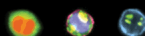


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NK Cells Are Effectors for Resolvin E1 in the Timely Resolution of Allergic Airway Inflammation

Oliver Haworth, Manuela Cernadas, and Bruce D. Levy

Immune responses are pathologically sustained in several common diseases, including asthma. To determine endogenous proresolving mechanisms for adaptive immune responses, we used a murine model of self-limited allergic airway inflammation. After cessation of allergen exposure, eosinophils and T cells were cleared concomitant with the appearance of increased numbers of NK cells in the lung and mediastinal lymph nodes. The mediastinal lymph node NK cells were activated, expressing CD27, CD11b, CD69, CD107a, and IFN- γ . NK cell depletion disrupted the endogenous resolution program, leading to delayed clearance of airway eosinophils and Ag-specific CD4⁺ T cells. NK cell trafficking to inflamed tissues for resolution was dependent upon CXCR3 and CD62L. During resolution, eosinophils and Ag-specific CD4⁺ T cells expressed NKG2D ligands, and a blocking Ab for the NKG2D receptor delayed clearance of these leukocytes. Of interest, NK cells expressed CMKLR1, a receptor for the proresolving mediator resolvin E1, and depletion of NK cells decreased resolvin E1-mediated resolution of allergic inflammation. Resolvin E1 regulated NK cell migration *in vivo* and NK cell cytotoxicity *in vitro*. Together, these findings indicate new functions in catabasis for NK cells that can also serve as targets for proresolving mediators in the resolution of adaptive immunity. *The Journal of Immunology*, 2011, 186: 000–000.

Resolution of inflammation is fundamental to health and tissue homeostasis. Failure of inflammatory responses to resolve in a timely manner can lead to pathological inflammation, a feature of several common human diseases (1, 2). Asthma is a chronic inflammatory disease of the airways that is characterized by infiltration of eosinophils and Th2 cells and release of proinflammatory cytokines and lipid mediators (3). The cellular and molecular regulators of adaptive immunity are of great interest.

Resolution of acute tissue inflammation is now appreciated to entail an active process with distinct cellular effectors and biochemical signaling pathways that are regulated by a new genus of specialized proresolving mediators, including resolvins (4). Resolvin E1 (RvE1; 5S, 12R, 18R-trihydroxy-6Z, 8E, 10E, 14Z, 16E-eicosapentaenoic acid) is an endogenous proresolving mediator for allergic airway responses (5) that serves as an agonist at CMKLR1 receptors (6). In contrast to pathogenic roles for T cells

in chronic inflammation, select populations of lymphocytes can display counterregulatory actions to dampen inflammation (7).

In this study, we used a murine model of asthma exacerbation with self-limited adaptive inflammation to determine the natural time course for leukocyte trafficking during tissue catabasis (as defined in Ref. 8) and identified an integral role for NK cells in mediating resolution. During resolution, NK cells accumulated in the lung draining lymph nodes and depleting NK cells, blocking their ability to interact with target cells, or disrupting their migration to target tissues delayed resolution of allergic inflammation, implicating these innate lymphocytes as pivotal cellular, proresolving effectors for adaptive immune responses.

Materials and Methods

Sensitization and challenge protocols

Allergic airway inflammation and resolution was modeled in allergen (OVA)-sensitized and aerosol-challenged FVB and BALB/cj mice (as in Ref. 5) (Fig. 1A) using a protocol approved by the Harvard Medical Area Institutional Review Board (Protocol 03618). Select mice were given RvE1 methyl-ester (100 ng, i.v.) once a day for 3 consecutive d (as in Ref. 5). To measure the impact of perturbations on the pace of resolution, the time from maximal to half-maximal responses was determined, defined as the resolution interval (R_i) (Supplemental Fig. 1).

In vivo depletion of NK cells

To deplete NK cells, mice were given anti-asialo GM1 Ab (aGM1) (9) (50 μ l/mouse, i.p.; Wako) or control IgG (rabbit) at the peak of allergic inflammation (protocol day 18) (Supplemental Figs. 2, 3). Although aGM1 can interact with other cell types, such as T cells (10, 11), only NK cells were significantly decreased with aGM1 in this study, as the numbers of CD4⁺ T cells from the bronchoalveolar lavage fluid (BALF) were increased (see *Results*), and no significant changes in CD8⁺ T cells were observed with aGM1.

Tracking OVA-specific (KJ1-26) CD4⁺ T cells *in vivo*

Ag-specific CD4⁺ T cells were isolated (Miltenyi Biotec) from DO11.10 BALB/cj mice (The Jackson Laboratory), and $\sim 2 \times 10^6$ cells were injected into recipient BALB/cj mice (as in Ref. 12). The next day, mice started the sensitization and challenge protocol. CD4⁺ KJ1-26⁺ T cells were detected using an Ab against the DO11.10 TCR (KJ1-26), and CD4⁺ KJ1-26⁺ T cell numbers were calculated as: % KJ1-26 cells $\times$ total CD4⁺ T cells (% CD4⁺ T cells $\times$ total number of lymphocytes).

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O.H. designed and performed experiments, analyzed data, and wrote the manuscript. M.C. provided reagents, analyzed data, and was involved in experimental design. B.D.L. designed and performed experiments, analyzed data, and wrote the manuscript.

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Abbreviations used in this article: aGM1, asialo GM1; aNKG2D, anti-NKG2D; BALF, bronchoalveolar lavage fluid; C_T, cycle threshold; LTB₄, leukotriene B₄; LXA₄, lipoxin A₄; MLN, mediastinal lymph node; PB, peripheral blood; R_i , resolution interval; RvE1, resolvin E1.

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Adoptive transfer of NK cells

NK cells were isolated (Miltenyi Biotec) from spleens of donor mice at protocol day 19 (to coincide with the recipient mice) (13), labeled with CFSE (5 μ M; Invitrogen) according to the manufacturer's instructions, and incubated (30 min, 37°C) with 10 μ g anti-mouse CXCR3, anti-mouse NKG2D-blocking Abs, or IgG control (rat IgG) before washing twice with RPMI 1640 medium supplemented with 10% FCS, antibiotics, and 50 μ M 2-ME (Sigma-Aldrich). The NKG2D Ab clone (C7) blocks the actions of NKG2D in vivo (14). On protocol day 19, NK cell-depleted recipient mice were reconstituted (i.v.) with $\sim 2 \times 10^6$ donor NK cells. After aGM1, endogenous NK cells are decreased for ~ 48 h, providing a window for administration of NK cells labeled with CFSE, which were readily detected in inflamed tissues and draining lymph nodes on day 21. The percentage of CFSE⁺ cells was determined in tissues at day 21 (Supplemental Fig. 4).

Abs and flow cytometry

Single-cell suspensions were generated with a 70- μ m cell strainer (Fisher Scientific). Lung and peripheral blood (PB) lymphocytes were enriched using Ficol (Sigma-Aldrich). NK cells were identified as being NKp46⁺ CD3⁻ (15). Abs were obtained from eBioscience: CD4 (L3T4), CD8 (53-6.7), CD3e (145-2C11), NKp46 (29A1.4), CD27 (LG 7F9), CD69 (H12F3), CXCR3 (CXCR3-173), CD62L (MEL-14), CMKLR1 (BZ194), KJ1-26 (KJ1-26), NKG2D (C7), and CD107a (1D4B); Invitrogen: CD11b (M1/70.15); Biolegend: CD11b (M1/70) and CD27 (LG.3A10); and BD Pharmingen: IFN- γ (XMG1.2). Blocking Abs were obtained for anti-mouse NKG2D (C7; eBioscience), anti-mouse CXCR3 (CXCR3-173; Biolegend), and anti-CD62L (MEL-14; Biolegend). Rat IgG (Biolegend) and hamster IgG (eBioscience) were used as controls. To detect NKG2D ligands, recombinant mouse NKG2D-human Fc fusion protein (R&D Systems) was used followed by an anti-human-IgG Fc (eBioscience). As a control, the secondary Ab was used alone. FACSCanto II (BD Biosciences) and FlowJo software (Tree Star) were used for analyses.

