Chronic Endotoxin Exposure Produces Airflow Obstruction and Lung Dendritic Cell Expansion

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Little is known about the mechanisms of persistent airflow obstruction that result from chronic occupational endotoxin exposure. We sought to analyze the inflammatory response underlying persistent airflow obstruction as a result of chronic occupational endotoxin exposure. We developed a murine model of daily inhaled endotoxin for periods of 5 days to 8 weeks. We analyzed physiologic lung dysfunction, lung histology, bronchoalveolar lavage fluid and total lung homogenate inflammatory cell and cytokine profiles, and pulmonary gene expression profiles. We observed an increase in airway hyperresponsiveness as a result of chronic endotoxin exposure. After 8 weeks, the mice exhibited an increase in bronchoalveolar lavage and lung neutrophils that correlated with an increase in proinflammatory cytokines. Detailed analyses of inflammatory cell subsets revealed an expansion of dendritic cells (DCs), and in particular, proinflammatory DCs, with a reduced percentage of macrophages. Gene expression profiling revealed the up-regulation of a panel of genes that was consistent with DC recruitment, and lung histology revealed an accumulation of DCs in inflammatory aggregates around the airways in 8-week-exposed animals. Repeated, low-dose LPS inhalation, which mirrors occupational exposure, resulted in airway hyperresponsiveness, associated with a failure to resolve the proinflammatory response, an inverted macrophage to

CLINICAL RELEVANCE

Occupational exposure to endotoxin can result in a significant, increasing burden of disease, with a development of airflow obstruction similar to that seen in chronic obstructive pulmonary disease and bronchiolitis obliterans. The underlying mechanism of the development of this airflow obstruction is not fully understood, and the inflammatory response is not well characterized. Previous studies did not fully address the innate immune response in this disease process. In a murine model of occupational endotoxin exposure, we correlated increases in airflow obstruction with pulmonary gene expression profiling, flow cytometry studies, and histologic analysis of the lung, to discover a novel underlying mechanism for persistent neutrophilic inflammation as a result of chronic endotoxin exposure. We report for the first time, to the best of our knowledge, that persistent lung neutrophilic inflammation in a murine model of occupational endotoxin exposure is correlated with an expansion of lung inflammatory dendritic cells. This mechanism may also be relevant to other chronic airway diseases.

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DC ratio, and a significant rise in the inflammatory DC population. These findings point to a novel underlying mechanism of airflow obstruction as a result of occupational LPS exposure, and suggest molecular and cellular targets for therapeutic development.

Keywords: airway resistance; inhalation; neutrophils; macrophages; dendritic cells; endotoxin

Endotoxin (lipopolysaccharide, or LPS) is a cell-wall component of Gram-negative bacteria, and is ubiquitous in the environment. Occupational settings in which the ambient concentration of endotoxin is markedly increased include swine farms, sewage treatment plants, humidified buildings, and the processing of organic materials (in particular, cotton), where exposure levels can exceed 5,900 EU/m³ (1). Endotoxin is also an important component of indoor air pollution in homes burning biomass fuel (2).

Human studies have demonstrated a relationship between occupational endotoxin exposure and the development of airflow obstruction, and suggest that sufficient endotoxin exists in cotton dust to cause respiratory symptoms (often as part of a syndrome termed “byssinosis”) and a decline in pulmonary function test results (3, 4). Importantly, the endotoxin concentration in cotton dust, and not the dust concentration, is correlated with the decline in lung function (5). Although most studies in humans examined the acute response to airborne endotoxin, some studies suggest that long-term exposure to cotton dust is associated with a loss of lung function, with symptoms and pulmonary function test results similar to those observed in chronic obstructive

pulmonary disease. Respiratory dysfunction often persists after cessation of the workplace exposure (6–8).

The biological effects of chronic endotoxin exposure have been studied in several animal models (9–12), and emphysematous changes (13) and airway obstruction (14) were reported. However, the role of specific inflammatory subsets in the mechanism of endotoxin-induced chronic airflow obstruction remains unclear, and few data are available on “early” (i.e., 1-week) versus “late” (i.e., 8-week) inflammatory cell subset responses attributable to recurrent endotoxin exposure. Moreover, previously described models of chronic endotoxin exposure in mice have proved challenging for many laboratories to implement, given the need to expose mice to aerosolized endotoxin for prolonged periods (e.g., 8 hours).

In this study, we developed a murine model of endotoxin exposure, using brief daily exposures of nebulized endotoxin to analyze the mechanisms that underlie the host response, using physiologic, gene expression, histologic, and flow cytometry studies. We demonstrate for the first time, to the best of our knowledge, that chronic, inhaled endotoxin results in airflow obstruction and airway hyperreactivity that correlates with a dysregulated balance of lung antigen-presenting cells (APCs) and a marked amplification of the proinflammatory response.

