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Authors
Li, John T
Melton, Andrew C
Su, George
et al.

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Unexpected Role for Adaptive αβTh17 Cells in Acute Respiratory Distress Syndrome

John T. Li,*,† Andrew C. Melton,* George Su,‡ David E. Hamm,§ Michael LaFemina,¶ James Howard,‖ Xiaohui Fang,#,** Sudarshan Bhat,†† Kieu-My Huynh,* Cecilia M. O'Kane,‡‡ Rebecca J. Ingram,‡‡ Roshell R. Muir,‡‡ Daniel F. McAuley,‡‡ Michael A. Matthay,#,** and Dean Sheppard*

Acute respiratory distress syndrome (ARDS) is a devastating disorder characterized by increased alveolar permeability with no effective treatment beyond supportive care. Current mechanisms underlying ARDS focus on alveolar endothelial and epithelial injury caused by products of innate immune cells and platelets. However, the role of adaptive immune cells in ARDS remains largely unknown. In this study, we report that expansion of Ag-specific αβTh17 cells contributes to ARDS by local secretion of IL-17A, which in turn directly increases alveolar epithelial permeability. Mice with a highly restrictive defect in Ag-specific αβTh17 cells were protected from experimental ARDS induced by a single dose of endotracheal LPS. Loss of IL-17 receptor C or Ab blockade of IL-17A was similarly protective, further suggesting that IL-17A released by these cells was responsible for this effect. LPS induced a rapid and specific clonal expansion of αβTh17 cells in the lung, as determined by deep sequencing of the hypervariable CD3RβVJ region of the TCR. Our findings could be relevant to ARDS in humans, because we found significant elevation of IL-17A in bronchoalveolar lavage fluid from patients with ARDS, and rIL-17A directly increased permeability across cultured human alveolar epithelial monolayers. These results reveal a previously unexpected role for adaptive immune responses that increase alveolar permeability in ARDS and suggest that αβTh17 cells and IL-17A could be novel therapeutic targets for this currently untreatable disease. *The Journal of Immunology*, **2015, 195: 87–95.**

Acute respiratory distress syndrome (ARDS), a frequent cause for respiratory failure in patients admitted to intensive care units, is characterized by diffuse alveolar filling with protein-rich fluid as a consequence of increased permeability of the alveolar–capillary barrier (1). ARDS can be a consequence of trauma, hemorrhagic shock, or toxic inhalation, but the most common cause is bacterial sepsis (2). The prevailing explanation is that the increase in permeability that underlies this disorder is a consequence of direct injury to the alveolar epithelium and endothelium. It is widely recognized that innate immune cells, principally neutrophils, are rapidly recruited to lung alveoli in response to injury or bacterial products, such as LPS. These neutrophils contribute to alveolar injury by secreting oxidants and proteases, as well as by promoting the formation of neutrophil extracellular traps (3, 4). However, until now there has been little evidence implicating direct effects of the adaptive immune system in the development of ARDS.

The time course of increased alveolar permeability in response to sepsis or experimental exposure to LPS calls into question the simple explanation of direct injury by neutrophils. For example, neutrophil recruitment peaks within the first few days after a single dose of endotracheal LPS, whereas the peak increase in alveolar permeability does not occur until 4–5 d later, at a time when lymphocytes are replacing neutrophils as the prominent hematopoietic cell in the airspaces (5). Recent work suggests that regulatory T cells contribute to the resolution phase of ARDS, but it is not clear whether this effect is Ag-dependent (6). We hypothesized that Ag-specific adaptive immune cells contribute to the induction of increased permeability in ARDS.

*Lung Biology Center, Department of Medicine, University of California, San Francisco, San Francisco, CA 94143; †Department of Pediatrics, University of California, San Francisco, San Francisco, CA 94110; ‡Department of Medicine, San Francisco General Hospital, San Francisco, CA 94110; §Adaptive Biotechnologies, Seattle, WA 98102; ¶Department of Medicine, San Francisco Veterans Affairs Medical Center, San Francisco, CA 94121; ‖Department of Pediatrics, UCSF Benioff Children's Hospital Oakland, Oakland, CA 94609; #Department of Anesthesia, Cardiovascular Research Institute, San Francisco, CA 94158; **Department of Medicine, Cardiovascular Research Institute, San Francisco, CA 94158; ††University of California, Berkeley, Berkeley, CA 94720; and ‡‡Center for Infection and Immunity, Queen's University of Belfast, Belfast BT7 1NN, United Kingdom

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J.T.L. designed the studies; performed the lung injury work, flow cytometry analysis, cultured epithelial cell studies, and deep sequencing experiments; and wrote the manuscript. A.C.M. performed the flow cytometry work and provided intellectual contribution to the experimental design; G.S. performed the lung injury work; D.E.H. supervised deep sequencing work, analyzed deep sequencing data, and contributed to writing portions of the manuscript related to deep sequencing; M.L., J.H., and X.F. provided intellectual contribution to study design and performed experiments related to cultured epithelial cells; S.B. performed and analyzed data related to survival studies; K.-M.H. managed mouse colonies and contributed to the lung injury experiments; C.M.O. and D.F.M. provided intellectual contribution and performed and analyzed data for studies related to human ARDS; R.J.I. and R.R.M. provided intellectual contribution and performed cytokine studies related to human ARDS; M.A.M. provided intellectual contribution and expertise in experiments related to cultured epithelial cells; and D.S. oversaw writing of the manuscript, design of experiments, and interpretation of all data in this manuscript.

Address correspondence and reprint requests to Dr. Dean Sheppard, Lung Biology Center, University of California, San Francisco, 1550 4th Street, Mission Bay, San Francisco, CA 94143. E-mail address: dean.sheppard@ucsf.edu

Abbreviations used in this article: ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; IL-17RC, IL-17 receptor C; KO, knockout.

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αβTh17 cells are Th cells that secrete a distinct subset of T cell cytokines, including IL-17A, IL-17F, IL-21, and IL-22 (7). IL-17A and IL-17F, in particular, are released in the setting of bacterial infections and have specific roles in response to bacterial and fungal pathogens (7). Nearly all cells express members of the IL-17 receptor family; therefore, IL-17, once released, can affect tissue injury by directly modulating the behavior of a variety of tissue cells (8, 9). IL-17 has been shown to have important roles in numerous models of inflammatory diseases, including experimental autoimmune encephalomyelitis, psoriasis, inflammatory arthritis, and allergic asthma (10–16). Most of these experimental models are driven by introducing known specific Ags, suggesting that the IL-17–dependent effects are a consequence of clonal expansion of Ag-specific αβTh17 cells. Current understanding of IL-17–mediated pathways in experimental ARDS is much more limited. In models of influenza and LPS-induced lung injury, IL-17 was linked to neutrophil recruitment (17, 18). The potential roles of pulmonary Ags driving an IL-17–mediated adaptive immune response or direct effects of IL-17 on alveolar epithelial barrier function that is independent of neutrophil recruitment have not been previously examined. We took advantage of mice we have developed with a specific defect in Ag-dependent αβTh17 cells to address these issues.

Materials and Methods

Animals

Mice were bred and maintained according to approved protocols by the University of California, San Francisco, Institutional Animal Care and Use Committee. *Itgb8*$^{fl/fl}$ × CD11c-cre mice were bred on a C57BL/6 background as previously described (19). IL-17 receptor C (IL-17RC) knockout (KO) mice were generated in the C57BL/6 background as previously described (20). IL-17A enhanced GFP knock-in reporter mice were obtained from The Jackson Laboratory (no. 018472).

