Ann Allergy Asthma Immunol xx (2012) xxx

Contents lists available at SciVerse ScienceDirect

$\alpha\beta$ T cells and a mixed Th1/Th17 response are important in organic dust-induced airway disease

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ARTICLE INFO

Article history:
Received for publication April 5, 2012.
Received in revised form June 20, 2012.
Accepted for publication June 24, 2012.

ABSTRACT

Background: Organic dust exposure in agricultural environments induces an inflammatory response that attenuates over time, yet repetitive dust exposures result in chronic lung diseases. Animal models resembling this chronic lung inflammatory response have been developed, yet the underlying cellular mechanisms are not well defined.
Objective: Because mice repetitively exposed to organic dust extracts (DE) display increased CD3+ T cell lung infiltrates, we sought to determine the phenotype and importance of these cells.
Methods: Mice received swine confinement DE repetitively for 3 weeks by established intranasal inhalation protocol. Studies were conducted with peptidoglycan (PGN) because it is a major DE component in large animal farming environments and has shared similar biologic effects with DE. Enumeration of T cells and intracellular cytokine profiles were conducted by flow cytometry techniques. Whole lung homogenate cytokines were analyzed by multiplex immunoassay. T cell receptor (TCR) $\alpha\beta$ knockouts were used to determine the functional importance of $\alpha\beta$-expressing T cells.
Results: DE increased lung-associated CD3+CD4+ T cells and interleukin (IL)-17 (but not IL-4, interferon [IFN]-γ, IL-10) producing CD4+ T cells. PGN treatment resulted in increased IL-17 and IFN-γ producing CD4+ T cells and IFN-γ producing CD8+ T cells. Both DE and PGN augmented expression of cytokines associated with Th1 and Th17 polarization in lung homogenates. DE-induced lung mononuclear aggregates and bronchiolar compartment inflammation were significantly reduced in TCR knockout animals; however, neutrophil influx and alveolar compartment inflammation were not affected.
Conclusion: Studies demonstrated that DE and PGN exposure promote a Th1/Th17 lung microenvironment and that $\alpha\beta$-expressing T cells are important in mediating DE-induced lung pathologic conditions.
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Introduction

Agricultural workers, particularly swine confinement operators, are routinely exposed to organic dust environments and display a high prevalence of lung disease, including chronic bronchitis, exacerbation of asthma, and obstructive lung disease.[1] Exposure of naïve individuals to organic dust results in an intense systemic and pulmonary inflammatory response that attenuates over time, suggestive of adaptation.[2,3] However, despite adaptation, exposed workers are at an increased risk of lung function decline, persistent airway inflammation, and progressive respiratory impairment.[1,3,4] Although several studies have defined the acute dust-induced inflammatory response, less is known about the underlying cellular and immunologic mechanisms resulting in the chronic inflammatory adaptation-like response to repetitive organic dust exposures.

T cells are potential candidates in mediating the chronic inflammatory response. Lymphocytes are elevated in bronchoalveolar lavage fluid (BALF) sampling of animal farm operators as compared with healthy volunteers.[5,6] Moreover, in repetitive swine confinement organic dust extract (DE) animal exposure models, there is an influx of lymphocytes (primarily $CD3^+$) within the lung.[7] However, the phenotype and importance of the recruited T cells in humans

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Disclosures: Authors have nothing to disclose.
Funding Sources: Supported by grants from the National Institute of Environmental Health Sciences (R01: ES019325; K08: ES015522-01 and [ARRA] ES015522-03S1 to JAP), National Institute of Occupational Safety Health (1R01OH008539-01 to DJR and 5U50 OH008085 to SJR), and the National Institute of Neurological Disorders and Stroke (3R01NS040730 to TK).

1081-1206/12/$36.00 - see front matter © 2012 American College of Allergy, Asthma & Immunology. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.anai.2012.06.015

and animals after repetitive organic dust exposures has not been defined. Numerous studies demonstrate that farm exposure is protective of childhood allergy because, in part, farming exposures have been associated with lower T-helper type 2 (Th2) cytokine profiles as determined by various sampling strategies.[8–10] Moreover, circulating Th1 and Treg cells have been found in studies focused on farm mothers or farm children.[11] However, pig farmers demonstrate increased circulating interleukin (IL)-13– and IL-4–producing Th2 cells when compared with healthy volunteers.[12] Interestingly, in the lung, Th17 skewing after acute organic dust exposure has been proposed because soluble IL-17 and IL-17A–expressing lymphocytes were increased in BALF from healthy volunteers exposed once to a swine confinement facility.[6] This latter observation may be important because lung neutrophil accumulation is a hallmark of organic dust-induced lung disease in humans and animals,[1] and IL-17 promotes neutrophil recruitment.[13,14]

To understand the role of T cells in the lung in the context of repeated organic dust exposures, mice were treated daily with DE for 3 weeks per an established animal model,[7] and lung-associated T cell populations and cytokine expression profiles were determined. We also evaluated responses to repetitive treatment with peptidoglycan (PGN), because recent studies demonstrate that PGN is a major component of large animal farming organic dusts.[15,16] Moreover, prior evidence suggests that PGN shares similar biologic and physiologic responses as observed with DE.[17,18] Finally, we assessed the functional role of T cells by usin T cell receptor (TCR) $\alpha\beta$ knock-out (KO) mice. Our studies demonstrate that both DE and PGN exposure elicited both a Th1 and Th17-polarized lung response, and that DE-induced cellular aggregates and bronchiolar inflammation were reduced in TCR $\alpha\beta$ KO mice, but neutrophil influx and alveolar inflammation were not altered. Collectively, these studies demonstrate that repetitive exposure to organic dust and PGN results in a $\alpha\beta$ T cell– dependent and –independent lung inflammatory response in a mixed Th1 and Th17 environment.

Methods

Dust extract

Dust extract (DE) was prepared as previously described[7,15] from settled surface dust samples collected 3 feet above floor level from swine confinement animal feeding operation facilities (~750 animals). Before extraction and filter sterilization, the dust was analyzed by gas chromatography-tandem mass spectrometry for muramic acid (PGN component), 3-hydroxy fatty acid (endotoxin component), and ergosterol (fungi component) according to previously published methods.[15] Results revealed high muramic acid (424.0 pmol/mg ± 17.7 pmol/mg) and 3-hydroxy fatty acid (3,109.8 ng/mg ± 152.6 ng/mg), but low ergosterol (9.3 pmol/mg ± 0.4 pmol/mg), which is consistent with previous dust sampling findings.[15,18] Dust was placed into solution and centrifuged, and the supernatant was filter-sterilized (0.22 μm; a process that also removes coarse particles). The aqueous DE was diluted to a final concentration of 12.5% (vol/vol) in sterile saline, which contained 2.91 to 3.88 mg/mL of total protein as measured by nanodrop spectrophotometry (NanoDrop Technologies, Wilmington, Delaware). DE (12.5%) has been previously shown to elicit optimal lung inflammation and is well tolerated.[7] The endotoxin levels in the 12.5% DE ranged from 22.1 to 91.1 EU/mL as assayed using the limulus amebocyte lysate assay according to the manufacturer's instruction (Sigma, St. Louis, Missouri). Peptidoglycan levels were not quantitated because commercially available measurement assays do not exist, to the best of our knowledge.