Measurement of peptide and lipid mediators

Select mediators were measured in aliquots of cell-free BALFs (2000 $\times$ g, 10 min, 4°C) by protein bead array (Aushon Biosystems) or ELISA (lipoxin A₄ [LXA₄; Neogen], PGE₂, and leukotriene B₄ [LTB₄; Cayman Chemical]).

Immunohistology

Lungs were fixed, sectioned, and stained by H&E or periodic acid-Schiff. Select images were acquired using a Leica microscope (model DMLB; Leica Microsystems).

Gene expression

Mediastinal lymph nodes (MLNs) and lungs were obtained and snap frozen. RNA was extracted with TRIzol (Invitrogen) and reverse transcribed. The cDNA was used as a template for the amplification of *cxc19* (GeneID: 17329), *cxc110* (GeneID: 15945), *cxc11* (GeneID: 56066), and a control gene *ppia* (GeneID: 268373) by real-time PCR using a Stratagene real-time PCR machine (model number Mx 3005; Stratagene). Fold change was calculated as $2^{-\Delta\Delta C_T}$ for the difference between the cycle threshold (C_T) value for the gene of interest and the respective C_T value for *ppia* (ΔC_T) compared with day 0.

NK cell cytotoxicity assay

NK cells were isolated from mouse spleens (Miltenyi Biotec) and incubated with vehicle or RvE1 free-acid (10 nM) for 15 min prior to the addition of RMA/S target cells labeled with Cell Tracker Orange (Invitrogen). Cytotoxicity was assessed after 4 h (37°C, 5% CO₂) using 7-aminoactinomycin (BD Pharmingen).

Statistical analysis

Results are expressed as the mean $\pm$ SEM. Statistical significance of differences was assessed by a one-tailed unpaired Student *t* test and a one-way ANOVA with Bonferroni's multiple comparison posttest to compare selected pairs. A *p* value < 0.05 was set as the level of significance.

Results

NK cells increase in tissue draining lymph nodes during resolution of allergic inflammation

To identify proresolving mechanisms in adaptive inflammation, we used a self-limited model of allergic airway inflammation (Fig. 1A).

After four daily aerosol allergen challenges, sensitized mice developed marked lung inflammation with increased airway eosinophils and lymphocytes. After cessation of allergen exposure, the lung inflammation largely resolved over a 7-d interval (Fig. 1B, Supplemental Fig. 1A, 1B). During this natural resolution process, the number of eosinophils (Fig. 1C) and T cells (Supplemental Fig. 1B) decreased in a linear trend in BALFs with an R_i of ~ 4 d (Supplemental Fig. 1A, 1B). T cell numbers also decreased in MLNs, but with a longer R_i (~ 5 d for CD8⁺ and ~ 6 d for CD4⁺ T cells) (Supplemental Fig. 1C). In contrast, NK cells (NKp46⁺ CD3⁻) in the lung draining MLNs and lung displayed markedly different kinetics (Fig. 1D–F). The number of NK cells in MLNs increased ~ 2.5 -fold at peak inflammation (24 h after last challenge on protocol day 18) ($p \geq 0.01$) (Fig. 1D). There was a further accumulation of NK cells in MLNs during early resolution (day 21) (~ 1.7 -fold increase compared with peak inflammation) ($p \geq 0.01$) that persisted in late resolution (day 25) (~ 1.3 -fold increase compared with peak inflammation) ($p = 0.05$) (Fig. 1E). NK cell numbers in the lung were similarly increased at day 18 compared with day 0 (Fig. 1F). The increased MLN NK cells in resolution temporally overlapped with stable to declining NK cell numbers in the lung (Fig. 1E, 1F), suggesting that NK cells were recruited to MLNs. In addition, MLN NK cells did not show evidence of proliferation by BrdU incorporation (data not shown).

To determine if MLN NK cell phenotype was dynamically regulated during resolution, we next examined NK cell subsets defined by CD27 and CD11b (Fig. 1G). CD27⁺ CD11b⁺ NK cells have been reported to display increased cytotoxicity (16), and there was a transient increase in the CD27⁺ CD11b⁺ population in MLNs during peak inflammation and early resolution (day 21) (Fig. 1G, 1H). During early resolution, MLN NK cells also expressed the cell activation markers CD69 and CD107a (lysosomal associated membrane protein-1) and could produce IFN- γ upon stimulation with PMA and ionomycin (Fig. 1I). In addition to increased cell number (Fig. 1D), expression of these molecules indicated that MLN NK cells were activated during resolution and suggested their involvement in the clearance of adaptive inflammation.

Decreased NK cells delays resolution

To investigate if these changes in NK cell number and phenotype had an impact on resolution, NK cells were decreased in number after lung inflammation was established using anti-aGM1 Ab (Supplemental Fig. 2). Administration of aGM1 significantly reduced NK cells in MLNs, lung, and spleen during resolution (Supplemental Fig. 2C, 2D). NK cell depletion led to persistent allergic airway inflammation with a relative increase in peribronchial leukocyte infiltration and inflammatory changes to the airway epithelium with associated mucus metaplasia (Fig. 2A, Supplemental Fig. 3). Levels of BALF lipid mediators were impacted by aGM1, including significant increases in LTB₄ ($p < 0.001$) (Fig. 2B) and decreases in LXA₄ ($p = 0.001$) (Fig. 2C) and PGE₂ ($p = 0.022$) (Fig. 2D). aGM1 also led to increases in BALF levels of IL-23 and IL-17 (Fig. 2E, 2F) and modest increases in IFN- γ (Fig. 2G). No significant changes were noted in BALF levels of the Th2 cytokines IL-5 and IL-13 (Fig. 2H, 2I). Of note, MLN and lung NK cells generated IL-13, and the percentage of IL-13-producing NK cells in both sites significantly decreased from peak inflammation to resolution (MLN, $38.6 \pm 2.3\%$ [day 18] to $24.4 \pm 2.8\%$ [day 21]; lung, $36.7 \pm 1.9\%$ [day 18] to $16.6 \pm 2.7\%$ [day 21]; mean $\pm$ SEM for $n \geq 6$; $p < 0.01$). There was no significant change in the BALF levels of T cell chemokines CCL17 (TARC) and CCL22 (MDC) (Fig. 2J, 2K).

