

Original Paper

Natural Killer Cell Deficits Aggravate Allergic Rhinosinusitis in a Murine Model

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Key Words

Chronic rhinosinusitis · Allergy · Natural killer cell · Eosinophil

Abstract

Objective: Defective innate immune functions can contribute to chronic rhinosinusitis (RS). Recently, it has been reported that chronic RS patients show impaired function of natural killer (NK) cells. We investigated the role of NK cells in eosinophilic inflammation in an allergic RS mouse model. **Methods:** Mice sensitized to ovalbumin (OVA) by intraperitoneal injection received nasal challenges with OVA for 5 weeks. NK cell depletion was achieved by intraperitoneal injections of anti-asialo ganglio-N-tetraosylceramide (ASGM1) antibodies 10 days before OVA sensitization and every 5 days thereafter until sacrifice. Sinonasal complex samples were evaluated histologically, and IL-4, IL-5, IL-13, IFN- γ , MIP-2, and eotaxin levels were measured in the nasal lavage fluid. Differential white blood cell counts were also obtained. **Results:** Allergic RS mice showed significantly more eosinophilic inflammation in the sinonasal mucosa, elevated levels of IL-4, IL-5, IL-13, and eotaxin in the nasal lavage fluid, and peripheral blood eosinophilia compared to control mice. The depletion of NK cells by anti-ASGM1 treatment induced more prominent eosinophilic inflammation and increased secretion of IL-5 and peripheral blood eosinophilia in allergic RS mice. **Conclusion:** The depletion of NK cells aggravates allergen-induced sinonasal eosinophilic inflammation, suggesting that impaired NK cell activity may be an exacerbating factor in eosinophilic chronic RS.

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Introduction

Innate immunity provides multiple first lines of defense against airborne microorganisms and particulates, and adaptive immunity develops on the foundation of innate immunity [1, 2]. The innate and adaptive immune systems cooperatively identify and eliminate infectious agents from the sinonasal cavity [3]. Defects in the innate immune system regulation of inflammation and restoration of homeostasis have recently been proposed as important pathogenic factors of persistent sinonasal inflammation of chronic rhinosinusitis (CRS) [1, 4, 5]. Although the pathognomonic features of CRS are mainly attributed to sustained inflammation associated with an activated local adaptive immune system [1], CRS-induced inflammation has multifactorial causes that are related to overlapping host reaction and environmental triggers [3]. The complex interaction between the environment and the mucosal immune system is magnified by factors that drive persistent sinonasal inflammation [1, 3].

Natural killer (NK) cells, a component of the innate immune system, mediate cytotoxicity, produce cytokines and chemokines, and provide cell contact-dependent co-stimulation, thereby serving as a bridge in an interactive loop between innate and adaptive immunity [6–8]. Eosinophilic inflammation is a major pathological hallmark of CRS, especially when it is associated with allergy and/or nasal polyps [4, 9]. In our previous study, we found that the NK cells of CRS patients were dysfunctional and showed an impaired ability to degranulate and produce interferon (IFN)- γ and tumor necrosis factor (TNF)- α . In addition, we found that decreased NK cell function was most severe in recalcitrant CRS patients and was associated with poor prognostic factors such as concomitant asthma and peripheral blood eosinophilia [10]. Thus, current data strongly suggest that NK cells may be involved in CRS-induced chronic eosinophilic inflammation, but this has not been assessed experimentally. In order to further confirm the presumed functional role of NK cells in sinonasal eosinophilic inflammation, an animal model demonstrating NK cell dysfunction is needed. However, currently, there is no induction method of NK cell dysfunction in mice; instead, selective depletion of NK cells using anti-asialo ganglio-N-tetraosylceramide (ASGM1) treatment in mice can serve as an alternative method of studying NK cell dysfunction. Hence, to verify the presumed functional role of NK cells in the pathogenesis of eosinophilic CRS, we assessed the consequences of NK cell depletion in a murine model of allergic rhinosinusitis (RS) in this study.

