Cytokine trafficking of IL-9 and IL-13 through TfnRc+ vesicles in activated human eosinophils

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Abstract Eosinophils are granulocytes that are elevated in lung mucosa in approximately half of patients with allergic asthma. These highly granulated cells can synthesize and secrete many cytokines, including IL-9 and IL-13. We hypothesized that IL-9 and IL-13 are found as preformed mediators in crystallloid granules and secreted using distinct trafficking pathways. Human eosinophils were purified from peripheral venous blood, adhered to coverslips, and stimulated with platelet activating factor (PAF). Cells were immunolabeled with antibodies to IL-9 or IL-13 and colocalized with markers for secretory organelles, using CD63 for crystallloid granules and transferrin receptor (TfnRc) for vesicles. Fixed cells were imaged using super-resolution microscopy and quantified by colocalization using Pearson’s correlation coefficient. IL-9 immunofluorescence increased in a time-dependent manner to PAF, whereas colocalization of IL-9 and CD63 significantly increased from 0.52 to 0.67 after 5 min PAF. Colocalization of IL-9 with TfnRc significantly increased at 60 min of stimulation with PAF (0.54 at 0 min to 0.60 at 60 min). IL-13 showed lower colocalization with CD63 (0.55) than TfnRc (0.63) in unstimulated cells. Upon PAF stimulation, IL-13 intensity transiently decreased at 5 and 60 min, whereas colocalization of IL-13 with CD63 decreased throughout stimulation to 0.43. While colocalization of IL-13 with TfnRc transiently increased to 0.66 at 5 min PAF, it returned to near baseline levels (0.64) after 15 min PAF. Our results suggest that IL-9 and IL-13 are stored in crystallloid granules as well as endosomal structures, and that IL-9 is primarily trafficked to the cell surface via TfnRc+ endosome-like vesicles.

KEYWORDS allergy/asthma, cytokines, degranulation, secretory granules, super-resolution microscopy, vesicles

1 INTRODUCTION Eosinophils are highly granulated white blood cells and are prominent in asthma.1 These granulocytes are rarely observed in healthy airways, but in approximately 50% of asthmatics, they become substantially elevated in blood and tissue.2,3 Increased eosinophilic inflammation of the airways broadly correlates with increased asthma severity and is often accompanied by degranulation.4 When activated, eosinophils rapidly release immunomodulatory cytokines.5 A well-known stimulant of eosinophil degranulation is platelet activating factor (PAF), a highly active lipid mediator that acts on PAF receptor and other mechanisms to induce eosinophil activation.6

Eosinophils synthesize and store several Th2 cytokines, including IL-4, IL-5, IL-9, and IL-13, many of which exhibit granular distribution in immunostained cells.5 Both IL-9 and IL-13 skew the immune response to a Th2 phenotype in eosinophil inflammatory diseases.7–10 Although the importance of these Th2 cytokines in immunity and asthma is well established, the specific intracellular trafficking mechanisms of these cytokines in eosinophils have not been characterized.

Cytokine secretion pathways in innate immune cells are dependent on soluble NSF attachment protein receptors (SNAREs) that are involved in the docking and fusion of vesicles to the cell membrane for...
cargos.\textsuperscript{11–14} A SNARE complex comprising vesicle-associated membrane protein (VAMP)-7 on crystalloid granules and SNAP23 and syntaxin-4 at the plasma membrane has been proposed to mediate the release of cargo in cytoplasmic eosinophil compartments.\textsuperscript{15–17} Our recent study demonstrated that eosinophils from an eoCRE transgenic mouse model with eosinophil-specific VAMP-7 gene deficiency showed decreased degranulation in correlation with diminished airway hyperresponsiveness in allergen-sensitized and challenged mice.\textsuperscript{18} Eosinophils from eoCRE/V7 mice also exhibited selectively decreased secretion of IL-9 but not other cytokines, suggesting that VAMP-7-mediated exocytosis contributes to IL-9 release.\textsuperscript{18} Immunolabeling of IL-9 in eosinophils exhibits a granular pattern of staining.\textsuperscript{19} and we have previously reported that NK-13 is stored in crystalloid granules in human eosinophils.\textsuperscript{20} However, the membrane transport pathways responsible for IL-9 and IL-13 trafficking and release have not been determined. In macrophages, recycling endosomes have been shown to be important traffickers of newly synthesized cytokines from the trans-Golgi network.\textsuperscript{11,12} Recycling endosomes, bearing membrane bound markers such as Arf6, Rab11, VAMP-3, and transferrin receptor (TfnRc), canonically recycle membrane and receptors such TfnRc to the cell surface.\textsuperscript{11,12} These recycling endosomes have also been implicated in cytokine trafficking in NK and micropolial cells,\textsuperscript{21,22} but no reports have yet suggested a role for them in eosinophil cytokine trafficking.