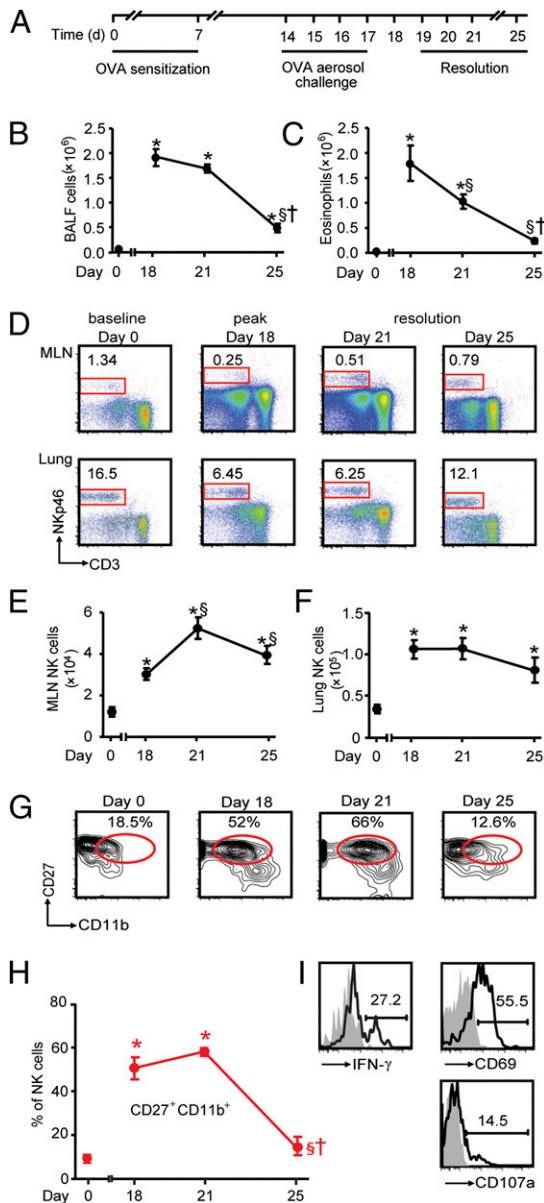


FIGURE 1. NK cells increase in local lymph nodes during resolution. *A*, Mice were sensitized and aerosol challenged with OVA (as in Ref. 5), and the extent of inflammation was determined on protocol days 18 (peak inflammation), 21, and 25 (resolution). Total number of BALF cells (*B*) and BALF eosinophils (*C*) were enumerated. *D*, Representative flow cytometry plots from MLNs and lung at baseline (day 0), peak inflammation (protocol day 18), and resolution (protocol days 21 and 25). Insets show percentage of lymphocytes that are NK cells (NKp46⁺CD3⁺). Time course for total NK cells in the MLNs (*E*) and lung (*F*). *G*, Flow cytometry plots for NK cell CD27⁺CD11b⁺ subset during inflammation and resolution. Insets show percentages. *H*, Percentage of CD27⁺CD11b⁺ NK cells during the onset and resolution of allergic airway inflammation. *I*, Representative flow cytometry plots showing the expression of IFN- γ , CD69, and CD107a on NKp46⁺CD3⁺ NK cells from the MLNs obtained in early resolution (day 21). Inset, Numbers indicate the percentage positive. Data (mean $\pm$ SEM) are representative of more than three independent experiments with $n \geq 4$ FVB mice in each group. * $p < 0.05$ (day 0), $^{\S}p < 0.05$ (day 18), $^{\dagger}p < 0.05$ (day 21).

NK cell depletion reduces resolvins E1-mediated resolution of airway inflammation

As NK cells appeared to have a pivotal role in the endogenous resolution program for adaptive immune responses, their contri-

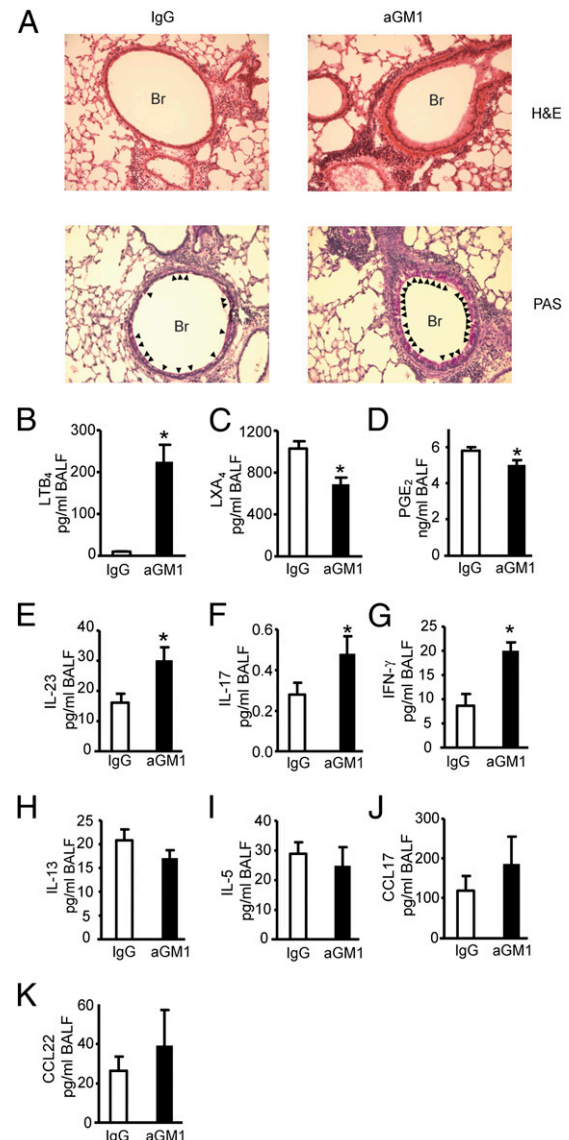


FIGURE 2. NK cells contribute to tissue catabasis and inflammatory mediator release. *A*, Representative ($n \geq 3$) lung tissue sections from day 21 FVB mice given aGM1 Ab or IgG were stained with H&E or periodic acid-Schiff (PAS). Original magnification $\times 200$. Arrowheads denote mucus (magenta)-containing Goblet cells. BALF lipid mediators: LTB₄, LXA₄, and PGE₂ (*B–D*); BALF cytokines and chemokines (*E–K*). Data (mean $\pm$ SEM) are representative of more than three independent experiments with $n \geq 3$ mice in each group. * $p < 0.05$. Br, bronchus.

bution to RvE1-mediated resolution was next determined. Administration of RvE1 at the peak of inflammation (Supplemental Fig. 4A) accelerated resolution by day 21 with decreased BALF total cells and eosinophils by $\sim 40\%$ (Fig. 3A, 3B). These protective actions for RvE1 were blocked by administration of aGM1 (Fig. 3A, 3B). At peak inflammation (day 18), the percentage of NK cells decreased relative to baseline (day 0) in both lung and PB (Fig. 3C, 3D). Administration of RvE1 hastened a return of NK cells to baseline levels in these tissues (Fig. 3C, 3D). No significant changes in the already increased MLN NK cell numbers were observed with RvE1 (Fig. 3E).

NK cells clear eosinophils and Ag-specific T cells during resolution

Depletion of NK cells with aGM1 during resolution significantly increased the number of inflammatory cells in BALFs at day 21

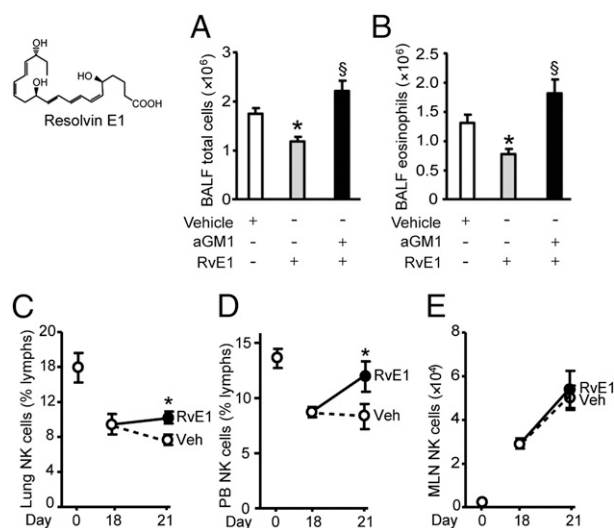


FIGURE 3. RvE1-mediated resolution of airway inflammation is blocked by NK cell depletion. BALF total cells (A) and eosinophils (B) at day 21 from mice given rabbit IgG or aGM1 plus vehicle or RvE1 (100 ng; inset). The percentage of NK cells in lung (C), PB (D), and total NK cell number (E) in MLNs from mice given vehicle or RvE1 (100 ng). Data (mean $\pm$ SEM) are representative of more than three independent experiments with $n \geq 3$ mice in each group. * $p < 0.05$ (vehicle), $^{\S}p < 0.05$ (RvE1).

(Fig. 4A, 4B). The total number of BALF cells and eosinophils significantly increased ($p < 0.001$) in mice depleted of NK cells (Fig. 4A). Total CD4⁺ T lymphocytes were increased in BALFs ($3.06 \pm 0.45 \times 10^4$ [IgG] versus $4.5 \pm 0.59 \times 10^4$ [aGM1]; $p < 0.05$) and MLN ($3.9 \pm 0.26 \times 10^6$ [IgG] versus $4.8 \pm 0.32 \times 10^6$ [aGM1]; $p < 0.05$), and the R_i for BALF lymphocytes nearly doubled from ~ 3 to ~ 6 d ($p < 0.001$) (Fig. 4B). In view of this increase in T cells, interactions between NK cells and CD4⁺ T cell responses in vivo were investigated using an adoptive transfer model of allergic airway inflammation with CD4⁺ T cells from transgenic DO11.10 mice with a TCR that is specific for chicken OVA (KJ1-26⁺ T cells) (12). Recipient BALB/cj mice given CD4⁺ KJ1-26⁺ T cells were sensitized and aerosol challenged with OVA, and then aGM1 or an IgG control Ab was administered to investigate the impact of NK cells on clearance of Ag-specific CD4⁺ KJ1-26⁺ T cells. NK cell depletion led to increased CD4⁺ KJ1-26⁺ T cells in MLNs ($p = 0.03$), lung ($p = 0.01$), and BALFs ($p = 0.03$) (Fig. 4C, 4D). During resolution, RvE1 accelerated the removal of Ag-specific CD4⁺ KJ1-26⁺ T cells in the MLNs and lung in an NK cell-dependent manner (Fig. 4E–G).

