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

Gregory T. Motz,* Bryan L. Eppert,* Brian W. Wortham,* Robyn M. Amos-Kroohs,* Jennifer L. Flury,* Scott C. Wesselkamper,* and Michael T. Borchers*†,‡

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 Raet1 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. The Journal of Immunology, 2010, 184: 4460–4469.

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hronic obstructive pulmonary disease (COPD) is expected to be the third leading cause of worldwide deaths within 10 y (1). The economic burden of COPD is measured by the billions of dollars in the United States, and increasing worldwide prevalence will place additional strains on the global economy (1). The chief cause of COPD is long term cigarette smoking, and most smokers will develop COPD if smoking-related, extrapulmonary diseases do not claim their lives beforehand (2). In conjunction with smoking, the development of COPD is modified by genetics, environmental exposures (e.g., air pollution and occupational exposures), and infections (3, 4).

COPD is ultimately a failure of proper breathing, and most patients initially seek doctor consultation for dyspnea (3). Airflow restriction is brought about by a combination of chronic bronchitis and emphysema, although the extent of either varies within individual patients (4). These pulmonary maladies result from inflammation, mucus hypersecretion, and alveolar destruction (4). The inflammation is strong correlate of disease severity in patients and is critically involved in disease development experimentally (2, 4, 5). Attention has traditionally been centered on the roles of macrophages and neutrophils in disease development (4). However, lymphocytes have received increased attention, and reports from our laboratory have provided insight into functions of T cells and NK cells in COPD (6–8).

The underlying COPD pathologies are punctuated by exacerbations of symptoms. Standard criteria for COPD exacerbations do not exist, but they are generally characterized by increased dyspnea, enhanced sputum, enhanced inflammation, and decline in lung function (9, 10). Consequently, exacerbations accelerate disease progression and are an integral part of disease pathogenesis. The toll of COPD exacerbations is also psychologically destructive; patients with frequent exacerbations have a lower quality of life and decreased mobility, leading to increased depression (11). Most importantly, exacerbations that require hospitalization result in death 8–11% of the time, and the remaining patients have a mortality rate of 23–43% y following admission (12, 13).

Between 40 and 60% of exacerbations are believed to be caused by viral infections (9, 14). The remaining exacerbations are likely the result of nonviral infections and pollution, although ~20% have an unknown etiology (14). Exacerbations associated with viral infections are severe; viral exacerbations lead to hospitalization more often than other causes and have a longer recovery time (15). The connection between viruses and COPD exacerbations remained mostly associative until, in a recent landmark study, Kang et al. (16) experimentally demonstrated that exacerbations of symptoms following cigarette smoke (CS) exposure can be caused by viral infection. Key to the progression of COPD, viral infection following CS exposure led to enhanced pulmonary inflammation, increased alveolar apoptosis, accelerated emphysema, and airway fibrosis.

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Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; CS, cigarette smoke; DC, dendritic cell; DOX, doxycycline; FA, filtered air; LN, lymph node; MFI, mean fluorescence intensity; PAMP, pathogen-associated molecular pattern.

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(16). However, the underlying cellular mechanisms responsible for these effects remain undiscovered.

The early immune response following viral infection relies on the recognition of viral pathogen-associated molecular patterns (PAMPs) through TLR3, TLR7, or TLR9 (17, 18). Stimulation of TLR3, TLR7, or TLR9 found on dendritic cells (DCs) and other accessory cells leads to the activation of NK cells through production of type I IFNs, IL-12, IL-18, and IL-15 (17–20). NK cell activation is critically important to the early control of viral infections, and NK cell functions typically peak several hours to a few days following infection (21). Traditionally, NK cells were appreciated for their ability to directly kill virus-infected cells (21). More recently, attention has been given to the noncytotoxic functions of 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 activation 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). B6-129S1-Raet1a<teto> tm9Sor-2tm1Ogle (FVB/NJ, 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.01306 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 sidestream smoke that is mixed with ambient, filtered air and introduced into a closed chamber containing mice. The concentration of the smoke/air mixture was maintained at 150 mg/m3 total suspended particulates (corresponding to five cigarettes every 10 min in 0.53 m3). Particulate threshold were determined from appropriate isotype control staining. Using the FlowJo software (Tree Star, Ashland, OR). Positive staining was determined by intracellular flow cytometry following the protocol provided by the manufacturer’s protocol. 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. IL-18 was measured 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), recombinant mouse IL-18 (MBL International) for generation of the standard curve. IFN-α was measured by ELISA using rat anti-mouse IFN-α capture Ab (8 μg/ml; clone RMMA-1; BBL Biomedical Laboratories, Piscataway, NJ), and goat anti-rabbit Ab conjugated with HRP (1:500 dilution; R&D Systems), and recombinant mouse IFN-α–A (BBL Biomedical Laboratories) for generation of the standard curve. All of the ELISAs were read using a Tecan Sunrise plate reader (Durham, NC).