Human bronchoalveolar lavage studies

Human bronchoalveolar lavage (BAL) fluid was collected with informed consent and analyzed in accordance to guidelines and approval by the Office of Research Ethics Committees, Northern Ireland. Details of patient demographics and inclusion criteria for developing the sample repository have been previously published (21).

LPS lung injury studies

Itgb8$^{fl/fl}$ × CD11c-cre and littermate control mice were used for the *Itgb8*$^{fl/fl}$ × CD11c-cre LPS-induced ARDS studies. C57BL/6 mice were used for the IL-17A blocking Ab studies. C57BL/6 mice were used for controls in IL-17RC KO lung injury studies. Mice were randomly assigned four-digit ear tag numbers and the operator was blinded to the genotype or treatment received prior to initiation of ARDS. Male and female mice between 8 and 12 wk of age, weighing 20–25 g, were used for the lung injury studies. Sample size was selected based on previous lung injury studies using similar models. The inclusion/exclusion criteria were based on age, weight, and genotype of the mice. Mice were anesthetized with inhaled isoflurane and placed in a supine position at a 60° incline. Endotracheal LPS (List Biological Laboratories, no. 201) (100 μg) dissolved in 100 μl distilled water was administered via direct endotracheal intubation of the trachea using P-10 polyethylene tubing (Becton Dickinson, no. 427400) attached to a 28-gauge insulin syringe (Becton Dickinson, no. 329461) followed by 150 μl air to improve distribution. In the IL-17A blocking Ab experiments, IL-17A Ab (eBioscience, no. 16-7173-85) or isotype control IgG1K Ab (eBioscience, no. 16-4714-85) was administered i.v. at a dose of 100 μg per mouse 2 d after treatment with LPS or H_2O. Lung injury was measured by permeability of Evans blue (Sigma-Aldrich, no. E2129)–labeled serum albumin as previously described in detail (22). In brief, Evans blue was injected i.v. at a dose of 25 μg/g via retro-orbital injection 96 h after endotracheal LPS instillation. Mice were euthanized 2 h after Evans blue administration, and serum was collected for total serum Evans blue measurement. The lungs were perfused free of blood with 10 ml PBS with 10 U/ml heparin (Sigma-Aldrich, no. 078K1610) under 20 cm H_2O of pressure by direct cannulation of the pulmonary artery via right ventriculotomy. Lungs were harvested and Evans blue was extracted by incubation in 1 ml formamide (Fisher Scientific, no. BP228-100) at 65°C for 48 h. Evans blue concentration was determined by measurement at 620 nm (Beckman Coulter spectrophotometer DU 800) and corrected for minor blood contamination by using a formula previously described (23). The Evans blue extravasation is reported as lung divided by serum Evans blue.

Flow cytometry

Whole lungs were isolated and minced with blunt scissors. Minced lung was passed through a 70-μm mesh strainer (BD Falcon, no. 352350) in RPMI 1640 (Cellgro Mediatech, no. 10-040CV). Red cell lysis was performed with red cell lysis buffer (Sigma-Aldrich, no. R7757). Isolated lung cells were incubated in the presence of 1 μM ionomycin (Sigma-Aldrich, no. I9657), PMA 50 μM (Sigma-Aldrich, no. P8139) and 2 μM monensin (eBioscience, no. 00-4505-51) at 37°C for 4 h. Fc receptor and nonspecific binding were blocked with anti-CD16/30 (eBioscience, no. 14061) and 10% rat serum (Sigma-Aldrich, no. R9759) for 20 min at 4°C. Live/dead staining was performed with Aqua Live/Dead stain (Invitrogen, no. L34957) in PBS for 30 min at 4°C. Single-cell suspension was stained with anti-CD4 (eBioscience, no. 48-0041-82), anti-γδ TCR (eBioscience, no. 12-5711-82), anti-CD44 (eBioscience, no. 12-04441-81), and anti-CD62L (eBioscience, no. 17-0621-81). Cells were fixed and permeabilized with a Fix and Perm kit (eBioscience, no. 00-5523-00) per the manufacturer's instructions. Intracellular staining was performed with anti–IL-17 Ab (eBioscience, no. 17-7177-81). Flow cytometry was performed using an LSR II (BD Biosciences) and analyzed using FlowJo (Tree Star). The isolated cells were gated for live cells from live/dead staining and these live cells were gated for $CD4^+$ and γδ TCR^+ cells. The $CD4^+$ and γδ TCR^+ cells were then gated for cells that stained for intracellular IL-17.

Cell count

BAL was performed by cannulating the trachea using a 20-gauge Intramedic Luer–Stub Adapter (Becton Dickinson, no. 427564), and lungs were lavaged twice with 0.8 ml PBS. Cell pellets were collected and resuspended in 1 ml PBS. Cells were counted using a Bright-Line hemacytometer (Hausser Scientific). Differential cell count was performed manually on cells harvested by cytospin using a Diff-Quick staining kit (Andwin Scientific, no. NC9943455) per the manufacturer's instructions.

IL-17A cytokine studies

For lung injury induced by LPS in mice, BAL fluid was collected in ice-cold PBS with Halt protease inhibitor cocktail (Thermo Scientific, no. 78430). Mouse IL-17A cytokine levels were measured using mouse IL-17A DuoSet kits (R&D Systems, no. DY421) according to the manufacturer's protocol. Human IL-17A cytokine levels were measured using cytometric bead arrays (R&D Systems Europe) per the manufacturer's recommendations.

Epithelial cell culture

Rat type II alveolar epithelial cells were isolated from male Sprague-Dawley rats weighing between 150 and 200 g as previously described (24, 25). Isolated rat type II epithelial cells were cultured on 96-well 0.4-μm pore size polycarbonate cell culture insert plates with receiver plates (Millipore, no. PSHT004R1) in DMEM/Ham's F12 50/50 media with 10% FBS at a density of 1.5×10^6 cells/cm^2. Human type II alveolar epithelial cells were isolated from human donor lungs declined for transplantation as previously described (25). Isolated human type II epithelial cells were cultured on 24-well 0.4-μm pore size Transwell plates (Corning, no. 3495) in DMEM/Ham's F12 50/50 media with 10% FBS at density of 1×10^6 cells/cm^2. Cells were cultured for 5 d until the confluent monolayer could maintain an air–liquid interface.

Epithelial barrier injury

Serum-starved cultured rat type II alveolar epithelial monolayers were stimulated with 100 ng/ml recombinant rat IL-17A (Prospec, no. CYT-542) for 24 h. Serum-starved human type II alveolar epithelial cells were stimulated with 100 ng/ml recombinant human IL-17A (Cell Signaling Technology, no. 8928). Transepithelial electrical resistance was measured using an STX100 electrode and ohm meter (World Precision Instruments). Permeability across Transwells was measured by diffusion of 4-kDa FITC-dextran (1 mg/ml; Sigma-Aldrich, no. FD4) during 2 h from the top to bottom well. Concentration of FITC-dextran was measured using a top plate reader with excitation of 488 nm, and emission was detected at 520 nm (Tecan GENios Pro).