Methods

Allergic RS Animal Model

BALB/c mice (6–7 weeks old) were purchased from Orient Bio Inc. (Sunngnam, Korea). Ovalbumin (OVA; grade V; Sigma, St. Louis, Mo., USA) was used to sensitize and challenge the mice. The Allergic RS mouse model was adapted from that used in previous studies [11, 12]. Briefly, the study mice were immunized by an intraperitoneal (i.p.) injection of 25 μ g of OVA absorbed into 2 mg of an aluminum hydroxide gel on days 0 and 4, followed by daily intranasal challenge (from day 7 to 11) with 3% wt/vol OVA diluted in 30 μ l of sterile normal saline. Subsequently, the study mice were exposed to 3% wt/vol OVA nasal allergen three times a week. Control mice received an i.p. injection of 2 mg alum in 0.5 ml of phosphate-buffered saline (PBS) solution. The control animals subsequently received 30 μ l of nasal saline solution at the same time intervals. All mice were euthanized 5 weeks after initial sensitization to allow sufficient time for allergic inflammatory changes to occur in the CRS murine model [13, 14]. All experimental protocols were approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Science.

Study Groups and in vivo Depletion of NK Cells

The control groups were designated as follows: PBS instillation only (Control, $n = 5$) and PBS instillation with NK cell depletion (NK[−] + Control, $n = 5$). The study groups of the OVA-challenged mice were designated as instillation with OVA only (Allergic RS, $n = 5$) and OVA instillation with NK cell depletion (NK[−] + Allergic

RS, n = 5). NK cell depletion was performed by i.p. injections of rabbit anti-ASGM1 antibodies (Ab) (10 µg/mouse; Cedarlane Laboratories Ltd., Ontario, Canada). To determine whether impaired function of NK cells is the predisposing factor in eosinophilic CRS patients, the first injections of anti-ASGM1 were performed 10 days before OVA sensitization or PBS i.p. injection (day –10), and then every 5 days until sacrifice. These experiments were repeated to verify that our results were reliable.

FACS Analysis for NK Cell Counts

To assess whether NK cells were depleted by anti-ASGM1 treatment, cells isolated from mouse spleens were resuspended at a concentration of 1×10^7 cells/ml in PBS containing 2% fetal calf serum. A total of 1×10^6 cells were incubated with rat anti-mouse CD16/CD32 (2.5 µg/ml; BD Pharmingen, San Diego, Calif., USA) for 10 min. Surface staining was performed with FITC-conjugated anti-CD3ε (145-2C11; BD Pharmingen), allophycocyanin conjugated anti-CD49b (HMα2; BD Pharmingen), phycoerythrin conjugated anti-mouse FcεRIα (MAR-1; eBioscience, San Diego, Calif., USA), or its isotype control Ab (eBioscience) in accordance with the manufacturer's instructions. Stained cells were immediately analyzed by flow cytometry (BD FACS-CantoII™; BD Biosciences, San Diego, Calif., USA). NK cells were identified as CD3[–]CD49b⁺ FcεRIα[–] cells [15].

Histological Analysis

Coronal paraffin-embedded sinonasal cavity sections were obtained by continuous microtome sectioning at a thickness of 4 µm [12, 16]. Tissue sections were stained with hematoxylin and eosin to evaluate general morphology, inflammatory cell infiltration in the lamina propria, and goblet cells counts in the epithelium. Sirius red stain was also performed to assess eosinophil infiltration. Quantitative histological analysis was performed by two pathologists in the Department of Pathology at Asan Medical Center who were blind to the group assignments. Of the five coronal sections that included the sinus cavity, the two best sections were used for analysis. Eosinophils and neutrophils in the lamina propria were determined and the measurements expressed as the number of cells/mm² of the lamina propria. Goblet cells were counted in the epithelium and these values were expressed as the number of cells/mm of epithelium.

Cytokines in Nasal Lavage Fluid

As previously described [12], a 22-gauge catheter was inserted into the tracheal opening in the direction of the choana after a partial tracheal resection under anesthesia. Nasal cavities were gently washed with 0.5 ml of normal saline, and fluid from the nostril was collected. Interleukin (IL)-4, IL-5, IL-13, IFN-γ, macrophage inflammatory protein (MIP) 2, and eotaxin concentrations in the nasal lavage fluid were measured by enzyme-linked immunosorbent assay kits (R&D systems, Minneapolis, Minn., USA) in accordance with the manufacturer's protocols.

White Blood Cell Counts

Blood samples were taken by cardiac puncture and fixed and processed for microscopic examination. Differential cell counting was performed after Wright-Giemsa staining. Percentages of lymphocytes, neutrophils, monocytes, and eosinophils were determined by counting in five randomly selected high-power fields (HPFs; magnification, 400×) and dividing this number by the total number of cells per HPF. To obtain the absolute number of each leukocyte subtype in the blood samples, these percentages were multiplied by the total number of cells recovered from the blood.

