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Authors

Ge, Moyar Qing
Kokalari, Blerina
Flayer, Cameron H
et al.

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Role of natural killer cells and surfactant protein D in dendritic cell lymph node homing: Effects of ozone exposure

Moyar Qing Ge[1,2,3], **Blerina Kokalari**[2], **Cameron H. Flayer**[1], **Sarah S. Killingbeck**[1], **Imre G. Redai**[2], **Alexander Macfarlane**[4], **Jin W. Hwang**[2], **Anisha Kolupoti**[2], **Michael D. Kemeny**[3], **Kerry Campbell**[4], and **Angela Haczku**[1,2]

[1]University of California, Davis, CA

[2]University of Pennsylvania, Philadelphia, PA

[3]National University of Singapore, Singapore

[4]Fox Chase Cancer Center, Philadelphia, PA

Abstract

The role of natural killer (NK) cells, surfactant protein D (SP-D), IFN-γ and the effect of ozone (O_3) inhalation was studied on recirculation of pulmonary dendritic cells (DC) to the mediastinal lymph nodes. Both O_3 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 naïve 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.

INTRODUCTION

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 or CCL21 (1). We previously demonstrated that NK-cell derived IFN-γ was necessary for CCL21 expression and DC lymph node homing in influenza A virus infected mice (2). Pulmonary NK cells are capable of releasing large quantities of IFN-γ both in homeostatic and inflammatory conditions (3). While the trigger for this NK cell function is not known, lung-specific factors were suggested to be important. SP-D, an epithelial pulmonary collectin with prominent immune regulatory properties, is a likely local candidate to regulate NK cells. SP-D expression increases during airway inflammation and plays an important role in

Correspondence to: Angela Haczku, MD, PhD, Professor of Medicine, Director, Translational Lung Biology Center, Pulmonary, Critical Care & Sleep Medicine, UC Davis, One Shields Avenue, Davis, CA 95616-5270, haczku@ucdavis.edu, phone: 530-754-6932, fax: 530-752-8632.

inflammatory resolution. SP-D$^{-/-}$ mice spontaneously develop an abnormal pulmonary immune phenotype (4) and show impaired pulmonary DC lymph node homing (5).

We chose to study O_3-induced airway inflammation because in this model there is no specific antigen uptake involved. Therefore, following O_3 inhalation DC migration should be primarily modulated by tissue changes. O_3 mainly affects the distal air spaces, inducing epithelial damage with release of pro-inflammatory mediators and cellular infiltration of the lung of both humans and mice (6) within a few hours of exposure.

Based upon the proximity of lung residential DC, NK cells and SP-D in the distal air spaces, we hypothesized that NK cell-SP-D interactions activate IFN-γ release at the peripheral ends of lymphatic vessels that facilitates DC lymph node homing. We further hypothesized that this process is altered upon O_3 exposure.

MATERIALS AND METHODS

Mice and model of O3 exposure

Seven-to ten week old male C57BL/6 mice (Jackson Laboratory, ME) SP-D$^{-/-}$ mice (a gift from Drs. Samuel Hawgood UCSF and Francis Poulain, UC Davis) and NKp46$^{-/-}$ mice (a gift from Dr. O. Mandelboim, Hebrew University and Dr. Wayne Yokoyama, Washington University) were exposed to 3 parts per million (ppm) O_3 or filtered air for 2 h and sacrificed at different time points to obtain BAL, lung tissue and mediastinal lymph nodes (6). All procedures were approved by the IACUC of the University of Pennsylvania.

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

Pulmonary dendritic cell migration assay

Mice were anesthetized (ketamine/xylazine, i.p., 100/20 mg/kg; Butler, OH; Akorn, IL) and given 50 μl of CFSE (8 mM; Fluka, MO; i.t.), then exposed to O_3 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 naïve wild type (WT) mice and cultured with or without rSP-D (1μg/ml, Sino Biological Inc. China) for 48 h. Released IFN-γ was assayed by ELISA.