Survival studies

IL-17RC KO and C57BL/6 wild-type mice were treated with 100 μl endotracheal H_2O or 125 μg LPS in the same volume. Mice were between 8 and 12 wk old weighing between 20 and 25 g. Mouse weights are reported as percentage weight change based on baseline body weight determined prior to treatment.

High-throughput TCR deep sequencing

CDR3β regions were amplified and sequenced by Adaptive Biotechnologies (Seattle, WA) using the immunoSEQ assay. In brief, a multiplex PCR system was used to amplify CDR3β sequences from DNA samples. The immunoSEQ assay can amplify all 35 V segments, both D genes, and the 14 functional J segments. This approach generates an 87-bp fragment capable of identifying the VDJ region spanning each unique CDR3β (26). Amplicons were sequenced using the Illumina HiSeq platform. Using a baseline developed from a suite of synthetic templates, primer concentrations and computational corrections were used to correct for the primer bias common to multiplex PCR reactions. Raw sequence data were filtered based on the TCRβ V, D, and J gene definitions provided by the IMGT database (http://www.imgt.org) and binned using a modified nearest neighbor algorithm to closely merge related sequences and remove both PCR and sequencing errors. Data were analyzed using the immunoSEQ analyzer tool.

Statistical analysis

Experimental ARDS studies. Lung permeability, lung cytokine, and BAL cell count studies are reported as means ± SEM. One-way ANOVA was applied to compare three or more groups and and post hoc Tukey–Kramer tests were used to identify specific differences.

Barrier integrity studies. For alveolar epithelial barrier Transwell studies, an unpaired two-tailed Student *t* test was applied.

Survival studies. Sample size for survival study was determined by sample size calculations for time to an event to detect a difference in survival of >30% (27) as: $n = C[(p_c q_c + p_e q_e)/d^2] + 2/d + 2$, where p_c indicates proportion of control group exhibiting event, p_e indicates desired proportion of experimental group exhibiting event, $q_c = 1 - p_c$, $q_e = 1 - p_e$, $d = |p_c - p_e|$, and C is a constant of 7.85, which was used in our calculations as determined by α and 1 − β for power of 80% and significance level of 5%. Comparisons of survival curves were calculated based on a log-rank Mantel–Cox test.

Weight change. The percentage weight change relative to initial weight was calculated for each mouse. Mean values ± SEM for each group of mice were plotted against the number of days after endotracheal LPS instillation. One-way ANOVA and a Bonferroni multiple comparison test were applied to determine differences between means on each day after endotracheal LPS instillation.

Clonality. The degree of clonal expansion within a sample and a direct measure of sample diversity were calculated as the inverse of normalized H (Shannon–Wiener index). The Shannon–Wiener index is normalized by dividing H by the $\log_2$ of the number of elements being measured (Pielou's evenness). The inverse of this result is a metric that varies between 0 and 1 where 0 is equal to all sequences being equally abundant and 1 is when a single sequence makes up the entire sample.

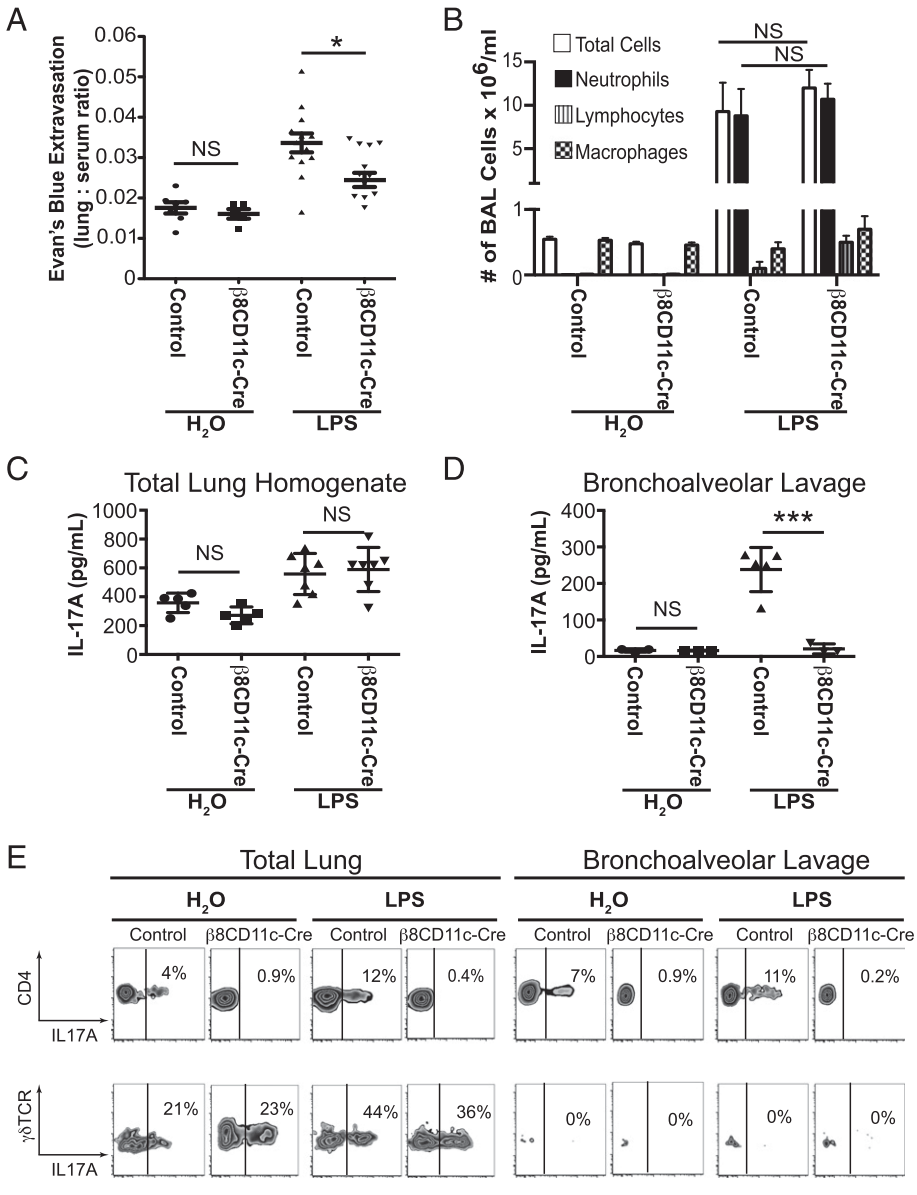

FIGURE 1. *Itgb8*$^{fl/fl}$ × CD11c-cre mice that fail to differentiate αβTh17 cells are protected from LPS-induced increases in lung permeability. (**A**) Endotracheal LPS (100 μg/mouse) or vehicle control (H_2O) was administered to *Itgb8*$^{fl/fl}$ × CD11c-cre and littermate control mice. Lung permeability was measured by extravasation of intravascular albumin labeled with Evans blue dye into the lung alveolar space and parenchyma; $n = 12$ in *Itgb8*$^{fl/fl}$ × CD11c-cre LPS group, $n = 13$ in littermate control LPS group, $n = 5$ in H_2O groups. $^*p < 0.05$, NS = $p > 0.05$ by one-way ANOVA and Tukey–Kramer test. (**B**) Total cell count and differential analysis of BAL fluid showed no difference in total cell count and neutrophil count 2 d after administration of H_2O or LPS (the peak time for neutrophil influx) between *Itgb8*$^{fl/fl}$ × CD11c-cre and littermate control mice; $n = 5$ per group. NS = $p > 0.05$, by Student *t* test. (**C**) IL-17A protein levels measured by ELISA in total lung homogenates 4 d after treatment with LPS or H_2O; $n = 5$ per group. (**D**) IL-17A protein levels in BAL fluid 4 d after treatment with LPS or H_2O; $n = 3$–5 per group. $^{***}p < 0.001$, NS = $p > 0.05$ by one-way ANOVA and Tukey–Kramer test. (**E**) Flow cytometry analysis of total lung isolates and BAL fluid obtained 4 d after treatment with LPS or H_2O. PMA- and ionomycin-stimulated and monensin-treated cells were stained for anti-CD4, anti-γδTCR, and anti–IL-17A. *Top row* shows CD4$^+$ gated cells and the bottom row shows γδTCR$^+$ gated cells. Data are representative flow cytometry plots with percentage of IL-17A$^+$ cells from four per group. Data reported as means ± SEM for (A)–(D).