Statistical Analysis

All data were analyzed with IBM® SPSS® Statistics for Windows, Version 20.0 (IBM, Armonk, N.Y., USA) and expressed as the mean ± standard deviation. Comparisons between the groups were performed with a nonparametric Mann-Whitney U test (two-tailed) and Kruskal-Wallis test. Statistical significance was defined as $p < 0.05$.

Results

NK Cell Population in the Spleen

The number of NK cells was lower in control mice treated with anti-ASGM1 (NK[–] + Control, $3.07 \pm 0.29\%$) than in control mice (Control, $4.10 \pm 0.43\%$), while the basophil population was not significantly affected by anti-ASGM1 treatment (Control, $0.29 \pm 0.82\%$ vs.

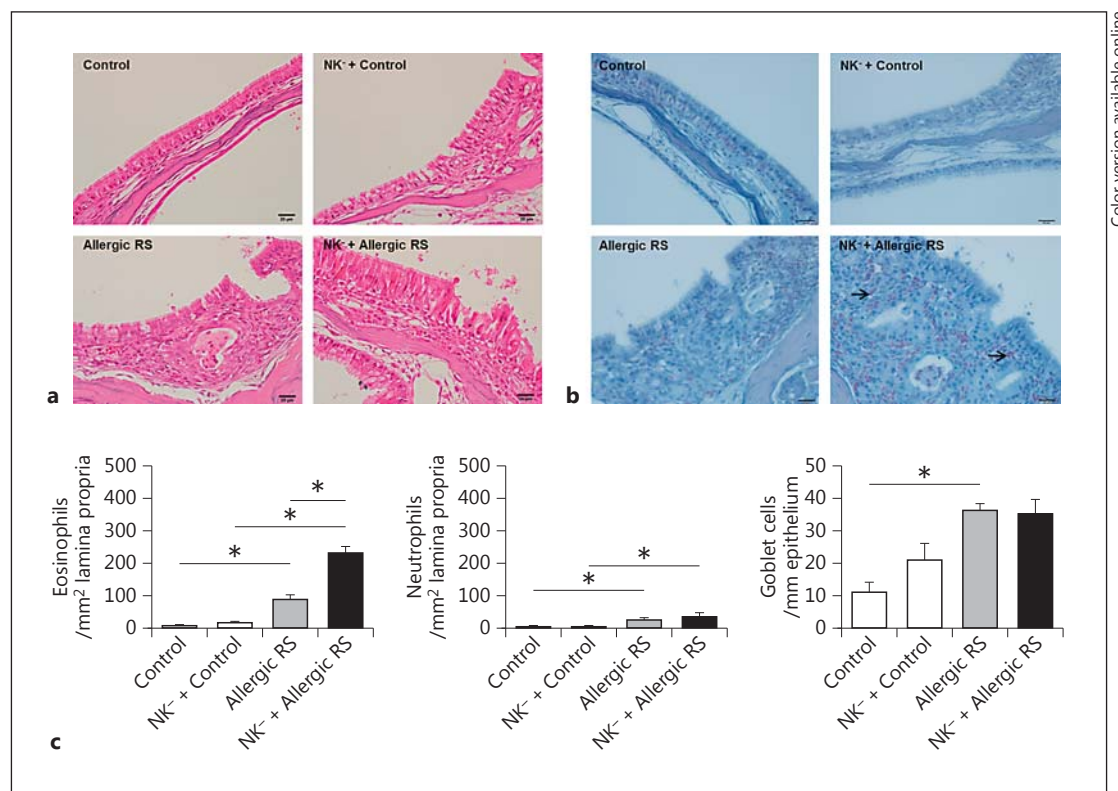


Fig. 1. **a** Allergic RS and NK⁻ + Allergic RS groups showed greater inflammatory cell infiltration and epithelial and goblet cell hyperplasia compared to the control groups (H&E stain; original magnification 400×). Scale bars = 20 μm. **b** Severe eosinophil infiltration was noted in the NK⁻ + Allergic RS group (Sirius red-stain; original magnification 400×). Scale bars = 20 μm. **c** The number of eosinophils was increased in the NK⁻ + Allergic RS group compared to the Allergic RS group. NK⁻, 10 μg/mouse of anti-ASGM1 treatment. * p < 0.05.

