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Chronic Cigarette Smoke Exposure Primes NK Cell Activation in a Mouse Model of Chronic Obstructive Pulmonary Disease

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Chronic obstructive pulmonary disease (COPD) is a debilitating, progressive lung disease punctuated by exacerbations of symptoms. COPD exacerbations are most often associated with viral infections, and exposure to cigarette smoke (CS) followed by viral infection has been shown experimentally to enhance lung inflammation, tissue destruction, and airway fibrosis. Despite this, however, the cellular mechanisms responsible for this effect are unknown. In this study, we examined NK cell function in a mouse model of COPD given the vital role of NK cells following viral infection. Ex vivo stimulation of lung leukocytes with poly(I:C), ssRNA40, or ODN1826 enhanced production of NK cell-derived IFN-γ in CS-exposed mice. NK cells from CS-exposed mice exhibited a novel form of priming; highly purified NK cells from CS-exposed mice, relative to NK cells from filtered air-exposed mice, produced more IFN-γ following stimulation with IL-12, IL-18, or both. Further, NK cell priming was lost following smoking cessation. NKG2D stimulation through overexpression of Rael1 on the lung epithelium primed NK cell responsiveness to poly(I:C), ssRNA40, or ODN1826 stimulation, but not cytokine stimulation. In addition, NK cells from CS-exposed mice expressed more cell surface CD107a upon stimulation, demonstrating that the NK cell degranulation response was also primed. Together, these results reveal a novel mechanism of activation of the innate immune system and highlight NK cells as important cellular targets in controlling COPD exacerbations.

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Lung and spleen leukocytes were cultured in cRPMI (RPMI 1640 with 2.05 mM t-glutamine [HyClone, Waltham, MA] containing 10% FBS, 1% sodium pyruvate, 100 µg/ml kanamycin, 0.05 mM 2-ME, and 1X non-essential amino acids [MP Biomedicals, Solon, OH]). A total of $5 \times 10^5$ cells per well ($2.5 \times 10^5$ cells per milliliter) in cRPMI containing 20 U/ml mouse rIL-2 (PeproTech, Rocky Hill, NJ) were aliquoted into a 96-well round-bottom culture plate (Costar, Cambridge, MA) and cultured at 37°C and 5% CO₂. Leukocytes were rested 4 h following isolation prior to stimulation. Depending on the experiment, leukocytes were stimulated with TLR ligands [endotoxin-free water (vehicle control), poly(I:C), ssRNA40, ODN1826, ODN1585, LPS-EK, PAM3CSK4, HKLm, FSL1, or ST-FLA] and splenic NK cells (20). Activated NK cells produce large amounts of IFN-γ, the effects of which are manifold (22), and NK cell-derived IFN-γ is critical to the inflammatory processes that control viral infections (20, 23, 24). Thus, NK cells are attractive mediators of the enhanced inflammatory response that occurs during COPD exacerbations.

We hypothesized that immune system activation in COPD patients is somehow “primed,” and that, following an infection, enhanced inflammation and tissue destruction occur. Specifically, we chose to examine NK cell function in a mouse model of COPD given that NK cells are critical effector cells following viral infection. We found that NK cells from CS-exposed mice were preactivated or primed and that, upon stimulation, they produced more IFN-γ and had an enhanced degranulation response. These results reveal a novel ac-
tivation of the innate immune system and highlight NK cells as important cellular targets in controlling COPD exacerbations.

Materials and Methods

Mice

C57BL/6J mice (female, 8 to 10 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). B6,129S-Ccr-rtta (× tetO)-CMV-Raet1a (FVB/NJ background, henceforth referred to as Raet1a-tg mice) were generated as described (6, 25). Raet1a-tg mice conditionally express Raet1a on the alveolar and airway epithelium in a doxycycline (DOX)-inducible manner (6, 25). Raet1a-tg mice (male and female, 8 to 10 wk old) were administered DOX in the diet for 10 d before experimental use (TD:03106 rodent diet [2018, 625 DOX]; Harlan Teklad, Indianapolis, IN). All of the experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Cincinnati Medical Center.

