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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: 6129–6135.

Resolution of allergic airway 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 (Ri) (Supplemental Fig. 1).

In vivo depletion of NK cells

To deplete NK cells, mice were given anti-asialo GM1 Ab (aGM1) (9) (50 µg/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 ~2 × 10^4 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 × total CD4+ T cells (% CD4+ T cells × total number of lymphocytes).
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 μM; Invitrogen) according to the manufacturer’s instructions, and incubated (30 min, 37°C) with 10 μg anti-mouse CXCX3, anti-mouse NK2GD-blocking Abs, or IgG control (rat IgG) before washing twice with RPMI 1640 medium supplemented with 10% FCS, antibiotics, and 50 μM 2-ME (Sigma-Aldrich). The NK2GD Ab clone (C7) blocks the actions of NK2GD in vivo (14). On protocol day 19, NK cell-depleted recipient mice were reconstituted (i.v.) with ∼2 × 10^6 donor NK cells. After aGMI, endogenous NK cells are decreased for ~48 h, providing a window for administration of NK cells labeled with CFSE, which are 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-μm cell strainer (Fisher Scientific). Lung and peripheral blood (PB) lymphocytes were enriched using Ficoll (Sigma-Aldrich). NK cells were identified as being NKp46^+ (15). Abs were obtained from eBioscience: CD4 (L3T4), CD8 (53-6-7), CD3e (145-2C11), NKp46 (29A1.4), CD27 (LG 7F9), CD27 (KJ1-26), NKG2D (C7), and CD107a (1D4B); Invitrogen: CD11b (H12F3), CXCR3 (CXCR3-173), CD62L (MEL-14), CMKLR1 (BZ194), KJ1-26 (KJ1-26), NK2GD (C7), and CD107a (1D4B); Invitrogen: IFN-γ, IL-12, IL-23. M2-ME (Sigma-Aldrich). The NKG2D Ab clone (C7) blocks the actions of NK2GD in vivo (14). Blocking Abs were used for anti-mouse NK2GD (C7; eBioscience), anti-mouse CXCX3 (CXCX3-173; Biologend), and anti-CD27 (Biotrend). Rat IgG (Biologend) and hamster IgG (eBioscience) were used as controls. To detect NK2GD ligands, recombiant mouse NK2GD-human Fc fusion protein (R&D Systems) was used followed by an anti-human-IgG Fc (eBioscience). As 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. FACSComp 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 × g 10 min, 4°C) by protein bead array (Aushon Biosystems) or ELISA (lipoxin A4 [LXA4; Neogen], PGF2α, and leukotriene B4 [LTB4; Cayman Chemical]).

Immunohistology

Lungs were fixed, sectioned, and stained by H&E or periodic acid-Schiff. Lungs were fixed, sectioned, and stained by H&E or periodic acid-Schiff. Lungs were fixed, sectioned, and stained by H&E or periodic acid-Schiff. Lungs were fixed, sectioned, and stained by H&E or periodic acid-Schiff. 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 ccl29 (GeneID: 17329), ccl10 (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^ΔΔCT for the difference between the cycle threshold (Ct) value for the gene of interest and the respective Ct value for ppir (ΔCt) 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 ± 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^2 of ~24.4 (Supplemental Fig. 1A, 1B). T cell numbers also decreased in MLNs, but with a longer R^2 (−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 < 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 < 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 PGF2α (p = 0.02) (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 ± 2.3% [day 18] to 24.4 ± 2.8% [day 21]; lung, 36.7 ± 1.9% [day 18] to 16.6 ± 2.7 [day 21]; mean ± SEM for n = 6; p < 0.01). There was no significant change in the BALF levels of T cell chemokines CCL17 (TARC) and CCL22 (MDC) (Fig. 2F, 2K).
NK cell depletion reduces resolvin 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 contribution 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 ∼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 ± SEM) are representative of more than three independent experiments with n = 3 mice in each group. *p < 0.05 (vehicle); †p < 0.05 (RvE1).

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 ∼50% (Fig. 5C) and disrupted resolution, leading to increased MLN (p = 0.03) and BALF total cells (p = 0.02) and BALF eosinophils (p = 0.007) (Fig. 5D).

The CXCR3 ligands cxcl9, cxcl10, and cxcl11 were all expressed in murine lung and MLNs (cxcl10 and cxcl11 data not shown). There was a marked decrease in MLN cxcl9 and increase in lung cxcl9 at peak inflammation that returned toward basal expression during resolution (day 21) (Fig. 5E, 5F). RvE1 increased cxcl9 expression in both tissues during resolution (Fig. 5E, 5F).
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 NK cell killing of Ag-specific CD4+KJ1-26+T cells (Fig. 6D). 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
NK cells that were exposed ex vivo to aNKG2D.

and the percentage of cells positive for NKG2D ligands. Mice were de-

eosinophils were enumerated after aNKG2D or IgG control Ab.

cytometry plots from CD4+ T cells in MLNs, BALFs, spleen, and lung.

Data (mean ± SEM) are representative of more than three independent experiments with n ≥ 4 BALB/c mice in each group. *p < 0.05 (vehicle).

addition to RvE1’s in vivo actions, RvE1 increased NK cell cy-
totoxicity 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 cxcl9. Of note, CXCR3-deficient mice have delayed wound healing (25). Together, these results
indicate that RvE1 selectively regulates tissue chemokines to

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 infec-
tion (30). When provided in a therapeutic dosing strategy, NK
cells can diminish allergic airway responses in mice (31). More-
over, 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 tran-
siently increased and was associated with expression of IFN-γ,
CD69, and CD107a (lyosomal 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
aGM1 depleted NK cells and delayed resolution. With aGM1,
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 eosino-
phils (34, 36). In addition to changes in these prophlogistic sig-
nals, levels of PGE2 and LXA4, an endogenous anti-inflammatory/
proresolution lipid mediator, were decreased. The distinct mecha-
nisms 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 PartnersHealthCare on the uses and clinical development of anti-inflammatory and proresolving lipid mediators. These are licensed for clinical development.

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