NK⁻ + Control, 0.29 ± 0.04%; p = 0.818) (online suppl. fig. 1; for all online suppl. material, see www.karger.com/doi/10.1159/000445775). The number of NK cells was slightly lower in OVA only-challenged mice (Allergic RS, 3.84 ± 0.67%) compared to control mice (Control), but this difference was not statistically significant (p = 0.588). The number of NK cells was lower in Allergic RS mice treated with anti-ASGM1 (NK⁻ + Allergic RS, 2.10 ± 0.58%) than in the Allergic RS group (3.84 ± 0.67%; p = 0.010).

Histology of Sinonasal Mucosa

The level of infiltration of inflammatory cells and of goblet cell hyperplasia in the sinonasal mucosa were not remarkably different between the control groups with and without treatment with anti-ASGM1 (fig. 1). The sinonasal mucosa from Allergic RS mice exhibited higher infiltration of inflammatory cells, including eosinophils and neutrophils, and goblet cell hyperplasia compared to the control groups (p < 0.05 for each control group; fig. 1). Epithelial hyperplasia and disruption and increased subepithelial thickness were also noted in Allergic RS mice. In particular, the number of eosinophils was significantly higher in the NK⁻ + Allergic RS group than in the Allergic RS group (p = 0.029), whereas the number of neutrophils was unchanged by treatment with anti-ASGM1. Goblet cell hyperplasia was unaffected by anti-ASGM1 treatment in the OVA-challenged study mice.

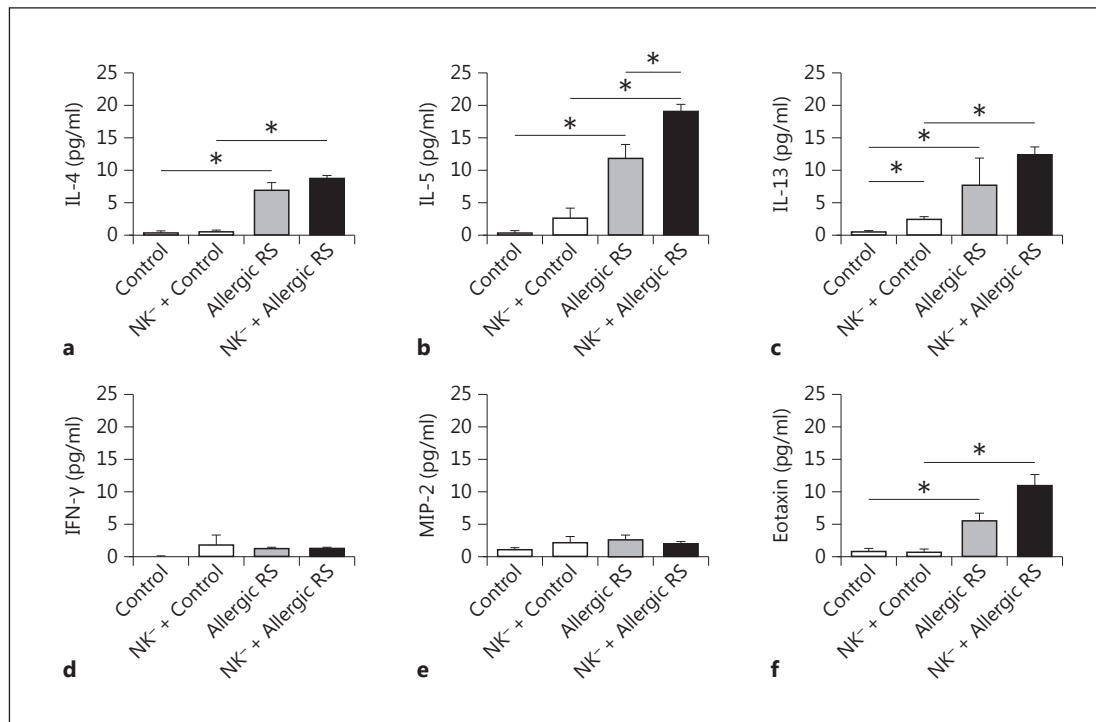


Fig. 2. IL-4 (a), IL-5 (b), IL-13 (c), IFN-γ (d), MIP-2 (e), and eotaxin (f) in the nasal lavage fluid were measured by ELISA. The level of IL-5 was found to be significantly higher in the NK⁻ + Allergic RS group compared to the Allergic RS groups. NK⁻, 10 μg/mouse of anti-ASGM1 treatment. * p < 0.05.