CS exposure

Age-matched C57BL/6J mice were exposed to either filtered, room air or smoke generated from 3R4F Kentucky Reference Cigarettes (University of Kentucky, Lexington, KY). CS exposures were carried out with a TE-10z smoking machine attached to an exposure chamber (Teague Enterprises, San Diego, CA) or cytotoxicants (recombinant mouse IL-12 and IL-18 [R&D Systems, Minneapolis, MN]) with doses described in the text. Leukocytes were stimulated for 20 h overnight, and brefeldin A (10 µg/ml; eBioscience, San Diego, CA) was added during the final 4 h of incubation. Leukocytes were harvested and washed, resuspended in FACS buffer (1X PBS, 0.5% BSA, and 0.05% NaN₃), and in-
cubated with mouse Fc block (anti-mouse FcγIII-II/CD16/CD32; eBio-
sience). Surface staining of NK cells was carried out on ice for 30 min using rat anti-mouse PE-NKp46 (clone 29A1.4; eBioscience). Leukocytes were then fixed in 2% paraformaldehyde and flow cytometry was performed using a FACS Calibur (BD Biosciences, San Jose, CA). The data were analyzed using the FlowJo software (Tree Star, Ashland, OR). Positive staining thresholds were determined from appropriate isotype control staining.

ELISAs

Ex vivo leukocyte culture supernatants were assayed for inflammatory mediators by ELISA. IFN-γ and IL-12p40 were detected using Duosets (R&D Systems) following the manufacturer’s protocol. DCs were isolated by ELISA using rat anti-mouse IL-18 capture Ab (2.5 µg/ml; clone 74; MBL International, Woburn, MA), biotinylated rat anti-mouse IL-18 detection Ab (1:2000 dilution; clone 93-10C; MBL International), streptavidin–HRP (R&D Systems), and recombinant mouse IL-18 (MBL International) for generation of the standard curve. IFN-α was measured by ELISA using a rat anti-mouse IFN-α capture Ab (8 µg/ml; clone RMMA-1; BPL Biomedical Laboratories, Piscataway, NJ), a monoclonal rabbit anti-mouse IFN-α de-
tection Ab (200 ng/ml; BPL Biomedical Laboratories), and goat anti-rabbit Abs conjugated with HRP (1:500 dilution; R&D Systems), and recombinant mouse IFN-α-A (BPL Biomedical Laboratories) for generation of the standard curve. All of the ELISA plates were read using a Tecan Sunrise plate reader (Durham, NC).

DC and NK cell coculture

For DC enrichment, spleen leukocytes from four mice were isolated as above and pooled. Leukocytes were resuspended in 1X PBS, 0.5% BSA, and 2 mM EDTA (MACS buffer) and incubated 15 min with functional grade mouse Fc block (anti-mouse FcγIII-II/CD16/CD32; eBioscience). DCs were then enriched following two rounds of positive selection using a pan-DC isolation kit (Miltenyi Biotec, Auburn, CA) and magnetic column separation. These DCs were isolated as above and pooled, followed by a 3 h plastic adherence plating step in cRPMI at 37°C and 5% CO₂. This step significantly reduces the presence of contaminating adherent cells. Following plating, nonadherent spleen leukocytes were enriched by negative selection using an NK cell isolation kit (Miltenyi Biotec) and magnetic column separation. NK cells were >80% pure following separation as determined by flow cytometry. For DC cell stimulation, spleen leukocytes were frozen as isolated above and pooled, followed by a 3 h plastic adherence plating step in cRPMI containing 20 U/ml mouse rIL-2 (PeproTech) in a 96-well round-bottom culture plate (Costar) at 37°C and 5% CO₂. Cells were stimulated with the indicated TLR ligands for 20 h, culture plates were spun at 400 × g for 5 min, cell culture supernatants were removed, and aliquots were frozen at −80°C until assayed. In some instances, DC–NK cell cocultures were assayed for IFN-γ by intracellular flow cytometry following the protocol described above.
NK cell purification and stimulation

NK cells from four to five pooled spleens were enriched by negative selection as described above. Enriched NK cells were then stained with rat anti-mouse allophycocyanin–DX5 (eBioscience) and hamster anti-mouse allophycocyanin–CD3 (clone 145-2C11; eBioscience). DX5–CD3+ NK cells were purified by FACs at the Cincinnati Children’s Hospital Research Flow Cytometry Core using a FACSArIA II. NK cells were ~99% pure as determined by flow cytometry. A total of 2 × 10⁵ purified NK cells per well (1 × 10⁶ cells per milliliter) were cultured in cRPMI containing 10 ng/ml IL-15 for viability (R&D Systems) in a 96-well round-bottom culture plate (Costar) at 37°C and 5% CO₂. NK cells were stimulated with recombinant mouse IL-12 or IL-18 (R&D Systems), or both, for 20 h. Cell culture plates were spun at 400 × g for 5 min, cell culture supernatants were removed, and aliquots were frozen at −80°C until being assayed.