Top 10 clones and pairwise sharing. A two-tailed Wilcoxon rank sum test was used to compare the sums of the average frequencies for the top 10 clones and fraction of shared sequences in pairwise sharing analysis.

Statistical software. Statistical analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and R statistical programming language 3.0.2.

Results

Itgb8$^{fl/fl}$ × *CD11c-cre mice are protected from experimental ARDS*

IL-17 can be released by a number of immune cells, including γδ T cells and NKT cells, and there is no specific genetically altered KO model in which IL-17 deletion is restricted to Ag-specific αβTh17 cells. However, we previously reported that mice deficient in the integrin β_8 subunit in CD11c-expressing cells (*Itgb8$^{fl/fl}$* × CD11c-cre) have a remarkably specific defect in αβTh17 cells owing to an inability to activate TGF-β during Ag presentation by dendritic cells (28, 29). We used these mice as the best available model of αβTh17–deficient mice, because mice lacking IL-17A and/or IL-17F and mice lacking IL-17 receptors have defects in responses to IL-17 cytokines from all cellular sources (both innate and adaptive). *Itgb8$^{fl/fl}$* × CD11c-cre allowed us to determine the specific importance of αβTh17 cells in ARDS. We assessed the peak increase in alveolar permeability in a model of ARDS (determined by extravasation of albumin-binding Evans blue dye) 4 d after endotracheal instillation of LPS into control or *Itgb8$^{fl/fl}$* × CD11c-cre mice. There was no baseline difference in permeability between *Itgb8$^{fl/fl}$* × CD11c-cre mice and littermate control mice treated with vehicle (H_2O), but *Itgb8$^{fl/f\ l}$* × CD11c-cre mice had a significantly reduced increase in Evans blue extravasation after treatment with LPS (Fig. 1A).

Previous studies identified an important function of IL-17A in host defense against various pathogens that was due to IL-17A–mediated neutrophil recruitment (30). Because neutrophils contribute to the pathogenesis of ARDS, we wanted to determine whether the protective effect observed in *Itgb8$^{fl/f\ l}$* × CD11c-cre mice could be secondary to a defect in neutrophil recruitment. The total cell count and neutrophil count in BAL fluid were similar in *Itgb8$^{fl/fl}$* × CD11c-cre mice and littermate control mice, suggesting that protection from increased lung permeability was not a consequence of reduced neutrophil recruitment (Fig. 1B).

Both αβTh17 and γδ T cells are important sources of IL-17A in the lung. We previously reported that *Itgb8$^{fl/fl}$* × CD11c-cre mice have a dramatic defect in pulmonary αβTh17 cells, but no defect in IL-17–producing γδ T cells in a model of allergic airway inflammation (29). Interestingly, there was no difference in IL-17A