Our study investigated intracellular storage sites of cytokines IL-9 and IL-13 in eosinophils and pathways involved in their release. We hypothesized that the Th2 cytokines, IL-9 and IL-13, are found as preformed cytokines stored in eosinophil crystalloid granules, and other intracellular organelles, such as small endosome-like vesicles, and are secreted using distinct trafficking pathways. We propose to examine the presence of TfnRc\textsuperscript{+} vesicles in eosinophils, which have not been explored in these cells before. In this study, we used human eosinophils isolated from venous blood of allergic and/or asthmatic voluntary donors. Immunofluorescence analysis was coupled with super-resolution microscopy to determine IL-9 and IL-13 trafficking following stimulation with PAF.

2 | MATERIALS AND METHODS

2.1 | Donor recruitment
Volunteers with self-reported asthma and/or allergies not taking any form of medication for at least 2 wk prior to sampling were recruited. All participants were 18 yr or older and provided informed consent prior to blood collection. Donors with over 3% circulating eosinophils were selected for the study. This study was approved by the University of Alberta human ethics review board.

2.2 | Human whole blood collection
A total of 30 ml of human blood was collected from each donor in EDTA (K2) vacutainer blood collection tubes (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA). Differential counts of whole blood were performed using Kimura stain and C-Chip hemocytometers (Bulldog Bio-NanoEnTek, Portsmouth, NH, USA).

2.3 | Human blood eosinophil isolation and preparation for immunolabeling
Eosinophils were purified from whole blood using MACSxpress human eosinophil isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany).\textsuperscript{23} Isolations resulted in $\geq$ 95% purity and $\geq$ 99% viability, as measured with Kimura stain and Trypan Blue stain, respectively. Puriﬁed eosinophils were then washed once with serum-free RPMI 1640 (Gibco Life Technologies, Waltham, MA, USA), centrifuged, and resuspended in this media. Borosilicate glass coverslips (#1, Thermo Fisher Scientific, Waltham, MA, USA) were cleaned with 95% ethanol and placed in 6-well plates (Thermo Fisher Scientific). Per well, 1 $\times$ 10\textsuperscript{6} eosinophils/ml were added. Cells were incubated at 37°C for 30 min to allow for adherence to coverslips. To stimulate cells, 5 $\mu$M PAF (C18; Sigma-Aldrich, St. Louis, MO, USA) was added for 0, 5, 15, and 60 min. Next, coverslips were washed thrice with PBS (HyCline Labs, GE Healthcare Life Sciences, Chicago, IL, USA). Eosinophils were then ﬁxed with 4% PFA for 30 min, washed, and stored in PBS at 4°C.

2.4 | Immunofluorescence staining
Cells were permeabilized and blocked with 0.1% saponin (Sigma-Aldrich), 2% BSA, and 10% heat-inactivated human serum in PBS for 30 min. Eosinophils were immunolabeled with Alexa Fluor 647 mouse $\alpha$-human IL-9 (BD Biosciences) or Alexa Fluor 647 mouse $\alpha$-human IL-13 (R&D Systems, Minneapolis, MN, USA) and Alexa Fluor 488 rabbit $\alpha$-human TfnRc (Bioss, Woburn, MA, USA) or Alexa Fluor 488 anti-human CD63 (Invitrogen, Waltham, MA, USA)/FITC anti-human CD63 (Bio-Rad, Hercules, CA, USA). Isotype controls were included for all antibodies (Alexa Fluor 647-conjugated mouse IgG1 [BD Biosciences], rabbit IgG Alexa Fluor 488-conjugated [Bioss], mouse IgG1 Alexa Fluor 488-conjugated [R&D Systems], and mouse IgG1 FITC-conjugated [Bio-Rad], respectively) (Supplementary Fig. S1). Rhodamine phalloidin (Thermo Fisher Scientific) was used to label F-actin and DAPI (Thermo Fisher Scientific) for nuclear staining. After labeling, coverslips were washed and mounted to slides using ProLong Glass Antifade Mountant (Thermo Fisher Scientific). Eosinophil immunofluorescence analysis was conducted in cells obtained from at least three donor samples.