MATERIALS AND METHODS

Murine Model

All animal studies were performed with the approval of our Institutional Animal Care and Use Committees. Sedation was undertaken with pentobarbital 70 mg/kg subcutaneously. Terminal experiments were performed in concordance with the “Report of the American Veterinary Medicine Association Panel on Euthanasia” (15). To model daily, low-dose occupational endotoxin exposure, 6- to 8-week-old male C57BL/6 mice (Charles River, Wilmington, MA) were treated with 2 mg nebulized LPS (*Pseudomonas*, L9143; Sigma Chemical Company, St. Louis, MO) or PBS for 15 minutes daily (16). Mice were exposed 5 days/week (Monday–Friday) for 5 days (“5-day”), 4 weeks (“4-week”), or 8 weeks (“8-week”), and were studied 24 hours after the final exposure. Lung physiology was measured with a Flexivent ventilator (Scireq, Montreal, PQ, Canada), followed by bronchoalveolar lavage (BAL) fluid and lung tissue collection (16). In separate studies, airway hyperreactivity was measured after methacholine challenge, and the methacholine dose required to produce a 50% increase in baseline central airways resistance (ED_{50}) was calculated by linear interpolation (17, 18).

Isolation of Murine Lung Inflammatory Cells from Whole Lungs

The right ventricle was perfused with PBS to remove the circulating blood pool. Lungs were removed, minced, and digested with 300 U/ml collagenase Type IV (Worthington Biochemical Corporation, Lakewood, NJ) and 100 U/ml DNase I (Boehringer Mannheim, Ingelheim am Rhein, Germany) in PBS. Tissue was passed over a 100- μ m nylon mesh sieve. Red blood cell lysis was performed (ammonium-chloride-potassium buffer) and the washed cell pellet was resuspended in flow cytometry staining buffer (PBS with 2.5% murine serum, and 2.5% fetal bovine serum) and enumerated.

Flow Cytometry

The isolated lung cell suspension was incubated with the monoclonal antibody (mAb) 2.4G2 (10 μ g/ml) to block Fc- γ receptors. Cells were stained with a combination of directly conjugated fluorescent anti-mouse mAbs, including CD45-PE-Cy7, CD4-PE, CD8-PerCP-Cy5.5, CD19-APC, CD11c-APC, Gr-1-APC, CD103-APC, F4/80-PE, rat IgG2b-PerCP-Cy5.5, rat IgG2a-APC, rat IgG2b-APC, rat IgG2a-FITC, rat IgG2a-PE-Cy7, rat IgG2a-PE, and rat IgG2b-PE (eBioscience, San Diego, CA), CD11c-FITC, CD11c-PE-Cy7, major histocompatibility

Class II (MHC Class II)–FITC, Ly6C-FITC (clone AL-21), Ly6G-PE, Siglec-F-PE, CD11b-PerCP-Cy5.5 (BD Biosciences, Franklin Lakes, NJ), and MHC Class II-PerCP-Cy5.5 (BioLegend, San Diego, CA). Cells were analyzed with a BD Biosciences Canto II flow cytometer and Flow Jo software (Tree Star, Ashland, OR). Live cell and CD45⁺ cell gating was used to exclude dead and nonhematopoietic cells.

CD4⁺ T-Cell Activation and Proliferation by Lung APC Subsets and Immunohistochemistry

Please see the online supplement for details.

RNA Sample Preparation, Microarray Hybridization, and Data Analysis of Lung Homogenates

Please see the online supplement for details.

ELISA Assays

ELISA was performed using BAL fluid (normalized to recovered BAL volume) and lung-tissue homogenates (normalized to grams of tissue), as described previously (19), after a multiplex Luminex assay screen (Luminex Corporation, Austin, TX) for cytokine elaboration, using lung-tissue homogenates (see the online supplement).

Statistical Analysis

Analyses were performed using SAS software, version 9.2 (SAS Institute, Inc., Cary, NC) and BioConductor/R, version 2.11.1 (www.Bioconductor.org). Values are reported as means $\pm$ standard errors. For analyses of the methacholine challenge data, a repeated-measures ANOVA was performed to account for correlated observations, followed by one-degree-of-freedom tests for group by dose interactions (17, 20, 21). Otherwise, ANOVA was performed, followed by appropriate comparisons to test for differences between the means of groups. Values not following a normal distribution were log-transformed. Differences in means were considered significant at $P < 0.05$.

RESULTS

Eight-Week LPS Mice Exhibit Increased Central Airway Resistance at Baseline and after Methacholine