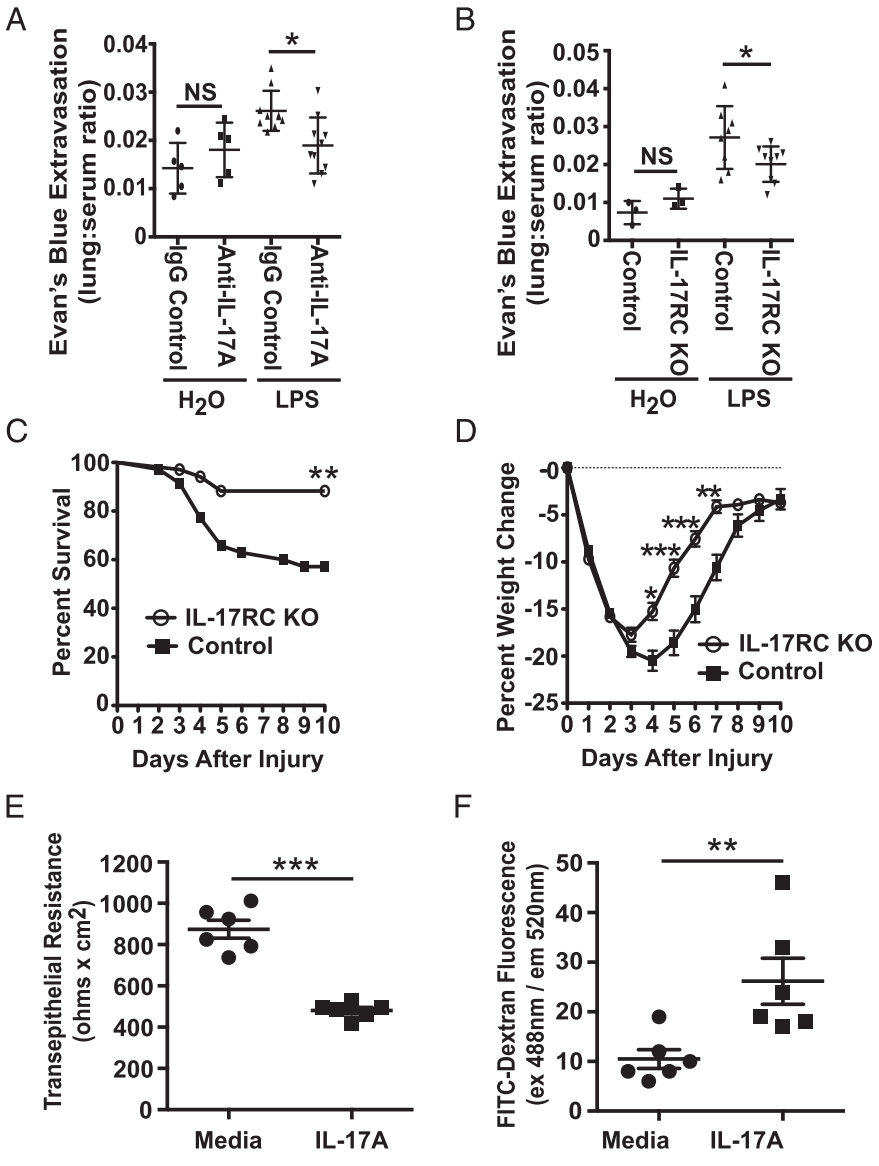

FIGURE 2. IL-17A inhibition and loss of IL-17RC protect mice against LPS-induced increased lung permeability. (**A**) Anti–IL 17A Ab or control IgG was administered i.v. 2 d after endotracheal administration of H_2O or LPS (100 μg/mouse) to C57BL/6 wild-type mice. Permeability was measured by leakage of Evans blue–labeled intravascular albumin into the lung at 4 d after LPS or H_2O administration. Data reported as means ± SEM; $n = 10$ in LPS groups and $n = 5$ in H_2O groups. $^*p < 0.05$ by one-way ANOVA and Tukey–Kramer test. (**B**) LPS or H_2O was administered to control or IL-17RC KO mice and permeability was assessed at 4 d, as in (A). Data are reported as means ± SEM; $n = 9$ in LPS groups and $n = 3$ in H_2O groups. $^*p < 0.05$ by one-way ANOVA and Tukey–Kramer test. (**C**) Higher dose LPS (125 μg per mouse) was administered endotracheally to control or IL-17RC KO mice and survival was assessed during the next 10 d. Data are reported as percentage survival; $n = 35$ in each group. $^{**}p < 0.01$ by log-rank Mantel–Cox test. (**D**) Body weight was measured daily in all surviving high-dose LPS-treated control and IL-17RC KO mice for 10 d. Data are reported as percentage weight change from initial weight. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ by one-way ANOVA and Bonferroni multiple comparison test. Confluent rat type II alveolar epithelial monolayers were cultured on collagen-impregnated semipermeable membrane inserts in the presence of serum-free media with and without recombinant rat IL-17A for 24 h. (**E**) Transepithelial electrical resistance and (**F**) permeability to FITC-dextran were measured. Data are reported as means ± SEM; $n = 6$ per group. $^{**}p < 0.01$, $^{***}p < 0.001$ by Student t test.

protein concentration in whole-lung lysates from LPS-challenged *Itgb8*$^{fl/fl}$ × CD11c-cre mice (Fig. 1C), but a dramatic decrease in IL-17A protein concentration in BAL fluid (Fig. 1D). After treatment with endotracheal LPS, we also found a dramatic defect in whole lung αβTh17 cells in *Itgb8*$^{fl/fl}$ × CD11c-cre mice, but normal numbers of IL-17–producing γδ T cells (Fig. 1E). αβTh17 cells were easily detected in BAL fluid from LPS-treated control mice, but were absent from BAL fluid of *Itgb8*$^{fl/fl}$ × CD11c-cre mice. IL-17–producing γδ T cells were not detected in BAL fluid (Fig. 1E). These results suggest tight spatial control of IL-17A production from distinct populations of T cells in the lung and raise the possibility that local alveolar production of IL-17A by αβTh17 cells could play an important role in the induction of lung permeability in response to LPS.

Disruption of IL-17A signaling protects against experimental ARDS

To determine whether the protection seen in mice with a defect in αβTh17 cells was due to reduced levels of locally secreted IL-17A, we evaluated the effects of an IL-17A blocking Ab in wild-type C57BL/6 mice in the same model (31). Anti–IL-17A blocking Ab was delivered i.v. 2 d after treatment with LPS to circumvent concerns of blocking IL-17A during the early phase of injury and affecting neutrophil recruitment. IL-17A blocking Ab protected mice from LPS-induced ARDS to a similar degree to that seen for *Itgb8*$^{fl/fl}$ × CD11c-cre mice (Fig. 2A). To further evaluate the role of IL-17A in ARDS, we examined the effects of LPS in mice deficient in IL-17RC (IL-17RC KO), the component of heterodimeric IL-17 receptors that is most specific for responses to IL-17A and IL-17F (32). IL-17RC KO mice were also protected against LPS-induced ARDS (Fig. 2B). Furthermore, when the LPS dose was increased to a dose that caused significant lethality in control mice, IL-17RC KO mice had a significant improvement in survival (Fig. 2C). Weight loss was similar during the first 3 d after initiation of injury, but IL-17RC KO mice had a significant decrease in severity of weight loss from 4 to 7 d (Fig. 2D).

Disruption of the alveolar epithelial barrier is a hallmark of ARDS. The direct effect of IL-17A on alveolar epithelial barrier function is unknown. We modeled the alveolar epithelial barrier by culturing primary rat type II epithelial cells in an air–liquid interface to form monolayers on semipermeable membrane filters. We examined the in vitro effects of IL-17A on the barrier integrity of the alveolar epithelial cell monolayers. Recombinant rat IL-17A disrupted the alveolar epithelial barrier as determined by decreased transepithelial resistance across confluent monolayers and increased permeability to FITC-dextran (Fig. 2E, 2F).

IL-17A levels are elevated in patients with ARDS, and IL-17A directly disrupts human alveolar epithelial barrier integrity

In human ARDS patients, very little has been reported related to IL-17A. During the H1N1 influenza outbreak, there were reports of elevated IL-17 levels in BAL fluid, but this was not a direct report of patients diagnosed with ARDS (33). To determine the relevance of our findings to humans, we measured IL-17A levels in BAL fluid samples collected 2–5 d after initial diagnosis of ARDS. IL-17A levels increased significantly in patients with ARDS (Fig. 3A). Similar to our findings in experimental ARDS, lymphocytes were readily detected in human BAL fluid samples from patients with ARDS. Human BAL fluid samples from control patients had a predominance of macrophages (Fig. 3B). Next, we sought to model the human alveolar epithelial barrier. Commercially available immortalized human lung epithelial cells derived from lung carcinoma cells do not provide the best model for alveolar epithelial cell barrier studies. Owing to this limitation, we undertook the task of isolating primary human alveolar epithelial cells from cadaveric donor lungs. Primary human alveolar epithelial cells

