NK cells and Lipoxin A 4 promote resolution of eosinophilic inflammation after nasal allergen challenge

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To the Editor:

Allergic airway diseases, such as rhinitis and asthma, are one of the most common chronic inflammatory respiratory diseases in the world (1). Although the mechanisms underlying the pathology and treatment of allergic airway inflammation have been widely studied, many aspects remain unclear, including how allergic eosinophilic inflammation effectively resolves in the airways (2). Recent studies implicated natural killer (NK) cells in the regulation of eosinophilic airway inflammation, notably by inducing apoptosis of autologous eosinophils*in vitro* (3). Moreover, lipoxin A_4(LXA_4), a lead member of a larger family of specialized pro-resolving lipid mediators, can enhance the ability of NK cells to cause eosinophil-apoptosis i*n vitro* (3) and decrease allergic eosinophilic inflammation in animal models (4).

In this study, twenty subjects (mean age 28.07 ± 7.1, ten men and ten women) with confirmed grass pollen allergic rhinitis were included. All the subjects underwent two standardized nasal allergen challenges with either a single pre-titrated threshold dose of a grass pollen allergen extract or a diluent in a randomized order and at least 4 weeks apart (for details see Supp. Material and Methods). Nasal lavage fluid and cells were collected at baseline and at different time points after challenge. The study was approved by the local ethics committee and all subjects gave informed written consent.

The nasal allergen challenge induced typical allergic symptoms and a local inflammatory response in all patients that resolved spontaneously within 72 hrs (Figure S1). No significant response was observed after the diluent challenge. Leukocyte sub-populations were further identified in nasal lavage samples by FACS (Figure 1A). Neutrophils were the most abundant population recruited after allergen challenge, peaking 1 hr after. As expected, eosinophils were also rapidly recruited. Interestingly, NK cells were recruited as early as 1 h after the challenge, persisted for 6 hrs before falling to their baseline levels. Monocyte and lymphoid cell recruitment was also observed (Figure 1B). There was a positive relationship between the total number of nasal lavage NK cells and that of eosinophils 1, 6 and 24 hrs after allergen challenge (Figure 1C).

As LXA_4 can decrease allergic eosinophilic inflammation and regulate NK cell-eosinophil interaction (3, 4), we next quantified LXA_4 levels in nasal lavage samples after the allergen challenge. We observed that LXA_4 was produced in the nasal mucosa at baseline and significantly increased in the nasal lavage fluid 1 hr after allergen challenge (Figure 2A). LXA_4 is produced by multistep enzymatic processes in different cell types, including neutrophils (6). Interestingly, increased LXA_4levels 1 hr after allergen challenge correlated with the peak of nasal neutrophil infiltration, suggesting a potential role of neutrophils in LXA_4 biosynthesis during

the early phase of the allergic inflammatory response (Figure 2B). LXA_4 levels also significantly increased at 48 and 72 hrs post-allergen challenge when compared to baseline levels and correlated at these time points to monocyte recruitment.

As previously shown, we observed that peripheral blood NK cells isolated from healthy donors induce apoptosis of autologous blood eosinophils*in vitro* (Figure S2). Moreover, to induce eosinophil apoptosis a direct contact and a combined action of $CD56^{bright}$ and $CD56^{dim}$ NK cells were needed (Figure S2). Besides a potential pro-resolving effect of NK cells on eosinophils, recent data has shown that NK cells can also trigger superoxide release by eosinophils, that can worsen inflammation (5). In our study, eosinophils isolated from healthy donors were by far the most important producers of superoxide anion among different leukocyte populations (Figure 2C). In the presence of NK cells, superoxide release from eosinophils was significantly reduced after 1 hr of co-incubation. In contrast, this inhibitor effect was no longer present after 4 hrs of co-incubation (Figure 2D). When eosinophils from healthy donors were exposed to LXA_4, they significantly reduced their superoxide release in a dose-dependent manner (Figure 2E). Superoxide release by eosinophils co-incubated with NK cells in the presence of LXA_4 was still inhibited in a dose-dependent manner 4 hrs later (Figure 2F) in contrast without LXA_4 (Figure 2D).

Our study underlines the complex network between cellular and molecular actors during resolution of allergic airway inflammation. Here we report for the first time that NK cells are recruited to the nasal mucosa of subjects with allergy in response to nasal allergen challenge and correlate with eosinophilic inflammation. The accumulation of neutrophils along with monocytes during the allergic inflammatory response may furthermore be an important regulatory feedback to initiate and promote resolution of allergic inflammation as our data suggest involvement of these cells in LXA_4 biosynthesis. Moreover, we identified a combined role for NK cells and LXA_4 in mediating resolution of eosinophilic inflammation *in vitro* .

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Conflict of interest

The authors declare that there is no conflict of interest.

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Figure 1. NK cells appear early during the allergic inflammatory response and correlate with eosinophils. Subjects (n=20) suffering from confirmed seasonal grass pollen rhinitis were challenged intranasally with a single threshold dose of a grass pollen extract or diluent.**(A)** Representative dot plots for flow cytometric sequential gating of leukocyte populations in nasal lavage. **(B)** Time course of neutrophils, eosinophils, NK cells, lymphoid cells, T lymphocytes and monocytes following allergen nasal challenge or diluent. Data are displayed as mean ± SEM; *P < 0.05; **P < 0.01; ***P < 0.001; 2-way ANOVA with Sidaks's multiple comparison test was used to compare allergen vs diluent at various time points. **(C)** Correlation between total NK cells and eosinophils 1, 6 and 24h after the allergen challenge (Pearson correlation $r$value and significance are noted).

Figure 2. LXA_4 is produced in the nasal mucosa after nasal allergen and is essential to inhibit NK cells triggered eosinophil superoxide release *in vitro*. **(A)**LXA_4 concentrations in nasal lavage samples; $*P< 0.05$ compared to diluent, $^{\$}P< 0.05$ compared to baseline levels; allergen *vs* diluent: 2-way ANOVA with Sidaks's multiple comparison test, levels *vs* baseline levels: 2-way ANOVA with Tukey's multiple comparison test. **(B)**Correlation of LXA_4 levels and neutrophil and monocyte counts after allergen challenge; Pearson correlation r value and significance are noted). **(C)** Superoxide release by different leukocyte population at rest (vehicle) and when activated by phorbol myristate acetate (PMA), $n = 5$ healthy donors, *P < 0.05, at rest (vehicle) when cells are compared to each other; **P < 0.05, after PMA when cells are compared to each other;$^{£}P < 0.05$, when cells are compared between rest (vehicle) and PMA; multiple t -tests with Bonferroni correction. **(D)** Superoxide release from eosinophils co-incubated with NK cells, $n = 7$ healthy donors, *p<0.05, paired Student's t test. **(E)**Superoxide release from eosinophils pre-treated with increasing doses of LXA_4 and stimulated with PMA; $n = 6$ healthy donors; *p<0.05 compared to

vehicle;$^{£}$p<0.05 compared to 1 nM; one-way ANOVA with Tukey's multiple comparison test. **(F)** , Superoxide release from eosinophils pre-treated with LXA_4 and co-incubated with NK cells, $n = 3$ healthy donors; *P < 0.05; multiple$t$ -tests with Bonferroni correction. Results are expressed as means ± SEM.

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