Mice were exposed to an inhalation protocol that mirrored occupational endotoxin exposure for 5 days/week for 5 days, 4 weeks, or 8 weeks. At 24 hours after the final exposure, mice were analyzed on a Flexivent ventilator. All LPS-exposed mice had increased central airways resistance (R_n), with a statistically significant increase in the 4-week and 8-week LPS-exposed mice compared with time-matched PBS control mice (Figure 1A). Separate groups of mice underwent methacholine challenge, and we observed increased airway hyperresponsiveness in all LPS-treated mice compared with control mice ($P = 0.0098$ and 0.003 at 5 days and 8 weeks, respectively, according to repeated-measures ANOVA; Figures 1B and 1C). Thus, the 8-week LPS exposure protocol resulted in increased airway resistance, both at baseline and after the methacholine challenge. Interestingly, for the 5-day LPS mice, a significant increase in airway resistance was also evident at 30, 60, and 120 mg/ml of methacholine (Figure 1B), and a significant difference in airway sensitivity was evident compared with PBS control mice (ED_{50} for PBS, 44.8 ± 17.9 mg/ml; ED_{50} for LPS mice, 11.9 ± 4.0 mg/ml; $P = 0.034$). Although the 8-week LPS mice exhibited an overall increase in airway reactivity compared with PBS control mice, we did not detect significant differences at individual doses of methacholine, and no significant difference was evident in airway sensitivity compared with PBS control mice (ED_{50} for PBS control mice, 25.0 ± 9.3 mg/ml; ED_{50} for LPS mice, 22.0 ± 11.1 mg/ml; $P = 0.87$).

Figure 1. Mice with low-dose chronic LPS exposure exhibit an increase in central airway resistance at baseline after 8 weeks of exposure and after methacholine challenge. (A) Mice were exposed to nebulized LPS (or PBS control) for 5 days per week, for 5 days, 4 weeks, or 8 weeks. Twenty-four hours after the final exposure, the mice were anesthetized, sedated, and analyzed on a Flexivent ventilator. All groups of LPS-exposed mice exhibited an increase in central airway resistance (Rn), with a statistically significant increase in 4-week and 8-week LPS-exposed mice, compared with PBS control mice (* $P < 0.05$ compared with PBS, $n = 8$ /group). Separate groups of 5-day mice (B) and 8-week mice (C) underwent methacholine challenge (doses of 0.9–120 mg/ml), with lung physiology measurements after each methacholine exposure. (* $P < 0.05$, LPS-treated versus PBS-treated groups by repeated-measures ANOVA for 5-day and 8-week mice, $n = 5$ /group; * $P < 0.05$ versus PBS-treated mice at discrete methacholine doses of 30, 60, and 120 mg/ml). Values plotted represent means $\pm$ standard errors.

Eight-Week LPS Mice Exhibit Increased BAL Neutrophils and IL-6 Concentrations, and Decreased IL-10 Concentrations, Consistent with a Persistent Proinflammatory Profile

We next asked whether the enhanced airway resistance observed in the 8-week LPS mice correlated with increased indices of inflammation. We observed an increase in total cells at 5 days, 4 weeks, and 8 weeks after LPS exposure, as well as an increased percentage of BAL neutrophils in all LPS mice, although unexpectedly, the percentage of BAL neutrophils statistically increased over time (Figure 2A). This observation was in contrast to those previously reported with other chronic LPS exposure protocols

Figure 2. Eight-week LPS-exposed mice develop a profile consistent with a failure to resolve the acute inflammatory response. (A) Cellularity of bronchoalveolar lavage (BAL) fluid was assessed after the exposure protocol ($n = 8$ /group). Increased total cell numbers with an increasing percentage of neutrophils (Neuts) were observed during the 8-week exposure protocol (* $P < 0.05$ for total cells versus PBS-treated mice at each time point; $P < 0.05$ for percentages of neutrophils at 8 weeks versus 5 days; Macs, macrophages). (B and C) Concentrations of a panel of cytokines were measured in BAL fluid collected from mice subjected to the exposure protocol (see MATERIALS AND METHODS; $n =$ at least 4/group), and the fold reduction in IL-10 concentrations in the LPS-exposed mice compared with PBS control mice is shown for each time point (B), along with the BAL concentrations of IL-6 in PBS-treated and LPS-treated mice at 5 days and 8 weeks (C). Increasing suppression of IL-10 with increased length of LPS exposure was evident (* $P = 0.003$ according to ANOVA) and an increase in BAL IL-6 levels over time in LPS-exposed mice (* $P < 0.05$ versus 5 days of PBS; ** $P < 0.05$ versus 8 weeks of PBS and 5 days of LPS).

that produced more macrophage-predominant inflammation (14). Consistent with a failure to “resolve” the proinflammatory phenotype over the 8-week exposure, a decrease in BAL “anti-inflammatory” IL-10 concentrations was observed over time, with the greatest reduction in the 8-week LPS mice (Figure 2B). Moreover, BAL concentrations of the “proinflammatory” cytokine IL-6 were increased in the 5-day and 8-week LPS mice

compared with PBS control mice, with a significantly greater increase in the 8-week LPS mice compared with the 5-day LPS mice (Figure 2C). Thus, the 8-week LPS exposure protocol produced increased lung inflammation and a proinflammatory phenotype that correlated with lung dysfunction.

