Human Eosinophils Secrete Preformed, Granule-Stored Interleukin-4 Through Distinct Vesicular Compartments

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Abstract

Secretion of interleukin-4 (IL-4) by leukocytes is important for varied immune responses including allergic inflammation. Within eosinophils, unlike lymphocytes, IL-4 is stored in granules (termed specific granules) and can be rapidly released by brefeldin A (BFA)-inhibitable mechanisms upon stimulation with eotaxin, a chemokine that activates eosinophils. In studying eotaxin-elicited IL-4 secretion, we identified at the ultrastructural level distinct vesicular IL-4 transport mechanisms. Interleukin-4 traffics from granules via two vesicular compartments, large vesiculotubular carriers, which we term eosinophil sombrero vesicles (EoSV), and small classical spherical vesicles. These two vesicles may represent alternative pathways for transport to the plasma membrane. Loci of both secreted IL-4 and IL-4-loaded vesicles were imaged at the plasma membranes by a novel EliCell assay using a fluoronanogold probe. Three dimensional electron tomographic reconstructions revealed EoSVs to be folded, flattened and elongated tubules with substantial membrane surfaces. As documented with quantitative electron microscopy, eotaxin-induced significant formation of EoSVs while BFA pretreatment suppressed eotaxin-elicited EoSVs. Electron tomography showed that both EoSVs and small vesicles interact with and arise from granules in response to stimulation. Thus, this intracellular vesicular system mediates the rapid mobilization and secretion of preformed IL-4 by activated eosinophils. These findings, highlighting the participation of large tubular carriers, provide new insights into vesicular trafficking of cytokines.

Keywords
electron tomography; eosinophil secretion; eosinophil sombrero vesicles; eotaxin; IL-4; piecemeal degranulation; secretory pathway; ultrastructure; vesicular transport

Interleukin-4 (IL-4) is a hallmark cytokine produced by polarized T-helper cell type II (Th2) lymphocytes typical of allergic and anthelmintic parasite immune responses. Interleukin-4 directs the differentiation of Th2 cells (1,2), provides a priming signal for naïve B cells (3) and

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Supplementary Material: Two supplemental videos are available. Video 1 shows electron tomographic reconstructions from an emptying granule within a representative eotaxin-activated eosinophil. The internal vesiculotubular network and forming EoSVs are visualized. Video 2 is a 3D model from the tomographic slices shown in video 1. Both videos correspond to Figure 4. These materials are available as part of the online article from http://www.traffic.dk/videos/6_12.asp
induces immunoglobulin E production from B cells (4). In addition to Th2 cells, eosinophils, white blood cells with functions in health, anthelminthic immunity and the pathogeneses of asthma, allergies and other diseases (5–8), are able to secrete IL-4. In parasitic infection models, innate immune, eosinophil-derived IL-4 has been linked with subsequent establishment of Th2-acquired immunity (9). Interleukin-4-producing eosinophils are also the major source of Th2 cytokines in late-phase responses of allergic airway inflammation (10).

In contrast to Th2 lymphocytes, in which de novo IL-4 synthesis must be induced, eosinophils can rapidly release IL-4 (11). This special capacity of eosinophils is based on their content of IL-4 that is both present as preformed protein within specific granules and can be mobilized for quick release upon stimulation (11). Using a sensitive, dual Ab capture and detection immunofluorescent microscopic assay (the EliCell assay) (12), extracellular IL-4 secretion has been demonstrated from eosinophils stimulated with eotaxin (13), a chemokine active on eosinophils (5). Eotaxin-elicited IL-4 release did not require new protein synthesis and was suppressed by brefeldin A (BFA), a candidate inhibitor of vesicular transport (14). These findings implicated vesicular compartments in immune responses involving secretion of preformed IL-4 from eosinophils.

Ultrastructural observations of losses within the core and/or matrix contents of eosinophil specific granules have identified that secretion in eosinophils from subjects suffering from a range of inflammatory and allergic disorders is based on vesicular transport of granule contents, termed piecemeal degranulation (PMD) (15–19). Recently, we have demonstrated that the ultrastructural granule changes of PMD are also identifiable in human eosinophils physiologically stimulated with the chemokines, eotaxin and regulated on activation, normal, T-cell expressed and secreted, and the lipid, platelet-activating factor (20). Moreover, intragranular vesiculotubular compartments were implicated in the mechanism of relocation and sequestration of granule products before release (20).

Utilizing immunonanogold and immunofluoronanogold ultrastructural localizations of IL-4, a novel EliCell assay and electron tomographic analyses, we demonstrate that in eotaxin-stimulated eosinophils, IL-4 is transported from eosinophil granules for extracellular secretion by a complex vesicular system involving both small spherical vesicles and distinct large vesiculotubular carriers, which we term ‘eosinophil sombrero vesicles’ (EoSV). Both vesicular compartments can interact with and arise from eotaxin-mobilized specific granules. Furthermore, we show that EoSVs constitute a morphologically unique vesicle population involved in eosinophil secretory responses and significantly formed upon eotaxin stimulation but suppressed when eosinophils are pretreated with BFA. These findings reveal insights into the regulated secretion of preformed cytokines by eosinophils and demonstrate, for the first time, the participation of large tubular carriers in the eosinophil secretory pathway.

