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Expression and subcellular localization of the Qa-SNARE syntaxin17 in human eosinophils

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Abstract

Background—SNARE members mediate membrane fusion during intracellular trafficking underlying innate and adaptive immune responses by different cells. However, little is known about the expression and function of these proteins in human eosinophils, cells involved in allergic, inflammatory and immunoregulatory responses. Here, we investigate the expression and distribution of the Qa-SNARE syntaxin17 (STX17) within human eosinophils isolated from the peripheral blood.

Methods—Flow cytometry and a pre-embedding immunogold electron microscopy (EM) technique that combines optimal epitope preservation and secondary Fab-fragments of antibodies linked to 1.4-nm gold particles for optimal access to microdomains, were used to investigate STX17.

Results—STX 17 was detected within unstimulated eosinophils. Immunogold EM revealed STX17 on secretory granules and on granule-derived vesiculotubular transport carriers (Eosinophil Sombrero Vesicles - EoSVs). Quantitative EM analyses showed that 77.7% of the granules were positive for STX17 with a mean $\pm$ SEM of 3.9 ± 0.2 gold particles/granule. Labeling was present on both granule outer membranes and matrices while EoSVs showed clear membrane-associated labeling. STX17 was also present in secretory granules in eosinophils stimulated with the cytokine tumor necrosis factor alpha (TNF- α) or the CC-chemokine ligand 11 CCL11 (eotaxin-1), stimuli that induce eosinophil degranulation. The number of secretory granules labeled for STX17 was significantly higher in CCL11 compared with the unstimulated group. The level of cell labeling did not change when unstimulated cells were compared with TNF- α -stimulated eosinophils.

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Conclusions—The present study clearly shows by immunogold EM that STX17 is localized in eosinophil secretory granules and transport vesicles and might be involved in the transport of granule-derived cargos.

Graphical Abstract

Keywords

SNARES; syntaxin17; human eosinophils; transmission electron microscopy; immunogold electron microscopy; secretory granules; eosinophil sombrero vesicles; vesicular trafficking

Introduction

Secretion is an essential biological activity of all eukaryotic cells by which they release specific products in the extracellular space during physiological and pathological events. In cells from the immune system, such as eosinophils, basophils, neutrophils and macrophages, secretory mechanisms underlie the functions of these cells during allergic, inflammatory and immunoregulatory responses (reviewed in [1, 2]).

Our Group has been studying mechanisms of intracellular trafficking and secretion in human eosinophils [3-7]. Eosinophil responses involve secretion of distinct cationic proteins and numerous cytokines with multiple functional activities. These mediators are released in a tightly orchestrated manner to regulate the progression of immune responses (reviewed in [8-10]). Different from lymphocytes that must exclusively synthesize proteins prior to secretion and similar to neutrophils [11] and mast cells [12], both cationic proteins and cytokines are additionally stored as preformed pools within eosinophil secretory granules [13].

In human eosinophils, vesicle-mediated transport of proteins from secretory granules is commonly described both *in vitro* and *in vivo* during different conditions, including inflammatory and allergic disorders [14-20]. Large carriers, identified as vesiculotubular structures of complex plasticity, termed Eosinophil Sombrero Vesicles (EoSVs), in addition to small vesicles, participate in the vesicular trafficking of eosinophil granule-stored mediators, such as IL-4 [3, 4] and major basic protein (MBP) [7]. EoSVs are constantly found in biopsies of patients with inflammatory diseases such as eosinophilic esophagitis [20] and bowel disease [8].

The volume and complexity of vesicular traffic in eosinophils and other cells from the immune system require a selective machinery to ensure the accurate docking and fusion of carrier vesicles at their designated target membranes. SNARE proteins (N-ethylmaleimide

sensitive factor attachment protein receptors) that are present on secretory granule and plasma membranes likely mediate this fusion.

SNAREs are generally small (14-40 KDa), coiled-coil forming proteins that are anchored to the membrane via a C-terminal anchor. They were originally classified as v- (vesicle-associated) or t- (target-membrane) SNAREs, on the basis of their locations and functional roles in a typical trafficking step. However, this orientation is not always maintained and an alternative structure-based terminology has now been used, wherein the family is divided into R-SNAREs and Q-SNAREs, on the basis of whether the central functional residue in their SNARE motif is arginine (R) or glutamine (Q). Q-SNAREs are then further classified into Qa, Qb, Qc and Qb,c subtypes based on where their SNARE domain(s) would sit in an assembled trans-SNARE complex (reviewed in [1, 21]).

