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Cutting Edge: Role of NK Cells and Surfactant Protein D in Dendritic Cell Lymph Node Homing: Effects of Ozone Exposure

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The roles of NK cells, surfactant protein D (SP-D), and IFN-γ, as well as the effect of ozone (O₃) inhalation, were studied on recirculation of pulmonary dendritic cells (DC) to the mediastinal lymph nodes. O₃ exposure and lack of SP-D reduced NK cell IFN-γ and lung tissue CCL21 mRNA expression and impaired DC homing to the mediastinal lymph nodes. Notably, addition of recombinant SP-D to naive mononuclear cells stimulated IFN-γ release in vitro. Because Nkp46, a glycosylated membrane receptor, was necessary for dose-dependent SP-D binding to NK cells in vitro and DC migration in vivo, we speculate that SP-D may constitutively stimulate IFN-γ production by NK cells, possibly via Nkp46. This mechanism could then initiate the IFN-γ/IL-12 feedback circuit, a key amplifier of DC lymph node homing. Inhibition of this process during an acute inflammatory response causes DC retention in the peripheral lung tissue and contributes to injury. The Journal of Immunology, 2016, 196: 000–000.

Dendritic cell (DC) lymph node homing is required to achieve optimal host defense, inflammatory resolution, and immune homeostasis in the lung. To reach the draining mediastinal lymph nodes, DC are driven by CCR7, activated by its ligands CCL19 and CCL21 (1). We previously demonstrated that NK cell–derived IFN-γ and lung tissue CCL21 mRNA expression and impaired DC homing to the mediastinal lymph nodes. Notably, addition of recombinant SP-D to naive mononuclear cells stimulated IFN-γ release in vitro. Because Nkp46, a glycosylated membrane receptor, was necessary for dose-dependent SP-D binding to NK cells in vitro and DC migration in vivo, we speculate that SP-D may constitutively stimulate IFN-γ production by NK cells, possibly via Nkp46. This mechanism could then initiate the IFN-γ/IL-12 feedback circuit, a key amplifier of DC lymph node homing. Inhibition of this process during an acute inflammatory response causes DC retention in the peripheral lung tissue and contributes to injury. The Journal of Immunology, 2016, 196: 000–000.

Materials and Methods

Mice and model of O₃ exposure

Seven- to ten-week-old male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME), SP-D−/− mice (a gift from Drs. Samuel Hawgood, University of California, San Francisco, and Francis Poulin, University of California, Davis), and Nkp46−/− mice (a gift from Drs. O. Mandelboim, Hebrew University, and Wayne Yokoyama, Washington University) were exposed to 3 ppm O₃ or filtered air for 2 h and sacrificed at different time points to obtain bronchoalveolar lavage (BAL), lung tissue, and mediastinal lymph nodes (6). All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Tissue processing and analysis, RNA isolation, real-time quantitative PCR (qPCR), and flow cytometry were performed as described previously (2, 6, 7).

Pulmonary DC migration assay

Mice were anesthetized (ketamine/xylazine, i.p., 100/20 mg/kg; Butler, Dublin, OH; Akorn, Lake Forest, IL), given 50 μl CFSE (8 mM, intra-
tracheally [i.t.; Fluka, St. Louis, MO], and exposed to O₃ or filtered air 6 h later. Lymph nodes were harvested and processed for FACS analysis (2).

Ex vivo SP-D binding to NK cells

Splenocytes were isolated from naive wild-type (WT) mice and cultured or not with recombinant SP-D (rSP-D; 1 μg/ml; Sino Biological, Shanghai, China) for 48 h. Released IFN-γ was assayed by ELISA.

Pulmonary lymphocytes were enriched from naive SP-D−/− mice and NKp46−/− mice. NKp46 expression was determined using FACS gated on NK cells (NK1.1+CD3+).

Cells were incubated with rSP-D (0 [control], 2, or 20 μg/ml) for 2 h at 4°C. Surface binding of SP-D on NKp46+ cells (from SP-D−/− lungs) and on NK1.1+CD3+ cells (from NKp46−/− lungs) was assessed by FACS.

Data analysis

A Student t test with Welch correction (unpaired, one-tailed) was performed (Prism 6 software; GraphPad, La Jolla, CA), with the Bonferroni correction for multiple comparisons, unless otherwise indicated. A p value <0.05 was considered statistically significant.