**Results**

**Small vesicles and large tubular carriers transport IL-4 within human eosinophils**

In previous work, we demonstrated extracellular IL-4 release from eotaxin-stimulated eosinophils using immunofluorescent EliCell assays (12,13). When imaged using transmission electron microscopy (TEM), these cells show the morphological pattern of PMD (20) characterized by progressive emptying of contents from granule cores and/or surrounding matrices in the absence of granule fusions (15). In addition, with granule emptying an extensive vesiculotubular network is unmasked and revealed to be present within eosinophil granules (20).

To address the specific mobilization and trafficking of IL-4 within eotaxin-stimulated eosinophils, we performed labeling of this cytokine using pre-embedding immunonanogold
EM (labeling before standard EM processing including dehydration, resin embedding and resin sectioning) for precise epitope preservation and subcellular localization. The use of a secondary antibody (Fab fragment) conjugated to 1.4 nm gold particles (Nanogold) enabled us to localize IL-4 to cytoplasmic vesicle populations (Figure 1A–E) as well as to the matrices, cores and membranes of specific granules (Figure 1C,F) from stimulated cells. Secretory vesicles showed distinct labeling for IL-4. Notably, IL-4 was detected not only on classical small spherical vesicles (Figure 1E) but also on large vesiculotubular structures distributed throughout the cytoplasm (Figure 1A–D). Vesiculotubular structures, reported as microgranules in the earlier eosinophil literature, are considered morphologically unique to eosinophils and have been used as a lineage assignment in granule-poor cells (15). These distinct vesicles are easily identifiable 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 (Figure 1A,B). They also can show a ‘C’ shaped morphology. The recognition of these structures to-date solely in eosinophils is intriguing, especially because their functional role remains unknown. We term these morphologically distinct vesicles eosinophil sombrero vesicles (EoSV). Eosinophil sombrero vesicles are larger (approximately 150–300 nm in diameter) than classical small spherical vesicles (approximately 50 nm in diameter).

Interleukin-4 labeling was preferentially associated with membranes of both EoSVs (Figure 1A–C) and small vesicles (Figure 1E) and not their internal content. In unstimulated cells, IL-4-positive vesicles were also observed (Figure 1G, arrowheads) but in lower numbers compared with agonist-stimulated eosinophils [12.3 ± 2.4 IL-4-labeled EoSVs per section in eotaxin-stimulated versus 3.5 ± 0.8 IL-4-labeled EoSVs in unstimulated eosinophils (mean ± SEM), n = 12 cells]. Additionally, we applied immunonanogold labeling to detect extracellular IL-4 on stimulated eosinophils that were neither sectioned nor permeabilized. Labeling of this cytokine was found at the cell surface at a very low density (not shown). This indicates that secreted IL-4 was not retained at the cell surface but was released into the extracellular medium, as suggested by our prior EliCell assays (12,13,21).

**Extracellular eotaxin-induced release of IL-4 is mediated through vesicular compartments**

To provide additional evidence that distinct vesicular compartments mediate release of IL-4 in eosinophils in response to eotaxin stimulation, we used a single probe consisting of an affinity-purified Fab fragment covalently labeled with both 1.4 nm gold and a fluorochrome (Fluoronanogold) to perform comparative microscopy studies. Eosinophils were prepared for both EliCell assays (12) and immunoEM using the same immunoprobe for IL-4 detection. The EliCell assay was modified to work with a triple antibody system. After 1 h of stimulation, individual eotaxin-stimulated, but not unstimulated, eosinophils showed IL-4 labeling imaged in EliCell assays as intense speckled fluorescence at the cell surfaces (Figure 2Aa). Images from deconvolution microscopy (Figure 2Ba) and three-dimensional (3D) reconstructions of antibody-captured and detected IL-4 (Figure 2Bb and Bc) showed discrete loci of IL-4 secreted outside of eosinophil plasma membranes. Control cells, either unstimulated or assayed with an irrelevant capture antibody, were negative (Figure 2Ab). Cells stimulated with calcium ionophore, a nonphysiological cytolytic stimulus, exhibited diffuse fluorescence indicative of cytolytic detection of IL-4 (not shown). By TEM, the use of immunofluoronanogold enabled us to detect a pool of IL-4-loaded vesicles distributed in the cytoplasm and underneath the plasma membrane (Figure 2Ca) as observed when we used immunonanogold alone. Again, the membranes of both small vesicles and EoSVs (Figure 2Ca and Cb, arrows) were consistently and preferentially labeled for IL-4. In all immunogold experiments, controls treated with an irrelevant antibody or secondary antibody alone were negative.