Animal model

C57BL/6 wild-type (WT) and TCR $\alpha\beta$ KO mice (mice homozygous for the *Tcrb*^{tm1Mom} targeted mutation; stock number 002118) on a C57BL/6 background (6–8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, Maine). TCR $\alpha\beta$ KO mice were selected to study the potential functional role of T cells, because most (>90%) blood T cells are $\alpha\beta$+.[19] Using an established intranasal inhalation murine model of ODE- and PGN-induced lung inflammation, mice were treated daily with 12.5% DE, PGN (10 or 100 μg), or sterile phosphate-buffered saline (PBS; diluent) for 3 weeks.[7,17] Mice were killed 24 h after the last treatment. *Staphylococcus aureus* PGN was purchased from Sigma (St. Louis, MO). PGN (100 μg), which is approximately half the protein concentration in 12.5% DE, is known to elicit airway inflammatory responses similar to DE and is well tolerated.[17] In this current study, 2 concentrations of PGN were investigated to determine a dose-dependent response. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center according to National Institutes of Health guidelines for the use of rodents.

Lung-associated cell collection

Cells were isolated from whole lungs as previously described.[20] Briefly, after opening the chest cavity, the right ventricle was infused with 10 mL of sterile PBS with heparin to remove blood from the pulmonary vasculature. Whole lung tissues were harvested and subjected to an automated dissociation procedure using a gentleMACS Dissociator instrument according to the manufacturer's instructions (Miltenyi Biotech, Auburn, California) in a solution containing collagenase type I (324 U/mL; Fisher, Pittsburgh, Pennsylvania), bovine DNase (75 U/mL), porcine heparin (25 U/mL), and PBS with Ca^{2+} and Mg^{2+}. To remove any large fragments, the cell solution was passed through nylon mesh (40 μM; Fisher), and cell pellets were briefly resuspended in cold red blood cell (RBC) lysing solution, centrifuged. Cell counts were obtained by hematocytometer and recorded. Viability was assessed and assured by trypan blue exclusion.

Cell surface phenotype and intracellular cytokine staining by flow cytometry

Dissociated lung-associated cells from each animal were incubated with anti-CD16/32 (Fc Block, BD Biosciences) for 10 minutes to minimize nonspecific antibody binding, and then stained with monoclonal antibodies (mAbs) directed against CD3, CD4, CD8, and appropriate isotype controls (BD Biosciences, San Jose, California) for 30 minutes at 4°C. Compensation was performed with antibody capture beads (BD Biosciences) stained separately with individual mAbs used in the test samples. Lymphocyte populations were gated by characteristic forward and side scatter properties and antibody-specific staining fluorescence intensity using a FACSAria (BD Biosciences) with gating strategy depicted in Figure 1A. Lymphocyte numbers were calculated by multiplying the percentage of each cell type measured by flow cytometry by the original hemocytometer count of total cells recovered from each animal.

Cytokine expression profiles of T cells that infiltrated the lungs were evaluated by intracellular cytokine staining and fluorescence-activated cell sorting (FACS) analysis. Single-cell suspensions of whole lung cells were underlaid with lymphocyte separation solution (Fisher Scientific, Pittsburgh, Pennsylvania) and centrifuged at 400*g* for 20 minutes. Collected mononuclear cells were incubated in PBS containing 0.1% bovine serum albumin with anti-CD16/32 (Fc Block). Next, cells were stained with mAbs directed against CD4 and CD8 and isolated by fluorescence-activated cell sorting using a FACSAria (BD Biosciences). Post-sort, flow cytometry confirmed greater than 95% purity for each T cell population. Subsequently, T cells were cultured at 5×10^4 cells/mL in complete L-glutamine-RPMI 1640 (Life Technologies, Grand Island, New York) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, California), 2-mercaptoethanol (5×10^{-5} M), and 50 μg/mL of penicillin/streptomycin (Invitrogen) in the presence of the protein transport inhibitor, GolgiPlug (BD Biosciences), and

