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NKG2D Mediates NK Cell Hyperresponsiveness and Influenza-Induced Pathologies in a Mouse Model of Chronic Obstructive Pulmonary Disease

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Chronic obstructive pulmonary disease (COPD) is characterized by peribronchial and perivascular inflammation and largely irreversible airflow obstruction. Acute disease exacerbations, due frequently to viral infections, lead to enhanced disease symptoms and contribute to long-term progression of COPD pathology. Previously, we demonstrated that NK cells from cigarette smoke (CS)-exposed mice exhibit enhanced effector functions in response to stimulating cytokines or TLR ligands. In this article, we show that the activating receptor NKG2D is a key mediator for CS-stimulated NK cell hyperresponsiveness, because CS-exposed NKG2D-deficient mice (*Klrk1*$^{-/-}$) did not exhibit enhanced effector functions as assessed by cytokine responsiveness. NK cell cytotoxicity against MHC class I-deficient targets was not affected in a COPD model. However, NK cells from CS-exposed mice exhibit greater cytotoxic activity toward cells that express the NKG2D ligand RAET1ε. We also demonstrate that NKG2D-deficient mice exhibit diminished airway damage and reduced inflammation in a model of viral COPD exacerbation, which do not affect viral clearance. Furthermore, adoptive transfer of NKG2D$^+$ NK cells into CS-exposed, influenza-infected NKG2D-deficient mice recapitulated the phenotypes observed in CS-exposed, influenza-infected wild-type mice. Our findings indicate that NKG2D stimulation during long-term CS exposure is a central pathway in the development of NK cell hyperresponsiveness and influenza-mediated exacerbations of COPD. ***The Journal of Immunology*****, 2012, 188: 4468–4475.**

Chronic obstructive pulmonary disease (COPD) is characterized by peribronchial and perivascular inflammation and largely irreversible airflow obstruction (1). Long-term cigarette smoking is the major cause of COPD; although the severity of the disease may vary, all long-term smokers will develop some degree of lung function impairment (2). In addition to cigarette smoke (CS) exposure, acute disease exacerbations due to infection contribute significantly to COPD progression. COPD exacerbations are characterized by a worsening of the patient's condition, including changes in symptoms, such as dyspnea, cough, and sputum production (3). These exacerbations are typically marked by a visit to healthcare providers, often leading to long hospital stays resulting in billions of dollars a year in direct costs (4). The majority of exacerbations are believed to be caused by viral infections that result in more severe symptoms and longer recovery times (5, 6).

The potential role of NK cells in the development and progression of COPD pathologies has received little attention even though NK cells are known to contribute to tissue remodeling through their potent cytolytic potential and elaboration of high levels of proinflammatory cytokines (7). NK cell functions are tightly controlled by a balance of activating and inhibitory receptors. Of the activating receptors, NKG2D (*Klrk1*) is distinguished because it is present on virtually all NK cells, recognizes stress- and infection-induced ligands, and can overcome inhibitory signals to evoke cytotoxicity and cytokine release (8). NKG2D is expressed almost exclusively on cytotoxic lymphocytes (i.e., NK cells, NKT cells, CD8$^+$ T cells, γδ$^+$ T cells) and recognizes a diverse array of ligands on the cell surface. NKG2D ligands include MICA, MICB, and the UL-16–binding proteins in humans, as well as the retinoic acid-inducible early genes (*Raet1α–Raet1ε*), *H60*, and *Mult1* in mice (8). In the case of NK cells, engagement of NKG2D causes lysis of ligand-expressing cells and cytokine production, most notably IFN-γ (8). NKG2D ligand expression is absent on cell surfaces under normal conditions; however, ligands are induced under conditions of stress, DNA damage, infection, and transformation (9, 10). In the context of CS exposure, we showed that NKG2D ligands are induced on stressed human airway epithelial cells (11) and persistently expressed on pulmonary epithelial cells of smokers with and without COPD (12). RAET1 is also expressed in the alveolar and airway epithelium in a mouse model of COPD (12). Using data obtained from COPD patients

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; CCSP, Clara cell secretion protein; COPD, chronic obstructive pulmonary disease; cRPMI, complete RPMI; CS, cigarette smoke; FA, filtered, room air; HS, horse serum.

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and a mouse model of lung-specific NKG2D ligand expression, we also confirmed that activation of the NKG2D receptor by CS-induced ligands is a potentially important contributor to lung inflammation and tissue destruction in COPD (12).

Recently, we demonstrated that chronic CS exposure primes NK cells to produce more IFN-γ after stimulation by ligands for TLR3, 7, and 9, as well as with IL-12, IL-18, or both (13). Excessive NK cell activation postinfection could worsen the outcomes associated with CS exposure. In the context of COPD, the presence of NK cells hyperresponsive to viral provocation could increase inflammation and contribute to symptomatic exacerbations and disease progression. In this study, we examined the roles of NK cells and the NKG2D activating receptor in viral exacerbations of COPD. We show that purified NK cells from CS-exposed mice are more cytotoxic toward NKG2D ligand-expressing target cells. Furthermore, we show that NKG2D is necessary for the development of enhanced NK cell hyperresponsiveness to activating cytokines. Moreover, we demonstrate that NKG2D is required for exaggerated pathologies resulting from influenza infection in a mouse model of COPD. These studies reveal potentially important roles for NK cells in the development and progression of COPD and indicate that these mechanisms are controlled by the presence and function of NKG2D.

Materials and Methods

Mice

C57BL/6J and C57BL/6J *B2m*$^{-/-}$ mice (female, 8–10 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME). NKG2D-deficient (*Klrk1*$^{-/-}$) mice were generated as described (14). All of the experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Cincinnati Medical Center.

Reagents

The following Abs and immunological reagents were used: NKp46 (29A1.4), NK1.1 (PK136), CD49b (DX5), CD94 (18d3), streptavidin-allophycocyanin, NKG2D (CX5), 2B4 (eBio244F4), 7-aminoactinomycin D (7-AAD), CFSE, and IFN-γ ELISA (eBioscience); IL-2 and IL-12 (PeproTech, Rocky Hill, NJ); IL-18 (R&D Systems); Dynabead CD49b^{+} NK cell isolation kit (Invitrogen); polyinosinic-polycytidylic acid (high molecular weight) (InvivoGen); anti-asialo GM1 (Wako); and α-Clara cell secretory protein (CCSP) polyclonal Ab (Seven Hills Bioreagents).

Mouse model of COPD

Mice were exposed to either filtered, room air (FA) or CS generated from 3R4F Kentucky Reference Cigarettes (University of Kentucky, Lexington, KY), as previously described (15). CS exposures were carried out with a TE-10z smoking machine attached to an exposure chamber (Teague Enterprises, Woodland, CA). The concentration of the smoke/air mixture was maintained at 150 ± 15 mg/m^3 total suspended particulates. Particulate concentrations were determined by weighing vacuum-drawn total particulate deposition onto filters connected to the chambers. Mice were exposed whole body in the exposure chamber for 4 h/d, 5 d/wk for 6 mo.

Leukocyte isolation

Mice were euthanized with an i.p. injection of sodium pentobarbital, followed by exsanguination. Lungs were perfused with 6 ml 1× PBS containing 0.6 mM EDTA. Lungs and spleens were withdrawn aseptically, and leukocytes were isolated, as previously described (15).