Our data support the hypothesis that small packets of granule-derived IL-4 are released at the cell surface through a vesicular transport mechanism when eosinophils are physiologically
stimulated. Altogether, our results provide, for the first time, direct evidence that a cytokine is mobilized from eosinophil granules through morphologically distinct vesicular compartments.

**Eotaxin induces formation of a morphologically distinct, granule-associated vesicular compartment, involved in the IL-4 secretory pathway**

Because IL-4 was densely localized not only to small spherical vesicles (Figure 1E), classically associated with intracellular transport, but also to EoSVs (Figure 1A–C), we next investigated the distribution of EoSVs in eotaxin-stimulated eosinophils using conventional TEM. Our EM quantitative analyses showed that in eotaxin-stimulated eosinophils (Figure 3A), the total number of EoSVs increased significantly [20.4 ± 1.8 per section for eotaxin-stimulated versus 14.3 ± 1.3 for unstimulated eosinophils (mean ± SEM), n = 50 cells, p < 0.05] (Figure 3B). The numbers of EoSVs in contact with granules also increased significantly in eotaxin-stimulated eosinophils compared with nonstimulated eosinophils [6.0 ± 0.9 per section in eotaxin-stimulated eosinophils versus 1.7 ± 0.5 in unstimulated cells (mean ± SEM), n = 50 cells, p < 0.05] (Figure 3B). Ninety percent of EoSVs that were in contact with granules in stimulated cells were associated with granules undergoing PMD. Indeed, TEM images strongly suggested a structural connection between EoSVs and emptying granules [Figure 3Aa (circles) and Ab (arrowheads)]. Emptying granules often displayed an extensive membranotubular network (Figure 3C) as recently reported (20). Of note, vesiculotubular structures were also seen projecting from mobilized granules (Figure 3C).

In previous work, we have demonstrated BFA-inhibitable IL-4 cytokine release from eotaxin-stimulated human eosinophils (13) and that BFA acts within eosinophil granules to collapse the extensive intragranular membranotubular networks (20). Here, we have used BFA, a potential inhibitor of vesicular transport (14), to investigate its effect on vesicular compartments. Pre-treatment with BFA prior to eotaxin stimulation dramatically suppressed the total numbers of EoSVs (Figure 3B). Also, the numbers of EoSVs in contact with emptying granules were substantially reduced, by 94%, by BFA treatment (Figure 3B). These BFA effects, as well their previously demonstrated collapsing actions on intragranular membrane domains (20), have potential implications that EoSV might be formed from the membranous tubular system, prominently observed within granules after stimulation with eotaxin (20). Thus, while the Golgi, a classical target of BFA (22), is also morphologically altered by BFA treatment in eosinophils (20), our EM findings provide novel insights into the alternative sites and means whereby BFA can inhibit eosinophil-mediated secretion of preformed granule-derived proteins.

To address whether EoSVs could be isolated as a distinct population, we processed unstimulated eosinophils by subcellular fractionation and analyzed by TEM. In contrast to small vesicles, which localize to more buoyant light fractions, EoSVs were largely localized in fractions slightly less dense than granule-containing fractions. In addition, in granule-enriched fractions, a small population of EoSVs was observed, frequently in close association with granules (Figure 3D). Isolated EoSVs, analyzed by conventional TEM after optimal fixation, showed the same morphology (Figure 3E) as seen in intact cells (Figure 3A). By immunonanogold EM, we found that isolated EoSVs were positive for major basic protein (MBP), one of the most abundant cationic proteins stored within and recognized as a marker of eosinophil specific granules (23). In contrast to membrane-associated labeling of IL-4, MBP was localized within the tubular lumen of EoSVs (Figure 3F).

**Secretory vesicles can be directly formed from granules**

To delineate the spatial organization of EoSVs and to track vesicle formation from granules, we utilized high-resolution 3D analysis by electron tomography (24,25). We analyzed by dual-axis electron tomography the same samples of eotaxin-activated eosinophils evaluated by
conventional TEM (Figure 3A). While conventional TEM studies are usually performed on approximately 80-nm thick sections, because the tomographic slices used in this work were only 4-nm thick, they offer a significant advantage over 80-nm serial ‘thin sections’ for tracking cell structures in 3D. Three dimensional reconstructions and models generated from serial sections revealed that individual EoSVs consisted of curved tubular structures, with cross-sectional diameters of approximately 150–300 nm, surrounding a cytoplasmic center (Figures 3G–H, 4A–H and 5D–E). As visualized by 3D models, along the length of EoSVs, there were both continuously fully connected cylindrical and circumferential domains and incompletely connected and only partially circumferential curved domains (Figures 3G–H and 5D–E, and see Supplementary Information Movies 1 and 2 available online at http://www.traffic.dk/videos/6_12.asp). These two domains explain both the ‘C’ shaped morphology of these vesicles and the presence of elongated tubular profiles very close to typical EoSV, as seen in 2D cross-sectional images of both entire eosinophils (Figure 3A) and isolated EoSVs (Figure 3E). While tubular extensions from the surfaces of eosinophil granules were documented by conventional TEM (Figure 3C), the contributions of these tubular extensions to forming EoSVs have not been recognized. Notably, electron tomography also revealed that EoSV can emerge from mobilized granules through a tubulation process (Figure 4 and see Supplementary Information Movies 1 and 2 available online at http://www.traffic.dk/videos/6_12.asp). This may explain, in part, why EoSVs are seen frequently attached to emptying granules [Figure 3Aa (circles) and Ab (arrowheads)].