Lung and spleen leukocyte isolation

Mice were euthanized with an i.p. injection of sodium pentobarbital following exsanguination. Lungs were perfused with 6 ml 1× PBS containing 0.6 mM EDTA. Lungs were withdrawn aseptically from the chest cavity, and leukocytes were isolated in a manner previously described (7). Tissue leukocytes were prepared by gently pressing spleens through a 100-μm cell strainer. Residual RBCs from lungs and spleens were lysed with RBC lysis solution (Qiagen, Valencia, CA), and leukocytes were then centrifuged in a continuous 33% Percoll solution to remove dead cells and debris (Sigma-Aldrich, St. Louis, MO). All of the procedures were performed steriley.

**Lung and spleen leukocytes were cultured in cRPMI (RPMI 1640 with 2.05 mM 1-glutamine [HyClone, Waltham, MA] containing 10% FBS, 1% sodium pyruvate, 100 μg/ml kanamycin, 0.05 mM 2-ME, and 1× nonessential amino acids [MP Biomedicals, Solon, OH]). A total of 5×106 cells (2.5×106 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 for 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, HKL3, FSL1, or S Rath (InvivoGen, San Diego, CA)] or cytokines (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 containing 0.1% saponin and 10% mouse serum (MP Biomedicals). After being washed, cells were resuspended FACS buffer containing 0.1% saponin and stained intracellularly with anti-rat-mouse allophycocyanin–IFN-γ (clone XM1G1.2; eBioscience). Stained leukocytes were washed and fixed in 2% paraformaldehyde, and flow cytometry was performed using a FACSCalibur (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. IL-18 was measured 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 rat anti-mouse IFN-α capture Ab (8 μg/ml; clone RMMA-1; BBL Biomedical Laboratories, Piscataway, NJ), and goat anti-rabbit Ab conjugated with HRP (1:500 dilution; R&D Systems), and recombinant mouse IFN-α–A (BBL Biomedical Laboratories) for generation of the standard curve. All of the ELISAs 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 1× 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. This kit enriches both classical DCs (CD11c+) and plasmacytoid DCs (CD11c–, PDCA–). DCs were >90% pure following separation as determined by flow cytometry. For NK cell enrichment, spleen leukocytes were resuspended 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. The remaining 20% of total post-NK cells were labeled with macrophages (mostly B cells, ~6%), CD3+ T cells (~2%), and uncharacterized cells (~2%). A total of 0.75×106 DCs per well and 1.5×105 NK cells per well were cultured either alone or in combination (1:2 ratio of DCs to NK cells) 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 and pooled. Leukocytes were resuspended in 1× PBS, 0.5% BSA, 2 mM EDTA, and 0.05% NaN3, and incubated with mouse Fc block (anti-mouse FcγIII/II/CD16/CD32; eBioscience). 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 for 20 min at room temperature. Leukocytes were washed and permeabilized for 15 min at 4°C with FACS buffer containing 0.1% saponin and 10% mouse serum (MP Biomedicals). After being washed, cells were resuspended FACS buffer containing 0.1% saponin and stained intracellularly with anti-rat-mouse allophycocyanin–IFN-γ (clone XM1G1.2; eBioscience). Stained leukocytes were washed and 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.

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NK cell priming 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 × 10^5 purified NK cells per well (1 × 10^5 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 × 10^5 lung and 1 × 10^5 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/well; clone eBio1D4B; eBioscience) was added to each well with 2 µM monensin, and cells were incubated for 4 h at 37°C and 5% CO2. Leukocytes were collected and washed in FACS buffer followed by staining with FITC–NKp46 (clone 29A1.4; eBioscience) as described above. Flow cytometry was performed as described above.

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^+CD44^hi 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 resulted 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).