NK cell degranulation CD107a assay

Round-bottom, Nunc MaxiSorp immunoplates (Rochester, NY) were coated overnight at 4°C with 10 μg/ml rat anti-mouse Ly49D (clone 4E5; BD Biosciences), 50 μg/ml mouse anti-mouse NK1.1 (clone PK136; eBioscience), and their respective isotype controls at the same concentrations. Wells were washed three times using 1× PBS prior to use. Lung and spleen leukocytes were isolated as indicated above and resuspended in cRPMI. A total of 5 × 10⁶ lung and 1 × 10⁶ spleen leukocytes were added to wells with plate-bound Abs. Functional grade Fc block was added to the wells, and leukocytes were incubated for 15 min at 37°C and 5% CO₂. Rat anti-mouse PE–CD107a (0.05 μg/ml IL-15 for viability (R&D Systems) in a 96-well round-bottom culture plate (Costar) at 37°C and 5% CO₂. NK cells were stimulated with recombinant mouse IL-12 or IL-18 (R&D Systems), or both, for 20 h. Cell culture supernatants were spun at 400 × g for 5 min, cell culture supernatants were removed, and aliquots were frozen at −80°C until being assayed.

Statistical analysis

Significant differences among groups were identified by ANOVA wherever appropriate, and individual comparisons between groups were confirmed by a post hoc Bonferroni–Dunn test. Differences between means were considered significant when the p value was <0.05.

Results

IFN-γ+ cells are predominantly NK cells following stimulation with viral PAMPs

We chose to study the NK cell response following stimulation with viral PAMPs because COPD exacerbations are most often associated with viral infection. NK cells comprised the majority of IFN-γ+ cells following ex vivo stimulation of lung leukocytes with viral PAMPs (Fig. 1A). Flow cytometric analysis revealed that 82, 61, and 65% of IFN-γ+ cells were NKP46+ NK cells following 20 h ex vivo treatment of lung leukocytes with poly(I:C), ssRNA40, and ODN1826, respectively (Fig. 1B). These data are in agreement with numerous reports that NK cells are critical producers of early IFN-γ following viral infection (20, 21). The second largest population of IFN-γ+ cells following treatment with viral PAMPs was predominantly CD8+CD44hi cells (data not shown), followed by nominal contributions from uncharacterized cell types that are likely CD4+ T cells, NKT cells, B cells, monocytes, or γδ T cells (22, 26). Stimulation of control lung leukocytes with vehicle control, poly(I:C), ssRNA40, and ODN1826 resulted in 2.1 ± 0.5, 21.4 ± 2.6, 24.8 ± 1.2, and 47.3 ± 4.2% of NK cells that were IFN-γ+, respectively (Fig. 1C).

CS exposure enhances production of NK cell IFN-γ following stimulation with viral PAMPs

We first determined whether chronic CS exposure caused an increase in lung NK cells. There were no increases in lung NK cells following CS exposure (Fig. 2A). We next examined NK cell IFN-γ following ex vivo lung leukocyte stimulation with viral PAMPs in mice exposed to filtered air (FA) or CS. There were no intrinsic differences in IFN-γ+NKP46+ cells between FA- and CS-exposed mice in unstimulated lung leukocytes (Fig. 2B). However, treatment of lung leukocytes from CS-exposed mice with viral PAMPs resulting in an increase in the percentage of IFN-γ+NKP46+ cells relative to FA-exposed mice (Fig. 2B, 2C). In addition, the mean fluorescence intensity of IFN-γ in IFN-γ+NKP46+ cells was higher in CS-exposed mice following treatment with viral PAMPs (Fig. 2B). Similar results (increased percentage and mean fluorescence intensity [MFI] of IFN-γ+NKP46+ cells) were obtained using either type A (ODN1585) or type B (ODN1826) CpG ligands (data not shown). The increase in IFN-γ of CS-exposed mice following stimulation of lung leukocytes with viral PAMPs detected by flow cytometry was confirmed by ELISA (Table 1).

The increase of IFN-γ+NKP46+ cells in CS-exposed mice following treatment with viral PAMPs was dependent on the duration of CS exposure. There was no detectable difference in IFN-γ+NKP46+ cells between FA- and CS-exposed mice following stimulation with poly(I:C), ssRNA40, or ODN1826 after a 2 wk acute exposure, but the difference emerged after 8 wk of chronic exposure (Fig. 2C). In mice, 8 wk of CS exposure does not induce the full spectrum of pathologic changes associated with COPD (e.g., robust inflammation, emphysema, and mucus production) and may reflect a toxicological response to CS exposure. Therefore, we stimulated lung leukocytes with viral PAMPs after 24 wk of exposure, a well-established time point that induces COPD-like disease in mice. Similar to the effects observed after 8 wk of CS exposure, stimulation of lung leukocytes from mice exposed to CS
for 24 wk with viral PAMPs resulted in an increase in the percentage NKp46+ NK cells that were IFN-γ+. Numbers within boxes following the percentages are the MFIs of NKp46+IFN-γ+ cells. Black line histogram is FA-exposed; gray line histogram is CS-exposed. C, Mice were exposed to FA or CS for 2, 8, and 24 wk, and lung leukocytes were stimulated ex vivo with the indicated ligands. Data are presented as the percentage increase in NKp46+IFN-γ+ cells of CS-exposed mice relative to FA-exposed mice following ligand stimulation. Values are presented as means ± SEM (n = 3–6 mice per group). *Denotes values that are significantly different from those of FA-exposed controls. p < 0.05. Data are representative of two independent experiments per time point.