The tomographic reconstructions have also shown that classical small spherical vesicles, which are substantially smaller than EoSVs, can be formed from emptying granules. These vesicles were seen budding from the limiting membrane of enlarged granules that had reduced electron density (Figure 5), an indication of their mobilization and release of granule contents. Our data provide strong evidence for the origin of vesicular compartments from granules undergoing PMD.

Discussion

We have previously reported that eotaxin-stimulated human eosinophils secrete preformed IL-4 by BFA-inhibitable and likely vesicular transport-mediated processes (13). We have now evaluated immunolocalization techniques and have utilized methods that both optimally preserve intracellular vesicular structures and enable full immunolocalization of IL-4 within eosinophils, heretofore only recognized within cores of eosinophil granules (26,27). In addition, we have utilized additional analytic methods, including electron tomography, to delineate compartments, including those that are the BFA-inhibitable, involved in the sequential mobilization and secretion of IL-4 by eotaxin-stimulated eosinophils.

Eotaxin stimulation of eosinophils in vitro elicits gross alterations in granule ultrastructure typical of PMD (20), as well-recognized to occur within activated eosinophils in vivo (15,16, 19), including the mobilization of granule core and/or matrix contents. Within eosinophil granules, we confirmed the immunolocalization of IL-4 and further localized IL-4 to more than granule cores to include granule matrices and membranes. In addition, we have demonstrated an excellent correlation between electron microscopy and the EliCell assay to visualize eotaxin-elicited vesicular transport and release of IL-4. Our results, using the same probe for both experimental approaches and analyses by 3D deconvolution microscopy reconstructions provide additional strong support for a secretory route from specific granules to cell membrane based on vesicular trafficking. Mobilization of IL-4 from granules requires its transport within cytoplasmic vesicles. By immunonanogold EM, with its resolution sufficient to localize IL-4 at small membrane microdomains, we identified two morphologically distinct vesicles as transporters of IL-4, small, more classical spherical vesicles and larger vesicles (EoSVs). Small spherical vesicles have been recognized around granules in eosinophils undergoing PMD.
Heretofore, however, most immunoEM studies have failed to identify cytokine-loaded vesicles and were able only to demonstrate cytokines within cores and/or matrices of eosinophil granules (26,27,29–31). To date, small spherical vesicles in eosinophils have been the ultrastructurally documented, nongranule sites of localization for only two cytokines, transforming growth factor (TGF)-α (32) and IL-13 (33). With specific pre-embedding fixation techniques and an antibody conjugated to very small 1.4 nm gold particles, IL-4 was abundantly detectable on small vesicles.

In addition to small vesicles, IL-4 was prominently localized to the membranes of larger EoSVs (approximately 150–300-nm cross sectional diameters). Moreover, EoSVs contained granule core-derived MBP, providing additional evidence that these vesiculotubular structures have roles in the secretion of eosinophil granule-stored proteins. Although EoSVs are a distinct morphological hallmark of eosinophils, to date, their functional roles remain poorly understood. In eotaxin-activated eosinophils, there was a significant increase of EoSV numbers, both in the cytoplasm and proximate to granules. This indicates a consistent functional interaction between specific granules and EoSVs. Electron tomography revealed that EoSVs are folded, flattened tubular carriers, larger and more pleiomorphic than the conventional small (approximately 50-nm diameter) spherical vesicles. The curved morphology of EoSV might offer several advantages to eosinophil secretion. First, it would provide a higher surface-to-volume ratio system for specific transport of membrane-bound proteins. Second, tubular carriers are more effective in dealing with the long distances that must be traversed in the cytoplasm until the cell surface is reached (34). This fact might be particularly relevant for rapid delivery of IL-4 and other eosinophil-preformed cytokines or proteins. In addition, because IL-4-loaded EoSVs were also seen in unstimulated eosinophils, they may represent storage pools of IL-4 for rapid mobilization under stimulation. Third, EoSV might provide a more effective means to recycle granule membrane after the mobilization of granule products. Emerging evidence has pointed to the participation of vesiculotubular carriers in cell secretory pathways. Well-documented examples are the transport system between the ER and Golgi complex, from the endosomal system or the trans Golgi network to the plasma membrane, and along axons (24,34,35). These carriers can appear as vesicles, tubules or vesiculotubular structures of various shapes and sizes. They show considerable plasticity, often changing shapes or dividing during transport (24,34,35). It has been suggested that large transport carriers could form either by fusion of small spherical vesicles or by direct budding from donor organelles (34). Our results strongly suggest that EoSVs not only frequently interact with but also may be directly formed from specific granules. Tubular structures were seen projecting from granule boundaries by conventional EM, after 1 h of agonist stimulation (Figure 3C). The clearest evidence for EoSVs originating from granules was provided by electron tomographic analyses (Figure 4) of activated granules demonstrating formation and release of EoSVs. Studies with BFA were also informative. Brefeldin A dramatically suppressed EoSV numbers, perhaps by its recently demonstrated direct action within eosinophil granules to collapse the intragranular membranotubular networks with formation of lipid-rich deposits (20) and to inhibit tubulation needed for EoSV formation. As a probable result, another ultrastructurally documented action of BFA was the significant inhibition of granule emptying when eosinophils were treated with BFA before stimulation with agonists (20). Therefore, in the time frame of our observed agonist-elicited mobilization of granule contents for secretion (20), it is highly likely that the pre-existing vesiculotubular network within granules was critically involved in the EoSV formation and not the attributable inhibition of trafficking from Golgi-derived vesicles. In addition, it is possible that BFA may act on cytoplasmic EoSVs to contribute to their diminution in stimulated eosinophils.