So far, few studies have documented SNAREs at subcellular sites of human eosinophils. Only three SNAREs, all R-SNAREs members, were characterized in intracellular locations: the vesicle-associated membrane protein (VAMP)2, found predominantly in vesicles [22-24], and VAMP7 and VAMP8, which were documented in granule-enriched fractions [25].

Here, we investigate the expression and subcellular localization of the Qa-SNARE syntaxin17 (STX17) within human eosinophils. By using flow cytometry and an immunogold electron microscopy technique that combines different strategies for optimal labeling and morphology preservation [26], we provide the first identification of STX17 in human eosinophils. This SNARE is localized in eosinophil secretory granules and EoSVs from both unstimulated and stimulated eosinophils and might be involved in the transport of granule-derived specific cargos.

Material and Methods

Eosinophil Isolation, Stimulation and Viability

Granulocytes were isolated from the blood of different healthy donors. Eosinophils were enriched and purified by negative selection using human eosinophil enrichment cocktail (StemSep™, StemCell Technologies, Seattle WA, USA) and the MACS bead procedure (Miltenyi Biotec, Auburn, CA, USA), as described [57], with the exception that hypotonic red blood cell (RBC) lysis was omitted to avoid any potential for RBC lysis to affect eosinophil function. Eosinophil viability and purity were greater than 99% as determined by ethidium bromide (Molecular Probes, OR, USA) incorporation and cytocentrifuged smears stained with HEMA 3 stain kit (Fisher Scientific, TX, USA), respectively. Experiments were approved by the Beth Israel Deaconess Medical Center Committee on Clinical Investigation, and informed consent was obtained from all subjects. Purified eosinophils (10^6 cells/mL) were stimulated with TNF- α (200 ng/mL; R&D Systems, USA) or recombinant human CCL11 (eotaxin-1) (100 ng/mL; R&D Systems, Minneapolis, MN) in RPMI-1640 medium plus 0.1% ovalbumin (OVA) (Sigma, St. Louis, MO, USA), or medium alone at 37°C, for 1 h as before [27].

Antibody Reagents

Antibodies for STX17 detection in eosinophils were an affinity-purified goat polyclonal antibody raised against a peptide mapping within a cytoplasmic domain of STX17 of human origin (Santa Cruz Biotechnology, TX, USA, sc-107095) used in parallel with control goat IgG (Santa Cruz Biotechnology) at concentrations of 5 µg/mL (immunoEM) or 10 µg/mL (flow cytometry). Secondary antibody for immunoEM studies was an affinity-purified rabbit anti-goat Fab fragment conjugated to 1.4-nm gold particles (1:100, Nanogold®, cat. # 2006, Nanoprobes; Stony Brook, NY). Secondary antibodies for flow cytometry were anti-goat antibodies conjugated to FITC (10 µg/mL, Jackson ImmunoResearch laboratories Inc., West Grove, PL, USA).

Conventional TEM

For conventional TEM, isolated eosinophils were fixed in a mixture of freshly prepared aldehydes (1% paraformaldehyde and 1.25% glutaraldehyde) in 1 M sodium cacodylate buffer for 1 h at room temperature (RT), embedded in 2% agar [19] and kept at 4 °C for further processing. Agar pellets containing eosinophils were processed as described. Briefly, samples were post-fixed in 1% osmium tetroxide in Sym-Collidine buffer, pH 7.4, for 2 h at RT. After washing with sodium maleate buffer, pH 5.2, they were stained en bloc in 2% uranyl acetate in 0.05 M sodium maleate buffer, pH 6.0 for 2 h at RT and washed in the same buffer as before prior to dehydration in graded ethanols and infiltration and embedding with a propylene oxide-Epon sequence (Eponate 12 Resin; Ted Pella, Redding, CA, USA) [19]. Specimens were examined using a transmission electron microscope (CM10, Philips) at 60 kV.