Results and Discussion

O₃-induced pulmonary inflammation was associated with impaired DC lymph node homing

Neutrophilic granulocyte infiltration to the airways, together with IL-6 and KC release, was accompanied by the accumulation

![Image of a graph showing BAL differential cell count, BAL cytokines, and BAL DCs.](http://www.jimmunol.org/)

**FIGURE 1.** O₃-induced neutrophilic airway inflammation was associated with suppression of DC lymph node homing, inhibition of CCL21 mRNA, and reduced IFN-γ production by NK cells. C56BL/6 mice exposed to O₃ or filtered air (for 2 h) were sacrificed 12 h later or as indicated. (A) BAL differential cell counts of macrophages (MP), eosinophils (EP), neutrophils (NP), and lymphocytes (LC) (Diff-Quik–stained cytospin preps). (B) BAL KC and IL-6 measured by ELISA. Data in (A) and (B) are mean ± SEM (n = 8–10). Representative FACS plots of TNF-α+ DC% in CD11c+ BAL cells (n = 12, *p < 0.05, Student t test) (C) and lymph node DC (left panels) and CD11b+CD103− and CD11b−CD103+ subsets (D). (E) CFSE was instilled (i.t.) 6 h before O₃ exposure. Percentage of DC of all cells, percentage of CFSE+ of total DC and CD11b+ or CD103+ total DC number in the mediastinal lymph node. (F and G) Lung mRNA was quantified by a qPCR microarray. Data points in (F) and (G) (n = 6–8) were obtained from three independent experiments. *p < 0.05, **p < 0.01, versus 0 h, ANOVA with Bonferroni correction. (H) Intracellular IFN-γ in pulmonary NK cells. Representative FACS plot from three independent experiments. (I) Number of IFN-γ+ lung NK cells. Data in (E) and (I) are mean ± SEM of n = 6–8 mice pooled from two or three independent experiments. *p < 0.05, Student t test with Welch correction; filtered air versus O₃.
of activated TNF-α+ DC in BAL in O3-exposed mice 12 h after exposure (Fig. 1A–C). According to dogma, DC lymph node homing is significantly induced during the inflammatory airway response (1). However, distinct from allergen challenge (5), we saw no increase in DC numbers in draining mediastinal lymph nodes harvested 12 h after O3 exposure. In fact, the proportion of DC (CD11c^{high}MHCII^{high}), especially the CD103+ population (denoting mucosal DC that migrated from the distal air spaces), was decreased in the posterior mediastinal lymph nodes of the O3-exposed group (Fig. 1D, 1E). To confirm that such a decrease reflected impaired DC migration, we labeled airway cells by instilling CFSE prior to O3 exposure of mice and found that the CFSE+ migratory DC proportion was significantly reduced in the lymph nodes (Fig. 1E, left panel). This change did not reflect an influx of unlabeled DC from the circulation, because circulatory DC retain an immature phenotype with low expression of CCR7 that is required for thoracic lymph node entry through the high endothelial venules. Indeed, the total numbers of CD11b–CD103+ DC in the lymph node 12 h post-O3 was also significantly decreased (Fig. 1E, right panel). Thus, O3-induced airway inflammation was associated with the increased presence of TNF-α+ DC in the air spaces and decreased mucosal DC homing to the mediastinal lymph nodes, suggesting that impaired regulation of DC migration could amplify airway inflammation.

A dynamic shift in DC numbers and phenotype, including reduced CCR7 expression, was found upon chronic O3 exposure in a different study (8). CCR7 activation by the specific ligands CCL19/21 is essential for lymph node homing (1). Although the mechanisms that regulate CCL19/21 expression are unclear, NK cell–derived IFN-γ was implied (2). In this study, we found that O3-suppressed DC lymph node homing was associated with inhibition of mRNA for CCL21 and IFN-γ (Fig. 1F, 1G). Because naive T lymphocytes are incapable of producing IFN-γ (9), the likely source of IFN-γ in the normal lung is the NK cell. Notably, the level of NK cell IFN-γ expression, as well as the total number of IFN-γ+ NK cells, was reduced in O3-treated mice (Fig. 1H, 1I), implying NK cell–derived IFN-γ in the underlying pathways of O3-inhibited DC lymph node homing. Our data were corroborated by another study in which chronic O3 exposure suppressed IFN-γ production by NKT cells (10). NK cell IFN-γ expression was also dampened upon coculture with O3-exposed epithelial cells (11), suggesting that epithelial-derived mediators may play a regulatory role in this process.