Pulmonary lymphocytes were enriched from naïve 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 [ctrl], 2 or 20μg/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

Student's t-test with Welch's correction (unpaired, one-tailed) was performed (Prism 6 software, GraphPad Inc., CA) with Bonferroni's correction for multiple comparisons unless otherwise indicated. Data are expressed as mean±SEM; $p<0.05$ 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 accumulation of activated, TNF-α^+ DC in the bronchoalveolar lavage in O_3 exposed mice 12 h after exposure (Figure 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 O_3 exposure. In fact, the proportion of dendritic cells ($CD11c^{high}MHCII^{high}$), especially the $CD103^+$ population (denoting mucosal dendritic cells that migrated from the distal air spaces) was decreased in the posterior mediastinal lymph nodes of the O_3 exposed group (Figure 1D, E). To confirm that such decrease reflected impaired DC migration, we labeled airway cells by instilling CFSE prior to O_3 exposure of mice and found that the $CFSE^+$ migratory DC proportion was significantly reduced in the lymph nodes (Figure 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 the $CD11b^-CD103^+$ DC in the lymph node 12 h post O_3 was also significantly decreased (1E right panel). Thus, O_3 induced airway inflammation was associated with 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 CCR7expression was found upon chronic O_3 exposure in a different study (8). CCR7 activation by the specific ligands CCL19/21 is essential for lymph node homing (1). While the mechanisms that regulate CCL19/21 expression are unclear, NK cell derived IFN-γ was implied (2). Here we found that O_3-suppressed DC lymph node homing was associated with inhibition of mRNA for both CCL21 and IFN-γ (Figure 1F–G). As naïve T lymphocytes are incapable of producing IFN-γ (9), the likely source of IFN-γ in the normal lung is the NK cell. Notably, not only the level of NK cell IFN-γ expression but also the total number of IFN-γ^+ NK cells were reduced in the O_3 treated mice (Figure 1H–I) implying NK cell-derived IFN-γ in the underlying pathways of O_3-inhibited DC lymph node homing. Our data were corroborated by another study in which chronic O_3 exposure suppressed IFN-γ production by NK T cells (10). NK cell IFN-γ expression was also dampened upon co-culture with O_3 exposed epithelial cells (11), suggesting that epithelial-derived mediators may play a regulatory role in this process.

Surfactant protein D (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 of inducing IFN-γ in immune cells (7). Naïve splenocytes cultured with murine recombinant SP-D significantly increased IFN-γ release (Figure 2A). This effect was specific and selective because SP-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 wild type mice, SP-$D^{-/-}$ mice had reduced pulmonary IFN-γ and CCL21 mRNA expression (Figure 2B). Thus, while NK cells were activated in the lung of SP-$D^{-/-}$ mice, their capacity to produce IFN-γ was diminished.

Compared to WT mice, O_3 induced neutrophilia and DC numbers (with a markedly elevated proportion of activated, TNF-α^+ cells) were enhanced in the BAL of SP-$D^{-/-}$ mice (Figures 2C, D). After CFSE treatment, the $CFSE^+$ DC count was reduced in the mediastinal lymph nodes of SP-$D^{-/-}$ mice (Figure 2E). Pulmonary IFN-γ expression also showed a trend of reduction post O_3 (Figure 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 producer BALB/c or SP-$D^{-/-}$ mice were highly susceptible to O_3-induced airway inflammation with a prolonged and heightened neutrophilia (6). In our study, O_3-induced neutrophilia, TNF-α^+ DC accumulation in the airways and the 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 (Figure 3A–C), bound SP-D dose dependently. 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: While the total percentages of DC remained similar between wild type 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 (Figure 3D). This defect was accompanied by a significant reduction in lung IFN-γ expression (Figure 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. It could also provide the initial activation of T cell independent IFN-γ release. Our study suggests that O_3 inhalation suppresses the 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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Abbreviations

BAL	Bronchoalveolar lavage
DC	Dendritic cell(s)
EP	eosinophil
LC	lymphocyte
MP	macrophage
NK	Natural Killer
NP	neutrophil
rSP-D	recombinant surfactant protein D
WT	wild type
i.t	intratracheal