NK purification and cell stimulation

Spleens from five mice were pooled, and splenocytes were isolated as described above and resuspended in complete RPMI (cRPMI; RPMI 1640 with 2.05 mM L-glutamine [HyClone, Waltham, MA] containing 10% FBS, 1% sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1× nonessential amino acids [MP Biomedicals, Solon, OH]). Cell resuspension was followed by a 20-min plastic adherence plating step at 37°C and 5% CO_2 that greatly reduces the presence of contaminating adherent cells. After plating, the remaining leukocytes were enriched for NK cells by positive selection, following the manufacturer's protocol for the Dynabead FlowComp Mouse CD49b NK isolation kit (Invitrogen). After enrichment, NK cells were >60% pure. The remaining cells were stained with NKp46 and sorted by flow cytometry for NK cells, resulting in a purity >99%. A total of 2.0 × 10^5 cells in 100 μl cRPMI containing 20 U/ml mouse rIL-2 (PeproTech) was aliquoted per well into a 96-well round-bottom culture plate (Costar, Cambridge, MA) and cultured at 37°C and 5% CO_2. Cells were rested 2 h and then stimulated for 16 h with IL-18 (10 μg/ml) or IL-12 + IL-18 (10 μg/ml). Supernatants were harvested and assayed for IFN-γ production by ELISA (eBioscience).

In vivo cytotoxicity

In vivo cytotoxicity was assessed according to a modified procedure described previously (16). Splenocytes were isolated from wild-type C57BL/6J mice or C57BL/6J *B2m*$^{-/-}$ mice deficient in MHC class I molecule expression. Cells were washed twice with PBS + 0.2% horse serum (HS) and resuspended at 10 × 10^6 cells/ml. The C57BL/6 *B2m*$^{-/-}$ cells were stained with 5 μM CFSE (eBioscience), and the wild-type C57BL/6 cells were stained with 0.5 μM CFSE. Cells were incubated for 8 min at 37°C, and the reaction was stopped by adding ice-cold PBS + 20% HS. Cells were washed twice with PBS + 0.2% HS and resuspended at 3 × 10^7 cells/ml in PBS. Equal numbers of cells (3.0 × 10^6 in 100 μl each) were combined and administered by tail vein injection into FA- or CS-exposed mice. After 16 h, ∼200 μl blood was collected via mandibular bleed using a 4-mm animal lancet (Goldenrod). RBCs were lysed using 1× RBC Lysis solution (QIAGEN) and then fixed in 1% paraformaldehyde for 20 min at 4°C. Cells were spun at 300 × *g*, and the supernatant was discarded. Pellets were resuspended in a 100 μl 2.5% saponin solution. The cells were analyzed by flow cytometry. Cytotoxicity was calculated as follows: (number of CFSE high cells in sample/number of CFSE low cells in sample)/(number of CFSE high cells injected/number of CFSE low cells injected). Flow cytometry was performed using a FACSCalibur (BD Biosciences, San Jose, CA). The data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Ex vivo cytotoxicity

For NK cell enrichment, spleen leukocytes from two mice were isolated, pooled, and enriched using a Dynabead FlowComp Mouse CD49b NK isolation kit, as described above (Invitrogen). Cells were resuspended in cRPMI containing 20 U/ml mouse rIL-2 and added to 5 ml polystyrene round-bottom tubes. Cells were allowed to rest for 2 h at 37°C and 5% CO_2. RMA-Mock and RMA-Raet1ε cells (kindly provided by Dr. Lewis Lanier, University of California, San Francisco) were stained with 0.5 μM CFSE (eBioscience), as described above. RMA cells were resuspended in cRPMI, and ∼20,000 stained cells were added to tubes containing purified NK cells for a total volume of 400 μl. Combined cells were spun at 300 × *g* and allowed to incubate at 37°C and 5% CO_2 for 4 h. After incubation, cells were washed in FACS buffer and resuspended in 300 μl buffer. Prior to flow cytometry analysis, 2.5 μl 7-AAD (eBioscience) was added to each tube. Cytotoxicity was calculated with the following equation using the percentage of 7-AAD$^-$ cells for each group: [(background − test)/background] × 100 = % cytotoxicity.

RT-PCR

Frozen tissue was homogenized using a Tissumizer (Tekmar), and total RNA was isolated with TRIzol reagent (Invitrogen). DNase treatment to remove residual DNA was performed using the Turbo DNA-free kit (Ambion). Reverse transcription of total RNA was performed using the high-capacity cDNA Archive kit (Applied Biosystems). FAM-labeled probes used for RT-PCR were Ulbp1 (Mult1) (Mm01180648_m1), Raet1 (Mm04206137_gh), and Rpl32 (Mm02528467_g1) (Applied Biosystems). Quantitative RT-PCR was performed using TaqMan universal PCR master mix (Applied Biosystems) on an Applied Biosystems 7300 real-time PCR system. Expression of mRNA was quantified by the $\Delta\Delta C_T$ method using *Rpl32* as the endogenous control.

Virus infection

The influenza A virus HKx31 (H3N2) was used in this study (17). The virus was passaged in embryonated chicken eggs by standard procedures and titrated on MDCK cells. Mice were infected with 2 × 10^3 PFU virus through noninvasive oral aspiration, as described previously (18). Infection was allowed to proceed for 4 d, at which time the mice were euthanized. Lungs were clamped at the left bronchus, and the left lung was frozen for viral titer, whereas the right lung was inflation formalin-fixed and paraffin-embedded, as previously described (11).

Pathology assessment and immunohistochemistry

To assess pulmonary inflammation, paraffin sections of lungs were stained with H&E. Histological scores for alveolar, peribronchial, and perivascular inflammation were semiquantitatively scored as the sum of the severity [absent (0), minimal (1), slight (2), moderate (3), strong (4), or severe (5)] and distribution [no inflammation (0) to diffuse (5)] by two analysts who were blinded to the treatment groups. At least four nonsequential sections were used for each mouse. Airways obstruction was quantitated as the percentage of intrapulmonary airways exhibiting any degree of coalesced luminal debris (i.e., epithelial cells, leukocytes, mucus). CCSP immunohistochemistry was performed on slides to assess airway damage after influenza infection using a polyclonal Ab to mouse CCSP (Seven Hills Bioreagents). The primary Ab was used at 1:20,000 dilution with a goat anti-rabbit biotinylated secondary Ab at 1:200 dilution (Vector Laboratories) after citrate Ag retrieval.

NK cell transfer

For NK cell enrichment, spleen and lung leukocytes from six FA- or CS-exposed mice were isolated, pooled, and enriched using the Dynabead FlowComp Mouse CD49b NK isolation kit described above (Invitrogen). Cells were then stained and sorted for $CD3^-$ $CD49b^+$ populations by flow cytometry. Cells were checked by NKp46 stain to confirm >90% NK cells. Cells were washed and resuspended in PBS at a concentration of 2.5×10^6 cells/ml, and 200 μl was administered through tail vein injection into CS $Klrk1^{-/-}$ recipient mice. Mice were allowed to rest for 24 h before viral infection, as described.

Plaque assay

Monolayers of MDCK cells were infected with serial dilutions of lung supernatant after homogenization of frozen lung. The infected cell monolayers were incubated for 1 h at 37°C to facilitate viral adsorption and then washed with PBS. After washing, the MDCK cell monolayers were treated with MEM containing 1 mg/ml trypsin and 0.8% agarose. The infected monolayers were incubated for 72 h at 37°C. At 72 h postinfection, the agarose-containing medium was gently removed, and the monolayers were stained with crystal violet to visualize the influenza virus plaques.

Statistics

Significant differences among groups were identified by *t* test or one-way ANOVA, wherever appropriate. Significant differences in histological comparisons were determined using the Mann–Whitney *U* test. Differences between means were considered significant at $p < 0.05$.