Cell Preparation for immunogold EM

For immunoEM, purified eosinophils were immediately fixed in fresh 4% paraformaldehyde in 0.02 M phosphate-buffered saline (0.15 M NaCl) (PBS), pH 7.4 [26]. Cells were fixed for 30 min at room temperature (RT), washed in PBS and centrifuged at 1500 g for 1 min. Samples were then resuspended in molten 2% agar in PBS and quickly recentrifuged. Pellets were immersed in 30% sucrose in PBS overnight at 4°C, embedded in OCT compound (Miles, Elkhart, IN, USA), and stored in -180°C liquid nitrogen for subsequent use.

Pre-embedding immunogold EM

Pre-embedding immunolabeling was carried out before standard EM processing (postfixation, dehydration, infiltration, resin embedding and resin sectioning). Immunogold was performed on cryostat 10 µm sections mounted on glass slides. After testing different section thicknesses, we found that 10 µm enabled optimal penetration of the antibodies [26]. All labeling steps were carried out at RT as before [26] as follows: a) one wash in 0.02 M PBS, pH 7.6, 5 min; (b) immersion in 50 mM glycine in 0.02 M PBS, pH 7.4, 10 min; (c) incubation in a mixture of PBS and bovine serum albumin (PBS-BSA buffer; 0.02 M PBS plus 1% BSA) containing 0.1% gelatin (20 min) followed by PBS-BSA plus 10% normal goat serum (NGS) (30 min). This step is crucial to block non-specific binding sites; (d) incubation with primary antibody (1 h); (e) blocking with PBSBSA plus NGS (30 min); (f) incubation with secondary antibody (1 h); (g) washing in PBS-BSA (three

times of 5 min each); (h) postfixation in 1% glutaraldehyde (10 min); (i) Five washings in distilled water; (j) incubation with HQ silver enhancement solution in a dark room according to the manufacturer's instructions (Nanoprobes) (10 min). This step enables a nucleation of silver ions around gold particles. These ions precipitate as silver metal and the particles grow in size facilitating observation under TEM; (k) Three washings in distilled water; (l) immersion in freshly prepared 5% sodium thiosulfate (5 min); (m) postfixation with 1% osmium tetroxide in distilled water (10 min); (n) staining with 2% uranyl acetate in distilled water (5 min); (o) embedding in Eponate (Eponate 12 Resin; Ted Pella, Redding, CA, USA); (p) after polymerization at 60°C for 16 h, embedding was performed by inverting eponate-filled plastic capsules over the slide-attached tissue sections. (q) separation of eponate blocks from glass slides by brief immersion in liquid nitrogen. Thin sections were cut using a diamond knife on an ultramicrotome (Leica, Bannockburn, IL, USA). Sections were mounted on uncoated 200-mesh copper grids (Ted Pella) before staining with lead citrate and viewed with a transmission electron microscope (CM 10; Philips, Eindhoven, the Netherlands) at 60 kV. Two controls were performed: (1) primary antibody was replaced by an irrelevant antibody, and (2) primary antibody was omitted. Electron micrographs were randomly taken at different magnifications to study the entire cell profile and subcellular features.

Flow cytometry

Unstimulated human eosinophils were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.1% saponin and blocked with 2.5% human serum in 0.1% BSA/PBS. Cells were incubated with anti-STX17 or isotype control antibodies, followed by secondary antibodies as described above. Data were acquired using the LSRII flow cytometer (BDBiosciences) and the analysis software, Flow Jo (Tree Star Inc., Ashland, OR).

Statistical Analysis

For quantification studies by conventional TEM (enumeration of the total number of specific granules undergoing morphological changes in TNF- α -stimulated and unstimulated cells), we randomly took electron micrographs of cell sections showing the entire cell profile and nucleus. A total of 59 electron micrographs (26 from unstimulated and 33 from stimulated cells) and 2346 secretory granules (1069 from unstimulated cells and 1277 from TNF- α -stimulated eosinophils) were counted and the number of intact granules as well as the number of granules undergoing losses of their contents (with lucent areas in their cores, matrices or both; reduced electron density and disassembled matrices and cores) was established [19].

For the immunolabeling studies, a total of 53 electron micrographs from TNF- α -, CCL11-stimulated or controls were evaluated and the numbers of secretory granules and EoSVs (labeled and not labeled) as well as the numbers of gold particles/subcellular compartment were counted using the software ImageJ (National Institutes of Health, Bethesda, MD, USA). A total of 1088 granules and 1106 EoSVs were counted. Data were compared using the Mann–Whitney U-test ($P < 0.05$).