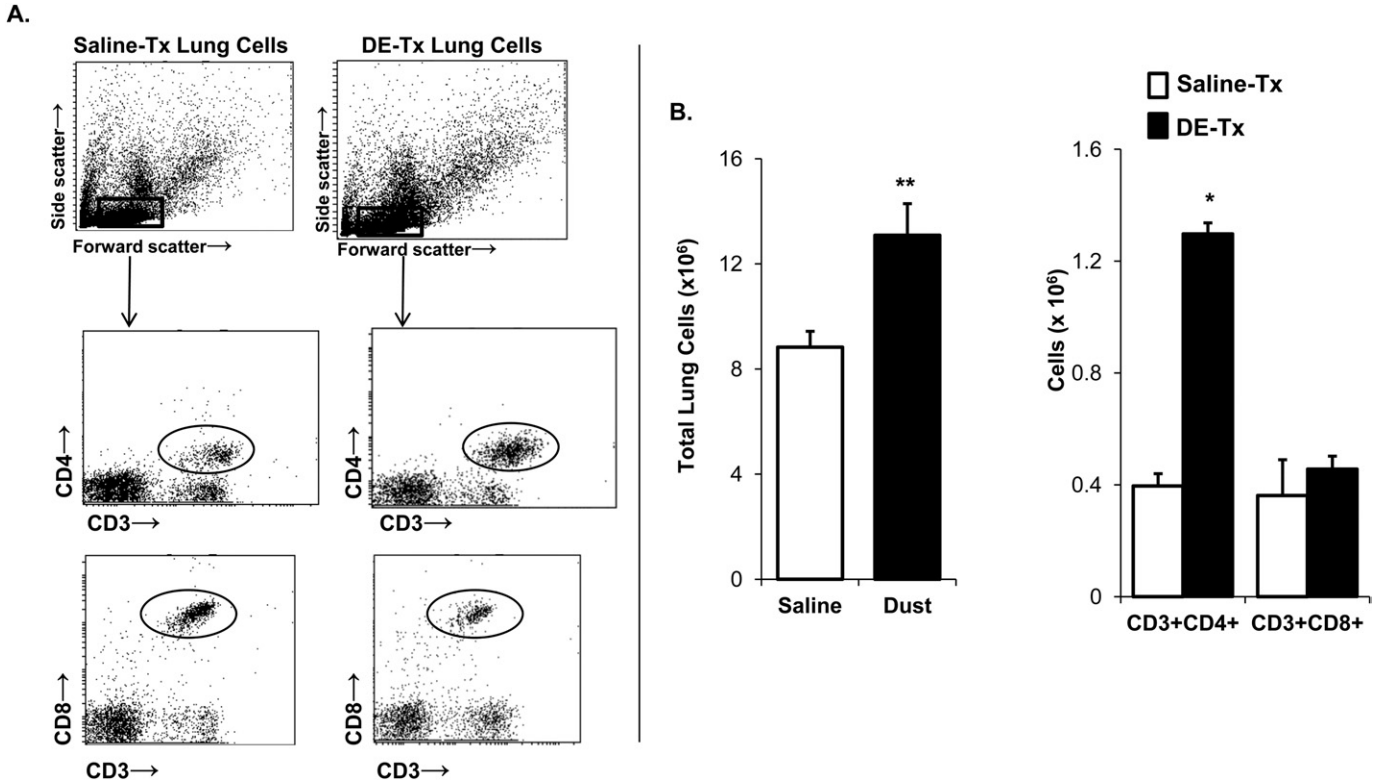

Fig. 1. Lung CD4$^+$ T cells are increased after repetitive DE exposure. C57BL/6 mice were repetitively treated with organic DE or saline for 3 weeks, whereupon cells were isolated from whole lungs. Lung-associated cells were stained for CD3, CD4, and CD8, and T cell populations were selected by characteristic forward and side scatter properties and specific antibody staining fluorescence intensity. Absolute CD4$^+$ T cells, but not CD8$^+$ T cells, were increased after repetitive DE exposure. **A,** A representative dot blot of saline-treated (tx) and dust extract (DE)-treated lung-associated cells is shown. **B,** Results represent mean ± SEM (n = 6 mice/group) of total lung-associated cells, CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ T cells (percentage of cell type × total lung cell count), with statistical difference denoted by asterisks (**$P < .01$).

stimulated with a mixture of 50 ng/mL phorbol myristate acetate (Sigma-Aldrich) and 1 μg/mL ionomycin (Sigma-Aldrich) at 37°C for 4 h. After activation, cells were washed and fixed in 4% paraformaldehyde for 15 minutes and stored overnight in staining buffer. Fixed cells were permeabilized for 15 minutes on ice in CytoPerm (BD Biosciences) and stained with mAbs directed against interleukin (IL)-17A, interferon (IFN)-γ, IL-4, IL-10, Foxp3, and appropriate isotype controls (all from BD Biosciences) for 30 minutes at 4°C. Fluorescence intensity was analyzed using a BD FACSAria, and data files were analyzed using DIVA software.

Cytokine assays

Cytokine profiles were also characterized in total lung homogenates. Briefly, total lung homogenates were prepared by homogenizing whole-lung samples in 500 μL of sterile PBS, and 100 μL of cell-free homogenate was analyzed by a custom SearchLight protein multiplex immunoassay array at Aushon Biosystems, Inc (Billerica, Massachusetts) for IL-1β, IL-6, tumor necrosis factor alpha, IL-17A, IL-23, IFN-γ, IL-12p40, IL-4. IL-22 and IL-2 levels were determined according to manufacturer's instruction using a quantikine enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, Minnesota).

BALF

BALF was collected as previously described.[7] Briefly, the total cell number recovered from pooled lavages (3 × 1 mL lavages) was enumerated and differential cell counts determined using cytospin-prepared slides (Cytopro Cytocentrifuge, Wescor Inc, Logan, Utah) stained with DiffQuick (Dade Behring, Newark, Delaware). Cell counts determined the differential ratio of cell types in 400 cells per mouse.

Lung histology and semiquantitative evaluation of inflammation

For histology studies, whole lungs were excised after lavage and inflated to 10 cm H_2O pressure with 10% formalin (Sigma) to preserve pulmonary architecture. Next, lungs were processed, embedded in paraffin, and sections (4–5 μm) were cut and stained with hematoxylin and eosin. Slides were microscopically reviewed and semiquantitatively assessed by a pathologist who was blinded to the treatment conditions using a previously published scoring system[7] for the distribution of inflammatory changes for each parameter that included alveolar compartment inflammation, bronchiolar compartment inflammation, and intrapulmonary cellular aggregates. Each parameter was independently assigned a value from 0 to 3, with the greater the score, the greater the inflammatory changes in the lung.

Statistical methods

Data are presented as the mean ± standard error of mean (SEM). Statistics were performed using a two-tailed, nonpaired or paired t-test where appropriate to determine significant changes between 2 treatment groups. One-way analysis of variance with Tukey multi-comparison post-test was employed to compare differences among 3 or more treatment groups using GraphPad (version 5.01) software. In all analyses, P values less than .05 were considered statistically significant.

Results

DE exposure elicits elevated CD4$^+$ infiltrates that are predominately Th17-polarized

The total number of lung-associated cells was increased after repetitive DE exposure as compared with saline control concomi-