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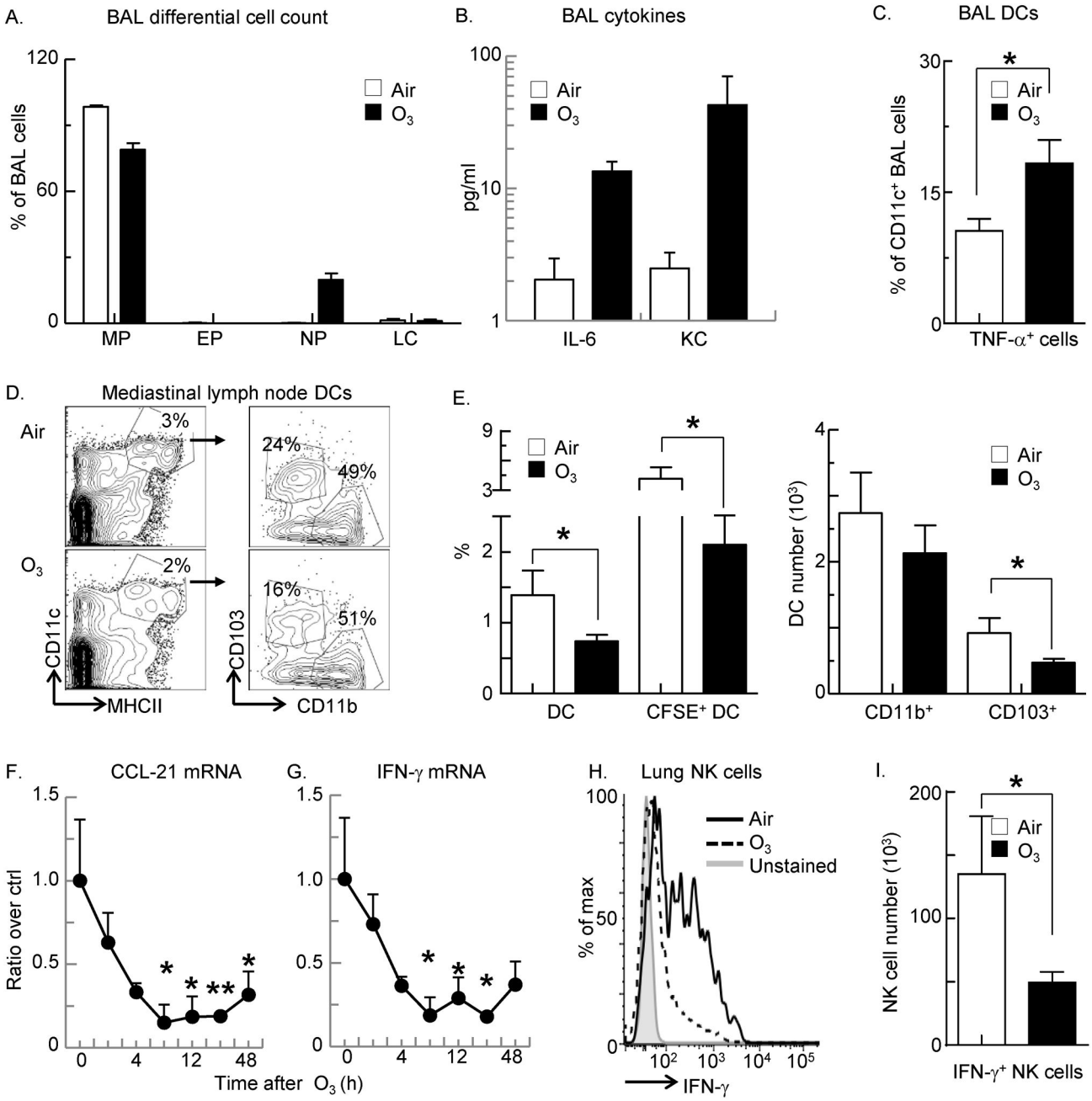

Figure 1. O_3 induced neutrophilic airway inflammation was associated with suppression of DC lymph node homing, inhibition of CCL21 mRNA and reduced IFN-γ production by NK cells

C56BL/6 mice exposed to O_3 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) (DiffKwik stained cytospin preps). **(B):** BAL KC and IL-6 measured by ELISA. Representative FACS plots of **(C):** TNF-α^+ DC% in $CD11c^+$ BAL cells (n=12, *p<0.05; Student's t test), **(D):** lymph node DC (left panels) and $CD11b^+CD103^-$ and $CD11b^-CD103^+$ subsets. **(E):** CFSE was instilled (i.t.) 6 h *before* O_3 exposure. % DC of all cells or % $CFSE^+$ of total DC. **(F–G):** Lung mRNA was quantified

by a qPCR microarray. **(H):** Intracellular IFN-γ in pulmonary NK cells. Representative FACS plot from three independent experiments. **(I):** Number of IFN-γ positive lung NK cells. (A, B): Mean±SEM of n=8–10. (E, I): Mean±SEM of n=6–8 mice pooled from 2–3 independent experiments. *p<0.05; Student's t test with Welch's correction; Filtered air *vs.* O_3. (F–G): data points (n=6–8) were obtained from three independent experiments; *p< 0.05; **p<0.01 vs. 0 h (ANOVA with Bonferroni correction).