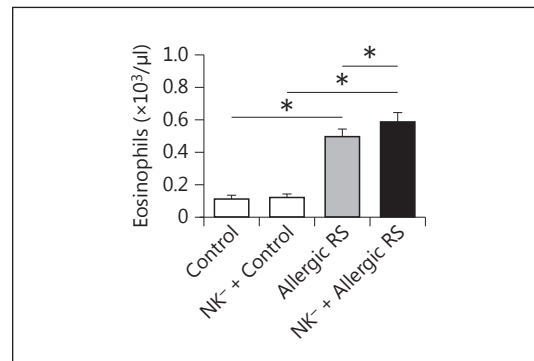


Fig. 3. The number of eosinophils was found to be higher in the Allergic RS groups, with a higher number seen in the NK⁻ + Allergic RS group compared to the Allergic RS group. NK⁻, 10 μg/mouse of anti-ASGM1 treatment. * p < 0.05.

Cytokines in Nasal Lavage Fluid

The levels of Th2 cytokines, including IL-4, IL-5, and IL-13, were higher in OVA-challenged study mice than in the control groups (p < 0.05 for each control). The level of IL-5 in the NK⁻ + Allergic RS group (19.10 ± 2.10 pg/ml) was higher following anti-ASGM1 treatment compared to the Allergic RS group (11.93 ± 5.51 pg/ml) (p < 0.05 for each; fig. 2b). However, IL-4 and IL-13 concentrations were largely unchanged by anti-ASGM1 treatment in Allergic RS mice (p > 0.05 for each; fig. 2a, c, respectively). The secretion of IFN-γ and MIP-2 was not affected by OVA-challenge and anti-ASGM1 treatment (p > 0.05 for each; fig. 2d, e, respectively). The eotaxin level was higher in the Allergic RS (5.54 ± 3.12 pg/ml) and NK⁻ + Allergic RS (10.97 ± 3.33 pg/ml) groups compared to the corresponding control group (p = 0.029 for each; fig. 2f).

White Blood Cell Counts

There were no differences found in the total leukocyte, lymphocyte, neutrophil, and monocyte counts between the control and OVA-challenged mice irrespective of anti-ASGM1 treatment (online suppl. fig. 2A–D). Interestingly, OVA challenge led to an increase in the eosinophil levels in the blood ($p < 0.05$ for each). This eosinophilia in Allergic RS mice was more pronounced in the animals that received anti-ASGM1 treatment ($p = 0.029$; fig. 3).

Discussion

Some CRS patients are recalcitrant or unresponsive to current medical and surgical treatments. Because the pathogenesis of CRS remains incompletely understood, imbalances in the innate immune system have been proposed as a mechanism to explain these persistent sinonasal inflammations [1]. Eosinophilic CRS can be particularly unresponsive to medical and surgical treatment [9, 17–19]. This suggests that an abnormal innate immune system and/or dysregulated inflammatory responses probably occur during eosinophilic CRS.

The allergic CRS mouse model is considered an appropriate model for studying the pathophysiology of eosinophilic CRS, which is recognized to be the result of immunological dysfunction and not anatomical obstruction [11, 12]. In our present study, we report that the depletion of NK cells, which are effector lymphocytes of the innate immune system that control microbial infections, leads to severe sinonasal mucosal inflammation, particularly eosinophilic inflammation, increased secretion of IL-5, and peripheral blood eosinophilia in a previously established murine model of Allergic RS.

NK cells are prototypical members of the innate lymphoid cell family and appear to have crucial roles in innate immunity against pathogenic and nonpathogenic microorganisms and in tissue remodeling [20, 21]. Conflicting results have been reported regarding the role of NK cells in eosinophilic inflammation of the airway mucosa. In a recent study, NK cells of patients with CRS showed impaired effector functions, including cytotoxic degranulation and IFN- γ and TNF- α production, which were inversely correlated with eosinophilia [10]. In contrast, it has been reported that NK cells may promote allergic lung inflammation by producing Th2 cytokines, whilst IFN- γ -secreting NK cells can inhibit eosinophilic airway inflammation and pulmonary fibrosis [22, 23]. Thus, NK cells may contribute to the balance of Th1 and Th2 cytokines in asthma and allergy. In particular, conflicting results on the crosstalk between NK cells and eosinophils have indicated that NK cells can inhibit or promote eosinophilic airway inflammation [24–26].