**FIGURE 2.** Chronic CS exposure enhances NK cell IFN-γ following ex vivo stimulation with viral PAMPs. Lung leukocytes were isolated from FA- and CS-exposed mice and stimulated with endotoxin-free water (control), 50 μg/ml poly(I:C), 1 μg/ml ssRNA40, or 1 μM ODN1826 for 20 h, and NKp46+ IFN-γ+ cells were assessed by flow cytometry. A, Percentage of freshly isolated lung leukocytes that are NKp46+ NK cells following 8 wk FA or CS exposure. B, Representative histograms following 8 wk of FA or CS exposure. Shown are histograms of IFN-γ gated on lung NKp46+ NK cells. Percentages within boxes are the percentages of NKp46+ NK cells fol-

**Chronic CS exposure primes NK cells for activation**

We next wanted to determine whether NK cells were truly pre-activated, or “primed,” following chronic CS exposure. Therefore, we used a DC–NK cell coculture system to address this question. In order to recover sufficient cells for experimentation and given that similar IFN-γ results were obtained in the spleens of CS-exposed mice (Fig. 3A), we enriched DCs and NK cells from spleens. In normal unexposed mice, neither DCs nor NK cells alone were sufficient for generation of IFN-γ following stimulation with viral

**Table 1. IFN-γ production following viral PAMP stimulation**

<table>
<thead>
<tr>
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<th>FA</th>
<th>CS</th>
<th>p Value</th>
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<tr>
<td>Control</td>
<td>Lung ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Spleen</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>poly(I:C) Lung</td>
<td>1982 ± 448</td>
<td>4,425 ± 705*</td>
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<td>Spleen</td>
<td>345 ± 14.6</td>
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<td>ssRNA40  Lung</td>
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<td>18,476 ± 2,557</td>
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<tr>
<td>Spleen</td>
<td>920 ± 537</td>
<td>4,338 ± 1,288*</td>
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<tr>
<td>ODN1826  Lung</td>
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<td>21,775 ± 1,696*</td>
<td>0.015</td>
</tr>
<tr>
<td>Spleen</td>
<td>2224 ± 244</td>
<td>12,079 ± 2,240*</td>
<td>0.014</td>
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Lung or spleen leukocytes from mice exposed to FA or CS for 8 wk were isolated as described in Materials and Methods. Leukocytes were stimulated in duplicate with endotoxin-free water (control), 50 μg/ml poly(I:C), 1 μg/ml ssRNA40, or 1 μM ODN1826 for 20 h, and supernatants were analyzed by ELISA for the indicated cytokines. Values are expressed in picograms per milliliter and presented as means ± SEM. n = 4 mice per group.

*Denotes values that are significantly different from FA-exposed controls.
NK cells were cocultured in a 1:2 ratio, a significant IFN-γ production following poly(I:C) and ODN1826 treatment was also lost (data not shown). However, the ssRNA40 response remained, with poly(I:C), or 1 μM ODN1826 for 20 h. IFN-γ was measured in cell culture supernatant by ELISA. The DC–NK cell coculture system established above allowed us to specifically address whether NK cells were primed for activation following CS exposure. IFN-γ production following poly(I:C) and ODN1826 treatment was also lost (data not shown). However, the ssRNA40 response remained, indicating that DCs are dispensable for IFN-γ production following ssRNA40 treatment of spleen leukocytes. When enriched DCs and NK cells were cocultured in a 1:2 ratio, a significant IFN-γ response was detected following poly(I:C) and ODN1826 treatment, but only modestly following ssRNA40 treatment (Fig. 3B). Significantly, in this DC–NK cell coculture, IFN-γ production was entirely restricted to NK cells (Fig. 3C).