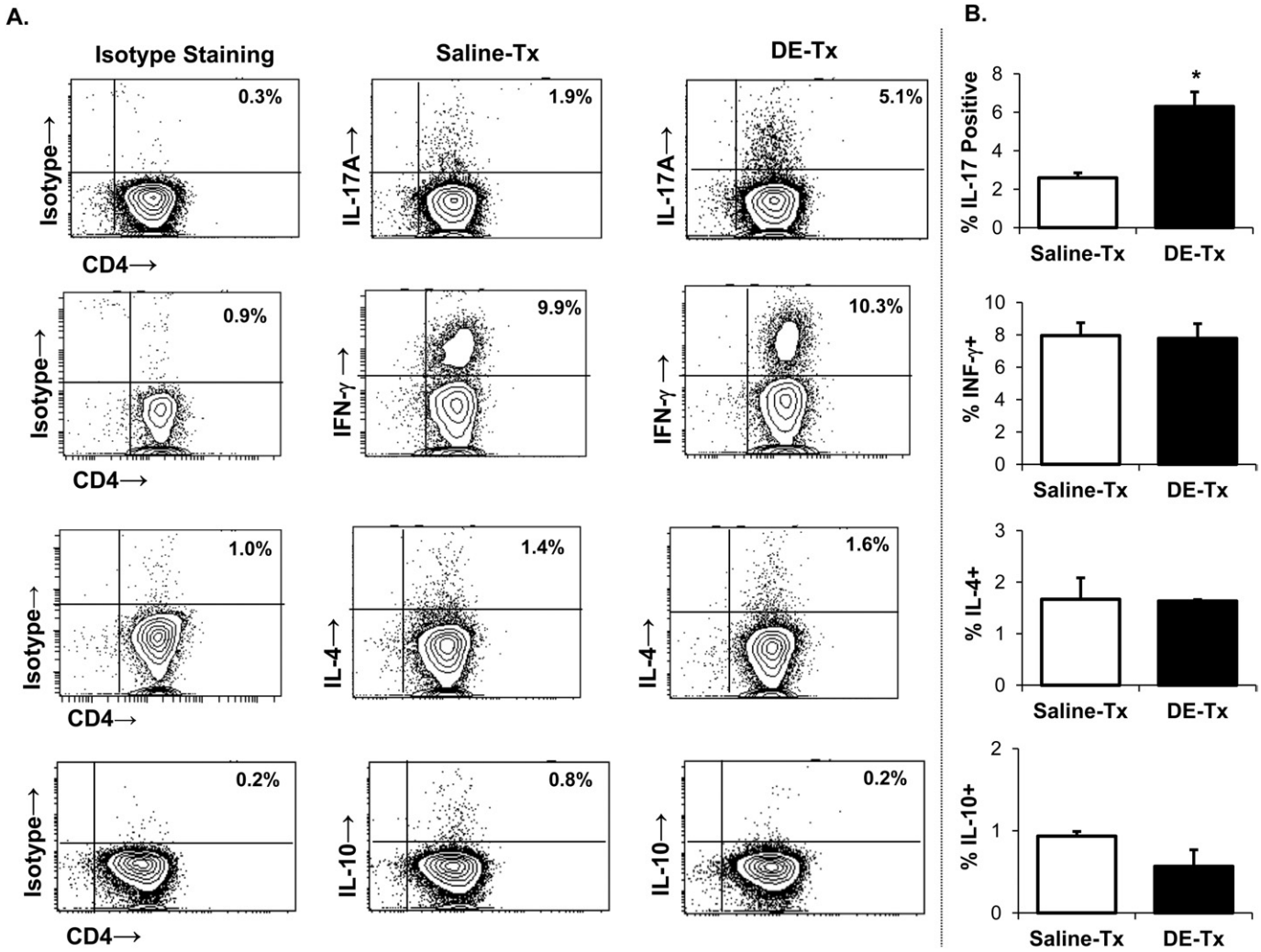

Fig. 2. IL-17–producing lung CD4$^+$ T cells are increased after repetitive DE exposure. C57BL/6 mice were repetitively exposed to DE and saline for 3 weeks, whereupon CD4$^+$ T cells pooled from 3 animals and isolated by FACS were immediately stimulated *ex vivo* with phorbol myristate acetate + ionomycin for 4 hours, and stained for IL-17 (Th17), IFN-γ (Th1), IL-4 (Th2), and IL-10 (Treg) to demonstrate cytokine profiles. **A**, A representative contour plot depicting cytokine staining of 1 of 4 independent experiments is shown. Left column depicts background or isotype staining for each respective cytokine. Middle and right columns depict saline- and DE-treated mice, respectively. **B**, Results represent mean ± SEM of the percentage positive cytokine staining of CD4$^+$ T cells, with statistical differences denoted by asterisks (*$P < .05$).

tant with significant elevations in the absolute number of lung CD4$^+$ T cells, but not CD8$^+$ T cells (Fig 1B).

Next, CD4$^+$ and CD8$^+$ T cells were isolated by FACS and stimulated with phorbol myristate acetate and ionomycin to determine cytokine expression profile. We found across 4 independent experiments a consistent 2- to 3-fold increase in the number of IL-17–producing CD4$^+$ cells with no major changes in IFN-γ–, IL-10–, or IL-4–producing CD4$^+$ T cells after DE exposure as compared with saline (Fig 2A and 2B). There were also no significant differences in Foxp3$^+$ Treg infiltrates after DE or saline treatment (eFig 1). Likewise, no significant differences were found in the frequency of IFN-γ– or IL-17–producing CD8$^+$ T cells in the lungs of DE- and saline-treated mice (eFig 2).

DE exposure induces a cytokine milieu that favors Th1/17-polarization in whole lung

In the lung homogenates from DE-treated mice there was evidence of a cytokine milieu that would promote both Th17 and Th1 responses. First, there was significant increase in the cytokines that impact Th17 development and function. Namely, IL-6, IL-1β, IL-17, and IL-22 levels were significantly increased in DE-treated mice (Fig 3). However, there were also significant increases in IFN-γ, tumor necrosis factor alpha, and IL-12p40, consistent with a Th1 phenotype. There was no difference in IL-23 levels in saline- and DE-treated lungs. IL-4, a prototypical Th2 cytokine, was at the lower limit of detection and not significantly different between groups (data not shown).

Repetitive intranasal challenge with PGN induces a Th1/Th17 polarized lung cell phenotype

Because recent studies suggest that bacterial PGN in industrial animal farming environments are highly prevalent and may be responsible for mediating DE-induced airway disease,[15,21] we investigated the effects of repetitive PGN challenges in separate experiments to determine whether PGN shared similar effects with DE on T cell development and accumulation. Similar to what was observed with DE-treated mice, PGN exposure resulted in a dose-dependent increase in total lung cell infiltrates and CD4$^+$ T cells as compared with saline (Fig 4A). Interestingly, the highest dose of PGN tested caused an increase in CD8$^+$ T cells infiltrating the lung (Fig 4A).

There was a consistent and robust increase in IL-17–producing CD4$^+$ T cells (ie, 6-fold to 9-fold across 3 independent experiments) after repetitive PGN exposure (Fig 4B). In addition, PGN challenge augmented the percentages of IFN-γ–producing CD4$^+$ T cells, but not IL-4 or IL-10 (Fig 4B and data not shown). Moreover, PGN