Results

STX17 is localized on eosinophil secretory granules and EoSVs in unstimulated cells. First, we investigated whether human unstimulated eosinophils express STX17 protein by flow cytometry. This technique demonstrated intracellular STX17 in these cells (Fig. 1A). The subcellular localization of STX17 in human eosinophils was next investigated with pre-embedding immunogold EM for precise subcellular localization [26]. STX17 labeling was clearly identified on secretory granules (Fig. 1B and Bi) and EoSVs (Fig. 1B and Bii). These organelles/structures have a typical morphology, which enables unambiguous identification by TEM (Fig. 1Bii). Secretory granules have an internal often electron-dense crystalline core and an outer electron-lucent matrix surrounded by a delimiting trilaminar membrane (Fig. 1B). STX17 labeling was associated with both granule matrices and outer membranes (Figs. 1B and 1Bi). EoSVs are easily identifiable within human eosinophils because of their typical 'mexican hat' (sombrero) appearance in cross sections with a central area of cytoplasm and a brim of circular membrane-delimited vesicle and large size (150-330 nm in diameter) compared to small, round transport vesicles (~50 nm in diameter) [3]. They also can show a "C" shaped morphology [3]. These vesicular compartments exhibited membrane-associated labeling for STX17 (Fig. 1Bii).

STX17 is concentrated on secretory granules in stimulated eosinophils

We next investigated the subcellular localization of STX17 in eosinophils stimulated with physiologic agonists, which are known to induce eosinophil activation and secretion: TNF- α and CCL11. TNF- α is an inflammatory stimulus that induces robust eosinophil cytokine secretion [13] and clear morphological changes of secretory granules associated with degranulation (Supplementary Fig. 1). Emptying of crystalloid granules is also noted after CCL11 stimulation of eosinophils from both humans [7, 19] and experimental models [28].

In the present work, eosinophils stimulated with TNF- α (Fig. 2) or CCL11 (Fig. 3) showed STX17 mostly localized in secretory granules (outer membranes and matrices) and also in EoSVs (Fig. 2). Control cells in which the primary antibody was replaced by an irrelevant antibody were negative (Supplementary Fig. 2).

To evaluate the level of STX17 labeling on secretory granules and EoSVs, eosinophil sections showing the entire cell profile and nucleus were analyzed and the total number of secretory granules and EoSVs, the number of STX17-labeled granules and EoSVs and the number of gold particles per granule or per EoSV were counted using the ImageJ software. Our quantitative EM analyses showed that 77.7 ± 1.7 % of secretory granules in unstimulated cells were positive for STX17 and that each granule had 3.9 ± 0.2 gold particles (mean $\pm$ SEM, $n = 321$ granules) (Fig. 4A). Vesicular compartments within unstimulated cells showed a level of STX17 labeling of 1.0 ± 0.2 gold particles per vesicle (mean $\pm$ SEM, $n = 333$ EoSVs) (Fig. 4B).

We next evaluated if cell stimulation would induce changes in the level of STX17 labeling within human eosinophils. After stimulating with TNF- α or CCL11 for 1 h and applying immunogold EM, we did not find a significant difference when the number of gold particles per secretory granule of unstimulated and stimulated cells was quantitated and

compared (Fig. 4A, left panel). Each granule exhibited 4.0 ± 0.2 gold particles/granule for TNF- α and 3.5 ± 0.4 gold particles/granule for CCL11- stimulated cells (mean $\pm$ SEM, n = 767 granules). However, in CCL11-stimulated cells, the number of secretory granules positive for STX17 significantly increased compared to unstimulated cells (Fig. 4A, right panel). The labeling level on EoSVs (number of gold particles/vesicles and percentage of labeled vesicles/cell section) did not change when EoSVs from unstimulated eosinophils were compared with stimulated cells (Fig. 4B).

ER cisternae and Golgi complex regions showed negligible or no labeling for STX17 in both unstimulated and stimulated cells (Fig. 5), indicating that this SNARE likely does not take part in constitutive secretion.

Discussion

SNARE members mediate membrane fusion during all steps of intracellular trafficking, and function in almost all aspects of innate and adaptive immune responses from different cells [21]. However, little is known about the expression and function of these proteins in human eosinophils. Here we demonstrate, for the first time, that these cells constitutively express STX17 and that this SNARE is localized in both secretory granules and granule-derived transport carriers (EoSVs). Stimulated eosinophils did not clearly change the levels of intracellular STX17.