The DC–NK cell coculture system established above allowed us to specifically address whether NK cells were primed for activation following CS exposure. Enriched NK cells from FA- or CS-exposed mice were cultured with DCs from both FA- and CS-exposed mice. The 1:2 DC–NK cell cocultures were then stimulated with poly(I:C) or ODN1826, and IFN-γ was measured by ELISA. Stimulation of cocultures with NK cells from CS-exposed mice consistently produced more IFN-γ than those containing NK cells from FA-exposed mice (Fig. 3D). This occurred regardless of whether they were cultured with DCs from FA- or CS-exposed mice (Fig. 3D). Interestingly, we observed a decrease in IFN-γ production in all of the cultures using DCs from CS-exposed mice. This observation is in agreement with reports that DCs from CS-exposed mice are hyporesponsive to TLR stimulation (29). This is the first demonstration that these observations extend to splenic DCs.

We wanted to unquestionably determine whether NK cells were indeed primed for activation. Therefore, we highly purified NK cells (99%) from the spleens FA- and CS-exposed mice. There were no intrinsic differences in the production of IFN-γ between the two exposure groups in the absence of stimulation (Fig. 4). However, highly purified NK cells from CS-exposed mice produced significantly more IFN-γ following stimulation with the IFN-γ–inducing cytokines IL-12 or IL-18, or both (Fig. 4). Similar results were obtained using lung NK cells (data not shown).

**NK cell activation following stimulation with bacterial PAMPs**

There are numerous reports implicating bacteria in exacerbations in both patients with COPD and mouse models of COPD (14, 30, 31). We hypothesized that NK cell priming may be intimately involved in both viral and bacterial exacerbations. Therefore, we isolated lung leukocytes from mice exposed to either FA or CS for 8 wk and stimulated them with PAMPs associated with bacteria. Relative to FA-exposed mice, stimulation of lung leukocytes from CS-exposed mice with bacterial PAMPs resulted in modest increases (20.0 ± 2.8% for LPS and 15.4 ± 3.3% for FSL-1), no difference (HKLM and ST-FLA), or even a decrease (12 ± 3.7% for Pam3CSK4) in the percentage of IFN-γ+NKp46+ NK cells (Fig. 5).

**Raet1 ligand expression enhances NK cell responsiveness to viral PAMPs but not cytokines**

We have recently demonstrated that chronic CS exposure leads to the overexpression of a stress-inducible ligand for NKGD2, Raet1, on the lung alveolar and airway epithelium (6). NKGD2 is almost exclusively found on NK cells in mice, and we have shown that Raet1 overexpression, alone, enhances NK cell responsiveness to LPS stimulation (25). We observed increases in NK cell IFN-γ in CS-exposed mice following LPS stimulation (Fig. 5). On the basis of these studies, we hypothesized that Raet1 overexpression and NKGD2 stimulation may be involved in NK cell priming. We used a previously described Raelta-tg mouse that has DOX-inducible, lung-specific expression of Raet1 on the airway and alveolar epithelium (6, 25). We first addressed whether Raet1 overexpression altered the response to viral PAMP stimulation. Raelta-tg mice were administered NO DOX or DOX for 10 d, and lung leukocytes were stimulated with poly(I:C), ssRNA40, and ODN1826 (Fig. 6A). Similar to the effect observed following LPS stimulation (25), Raet1 overexpression increased the percentage of IFN-γ+NKp46+ cells following stimulation with viral PAMPs (Fig. 6A). We next wanted to determine whether Raet1 overexpression was involved in NK cell hyperresponsiveness to cytokine stimulation. Raelta-tg mice were administered NO DOX or DOX for 10 d, and lung leukocytes were stimulated with IL-12. Contrary to the effect observed following viral PAMP stimulation, there was no difference in the percentage of IFN-γ+NKp46+ cells in NO DOX and DOX Raelta-tg mice following IL-12 treatment (Fig. 6B).

**NK cell priming is lost following CS cessation**

Several reports indicate that the numbers of COPD exacerbations in patients are reduced following cigarette smoking cessation (32, 33). We therefore examined whether NK cell priming persisted after smoking cessation. C57BL/6 mice were exposed to either FA...
Materials and Methods

In this study, we showed that CS exposure primed enhanced NK cell activation in response to subsequent PAMP or cytokine stimulation. This response is likely integral to the enhanced inflammation, protease activation, alveolar epithelial cell apoptosis, and lung function decline that characterize COPD and is accelerated during COPD exacerbations. NK cells are the primary source of early IFN-γ following viral infection and are critical to the orchestration of the inflammatory response that controls infection (23, 24). IFN-γ production is responsible for the generation of a proinflammatory state in the host (22), and pulmonary overexpression of IFN-γ, alone leads to a mouse model of COPD, and we have shown that NK cells are preferentially activated following chronic CS exposure. As described above, the innate immune response following viral infection is enhanced by prior CS exposure (16), and we believe that primed NK cells play an integral role in this process. However, the in vivo relevance of NK cell priming is a limitation of this work and requires further study. Additional evidence implicating NK cell in the development of COPD comes from studies on immunocompromised mice exposed to CS (35, 36). It has been demonstrated that, in the absence of T cells and B cells, the development of COPD phenotypes still occurred following chronic CS exposure (35, 36). In addition, we have recently demonstrated a role for the innate functions of cytotoxic lymphocytes in the pathogenesis of COPD (6). CS exposure drives the expression of stress-inducible ligands for NKG2D, an activating receptor found on NK and CD8⁺ T cells, and chronic activation of NKG2D is sufficient for development of certain COPD phenotypes, such as emphysema (6). Together, these data highlight the vital importance of the innate immune system, and NK cells in particular, in COPD pathogenesis.