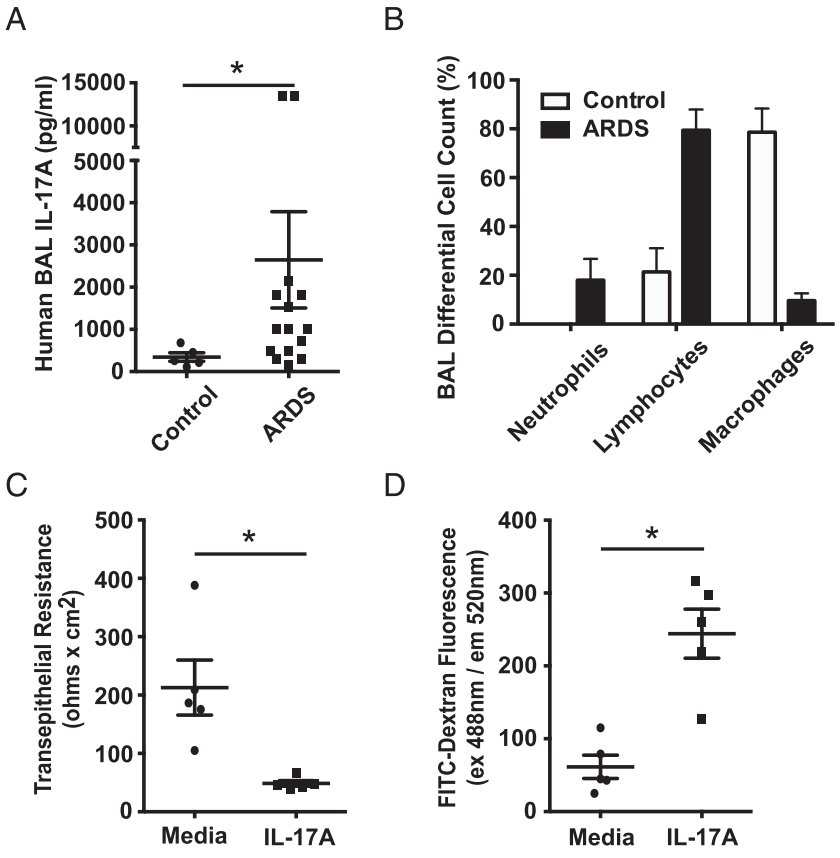

FIGURE 3. IL-17A levels are significantly elevated in patients with ARDS, and IL-17A increases permeability across human alveolar epithelial cell monolayers. (**A**) BAL fluid was collected by bronchoscopy in patients diagnosed with ARDS at 2–5 d after initial diagnosis; $n = 15$. Control patients were healthy volunteers; $n = 5$. *$p < 0.05$ by Student t test. (**B**) Cell differential from BAL fluid collected in the same patients diagnosed with ARDS demonstrated readily detectable lymphocytes (74 ± 8.8), neutrophils (18 ± 8.7), and macrophages (9.6 ± 3.0). Control patients had lymphocytes (21 ± 9.7), no detectable neutrophils, and macrophages (79 ± 10); $n = 5$ patients per group. Data for absolute cell counts were not available. (**C**) Primary isolated human alveolar type II cells from cadaveric lungs were cultured to confluency on semipermeable membrane inserts. Transepithelial resistance was measured after 24 h of incubation in the presence of serum-free media with and without recombinant human IL-17A; $n = 5$ per group. *$p < 0.05$ by Student t test. (**D**) Permeability to FITC-dextran was measured in confluent monolayers of primary cultured human alveolar type II cells after 24 h incubation with recombinant human IL-17A or vehicle; $n = 5$ per group. All data reported as means ± SEM. *$p < 0.05$ by Student t test.

were cultured in an air–liquid interface to form monolayer barriers on semipermeable membranes. Recombinant human IL-17A decreased transepithelial resistance of primary human alveolar epithelial monolayers and increased permeability to FITC-dextran (Fig. 3C, 3D).

Clonal expansion of αβTh17 cells in experimental ARDS

The contribution of αβTh17 cells to LPS-induced ARDS raised the possibility that this could be an Ag-driven process. Because this is a new line of investigation and without a priori knowledge of the pulmonary Ags associated with ARDS, we sought to determine whether there is evidence suggesting that specific Ags could contribute to expansion of pathogenic αβTh17 cells in response to a single dose of endotracheal LPS. We employed high-throughput sequencing to characterize the diversity of αβTh17 cells based on the unique VDJ sequences of each TCR. Quantitative sequencing of the diverse hypervariable region of the β-chain V and J regions of the TCR (CD3RβVJ) allows determination of whether the increase in αβTh17 cells that we found in response to a single dose of endotracheal LPS was due, at least in part, to clonal expansion. We performed TCR sequencing on genomic DNA derived from either αβTh17 cells (identified by knock-in of GFP into the IL-17A locus in $CD4^+$ cells) or non–IL-17A-expressing $CD4^+$ T cells, isolated from lungs and spleens of mice treated with endotracheal LPS or H_2O. An average of 137,940 sequencing reads and 15,636 unique TCR sequences were obtained for each sample. Average V and J gene usage and the distribution of CDR3 lengths were calculated. To characterize the degree of oligoclonality for each sample, we calculated H, the Shannon–Wiener index for each sample based on the frequency of each unique CD3RβVJ sequence (34). The results represent both a diversity metric and a quantification of the degree of clonal expansion within each sample. H is normalized by the log of the number of unique TCR sequences observed by Pielou's evenness index to produce a metric that varies between 1 for an oligoclonal sample to 0 for a fully polyclonal sample (35). Clonality calculated in this manner is independent of the number of T cells sequenced and sampling depth.

Our results show that clonal expansion was significantly greater in αβTh17 ($CD4^+IL\text{-}17^+$) cells isolated from the lungs of mice treated with LPS. We also sought to determine whether clonal expansion was restricted to αβTh17 cells or whether this phenomenon would be observed in $CD4^+$ T cells that do not express IL-17A ($CD4^+IL\text{-}17^-$). Endotracheal LPS slightly increased clonal expansion of $CD4^+IL\text{-}17^-$ T cells isolated from the lungs, but the increase was significantly less than that seen for αβTh17 cells. There was no detectable clonal expansion in both αβTh17 and $CD4^+IL\text{-}17^-$ T cells isolated from the lungs of mice treated with endotracheal H_2O (Fig. 4A).

In the same mice treated with endotracheal LPS or H_2O, we measured the CD3RβVJ sequences of αβTh17 and $CD4^+IL\text{-}17^-$ T cells isolated from the spleens. There were no significant differences in clonal expansion of either αβTh17 or $CD4^+IL\text{-}17^-$ T cells isolated from the spleens (Fig. 4B).