Gene Expression Profiling of Myeloid and APC Markers Are Up-Regulated in Lung Homogenates with 8 Weeks of LPS

Given our observation of increased proinflammatory indices in the 8-week LPS mice, we set out to evaluate whether a change occurred in differential gene expression between the LPS-treated and PBS-treated mice at 5 days and 8 weeks, and whether a gene expression pattern or “signature” existed that would inform the underlying mechanism of dysregulated inflammation. Hierarchical clustering and principal components analyses correctly clustered PBS-treated and LPS-treated mice into discrete groups (see Figure E1 in the online supplement). Using a false discovery rate of 5%, 325 unique and annotated genes (mapped by 399 Affymetrix probes [Affymetrix, Santa Clara, CA]) were differentially expressed between LPS-treated and PBS-treated mice in the 5-day group, and 806 unique and annotated genes (1,027 probes) were differentially expressed in the 8-week group (Table E1). Consistent with our findings of increased inflammation in the LPS-treated mice, we observed a significant up-regulation in both 5-day and 8-week LPS mice of a number of genes important for the proinflammatory response. The intersection of differentially expressed genes between the 5-day and 8-week mice revealed an overlap of 209 genes, suggesting that longer durations of LPS exposure lead to additional alterations in gene expression. We examined this phenomenon at the functional level, using enrichment analysis. Interestingly, we found that differentially expressed genes in the 8-week LPS group were overrepresented in two pathways

involving APCs that were not enriched in the 5-day LPS mice (Table E2).

We next asked whether genes important in myeloid APC development might be differentially expressed between the 5-day and 8-week LPS mice compared with their PBS control mice (see Table E3 for average expression intensities of candidate genes). We found 10 genes that were differentially expressed in the 8-week LPS mice compared with their PBS control mice, and of these, four genes were differentially expressed exclusively in the 8-week LPS mice versus PBS-treated mice (and not in the 5-day LPS-treated versus PBS-treated mice): *Ccr2* (chemokine receptor 2), *Csf1r* (also known as M-CSFR, or macrophage colony stimulating factor receptor), *Csf2* (also known as GM-CSF or granulocyte-macrophage colony stimulating factor), and *Itgam* (α M, the α subunit of CD11b) (Figure 3A). Interestingly, we did not observe any genes within this subgroup that were up-regulated exclusively after 5 days of LPS (but not after 8 weeks of LPS). Finally, we tested whether a significant up-regulation of these genes occurred after 8 weeks of LPS exposure compared with 5 days of LPS exposure, and we observed increased expression of *Ccl8* ($P = 0.0355$), *Cx3cr1* ($P = 0.008$), *Itgax* ($P = 0.006$), and *Ly6i* ($P = 0.0015$). We validated *Ly6i* gene expression using quantitative RT-PCR (Figures 3B and 3C), and found more enhanced changes in gene expression after 8 weeks of LPS than were observed in the microarray study (fold -change in LPS/PBS expression at 8 weeks, 52.8 ± 5.4 , versus 5 days, 11.1 ± 1.3 ; $P < 0.05$, for 8 weeks versus 5 days). We also validated *Ccl8*, *Cx3cr1*, and *Itgax* gene expression using quantitative RT-PCR (Table E4). These gene expression studies suggest that accentuated changes occur in the lung myeloid cell compartment after 8 weeks of LPS exposure, compared with shorter-term LPS exposure. Given these findings, we next analyzed changes in the lung myeloid cell compartment after prolonged LPS exposure.

Figure 3. After long-term LPS exposure, mice exhibit a gene signature profile consistent with the recruitment of dendritic cells (DCs) to the lung and persistence of the inflammatory response. (A) Analysis of a subset of genes involved in myeloid antigen-presenting cell (APC) development demonstrated differential expression in total lung homogenates of 10 markers in 8-week LPS mice compared with PBS control mice, using a false discovery rate (FDR) of 5%. Four genes were differentially expressed exclusively in the 8-week LPS-treated versus PBS-treated mice (and not in the 5-day LPS-treated versus PBS-treated mice): *Ccr2*, *Csf1r*, *Csf2*, and *Itgam* ($^{\#}$ FDR < 0.05 for 8-week LPS versus PBS, but not 5-day LPS versus PBS). A significant up-regulation of *Ccl8*, *Ly6i*, *Cx3cr1*, and *Itgax* occurred at 8 weeks compared with 5 days of LPS exposure ($^*P < 0.05$ and $^{**}P < 0.01$ for 8-week versus 5-day LPS gene expression). (B and C) Quantitative RT-PCR was performed on total lung homogenates ($n = 4$ per group) to validate changes in *Ly6i* gene expression. We observed an increase in absolute expression (B) of *Ly6i* after both 5 days and 8 weeks of LPS expression ($^*P < 0.05$ versus PBS control mice; $^{**}P < 0.05$ versus PBS control mice and versus 5 days of LPS), and an increase in fold-change in expression (C) after 8 weeks of LPS compared with 5 days of LPS ($^*P < 0.05$ versus LPS; PBS fold-change after 5 days of LPS).