In contrast to the immunonanogold localization of MBP within the lumen of EoSVs (Figure 3F), an interesting finding was the association of IL-4 with the membranes of both small spherical vesicles and EoSVs as also shown for the cytokine TGF-α in eosinophil small vesicles.
If IL-4 and other cytokines are transported bound to the membranes of vesicles, as we suggest, the large surface to volume ratio of tubular carriers, i.e. EoSVs, would thus prove ideal for participation of these structures in eosinophil vesicular transport. In addition, IL-4 labeling was also detected on the peripheral delimiting membranes of granules. This implies that IL-4 can be relocated to granule-limiting membranes in order to be delivered to the cell surface by budding vesicles. In fact, we have recently shown that eosinophil specific granules are not merely storage organelles. They are elaborate and compartmentalized organelles with internal membranous vesiculotubular domains able to sequester and relocate granule products upon stimulation (20). A functional implication of this finding is that it adds support to the occurrence of selective release of products from eosinophils as previously indicated (13,30), although the mechanisms of intragranular membrane dynamics remain to be delineated. Structural connections between the intragranular membranous network and granule-limiting membranes, as visualized using electron tomography (20), may contribute to the mobilization of granule proteins for their release into vesicles derived from granules.

Therefore, our results may be indicative of a membrane-bound mechanism, involving the presence of a yet unidentified membrane-docking protein, in the specific trafficking of IL-4. In fact, our studies in progress implicate the transmembrane IL-4 receptor α chain in cytokine-specific vesicle membrane transport (unpublished observations). Collectively, our observations reveal the complex vesicular trafficking of IL-4 from within granules to the plasma membrane in eotaxin-stimulated human eosinophils. This dynamic secretory pathway is associated with the formation of different vesicular compartments, i.e. cytoplasmic small spherical vesicles and vesiculotubular carriers (EoSVs) that interact with and traffic from specific granules. Thus, this intracellular vesicular system provides transport for the rapid secretion of IL-4 by activated eosinophils.

Materials and Methods

Eosinophil isolation, stimulation and viability

Granulocytes were isolated from the blood of healthy donors as described (21), and eosinophils were purified by negative selection using human eosinophil enrichment cocktail (StemSep™, StemCell Technologies, Vancouver, Canada) and the MACS bead procedure (Miltenyi Biotec, Auburn, CA, USA). Experiments were approved by the Committee on Clinical Investigation, and informed consent was obtained from all subjects. Eosinophil purity was >99%. For different approaches, eosinophils (10⁶ cells/mL) were stimulated with 100 ng/mL of recombinant human eotaxin (R&D Systems, Minneapolis, MN, USA) for 1 h, 1 μg/mL of BFA (Biomol, Plymouth Meeting, PA, USA) for 30 min prior to eotaxin or medium alone (RPMI-1640 medium plus 0.1% OVA) (Sigma, St. Louis, MO, USA) at 37 °C. Cell viability after stimulation was greater than 95% as determined by ethidium bromide incorporation.

Antibody reagents

Anti-human mouse IL-4 (clone 3010.211) and irrelevant isotype control (clone 11711.11) monoclonal antibodies (R&D) were used for immunonanogold EM (2 μg/mL) and EliCell (5
A biotinylated goat polyclonal anti-human IL-4 IgG (R&D) was used as a capturing antibody for EliCell (20 μg/mL). Secondary antibody for immunonanogold EM was an affinity-purified goat anti-mouse Fab fragment conjugated to 1.4 nm gold (1:100) (Nanogold®, Nanoprobes, Stony Brook, NY, USA). Secondary antibody for comparative microscopy (EliCell and immunoEM) was an affinity-purified Fab fragment from goat anti-mouse IgG (1:25) conjugated to Alexa 488 and 1.4 nm gold (AlexaFluor®-488-Fluoronanogold™, Nanoprobes). Antibodies for MBP detection in eosinophil fractions were monoclonal mouse anti-human MBP IgG1 (clone AHE-2) and irrelevant isotype IgG1 (clone S1-68.1) control (2 μg/mL) (BD-PharMingen, San Diego, CA, USA).