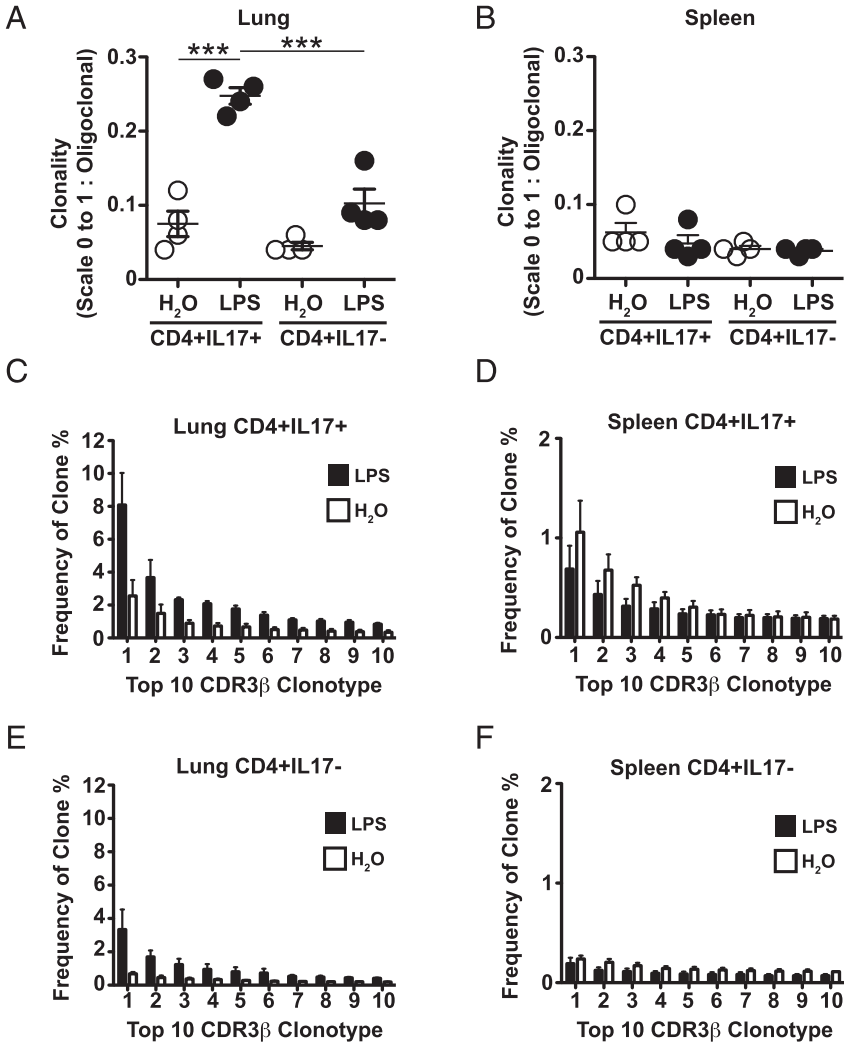

FIGURE 4. αβTh17 cells are clonally expanded in experimental ARDS. (**A**) Deep sequencing of the hypervariable CDR3βVJ region of αβTh17 cells ($CD4^+IL\text{-}17^+$) and non–IL-17-producing $CD4^+$ T cells ($CD4^+IL\text{-}17^-$) isolated from lungs 4 d after endotracheal LPS (100 μg/mouse) or H_2O. To characterize the polyclonal versus oligoclonal nature of the samples, we calculated the inverse of the normalized Shannon–Wiener index for each sample. This clonality metric varies from 0 to 1, with 1 being a completely monoclonal population. $^{***}p < 0.001$ by one-way ANOVA and Tukey–Kramer test. (**B**) Clonality of $CD4^+IL\text{-}17^+$ and $CD4^+IL\text{-}17^-$ T cells isolated from the spleens 4 d after endotracheal LPS or H_2O; $n = 4$ per group; $p > 0.05$ by one-way ANOVA and Tukey–Kramer test. (**C**) To further characterize the difference in clonal expansions between the two treatment groups, the frequency of the top 10 individual clones was summed and compared to determine whether the difference in clonality between groups was due largely to the expansion of high-frequency clones. The 10 highest frequency $CD4^+IL\text{-}17^+$ T cell clones isolated from the lungs of mice 4 d after endotracheal LPS averaged 23% of the total repertoire compared with an average of 7.9% in the H_2O treatment group; $n = 4$ per group. $^{**}p < 0.01$ by two-tailed Wilcoxon sum rank test. There were no significant differences in the 10 highest frequency clones isolated from (**D**) splenic $CD4^+IL\text{-}17^+$ T cells after treatment with LPS (average, 1.0%) versus H_2O (average, 1.3%); (**E**) lung $CD4^+IL\text{-}17^-$ T cells after treatment with LPS (average, 11%) versus H_2O (average, 3.1%); (**F**) splenic $CD4^+IL\text{-}17^-$ T cells after treatment with LPS (average, 2.9%) versus H_2O (average, 3.9%); $n = 4$ per group; $p > 0.05$ by two-tailed Wilcoxon sum rank test. All data reported as means ± SEM.

To determine the differences in αβTh17 cells in ARDS, we examined a subset of the T cell repertoire from individual samples. A comparison of the fraction of each sample comprised of the 10 most frequent clones isolated from lungs of mice treated with either LPS or H_2O revealed a nearly 3-fold higher frequency in αβTh17 cells isolated from mice treated with endotracheal LPS (Fig. 4C). The fraction of the top 10 most frequent clones from $CD4^+IL\text{-}17^-$ T cells showed a nonsignificant increase in mice treated with LPS compared with those treated with H_2O (Fig. 4E). Furthermore, the fraction of each sample comprising the top 10 most frequent clones was notably lower in both αβTh17 and $CD4^+IL\text{-}17^-$ T cells obtained from the spleens and was not different between mice treated with LPS or H_2O (Fig. 4D, 4F).

The αβTh17 cells isolated from the lungs of mice treated with LPS or H_2O showed a predominantly memory T cell phenotype of $CD44^{high}CD62L^{low}$ (Fig. 5A). To assess the likelihood that a common Ag or restricted number of Ags might drive the convergence of the immune repertoire of individual mice after experimental ARDS, the degree of sample similarity among different mice was measured by calculating the fraction of pairwise sequences shared between all pairwise combinations of samples. αβTh17 cells isolated from the lungs of mice treated with LPS showed significantly more sharing than did αβTh17 cells isolated from mice treated with H_2O. Furthermore, pairwise sharing was lower and not significantly different in $CD4^+IL\text{-}17^-$ T cells isolated from the lungs of mice treated with LPS or H_2O (Fig. 5B).

We also evaluated CD3RβVJ sequences shared by all mice in a given treatment group and found 13 such sequences in αβTh17 cells isolated from the lungs of LPS-treated mice but only a single and unique shared sequence in αβTh17 cells from the lungs of H_2O-treated mice (Fig. 5C). There were no common sequences in αβTh17 cells isolated from the spleens of mice treated with either LPS or H_2O. There was only one common sequence in the $CD4^+$ $IL\text{-}17^-$ T cells isolated from the lungs of mice treated with LPS and one common sequence from mice treated with H_2O (Fig. 5D). These results suggest the possibility of clonal expansion of αβTh17 cells in response to common endogenous or exogenous pulmonary Ags.

Discussion

In this study, we found an important contribution of αβTh17 cells to an intermediate phase of ARDS that begins a few days after initial injury. Mice lacking αβTh17 cells, IL-17RC KO mice, and mice treated with Ab-blocking IL-17A were all similarly protected from experimental lung injury. IL-17A was readily detectible in the BAL fluid of LPS-treated mice. These experimental findings appear to translate into patients with ARDS where IL-17A was elevated in BAL fluid collected 2–5 d after initial diagnosis of ARDS. We also found that IL-17A directly increased the permeability of human alveolar epithelial cells, suggesting a possible mechanism for the effects of IL-17 in ARDS. This finding extends the role of IL-17A beyond tissue injury caused by neutrophil-related pathways. Because αβTh17 cells are known to expand and release IL-17A and other cytokines in response to specific Ags, we sought evidence of clonal expansion. We found a marked increase in oligoclonality and an expansion of shared CD3RβVJ

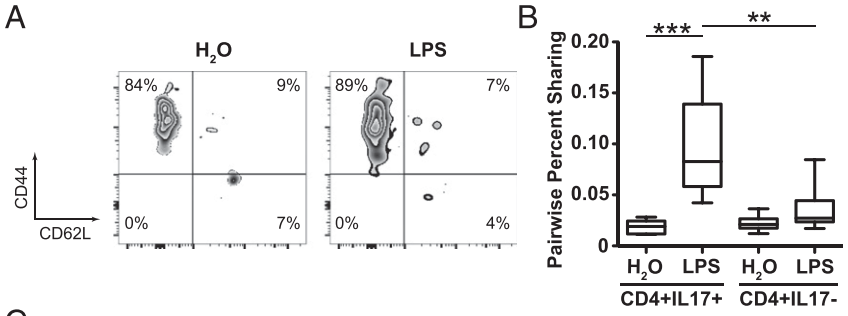

FIGURE 5. Expansion of common sequences of the hypervariable CDR3βVJ regions from predominantly memory αβTh17 cells isolated from the lung in experimental ARDS. (**A**) Flow cytometry analysis of $CD4^+IL\text{-}17^+$ T cells isolated from the lung stained for memory T cell markers CD44 and CD62L (memory T cells defined by $CD44^{high}$ $CD62L^{low}$). Representative flow cytometry plots are from mice treated with LPS or H_2O; $n = 3$ per group. (**B**) Percentage pairwise sharing of all CDR3βVJ sequences between different mice in each treatment group was calculated as the number of shared sequences in each sample divided by the total number of sequences for both samples. The TCR sequences among αβTh17 cells ($CD4^+IL\text{-}17^+$) were significantly more shared among lung samples treated with LPS. The mean fraction of shared sequences was 0.097 for $CD4^+IL\text{-}17^+$ T cells from mice treated with LPS compared with 0.018 for $CD4^+IL\text{-}17^+$ T cells from mice treated with H_2O, 0.035 for $CD4^+IL\text{-}17^-$ T cells from mice treated with LPS, or 0.022 from mice treated with H_2O. Data reported as means ± SEM; $n = 4$ mice per group with six unique pairwise comparisons between mice of each group. $^{**}p < 0.01$, $^{***}p < 0.001$ by Kruskal–Wallis rank sum test. (**C**) The list of common amino acid sequences from the CDR3βVJ region of $CD4^+IL\text{-}17^+$ T cells isolated from the lungs of mice treated with LPS or H_2O; $n = 4$ per group. (**D**) List of the common amino acid sequences from the CDR3βVJ region of $CD4^+IL\text{-}17^-$ T cells isolated from the lungs of mice treated with LPS or H_2O; $n = 4$ per group.