Prolonged Inhalational Endotoxin Exposure Results in the Expansion of Lung Mononuclear Cells and Neutrophils

Lung leukocytes isolated from whole lungs of 5-day and 8-week mice were examined using flow cytometry. As was observed in the BAL fluid (Figure 2), a significant increase was evident in the number of lung neutrophils in both the 5-day and 8-week LPS mice, with greater numbers observed in the 8-week LPS mice versus 5-day LPS mice (Figure 4A). This was observed when Gr-1^{hi} expression was used to quantify neutrophil numbers, as well as when more stringent phenotypic markers (CD45⁺CD11c⁻CD11b⁺Ly6G⁺) were used (Figure E2A). Moreover, increases in the numbers of other leukocyte subsets were observed. An approximately twofold increase occurred in the number of CD4⁺ and CD8⁺ T cells and B (CD19⁺) cells in the 5-day and 8-week LPS mice (versus PBS control mice), as well as significant increases in the number of lung monocytes in both the 5-day and 8-week LPS mice, with the most notable increase occurring in the 8-week LPS mice (data not shown). Given that the gene expression profiles suggested a shift in APCs within the lung, we focused our attention on APC subsets.

Eight Weeks of LPS Promotes Lung Dendritic-Cell Expansion and Inversion of the Ratio of Dendritic Cells to Macrophages

The most striking changes observed in lung leukocyte subsets occurred in the antigen-presenting cell compartment. Although many investigators used CD11c and MHC Class II staining and autofluorescence to discriminate between dendritic cells (DCs) and macrophages (22–24), we applied further characterization with Siglec F and F4/80 staining. DCs were enumerated using previously characterized phenotypic markers (CD11c⁺MHC

Class II^{hi}) and macrophages such as CD11c⁺MHC Class II^{int}. To ensure that changes to phenotypic cell surface marker expression in the setting of LPS exposure did not confound these analyses, staining for Siglec F and F4/80 (prominently expressed on lung macrophages) was also used to identify or exclude macrophages in these analyses (Figure E2D and Figure 5).

An approximately twofold increase was evident in the numbers of both lung macrophages and myeloid DCs after 5 days of LPS exposure compared with PBS control mice (Figure 4B). In the 8-week LPS mice, an even more striking increase in lung DC numbers occurred (~ 6.7-fold). However, in contrast to the 5-day LPS mice in which macrophage numbers increased, the numbers of lung macrophages in the 8-week LPS mice were unchanged compared with PBS control mice (Figure 4B). Consistent with this, the percentage of DCs and macrophages in the lung digests were not significantly changed after 5 days of LPS. However, a marked increase in the percentage of lung DCs after 8 weeks of LPS occurred (59.4% ± 8.3%), compared with PBS mice (28.2% ± 5.1%). A concomitant, striking decrease occurred in the percentage of APCs that were macrophages after 8 weeks of LPS (40.8% ± 8.1%), compared with PBS control mice (71.8% ± 5.1%) (Figures 4C and 4D).

The flow cytometry observations of expanded DCs after 8 weeks of LPS were supported by increases in IL-1-α and IL-12p40 in lung homogenates. These concentrations were significantly higher than those observed after 5 days of LPS exposure (Figure E2B). IL-12p40 is almost exclusively produced by myeloid lineage cells such as monocytes, macrophages, and DCs. IL-1-α can be produced by a variety of cells, but is also elaborated by myeloid lineage cells, including monocytes and DCs. In contrast, a variety of other cytokines were not similarly up-regulated at 8 weeks compared with 5 days of LPS, including IL-2, TNF-α, and IL-5.

Figure 4. Prolonged inhalational LPS exposure increases lung neutrophil and myeloid DC numbers, and alters the balance of antigen-presenting cell subsets. (A) Lung neutrophil numbers increased after 5 days, and more prominently after 8 weeks, of inhalational LPS exposure. Neutrophil numbers were quantified by Gr-1^{hi} staining (**P* < 0.05 versus 5 days of PBS; ***P* < 0.05 versus 8 weeks of PBS and versus 5 days of LPS) (*n* = 8/group). (B) Lung DC and macrophage numbers increased after 5 days of inhalational LPS versus PBS control mice, as determined by flow cytometry (**P* < 0.05 versus 5 days of PBS). After 8 weeks of LPS exposure, a marked increase in DC numbers was evident (***P* < 0.05 versus 8 weeks of PBS, and *P* < 0.05 versus 5 days of LPS; *n* = 8/group). (C) The percentage of DCs and macrophages (Mac) that make up the CD11c⁺ antigen-presenting cell compartment in the lung was not altered at 5 days. After 8 weeks of inhalational LPS exposure, a significant increase was evident in the percentage of lung CD11c⁺ APCs that were DCs (**P* < 0.05 versus 8 weeks of PBS), accompanied by a marked reduction in the percentage of macrophages (***P* < 0.05 versus 8 weeks of PBS; *n* = 8/group). (D) Representative flow cytometry plots of lung digests gated on CD11c⁺ cells demonstrate two populations of APCs, based on side scatter and major histocompatibility class (MHC) Class II expression. MHC Class II^{hi}, DCs; MHC Class II^{int}, macrophages (*n* = 8/group). After 8 weeks of inhalational LPS, a striking increase was observed in the percentage of lung DCs, with a concomitant reduction in the percentage of macrophages. (E) Myeloid DC subset analysis demonstrated a statistically significant increase in the percentage of inflammatory (CD11b⁺) DCs after 8 weeks of inhalational LPS exposure (*P* < 0.05 versus 8 weeks of PBS). A trend was evident toward an increase after 5 days of exposure that did not reach statistical significance (*n* = 8/group).