FIGURE 1. IFN-γ^+ cells following viral PAMP stimulation are predominantly NK cells. Lung leukocytes from unexposed C57BL/6 mice were isolated 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 IFN-γ^+ cells were assessed by flow cytometry. A, Representative flow plot following ssRNA40 stimulation indicating that the majority of IFN-γ^+ cells are NKp46^+. Percentages shown are the cells within the gates. B, Quantification of IFN-γ^+ cells following stimulation. C, Percentage of NKp46^+ NK cells that are IFN-γ^+ following stimulation with the indicated PAMPS. Values are presented as means ± SEM (n = 5).
for 24 wk with viral PAMPs resulted in an increase in the percentage NKP46+ NK cells that were IFN-γ+ (Fig. 2C).

Interestingly, the increase of IFN-γ+NKP46+ cells in CS-exposed mice following treatment with viral PAMPs occurred in the spleen as well (Fig. 3A). Similar to stimulation of lung leukocytes, this increase in IFN-γ was also detectable by ELISA (Table I).

**CS exposure does not enhance IFN-γ-inducing cytokines following TLR treatment**

The majority of cells expressing TLR3, TLR7, and TLR9 are thought to be DCs, monocytes/macrophages, and B cells (17, 18). A simple explanation for enhanced NK cell IFN-γ would be an increase in inflammatory cells that produce IFN-γ-inducing cytokines. There were no differences between 8 wk FA- and CS-exposed mice in lung DCs (classical or plasmacytoid DCs), B cells, or NK cells (determined by flow cytometry; data not shown; NK cells; Fig. 2A). As expected, chronic CS exposure did increase the amount of lung monocytes/macrophages to ∼2-fold that of FA-exposed mice (data not shown).

We next determined whether these cellular changes had a net effect on the IFN-γ-inducing cytokines following viral PAMP treatment. Stimulation of lung leukocytes with viral PAMPs did not increase the production of IFN-α, IL-12, or IL-18 in CS-exposed mice relative to that in FA-exposed mice (Table II). This indicates that although the proportions of some accessory cells are increased, they are hyporesponsive (27–29), resulting in no differences in the total amount of IFN-γ-inducing cytokines produced following stimulation.

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

We next wanted to determine whether NK cells were truly preactivated, or “primed,” following chronic CS exposure. Therefore, we used a DC–NK cell coculture system to address this question. 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 I. IFN-γ production following viral PAMP stimulation**

<table>
<thead>
<tr>
<th></th>
<th>FA CFU</th>
<th>CS CFU</th>
<th>p Value</th>
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<tbody>
<tr>
<td>Lung</td>
<td>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)</td>
<td>1982±448</td>
<td>4,425±705*</td>
<td>0.027</td>
</tr>
<tr>
<td>Spleen</td>
<td>343±14.6</td>
<td>1,966±613</td>
<td>0.057</td>
</tr>
<tr>
<td>ssRNA40</td>
<td>13,731±2272</td>
<td>18,476±2,537</td>
<td>0.050</td>
</tr>
<tr>
<td>Spleen</td>
<td>920±537</td>
<td>4,338±1,288*</td>
<td>0.016</td>
</tr>
<tr>
<td>ODN1826</td>
<td>14,192±1,554</td>
<td>21,775±1,696*</td>
<td>0.014</td>
</tr>
<tr>
<td>Spleen</td>
<td>2224±244</td>
<td>12,079±2,240*</td>
<td>0.014</td>
</tr>
</tbody>
</table>

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. *Denotes values that are significantly different from FA-exposed controls.
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 of 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 NK2GD, Raet1, on the lung alveolar and airway epithelium (6). NK2GD 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 NK2GD stimulation may be involved in NK cell priming. We used a previously described Raet1-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. Raet1-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. Raet1-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 Raet1-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