**Conventional TEM**

For conventional TEM, intact or fractionated 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 as described (28) and kept at 4 °C for further processing. Agar pellets containing intact or subcellular fractionated eosinophils were processed as described (28). Briefly, samples were postfixed 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). Specimens were examined using a Philips 300 electron microscope at 60 kV. At least 450 electron micrographs were analyzed. To quantify the total number of specific granules and EoSVs, we randomly took 75 electron micrographs of cell sections showing the entire cell profile and nucleus at ×12 000 and used at end magnification of ×33 000. A total of 2445 granules and 977 EoSVs were counted. Diameters were measured from the outer dimensions of EoSV external membranes. Data were compared using the Mann–Whitney U-test (p < 0.05).

**ImmunoEM**

For immunonanogold EM, cell suspensions or subcellular fractions containing EoSVs were fixed in fresh 4% paraformaldehyde in 0.02 M PBS, pH 7.4, (30 min) at RT, washed in PBS and embedded in 2% agar in PBS. Pellets were immersed in 30% sucrose in PBS overnight at 4 °C, embedded in optimal cutting temperature compound (Miles, Elkhart, IN, USA) and stored in −180 °C liquid nitrogen for subsequent use. Pre-embedding immunolabeling was done before standard EM processing (dehydration, infiltration, resin embedding and resin sectioning). Pre-embedding immunoEM optimizes antigen preservation and is more sensitive to detect small molecules than postembedding labelings, which are done after conventional EM processing. Moreover, to reach antigens at membrane microdomains such as small vesicles, we used labeling with very small gold particles (Nanogold). Pre-embedding immunonanogold was performed on frozen 10-μm sections. All steps were done at RT as described (36) and modified as follows: cells were incubated in PBS-BSA buffer (0.02 M PBS plus 1% BSA) containing 0.1% gelatin (Sigma) (20 min) followed by PBS-BSA plus 10% normal goat serum (NGS) and incubated with primary antibody (1 h). After blocking with PBS-BSA plus NGS (30 min), cells were incubated with secondary antibody (1 h), washed in PBS-BSA, postfixed in 1% glutaraldehyde (10 min) and incubated with HQ silver enhancement solution (Nanoprobes) (10 min). Cells were immersed in 5% sodium thiosulfate (5 min), postfixed with 1% osmium tetroxide in distilled water (10 min), stained with 2% uranyl acetate in distilled water (5 min) and embedded as described (36). Two controls were performed: (i) primary antibody was replaced by an irrelevant antibody and (ii) primary antibody was omitted. When AlexaFluor®-488-Fluoronanogold™ probe was used for comparative microscopy, the PBS-BSA buffer was replaced by PBS-milk buffer (PBS plus 5% nonfat dried milk and 2% NGS).
For cell surface immunolabeling, nonpermeabilized eosinophils were fixed in paraformaldehyde, as above, and kept in suspension for immediate staining. Immunolabeling was done using the same protocol above except that LI silver enhancement solution (Nanoprobes) was used. After the sodium thiosulfate step, cells were embedded in agar and processed as for conventional TEM. All specimens were examined as described for conventional TEM.

**Electron tomography, 3D reconstruction and modeling**

Eponate sections of 200 or 400 nm were collected from eotaxin-stimulated eosinophils for analysis using electron tomography. Tilt series were acquired fully automatically at 200 kV on a Tecnai Sphera microscope (FEI, Eindhoven, the Netherlands) using Xplore 3D software (FEI). Digital images of the structures of interest were recorded as they were tilted from −65° to +65° at 1° intervals on a 1K Gatan 794 slow scan CCD camera. The tomograms were generated using Xplore 3D software (FEI). All tilted images were aligned to a common tilt axis using cross correlation, and the volume was reconstructed by real space-weighted back projection. Five tomograms were analyzed. Modeling was carried out using IMOD software (37).

**EliCell assay**

EliCell was performed as described (12) and modified as follows. Briefly, a mixture of freshly purified eosinophils in melted Neutravidin-conjugated agarose matrix (Pierce, Rockford, IL, USA) plus biotinylated anti-IL-4 and eotaxin were gently spread onto a slide. The slide was covered with a perfusion chamber (CoverWell™, Grace Bio-Laboratories, Bend, OR, USA), overlaid with eotaxin and incubated (37 °C, 1 h). Unstimulated, eotaxin-stimulated and calcium ionophore-stimulated eosinophils were prepared as described (12). Cells were fixed and washed as before (12), blocked with 1% NGS plus 5% dried milk in calcium magnesium free HBSS (HBSS−/−) for 10 min and incubated with primary anti-IL-4 (1 h). After washing in HBSS−/−, the slides were incubated with secondary antibody (45 min), washed in blocking buffer followed by HBSS−/−, dried and mounted. Two controls were performed: (i) biotinylated anti-IL-4 antibody was replaced by an irrelevant biotinylated isotype antibody and (ii) biotinylated capture antibody was omitted. Analyses were performed using both phase contrast and fluorescence microscopy with a Provis AX-70 Olympus microscope.