Figure 5. Lung DCs and macrophages are morphologically and functionally distinct. (A) Lung DCs ($CD11c^{+}MHC\ Class\ II^{hi}SiglecF^{-}$) and macrophages ($CD11c^{+}MHC\ Class\ II^{int}SiglecF^{+}$) were isolated from LPS-treated C57BL/6 mice by flow cytometry (as shown in Figure 4 and Figure E2). Cytospins were prepared for the assessment of cell morphology. Representative images of each APC subset (a, macrophages; b, DCs) are shown. (B) In contrast to macrophages, lung DCs significantly augmented the proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled, ovalbumin (OVA)-specific $CD4^{+}$ T cells, as determined by flow cytometry (* $P < 0.05$ versus Mac). (C) Lung DCs differentially augmented OVA-specific $CD4^{+}$ T-cell activation as measured by IL-2 production, compared with lung macrophages (* $P < 0.05$ versus Mac).

Prolonged Inhalational LPS Exposure Promotes Accumulation of Lung Inflammatory Myeloid DCs

DC subset analyses were also performed to determine the impact of LPS exposure on myeloid DC development, recruitment, and numbers. $CD11c^{+}MHC\ Class\ II^{hi}$ DC subsets were identified by the expression of CD11b and CD103, as previously described (Figure E2D). The two main populations of myeloid lung DCs, namely, the $CD103^{+}$ and $CD11b^{+}$ subsets, were increased in

both the 5-day and 8-week LPS mice. No significant differences were observed in percentages of the $CD103^{+}$ and $CD11b^{+}$ DC subsets after 5 days of LPS. In contrast, in 8-week LPS mice, a significant increase was evident in the percentage of $CD11b^{+}$ DCs, a subset that was characterized as inflammatory DCs (8-week LPS mice, $47\% \pm 2.0\%$, versus 8-week PBS mice, $26\% \pm 3.1\%$) (Figure 4E) (25). The term “inflammatory DC” refers to the subpopulation of cells characterized in the literature that are $CD11b^{hi}$ and have been observed in increased numbers in the setting of lung inflammation after a variety of stimuli (apart from chronic endotoxin, as described in this study), including ovalbumin (OVA) allergic lung inflammation, house dust mite allergic lung inflammation, lung infections, and cigarette smoke (23, 26–31). These inflammatory DCs have been reported to involve distinct phenotypic and effector functions, and have been shown to arise from distinct blood precursors. Elegant work has shown that these inflammatory lung DCs arise from $CCR2^{+}Ly6C^{hi}$ monocytes in the setting of lung inflammation (32). Consistent with this, we also observed a marked increase in the number of lung $Ly6C^{hi}$ monocytes after inhalational LPS exposure, most notably at 8 weeks (data not shown).

Lung Macrophages and DCs Are Morphologically and Functionally Distinct

To assess the functional properties of lung APC subsets as already defined, lung DCs ($CD11c^{+}MHC\ Class\ II^{hi}SiglecF^{-}$) and macrophages ($CD11c^{+}MHC\ Class\ II^{int}SiglecF^{+}$) from LPS-treated mice and PBS control mice were sorted by flow cytometry. Lung macrophages demonstrated a characteristic morphology, with abundant cytoplasm and round nuclei (Figure 5A, a). Lung DCs were more heterogeneous and included cells with prominent dendrites (Figure 5A, b) and veiled cells. The lung APC subsets were cultured with carboxyfluorescein succinimidyl ester (CFSE)-labeled, OVA-specific $CD4^{+}$ T cells isolated from OT-II (ovalbumin-specific mouse T-cell receptor) transgenic mice and increasing concentrations of OVA. $CD4^{+}$ T-cell proliferation and activation were determined by flow cytometry and IL-2 production, respectively. Consistent with the well-described antigen-presenting properties of DCs, the sorted lung DCs from LPS-treated mice exhibited significantly augmented OT-II $CD4^{+}$ T-cell proliferation (Figure 5B) and IL-2 cytokine production (Figure 5C), in contrast to the macrophage subset with only a modest effect on T-cell proliferation. These results are in keeping with the differential ability of these cell types to process antigens, including OVA (33). In contrast to DCs, macrophages rapidly degrade internalized proteins, whereas DCs maximize the generation of immunogenic peptides for MHC Class II-peptide complex formation. Lung DCs from LPS-treated mice exhibited an augmented ability to stimulate OT-II $CD4^{+}$ T cells, compared with DCs isolated from PBS-treated mice (Figure E2C).