Blocking NK cell homing disrupts resolution

To determine if NK cell recruitment was an active step in resolution, the time course for expression of the NK cell homing receptors CXCR3 and CD62L (13) was measured by flow cytometry. During resolution, CXCR3 expression was selectively upregulated on NK cells from MLNs and PB (Fig. 5A). In contrast, CD62L was downregulated on NK cells from the MLNs and spleen during resolution and upregulated on lung NK cells (Fig. 5A, 5B). To test if CXCR3 and CD62L were required for NK cell recruitment to MLNs and lung during resolution, NK cells were labeled ex vivo with CFSE and incubated with a blocking Ab for CXCR3, CD62L, a combination of both, or control Ab prior to reconstitution by adoptive transfer into mice depleted of endogenous NK cells with aGM1 (Supplemental Fig. 4B, 4C). Blocking NK cell CXCR3 markedly inhibited recruitment into MLNs ($p = 0.01$) by $\sim 50\%$ (Fig. 5C) and disrupted resolution, leading to

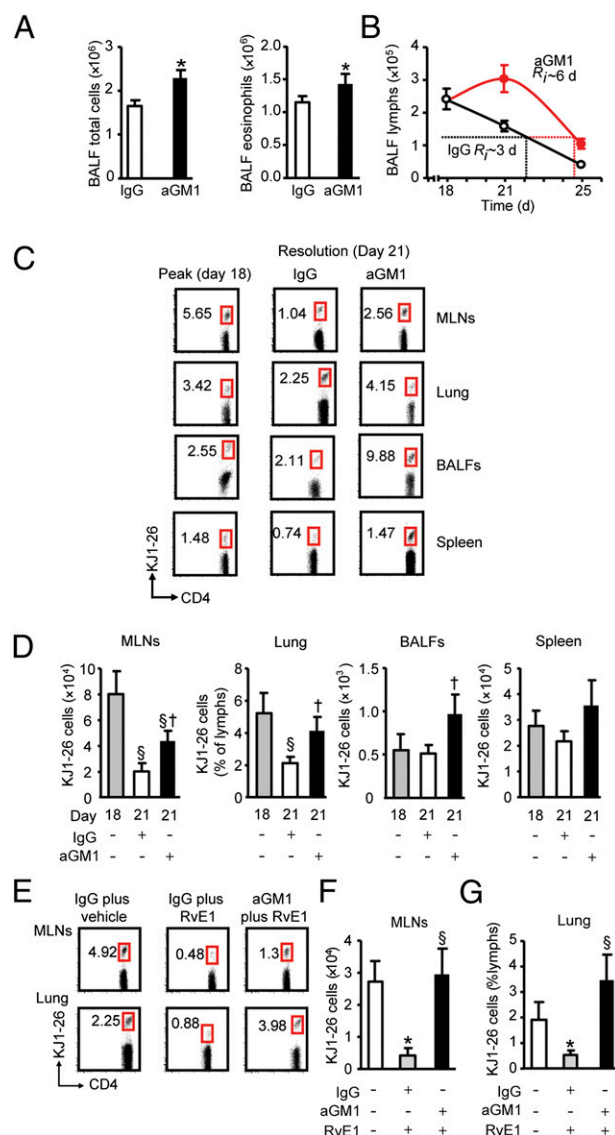


FIGURE 4. Depletion of NK cells delays resolution of allergic inflammation. A, Mice were given aGM1 Ab or control IgG at peak inflammation (protocol day 18), and the total number of BALF cells (left panel) and eosinophils (right panel) were enumerated during resolution (protocol day 21). B, The R_i was determined for BALF lymphocytes from FVB mice given aGM1 (red) or control IgG (white). C, Representative flow cytometry plots showing percentage of CD4⁺ KJ1-26⁺ cells (inserts) from MLNs, lung, BALFs, and spleen from peak inflammation (day 18) and early resolution (day 21) from mice given control IgG or aGM1. D, The total number of CD4⁺ KJ1-26⁺ T cells in MLNs, lung, BALFs, and spleen at peak inflammation (day 18) and following aGM1 or control IgG (day 21). E, Flow cytometry plots of KJ1-26 CD4⁺ T cells from MLNs and lung from mice given rabbit IgG and RvE1 (100 ng) or vehicle control or aGM1 and RvE1 (100 ng). Total number of CD4⁺ KJ1-26⁺ T cells in MLNs (F) and lung (G) from day 21 BALB/cj mice. Data (mean $\pm$ SEM) are representative of three experiments with $n \geq 3$ BALB/cj mice in each group. $^{\S}p < 0.05$ (day 18), $^{\dagger}p < 0.05$ (day 21).

increased MLN ($p = 0.03$) and BALF total cells ($p = 0.02$) and BALF eosinophils ($p = 0.007$) (Fig. 5D).

The CXCR3 ligands *cxc19*, *cxc110*, and *cxc111* were all expressed in murine lung and MLNs (*cxc110* and *cxc111* data not shown). There was a marked decrease in MLN *cxc19* and increase in lung *cxc19* at peak inflammation that returned toward basal expression during resolution (day 21) (Fig. 5E, 5F). RvE1 increased *cxc19* expression in both tissues during resolution (Fig. 5E,