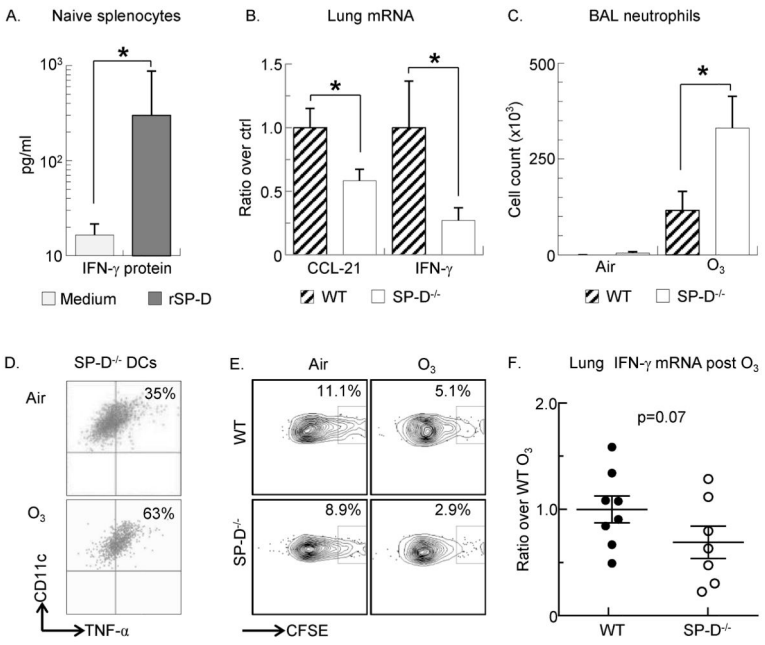

Figure 2. Presence of SP-D promoted IFN-γ release and DC migration to lymph nodes

(A): IFN-γ (ELISA) from naïve wild type (WT) splenocytes cultured with rSP-D (1μg/ml) for 48 h. **(B):** IFN-γ and CCL-21 mRNA in the lung from naïve WT and SP-$D^{-/-}$ mice (qPCR, normalized to 0hr) **(C–F):** WT and SP-$D^{-/-}$ mice were exposed to O_3 or filtered air for 2 h. BAL was obtained 12 h later. **(C):** BAL neutrophils were determined by total and differential cell counts using DiffKwik stained cytospin preparations. **(D):** Representative data of TNF-α^+CD11c^+ BAL cells (n=7–8). **(E):** % CD11c^+MHC-II$^+$ CFSE$^+$ DC in WT and SP-$D^{-/-}$ mediastinal lymph nodes 12 h post O_3. (CFSE instilled [i.t.] 6 h prior O_3). **(F):** IFN-γ mRNA in the lung from WT and SP-$D^{-/-}$ mice 12 h post O_3 (qPCR). (B, D, F): mean ±SEM of n=6 (3 repeat experiments); *p< 0.05 Student's t test with Welch's correction. rSP-D *vs.* medium (A); WT *vs.* SP-$D^{-/-}$ (B,D). (C, E–F): FACS plots representative of 2 independent experiments.