2.5 | Image collection and analysis
Cells were imaged using a DeltaVision OMX super-resolution microscope. In all experiments, images were collected from isotype and test antibodies to correct for endogenous fluorescence and nonspecific binding by antibodies. Colocalization was quantified through Pearson’s correlation coefficient using Velocity image analysis software (Version 6.5.1, Puslinch, Ontario, Canada). We found Pearson’s correlation
coefficients to be best suited to determine colocalization for our experiments. An automated process used to automatically define the region of interest and subsequent analysis was employed, as manual selection of cells yielded no overall statistical difference from the automated process (Supplementary Figs. S2 and S3). At least 50 cells from each time point were analyzed. Object-based colocalization of granules and vesicles was carried out using Imaris software (Version 8.1, Zurich, Switzerland) (Supplementary Fig. S4).

2.6 | Statistical analysis

All analysis was performed using GraphPad Prism software (Version 8, La Jolla, CA, USA). Statistical comparisons between time points were performed with a Kruskal-Wallis analysis followed by Dunn’s multiple comparisons test. Data from all experimental replicates were analyzed with a two-way ANOVA followed by Dunnett’s multiple comparisons test. A minimum of three donors were analyzed for all statistical tests. Significance was set at \( P < 0.05 \).

3 | RESULTS AND DISCUSSION

3.1 | IL-9 and IL-13 colocalize with eosinophil CD63+ crystalloid granules and TfnRc+ vesicles

To determine the intracellular distribution of IL-9 and IL-13 in eosinophils, adherent cells were fixed and immunolabeled for super-resolution microscopy imaging. Both IL-9 and IL-13 exhibited punctate staining throughout the cytoplasm in unstimulated cells, suggesting granular storage of preformed cytokines (Figs. 1–4). We assessed the colocalization of these Th2 cytokines with membrane-bound markers of intracellular organelles, CD63 and TfnRc. CD63 labeling represents crystalloid granules, whereas TfnRc staining includes endosomal populations (recycling, early, and late), referred to here as TfnRc+ vesicles. CD63 staining appeared doughnut shaped, indicative of membrane localization of this tetraspanin in crystalloid granules (Figs. 1, 3), as previously reported. The staining for TfnRc followed a dense, granular pattern throughout the cell cytoplasm with fewer ring-shaped structures (Figs. 2, 4). Pearson’s correlation coefficient was used to evaluate colocalization of IL-9 and IL-13 with CD63+ crystalloid granules and TfnRc+ vesicles upon PAF activation of eosinophils. Our findings confirm earlier observations that IL-9 and IL-13 are stored as preformed cytokines in eosinophils.

3.2 | IL-9 immunofluorescence and colocalization with TfnRc+ vesicles increase following PAF stimulation

Following stimulation by PAF (5 \( \mu \text{M} \)), we observed morphologic changes from compact, rounded eosinophils to stretched and elongated shapes by 15 and 60 min, as previously reported (Figs. 1–4, and Supplementary Figs. S5, S6). The stretched morphology of PAF-stimulated eosinophils was accompanied by the appearance of numerous filopodia that were often polarized at one end of the cell, particularly after 60 min of stimulation. Interestingly, we observed increased IL-9 immunofluorescence intensity at each time point of PAF stimulation (\( P < 0.05 \), Fig. 1B), which may be reflective of de novo synthesis. Although variability between different donors occurred, an overall increasing trend was consistently observed across five donors (Fig. 5A).

With 5, 15, and 60 min of PAF stimulation, colocalization of IL-9 with CD63 transiently increased at 5 min of stimulation before returning to baseline values (\( P < 0.05 \), Fig. 1C), and this trend was reflected in data from multiple donors (Fig. 5B). To explore this from a different angle, we used object-based colocalization method to determine proportions of IL-9 granule- or vesicle-associated cytokine pools (Fig. 6). Interestingly, object-based colocalization showed that greater proportions of CD63+ granules containing IL-9 were observed only at 5 min of PAF stimulation (Fig. 6C).