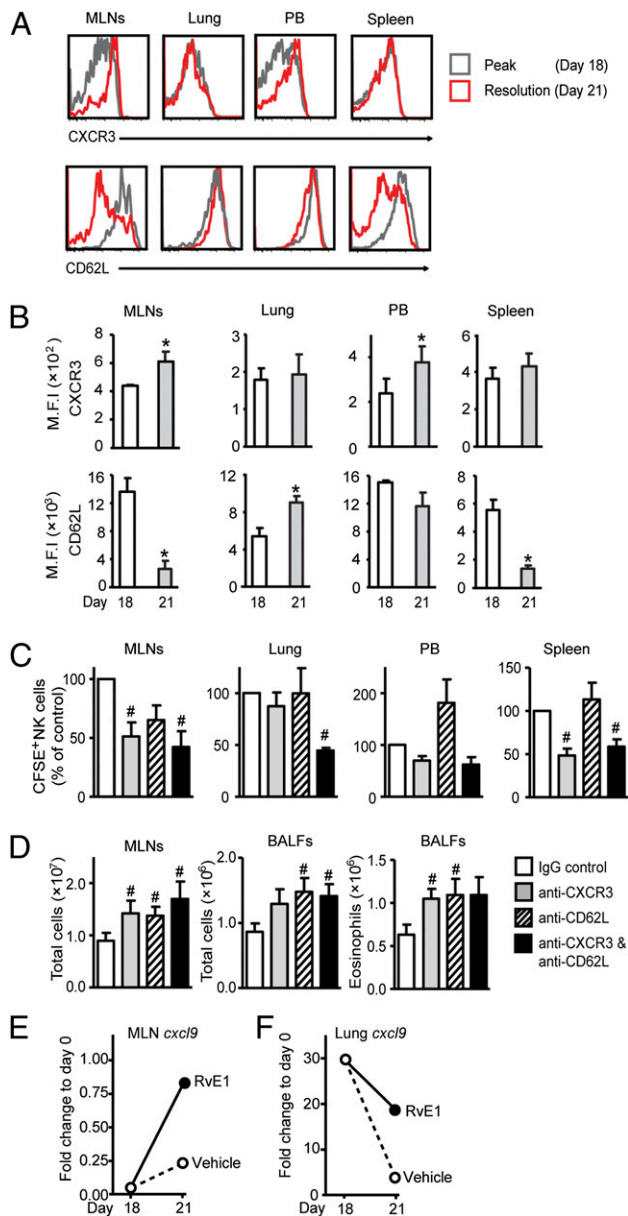


FIGURE 5. NK cells are recruited to MLNs and lung during resolution. *A*, Histograms show representative expression of CXCR3 and CD62L on NK cells (NKp46⁺CD3⁺) from MLNs, lung, PB, and spleen at peak inflammation (day 18, gray), and resolution (day 21, red). *B*, Median fluorescence intensity (M.F.I.) of CXCR3 and CD62L expression on NK cells from the MLNs, lung, PB, and spleen. *C*, After depletion of endogenous NK cells with aGM1, CFSE-labeled donor NK cells were adoptively transferred (day 19) after exposure ex vivo to anti-CXCR3, anti-CD62L, a combination of both, or control IgG and enumerated in recipient mouse tissues during resolution (day 21). *D*, MLN total cells and BALF total cell counts and eosinophils from mice reconstituted with adoptively transferred NK cells. Fold change in *cxcl9* expression in MLNs (*E*) and lung (*F*) during peak inflammation (day 18) and resolution (day 21) of airway inflammation in mice given vehicle or RvE1. Data (mean ± SEM) are representative of more than three independent experiments with $n \geq 3$ FVB mice in each group. * $p < 0.05$ (day 18), # $p < 0.05$ (percentage compared with IgG).

5F). In contrast, RvE1 did not significantly change NK cell CD62L or CXCR3 in MLNs or lung at day 21 (data not shown). In conjunction with the findings with the CXCR3-blocking Ab (Fig. 5C), these results indicate that CXCL9–CXCR3 interactions are important to timely resolution and that RvE1 can enhance NK cell

trafficking to target organs by upregulation of *cxcl9* expression during resolution. Together, these findings emphasize the importance of NK cell trafficking to target tissues and secondary lymphoid organs for resolution of adaptive inflammation.

NK cell recognition of eosinophils and Ag-specific CD4⁺ T cells

NK cells can target autologous CD4⁺ T cells via their NKG2D receptor (17), so the impact of this receptor system on leukocyte clearance was next determined. Because multiple ligands for the NKG2D receptor are upregulated during inflammation and cellular stress (18), their expression was measured using an NKG2D-Fc fusion protein (19). During resolution, NKG2D ligands were expressed on Ag-specific CD4⁺KJ1-26⁺ T cells from MLNs, BALFs, and lung, but not spleen (Fig. 6A). NKG2D ligands were not evident on KJ1-26[−] T cells (Fig. 6A). Of interest, NKG2D ligands were also detected on lung eosinophils (CD11b⁺CCR3⁺) (Fig. 6A). The functional impact of signaling at this receptor was determined by blocking the NKG2D receptor on NK cells. Endogenous NK cells were depleted with aGM1 and reconstituted from donor mice with NK cells that were exposed ex vivo to an NKG2D-blocking or IgG control Ab (Supplemental Fig. 4B). Mice given NK cells incubated with anti-NKG2D (aNKG2D) had significantly more inflammation at protocol day 21 with increased BALF total cells ($p = 0.02$) and eosinophils ($p = 0.02$) (Fig. 6B). In addition, blocking NK cell NKG2D impaired the clearance of KJ1-26⁺CD4⁺ T cells from MLNs ($p = 0.04$), BALFs ($p = 0.04$), and lung ($p = 0.02$) during resolution (Fig. 6C, 6D).

Of interest, day 21 NK cells expressed the RvE1 receptor CMKLR1 (Fig. 6E). Because RvE1 can accelerate catabasis by clearance of Ag-specific CD4⁺KJ1-26⁺ T cells (Fig. 4), the direct impact of RvE1 on in vitro NK cell cytotoxicity was determined. Exposure of NK cells to RvE1 (10 nM) significantly increased their ability to kill target RMA/S cells at E:T ratios of 3:1 and 6:1 (Fig. 6F). Together, these results suggest that NK cells were activated in vivo to use the NKG2D receptor to clear Ag-specific targets of allergic inflammation, such as CD4⁺ lymphocytes, to promote the timely resolution of adaptive immune responses.

Discussion

Clearance of leukocytes from inflamed tissues is fundamental to resolution and restoration of tissue homeostasis and can be impaired in diseases of chronic inflammation (1, 20). In this study, we investigated endogenous mechanisms for resolution of adaptive inflammation by using a self-limited experimental model of allergic airway inflammation. At peak inflammation in this model, eosinophils and activated T cells infiltrated the lung, and after cessation of allergen exposure, the leukocytes were cleared from the lung within ~1 wk. During this 1-wk resolution phase, there was an increase in the numbers of NK cells in the lung draining MLNs that temporally overlapped decrements in BALF eosinophils and T cells. In addition to accumulating in MLNs during resolution, NK cells acquired markers consistent with cell activation. Administration of aGM1 Ab that principally depletes NK cells led to delayed resolution for both eosinophils and CD4⁺ T cells. In addition to limiting the development of adaptive immune responses (21), the present results provide evidence that NK cells are important cellular effectors for promoting the resolution of established adaptive inflammation.

NK cells express the RvE1 receptor CMKLR1 (also known as ChemR23) (6, 22). RvE1 is a potent proresolving mediator for allergic airway inflammation (5), and NK cell depletion markedly impaired RvE1's protective actions. RvE1 regulated NK cell homing and in vivo clearance of Ag-specific CD4⁺ T cells. In