Eight-Week LPS Mice Exhibited Increased Lung Neutrophil Staining and Peri-Airway Inflammatory Aggregates that Stained Positive for APCs

To determine the localization of the increased cellularity observed in the flow cytometry experiments, we performed hematoxylin-and-eosin staining of lung sections from 5-day and 8-week mice. Although increased inflammation was evident in both LPS groups, the magnitude of cellular infiltration was more pronounced at 8 weeks than at 5 days. Moreover, the 8-week LPS mice exhibited peri-airway inflammatory aggregates that were not observed in the 5-day mice (Figure 6, d versus b). Gr-1 staining revealed increased parenchymal neutrophil infiltration at 5 days and 8 weeks of LPS, with a more pronounced

increase in neutrophils at 8 weeks (Figure E3, *d* versus *b*). However, increased Gr-1 staining was *not* observed to be localized to the peri-airway aggregates in the 8-week LPS mice.

Consistent with the marked increase in the number of APCs, an increase was evident in the number of lung CD11c⁺ cells in both the 5-day and 8-week LPS mice. However, the 8-week LPS mice exhibited more positive cells than did the 5-day LPS mice. Moreover, localized CD11c staining occurred in the peri-airway aggregates in the 8-week LPS mice (Figure 6, *h*). Cells with the morphologic characteristics of DCs, with long cytoplasmic and interdigitating processes and large, round macrophages with abundant cytoplasm (Figure 6, *i* and *j*, respectively), were observed, consistent with the flow cytometry data (Figure 4). Consistent with the inversion of DC/macrophage ratios observed, less pronounced Mac-3 staining was evident in the 8-week LPS mice compared with the 5-day LPS mice (Figure E3, *h* versus *f*).

DISCUSSION

In this study, we demonstrate that in a murine model mimicking the weekday cycle of occupational endotoxin exposure in cotton textile workers (34), increased airflow resistance and airway hyperreactivity (Figure 1) are accompanied by increased lung neutrophils with longer durations of exposure (Figures 2 and 4). These findings are associated with a striking inversion of the ratio of macrophages to DCs, with an increase in lung proinflammatory DCs after 8 weeks of LPS exposure (Figures 4–6). Gene expression data support the presence of a “gene signature” of APC recruitment in the 8-week mice (Figure 3). These findings support a novel mechanism in occupational endotoxin-related lung disease, and suggest that the persistent neutrophilic proinflammatory response we observed in the 8-week LPS mice is driven by an expansion of DCs in the lung. DCs are critical in antigen presentation and in modulating innate and adaptive immune responses. Hence, the failure to resolve the proinflammatory response may be explained by triggering APC pathways that provide an ongoing platform for neutrophil and other leukocyte recruitment.

In contrast to DCs, which are considered the most potent APCs, lung macrophages exhibit poor T-cell stimulatory capacity, and have been demonstrated to play an anti-inflammatory role in the lung. Lung macrophages that outnumber stimulatory DCs at steady state have been shown to inhibit DC antigen presentation

and airway hyperresponsiveness *in vivo* (35–38). The inversion of the ratio of lung macrophages to DCs observed in our study has been observed in a murine model of silicosis (39) and in an even more relevant human study of inhalational LPS (40), suggesting that the balance between these myeloid populations may play a critical role in the development of other environmental lung diseases. However, the associations between APCs, the persistence of proinflammatory responses, and the physiologic responses we report have not been previously described, to the best of our knowledge.

One potential explanation for the DC/macrophage inversion we observed is that the inflammatory environment provided by chronic inhaled endotoxin promotes the development of DCs over macrophages. Previous work demonstrated that resident alveolar macrophages are rapidly replaced by recruited monocytes after intratracheal instillations of LPS (41). This replacement is likely mediated by inflammatory mediators such as CCL2 and its receptor CCR2 that is highly expressed in inflammatory monocytes, as was shown in endotoxemia and microbial infection models (32, 41). Intriguingly, *Ccr2* was one of four genes that we found to be differentially expressed in lung homogenates after LPS exposure for 8 weeks, but not at 5 days (Figure 3). In addition, although we did not explore this possibility, macrophage apoptosis may also play a role in this model. Macrophage apoptosis was shown to be induced by LPS, and increased rates of apoptosis were observed in macrophages recruited to the lung in an inhalational LPS model of acute lung injury (42).

Further analysis of the significant increase in DC numbers observed at 8 weeks of LPS demonstrated a rise in both CD103⁺ and CD11b⁺ subsets of myeloid lung DCs. However, there was a greater increase in the inflammatory DC (CD11b⁺) subset that has been shown to develop from monocytes in the setting of inflammation (Figure 4E) (32, 43). This may be an important contributing mechanism to the airway and lung neutrophil recruitment observed at 5 days, and more prominently at 8 weeks. The intratracheal administration of LPS was previously shown to augment significantly the intracellular staining of keratinocyte-derived chemokine (KC) and macrophage inhibitory protein (MIP)-2, potent chemokines in neutrophil chemotaxis and activation, in lung CD11b^{hi} DCs (26). An important role for DCs in this chronic endotoxin exposure model is further supported by the expansion of the adaptive immune compartment, with a significant increase in CD4⁺ and CD8⁺ T and B cells. Monocyte-derived CD11b⁺ lung