**FIGURE 2.** Presence of SP-D promoted IFN-γ release and DC migration to lymph nodes. (A) IFN-γ (ELISA) from naive WT splenocytes cultured with rSP-D (1 μg/ml) for 48 h. (B) CCL-21 and IFN-γ mRNA in the lung of naive WT and SP-D^{−/−} mice (qPCR, normalized to 0 h). (C–F) WT and SP-D^{−/−} mice were exposed to O3 or filtered air for 2 h. BAL was obtained 12 h later. (C) BAL neutrophils were determined by total and differential cell counts using Diff-Quik–stained cytospin preparations. (D) Representative data for TNF-α+CD11c+ BAL cells (n = 7–8). (E) Percentage of CD11c+MHCII+CFSE+ DC in WT and SP-D^{−/−} mediastinal lymph nodes 12 h post-O3. CFSE was instilled (i.t.) 6 h prior to O3. (F) IFN-γ mRNA in the lung of WT and SP-D^{−/−} mice 12 h post-O3 (qPCR). Data in (A)–(C) and (F) are mean ± SEM of n = 6–8 (three repeat experiments). FACS plots in (D) and (E) are representative of two independent experiments. *p < 0.05, Student t test with Welch correction, rSP-D versus medium (A), WT versus SP-D^{−/−} (B and C).
SP-D promoted IFN-γ release and DC migration to lymph nodes

SP-D is an immune-regulatory product of the distal epithelium with the capability to induce IFN-γ in immune cells (7). Naive splenocytes cultured with murine rSP-D significantly increased IFN-γ release (Fig. 2A). This effect was specific and selective because surfactant protein A did not change IFN-γ expression, and other cytokines (IL-4 or IL-5) were not affected by SP-D (data not shown). Compared with WT mice, SP-D−/− mice had reduced expression of pulmonary IFN-γ and CCL21 mRNA (Fig. 2B). Thus, although NK cells were activated in the lung of SP-D−/− mice, their capacity to produce IFN-γ was diminished.

Compared with WT mice, O3-induced neutrophilia and DC numbers (with a markedly elevated proportion of activated TNF-α+ cells) were enhanced in the BAL of SP-D−/− mice (Fig. 2C, 2D). After CFSE treatment, the CFSE+ DC count was reduced in the mediastinal lymph nodes of SP-D−/− mice (Fig. 2E). Pulmonary IFN-γ expression also showed a trend toward reduction post-O3 (Fig. 2F). Previous studies showed that a differential ability of mice to develop inflammation was inversely associated with the amount of SP-D in the airways: genetically low SP-D–producing BALB/c or SP-D−/− mice were highly susceptible to O3-induced airway inflammation with a prolonged and heightened neutrophilia (6). In our study, O3-induced neutrophilia, TNF-α+ DC accumulation in the airways, and diminished CFSE+ DC migration to the lymph nodes were all amplified in SP-D−/− mice. These support that SP-D is necessary to maintain pulmonary DC lymph node homing. Our data also raised the possibility that SP-D plays a role through stimulation of IFN-γ by NK cells.

SP-D bound to NK cells in vitro through Nkp46

Whether SP-D can directly bind NK cells was not previously known. We hypothesized that one of the membrane receptors, Nkp46, may mediate specific SP-D binding and its effects on NK cells. We chose Nkp46 over the other natural cytotoxicity receptors (Nkp30 and 44) because it is evolutionarily conserved (the only member of the NCR family found in mice), constitutively expressed, and capable of inducing IFN-γ synthesis.
and release by NK cells (through activation of PI3K and PLC-γ) (12). SP-D can bind cell surface glycoconjugates (4), and NKp46 is a highly glycosylated receptor (13).

SP-D–/– pulmonary NK cells expressing NKp46, but not NK cells from NKp46–/– mice (14) (Fig. 3A–C), bound SP-D in a dose-dependent manner. This suggested that NKp46 could indeed mediate SP-D–induced IFN-γ production by NK cells. This was supported by our data on DC lymph node homing: although the total percentages of DC remained similar between WT and NKp46–/– mice, upon CFSE labeling, DC migration from the lungs to the posterior mediastinal lymph nodes was diminished in the absence of NKp46, indicating a migration defect (Fig. 3D). This defect was accompanied by a significant reduction in lung IFN-γ expression (Fig. 3E).

Interactions of SP-D with NK cells through NKp46 may play an important role in homeostatic DC lymph node homing and maintenance of an inflammation-free pulmonary environment. They could also provide the initial activation of T cell–independent IFN-γ release. Our study suggests that O3 inhalation suppresses SP-D/NKp46/IFN-γ– and CCL21-mediated DC recirculation to the mediastinal lymph nodes. This effect may contribute to acute lung injury.

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Disclosures
The authors have no financial conflicts of interest.

References
Corrections


The ninth author’s name was published incorrectly as Michael D. Kemeny. The correct name is David M. Kemeny.

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