**Subcellular fractionation**

Eosinophils were resuspended in disrupting buffer as described (30) and subjected to nitrogen cavitation under pressure of 800 psi for 10 min. Post-nuclear supernatants recovered after centrifugation (400 × g, 10 min) were ultracentrifuged (100 000 × g 1 h) at 4 °C in linear Accudenz® (Accurate Chemical and Scientific Corp., Westbury, NY, USA) gradients (0–45% in disrupting buffer). Fractions (20 × 0.5 mL) were collected with a peristaltic pump.

**Deconvolution microscopy**

Fluorescence images were acquired using a Retiga Exi-cooled CCD camera coupled to a Provis AX-70 Olympus microscope and an UPlanApo objective (100 × 1.35). The microscope, Z-motor drive, shutters and camera were controlled by IPLab 3.6 for Macintosh (Scanalytics, VA, USA). The acquired stacks were further processed for deconvolution and 3D rendering with Volocity 2.6 (Improvement, Lexington, MA, USA). Plasma membrane was stained by Texas Red®-X conjugate of wheat germ agglutinin (Molecular Probes, Eugene, OR, USA) as per the manufacturer’s instructions.

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References


Figure 1. Distinct vesicular compartments are labeled for interleukin 4 (IL-4) within human eosinophils
A–C) Immunonanogold electron microscopy revealed large (150–300 nm in diameter) IL-4-positive cytoplasmic vesicles (eosinophil sombrero vesicles) with membrane-associated labeling (box in Aa, Ab and arrows in B and C). Ab represents the boxed area of Aa. D, E) The membranes of a granule-associated vesiculotubular carrier (D, arrow) and small spherical vesicles (E, arrow) were labeled for IL-4. F) IL-4 labeling was also seen within granules and on the peripheral delimiting membranes of granules. G) In unstimulated eosinophils, some IL-4-positive vesicles (arrowheads) were observed in the cytoplasm. Eosinophils were isolated from the blood by negative selection, stimulated with 100 ng/mL eotaxin (A–F) or medium
alone (G) during 1 h at 37 °C and processed for pre-embedding immunonanogold labeling (before standard electron microscopy procedures) with mouse anti-IL-4 monoclonal antibody followed by 1.4 nm gold-conjugated goat-anti-mouse Fab fragments. Gr, granule. Scale bar, 400 nm (Aa), 150 nm (Ab), 300 nm (B–D), 150 nm (E), 500 nm (F) and 600 nm (G).
Figure 2. Vesicular trafficking and secretion of interleukin 4 (IL-4) from human eosinophils
A) Phase-contrast (left panels) and fluorescence (right panels) microscopy of identical fields of eosinophils incubated for 1 h in an IL-4 dual antibody (Ab) capture and detection EliCell assay. Eosinophils were embedded in a Neutravidin-linked gel matrix containing biotinylated goat anti-IL-4 Ab to capture released IL-4 at cell surfaces. Once captured, IL-4 was detected by mouse anti-IL-4 monoclonal Ab followed by goat-anti-mouse Fab fragments conjugated with both Alexa 488 and 1.4 nm gold (Fluoronanogold). Eotaxin-stimulated eosinophils (Aa) exhibited dotted green fluorescent immunoreactive staining for IL-4, while nonstimulated eosinophils were negative (Ab). B) Interleukin-4 secretion images from eosinophils prepared for EliCell as described in A and analyzed using deconvolution microscopy after plasma
membrane staining with Texas Red®-X conjugate of wheat germ agglutinin. In Ba, deconvolution images, including Y-Z and X-Z slices, reveal green IL-4 staining on the external surface of red-stained membrane. In Bb and Bc, three dimensional reconstructed images demonstrate released and captured IL-4 as focal fluorescent green spots outside of the cell membrane. C) Membrane-bound IL-4 on vesicles from a representative eosinophil prepared for pre-embedding immunoelectron microscopy (see Material and Methods for details) with the same probe used for EliCell in A. A typical eosinophil sombrero vesicle is seen underneath the cell membrane (Ca, arrow) and around a specific granule (Cb, arrow). In all experiments, eosinophils were stimulated as described in Figure 1. G, granule. Results are representative of at least three independent experiments. Scale bars, 10 μm (A), 6 μm (Ba), 4 μm (Bb), 6 μm (Bc), 240 nm (Ca) and 200 nm (Cb).
Figure 3. Eosinophil sombrero vesicles (EoSV) are distinct vesicular compartments, which associate with emptying granules