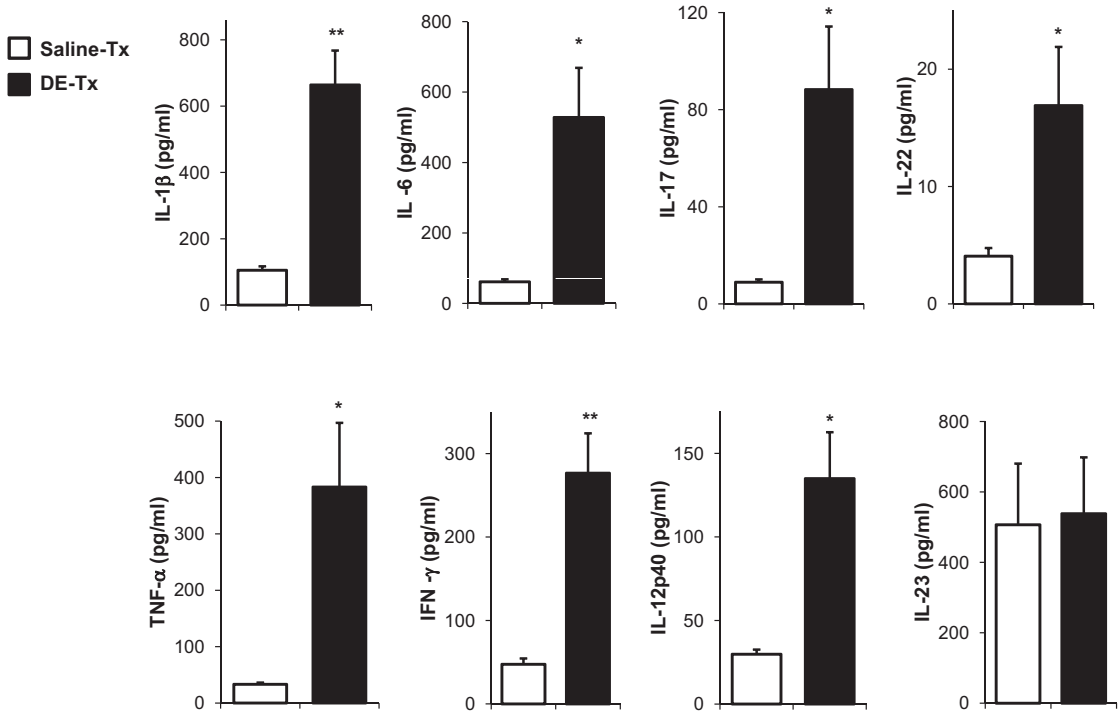

Fig. 3. DE exposure induces a Th1/17-polarized cytokine response in the whole lung. C57BL/6 mice were repetitively exposed to DE or saline for 3 weeks, and whole lung samples were homogenized and cytokine profiles associated with T cell subsets were analyzed from cell-free supernatants of total lung homogenates by protein multiplex immunoassay. Results represent the mean ± SEM of 6 mice from 2 independent experiments, with statistical differences denoted by asterisks (*$P < .05$, **$P < .01$).

treatment resulted in a consistent increase in IFN-γ–, but not IL-17–producing lung CD8$^+$ T cells as compared with saline control (Fig 4C). The cytokine profile of lung homogenates after repetitive PGN exposure resembled what was observed after DE and confirmed the establishment of a mixed Th1 and Th17-polarized immune response (eFig 3).

αβ-expressing T cells are essential for the formation of DE-induced peribronchiolar mononuclear aggregates, but not neutrophil infiltrates

To determine the functional importance of T cells in mediating repetitive DE-induced lung inflammation, αβ-expressing T cells were targeted through utilization of TCR αβ KO mice. There was an absence of DE-induced mononuclear aggregates and a significant reduction in peribronchiolar inflammation in the TCR αβ KO animals as compared with WT mice (Fig 5A and 5B). The number of neutrophils recovered from BALF did not differ between TCR αβ KO and WT mice after repetitive DE exposure (Fig 5C). These studies confirm that αβ-expressing T cells are critical for the genesis of the DE-induced mononuclear infiltrates, but do not appear to explain the persistent neutrophilic influx after repetitive DE exposure.

Discussion

Our studies demonstrate that repetitive exposure to swine confinement organic DE resulted in the influx of CD4$^+$ T cells concomitant with a mixed Th1 and Th17 lung microenvironment. Exposure to PGN, a major component of DE in porcine and other large animal confinement facilities,[15,16,21] also resulted in the accumulation of CD4$^+$ T cells and CD8$^+$ T cells, with a mixed Th1 and Th17-polarized lung cytokine profile. These studies established that the development of lung parenchymal mononuclear aggregates and peribronchiolar inflammation after DE exposure is dependent on αβ-expressing T cells, but that DE-induced airway neutrophil influx is independent of these cells. Collectively, these studies establish that T cells skewed to Th1/Th17 phenotype are important in the inflammatory murine lung response to repeated organic dust exposures.

Farming exposures have gained interest over the past decade, as early life exposures to farming-related environments have been associated with a protection against the development of atopy or IgE-mediated diseases.[8,9] Although this association is strong in European cohort studies, evidence suggests that farming exposures are protective against IgE-mediated disease development in the United States.[22] However, farming practices differ in the United States with the rise in large-scale, concentrated, closed animal feeding operations.[1] Moreover, despite a lower risk of atopy, children living on farms that raise swine in Iowa had a self-reported asthma prevalence of 46 to 56%.[22] In adults, repetitive or chronic farm exposure is well recognized to result in a high prevalence of airway inflammatory diseases, including workplace-exacerbated asthma, chronic bronchitis, and obstructive lung disease.[23] Differing lineage types of effector CD4$^+$ T cells induced by these farm organic dust exposures within the lung are likely responsible for skewing the adaptive immune response and regulating tissue inflammation and disease development.

We found that repetitive organic dust exposures induced both a Th1 and Th17 lung response, but not a Th2 or Treg response. Th1 and Th17 cell lineages are associated with promoting neutrophil, as opposed to eosinophil, accumulation in the lungs. Th17 influx was augmented in the lungs of mice after repetitive DE exposure, which was evident by enhanced CD4$^+$ IL-17–producing cells. However, cytokine array analysis of whole lung homogenates demonstrated a mixed Th1/Th17 response. Namely, IL-6, IL-1β, IL-17, and IL-22 were increased, which is consistent with driving a Th17 response; however, IL-12 and IFN-γ were also significantly increased, suggestive of a Th1-promoting environment. IL-23 was not different between groups, which may be attributable to the time interval investigated or represents a distinction from other lung inflammatory models such as asthma, whereby IL-23 and IL-17A are important in the enhancement and maintenance of eosinophilic and neutrophilic inflammatory responses.[24–27] A similar Th1/Th17 response was demonstrated after repetitive PGN exposures, supporting a role for

