an increase in total CD326⁺ cells when compared with the mice depleted of Tregs with no antibody administration (Figures E7D and E7E). However, the percentage of proliferating epithelial (CD326⁺Ki-67⁺) and proliferating endothelial (CD31⁺Ki-67⁺) cells was lower in Treg-depleted mice compared with *Foxp3^{EGFP}* mice (Figures E7F and E7G). These results demonstrate that Tregs enhance the proliferation of parenchymal cells. Neutralizing IFN- γ in the presence of Tregs led to fewer proliferating CD31⁺ cells (Figure E7G).

Thus, IFN- γ contributes to several of the changes induced by the absence of Tregs, as demonstrated by neutralizing IFN- γ in Treg-depleted mice. Neutralization reversed the effects of Treg depletion on inflammatory macrophages and B cells by preventing the increase in inflammatory macrophages and the decrease in B cells. IFN- γ did not regulate the inhibitory

effects of Tregs on neutrophil recruitment; CD3⁺, CD4⁺, and CD8⁺ cell numbers; the production of effector CD4⁺ and CD8⁺ T cells; or the proliferation of epithelial or endothelial cells. Interestingly, neutralizing IFN- γ results in a slight increase in the numbers of Tregs in Treg-depleted mice. IFN- γ also contributes to processes that are not mediated by Tregs, such as weight loss and numbers of macrophage subpopulations and $\gamma\delta$ ⁺ lymphocytes at a time point during resolution of ALI.

Discussion

In this report, we explored the transcriptional changes occurring in AT2 cells during the resolution of lung injury and the contribution of Tregs to these changes. We chose 7 days after LPS administration as an appropriate time to study resolution, as this was the peak of both AT2 proliferation and lung Treg numbers in our prior studies (15, 19). The results of our AT2 transcriptional profiling indicate that AT2 cells differentially express over 1,400 genes at Day 7 of resolution after LPS-induced lung injury compared with uninjured AT2 cells. Importantly, the presence of Tregs in this microenvironment affected the expression of 49 genes by AT2 cells. These effects of Tregs on the AT2 transcriptome could be either direct or indirect. Either

Figure 4. IFN- γ antibody-mediated neutralization partially reverses the effect of Treg depletion during resolution of acute lung injury. $Foxp3^{EGFP}$ or $Foxp3^{DTR}$ mice received intraperitoneal DT (50 μ g/kg or 10 μ g/kg) on Days -2, -1, 1, 3 and 5. Intratracheal LPS was administered on Day 0. A subset of $Foxp3^{DTR}$ mice were given intraperitoneal injections of 20 μ g/mouse of either an IFN- γ -neutralizing antibody (Ab) or an isotype control Ab at Days 1, 2, and 3 after LPS injury. Mice were weighed daily and examined at Day 7 after LPS for injury parameters or immunophenotyping in lung digests. (A) Body weight relative to baseline determined after injury ($n = 14$ –59/group, combined from at least two independent experiments). (B) BAL total protein and (C) BAL cell counts determined between conditions. (D) Total lung cell count obtained from lung digests. (E) Total lung $Foxp3^+$ lymphocyte numbers and (F) Percentage of CD4 $^+$ cells that are $Foxp3^+$ in lung single-cell suspensions. Immunophenotyping of (G) alveolar macrophages, (H) interstitial macrophages, (I) neutrophils, and (J) inflammatory macrophages as total numbers in single-cell suspensions determined by flow cytometry (27). (K–Q) Changes in CD3 $^+$, CD4 $^+$, CD8 $^+$, $\gamma\delta^+$, CD19 $^+$, NK, and NK T lymphocyte subsets as total numbers in single-cell suspensions determined by flow cytometry (19).

other mediators or other cell types may mediate the effects of Tregs. In fact, our previous *In vitro* studies demonstrated that contact-dependent and contact-independent effects of Tregs impact AT2 proliferation (15). Determining both the direct and indirect effects of Tregs on AT2 cells are essential issues that remain to be resolved.