A previous report demonstrated that TLR stimulation of lung DCs from CS-exposed mice resulted in blunted cytokine elaboration (29). In agreement with this finding, we demonstrated that DCs from CS-exposed mice, relative to DCs from FA-exposed mice, induced less NK cell IFN-γ following TLR stimulation. With the exception of a slight decrease in IL-12 production from unstimulated lung leukocytes and ssRNA40-stimulated lung leukocytes from CS-exposed mice, there were no differences in accessory cytokine production between FA- and CS-exposed mice following viral PAMP stimulation. Taken together with the data that demonstrate consistent increases in IFN-γ production following CS exposures, these findings provide strong evidence that NK cells represent the hyperresponsive cell population following viral PAMP exposures in the context of repeated CS exposure. The finding that CS exposure may suppress specific immune components while simultaneously activating other immune components accentuates the complexity of CS effects on pulmonary and systemic immunity.

In this study, we showed that CS exposure primed enhanced NK cell activation in response to subsequent PAMP or cytokine stimulation. This response is likely integral to the enhanced inflammation, protease activation, alveolar epithelial cell apoptosis, and lung function decline that characterize COPD and is accelerated during COPD exacerbations. NK cells are the primary source of early IFN-γ following viral infection and are critical to the orchestration of the inflammatory response that controls infection (23, 24). IFN-γ production is responsible for the generation of a proinflammatory state in the host (22), and pulmonary overexpression of IFN-γ, alone leads to
FIGURE 5. NK cell IFN-γ following ex vivo stimulation with bacterial PAMPs. Lung leukocytes were isolated from mice exposed to FA or CS for 8 wk and were stimulated with endotoxin-free water (control), 0.5 µg/ml Pam3CSK4, 10⁸ cells per milliliter HKLM, 0.5 µg/ml LPS-EK, 5 µg/ml ST-FLA, or 0.5 µg/ml FSL1 for 20 h, and NKp46⁺IFN-γ⁺ cells were assessed by flow cytometry. Data are presented as the percentage increase in NKp46⁺IFN-γ⁺ cells of CS-exposed mice relative to FA-exposed mice following stimulation. Values are presented as means ± SEM (n = 4 mice per group). *Denotes values that are significantly different from FA-exposed controls. p < 0.05.

FIGURE 6. NKG2D activation enhances stimulation with viral PAMPs but does not prime NK cells. Lung leukocytes were harvested from Ras-t1a-tg mice treated with or without DOX for 10 d and stimulated for 20 h followed by analysis by flow cytometry. A, Lung leukocytes were stimulated with endotoxin-free water (control), 50 µg/ml poly(I:C), 1 µg/ml ssRNA40, or 1 µM ODN1826 for 20 h, and the presence of IFN-γ⁺-producing NKp46⁺ NK cells was assessed. B, Lung leukocytes were stimulated with 1× PBS/0.5% BSA (control), 1 ng/ml IL-12, or 10 ng/ml IL-12 for 20 h, and the presence of IFN-γ⁺-producing NKp46⁺ NK cells was assessed. Data are presented as the percentage increases in NKp46⁺ IFN-γ⁺ cells of DOX-treated mice relative to mice without DOX treatment (no DOX), following leukocyte stimulation. Values are presented as means ± SEM (n = 3–5 mice per group). *Denotes values that are significantly different from no DOX controls. p < 0.05. Data are representative of two independent experiments.

Many of the phenotypic changes that occur in COPD (37). IFN-γ orchestrates trafficking of immune cells to sites of inflammation through upregulation of adhesion molecules (e.g., ICAM-1 and VCAM-1) and chemokines (e.g., MCP-1, IP-10, MIG, MIP-1β, and RANTES). The responding inflammatory cells enter the inflamed tissue through diapedesis and carry out effector functions, such as protease production (22). These processes become relevant when one considers that monocyte recruitment, macrophage differentiation, and subsequent protease induction are a well characterized mechanism of COPD pathogenesis involved in tissue destruction. Thus, enhanced production of NK cell-derived IFN-γ following stimulation is likely a critical mediator of COPD pathogenesis.