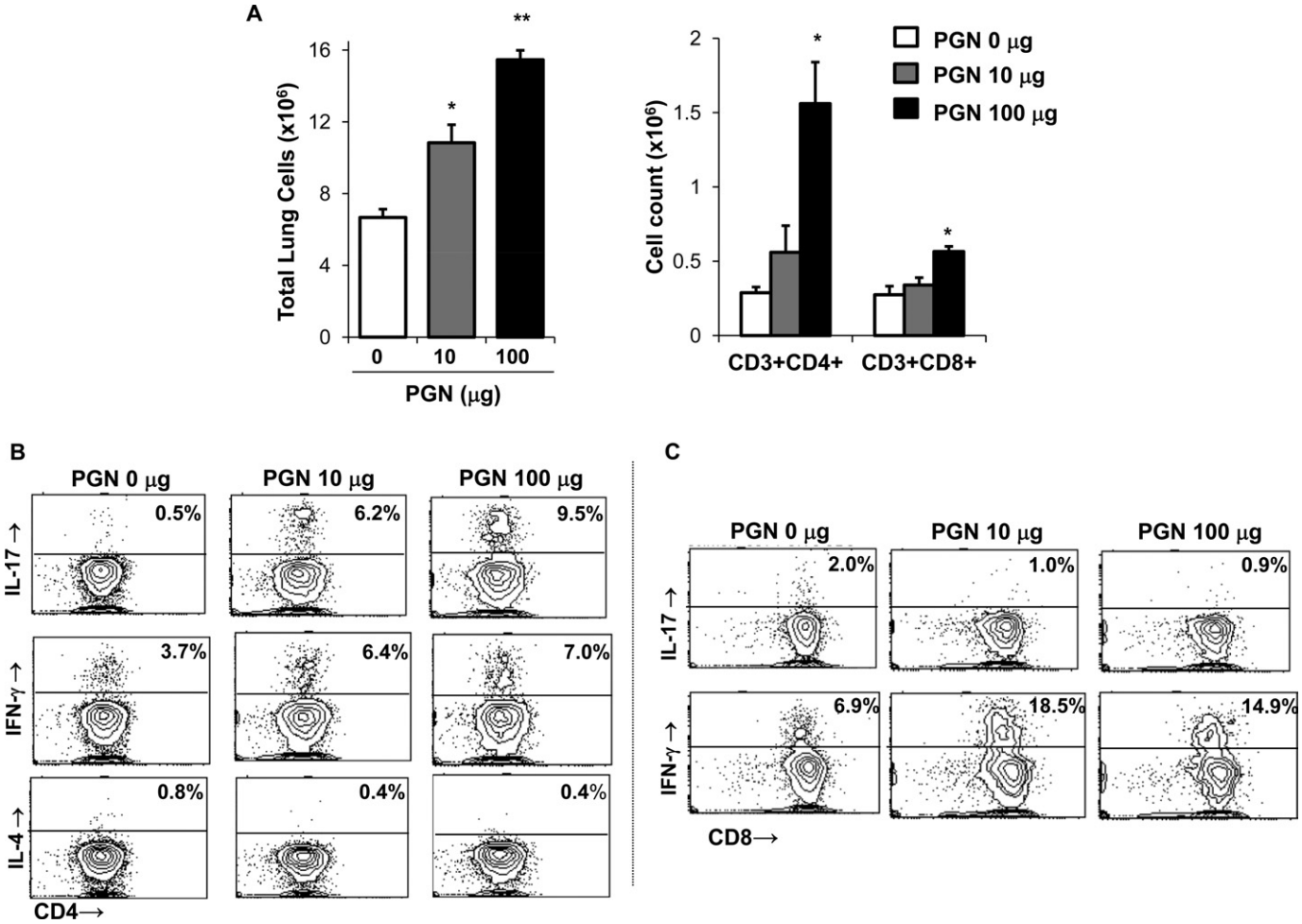

Fig. 4. Repetitive intranasal challenge with peptidoglycan (PGN) induces a Th1/Th17-polarized lung cell phenotype. C57BL/6 mice were repetitively exposed to PGN (10 and 100 μg) or saline for 3 weeks, whereupon lung-associated cells were stained for $CD3^+CD4^+$ and $CD3^+CD8^+$ and T cells and analyzed by FACS. **A,** Results represent mean ± SEM ($n = 6$ mice/group) of total lung cells, $CD3^+/CD4^+$ and $CD3^+/CD8^+$ T cells (percentage of cell type × total lung cell count). Statistical difference as compared with saline control (PGN 0 μg) is denoted by asterisk (*$P < .05$, **$P < .01$). $CD4^+$ and $CD8^+$ T cells pooled from 3 animals and isolated by FACS were immediately stimulated ex vivo with phorbol myristate acetate + ionomycin for 4 hours and stained for IL-17, IFN-γ, and IL-4 to demonstrate cytokine profiles. A representative contour plot depicting cytokine staining of isolated $CD4^+$ T cells (**B**) and $CD8^+$ T cells (**C**) of 1 of 3 independent experiments is shown.

microbial components such as PGN in driving organic dust–induced airway inflammatory responses. Our findings of a mixed Th1/Th17 response would be consistent with observations in smoking subjects with chronic obstructive lung disease.[28] However, these results differ from the murine model of hypersensitivity pneumonitis and lung fibrosis whereby Th17, but not Th1 or Th2, lung responses predominate.[29] It might be warranted to investigate the lung pathogenic response after organic dust exposure in mice deficient in IL-17R signaling as well as to investigate IL-17 receptor variant expression levels. However, our findings suggest to us that strategies to target a specific cytokine to reduce organic dust–induced airway disease might be difficult because of the myriad of cytokines involved. Another potential future approach could be to examine the alteration of Th1/Th17 transcription factors such as Tbet, GATA3, and retinoid-related orphan receptor (ROR)γt. However, our findings suggest to us that strategies to target a specific cytokine to reduce organic dust–induced airway disease might be difficult because of the multiple cytokines involved.

The numbers of $CD4^+$ T cells were increased in the lungs of mice repetitively exposed to organic dusts. Our studies also confirmed that $\alpha\beta$-expressing T cells are the critical T cell infiltrate after repetitive DE exposure, because cellular aggregates failed to form in TCR $\alpha\beta$ KO mice, and bronchiolar inflammation was significantly reduced after DE exposures. Interestingly, alveolar inflammation and neutrophil influx was found to be $\alpha\beta$ T cell independent. This later observation implies that alternative cell types, such as macrophages or airway epithelia, are also likely important contributors for sustaining neutrophil influx. A potential future approach to further understand the role of cellular players would be the generation of chimera mice or adoptive transfer models of TCR-deficient and healthy naïve mice. Alternatively, $CD8^+$, $\gamma\delta$ T cells, natural killer (NK) cells, and NKT cells are also important sources of a number of these lung-associated cytokines that promote airway inflammation.[24] However, repetitive DE exposure did not increase the number of $CD8^+$ T cells or IFN-γ– or IL-17–producing $CD8^+$ T cells, which suggests that they may not be as important in this model. In addition, the frequencies of $\gamma\delta$ T cells, NK cells, and NKT cells were quite low and did not differ among groups (data not shown). Although we did not quantitate $\gamma\delta$ T cells in the TCR $\alpha\beta$ KO mice, an earlier publication from our laboratory[7] demonstrated that T cell influx after organic dust treatment was clustered in cellular aggregates throughout the lung parenchyma. In the current study, these cellular aggregates were absent in the $\alpha\beta$ TCR-deficient mice after organic dust exposure, confirming that the T lymphocytes within the aggregates express $\alpha\beta$ TCR. It also suggests that a compensatory increase in other lymphocyte types, such as $\gamma\delta$T cells, did not occur because the aggregates were absent, although admittedly, a minor population may still be discernible by more sensitive detection