There are 38 known members of the mammalian SNARE family at present. Each cell type expresses different combinations of SNARE-family members that are selectively distributed on organelles and membrane domains. Therefore, defining the locations of individual SNAREs has emerged as a powerful initial approach for mapping intracellular pathways and manipulating both trafficking steps and cellular responses (reviewed in [21]). Specifically, eosinophil secretory vesicles, but not granules, express the SNARE VAMP2 [22, 24] while crystalloid granules, but not vesicles, express VAMP7 and VAMP8. The roles of these molecules in eosinophils have been investigated. VAMP2 colocalized with RANTES throughout interferon gamma (IFN- γ)-induced vesicle-mediated secretion of RANTES [22], and it was suggested to mediate specific membrane docking through interaction with plasma membrane SNAREs, SNAP23, and syntaxin4 [29]. Antibody inhibition of VAMP7 but not of VAMP8 impaired the release of secretory granules mediators, eosinophil peroxidase (EPO) and eosinophil-derived neurotoxin (EDN) and, thus, VAMP7 was considered as critical for mediator release from human eosinophils [25].

Our present results add a new member to the known repertoire of eosinophil SNAREs. By using different immunodetection approaches - flow cytometry and immunogold EM -, STX17 was clearly localized within human eosinophils.

Pre-embedding immunoEM optimizes antigen preservation and is more sensitive to detect small molecules than post-embedding labeling that is limited by poor preservation of the antigenicity. Here we used a pre-embedding approach combined with very small gold particles that facilitated both the protein visualization at specific intracellular sites and the study of cell morphology. The use of very small gold particles (1.4 nm) conjugated to

secondary antibodies has the advantage of greater tissue penetration to reach antigens at membrane microdomains [26]. We demonstrate by immunogold EM that STX17 is localized in crystalloid granules and EoSVs from human eosinophils. In contrast to the SNAREs already described in human eosinophils – VAMP2 (just in vesicles) [22] and VAMP7 (just in granules) [25], the presence of STX17 in both granules and in a population of granule-derived transport vesicles (EoSVs) indicate that this SNARE may be functionally implicated in membrane trafficking from secretory granules to the plasma membrane while VAMP2 and VAMP7 may be related to other eosinophil secretory pathways.

STX17 is ubiquitously expressed in human tissues [30] and was documented in the smooth ER of secretory cells and to some extent in the ERGIC (RER–Golgi intermediate compartment) [31, 32]. STX17 appears to be required for constitutive secretion [33] and to function as a receptor at the ER membrane that mediates trafficking between the ER and post-ER compartments [31].

Secretory processes have traditionally been classified as constitutive or regulated processes: constitutive secretion refers to vesicular secretory traffic directly from Golgi to the plasma membrane, and regulated secretion classically refers to exocytosis of storage granules, which fuse with the plasma membrane. Human eosinophils are not rich in ER [34] and we found negligible labeling for STX17 in the ER and Golgi compartments within both unstimulated and activated eosinophils (Fig. 5). This finding suggests that STX17 is likely not involved in constitutive secretion. On the other hand, the consistent localization of STX17 at secretory granules and EoSVs may be indicative of a role for this SNARE in regulated secretion and/or in the transport of a specific cargo through a process of secretion termed piecemeal degranulation (PMD). By PMD, a specific granule-stored cytokine or cationic protein is mobilized from secretory granules into budding vesicles, which travel to the plasma membrane for extracellular release (reviewed in [2]). Under physiological and pathological conditions, PMD is the most relevant and frequent mechanism of secretion of mediators from human eosinophils [2]. In the present work, stimulation with CCL11, which is known to induce granule emptying through PMD and increased formation of EoSVs in human eosinophils [3, 19] led to a higher number of granules labeled for STX17 (Fig. 4A), suggesting that STX17 may be involved in this secretory pathway.