Results

Expression of NK cell cytotoxicity receptors is unchanged in a mouse model of COPD

Previously, we showed that NK cells are the predominant producers of IFN-γ in response to viral ligands and that this production is enhanced after CS exposure (13). This enhanced NK cell response was independent of changes in IFN-α, IL-12, or IL-18. We explored the possibility that this altered NK cell phenotype is associated with changes in activating/inhibiting receptor expression on NK cells from mice exposed long-term to CS. We examined the levels of several key receptors, including NKp46, NK1.1, NKG2D, and CD244 (2B4), on NK cells in our mouse model of COPD. The total numbers of NK cells isolated from the lung and spleen were unaltered by long-term CS exposure (Fig. 1A, 1B). The percentage of NK cells (identified as $Nkp46^+$) (19) expressing the activating receptors NK1.1, NKG2D, and CD244 (2B4), and their receptor density were not different between exposure groups (Fig. 1C–F). We also assessed CD94 expression because it heterodimerizes with NKG2A/C subunits during NK cell activation. There was no difference in the number of $CD94^+$ cells between treatments, and no significant differences were found in the expression levels of CD94-mid and -hi cells. The expression of CD49b, a common marker for NK identification, similarly exhibited no significant differences in expression between treatment groups. Thus, NK cells isolated from multiple compartments of a mouse model of COPD were phenotypically indistinguishable from the FA-exposed controls.

NK cell cytotoxicity against MHC class I-deficient targets in a mouse model of COPD

We previously showed that CS exposure increases CD107a expression (a marker of degranulation) (20) on NK cells (13). In the current study, we specifically examined the function of NK cell cytotoxic activity in a mouse model of COPD. Using an in vivo model of NK cell cytotoxicity, splenocytes from C57BL/6 wild-type and $B2m^{-/-}$ mice, which lack surface MHC class I Ag, were differentially labeled with the fluorescent marker CFSE and injected i.v. The ratios of peripheral blood wild-type cells and $B2m^{-/-}$ cells recovered after the assay define NK cell cytotoxic effector function (Fig. 2A) (16). We found that NK cell cytotoxicity toward MHC class I-deficient cells was not different in our COPD model compared with mice exposed to FA for the same duration (Fig. 2B). Notably, we observed a similar significant decrease in cytotoxicity in mice exposed to CS or FA for 10 mo, indicating an age-dependent decrease in baseline NK cell function (Fig. 2B).

CS exposure enhances NKG2D-mediated cytotoxicity

Engagement of the NKG2D (*Klrk1*) receptor is a potentially important pathway for NK cell activation in the context of COPD. Previously, we demonstrated that NKG2D ligand expression is induced on airway epithelial cells of smokers and COPD patients and the alveolar and airway epithelium of mice exposed to CS (12). However, the specific function of NKG2D in COPD has not been examined. To investigate NKG2D function on NK cells, we performed a cytotoxicity assay using an RMA mouse T lymphoma cell line transfected to express Raet1ε (21). RMA cells tend to aggregate in organs making detection in blood difficult (22). Therefore, we used an ex vivo cytotoxicity assay to measure NK activity (16). As shown in Fig. 3, NK cells from mice exposed to CS were more cytotoxic toward RMA-Raet1ε cells than from mice exposed to FA. The finding that NKG2D function is enhanced in the COPD model is significant because it identifies a unique mechanism whereby CS exposure amplifies subsequent NK cell responses toward infected cells.

NKG2D deletion abolishes NK cell hyperresponsiveness in a mouse model of COPD

Previously, we reported NK cell hyperresponsiveness in the context of CS exposure and in an inducible, lung-specific Raet1-expressing transgenic mouse model (13). To investigate the role of NKG2D in mediating CS-induced NK cell hyperresponsiveness, we used mice deficient in NKG2D ($Klrk1^{-/-}$). NK cells were purified (~99%) from FA- and CS-exposed mice and stimulated with IL-18 or IL-12/IL-18. Consistent with previous data (13), CS exposure enhances NK cell IFN-γ production after stimulation with cytokines (Fig. 4). The increased IFN-γ production occurred absent significant differences in IL-12 or IL-18 receptor expression on NK cells between FA- and CS-exposed mice (data not shown). In contrast, NKG2D-deficient mice failed to exhibit increased cytokine responsiveness in a mouse model of COPD. Together with the current results, demonstrating enhanced NKG2D function in our CS model of COPD (Fig. 3), our findings suggest that a hyperresponsive NK cell phenotype may be achieved through chronic, lung-specific NKG2D stimulation.

NKG2D on NK cells is necessary for enhanced pulmonary inflammation and airway injury following influenza infection in COPD

Viral infections contribute significantly to the pathogenesis of COPD. The above findings that NKG2D is required for NK cell hyperresponsiveness led us to examine the role of NKG2D in influenza-induced exacerbations of COPD. Because we were in-