In our present study, whilst the number of NK cells in the Allergic RS group was similar to that of the control group, CRS-related pathophysiological changes such as eosinophilia in both the sinonasal mucosa and peripheral blood, and elevated levels of Th2 cytokines and eotaxin in the nasal lavage fluid, were observed in the Allergic RS group. These results suggest that OVA sensitization and challenge alone may have limited influence on NK cells. However, anti-ASGM1 Ab treatment before OVA sensitization significantly reduced the number of NK cells and subsequently enhanced eosinophilic inflammation in the sinonasal mucosa and the secretion of IL-5 in the nasal lavage fluid, and increased the eosinophil level in blood, compared to the Allergic RS group. These results indicate that depletion of NK cells is indeed involved in the enhancement of eosinophilic inflammation in Allergic RS mice, which mirrors the results of our previous clinical study showing impaired activity of NK cells in CRS patients, especially in recalcitrant cases [10]. Depletion of NK cells using anti-ASGM1 Ab in mice may appropriately reflect NK cell dysfunction in humans, as there is no current method to directly induce NK cell dysfunction in mice.

Interestingly, our current findings appear contrary to previously reported results of NK cell depletion in a mouse model of allergic asthma, which showed significant decreases in

pulmonary eosinophils and the levels of Th2 cytokines in bronchoalveolar lavage fluid [27]. These differences may result from the somewhat different experimental methods used between the two studies. In our current study, anti-ASGM1 Ab was used to deplete NK cells in BALB/c mice, whereas Korsgren et al. [27] used anti-NK 1.1 monoclonal Ab, which can deplete NK cells only in certain mouse strains such as C57BL/6 and SJL mice, but not in BALB/c mice [28]. Also, in our present study, BALB/c mice were challenged with 3% wt/vol OVA a total of 17 times to induce Allergic RS development. However, Korsgren et al. [27] challenged C57BL/6 mice with 1% wt/vol OVA a total of 7 times. Our current Allergic RS mouse model system may thus better reflect more severe allergic airway inflammation than that employed by Korsgren et al. The use of 10 μ l (approximately 370 μ g) anti-ASGM1 Ab has been reported to deplete not only NK cells but also basophils, which may abolish the development of chronic allergic skin inflammation and lead to a more Th1-dominant response [15]. However, in our present analyses, we used only 10 μ g anti-ASGM1 Ab to partially deplete NK cells and exclude the effect of basophil depletion on the eosinophilic inflammation of Allergic RS mice. We confirmed that the number of basophils (CD3⁻CD49b⁺Fc ϵ RI α ⁺) was not significantly reduced following treatment with 10 μ g anti-ASGM1 Ab (online suppl. fig. 1). Moreover, if anti-ASGM1 Ab depletes mainly basophils, our anti-ASGM1 Ab-treated Allergic RS mice would have shown Th1-dominant sinonasal inflammation rather than aggravation of eosinophilic inflammation. Therefore, eosinophilic sinonasal inflammation in our Allergic RS mice was likely affected by NK cells rather than basophils. Taken together, these results suggest that NK cells exhibit diverse regulatory functions on eosinophilic inflammation. In other words, NK cells may interact with T lymphocytes or other inflammatory cells to inhibit eosinophilic inflammation [29] or exacerbate the Th2 response and promote eosinophilic inflammation [27], depending on the animal model and experiment protocol employed. The mechanisms underlying these differences require further evaluation.

In our current study, the IL-5 concentrations in the nasal lavage fluid were significantly increased by the depletion of NK cells in our Allergic RS mice. A previous study has reported that IL-5 regulates eosinophil recruitment in the lung [30]. IL-5 also has important roles in eosinophilic accumulation and the activation of eosinophilic CRS [31, 32].

Our study has a limitation of note. We analyzed the effect of anti-ASGM1 treatment on spleen NK cells and not on sinonasal NK cells. However, the distribution of sinonasal NK cells in mice has not been investigated, and NK cell receptors have not been identified. Further studies are thus needed to investigate the distribution and function of sinonasal NK cells in humans as well as in mice. In addition, the underlying mechanism of the association between NK cells and eosinophils in eosinophilic inflammation in CRS needs further elucidation. In this respect, restoration of bacterial infection-mediated eosinophilic inflammation by NK cell depletion may potentially contribute to the aggravation of sinonasal eosinophilic inflammation [29]. We would evaluate this possible mechanism in the future study. Moreover, bacterial burden in mouse nasal airway may be different from that in human due to environmental differences between mouse and human, which should be assessed to determine whether the mouse ecology differs from that of human.