Emphysema, a key component of COPD, is characterized by destruction of the airways, leading to loss of elastic recoil and decline in lung function. Epithelial and endothelial cell apoptosis in the lung is critical to the development of emphysema (38–40). Mechanisms, such as oxidative stress, are well-recognized for their role in apoptotic cell death following smoke exposure (38, 41). We have recently described a novel mechanism of cytotoxic lymphocyte-mediated alveolar epithelial cell apoptosis in COPD (6). In this report, we demonstrated that chronic CS exposure causes enhanced NK cell degranulation capacity, a surrogate for cytotoxicity. Priming of NK cell cytotoxicity is potentially a new mechanism for cellular apoptosis in COPD. Further, NK cell-mediated cellular apoptosis may be responsible for the observation of increased lung tissue destruction in CS-exposed mice postinfection (16).

An emerging body of literature supports an autoimmune etiology in COPD (4, 42–44), and autoreactive T cells and autoantibodies directed against multiple cellular components have recently been demonstrated (43, 44). The mechanisms surrounding the development of autoimmunity in COPD remain unknown, but we believe that our demonstration of enhanced activation of primed NK cells is intimately involved in the autoimmune etiology of COPD. Following infection, the production of excessive levels of proinflammatory cytokines, such as IFN-γ, may aberrantly activate APCs, increase Ag presentation, costimulate T cells, or lead to an enhanced bystander activation of autoreactive T cells (45). Further, enhanced NK cytotoxicity following infection will undoubtedly release more intracellular components. This may contribute to the development of autoimmunity by exposing the immune system to molecules against which no self tolerance has been raised. Additionally, the release of damage-associated molecular patterns following cell death may activate the immune system, lowering the threshold for the development of autoimmunity (46).

NK cell priming is a term that has been used to describe a phenomenon where, following infection, DCs engage and activate naive NK cells in the lymph nodes (LNs) (19). Primed NK cells then exit the LNs and enter circulation where they execute effector functions upon secondary stimulation, presumably by infected cells (19). NK cell priming requires type I IFNs and IL-15, but IL-18 can also prime NK cells (19, 47). IL-12 is dispensable for NK cell priming, but in agreement with our data, IL-12 enhances production of IFN-γ from primed NK cells (19). It is unclear how chronic CS exposure primes NK cells in a mouse model of COPD. However, a similar priming mechanism may occur following chronic CS exposure, and this would provide a sufficient explanation for our observation that NK cells in both lungs and spleens exhibit signs of priming. The picture is incomplete (48), but several reports have demonstrated that DC activation occurs in COPD patients and in mouse models (49–51). Maturation of DCs has been correlated with disease severity, strongly suggesting a mechanistic role for DCs in COPD (49). Smoke exposure causes DCs to preferentially stimulate CD8⁺ T cells while suppressing CD4⁺ T cells, an interesting observation considering the functional similarities
between CD8+ T cells and NK cells (51). Thus, analogous to infection, we propose that activated DCs in CS-exposed individuals travel to the LNs and prime naive NK cells, leading to enhanced NK cell activation upon secondary stimulation.

An alternative explanation for the effects that we observed may be that the NK cells in CS-exposed mice are “memory-like” NK cells (52). NK cells previously exposed in vitro to IL-12 and IL-18 maintain a hyperresponsive state following secondary stimulation and survive up to 1 mo following in vivo injection (52). However, cytokine-induced, memory-like NK cells exhibit no differences in cytotoxicity relative to naive NK cells. This suggests that this mechanism is unlikely in our model. Further, memory-like NK cells persisted for several weeks following initial stimulation (52). Following smoking cessation, we observed that NK cell responsiveness returned to normal levels, indicating that the effect is transient and reminiscent of short-term priming rather than long-term memory. These data highlight the importance of smoking cessation as a therapy to prevent COPD exacerbations.

**FIGURE 7.** NK cell priming is lost following smoking cessation. Mice were exposed to either FA or CS for 8 or 24 wk. Mice undergoing cessation were exposed for 8 or 24 wk followed by 4 wk of smoking cessation. Lung leukocytes were stimulated with 10 ng/ml IL-12 for 20 h, and the presence of IFN-γ-producing NKp46+ NK cells was assessed by flow cytometry. Values are presented as means ± SEM (n = 3–4 mice per group). *Denotes values that are significantly different from FA-exposed controls. **Denotes cessation values that are significantly different from CS-exposed mice of the corresponding time point. p < 0.05.