Many of these 49 genes contributed to immune regulatory responses in the AT2 cell transcriptome. Several GO biological processes were enriched in the absence of Tregs, including “response to type I IFN” and “multiorganism intracellular transport.” Further comparison of the AT2 transcriptome with and without Tregs indicated that the majority of DEGs were increased in the absence of Tregs (45 of 49; Figure 2 and Table E1). These 45 were IFN-stimulated genes (either type I or type II IFN), as identified by the Interferome database (<http://www.interferome.org>) (28), and 40 of these were identified as stimulated by IFN- γ (28). The remaining four DEGs included *Entpd4*, *Entpd4b*, *Gm22771* (predicted gene 22771), and *Gm28177*.

The functions of Tregs during LPS-induced lung injury have been partially described in our previously published papers (15, 18, 19). On the basis of our array and immune-mediator data, we explored the contribution of IFN- γ to the effects of Treg depletion. These studies show that IFN- γ importantly contributes to several of the changes induced by the absence of Tregs, including the presence of CD19⁺ B cells and the influx of inflammatory macrophages. IFN- γ does not contribute to the inhibitory effects of Tregs on neutrophil recruitment, CD3⁺, CD4⁺, and CD8⁺ cell numbers, the production of effector CD4⁺ and CD8⁺ T cells, or the proliferation of epithelial or endothelial cells. Although our studies focus on the contributions of IFN- γ to Treg-directed resolution, IFN- γ also contributes to processes that are not mediated by Tregs, such as weight loss and numbers of alveolar, interstitial, and inflammatory macrophages in the lung.

Uncoupling the Treg-independent effects IFN- γ may exert on the rate and quality of resolution is left to be determined.

In mice depleted of Tregs, Tregs were more numerous when IFN- γ was neutralized through antibody-mediated blockade during LPS-induced ALI (Figures 4E and 4F). These findings suggest that Treg recruitment, proliferation, and/or retention are a crucial component of the reparative process and may be negatively regulated by IFN- γ and its downstream signaling (19). Other reports have shown that IFN- γ can mediate loss of Treg-suppressive function (30, 31).

The concept that dampening IFN- γ function may provide a benefit in ALI is very appealing. IFN- γ polymorphisms are reported to correlate with increased longevity (32). Other polymorphisms in the *IFNG* (INF- γ) gene may increase IFN- γ production and provide resistance to chronic pulmonary tuberculosis infections (33). In patients with ARDS, a panel of four mediators, one of which is IFN- γ , predicts a more reactive phenotype and is associated with higher mortality (31, 32). Other work also supports IFN- γ contributing to CD8⁺ cell-induced lung injury (34, 35). Furthermore, a recombinant IFN- γ blocking antibody (emapalumab) was recently approved in the United States for the treatment of patients with primary hemophagocytic lymphohistiocytosis (36). These studies, together with ours, demonstrate that potential immunotherapy to block or neutralize IFN- γ could be useful in controlling severe lung inflammation.

The depletion of Tregs during resolution decreased the expression of *Entpd4*, *Entpd4b*, *Lrmda*, and *Ide* by AT2 cells. Each of these has interesting potential functions in repair and resolution of injuries. *Entpd4* encodes for an apyrase, also called UDPase (uridine diphosphatase), and is a member of a family of enzymes that catalyze nucleotide triphosphates and diphosphates as part of the purinergic signaling pathway (37). The role of increased expression of UDPase in

AT2 cells during resolution is unknown; however, post-translational modifications of tight-junction proteins can affect epithelial barrier function (38). Interestingly, the GO nucleotide phosphate metabolic process (GO: 0006753), was enriched in resolving AT2 cells in the presence of Tregs compared with uninjured AT2 cells. Purinergic signaling can regulate immune-cell processes and can specifically regulate the cytokine CCL20, which was upregulated in AT2 cells when Tregs were depleted during injury (Figure 2 and Table E1) (37, 39, 40). Extracellular purinergic signaling can induce CCL20 expression through P2Y₂ (P2Y purinoceptor 2), which is also significantly upregulated in both resolving AT2 cell groups (37, 39, 40). Our study lends support to the postulate that AT2 purinergic signaling pathways play essential roles in ALI resolution. LRMDA is not well studied (41, 42), and its role in alveolar repair is left to be determined. IDE functions as a zinc metalloprotease, degrading insulin, TGF- α (transforming growth factor- α), β -endorphin, and β -amyloid (43, 44). *Ide* expression is stress inducible and may function in addition as a heat shock-like protein, affecting cell growth regulation (45). Thus, the differential expression of *Entpd4*, *Ide*, and *Lrmda* in AT2 cells is likely to have functional importance during the resolution of ALI.