Conclusion

NK cell deficiency may play an important role in the development of allergen-induced eosinophilic sinonasal inflammation in a mouse model of Allergic RS. In this respect, it is possible that defectively functioning NK cells play a role in eosinophilic CRS patients. Thus, NK cell activity may be a key factor governing disease progression in the pathogenesis of eosinophilic CRS.

Acknowledgments

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Disclosure Statement

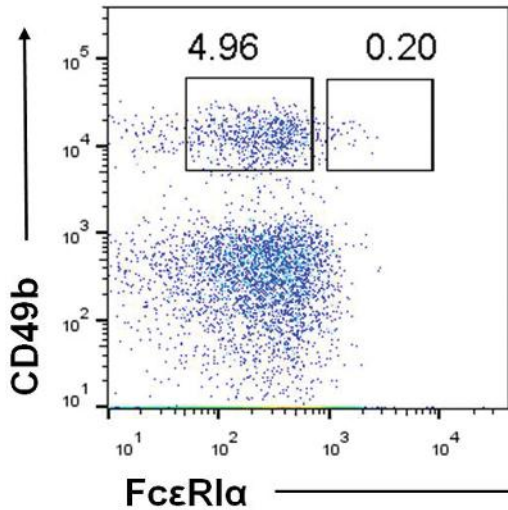
The authors declare no financial or other issues that might lead to a conflict of interest.