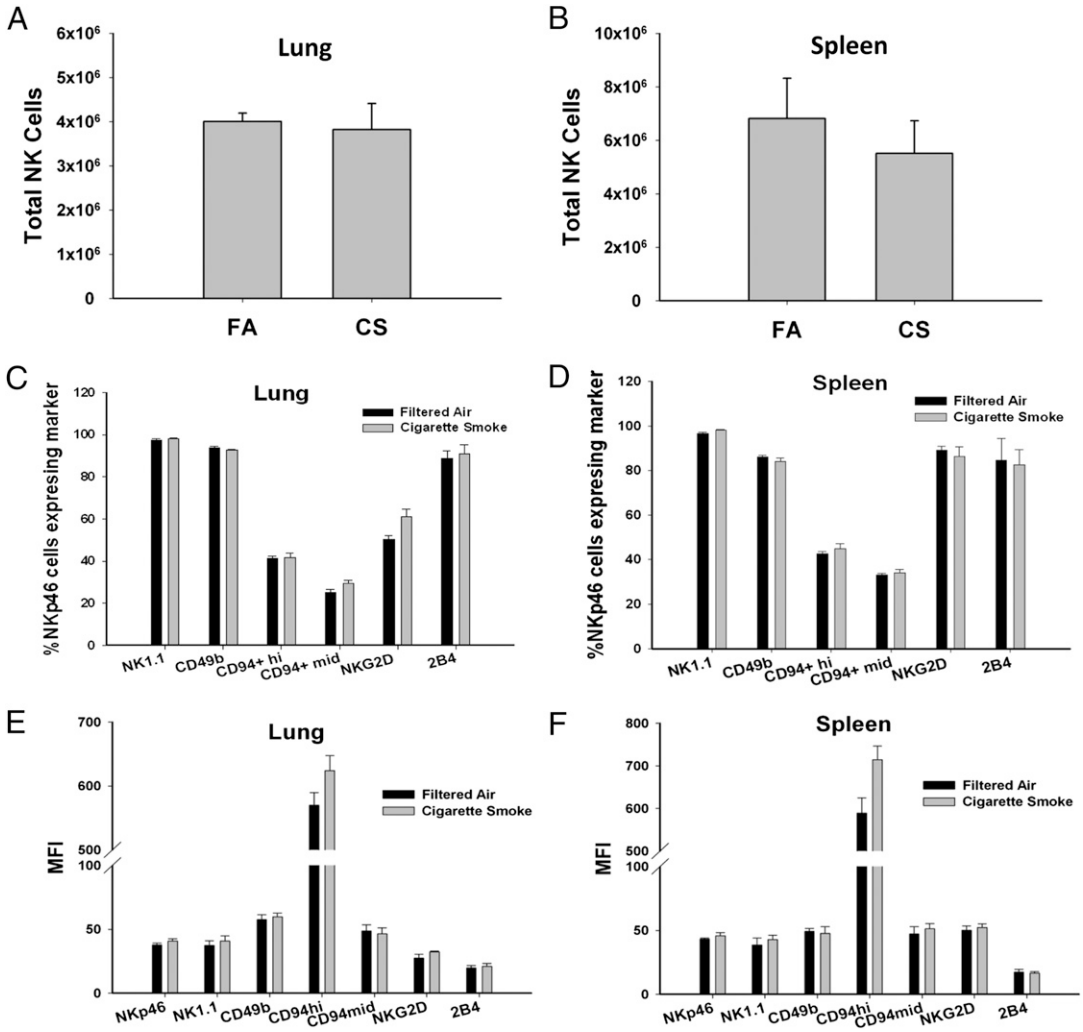

FIGURE 1. Characterization of NK cell markers in a mouse model of COPD. NK cells were isolated from C57BL/6 mice, exposed to FA or CS for 6 mo, and analyzed by flow cytometry. (**A** and **B**) Total NK cells enumerated by NKp46$^+$ expression. (**C** and **D**) The percentage of NKp46$^+$ cells expressing the indicated NK cell markers. (**E** and **F**) The geometric mean fluorescent intensity (MFI) of NK cell receptors. Values are presented as mean ± SEM (n = 5–6/group). All data are representative of two independent experiments.

terested in the role of NK cells, we chose to examine the early effects (i.e., 4 d postinfection) of influenza infection in our COPD model. Compared with influenza-infected mice exposed to FA, infection of mice exposed to CS augmented inflammation and airway epithelial injury that included both loss of epithelial integrity and differentiated epithelial cell morphology (Fig. 5A, 5C). Furthermore, we found that these exaggerated pathologies were reduced in mice lacking the NKG2D receptor (Fig. 5B, 5D).

By staining with Abs against CCSP, a specific marker for airway epithelial cells, we assessed the extent of damage to the airway epithelium. CCSP staining demonstrated increased disruption of epithelial integrity within the large airways of CS-exposed *Klrk1*$^{+/+}$ mice and that surviving epithelial cells had reduced expression of CCSP as a marker of epithelial differentiation relative to FA-infected *Klrk1*$^{+/+}$ mice (Fig. 5C). In contrast, the damage to the airway epithelium of *Klrk1*$^{-/-}$ mice was similar to FA-exposed *Klrk*$^{+/+}$ mice, regardless of exposure (Fig. 5D). Similarly, influenza infection caused blockage of many of the small airways of CS-exposed *Klrk1*$^{+/+}$ mice by large aggregates of cells that appeared to be a mix of denuded epithelial cells and leukocytes (Fig. 5A, 5C). These clumps of cells were infrequently observed in the FA-exposed *Klrk1*$^{+/+}$ mice. Such mixed aggregates of cells/cellular debris were infrequently seen in the influenza-infected *Klrk1*$^{-/-}$ mice, regardless of exposure (Fig. 5B, 5D).

To verify that the lack of the exaggerated response to influenza in the CS-*Klrk1*$^{-/-}$ mice was attributable to receptor function and not the lack of NKG2D ligand induction, we assessed the affects of CS and influenza infection on transcript levels of multiple NKG2D ligands. As previously reported by our group, CS exposure induces RAE1 expression in lung tissue (12). In this study, we demonstrate that CS exposure and influenza infections similarly upregulate *Raet1* expression. An analogous pattern of *Raet1* expression occurred in CS-exposed and influenza-infected *Klrk1*$^{+/+}$ and *Klrk1*$^{-/-}$ mice (Fig. 5E). Another NKG2D ligand, *Mult1*, demonstrated similar expression patterns to CS and influenza in both *Klrk1*$^{+/+}$ and *Klrk1*$^{-/-}$ mice (Fig. 5F).

We next sought to define the role of NKG2D specifically on NK cells in the exaggerated pathologies associated with influenza infection and COPD. We transferred purified lung NK cells from FA- and CS-exposed *Klrk1*$^{+/+}$ mice into CS-exposed *Klrk1*$^{-/-}$ recipients, which were then infected with influenza. Histopathological assessments were conducted to calculate inflammation severity and distribution, as well as to calculate the extent of airway obstruction. Influenza-infected *Klrk1*$^{+/+}$ mice exposed to

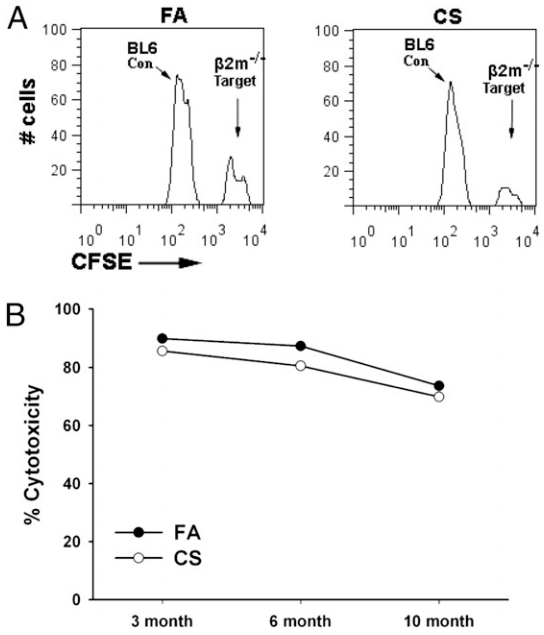

FIGURE 2. NK cell cytotoxicity against MHC class I-deficient and NKG2D ligand-expressing targets in mouse model of COPD. (**A**) Representation of flow cytometry analysis comparing in vivo clearance. CFSE-labeled C57BL/6 (CFSE low) cells and C57BL/6 *B2m*$^{-/-}$ (CFSE high) cells were injected i.v. into recipient mice exposed to FA or CS. Mice were bled for 16 h following injections, and labeled target cells were analyzed by flow cytometry. (**B**) Time course of NK cell cytotoxicity against *B2m*$^{-/-}$ target cells, as described in (A), in mice exposed to FA or CS for 3–10 mo. Data are representative of results from four independent experiments.

FA for 6 mo and influenza-infected *Klrk1*$^{-/-}$ mice exposed to FA or CS for 6 mo exhibited significantly less inflammation than did *Klrk1*$^{+/+}$ CS-exposed mice (Fig. 5H, 5I). Transfer of NK cells from CS-exposed *Klrk1*$^{+/+}$ donors into CS-exposed *Klrk1*$^{-/-}$ recipients nearly recapitulated the phenotype of influenza-infected CS-exposed *Klrk1*$^{+/+}$ mice (compare Fig. 5G and 5A). The pathologies elicited with the transfer of NK cells from CS-exposed

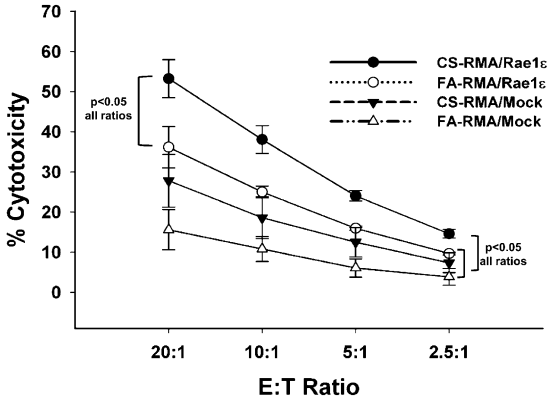

FIGURE 3. NK cells of CS-exposed mice demonstrate increased cytotoxic activity toward RAE1-expressing cells. NK cells were purified from pooled splenocytes of mice exposed to FA or CS for 6 mo, and cytotoxicity toward RAE1ε targets was assessed ex vivo. NK cells and CFSE-labeled RMA cells transfected to express RAE1ε (or mock transfected) were combined at increasing E:T ratios. Cells were incubated for 4 h, harvested, and analyzed by flow cytometry, as described in *Materials and Methods*. Data are representative of four independent experiments, each using NK cells pooled from two mice. Significant differences between groups at all E:T ratios are indicated.