C CD4+ IL17+

Common Sequences from Lungs Treated with LPS :

Amino Acid Sequence	Total	LPS#1	LPS#2	LPS#3	LPS#4
CASSPPGQSNERLFF	446	152	75	87	132
CASSQDNSGNTLYF	383	21	2	91	269
CTCSATGYEQYF	334	55	92	7	180
CAWSPGTGGDAEQFF	274	47	24	62	141
CASSQDGVNQDTQYF	199	11	21	67	100
CASSQSSYEQYF	194	11	143	29	11
CASSDWGGNYAEQFF	95	60	7	17	11
CASGDEQYF	91	26	38	19	8
CTCSGQSQNTLYF	69	20	5	25	19
CASSSQGSGNTLYF	66	18	31	11	6
CASSLDWGGYEQYF	55	15	10	9	21
CASSTGTGGYEQYF	39	10	7	17	5
CGARGQNTGQLYF	37	22	4	9	2

Common Sequences from Lungs Treated with H_2O :

Amino Acid Sequence	Total	H_2O#1	H_2O#2	H_2O#3	H_2O#4
CASSLDRGSYEQYF	323	14	100	190	19

D CD4+ IL17-

Common Sequences from Lungs Treated with LPS :

Amino Acid Sequence	Total	LPS#1	LPS#2	LPS#3	LPS#4
CAWSPGTGGDAEQFF	191	38	63	5	85

Common Sequences from Lungs Treated with H_2O :

Amino Acid Sequence	Total	H_2O#1	H_2O#2	H_2O#3	H_2O#4
CASSLDRGSYEQYF	332	77	110	41	104

sequences specifically in αβTh17 cells in the lungs (but not the spleens) of LPS-treated mice. Taken together, these results reveal an unexpected contribution of an adaptive immune response involving αβTh17 cells and IL-17A that develops within a few days in response to a single treatment with an activator of innate immunity (LPS).

It is well recognized that αβTh17 cells are important in host response to extracellular pathogens and play a key role in maintaining the integrity of the intestinal mucosa (30, 36). Our data suggest that αβTh17 cells in the alveoli contribute to barrier disruption, perhaps as a consequence of direct effects of IL-17A on alveolar epithelial cells. However, our data do not provide direct proof that the in vivo protection we observed was due to prevention of direct effects on the epithelium. This question would be best addressed through performing lung injury studies in mice specifically unable to respond to IL-17A in alveolar epithelial cells, but unfortunately no such mice have yet been described. In future work, it will also be important to identify the molecular mechanisms by which IL-17A increases alveolar epithelial permeability.

Furthermore, our findings that in lung digests there were large numbers of γδ T cells but that IL-17–expressing cells in the BAL fluid were all αβTh17 cells suggest that there could be spatial differences in the location of IL-17A–secreting cells in the lung. The paucity of γδ T cells in the BAL fluid is consistent with the possibility that γδ T cells are immobile around the conducting airways of the lung where they play a key role in innate host defense functions as previously described (37, 38). An increase in αβTh17 cells in the BAL fluid suggests that αβTh17 cells are recruited to the alveolar space in response to LPS-induced lung injury and are the likely source of tissue-injurious IL-17A. The predominance of memory αβTh17 cells in the lung raises the question of the origin of the αβTh17 cells in lung injury. Our data do not directly prove whether expansion of αβTh17 cells in response to LPS is a result of differentiation of naive T cells or expansion of pre-existing memory αβTh17 cells. However, the short time frame of our study (4 d after initiation of injury) seems more consistent with expansion of pre-existing memory αβTh17 cells. Vital imaging αβTh17 and γδ T cells in experimental lung injury could help us better understand their trafficking patterns in ARDS.

Our study used *Itgb8*$^{fl/fl}$ × CD11c-cre mice to examine the role of αβTh17 cells. We used this strategy instead of CD4-depleted mice because both beneficial and harmful effects of CD4$^+$ T cells have been described in ARDS. For example, CD4$^+$ T regulatory cells have been shown to be important in the resolution phase of ARDS and in mitigating injury to the alveolar–capillary barrier induced by LPS (6). Nakajima et al. (5) found that CD4$^+$ cells could be injurious in experimental ARDS, and blockade of CTLA-4 increased IL-17A levels, but this study did not directly test αβTh17 cells. Studying Rag$^{-/-}$ mice could have limitations where various CD4$^+$ T cell subsets may have different effects of protection and injury that nullify each other. Our *Itgb8*$^{fl/fl}$ × CD11c-cre mice are the only line of genetically manipulated mice we are aware of that specifically lacks Ag-dependent αβTh17 cells while all other sources of IL-17 remain intact.

Our findings of oligoclonality of sequences in the hypervariable CD3RβVJ region and the presence of a number of sequences shared in αβTh17 cells from all of the mice treated with a single dose of LPS strongly suggest that this clonal expansion is Ag driven. However, we cannot rule out the possibility that LPS could drive APCs to secrete inflammatory cytokines that stimulate αβTh17 cell proliferation (39). LPS could also serve as an adjuvant to stimulate αβTh17 cell response to peptides, such as previously described with myelin oligodendrocyte glycoprotein in experimental autoimmune encephalitis (40). MHC class II–dependent and –independent pathways have been previously explored in a mouse *Klebsiella pneumoniae* pneumonia model (41). In an MHC class II–dependent pathway, outer membrane proteins of *K. pneumoniae* were capable of stimulating memory αβTh17 cells (41). In the same study, a smaller percentage of αβTh17 cells was capable of being stimulated by MHC class II–deficient cells that implied an Ag-independent pathway driven by IL-1β could also exist (41). Our finding that the increase in αβTh17 cells in response to LPS was associated with a marked reduction in TCR sequence diversity leads us to favor the possibility that specific Ags, as yet unidentified, drive this expansion process.

This is one of the first studies, to our knowledge, that suggests pulmonary Ags could drive an injurious adaptive immune response in the devastating lung disorder of ARDS. However, this conclusion must remain speculative until we or others identify specific exogenous or endogenous Ags that drive this response. We hope our study will spawn future research to identify one or more relevant Ags that has the potential to shed new light on the link between innate and adaptive immunity in driving the pathogenic contributions of αβTh17 cells to ARDS, and it could also be relevant to other IL-17–dependent pathologies.

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Disclosures

The authors have no financial conflicts of interest.

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