In contrast, IL-9 increasingly colocalized with TfnRc over 60 min (\( P < 0.01 \), Figs. 2B and 5C). Similarly, greater proportions of TfnRc+ vesicles containing IL-9 were observed at 15 and 60 min of PAF stimulation (Fig. 6E).

3.3 | IL-13 colocalization with CD63+ crystalloid granules decreases throughout PAF stimulation

In marked contrast to IL-9, IL-13 immunofluorescence intensity consistently showed a modest decrease after 60 min of PAF stimulation, although in one donor it declined at 5 min as well (\( P < 0.01 \), Figs. 3B and 5D). Overall, our findings suggest that IL-13 was not newly synthesized during PAF stimulation. IL-13 colocalization with CD63 decreased over the entire PAF stimulation time course (Figs. 3C and 5E). This decrease in IL-13 colocalization with CD63 suggests that IL-13 may be shuttled from its storage site in crystalloid granules for mobilization to the cell surface as the cell is activated. Conversely, IL-13 colocalization with TfnRc+ vesicles transiently increased after 5 min PAF stimulation and then returned to baseline levels by 15 min (Figs. 4B and 5F). Although IL-13 colocalized with TfnRc+ vesicles, there was no overall change in colocalization during stimulation, suggesting that TfnRc+ vesicles are not the main trafficking compartment for this cytokine. As IL-13 did not follow the same pattern of colocalization compared to IL-9, our findings suggest an independent pathway of IL-13 trafficking to the cell periphery for release.

This is the first report of TfnRc+ vesicles in eosinophils, suggesting that eosinophils, like many other cells in the body, utilize membrane recycling pathways for trafficking of receptors and granule contents. Moreover, TfnRc+ vesicles colocalized with IL-9 and IL-13, suggesting a role for these vesicles in cytokine trafficking in eosinophils. We found increased colocalization of IL-9 with TfnRc following stimulation with PAF, indicating elevated IL-9 content in these vesicles following stimulation and that TfnRc+ vesicles play a key role in IL-9 trafficking for...
**FIGURE 1** IL-9 intensity and colocalization with CD63⁺ crystalloid granules changes dynamically in platelet activating factor (PAF)-stimulated eosinophils. (A) Human eosinophils were adhered to glass coverslips and stimulated with PAF for 0, 5, 15, and 60 min. Cells were fixed, permeabilized, and labeled with anti-IL-9 (red) and anti-CD63 (green) as a marker for crystalloid granules. Actin cytoskeleton was stained with rhodamine-phalloidin (gray) and nuclei with DAPI (blue). Yellow regions indicate colocalization between intracellular cytokine and crystalloid granules. High magnification insets of the merged images are shown at right. Scale bar represents 5 µm. Using Volocity software, immunofluorescence intensity values for intracellular cytokine levels (B) and Pearson's correlation coefficients for IL-9 and CD63 colocalization (C) were measured. A minimum of 50 eosinophils were analyzed in each plot. Data are shown from a representative donor. Means are indicated by red lines. Error bars indicate SD.*P < 0.05 and **P < 0.01.

A limitation of our study is that there is an inherent bias in our analysis, as only cells that adhered to coverslips were evaluated. This subset of adherent eosinophils may display different cytokine levels and trafficking compared to the population in suspension. However, it was not
feasible to employ a different technique to analyze the entire sample of cells, such as flow cytometry, as flow cytometry does not provide sufficient intracellular resolution required for colocalization of intracellular crystalloid granules and vesicles. Additionally, only atopic donors were tested in this study as healthy individuals do not provide sufficient numbers of eosinophils for isotype and test antibodies at all time points to conduct our experiments.