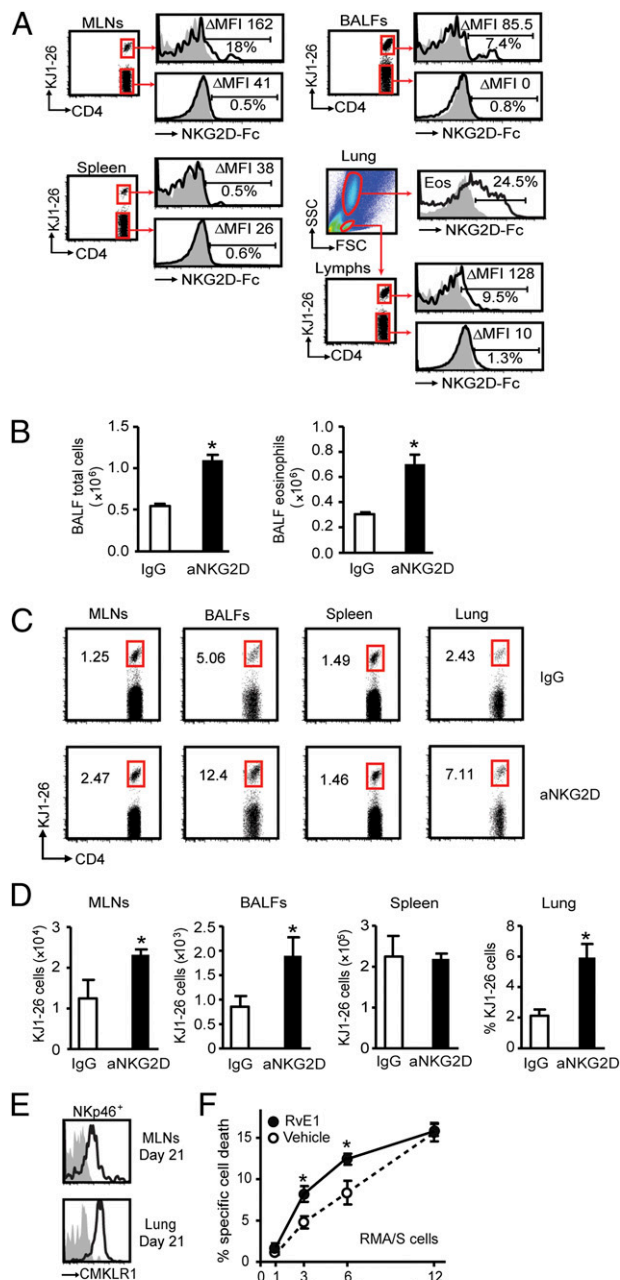


FIGURE 6. Ag-specific T cells and eosinophils express NKG2D ligands for clearance from inflamed lung. **A**, Expression of NKG2D ligands on KJ1-26⁺ and KJ1-26⁻CD4⁺ T cells from MLNs, BALFs, spleen, and lung eosinophils (Eos) was determined with NKG2D-Fc fusion protein. Histograms show secondary alone (gray) and expression of ligands (black). *Inserts* show the δ (Δ) median fluorescence intensity (MFI-MFI control) and the percentage of cells positive for NKG2D ligands. Mice were depleted of endogenous NK cells with aGMI and reconstituted with donor NK cells that were exposed ex vivo to aNKG2D. **B**, BALF total cells and eosinophils were enumerated after aNKG2D or IgG control Ab. **C**, Flow cytometry plots from CD4⁺ T cells in MLNs, BALFs, spleen, and lung. *Inserts* show percentages of CD4⁺KJ1-26⁺ T cells. **D**, The number of CD4⁺KJ1-26⁺ T cells in MLNs, BALFs, spleen, and lung (percentage) after aNKG2D or control Ab. **E**, Representative histograms show expression of the RvE1 receptor CMKLR1 on NK cells (NKp46⁺CD3⁻) from MLNs and lung (day 21). **F**, NK cell cytotoxicity toward RMA/S target cells was determined in the presence of RvE1 (10 nM) or vehicle control. Data (mean $\pm$ SEM) are representative of more than three independent experiments with $n \geq 4$ BALB/cj mice in each group. * $p < 0.05$ (vehicle).

addition to RvE1's in vivo actions, RvE1 increased NK cell cytotoxicity in vitro. The RvE1-mediated increase in PB and lung NK cells suggests that this mediator also increased NK cell transit through the MLNs. CXCL9 was originally identified as a chemokine regulated by IFN- γ (23). Blocking CXCL9 decreases allergic airway responses (24), and in this study, a neutralizing Ab for CXCL9's cognate receptor CXCR3 on NK cells impeded their ability to reach the MLNs and delayed resolution. These findings are consistent with a role for IFN- γ during resolution. In this model, RvE1 increases IFN- γ (5), and in this study increased both MLN and lung expression of *cxc9*. Of note, CXCR3-deficient mice have delayed wound healing (25). Together, these results indicate that RvE1 selectively regulates tissue chemokines to target NK cell homing for catabasis.

NK cells are innate lymphocytes that can play diverse roles in immunity, in particular in antiviral and antitumor host responses via the regulated killing of transformed cells and the release of immunomodulatory cytokines (26). They are also capable of influencing adaptive immunity (reviewed in Ref. 27), including for the development of allergic airway inflammation (28), contact hypersensitivity (29), and memory responses to murine CMV infection (30). When provided in a therapeutic dosing strategy, NK cells can diminish allergic airway responses in mice (31). Moreover, NK cells can regulate pathogen-mediated inflammation in the lung to facilitate the clearance of acute bacterial pneumonia, and their activation in murine pneumonia is dependent upon recognition of NKG2D ligands in the lung (32). In this study, the NKG2D receptor contributed to inflammatory cell removal in vivo. Eosinophils and Ag-specific CD4⁺ T cells expressed NKG2D ligands, and blocking the NK cell NKG2D receptor delayed clearance of these cells. In addition to these leukocyte effectors of adaptive immune responses, structural cells in the lung can also use the NKG2D system to regulate airway immune responses, as airway epithelial cells express low levels of NKG2D ligands that are increased upon exposure to oxidative stress (33).

During resolution, the CD27⁺CD11b⁺ NK cell population transiently increased and was associated with expression of IFN- γ , CD69, and CD107a (lysosomal associated membrane protein-1), demonstrating that the resolution NK cells were activated and not passively trafficking to the lymph nodes. These activated NK cells were potent regulators of the levels of inflammatory mediators in the lung. In this murine experimental model of asthma, Th2 cytokines, such as IL-5 and IL-13, are pivotal for the development of allergic airway responses (34), but in the resolution phase, IL-23 and IL-17 serve nonredundant roles (5). Administration of aGMI depleted NK cells and delayed resolution. With aGMI, airway inflammation and mucus metaplasia persisted, and levels of the Th2 cytokines IL-5 and IL-13 and the T cell chemokines CCL17 (TARC) and CCL22 (MDC) were not significantly changed. In contrast, IL-23 levels were increased, and there was a marked increase in LTB₄. These mediators can increase recruitment of CD4⁺ and CD8⁺ effector T cell populations (35), and LTB₄ in particular is also a chemoattractant and secretagogue for eosinophils (34, 36). In addition to changes in these proinflammatory signals, levels of PGE₂ and LXA₄, an endogenous anti-inflammatory/proresolution lipid mediator, were decreased. The distinct mechanisms for the development of inflammation and its resolution are further emphasized by depletion of NK cells during OVA challenge (rather than resolution) that ameliorates allergic airway responses (37), indicating the potential of NK cells to play significant roles in both the onset and downstroke of inflammatory responses.

In summary, findings presented in this study provide evidence for transient NK cell activation during the resolution of self-limited adaptive inflammation and that NK cells play an integral role in

RvE1-mediated catabasis. Regulation of NK cell trafficking to the target tissue and draining lymph nodes contributes to a resolution program for inflamed tissue via regulation of inflammatory mediators and clearance of eosinophils and Ag-specific T cells. Together, these results demonstrate pivotal proresolving roles for NK cells in tissue catabasis for adaptive immune responses.

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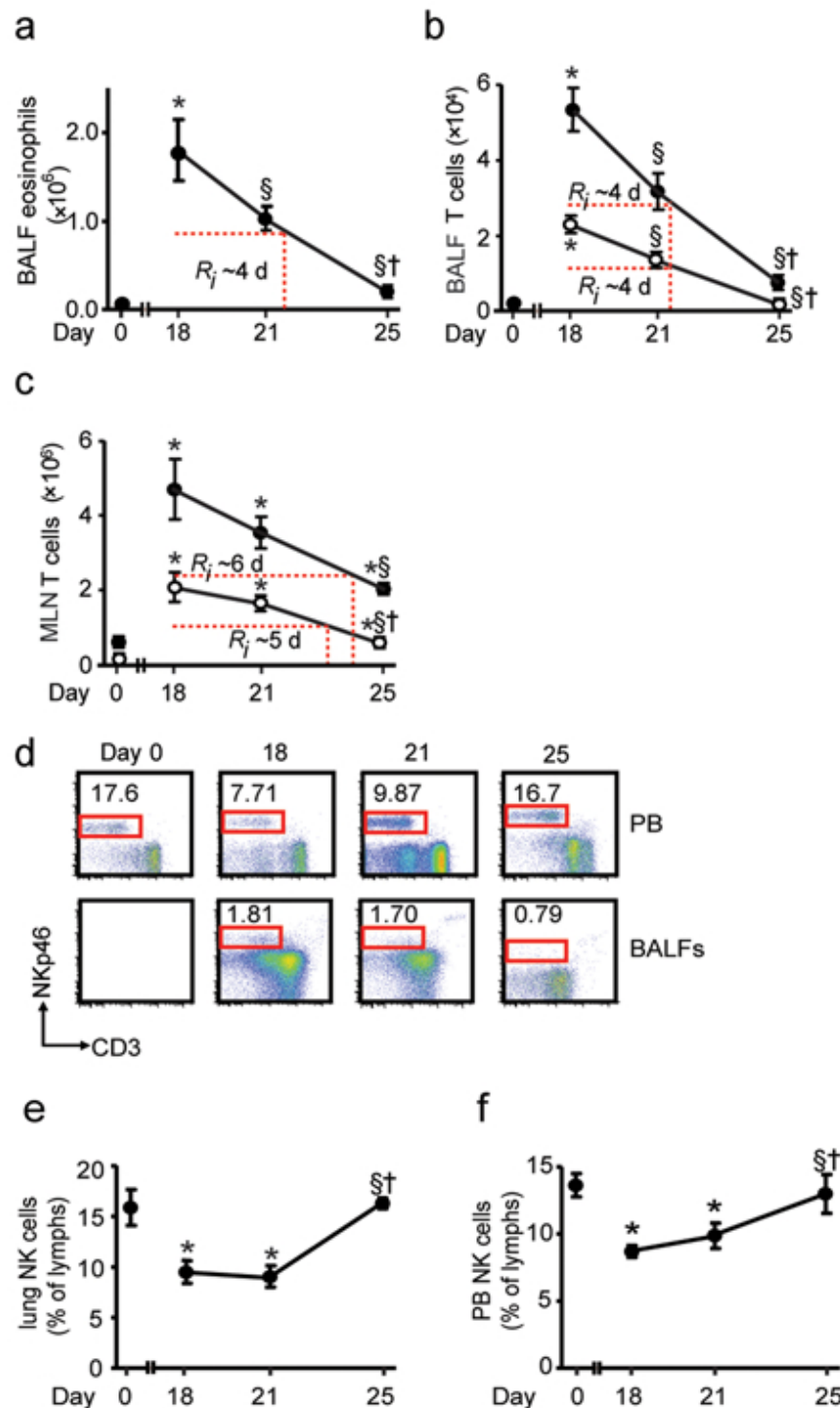
Disclosures