(Aa) Transmission electron microscopy (TEM) of an eotaxin-stimulated eosinophil reveals EoSVs in contact (white circles) with enlarged emptying granules and distributed in the cytoplasm (arrowheads). Ab corresponds to an area of Aa where EoSVs in contact with granules are seen in higher magnification (arrowheads). (B) Quantification of EoSV numbers by TEM. Eotaxin (Eot) induced both formation of EoSVs and association of these vesicular compartments with granules undergoing release of their contents (p < 0.05). Brefeldin-A (BFA) pretreatment dramatically suppressed all EoSV numbers (p < 0.05). (C) Membranous vesiculotubular structures (highlighted in pink in Cb) are seen within and projecting from the
surface of an emptying specific granule. (D) In a granule-enriched fraction, isolated by subcellular fractionation, an EoSV is seen in close association with the granule peripheral delimiting membrane (inset). In E and F, EoSVs isolated by subcellular fractionation and visualized by, respectively, conventional TEM and after immunonanogold labeling for major basic protein (MBP). Note the clear delimiting membrane unit (trilaminar aspect). (G, H) Three-dimensional models of the same sample as in A visualize EoSVs as curved tubular and open structures surrounding a cytoplasmic center. In H, the arrow points to the tubular lumen. Cells were stimulated as in Figure 1. A total of 977 EoSVs were counted in 75 cell sections showing the entire cell profile and nucleus. Unstimulated eosinophils were fractioned on Accudenz® gradients to separate EoSVs from other cellular components. Fractions containing granules or EoSVs were fixed, embedded in agar and processed using conventional TEM or pre-embedding immunonanogold electron microscopy as in Material and Methods. Three dimensional models were generated from approximately 4-nm thick serial slices obtained using automated electron tomography and are also shown in Figure 5 and Movies 1 and 2 (available online at http://www.traffic.dk/videos/6_12.asp). Results are representative of three independent experiments. NS: not stimulated. Gr, granule. Scale bars, 450 nm (Aa), 300 nm (Ab, C, D), 200 nm (E), 180 nm (F), 150 nm (G) and 100 nm (H).
Figure 4. Tomographic slices and three dimensional (3D) models of an emptying specific granule and eosinophil sombrero vesicles (EoSV)

A–F) The tomographic volume shows substantial changes within and at a granule surface associated with EoSV formation. Forming EoSVs are indicated in E and F (arrows). Several EoSV profiles are also seen close to the granule. Arrowheads indicate a similar EoSV imaged as an opened, tubular-shaped structure. Numbers on the lower right corners of the panels indicate slice numbers through the tomographic volume. In G, the granule peripheral delimiting membranes (blue), the membranes of emerging vesicles (yellow, green and red), proximate EoSVs (pink) and intragranular subcompartments (green) were drawn. (H) When all drawn lines in serial sections were combined, a 3D model was generated. (I) Detailed image of the
granule surface corresponding to the area outlined by the box in H. Arrows indicate progressive
tubular extensions (in yellow, green and red), involved in the formation of EoSVs. Gr, granule.
The slices (approximately 4 nm of thickness) were extracted from 3D reconstructions of a 400-
nm eosinophil section analyzed using automated electron tomography at 200 Kv. Some of
EoSVs shown close to the lower pole of the granule correspond to those in Figure 3G. Cells
were stimulated as in Figure 1, chemically fixed and processed for conventional transmission
electron microscopy. Scale bar, 500 nm (A–H), 120 nm (I). Also see Supplementary
Information, Movies 1 and 2 (available online at http://www.traffic.dk/videos/6_12.asp).
Figure 5. Small spherical vesicles are formed from mobilized eosinophil granules
A, B) Budding of small vesicles (circles in A and arrows in B) is seen at the surface of eosinophil specific granules. The slices (approximately 4 nm of thickness) were extracted from three dimensional (3D) reconstructions of a 400-nm eosinophil section analyzed using automated electron tomography. Numbers on the lower right corners of the panels indicate the slice number through the tomographic volume. In C, a series of consecutive granule membrane contours traced from 47 serial sections as shown in B. An eosinophil sombrero vesicle (EoSV) as seen in B (arrowhead) was also traced (pink). D–F) Meshed models of an eosinophil granule from contours shown in C. A progressive protrusion on the granule-limiting membrane is indicated in D (arrowheads). E) A separate spherical vesicle is shown in yellow. In F, the arrow points to the same spherical vesicle in a different model view. This model was meshed using contours traced from the serial sections where the vesicle appears separated from the granule outer membrane. In D and E, the 3D structure of EoSVs is also demonstrated. Cells were stimulated as in Figure 1, chemically fixed and processed for conventional transmission electron microscopy. Gr, granule. Scale bars, 500 (A), 250 (B, C), 150 (D, E) and 200 nm (F).