To further elucidate the trafficking pathways of IL-9 and IL-13, we attempted to stain for additional markers of recycling endosomes (VAMP-3 and Rab11) and crystalloid granules (Rab27a and VAMP-7), as well as other intracellular compartments, including the Golgi apparatus (giantin), endoplasmic reticulum (calreticulin), and SNARE proteins (SNAP-23 and syntaxin-4). However, these markers did not generate a signal greater than their respective isotype control when
FIGURE 3  IL-13 intensity and colocalization with CD63+ crystalloid granules changes in platelet activating factor (PAF)-stimulated eosinophils. Human eosinophils were stimulated and labeled as described in Figure 1 with anti-IL-13 (red) substituting for anti-IL-9 and anti-CD63 (green) as a marker for crystalloid granules. Scale bar represents 5 µm. Data are shown from a representative donor. Means are indicated by red lines. Error bars indicate SD.*P < 0.05 and **P < 0.01

labeled using our immunostaining protocol (data not shown). These challenges in immunostaining were likely due to high levels of endogenous eosinophilic autofluorescence and nonspecific antibody labeling.

Cytokine secretion could not be detected at levels above the limit of quantification (>30 pg/ml) for ELISAs when we examined supernatants from 1 x 10^6 eosinophils stimulated by 5 µM PAF or positive control calcium ionophore (data not shown). This was presumably due to limited numbers of eosinophils, which prevented detection of supernatant-derived cytokines by ELISA. Previous ELISA measurements of mouse eosinophil IL-9 and IL-13 used cells from IL-5 transgenic mice with 15-fold more cells per sample and showed higher levels of these cytokines, whereas assays on human eosinophils detected negligible quantities (<10 pg/ml) of released IL-9 and IL-13.6,18

Future studies will benefit from testing additional cytokines and chemokines such as CCL5/RANTES, a key proinflammatory chemokine...
FIGURE 4  Colocalization of IL-13 with transferrin receptor (TfnRc) vesicles in platelet activating factor (PAF)-stimulated eosinophils. Human eosinophils were stimulated and labeled as described in Figure 1 with anti-IL-13 (red) and anti-TfnRc (green). Scale bar represents 5 µm. Data are shown from a representative donor. Means are indicated by red lines. Error bars indicate SD. **P < 0.01

detected in eosinophils. Understanding the mechanisms for IL-9 and IL-13 release will contribute to our understanding of cytokine secretion from these cells. Coordinated efforts may link the mechanistic understanding of cytokine release with clinical practice in treating patients with eosinophilic asthma. Our current findings combined with future studies will provide insight into potential targets for therapeutic intervention.

AUTHORSHIP

S.A. designed, performed, and analyzed experiments, and wrote the original draft of the manuscript; N.F., O.S., and M.S. performed and analyzed experiments and reviewed and revised the manuscript; N.T. and S.A. designed the image analysis protocols; and P.L. supervised the study, designed experiments, and edited the manuscript.
**FIGURE 5**  Intensity and colocalization of IL-9 and IL-13 with CD63^+ granules and transferrin receptor (TfnRc)^+ vesicles demonstrates a similar pattern of release from all donors. Immunofluorescence (A and D) and Pearson’s correlation coefficients (B, C, E, and F) were measured on Velocity software from grouped donor data. Each plot had a minimum of three donors, from which 50 eosinophils were analyzed and averaged for each platelet activating factor (PAF) stimulation time points. Donor numbers represent replicates for each data set and are not assigned to an individual. Mean values of 250 measurements from each donor at each time point are shown by a data point. Error bars indicate SEM. *P < 0.05 and **P < 0.01

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**DISCLOSURES**

The authors declare no conflicts of interest.

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FIGURE 6 Greater proportion of intracellular IL-9 in transferrin receptor (TfnRc)+ endosomes compared to CD63+ crystalloid granules. Intracellular IL-9, CD63+ granules, and TfnRc+ endosomes were defined and quantified on a single cell basis as described in Supplementary Figure S4. IL-9-defined spots co-located within CD63+ granules or TfnRc+ endosomes were then quantified on a per cell basis. A number of IL-9-defined spots (A), percentage of total IL-9 within CD63+ granules (B) or TfnRc+ endosomes (D), and percentages of CD63+ granules or TfnRc+ endosomes containing IL-9 (C, E) were measured per cell. At each time point, 50 eosinophils were analyzed from an individual donor. Means are represented as red lines and error bars indicate SD. Kruskal-Wallis test followed by Dunn’s multiple comparisons test was performed. *P < 0.05 and **P < 0.01.

SUPPORTING INFORMATION
Additional information may be found online in the Supporting Information section at the end of the article.