A recent study reported several AT2 subpopulations during LPS-induced injury using single-cell RNA sequencing (46). Interestingly, our study revealed several DEGs from each of the AT2 subpopulations described in this study: the proliferating (*Cdc20* [cell division cycle 20], *Birc5* [baculoviral inhibitor of apoptosis repeat-containing protein 5], and *Mki67* [marker of proliferation Ki-67]), cell-cycle arrest (*Itgb6* [integrin- β 6], *Tgfb2* [TGF- β 2], and *Fn1* [fibronectin-1]) and transdifferentiating (*Akap5* [A-kinase anchoring protein 5], *Pdpr* [podoplanin], *Clic3* [chloride intracellular channel 3], and *Clic5*) subpopulations (46). Future work using other methods, such as single-cell RNA

Figure 4. (Continued). ($n = 9\text{--}17/\text{group}$, combined from two independent experiments). Data are expressed as the mean $\pm$ SEM. *P* values for body weights in A were determined by two-way ANOVA with the Tukey multiple-comparison test between conditions. Significance between comparisons and the day(s) are listed. *P* values for remaining figures (B–Q) were determined by one-way ANOVA with the Tukey multiple-comparison test between conditions. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$. *P* values for pairwise comparison between the IFN- γ antibody group and isotype antibody group were determined by Mann-Whitney rank sum test. # $P < 0.05$.

sequencing, may identify subpopulations of AT2 cells or changes in the rate of AT2 transdifferentiation to AT1 cells that are modulated by Tregs during resolution.

In summary, our results illustrate the complexity of alveolar epithelial function in resolution of lung injury and provide insight into the effects one immune-cell population exerts on an important alveolar epithelial-cell type.

Understanding the interactions in the reparative process is critical to determine which processes may be amenable to therapeutic interventions. These studies highlight the importance of Treg-directed lung repair and may further inform the development of short-term and appropriately timed immunotherapeutic approaches to inflammatory lung diseases such as ARDS (34, 47–49). ■

Author disclosures are available with the text of this article at www.atsjournals.org.

Acknowledgment: The authors thank the UNC Flow Cytometry Core Facility and Dr. Evan Trudeau for assistance with cell sorting, and Michael J. Vernon and the UNC School of Medicine Functional Genomics Core Facility for microarray processing. The Venn diagram was constructed with software from the Pacific Northwest National Laboratory available through the omics.pnl.gov website.