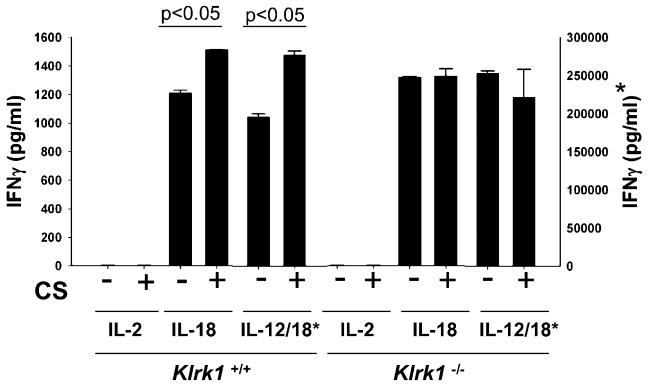

FIGURE 4. *Klrk1*$^{-/-}$ mice do not develop enhanced cellular responses in a mouse model of COPD. NK cells from the pooled spleens of five mice were highly purified (>99% NKp46^{+}) and stimulated overnight with cytokines, as labeled. IFN-γ was quantified by ELISA. The marked (*) axis represents levels of IFN-γ released by IL-12/18 stimulation. Values are presented as mean ± SEM. Relevant significant differences between groups are highlighted. Data are representative of three independent experiments.

donors was also significantly greater than the pathologies associated with the transfer of NK cells from FA-exposed donors into the CS-exposed *Klrk1*$^{-/-}$ recipients (Fig. 5H, 5I). These experiments demonstrate that NKG2D^{+} NK cells from CS-exposed mice play a major role in inflammation and airway obstruction in the mouse model of COPD viral exacerbation.

In addition to the inflammatory and histopathologic assays, we assessed the viral titers in the lungs of influenza-infected mice (Fig. 6). There were no significant differences among FA- and CS-exposed *Klrk1*$^{+/+}$ or *Klrk1*$^{-/-}$ mice or CS-exposed *Klrk1*$^{-/-}$ mice that received *Klrk1*$^{+/+}$ FA or CS NK cells. These studies demonstrate that NKG2D is necessary for the enhanced pulmonary inflammation and airway injury after influenza infection in COPD, independent of the ability of the immune system to eliminate the virus.

Discussion

A prevailing assumption is that CS is a toxic irritant that broadly inhibits leukocyte function (23, 24). However, a growing body of literature suggests that this perspective is too narrow and that chronic CS exposure can augment specific innate and adaptive immune functions in COPD patients (7, 25–27). In the course of infection, NK cells play an important dual role: the early release of cytokines and directed cytotoxicity. Previously, we showed that NK cells from CS-exposed mice have enhanced cytokine production in response to stimulating cytokines or to TLR ligands that mimic pathogen-associated molecular patterns (13). In the current study, we show that this enhanced function is dependent on the presence of NKG2D. NK cells from CS-exposed, NKG2D-deficient NK cells do not exhibit enhanced IFN-γ production when stimulated with IL-12 and/or IL-18. Excessive NK cell activation postinfection could worsen the outcomes associated with CS exposure. In the context of COPD, the presence of NK cells hyperresponsive to viral provocation could increase the inflammatory response and contribute to the progression of COPD.

Although there have been a number of studies in humans investigating the effect of CS exposure on cytotoxic lymphocyte effector functions, primarily cytotoxicity, the outcomes of these studies are equivocal. Several studies reported increased cytotoxic activity (28–32), whereas others showed decreased activity in smokers with and without COPD (27, 33–36). Analyses of NK cell function in mice exposed to CS show a similar variability in results