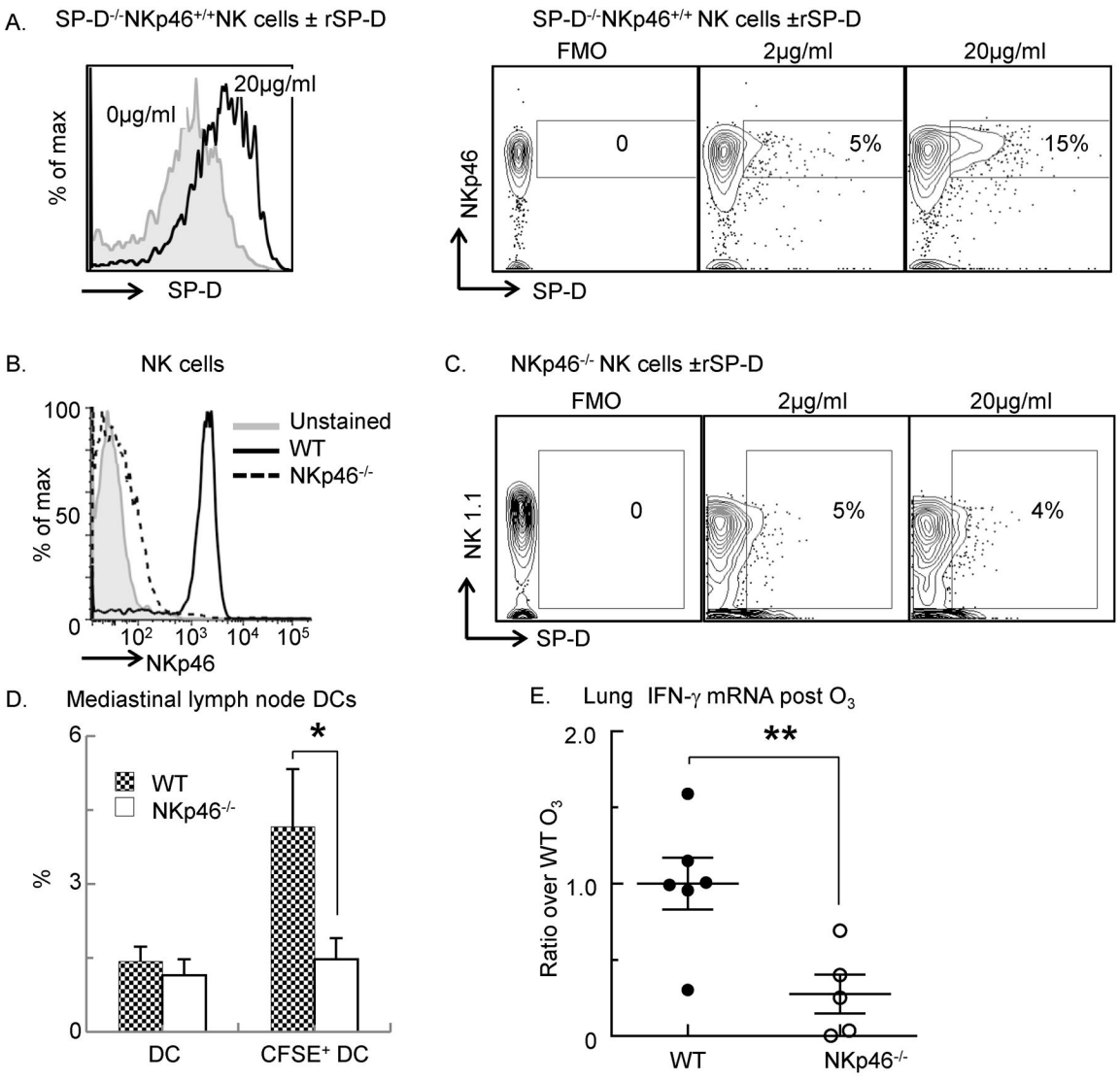

Figure 3. NKp46 was necessary for SP-D binding to NK cells and for DC lymph node homing

(A): Surface binding of SP-D on pulmonary NKp46^{+}NK1.1^{+}CD3^{-} cells from naïve SP-D$^{-/-}$ mice incubated with rSP-D *ex vivo*. FMO: Fluorescence Minus One: cells were stained with all antibodies except for anti SP-D. **(B):** NKp46 expression on pulmonary NK1.1^{+}CD3^{-} cells from naïve WT and NKp46$^{-/-}$ mice. **(C):** Surface binding of SP-D on pulmonary NK1.1^{+}CD3^{-} cells from NKp46$^{-/-}$ mice incubated with rSP-D *ex vivo*. **(D):** CFSE (i.t.) expressing DC in lymph nodes in WT and NKp46$^{-/-}$ mice 12 h post O_3. % DC of all cells or % CFSE^{+} of total DC **(E):** IFN-γ mRNA in the lung from WT and NKp46$^{-/-}$ mice 12 h post O_3 (qPCR). (A–C), 2 independent experiment. (D, E), Mean±SEM of n=6–10 mice from 2 independent experiments, *p<0.05; **p<0.01 Student's t test with Welch's correction. WT *vs.* NKp46$^{-/-}$.