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**FIGURE 3.** DC–NK cell coculture reveals that CS exposure activates NK cells. A, Representative histogram of spleen NKp46+ cells following 8 wk of FA or CS exposure. Histogram is generated from poly(I:C) stimulation. Shown are histograms of IFN-γ gated on spleen NKp46+ NK cells. Percentages within boxes are the percentages of NKp46+ cells that are IFN-γ−. Numbers within boxes following percentages are the MFIs of NKp46− and IFN-γ− cells. Black line is FA-exposed; gray line is CS-exposed. B, Splenic DCs (0.75 × 10^6 cells per well) or NK cells (1.5 × 10^6 cells per well) were enriched by MACS, cultured either alone or in combination (1:2 ratio), and stimulated with endotoxin-free water (control), 50 μg/ml poly(I:C), 1 μg/ml ssRNA40, or 1 μM ODN1826 for 20 h. IFN-γ was measured in cell culture supernatant by ELISA. C, Representative flow cytometry plots gated on IFN-γ+ cells following stimulation with poly(I:C) or ODN1826. Shown are plots of preseparated splenocytes and DC–NK cell coculture. Percentages shown are the amounts of IFN-γ+ cells that are NKp46+. D, Spleens of five mice were pooled, and DCs from 8 wk FA- and CS-exposed mice were enriched and plated at 0.75 × 10^6 cells per well. Spleens from four mice were pooled, NK cells from either 8 wk FA- or CS-exposed mice were enriched, and 1.5 × 10^6 NK cells per well were added to either the FA or CS DCs (1:2 ratio). Cells were then stimulated with endotoxin-free water (control produced no IFN-γ; data not shown), 50 μg/ml poly(I:C), or 1 μM ODN1826 for 20 h. IFN-γ was measured in cell culture supernatant by ELISA. *Not detected. Values are presented as means ± SEM. †Denotes values that are significantly different, p < 0.05. All of the data are representative of two independent experiments.

PAMPs (Fig. 3B). If DCs or NK cells were depleted from the ex vivo splenocyte culture by prior magnetic column separation, 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 of 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 NK2GD, Raet1, on the lung alveolar and airway epithelium (6). NK2GD 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 NK2GD stimulation may be involved in NK cell priming. We used a previously described Raet1-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. Raet1-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. Raet1-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 Raet1-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
Cytokine production of lung leukocytes following viral PAMP stimulation

<table>
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<tr>
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<th>FA</th>
<th>CS</th>
<th>p Value</th>
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<tr>
<td>Control</td>
<td>IL-12p40</td>
<td>144 ± 5.8</td>
<td>123 ± 3.9*</td>
</tr>
<tr>
<td>IL-18</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>IFN-α</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>poly(I:C)</td>
<td>IL-12p40</td>
<td>258 ± 14.7</td>
<td>222 ± 16.0</td>
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<tr>
<td>IL-18</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>IFN-α</td>
<td>204 ± 29.7</td>
<td>327 ± 90.2</td>
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</tr>
<tr>
<td>ssRNA40</td>
<td>IL-12p40</td>
<td>1299 ± 78.1</td>
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<tr>
<td>IL-18</td>
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<tr>
<td>IFN-α</td>
<td>36.9 ± 8.8</td>
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<tr>
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<td>5377 ± 625</td>
<td>6331 ± 1.284</td>
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<tr>
<td>IL-18</td>
<td>ND</td>
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<tr>
<td>IFN-α</td>
<td>194 ± 42.6</td>
<td>222 ± 101</td>
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Lung 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 filtered air-exposed controls.

or CS for 8 or 24 wk. Additional mice were exposed to CS for 8 or 24 wk and then exposed to FA for 4 wk (smoking cessation). Lung leukocytes from these mice were stimulated with IL-12, and IFN-γ was measured by flow cytometry. As expected, 8 or 24 wk of CS exposure increased the total IFN-γ/NKp46+ NK cells following IL-12 treatment by ~60% (Fig. 7). However, the amount of IFN-γ/NKp46+ NK cells after IL-12 treatment was the same as that in FA-exposed mice following smoking cessation (Fig. 7).

**Chronic CS exposure primes NK cell degranulation capacity**

In addition to NK cell IFN-γ production, we wanted to determine whether NK cell degranulation capacity was affected following chronic CS exposure. Following stimulation of NK cells with plate-bound Abs, we measured the surface expression of the lyosome-associated membrane protein 1 (CD107a). CD107a is expressed on the cell surface following exocytosis of cytotoxic granules, is a newly developed measure of NK cell degranulation, and is a surrogate for cytotoxic activity (34). Stimulation of NK cells with either Ly49D or NK1.1 led to enhanced CD107a expression in CS-exposed mice relative to that in FA-exposed mice (Fig. 8A). Both lung and spleen NK cells from CS-exposed mice displayed increased degranulation capacity following stimulation (Figs. 8B, 9).

**Discussion**

The effect of chronic CS exposure on the innate immune system, and NK cells in particular, is not well understood. In this paper, we provide the first comprehensive study of NK cell function in 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 cells 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...
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-1α/β, 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-appreciated 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. This is

![Figure 7](https://example.com/fig7.jpg)

**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](https://example.com/fig8.jpg)

**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 NKP46+ 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](https://example.com/fig9.jpg)

**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 transpresentation 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 utilizes markers to identify NK cells and 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).