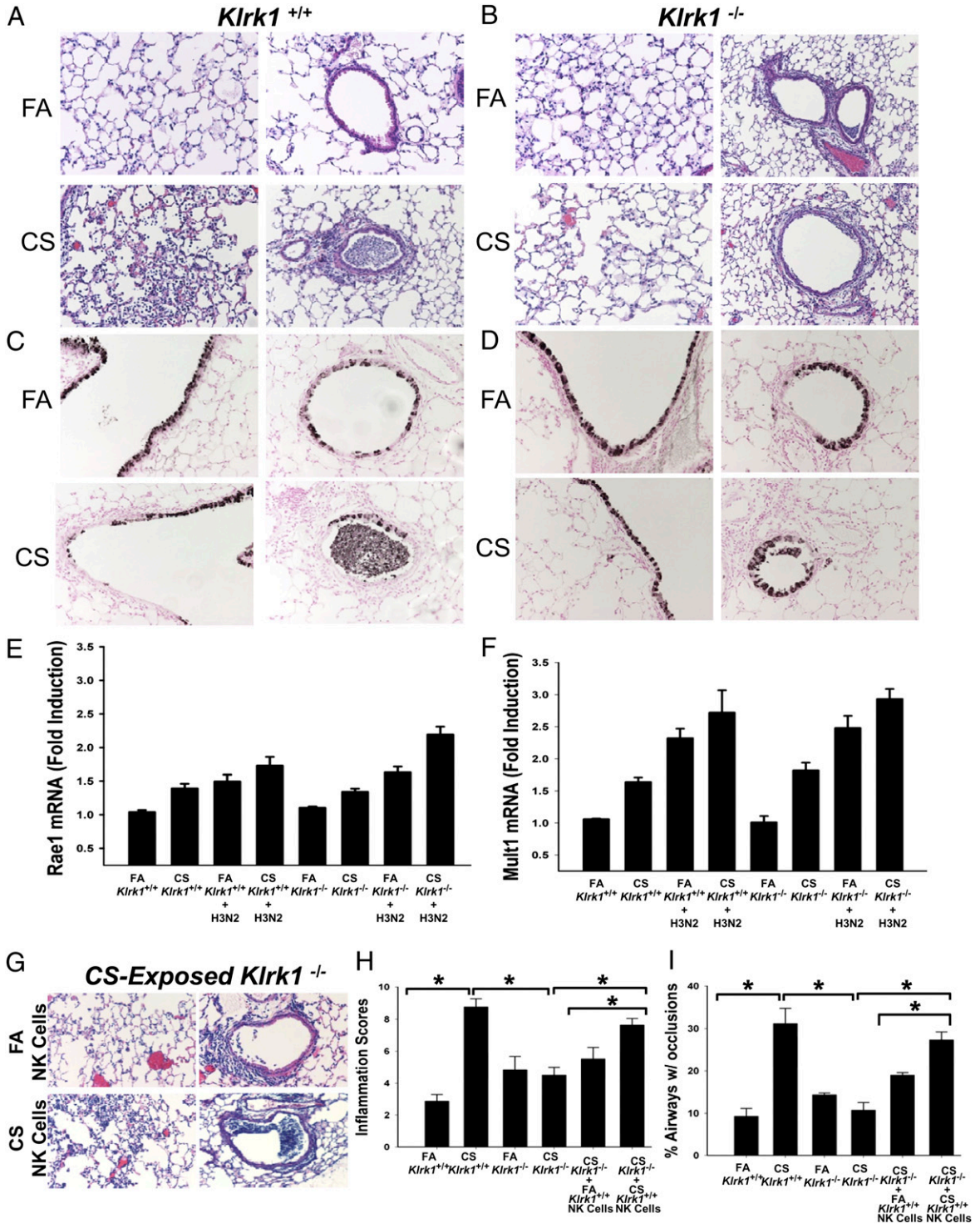

FIGURE 5. CS-exposed *Klrk1*$^{-/-}$ mice lack enhanced cellular responses associated with influenza infection. Mice were infected with 2×10^3 PFU influenza virus, and end points were measured 4 d postinfection. (**A** and **B**) H&E-stained lung sections representing changes in lung inflammation and airways obstruction between groups. Original magnification ×200. (**C** and **D**) CCSP immunohistochemistry staining of lung sections showing epithelial damage of large airways and small airways. Original magnification ×200. (**E** and **F**) Total RNA was isolated from lung homogenates of FA- or CS-exposed *Klrk1*$^{+/+}$ and *Klrk1*$^{-/-}$ mice with and without influenza infection. *Raet1* and *Mult1* transcripts were assayed by quantitative RT-PCR and normalized to *Rpl32*. (**G**) H&E-stained lung sections of influenza-infected CS-exposed *Klrk1*$^{-/-}$ mice that received NK cells from FA- or CS-exposed *Klrk1*$^{+/+}$ mice. Original magnification ×200. (**H**) Semiquantitative assessment of inflammation severity and distribution. (**I**) Quantitation of the percentage of intrapulmonary airways exhibiting any degree of airways obstruction. (n = 4–8 mice/group). *Significant differences between groups ($p > 0.05$).

(13, 37). The recent reports from Urbanowicz et al. (27, 32) indicate that compartmental differences in cytotoxic lymphocyte function may account for these discordant results. Those investigators found that NK cells collected from the peripheral blood of long-term smokers showed reduced cytotoxicity (27), whereas NK cells collected from the sputum of similar patient populations showed increased cytotoxicity (32). Ultimately, the reasons for the disparities are unclear, but the inconsistencies highlight the need

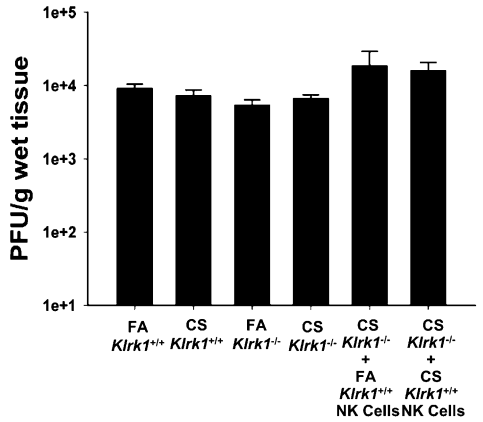

FIGURE 6. Long-term CS exposure does not affect viral clearance in *Klrk1*$^{+/+}$ or *Klrk1*$^{-/-}$ mice. Influenza titers in individual mice were determined by plaque assay, as described in *Materials and Methods*. Data are presented as PFU/g of wet lung tissue. All data are representative of two independent experiments (n = 4–8 mice/group).

for mechanistic studies to understand the effects of CS exposure on NK cell function and the role of NK cells in COPD pathogenesis.

NK cells interact with cells through an extensive array of stimulatory and inhibitory receptor–ligand interactions, including NKG2D (8, 38). In this study, we report increased NKG2D-mediated cytotoxicity in a mouse model of COPD. Previously, we demonstrated that inducible expression of RAET1 on mouse pulmonary epithelial cells, and subsequent activation of NKG2D, causes an emphysematous phenotype that was associated with increased epithelial cell apoptosis (12). Moreover, these mice exhibited enhanced cytokine release in response to TLR stimulation (13) and enhanced clearance of pulmonary bacterial infection (39). Our findings appear contradictory to reports that repeated activation of NKG2D in transgenic mice caused receptor downregulation and loss of function (8, 40–42). However, these studies examined the effects of constitutive expression of NKG2D ligands. In contrast, our mouse model conditionally expresses *Raet1α* in pulmonary epithelial cells, an exposure more comparable to the CS model of COPD, and did not exhibit downregulation of NKG2D expression (12). Together with the current results demonstrating enhanced NKG2D function in our CS model of COPD, our findings suggest that a hyperresponsive NK cell phenotype is achieved through chronic, lung-specific NKG2D stimulation.

CS exposure increases pulmonary inflammation and lung remodeling in response to viral infection. Earlier studies did not identify specific mechanisms that may contribute to CS-induced viral exacerbations (43–46), whereas Kang et al. (47) identified multiple soluble mediators (IL-18, IFN-γ) and signaling pathways (e.g., TLR3) that contribute to viral-induced pathologies in CS-exposed mice. However, the specific cell populations and pathways affected by CS exposure that, in turn, contribute to the heightened response to influenza have not been identified. This distinction is important because neither cytokine nor receptor pathways were altered by CS exposure in the absence of infection in the previous studies (43–47). The integrity of these pathways may reflect their importance for responses to pulmonary viral pathogens with established tobacco smoke-induced injury. In this study, we identified significant cell-specific alterations in response to CS exposure that directly contribute to the increased pathology after viral infection. In the context of influenza infection of CS-exposed mice, we demonstrate the requirement for NKG2D^{+} NK cells in the exacerbation of COPD pathologies. The CS-exposed NKG2D-deficient mice could not mount the prototypic injury response of CS-exposed wild-type mice that included the influx of inflammatory cells, the loss/destruction of an intact respiratory epithelium, and diminished expression of CCSP, a phenotypic marker of the protective Clara cell. However, reconstitution with NKG2D^{+} NK cells from CS-exposed wild-type mice in the NKG2D-deficient mice reestablishes the prototypic injury response. Of note, there were no differences in viral load between any groups of influenza-infected mice. This finding is important because it suggests that the exaggerated immune response to virus and the subsequent increased pathology in COPD, which is dependent on NKG2D, is not a function of immunosuppression and increased susceptibility to infection. Rather, the reported increased incidence and severity of symptoms in smokers is likely due to the increased inflammation and airway obstruction as a consequence of enhanced NK cell function in response to the viral infection.

Although not directly examined in this study, it is tempting to speculate that the diminished pathologies in CS-exposed, influenza-infected, NKG2D-deficient mice were due to the failure of these NK cells to develop a hyperresponsive phenotype in the COPD model. An alternative explanation for these observations is that there is a fundamental requirement for NKG2D in response to pulmonary influenza infection. If so, the FA-exposed *Klrk1*$^{+/+}$ or *Klrk1*$^{-/-}$ mice would reveal this requirement for homeostatic responses to influenza infection in terms of inflammation, injury, and clearance. However, the lack of effect of NKG2D deficiency on these basal responses strongly supports the concept that NKG2D-dependent, CS-induced, NK cell hyperresponsiveness is an important mechanism in disease pathogenesis.

Together with our previous report demonstrating CS-induced Raet1 expression in the lung (12), our new findings suggest a model whereby CS induces the chronic local expression of NKG2D ligands on resident pulmonary cells, which primes NK cells to independent stimulation by cytokines, TLR ligands, and NKG2D ligands. These NK-activating mediators are known to increase during viral infection and lead to enhanced inflammation and cytotoxicity. Important to the exacerbation of COPD, the enhanced NK cell activation leads to increased pulmonary injury and airway obstruction. Although we demonstrate a clear role for NKG2D in the priming of NK cell function in the context of long-term CS exposure, the mechanisms by which NKG2D mediates NK cell priming and the subsequent response to influenza remain to be fully elucidated.