Figure 6. After 8 weeks of LPS exposure, lung sections reveal increased overall inflammation with peri-airway inflammatory aggregates that stain positive for CD11c, consistent with the localized expansion of mononuclear and DC populations. Representative lung sections obtained from mice harvested after the exposure protocol were stained for hematoxylin and eosin and CD11c (from $n =$ at least 3 per group). Both LPS-exposed groups exhibited an overall increase in inflammatory cells, with a more pronounced increase at 8 weeks (*d*) compared with 5 days (*b*) of LPS. Moreover, 8-week LPS mice exhibited peri-airway inflammatory aggregates (*d*, arrow). CD11c staining revealed an increased number of positive cells in both LPS-exposed groups, with an increase in staining at 8 weeks (*h*) compared with 5 days (*f*) of LPS exposure. The 8-week LPS mice exhibited a localization of CD11c⁺ cells in the airway inflammatory aggregates (*h*, arrow), which upon further magnification appeared consistent with DCs (*i*, arrows pointing to dendrites) and macrophage (MAC) morphology (*j*).

DCs have been shown to play a critical role in the expansion of lung antigen-specific CD4⁺ T cells and the initiation of lung-specific adaptive immunity (32). The expansion of the lymphocyte compartment may be facilitated by the increased concentrations of IL-12p40 observed in lung homogenates from 8-week LPS-exposed mice (Figure E2). IL-12 is potently induced in DCs by LPS, and is critical in the coordination of cell-mediated innate and adaptive immune responses to microbial pathogens by DCs (44, 45). In addition, DCs were recently shown to remain persistently activated after the resolution of allergic inflammation and to promote the development of airway inflammation in response to neoantigens, lending further support to the relevance of this mechanism in environmental lung disease (46).

Additional contributors to the immune and physiologic phenotype observed are likely. DCs are critical for the development of regulatory T cells (Treg). In both the lung and gut, IL-10 and the CD103⁺ DC subset promote Treg development (47, 48). The expansion of the inflammatory CD11b⁺ DC subset, as well as the decreased IL-10 concentrations observed, may lead to decreased numbers of Tregs, contributing to the failure of resolution observed in the 8-week inhalational exposure. LPS can also affect the function of resident or recruited Tregs. The ligation of Toll-like receptors on DCs by LPS has been shown in elegant work to overcome the regulatory properties of CD4⁺ CD25⁺ Tregs (49), which could lead to further amplification of the inflammatory response. Additional studies will be necessary to characterize the specific roles of immune cell subsets and DCs in the development of airway hyperresponsiveness in this model of chronic endotoxin exposure, and these roles are the subject of ongoing study.

The interesting cellular subsets we identified in the 8-week LPS mice may reflect the unique exposure protocol that we developed to replicate occupational environmental exposure and that could be performed in our laboratory without specialized equipment. Although our microarray analysis produced an interesting, novel "gene signature" of APC recruitment, we were reassured to find that our lung physiology findings and patterns of up-regulated genes and pathways at 5 days and 8 weeks overlapped with those reported by investigators using previously published models of chronic endotoxin exposure (14, 50), even with the use of different types of endotoxin, murine strains, and exposure protocols. Further analyses of the similarities and differences in our microarray datasets comprise the subject of ongoing work. In addition, we believe that our observations regarding APC cell subsets were possible only with the use of flow cytometry of lung digests, because analyses of BAL fluid, as routinely performed in many endotoxin exposure studies, do not fully reflect the flux in inflammatory cell subsets.

The limitations of our study include our approach of studying only one component (endotoxin) in a complex environmental exposure, as well as the limitations of correlating any murine model with human disease. In addition, we observed a correlation between physiologic dysfunction and changes in inflammatory cell subsets, and further complex experiments will be necessary to demonstrate the causality of changes in cell populations in producing altered phenotypes. Additional contributors to the immune and physiologic phenotype observed are likely. The expansion of the inflammatory CD11b⁺ DC subset, as well as decreased IL-10 concentrations, observed after 8 weeks of LPS exposure may be important in the failure to resolve inflammation. Additional studies will be necessary to characterize the specific roles of immune cell subsets and DCs in the development of airway hyperresponsiveness in this model of chronic endotoxin exposure.

In conclusion, we demonstrated that a murine model of repeated endotoxin inhalation that mirrors human occupational

exposure produces airflow obstruction and airway hyperreactivity. These physiologic findings were correlated with increased lung neutrophils and an expansion of DCs in the lung, with a resultant inversion of the ratio of DCs to macrophages after 8 weeks of endotoxin exposure. Moreover, we observed localized peri-airway inflammation that stained positive for myeloid lineage cells that may contribute to the observed physiologic responses to chronic endotoxin inhalation. Finally, our microarray analysis produced a plausible gene signature of myeloid recruitment to the lung that has the potential to aid in the discovery of additional underlying mechanisms of occupational exposures, and ultimately in further phenotyping disease states. Future studies will be important for targeting lung APC recruitment, with the ultimate goal of ameliorating the effects of chronic endotoxin exposure.

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