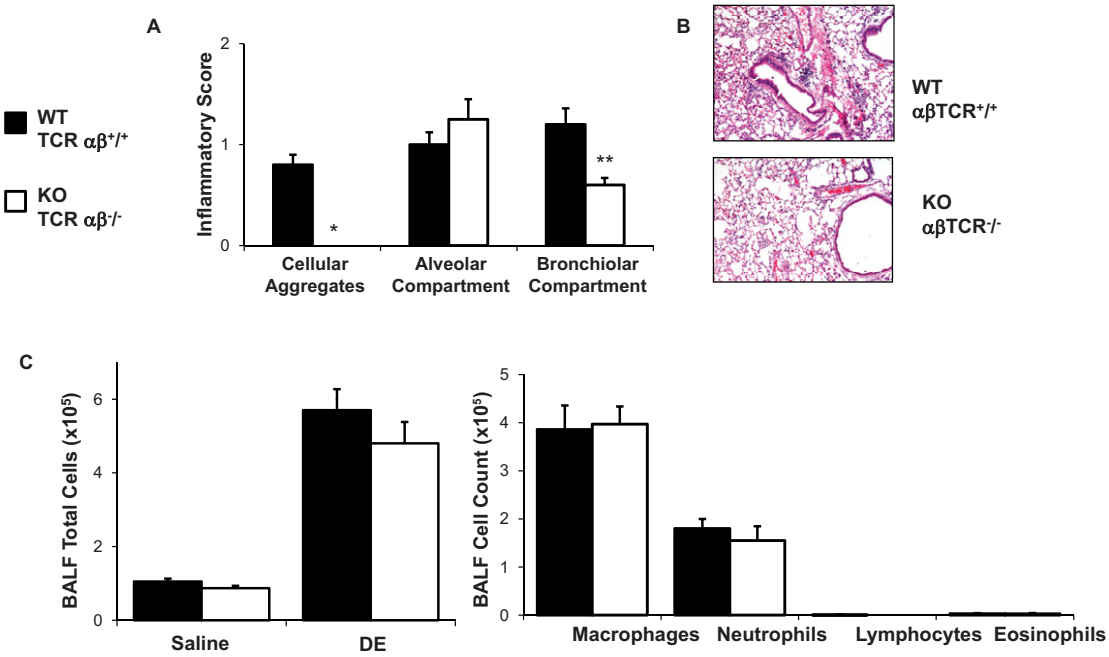

Fig. 5. $\alpha\beta$ T cells are essential for the formation of DE-induced peribronchiolar mononuclear aggregates, but not neutrophil infiltrates. C57BL/6 wild-type (WT, $^{+/+}$) and TCR $\alpha\beta$ knockout (KO, $^{-/-}$) mice on a C57BL/6 background were repetitively exposed to DE for 3 weeks. Whole lungs were excised and inflated to 10 cm H_20 pressure with 10% formalin to preserve pulmonary architecture, processed, and embedded in paraffin, and sections (4–5 μm) were cut and stained with hematoxylin and eosin. **A**, Results represent the mean ± SEM (n = 6 mice/group) of the semiquantitative degree and distribution of lung cellular aggregates, alveolar inflammation, and bronchiolar inflammation. **B,** A representative section of 1 of 6 mice per treatment group is shown at 20× magnification. **C**, Results represent the mean ± SEM of the total cells and cell differentiation recovered from the BALF of WT and TCR $\alpha\beta$ KO mice after repetitive DE exposure (n = 6 mice per group). Statistical difference is denoted with asterisks between groups (**$P < .01$).

methods such as FACS. The potential involvement of $\gamma\delta$T cells in this model can be investigated in future studies. Overall, because studies demonstrated an important role for T cells, future studies could investigate whether lymphocyte-targeted interventions with immunosuppressive drugs, including cyclosporine or mycophenolate mofetil, might be beneficial in reducing DE-induced airway disease. This may be a novel future direction because pharmacologic interventions that include inhaled sodium cromoglycate, corticosteroids, and long-acting beta agonists have shown modest and sometimes conflicting effects in the prevention or treatment of exposed agriculture workers.[30–32]

Research into respiratory disease exposure in various agricultural settings has primarily focused on gram-negative bacterial endotoxins. Endotoxins have also been recognized to induce Th1 and Th17 responses in the lung.[33,34] Although endotoxins are clearly important, they may explain only a relative small proportion of respiratory disease seen in agricultural workers.[35] Bioaerosols in current swine production facilities contain up to 80% gram-positive bacteria and high levels of PGN/muramic acid.[15,17,21,36] Moreover, our previous work has implicated non-endotoxin components, such as PGN, as a driver of swine facility DE-induced inflammatory consequences.[17,18] In this current study, similar to that observed with DE, PGN alone induced Th17/Th1-polarized $CD4^+$ T cells. However, a difference was the observation of increased $CD8^+$ T cells skewed toward a Th1 state. Whereas this could be related to biological potency, it also highlights that environmental organic dust exposures are inherently complex, and immune responses cannot simply be ascribed to 1 single agent. The observations with PGN alone might be broadly applicable to other environments whereby gram-positive bacteria or PGNs are detected, and this includes school rooms, domestic homes, and other animal farming environments.[15,37,38]

In conclusion, organic DE from porcine animal confinement facilities promotes a mixed Th1 and Th17 lung microenvironment with an important role for $\alpha\beta$-expressing T cells in mediating DE-induced lung pathologic conditions. Moreover, PGN, a component of organic dust, elicited a similar lung response, suggesting that this microbial motif is a major driver of pathogenic lung inflammation. Overall, this information could be important when investigating potential cellular-directed therapies to reduce organic dust–induced airway inflammatory consequences, particularly those related to chronic bronchitis and obstructive lung disease in exposed agricultural workers.

Acknowledgments

The authors thank Victoria Smith and Charles Kuszynski, PhD, of the UNMC Cell Analysis Facility for assistance with flow cytometric measurements, Greg Dooley, Ph.D., of the Colorado State University Center for Environmental Medicine for assistance with mass spectrometry analysis of dust samples, UNMC Tissue Science Facility for assistance with digital microscopy images prepared for the manuscript, and Elizabeth Klein at UNMC for technical assistance.