It has been accepted that distinct SNARE isoforms may, in part, determine the specificity of trafficking and membrane fusion between organelles or with the cell surface. More recently, using a model cell line (HeLa cells), STX17 was documented on the outer membrane of autophagosomes and considered essential for fusion between this compartment and the endosomal/lysosomal membrane [35]. Our present study has clearly identified STX17 on the outer membrane of eosinophil specific granules (Fig. 1Ai). As noted, these organelles store preformed immune mediators. We may speculate that STX17 takes part in specific trafficking events underlying the distinct eosinophil secretory pathway. However, further studies need to be undertaken to assign functions to STX17 in the eosinophil immune responses.

Taken together, our present results demonstrate, for the first time, sites of localization of STX17 within eosinophil leukocytes. The expression of this SNARE in secretory granules

and granule-associated vesicular compartments indicates that this molecule might mediate membrane trafficking from granules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- First demonstration of the Qa-SNARE syntaxin 17 (STX17) in human eosinophils
- High resolution immunogold EM shows STX17 in granules and tubular vesicles
- Unstimulated, TNF- α or CCL11-stimulated eosinophils express STX17
- Our findings suggest a role for STX17 in the transport of granule-derived cargos

Fig. 1.

STX17 is present on secretory granules and vesicular carriers within human eosinophils. (A) The intracellular content of STX17 after 1 h incubation at 37°C was measured by flow cytometry. (B) A representative ultra-thin section from an unstimulated eosinophil shows STX17 at secretory granules (Gr) and in association with Eosinophil Sombbrero Vesicles (EoSVs). (Bi) and (Bii) are boxed areas of (B) seen in high magnification. (Bi) STX17 is clearly labeled at granule outer membranes (arrows) and in the granule matrices. In (Bii), labeling is associated with the membrane of EoSVs (circles). Eosinophils from a healthy donor were isolated from peripheral blood and processed for pre-embedding immunonanogold electron microscopy as described [26, 34]. N, nucleus. IC, irrelevant antibody control. Scale bars: (B) 0.5 μ m; (Bi, Bii) 0.4 μ m.

Fig. 2. Subcellular localization of STX17 within human eosinophils stimulated with TNF- α . (A and Ai) STX17 is observed at secretory granules (Gr) and Eosinophil Sombbrero Vesicles (EoSVs) membranes (Aii and Aiii, arrowheads). Note in (Aii) that a labeled vesicle is seen in close apposition to the plasma membrane while in (Aiii) a vesicle is associated with a labeled granule. (Ai-Aiii) are boxed areas of (A) seen in high magnification. Eosinophils from a healthy donor were isolated, stimulated with TNF- α for 1h, fixed and processed for pre-embedding immunonanogold electron microscopy as described [26]. N, nucleus. Scale bars: (A) 0.7 μ m; (Ai) 0.3 μ m; (Aii and Aiii) 0.2 μ m.

Fig. 3. STX17 within human eosinophils stimulated with CCL11. (A) A representative electron micrograph shows the morphology of an activated eosinophil with emptying secretory granules labeled for STX17 (seen in high magnification in Ai). Arrowheads indicate gold particles in (Ai). Eosinophils from a healthy donor were isolated, stimulated with CCL11 for 1h, fixed and processed for pre-embedding immunonogold electron microscopy as described [26]. N, nucleus. Scale bars: (A) 0.7 μm ; (Ai) 0.4 μm .

Fig. 4. Quantitative STX17 immunolabeling in unstimulated, TNF- α - and CCL11-stimulated eosinophils. (A, left panel) Quantitative analysis showed similar numbers of gold particles/secretory granule in both unstimulated and stimulated cells. The percentage of labeled granules per cell section is shown in (A, right panel). The number of positive granules significantly increased in CCL11-stimulated cells compared with unstimulated and TNF- α groups (*, $P < 0.01$). In (B), the level of STX17 labeling in EoSVs did not change when all conditions were compared. Data shown represent the mean $\pm$ SEM. The total number of organelles/structures evaluated was as follows: 1088 secretory granules and 1106 vesicular compartments. Eosinophils from a healthy donor were isolated, stimulated with TNF- α or CCL11 for 1h, fixed and processed for pre-embedding immunogold electron microscopy as described [26].

Fig. 5. STX17 labeling is not associated with ER and Golgi compartments. While secretory granules (Gr) are strongly labeled for STX17, the ER and Golgi region (circle) shows no labeling for this SNARE. Eosinophils from a healthy donor were isolated and processed for pre-embedding immunonanogold electron microscopy as described [26]. N, nucleus. Scale bar: 1.6 μ m.