References

1. Yamamoto K, Ferrari JD, Cao Y, Ramirez MI, Jones MR, Quinton LJ, *et al.* Type I alveolar epithelial cells mount innate immune responses during pneumococcal pneumonia. *J Immunol* 2012;189:2450–2459.
2. Mizgerd JP. Respiratory infection and the impact of pulmonary immunity on lung health and disease. *Am J Respir Crit Care Med* 2012;186:824–829.
3. Bhattacharya J, Matthay MA. Regulation and repair of the alveolar-capillary barrier in acute lung injury. *Annu Rev Physiol* 2013;75:593–615.
4. Hong KU, Reynolds SD, Giangreco A, Hurley CM, Stripp BR. Clara cell secretory protein-expressing cells of the airway neuroepithelial body microenvironment include a label-retaining subset and are critical for epithelial renewal after progenitor cell depletion. *Am J Respir Cell Mol Biol* 2001;24:671–681.
5. Giangreco A, Arwert EN, Rosewell IR, Snyder J, Watt FM, Stripp BR. Stem cells are dispensable for lung homeostasis but restore airways after injury. *Proc Natl Acad Sci USA* 2009;106:9286–9291.
6. Reynolds SD, Giangreco A, Power JH, Stripp BR. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. *Am J Pathol* 2000;156:269–278.
7. Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, *et al.* Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005;121:823–835.
8. Chapman HA, Li X, Alexander JP, Brumwell A, Lorzio W, Tan K, *et al.* Integrin $\alpha 6 \beta 4$ identifies an adult distal lung epithelial population with regenerative potential in mice. *J Clin Invest* 2011;121:2855–2862.
9. McQuarrel JL, Yuen K, Williams B, Bertonecchio I. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. *Proc Natl Acad Sci USA* 2010;107:1414–1419.
10. Uhal BD. Cell cycle kinetics in the alveolar epithelium. *Am J Physiol* 1997;272:L1031–L1045.
11. Kauffman SL. Alterations in cell proliferation in mouse lung following urethane exposure. 3. Effects of chronic exposure on type 2 alveolar epithelial cell. *Am J Pathol* 1972;68:317–326.
12. Barkauskas CE, Crouse MJ, Rackley CR, Bowie EJ, Keene DR, Stripp BR, *et al.* Type 2 alveolar cells are stem cells in adult lung. *J Clin Invest* 2013;123:3025–3036.
13. Rojas M, Woods CR, Mora AL, Xu J, Brigham KL. Endotoxin-induced lung injury in mice: structural, functional, and biochemical responses. *Am J Physiol Lung Cell Mol Physiol* 2005;288:L333–L341.
14. Su X, Looney MR, Gupta N, Matthay MA. Receptor for advanced glycation end-products (RAGE) is an indicator of direct lung injury in models of experimental lung injury. *Am J Physiol Lung Cell Mol Physiol* 2009;297:L1–L5.
15. Mock JR, Garibaldi BT, Aggarwal NR, Jenkins J, Limjunyawong N, Singer BD, *et al.* Foxp3⁺ regulatory T cells promote lung epithelial proliferation. *Mucosal Immunol* 2014;7:1440–1451.
16. Haribhai D, Lin W, Relland LM, Truong N, Williams CB, Chatila TA. Regulatory T cells dynamically control the primary immune response to foreign antigen. *J Immunol* 2007;178:2961–2972.
17. Kim JM, Rasmussen JP, Rudensky AY. Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice. *Nat Immunol* 2007;8:191–197.
18. Dial CF, Tune MK, Doerschuk CM, Mock JR. Foxp3⁺ regulatory T cell expression of keratinocyte growth factor enhances lung epithelial proliferation. *Am J Respir Cell Mol Biol* 2017;57:162–173.
19. Mock JR, Dial CF, Tune MK, Norton DL, Martin JR, Gomez JC, *et al.* Transcriptional analysis of Foxp3⁺ Tregs and functions of two identified molecules during resolution of ALI. *JCI Insight* 2019;4:124958.
20. Dalton DK, Pitts-Meek S, Keshav S, Figari IS, Bradley A, Stewart TA. Multiple defects of immune cell function in mice with disrupted interferon-gamma genes. *Science* 1993;259:1739–1742.
21. D'Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, *et al.* 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. Lee JH, Kim J, Gludish D, Roach RR, Saunders AH, Barrios J, *et al.* Surfactant protein-C chromatin-bound green fluorescence protein reporter mice reveal heterogeneity of surfactant protein C-expressing lung cells. *Am J Respir Cell Mol Biol* 2013;48:288–298.
23. Fujino N, Kubo H, Ota C, Suzuki T, Suzuki S, Yamada M, *et al.* A novel method for isolating individual cellular components from the adult human distal lung. *Am J Respir Cell Mol Biol* 2012;46:422–430.
24. Vaughan AE, Brumwell AN, Xi Y, Gotts JE, Brownfield DG, Treutlein B, *et al.* Lineage-negative progenitors mobilize to regenerate lung epithelium after major injury. *Nature* 2015;517:621–625.
25. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, *et al.*; The Gene Ontology Consortium. Gene ontology: tool for the unification of biology. *Nat Genet* 2000;25:25–29.
26. Wu D, Lim E, Vaillant F, Asselin-Labat ML, Visvader JE, Smyth GK. ROAST: rotation gene set tests for complex microarray experiments. *Bioinformatics* 2010;26:2176–2182.
27. Misharin AV, Morales-Nebreda L, Mutlu GM, Budinger GR, Perlman H. Flow cytometric analysis of macrophages and dendritic cell subsets in the mouse lung. *Am J Respir Cell Mol Biol* 2013;49:503–510.
28. Rusinova I, Forster S, Yu S, Kannan A, Masse M, Cumming H, *et al.* Interferome v2.0: an updated database of annotated interferon-regulated genes. *Nucleic Acids Res* 2013;41:D1040–D1046.
29. Mock JR, Tune MK, Dial CF, Torres-Castillo J, Hagan RS, Doerschuk CM. Effects of IFN- γ on immune cell kinetics during the resolution of acute lung injury. *Physiol Rep* 2020;8:e14368.
30. Overacre-Delgoffe AE, Chikina M, Dadey RE, Yano H, Brunazzi EA, Shayan G, *et al.* Interferon- γ drives Treg fragility to promote anti-tumor immunity. *Cell* 2017;169:1130–1141, e11.
31. Delgoffe GM, Woo SR, Turnis ME, Gravano DM, Guy C, Overacre AE, *et al.* Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature* 2013;501:252–256.
32. Lio D, Scola L, Crivello A, Bonafè M, Franceschi C, Olivieri F, *et al.* Allele frequencies of +874T: >A single nucleotide polymorphism at the first intron of interferon-gamma gene in a group of Italian centenarians. *Exp Gerontol* 2002;37:315–319.
33. Lio D, Marino V, Serauto A, Gioia V, Scola L, Crivello A, *et al.* Genotype frequencies of the +874T: >A single nucleotide polymorphism in the first intron of the interferon-gamma gene in a sample of Sicilian patients affected by tuberculosis. *Eur J Immunogenet* 2002;29:371–374.
34. Włodarczyk MF, Kraft AR, Chen HD, Kenney LL, Selin LK. Anti-IFN- γ and peptide-tolerization therapies inhibit acute lung injury induced by cross-reactive influenza A-specific memory T cells. *J Immunol* 2013;190:2736–2746.
35. Ramana CV, DeBerge MP, Kumar A, Alia CS, Durbin JE, Enelow RI. Inflammatory impact of IFN- γ in CD8⁺ T cell-mediated lung injury is mediated by both Stat1-dependent and