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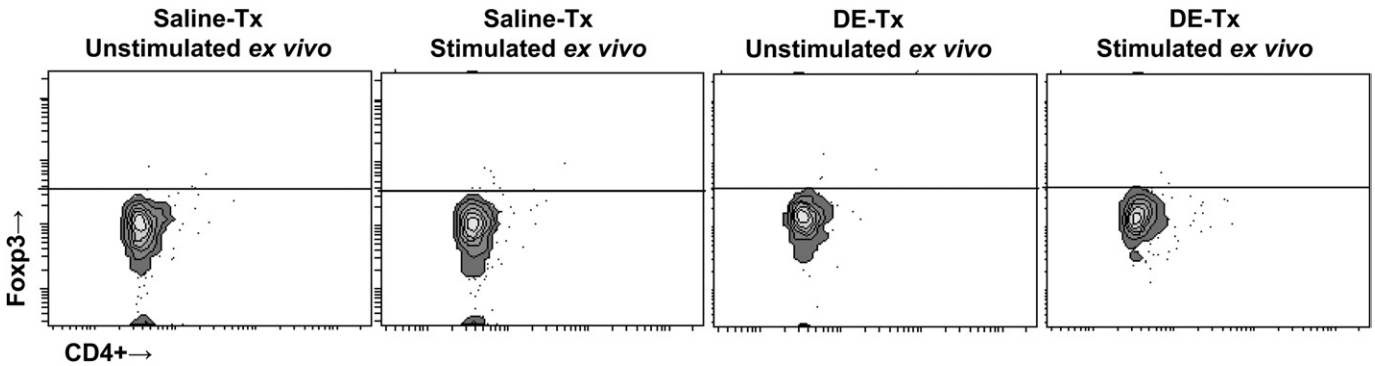
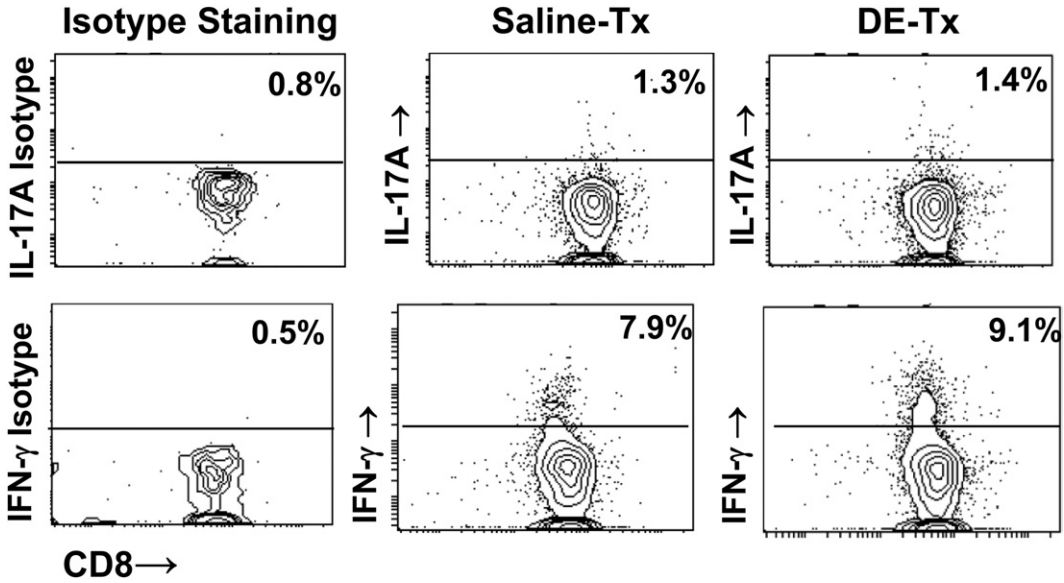
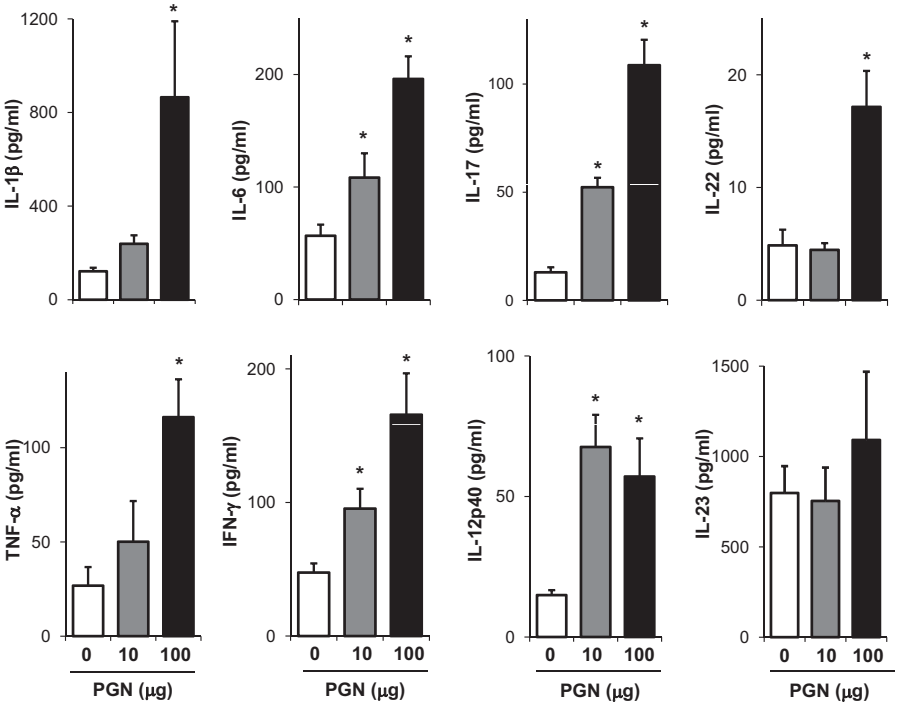


eFigure 1. Repetitive DE exposure does not induce Foxp3 expression of $CD4^+$ T cells. C57BL/6 mice were repetitively exposed to DE and saline for 3 weeks, and $CD4^+$ T cells pooled from 3 animals were isolated from lung tissue by FACS and immediately *ex vivo* stimulated with phorbol myristate acetate + ionomycin for 4 hours, and stained for Foxp3. As an additional control, $CD4^+$ T cells from saline and DE-treated mice were not *ex vivo* stimulated, but stained for Foxp3. A representative contour plot depicting Foxp3 staining for 1 of 3 independent experiments is shown.



eFigure 2. Repetitive DE exposure does not induce IFN-γ and IL-17 production of $CD8^+$ T cells. C57BL/6 mice were repetitively exposed to DE and saline for 3 weeks, and $CD8^+$ T cells pooled from 3 animals were isolated from lung tissue by FACS and immediately stimulated with phorbol myristate acetate + ionomycin for 4 hours, and stained for IL-17 and IFN-γ to demonstrate cytokine profiles. A representative contour plot depicting cytokine staining for 1 of 3 independent experiments is shown. Left column depicts background or isotype staining for each respective cytokine. Middle and right column depict saline- and DE-treated mice, respectively.



eFigure 3. PGN exposure induces a Th1/17-polarized cytokine response in the whole lung. Cytokine profiles associated with T cell subsets were analyzed from cell-free supernatants of total lung homogenates from C57BL/6 mice repetitively exposed with PGN (100 μg and 10 μg) or saline (PGN 0 μg) for 3 weeks. Results represent the mean ± SEM of 6 mice from 2 independent experiments with statistical significance denoted by asterisk ($^*P < .05$) as compared with saline (PGN 0 μg).