**FIGURE 8.** Chronic CS exposure enhances NK cell degranulation capacity. Lung or spleen leukocytes were stimulated with plate-bound Abs as described in Materials and Methods for 4 h, and CD107a expression on NKp46+ cells was assessed by flow cytometry. A, Representative histogram of CD107a expression on lung NK p46+ NK cells following stimulation with Ly49D or NK1.1. Percentages within boxes are the percentages of NKp46+ cells that are IFN-γ+. Numbers within boxes following percentages are the MFIs of NKp46+IFN-γ+ cells. Heavy black line is FA-exposed, gray line is CS-exposed, and light black line is CD107a expression following stimulation with IgG isotype control. B, Quantification of CD107a data following stimulation of lung and spleen leukocytes with Ly49D or NK1.1. Data are presented as the percent increases in NKp46+CD107a+ cells of 8 wk CS-exposed mice relative to FA-exposed mice following NK cell stimulation. Values are presented as means ± SEM (n = 3–5 mice per group). *Denotes values that are significantly different from FA-exposed controls. p < 0.05. Data representative of two independent experiments.

**FIGURE 9.** Potential mechanisms and consequences of NK cell priming in COPD. A, CS exposure may cause local production of type I IFNs, IL-12, IL-18, and IL-15 derived from lung epithelium, DCs, and macrophages. These cytokines may locally prime lung NK cells. Primed NK cells in the lung may then exit the lung and enter the peripheral blood. In conjunction, type I IFN production caused by CS exposure may induce IL-15 production from DCs. These activated DCs may traffic to the lymph nodes where they would prime NK cells through trans-presentation of IL-15. Primed NK cells would then leave the lymph and enter the periphery. B, CS exposure and viral or bacterial infection induce the expression of NKG2D ligands (e.g., RAET1) on the lung epithelium. Viral and bacterial PAMPs signal through TLRs expressed in NK cells in a cell-autonomous manner. Costimulation of NKG2D and TLRs may therefore enhance NK cell activation beyond stimulation with either agonist alone. At the same time, during infection, inflammatory cytokines act on NK cells primed by CS exposure and cause an enhanced inflammatory and degranulation response. Important to the pathogenesis of COPD, enhanced NK cell activation may lead to increased macrophage or monocyte and neutrophil recruitment and activation, destruction of lung tissue and alveolar apoptosis, and mucus production. Additionally, enhanced activation of primed NK cells following infection may activate the adaptive immune response.
supported by observations that COPD patients who stop smoking have significantly reduced frequency of exacerbations (32, 33), which we hypothesize to be due to loss of NK cell priming.

We have reported that chronic CS exposure induced NKG2D ligand expression on the airway and alveolar epithelium (6). Additionally, we have demonstrated that prior NKG2D stimulation primed NK cell responsiveness to TLR4 stimulation of lung leukocytes (25). In this study, we show that NKG2D ligation also primed NK cell responsiveness to viral PAMP stimulation of lung leukocytes. However, Rael1 overexpression and NKG2D ligation were not sufficient for priming the response to cytokine stimulation. TLR3, TLR7, and TLR9 are expressed in NK cells and are functionally dependent on the presence of accessory cytokines (53, 54). In agreement with our in vivo data, in vitro costimulation of human NK cells with viral PAMPs and NKG2D ligands, in the presence of accessory cytokines, led to more IFN-γ production than either stimulation alone (53). Therefore, we propose that prior NKG2D stimulation on NK cells primes cell-autonomous TLR signaling, and CS exposure-driven NKG2D activation of NK cells likely synergizes with the additional priming mechanisms described above.

In contrast to the data presented in this study and the observations of others (16, 35, 36), there is some data suggesting that NK cells may be functionally suppressed in COPD. However, the interpretation of these reports is limited by small samples sizes, the use of non-COPD smokers with unknown patient characteristics, or in vitro treatment of PBMCs with Cs extract (55, 56). The only report directly examining NK cell function in COPD used cells derived from patients using inhaled steroids (56), a variable known to impair functional assays, particularly in COPD (57). It has also been suggested that CS may impair NK cell function, leading to enhanced tumor burden following tumor challenge (58). Unfortunately, this report relied on the use of heterogeneous cell populations, so the contribution of NK cells remains undefined (58). The limited knowledge on NK cell function in COPD and the apparent dichotomy of results further highlight the need for more studies.

Together, our data demonstrate an entirely novel mechanism of NK cell activation resulting from chronic CS exposure. Current strategies for the prevention of COPD exacerbations have yielded few beneficial results. Therefore, NK cell priming provides a new therapeutic target for the prevention and treatment of COPD exacerbations. Finally, understanding the function of NK cells and NK cell priming in COPD will undoubtedly provide new insight into development and progression of COPD.

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