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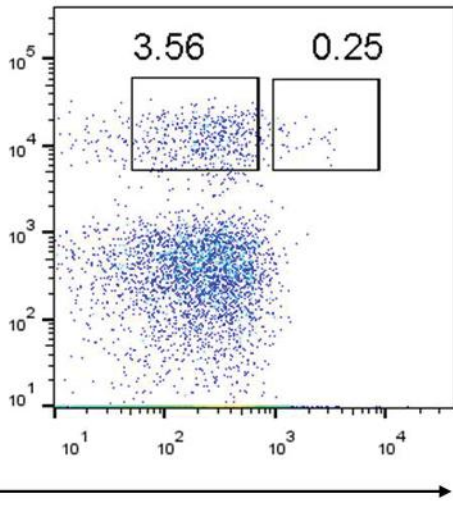
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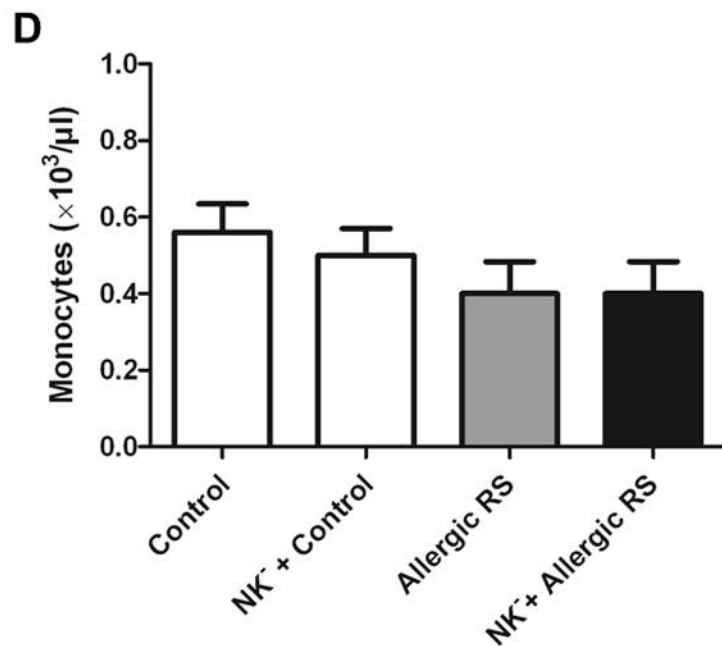
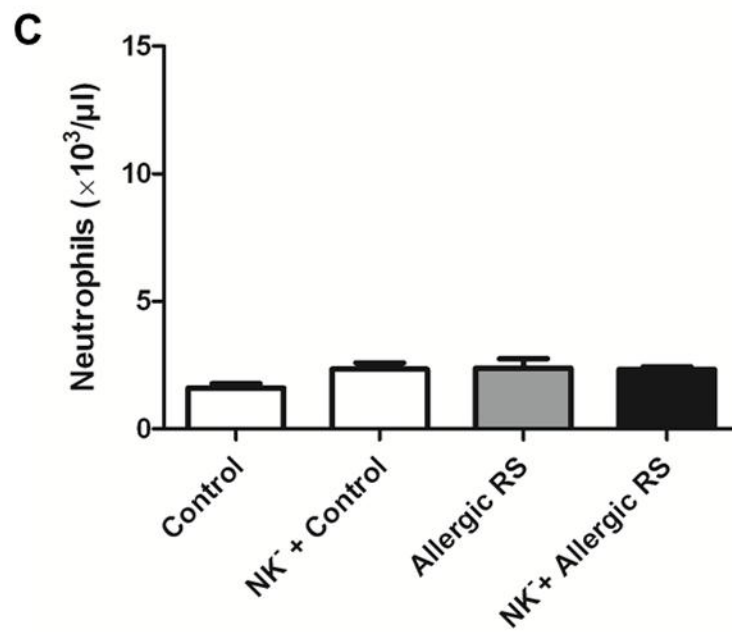
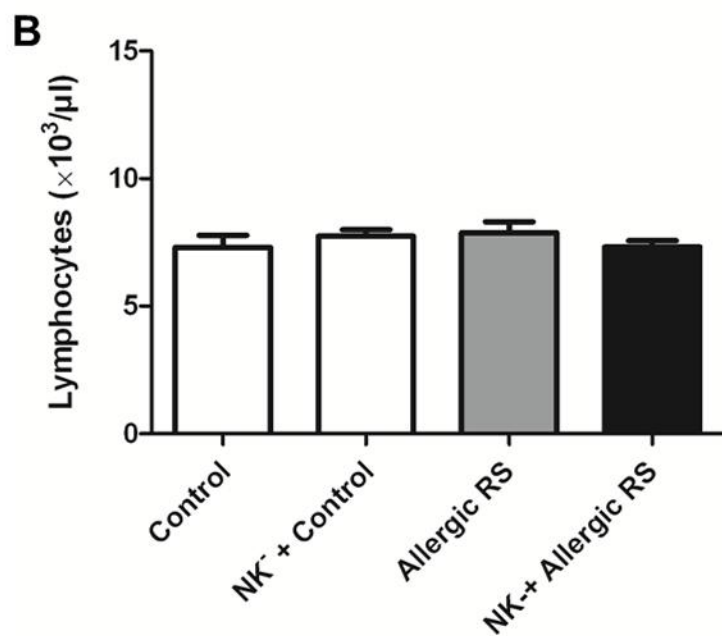
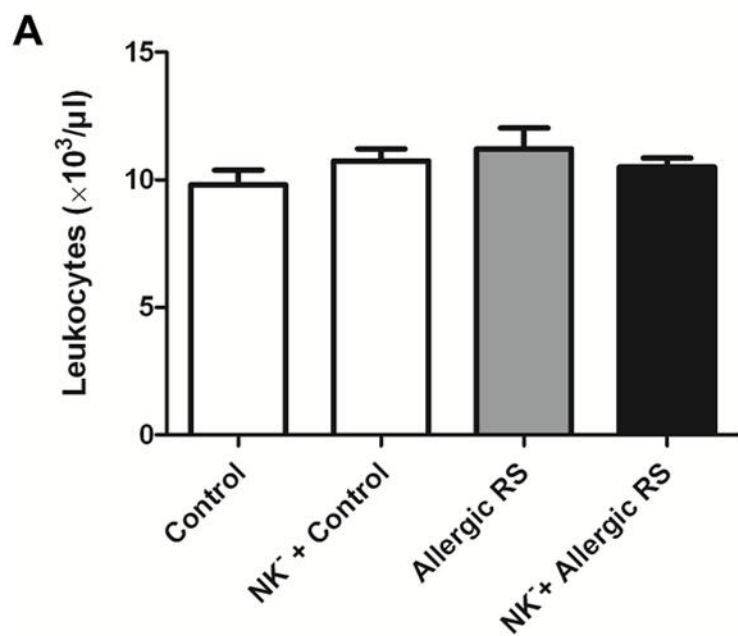
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Control



NK- + Control





Supplementary figure legends

Supplementary Fig. 1. Anti-ASGM1 treatment significantly reduced the number of NK cells (CD3⁺CD49b⁺ FcεRIα⁻) from 4.96% to 3.56%, but not of basophils (CD3⁺CD49b⁺ FcεRIα⁺; 0.20% vs. 0.25%) in control mice.

NK⁻, 10 µg/mouse of anti-ASGM1 treatment.

Supplementary Fig. 2. The number of leukocyte (A), lymphocyte (B), neutrophil (C), and monocyte (D) in blood collected from mice.

NK⁻, 10 µg/mouse of anti-ASGM1 treatment