B.D.L. is a coinventor on patents assigned to Brigham and Women's Hospital and Partners HealthCare on the uses and clinical development of anti-inflammatory and proresolving lipid mediators. These are licensed for clinical development.

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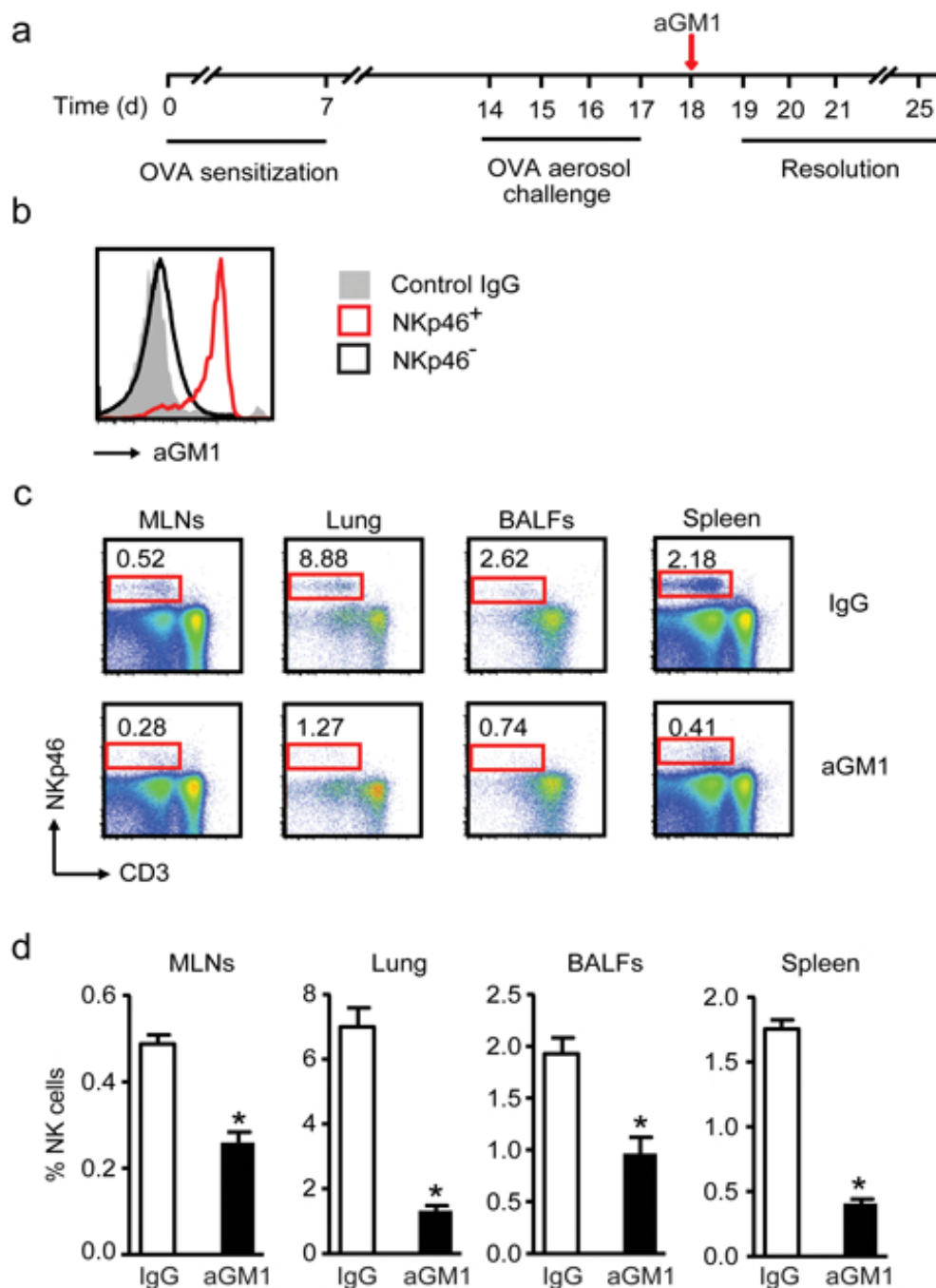
Supplementary Figure 1



Supplementary Fig. 1. Resolution of allergic airway inflammation.

a, BALF eosinophils were enumerated at days 0, 18, 21 and 25, and the resolution interval (R_i) was calculated. CD4 (black) and CD8 (white) T cells from **(b)** BALFs and **(c)** MLNs during inflammation and resolution were enumerated (see Methods). For each leukocyte subset and tissue, the R_i was calculated. **d** Representative FACS plots showing the percentage (insert) of NK cells (NKp46⁺, CD3⁻) in peripheral blood (PB), and BALFs on days 0, 18, 21 and 25 of the protocol. Time course for the percentage of **(e)** lung and **(f)** PB NK cells. Data (mean $\pm$ s.e.m) are representative of more than 3 independent experiments with $n > 4$ FVB mice in each group. * $P < 0.05$ (Days 0), $\S$ $P < 0.05$ (Day 18), $\dagger$ $P < 0.05$ (Days 21).