In the current study, we present evidence that NK cells regulate inflammation in a model of viral infection of COPD and that NKG2D mediates enhanced NK cell effector function and influenza-induced pathologies in a mouse model of COPD. These findings indicate that alterations in NK cell function will have significant consequences in COPD patients infected with virus. Although NK cells are by far the predominant cell type expressing NKG2D in mice, NKG2D is also expressed almost ubiquitously on human CD8 T cells (8). However, studies suggest that NKG2D plays a costimulatory role based on the relative inability to stimulate T cell responses with NKG2D ligands alone (48–50). This difference in NKG2D expression and function between mice and humans, coupled with evidence for a role of CD8 T cells in COPD pathogenesis (51–53), necessitates further examination of the contribution of NKG2D-bearing lymphocyte subpopulations in COPD pathogenesis.

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Disclosures

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References

1. Pauwels, R. 2001. Global initiative for chronic obstructive lung diseases (GOLD): time to act. *Eur. Respir. J.* 18: 901–902.
2. Rennard, S. I., and J. Vestbo. 2006. COPD: the dangerous underestimate of 15%. *Lancet* 367: 1216–1219.
3. Rodriguez-Roisin, R. 2000. Toward a consensus definition for COPD exacerbations. *Chest* 117(5, Suppl. 2)398S–401S.
4. Anzueto, A. 2010. Impact of exacerbations on COPD. *Eur. Respir. Rev.* 19: 113–118.
5. Wedzicha, J. A. 2004. Role of viruses in exacerbations of chronic obstructive pulmonary disease. *Proc. Am. Thorac. Soc.* 1: 115–120.
6. Wesseling, G. 2007. Occasional review: influenza in COPD: pathogenesis, prevention, and treatment. *Int. J. Chron. Obstruct. Pulmon. Dis.* 2: 5–10.
7. Freeman, C. M., M. K. Han, F. J. Martinez, S. Murray, L. X. Liu, S. W. Chensue, T. J. Polak, J. Sonstein, J. C. Todt, T. M. Ames, et al. 2010. Cytotoxic potential of lung CD8(+) T cells increases with chronic obstructive pulmonary disease severity and with in vitro stimulation by IL-18 or IL-15. *J. Immunol.* 184: 6504–6513.
8. Champsaur, M., and L. L. Lanier. 2010. Effect of NKG2D ligand expression on host immune responses. *Immunol. Rev.* 235: 267–285.
9. Gasser, S., and D. H. Raulet. 2006. The DNA damage response arouses the immune system. *Cancer Res.* 66: 3959–3962.
10. Pegram, H. J., D. M. Andrews, M. J. Smyth, P. K. Darcy, and M. H. Kershaw. 2011. Activating and inhibitory receptors of natural killer cells. *Immunol. Cell Biol.* 89: 216–224.
11. Borchers, M. T., N. L. Harris, S. C. Wesselkamper, S. Zhang, Y. Chen, L. Young, and G. W. Lau. 2006. The NKG2D-activating receptor mediates pulmonary clearance of *Pseudomonas aeruginosa*. *Infect. Immun.* 74: 2578–2586.
12. Borchers, M. T., S. C. Wesselkamper, V. Curull, A. Ramirez-Sarmiento, A. Sánchez-Font, J. Garcia-Aymerich, C. Coronell, J. Lloreta, A. G. Agusti, J. Gea, et al. 2009. Sustained CTL activation by murine pulmonary epithelial cells promotes the development of COPD-like disease. *J. Clin. Invest.* 119: 636–649.
13. Motz, G. T., B. L. Eppert, B. W. Wortham, R. M. Amos-Kroohs, J. L. Flury, S. C. Wesselkamper, and M. T. Borchers. 2010. Chronic cigarette smoke exposure primes NK cell activation in a mouse model of chronic obstructive pulmonary disease. *J. Immunol.* 184: 4460–4469.
14. Guerra, N., Y. X. Tan, N. T. Joncker, A. Choy, F. Gallardo, N. Xiong, S. Knoblaugh, D. Cado, N. M. Greenberg, and D. H. Raulet. 2008. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. [Published erratum appears in 2008 *Immunity* 28: 723.] *Immunity* 28: 571–580.
15. Motz, G. T., B. L. Eppert, G. Sun, S. C. Wesselkamper, M. J. Linke, R. Deka, and M. T. Borchers. 2008. Persistence of lung CD8 T cell oligoclonal expansions upon smoking cessation in a mouse model of cigarette smoke-induced emphysema. *J. Immunol.* 181: 8036–8043.
16. Barnes, M. J., H. Aksoylar, P. Krebs, T. Bourdeau, C. N. Arnold, Y. Xia, K. Khovananth, I. Engel, S. Sovath, K. Lampe, et al. 2010. Loss of T cell and B cell quiescence precedes the onset of microbial flora-dependent wasting disease and intestinal inflammation in Gimap5-deficient mice. *J. Immunol.* 184: 3743–3754.
17. Baez, M., P. Palese, and E. D. Kilbourne. 1980. Gene composition of high-yielding influenza vaccine strains obtained by recombination. *J. Infect. Dis.* 141: 362–365.
18. Glasser, S. W., T. L. Witt, A. P. Senft, J. E. Baatz, D. Folger, M. D. Maxfield, H. T. Akinbi, D. A. Newton, D. R. Prows, and T. R. Korfhagen. 2009. Surfactant protein C-deficient mice are susceptible to respiratory syncytial virus infection. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 297: L64–L72.
19. Walzer, T., M. Bléry, J. Chaix, N. Fuseri, L. Chasson, S. H. Robbins, S. Jaeger, P. André, L. Gauthier, L. Daniel, et al. 2007. Identification, activation, and selective in vivo ablation of mouse NK cells via NKp46. *Proc. Natl. Acad. Sci. USA* 104: 3384–3389.
20. Alter, G., J. M. Malenfant, and M. Altfeld. 2004. CD107a as a functional marker for the identification of natural killer cell activity. *J. Immunol. Methods* 294: 15–22.
21. Cerwenka, A., J. L. Baron, and L. L. Lanier. 2001. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc. Natl. Acad. Sci. USA* 98: 11521–11526.
22. Höglund, P., R. Glas, C. Ohlén, H. G. Ljunggren, and K. Kärre. 1991. Alteration of the natural killer repertoire in H-2 transgenic mice: specificity of rapid lymphoma cell clearance determined by the H-2 phenotype of the target. *J. Exp. Med.* 174: 327–334.
23. Sopori, M. 2002. Effects of cigarette smoke on the immune system. *Nat. Rev. Immunol.* 2: 372–377.
24. Stämpfli, M. R., and G. P. Anderson. 2009. How cigarette smoke skews immune responses to promote infection, lung disease and cancer. *Nat. Rev. Immunol.* 9: 377–384.
25. Feghali-Bostwick, C. A., A. S. Gadgil, L. E. Otterbein, J. M. Pilewski, M. W. Stoner, E. Csizmadia, Y. Zhang, F. C. Sciurba, and S. R. Duncan. 2008. Autoantibodies in patients with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 177: 156–163.