- independent pathways. *Am J Physiol Lung Cell Mol Physiol* 2015; 308:L650–L657.
36. Al-Salama ZT. Emapalumab: first global approval. *Drugs* 2019;79: 99–103.
 37. Deaglio S, Robson SC. Ectonucleotidases as regulators of purinergic signaling in thrombosis, inflammation, and immunity. *Adv Pharmacol* 2011;61:301–332.
 38. Shigetomi K, Ikenouchi J. Regulation of the epithelial barrier by post-translational modifications of tight junction membrane proteins. *J Biochem* 2018;163:265–272.
 39. Marcet B, Horckmans M, Libert F, Hassid S, Boeynaems JM, Communi D. Extracellular nucleotides regulate CCL20 release from human primary airway epithelial cells, monocytes and monocyte-derived dendritic cells. *J Cell Physiol* 2007;211: 716–727.
 40. Junger WG. Immune cell regulation by autocrine purinergic signalling. *Nat Rev Immunol* 2011;11:201–212.
 41. Grønskov K, Jespersgaard C, Bruun GH, Harris P, Brøndum-Nielsen K, Andresen BS, et al. A pathogenic haplotype, common in Europeans, causes autosomal recessive albinism and uncovers missing heritability in OCA1. *Sci Rep* 2019;9:645.
 42. Tanikawa M, Sanjiv K, Helleday T, Herr P, Mortusewicz O. The spliceosome U2 snRNP factors promote genome stability through distinct mechanisms; transcription of repair factors and R-loop processing. *Oncogenesis* 2016;5:e280.
 43. Wang DS, Dickson DW, Malter JS. beta-Amyloid degradation and Alzheimer's disease. *J Biomed Biotechnol* 2006;2006:58406.
 44. Affholter JA, Fried VA, Roth RA. Human insulin-degrading enzyme shares structural and functional homologies with *E. coli* protease III. *Science* 1988;242:1415–1418.
 45. Tundo GR, Sbardella D, Ciaccio C, Bianculli A, Orlandi A, Desimio MG, et al. Insulin-degrading enzyme (IDE): a novel heat shock-like protein. *J Biol Chem* 2013;288:2281–2289.
 46. Riemondy KA, Jansing NL, Jiang P, Redente EF, Gillen AE, Fu R, et al. Single cell RNA sequencing identifies TGFβ as a key regenerative cue following LPS-induced lung injury. *JCI Insight* 2019;5:123637.
 47. Tang Q, Vincenti F. Transplant trials with Tregs: perils and promises. *J Clin Invest* 2017;127:2505–2512.
 48. Singer BD. Opening the regulatory T cell toolbox. *Am J Respir Cell Mol Biol* 2017;57:137–138.
 49. Singer BD, King LS, D'Alessio FR. Regulatory T cells as immunotherapy. *Front Immunol* 2014;5:46.