Supplementary Figure 2



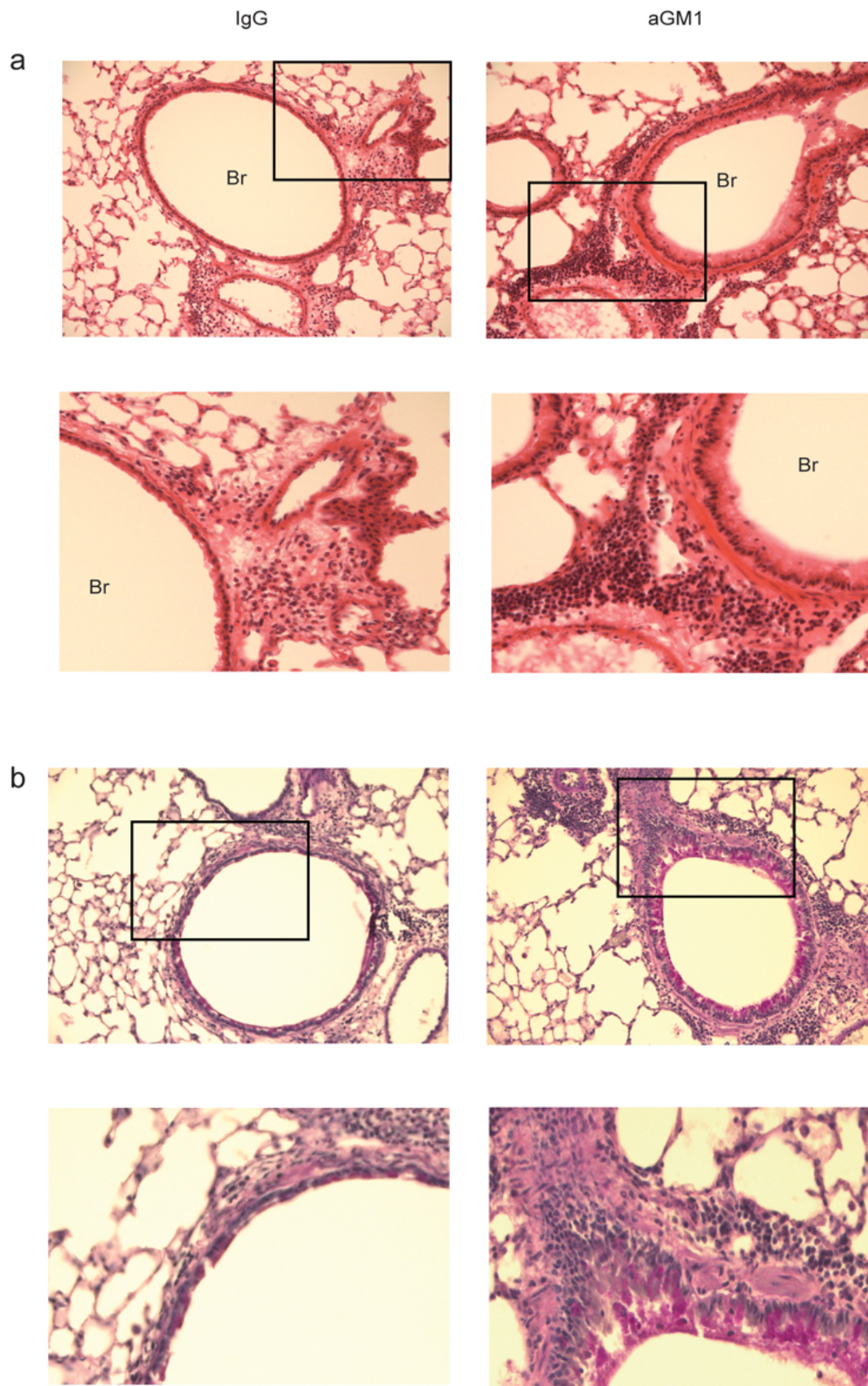
Supplementary Fig. 2. Reduction in NK cell number *in vivo* with anti-asialo GM1 antibody.

a, Schematic diagram showing the protocol used to reduce NK cells. At the peak of inflammation (day 18), mice were given an i.p. injection of anti-asialo GM1 antibody (aGM1) or control (rabbit) IgG.

b, Lymphocytes from the spleen that were NKp46 positive (red) or negative (black) were analyzed for their expression of aGM1.

c, Representative flow cytometry plots showing the reduction of NK cells at day 21 in the MLNs, lung, BALFs and spleen following control IgG (top) or aGM1 (bottom).

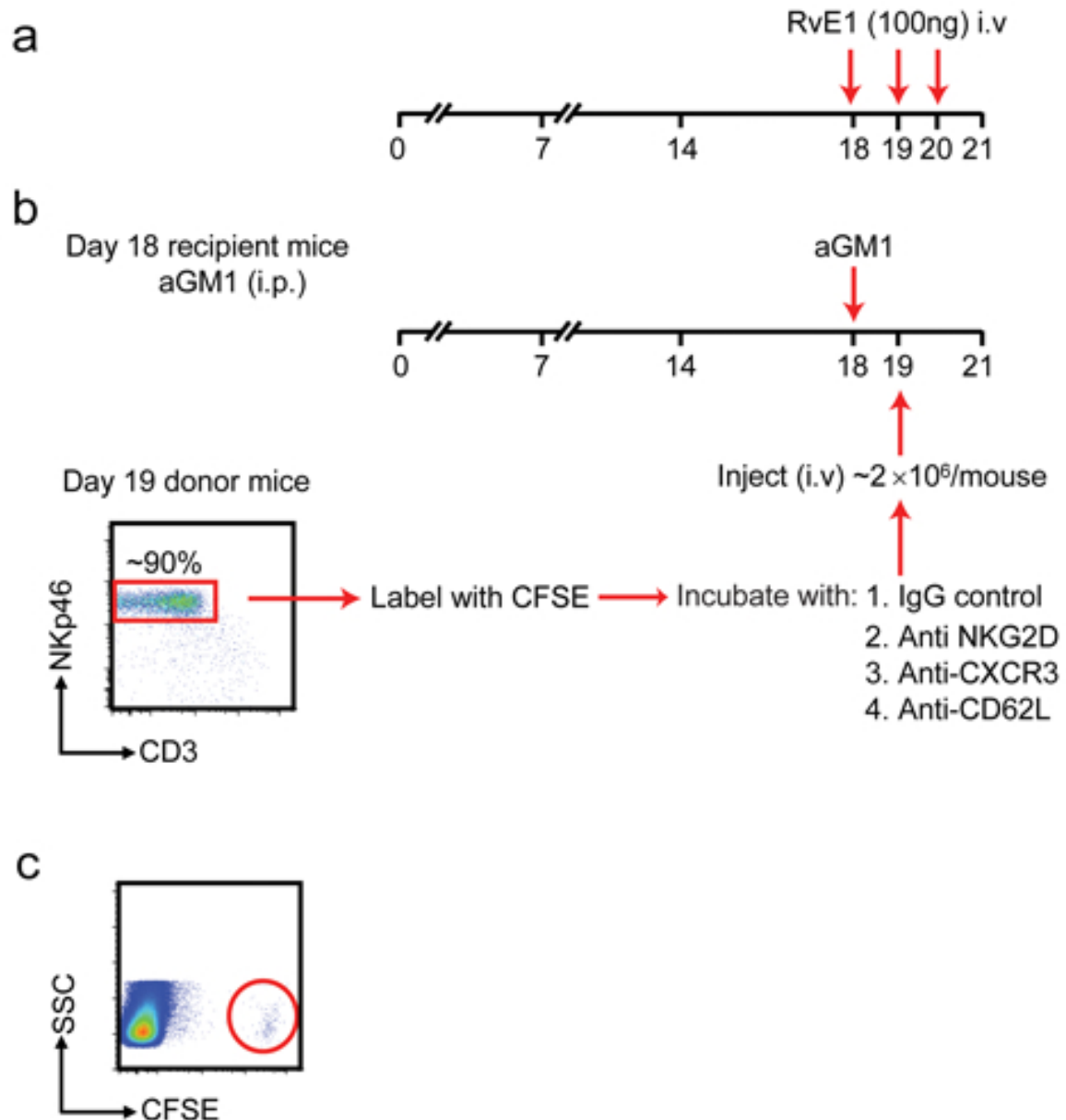
d, Percentage of NK cells on day 21 following aGM1 administration. Data (mean $\pm$ s.e.m) are representative of more than 3 independent experiments with $n > 4$ FVB mice in each group. * $P < 0.05$.



Supplementary Figure 3. aGM1 administration increases lung inflammation and mucus metaplasia.

Representative lung tissue sections from protocol day 21 after mice were given aGM1 antibody or IgG control antibody. Tissue sections were stained with (a) H&E and (b) PAS. Original magnification $\times 200$ (upper panels). Br, bronchus. Insert magnification $\times 400$.

Supplementary Figure 4



Supplementary Fig. 4. Protocol for RvE1 and the adoptive transfer and reconstitution of NK cells.

a, Schematic diagram showing the protocol used for the administration of RvE1.

b, Schematic diagram showing the adoptive transfer protocol. To decrease endogenous NK cells, recipient mice were given aGM1 at the peak of inflammation (protocol day 18). NK cells were isolated from donor mice spleen on day 19 of the protocol, and *ex vivo* labeled with CFSE and incubated with either control IgG or blocking antibodies against NKG2D or CXCR3 prior to reconstitution by intravenous administration to recipient mice.

c, Representative flow cytometry plot showing adoptively transferred CFSE⁺ NK cells from murine tissues at day 21 (red insert).