26. Lee, S. H., S. Goswami, A. Grudo, L. Z. Song, V. Bandi, S. Goodnight-White, L. Green, J. Hacken-Bitar, J. Huh, F. Bakaeen, et al. 2007. Antielastin autoimmunity in tobacco smoking-induced emphysema. *Nat. Med.* 13: 567–569.
27. Urbanowicz, R. A., J. R. Lamb, I. Todd, J. M. Corne, and L. C. Fairclough. 2009. Altered effector function of peripheral cytotoxic cells in COPD. *Respir. Res.* 10: 53.
28. Chrysofakis, G., N. Tzanakis, D. Kyriakoy, M. Tsoumakidou, I. Tsiligianni, M. Klimathianaki, and N. M. Siafakas. 2004. Perforin expression and cytotoxic activity of sputum CD8+ lymphocytes in patients with COPD. *Chest* 125: 71–76.
29. Kratikanont, P., R. D. deShazo, D. E. Banks, and Y. Chapman. 1987. Cytotoxic cell function in bronchogenic carcinoma. *Chest* 92: 90–94.
30. Lethbridge, M. W., D. M. Kemeny, J. C. Ratoff, B. J. O'Connor, C. M. Hawrylowicz, and C. J. Corrigan. 2010. A novel technique to explore the functions of bronchial mucosal T cells in chronic obstructive pulmonary disease: application to cytotoxicity and cytokine immunoreactivity. *Clin. Exp. Immunol.* 161: 560–569.
31. Newman, L. S., K. Kreiss, and P. A. Campbell. 1991. Natural killer cell tumoricidal activity in cigarette smokers and in silicotics. *Clin. Immunol. Immunopathol.* 60: 399–411.
32. Urbanowicz, R. A., J. R. Lamb, I. Todd, J. M. Corne, and L. C. Fairclough. 2010. Enhanced effector function of cytotoxic cells in the induced sputum of COPD patients. *Respir. Res.* 11: 76.
33. Hughes, D. A., P. L. Haslam, P. J. Townsend, and M. Turner-Warwick. 1985. Numerical and functional alterations in circulatory lymphocytes in cigarette smokers. *Clin. Exp. Immunol.* 61: 459–466.
34. Mian, M. F., N. M. Lauzon, M. R. Stämpfli, K. L. Mossman, and A. A. Ashkar. 2008. Impairment of human NK cell cytotoxic activity and cytokine release by cigarette smoke. *J. Leukoc. Biol.* 83: 774–784.
35. O'Shea, D., T. J. Cawood, C. O'Farrelly, and L. Lynch. 2010. Natural killer cells in obesity: impaired function and increased susceptibility to the effects of cigarette smoke. *PLoS ONE* 5: e8660.
36. Phillips, B., M. E. Marshall, S. Brown, and J. S. Thompson. 1985. Effect of smoking on human natural killer cell activity. *Cancer* 56: 2789–2792.
37. Lu, L. M., C. C. Zavitz, B. Chen, S. Kianpour, Y. Wan, and M. R. Stämpfli. 2007. Cigarette smoke impairs NK cell-dependent tumor immune surveillance. *J. Immunol.* 178: 936–943.
38. Lanier, L. L. 2008. Up on the tightrope: natural killer cell activation and inhibition. *Nat. Immunol.* 9: 495–502.
39. Wesselkamper, S. C., B. L. Eppert, G. T. Motz, G. W. Lau, D. J. Hassett, and M. T. Borchers. 2008. NKG2D is critical for NK cell activation in host defense against *Pseudomonas aeruginosa* respiratory infection. *J. Immunol.* 181: 5481–5489.
40. Coudert, J. D., L. Scarpellino, F. Gros, E. Vivier, and W. Held. 2008. Sustained NKG2D engagement induces cross-tolerance of multiple distinct NK cell activation pathways. *Blood* 111: 3571–3578.
41. Oppenheim, D. E., S. J. Roberts, S. L. Clarke, R. Filler, J. M. Lewis, R. E. Tigelaar, M. Girardi, and A. C. Hayday. 2005. Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. *Nat. Immunol.* 6: 928–937.
42. Wiemann, K., H. W. Mittrücker, U. Feger, S. A. Welte, W. M. Yokoyama, T. Spies, H. G. Rammensee, and A. Steinle. 2005. Systemic NKG2D down-regulation impairs NK and CD8 T cell responses in vivo. *J. Immunol.* 175: 720–729.
43. Bauer, C. M., C. C. Zavitz, F. M. Botelho, K. N. Lambert, E. G. Brown, K. L. Mossman, J. D. Taylor, and M. R. Stämpfli. 2010. Treating viral exacerbations of chronic obstructive pulmonary disease: insights from a mouse model of cigarette smoke and H1N1 influenza infection. *PLoS ONE* 5: e13251.
44. Gualano, R. C., M. J. Hansen, R. Vlahos, J. E. Jones, R. A. Park-Jones, G. Deliyannis, S. J. Turner, K. A. Duca, and G. P. Anderson. 2008. Cigarette smoke worsens lung inflammation and impairs resolution of influenza infection in mice. *Respir. Res.* 9: 53.
45. Mackenzie, J. S., and R. L. P. Flower. 1979. The effect of long-term exposure to cigarette smoke on the height and specificity of the secondary immune response to influenza virus in a murine model system. *J. Hyg. (Lond.)* 83: 135–141.
46. Robbins, C. S., C. M. Bauer, N. Vujicic, G. J. Gaschler, B. D. Lichty, E. G. Brown, and M. R. Stämpfli. 2006. Cigarette smoke impacts immune inflammatory responses to influenza in mice. *Am. J. Respir. Crit. Care Med.* 174: 1342–1351.
47. Kang, M. J., C. G. Lee, J. Y. Lee, C. S. Dela Cruz, Z. J. Chen, R. Enelow, and J. A. Elias. 2008. Cigarette smoke selectively enhances viral PAMP- and virus-induced pulmonary innate immune and remodeling responses in mice. *J. Clin. Invest.* 118: 2771–2784.
48. Groh, V., R. Rhinehart, J. Randolph-Habecker, M. S. Topp, S. R. Riddell, and T. Spies. 2001. Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat. Immunol.* 2: 255–260.
49. Maasho, K., J. Opoku-Anane, A. I. Marusina, J. E. Coligan, and F. Borrego. 2005. NKG2D is a costimulatory receptor for human naive CD8+ T cells. *J. Immunol.* 174: 4480–4484.
50. Roberts, A. I., L. Lee, E. Schwarz, V. Groh, T. Spies, E. C. Ebert, and B. Jabri. 2001. NKG2D receptors induced by IL-15 costimulate CD28-negative effector CTL in the tissue microenvironment. *J. Immunol.* 167: 5527–5530.
51. O'Shaughnessy, T. C., T. W. Ansari, N. C. Barnes, and P. K. Jeffery. 1997. Inflammation in bronchial biopsies of subjects with chronic bronchitis: inverse relationship of CD8+ T lymphocytes with FEV1. *Am. J. Respir. Crit. Care Med.* 155: 852–857.
52. Saetta, M., A. Di Stefano, G. Turato, F. M. Facchini, L. Corbino, C. E. Mapp, P. Maestrelli, A. Ciaccia, and L. M. Fabbri. 1998. CD8+ T-lymphocytes in peripheral airways of smokers with chronic obstructive pulmonary disease. *Am. J. Respir. Crit. Care Med.* 157: 822–826.
53. Zhu, X., A. S. Gadgil, R. Givelber, M. P. George, M. W. Stoner, F. C. Sciurba, and S. R. Duncan. 2009. Peripheral T cell functions correlate with the severity of chronic obstructive pulmonary disease. *J. Immunol.* 182: 3270–3277.